Human embryonic stem cells : advancing biology and cardiogenesis towards functional applications l

Braam, S.R.

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Advancing biology and cardiogenesis towards functional applications

STEFAN R. BRAAM

Colophon

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Advancing biology and cardiogenesis towards functional applications

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Human embryonic stem cells
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Contents

CHAPTER ONE

Introduction

Abstract

Human embryonic stem cells are pluripotent cells capable of sustained self-renewal and differentiation to derivates of the three germ layers ectoderm, endoderm and mesoderm. Because of these unique properties, the cells hold great potential as a model for human development, disease pathology, drug discovery and safety pharmacology. All these applications will depend on comprehensive knowledge of their biology and control of their signaling mechanisms and fate choices.

HUMAN EMBRYONIC STEM CELLS

Human embryonic stem cells (hESC) were first derived in 1998[1] under conditions similar to those developed for mouse embryonal carcinoma and embryonic stem cells (mESC). This involves co-culture with mitotically inactivated fibroblasts that function as 'feeder' layers[2,3]. Retrospectively it appears a lucky coincidence that these fibroblasts supported the undifferentiated growth of both mESC and hESC. Although mESC and hESC are both derived from the inner cell mass of blastocyst stage embryos there are clear differences in morphology, behavior and growth factor requirements. mESC self-renewal depends on leukemia inhibitory factor (LIF) supplemented with bovine serum, LIF and BMP4 or dual inhibition of GSK3 and MAPK[4,5]. By contrast, hESC rapidly differentiate upon exposure to BMP4[6] and require basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF-β) /Activin A signaling for undifferentiated growth[7]. The apparent differences between the two cell types have recently been attributed to their state of differentiation[8]. Cell lines from mouse epiblast can be isolated under conditions established for hESC culture[8,9]. These epiblast stem cells share signaling pathways for self-renewal, gene expression signatures and ability to differentiate with hESC.

PLURIPOTENCY AND EXIT FROM THE PLURIPOTENT STATE

Pluripotency of stem cells is regulated by a complex interplay of cell-matrix interaction, transcriptional regulation, chromatin-modifying enzymes, miRNAs and specific signal-transduction pathways[10]. Interestingly, the primary signaling pathway in hESC, FGF2, is highly dependent on the ECM in various cell systems[11]. However it remains to be investigated how growth factor signaling converges with matrix-integrin signaling in hESC and specifically which matrices and integrins are important for pluripotency. Furthermore it is presently unclear how external stimuli affect the pluripotency transcriptional regulators OCT4, SOX2, NANOG. However it is clear that SOX2 and OCT4 function by heterodimerization and act synergistically to activate Oct-Sox enhancers present in the core promoters of pluripotency genes. NANOG is thought to stabilize the pluripotent state, but is not essential for pluripotency[12]. Genome wide chromatin IP Chip experiments in hESC for OCT4, SOX2 and Nanog showed that OCT4, SOX2, and NANOG bind together at their own promoters to form an interconnected autoregulatory feed forward loop[13]. All three transcription factors co-occupy several hundreds of genes at overlapping sites in the genome. This strongly suggests that they act in a coordinated way to maintain the pluripotent transcriptional network. Interestingly, functional clustering of the putative genes regulated by OCT4, SOX2, and NANOG results in two distinct gene sets. The first set is actively expressed and includes transcription factors, signal transduction components and chromatin modifying enzymes that promote pluripotency. The second set of genes is not expressed but their expression is associated with lineage commitment and differentiation. Silencing of this class of genes is probably actively contributing to maintenance of the undifferentiated state. Exactly how hESC downregulate the core pluripotency network and initiate a differentiation program is unclear. Nevertheless differentiation protocols for directed differentiation to various lineages have been described. However differentiation is accompanied by severe heterogeneity and the amount of desired cells is typically limited to a few percent of the

total cell yield. Nevertheless, both gene expression as well as protein analyses confirm that differentiating cells follow a gene expression pattern during differentiation as observed in the developing embryo. For example; during gastrulation, cardiac progenitors form when they migrate laterally from the primitive streak. These cells express markers like Brachyury T and MESP1[14]. MESP1 is thought to be the first marker of cardiac committed mesoderm and directly induces epithelial–mesenchyme transition and activates many members of the core cardiac regulatory network including NKX2-5[15]. Whole genome micro-array analyses of differentiating hESC cultures to the cardiac lineage confirms these temporal gene expression patterns[16].

APPLICATIONS OF HESC AND THEIR DERIVATES

The isolation of hESC in 1998 led to great excitement in various areas of biomedical research[1]. For the first time, a non-transformed human cell capable of sustained self-renewal and directed differentiation to any cell type was available. These unique properties prompted fundamental research into translational medicine. Restoration of organ function by replacing damaged tissue from stem cells was among the most appealing applications. However, issues like effective transplantation techniques, immune rejection of foreign cells, proper functional integration, safety related to introduction of foreign dividing cells and teratoma formation by residual undifferentiated cells are considered to represent significant hurdles to clinical introduction for cell transplantation therapy[17]. Other applications like the use of differentiated hESC as human *in vitro* models for dissection of molecular pathways controlling differentiation, self renewal and biology of disease has a huge biomedical potential in the short term. hESC differentiated to neurons, hepatocytes or cardiomyocytes could be used for the pre-clinical safety evaluation of novel drug candidates[18]. Mutations observed in the clinic can be directly introduced in human stem cells, because there is no species difference. Disease models based on these mutant hESC lines may have huge potential as model for drug development and safety pharmacology. More recently, the landmark discovery of another source human pluripotent stem cells derived from somatic cells by reprogramming (induced pluripotency stem cells, iPS cells) greatly boosted this field of research[19,20]. Human iPS cells derived from patients with specific genetic diseases are now complementing hESC as a tool for basic research.

Differentiation of hESC to beating clusters of cardiomyocytes has always attracted significant attention and various methods have been described to induce cardiomyogenesis in pluripotent cell lines[21]. Cardiomyocytes from human stem cells are of great interest for the pharmaceutical industry as model for drug development and drug safety pharmacology[22]. The heart has proven to be particularly sensitive to off-target, toxic effects of drugs. Reports of unexpected drug-induced cardiac arrhythmias associated with sudden cardiac death, has led to the withdrawal of a number of these non-cardiac drugs from clinical use[23]. Currently, assessing risk for arrhythmias is part of the standard required pre-clinical evaluation of all drugs in development (ICH topic S7B)[24].

CHALLENGES

Progress in implementation of human embryonic stem cell technology in both academic research laboratories and the biopharmaceutical industry has lagged behind expectations. Basic technology available for research on mouse ESCs, like gene targeting and defined feeder-free culture and differentiation, has been challenging in hESC[25]. These techniques are essential for all further biomedical applications. Cell transplantation, basic studies on human developmental biology, drug target discovery and safety pharmacology all benefit from defined selected cell populations. However, the availability of lineage specific cell surface antigens for selection of a particular cell type is very limited[26]. hESC lines expressing fluorescent or selectable cassettes from lineage specific promoters will therefore be essential for pure populations of the desired cell type. Functional analyses of signaling pathways in human physiology and pathophysiology will require targeted gene disruption by RNA interference or homologous recombination. This will facilitate the development of human non-transformed cell models that can be cultured indefinitely.

One intrinsic problem that has turned out to underlie the majority of challenges associated with hESC is the culture system. hESC culture traditionally required undefined components like feeders and fetal calf serum. The undifferentiated cells are growing in multilayered colonies, expand quite slowly and are difficult to manipulate. Both of these issues hamper the understanding of self-renewal and further technology development based on these cells. It is therefore crucial to develop defined monolayer culture systems that are amenable to efficient genetic manipulation of hESC, and that support sustained self-renewal robustly. Targeted cell lines expressing fluorescent reporters from lineage specific promoters can be subsequently used for efficient (high-throughput) optimization of differentiation and selection of a desired cell type.

Aims and scope of this thesis

All future applications of human embryonic stem cells will depend on exquisite knowledge of their biology and control of their signaling and fate. Chapters in this thesis describe multiple aspects of human embryonic stem cell biology and technology development to achieve these goals. In **Chapter 2** a feeder-free human embryonic stem cell culture protocol is described, which has been optimized for 12 independent lines. The system is optimal for clonal growth and efficient gene transfer without loss of pluripotency. In **Chapter 3** detailed bench protocols for culture adaptation and genetic manipulation are presented. In **Chapter 4** the plasma membrane characteristics of feeder-free human embryonic stem cell cultures are further investigated. It is shown that these cells express a uniform epithelial plasma membrane profile and that VIMENTIN, normally associated with mesenchymal cells is also expressed. We show that this expression is related to stress and associated with hardness of the tissue culture plastic substrate rather than differentiation. In **Chapter 5** the plasma membrane of hESC is further investigated through functional analysis of the expression of integrins, the surface receptors of extracellular matrix proteins. Recombinant vitronectin was identified as

the first defined substrate that supports human embryonic stem cells in completely defined culture medium. In **Chapter 6** SILAC technology and quantitative phopspho-proteomics are used to investigate how human embryonic stem cells exit the pluripotent state upon BMP4 exposure. Approximately 50% of the 3067 identified phosphosites were regulated within 1 hr of differentiation induction, revealing a complex interplay of phosphorylation networks spanning different signaling pathways. Among the phosphorylated proteins was the pluripotency-associated protein SOX2, which was SUMOylated as a result of phosphorylation. Using the data to predict kinase-substrate relationships the hESC kinome is reconstructed; CDK1/2 emerged as central in controlling self-renewal and lineage specification. In **Chapter 7** gene targeting and defined culture systems are used to optimize and study the differentiation to the cardiac lineage. EGFP is targeted to the NKX2-5 locus – one of the earliest markers of cardiac commitment. The early NKX2-5 positive cell population contained multipotent progenitor cells capable of directed differentiation to the cardiac, endothelial and vascular smooth muscle cells. In **Chapter 8** human cardiomyocytes derived from hESC are described as a scalable reproducible system on which to base cardiac safety pharmacology assays. Evidence is provided that patient serum levels of drugs and known responses on QT interval overlap with field potential duration values derived from hESC-CM, as predicted. On this basis, field potential duration prolongation is proposed to be a directly applicable safety criterion for pre-clinical evaluation of new drugs in development. In **Chapter 9** we review the state of the art and discuss that the ability to reprogram adult cells to pluripotent stem cells and genetically manipulate stem cells present opportunities to develop human disease models. The availability of human cardiomyocytes from stem cell sources is now expected to accelerate cardiac drug discovery and safety pharmacology by offering more clinically relevant human culture models than presently available. Finally, in **Chapter 10** the results and conclusions of the previous chapters are discussed, together with their future implications.

References

1. Thomson, J.A., et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147 (1998).
2. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78, 7634-7638 (1981).
3. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154 (1981).
4. Ying, Q.L., Nichols, J., Chambers, I. & Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115, 281-292 (2003).
5. Ying, Q.-L., et al. The ground state of embryonic stem cell self-renewal. Nature 453, 519-523 (2008).
6. Pera, M., et al. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 117, 1269-1280 (2004).
7. Xu, R.-H., et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Meth 2, 185-190 (2005).

8. Tesar, P., et al. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448, 196-199 (2007).

9. Brons, I., et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448, 191-195 (2007).

10. Jaenisch, R. & Young, R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132, 567-582 (2008).

11. Saksela, O., Moscatelli, D., Sommer, A. & Rifkin, D.B. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. The Journal of Cell Biology 107, 743-751 (1988).

12. Chambers, I., et al. Nanog safeguards pluripotency and mediates germline development. Nature 450, 1230-1234 (2007).

13. Boyer, L.A., et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122, 947-956 (2005).

14. Kitajima, S., Takagi, A., Inoue, T. & Saga, Y. MesP1 and MesP2 are essential for the development of cardiac mesoderm. Development 127, 3215-3226 (2000).

15. Bondue, A., et al. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. Cell Stem Cell 3, 69-84 (2008).

16. Beqqali, A., Kloots, J., Ward-van Oostwaard, D., Mummery, C. & Passier, R. Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells 24, 1956-1967 (2006).

17. Passier, R., van Laake, L.W. & Mummery, C.L. Stem-cell-based therapy and lessons from the heart. Nature 453, 322-329 (2008).

18. Sartipy, P., Björquist, P., Strehl, R. & Hyllner, J. The application of human embryonic stem cell technologies to drug discovery. Drug Discov Today 12, 688-699 (2007).

19. Yu, J., et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917-1920 (2007).

20. Takahashi, K., et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861-872 (2007).

21. Passier, R. & Mummery, C. Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 16, 498-502 (2005).

22. Braam, S., Passier, R. & Mummery, C. Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery. Trends Pharmacol Sci 30, 536-45 (2009).

23. Redfern, W.S., et al. Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. Cardiovascular Research 58, 32-45 (2003).

24. Cavero, I. & Crumb, W. ICH S7B draft guideline on the non-clinical strategy for testing delayed cardiac repolarisation risk of drugs: a critical analysis. Expert Opinion on Drug Safety 4, 509-530 (2005).

25. Braam, S.R., et al. Improved genetic manipulation of human embryonic stem cells. Nat Methods 5, 389-392 (2008).

26. Dormeyer, W., et al. Plasma Membrane Proteomics of Human Embryonic Stem Cells and Human Embryonal Carcinoma Cells. J Proteome Res (2008).

CHAPTER TWO

Improved genetic manipulation of human embryonic stem cells

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Abstract

Low efficiency of transfection limits the ability to genetically manipulate human embryonic stem cells (hESC), and differences in cell derivation and culture methods require optimization of transfection protocols. We transiently transferred multiple independent hESC lines with different growth requirements to standardized feeder-free culture, and optimized conditions for clonal growth and efficient gene transfer without loss of pluripotency. Stably transfected lines retained differentiation potential, and most lines displayed normal karyotypes.

To realise the full potential of human embryonic stem cells (hESC), efficient methods to manipulate their genome are required. hESC lines expressing fluorescent reporters from lineage-specific promoters will be important for selecting specific lineages where no appropriate cell surface antigens are expressed and for *in vitro* toxicological screening. Targeted gene disruption by RNA interference or homologous recombination will facilitate *in vitro* modelling of human disease where clinically relevant mutations or deletions are known. However, progress has lagged behind expectations in part because of poor transfection and single cell cloning efficiencies. Lentiviral infection is presently the most efficient method for gene transfer but has major limitations including silencing of randomly integrated copies of the transgene[1], incompatibility with homologous recombination and costly, time-consuming large scale production of multiple constructs. Adenoviral constructs yield modest (~11%) infection efficiencies[2] and plasmid transfection shows highly variable transfection efficiencies, ranging from 3-35% in independent lines[3]. Furthermore, the most efficient transfection methods have been optimized using two WiCell (US) hESC lines, H1 and H9. For non-US or non-NIH funded researchers, >400 other lines are available. Although various stable[3-5] and inducible gene expression systems have been reported for hESC[6-8] none have yet been applied to multiple cell lines and growth conditions presently available. In addition, initial gene delivery was often inefficient. Low transfection efficiency in hESC lines therefore remains an unsolved problem.

hESC are usually cultured with mouse embryonic feeders (MEF) to support self renewal but more recently human feeders (HF) and feeder-free Matrigel™ substrates have been used in combination with enzymatic or non-enzymatic passage and growth factor-supplemented basal media.

To develop a generic method for ectopic gene expression in hESC, we investigated whether twelve independently derived cell lines (HES-2, Envy, HUES1,5,7,15, HESC-NL1,2,3,4 and NOTT1,2) could be transferred to common feeder-free culture conditions and undergo efficient transfection using electroporation, lipofection, lentivirus and adenovirus, without loss of pluripotency or karyotypic stability. The lines selected were derived and grown under the most diverse conditions we had available: mechanical passage on MEFs in serum containing medium, mechanical passage on HFs in KSR medium and enzymatic passage on MEFs in KSR medium (Supplementary Methods online). Transfer to feeder-free conditions on Matrigel in KSR-containing MEF-conditioned medium (CM)[9] was achieved in two stages: first adaptation to Matrigel; second, adaptation to trypsin (if necessary) (Figure 2.1A). Success, particularly for mechanically passaged lines, was critically dependent on very high density culture during the first passages. The combination of these two steps rapidly allowed plating of cells at low density for gene transfer without major loss of cells or pluripotency, as indicated by immunostaining/FACS analyses for cell surface markers Tra-1-60, GCTM2 and SSEA4, and transcription factors OCT4 and SOX2 (Figure 2.1B-I, Table S1 and Figure S1). After adaptation, all hESC cultures were easier to maintain than using their original culture method. To test the specific gene delivery methods functionally we selected three cell lines, HES2, HUES7 and

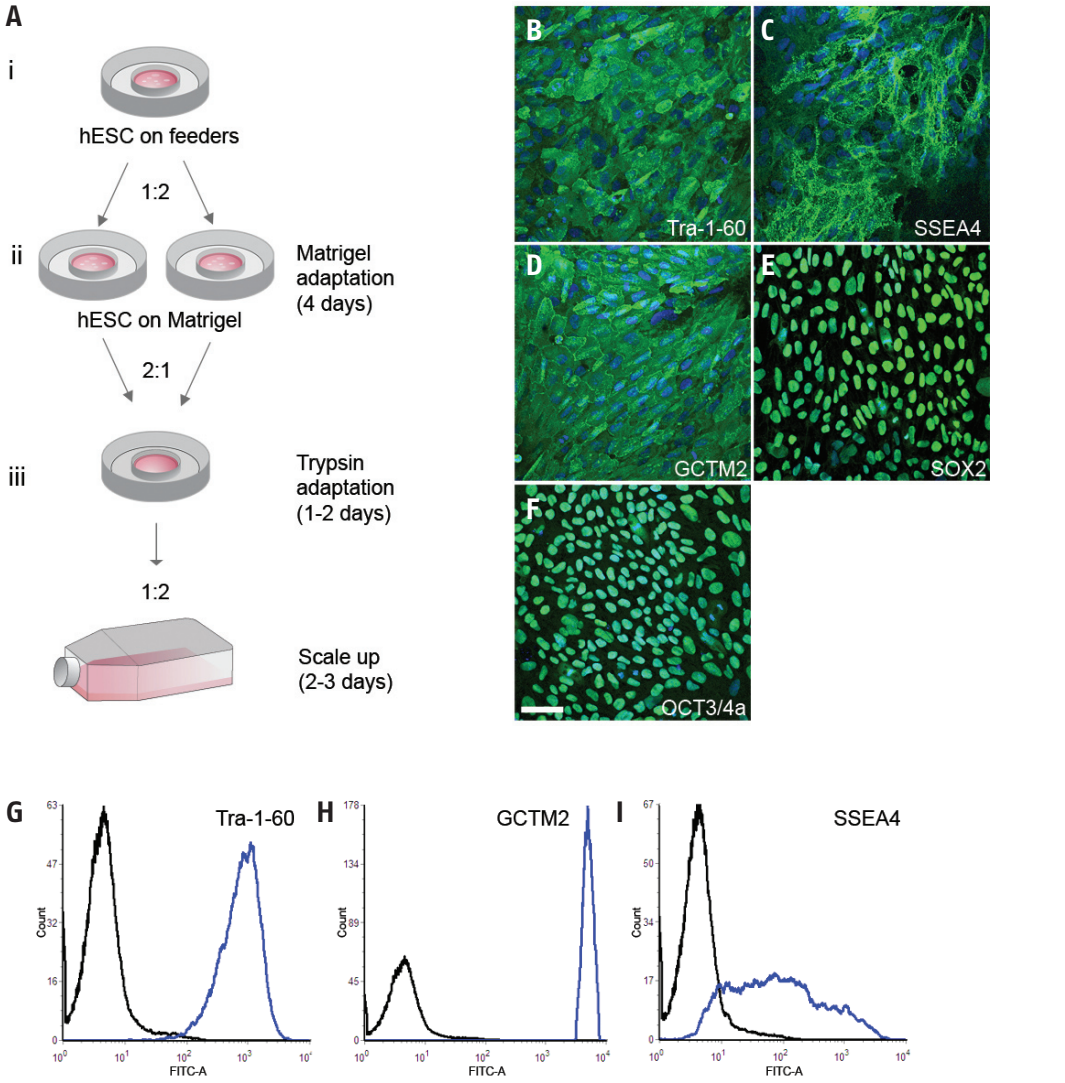

Figure 2.1

Procedure and characterization of cells adapted to trypsin based Matrigel culture

(A) Schematic representation of culture procedure: (I) hESC grown on feeders are mechanically or enzymatically passaged and plated at high density (ratio 1:2) on two Matrigel-coated IVF (*in vitro* fertilization) organ-dishes and grown for an additional 4 days. (II) Differentiated 3D-structures are removed and hESC are trypsinized for 3 min. hESC are replated in MEF-CM at high density by combining cells from two equivalent size plates (i.e. ratio 2:1). (III) hESC are then grown for another 2-3 days and split at a 1:2 ratio to scale-up the culture. (B-F) Immunostaining for stem cell markers and DNA for HES2 (overlay). (B) Tra-1-60, (C) SSEA4, (D) GCTM2, (E) SOX2, (F) OCT4. Scale bar, 50 µm. (G) FACS analyses of HES2 for Tra-1-60 97,2% pos, (H) GCTM2 99,5% pos and (I) SSEA4 75,3% pos.

HESC-NL4, again maintained on the widest range of conditions for detailed investigation but also included nine others in the efficiency analysis since the applicability of these methods to multiple lines is of utmost importance (Table S1).

Transfection was efficient in all twelve lines, independent of previous growth on MEFs or HFs, enzymatic or mechanical passage or maintainance in serum-containing or serum-replacement conditions (Table S1). As transfection of plasmid DNA is of interest for analyses of gene function. We used pCAG-GFP-IRES-Puro^r, a plasmid expressing GFP driven by a modified chicken actin promoter, to optimize transfection efficiency using the non-toxic polyamine-based reagent Genejammer[9]. Transfection efficiencies for all lines under their original conditions were extremely low (Figure 2.2A-D). However, once adapted, transfection efficiencies close to 80% were achieved by altering the ratio of DNA and transfection reagent, incubation time and volumes in the Matrigel cultures (Figure 2.2E, Table S1 and Supplementary Methods).

For homologous recombination, electroporation is the preferred method, since lipofection is less efficient in generating correctly gene-targeted clones[10,11]. We therefore used the same pCAG-GFP-IRES-Puro^r plasmid to optimize electroporation conditions by varying pulsation parameters, voltages and capacity. Optimal parameters for both cell survival and transfection efficiency resulted in a transfection efficiency of ~45% (Figure 2.2E, Table S1 and Supplementary Methods).

For high-throughput functional screening of gene or siRNA libraries, usually available in a viral background, transduction efficiencies of >80% are generally required. Using the original culture conditions, viral vectors driving GFP infected the feeder cells at high efficiency but left hESC colonies uninfected (data not shown). By varying the volumes, titres and incubation times we achieved efficiencies of up to 90% on the Matrigel cultures, using both adenoviral and lentiviral vectors, although lentiviral mediated gene transduction was more efficient than adenoviral using the same titre (Figure 2.2E and Supplementary Methods). Double stranded RNA is a widely-used tool for knocking down specific genes. Using an Alexa Fluor 488-conjugated negative control siRNA with Lipofectamine 2000, transfection efficiencies of ~90% were obtained (Figure 2.2E, Table S1 and Supplementary Methods).

The greatly enhanced transfection efficiencies for plasmid, siRNA and viruses using our protocol was in part attributable to the absence of feeder cells which sequester transfection reagents[9] but predominantly due to the low density plating in monolayer; this allowed cells to spread and interact optimally with the transfection agent. This was evident from the inverse correlation between cell density and plasmid transfection efficiency (Figure 2.2F). A similar inverse correlation was observed for adenoviral infections (Figure 2.2G). To show that the method supported production of stable hESC lines, HUES7 was transfected with the pCAG-GFP-IRES-Puro^r plasmid and selected for puromycin resistance. The combination of transfection at low cell density with culture for a few days, allowed reproducible expansion of targeted cells which formed small drug-resistant clusters. These represented a surrogate solution for

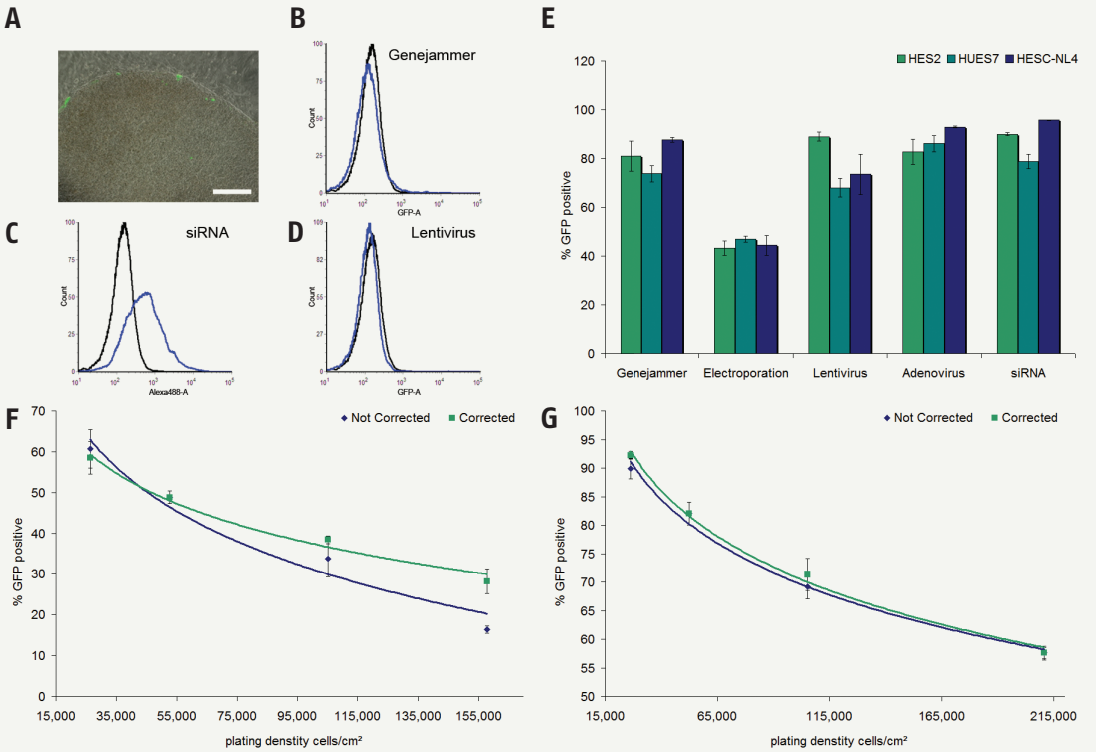

Figure 2.2

Low density Matrigel culture results in greatly enhanced transfection efficiencies of hESC

(A) Genejammer pCAG-GFP-IRES-Puro^r plasmid transfection in feeder co-culture. Scale Bar 250 µm. (B) SSEA4 positive gated HESC-NL4 cells cultured on feeders transfected with Genejammer and analysed by FACS for GFP expression, 1,5% pos. (C) idem, but transfected with siRNA, 46% pos (D) idem, but transfected with Lentivirus, 2.5% pos. (E) Summary of various transfection methods in HES2, HESC-NL4 and HUES7, data are presented as mean +/- S.D n=3. (F) HES2-Matrigel pCAG-GFP-IRES-Puro^r plasmid Genejammer transfection as a function of cell density; n.b. the slightly lower efficiency compared to figure 2c is due to a 4 hour incubation period instead of overnight. This was to prevent toxicity when the concentration of Genejammer:DNA was adjusted to higher cell numbers (corrected). Data are presented as mean +/- s.e.m n=3. Anova multiple comparison revealed statistically significant differences between groups ($p<0.01$). (G) HES2-Matrigel Adenovirus infection as a function of cell density; n.b. viral volume was kept constant with increasing cell densities (i.e. MOI 2500, 1250, 625, 312; not corrected) and adjusted to altered cell densities (MOI 1250 for all cell densities; corrected). Data are presented as mean +/- s.e.m n=3. Anova multiple comparison revealed statistically significant differences between groups ($p<0.01$).

the low clonal survival of hESC, although we cannot exclude multicellular contributions to colonies. In two independent experiments, the average stable cloning rate was $1.9x10^{-4}$ and $2.28x10^{-4}$, considerably more efficient than reported previously[12]. A pool of puromycin resistant, GFP-positive clones was trypsinized and further cultured on Matrigel in MEF-CM. This polyclonal line showed a normal 46,XY karyotype (Figure S2A). HESC-NL2, HESC-NL3 and HESC-NL4 were similarly stably transfected with the pCAG-GFP-IRES-Puro^r plasmid. To confirm quantitatively that stable lines were undifferentiated we determined SSEA3 expression by FACS in 5 independent randomly-selected HESC-NL2 clones (21 days after transfection). In agreement with their morphology, SSEA3 expression was high (Figure S3). Altogether, we generated stable hESC lines reproducibly in >10 independent experiments using the method described. Several transgenic lines were transferred back to their original (human) feeder fibroblasts. Culture on feeders is crucial for long term maintenance of a normal karyotype[13,14]. Transient transfer to feeder-free conditions had not caused chromosomal abnormalities in any of the HESC-NL2 and -3 clones (Figure 2.3L and Figure S2C), although exceptionally in HESC-NL4, the karyotype was 46,XYqh+,i(7)(p10) for all cells analyzed in three independent clones (Figures S2B).

All hESC clonal derivatives transferred to feeders grew out into colonies with undifferentiated cell morphology (large nuclei, high nucleus to cytoplasm ratio). We observed transgene silencing within 3 weeks of transfer to feeder culture in approximately 50% of the clones, as reported by others previously[3]. In the remaining clones, robust GFP expression driven by the transgene was sustained for at least 10 passages (Figure 2.3A-C). One transgenic line, GFP-HESC-NL3, was further characterized in more detail by FACS analyses for stem cell surface markers Tra-1-60, GCTM2 and SSEA4 (Figure 2.3D-F) and multi-lineage differentiation by co-culture with END2 cells[15]. Immunostaining with antibodies against cardiac myosin heavy chain (mesoderm) (Figure 2.3G) betaIII-tubulin, (ectoderm) (Figure 2.3H) and alpha-fetoprotein (endoderm) (Figure 2.3I) confirmed pluripotency. Next we used three siRNAs and a control to knockdown SOX2. Two of three siRNAs successfully knocked down SOX2 independent of general translational attenuation, as controlled by β-actin staining (Figure 2.3J,K and Figure S4).

Finally we used our improved method to target the POU5F1 3' UTR with IRES-GFP-IRES-Neo^r [10] by homologous recombination. Electroporation of $5x10^6$ HUES7 cells followed by G418 selection resulted in >150 colonies. PCR genotyping showed 4 positives in 48 clones analyzed. Targeting was confirmed by Southern hybridization (Figure S5). The targeting efficiency was 8.3%, lower than reported previously for H1.1. This may be related to the use of heterogenic rather than isogenic DNA, important for determining targeting efficiency in mouse ESCs although direct comparisons of isogenic versus heterogenic DNA in hESC have not yet been reported.

Further analyses of one clone showed homogenous GFP expression and immunoreactivity for OCT4 (data not shown). Upon differentiation, most cells became GFP negative and lost OCT4 immunoreactivity. Some cells remained GFP positive and showed overlapping immunofluorescent staining for OCT4, confirming the specificity of the OCT4-GFP reporter line

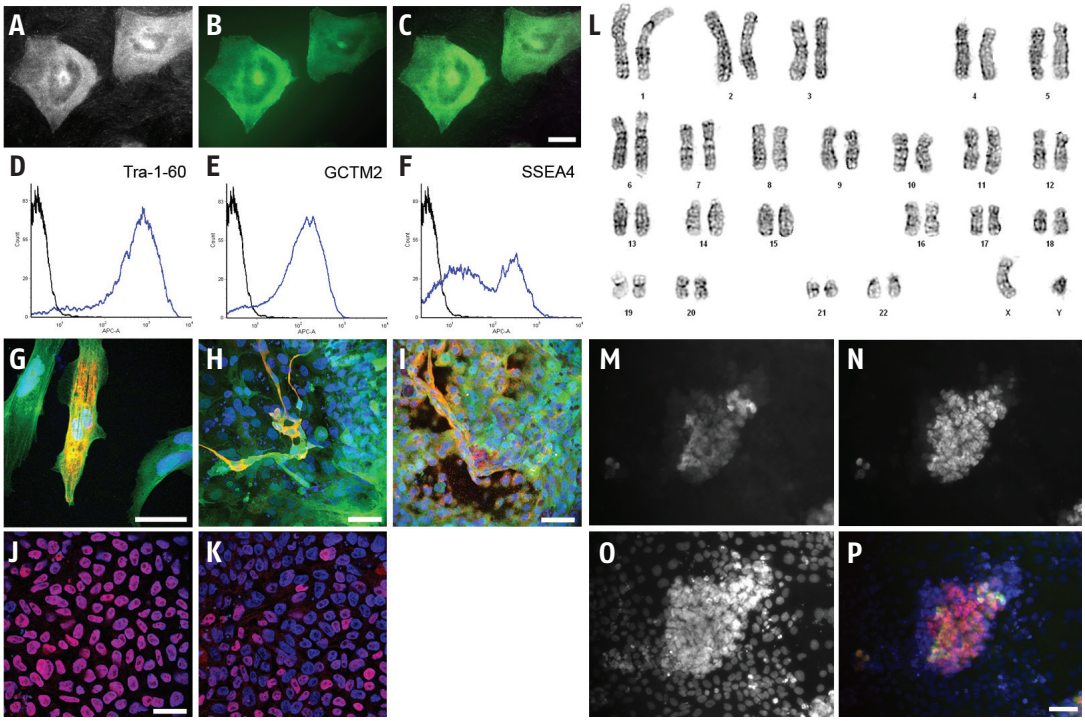

Figure 2.3

Functional validation of transfection methods

(A–C) Morphology of undifferentiated stable transfected pCAG-GFP-Puro^r GFP-HESC-NL3 lines. FACS analyses of stem cell markers (D) Tra-1-60 95,5% pos (E) GCTM2 91.5% pos. (F) SSEA4 76.2% pos. Immunostaining shows expression of GFP and specific lineage markers in red (G) β-MHC staining on dissociated cells (mesoderm) , (H) β III tubulin (ectoderm), (I) alpha-fetoprotein (endoderm), (J) control siRNA and (K) SOX2 siRNA knockdown stained for SOX2 (red), and DNA (blue). (L) Karyogram of GFP-HESC-NL3 showing a normal diploid karyotype after transfection and clonal growth. (M–P) Validation of an OCT4-GFP reporter line, (M) GFP epifluorescence, (N) endogenous OCT4 staining using antibodies, (O) DAPI staining, (P) overlay. Scale bars, 50 µm.

(Figure 2.3M-P). Karyotype analysis showed 47, XY, t(21;21), +12 and normal 46,XY cells in the culture indicating that the targeted cell was normal but a subset of daughter cells had undergone chromosomal changes during culture upscale. Karyotypic abnormalities have, however, been reported previously in this particular line[14].

Although recently other groups have shown the feasibility of transfecting hESC[3,8], rapid adaption to Matrigel monolayer cultures, combined with low cell densities described here, resulted in transfection efficiencies far higher and more consistent than conventional methods. Twelve independently derived hESC lines cultured under completely different conditions behaved very similarly, demonstrating that in contrast to previous reports this method is robust and widely applicable for multiple purposes to a variety of cell lines.

SUPPLEMENTAL DATA

Supplemental Data contains 5 Supplemental Figures, Supplemental Methods and Supplemental references which can be found on-line at http://www.nature.com/nmeth/journal/v5/n5/full/nmeth.1200.html

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References

1. He, J., Yang, Q. & Chang, L.J. Dynamic DNA methylation and histone modifications contribute to lentiviral transgene silencing in murine embryonic carcinoma cells. J Virol 79, 13497-508 (2005).

2. Smith-Arica, J.R. et al. Infection Efficiency of Human and Mouse Embryonic Stem Cells Using Adenoviral and Adeno-Associated Viral Vectors. Cloning and Stem Cells 5, 51-62 (2003).

3. Liew, C.-G., Draper, J.S., Walsh, J., Moore, H. & Andrews, P.W. Transient and Stable Transgene Expression in Human Embryonic Stem Cells. Stem Cells 25, 1521-1528 (2007).

4. Thyagarajan, B. et al. Creation of Engineered Human Embryonic Stem Cell Lines Using phiC31 Integrase. Stem Cells 26, 119-126 (2008).

5. Lombardo, A. et al. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotech 25, 1298-1306 (2007).

6. Vieyra, D.S. & Goodell, M.A. Pluripotentiality and Conditional Transgene Regulation in Human Embryonic Stem Cells Expressing Insulated Tetracycline-ON Transactivator. Stem Cells 25, 2559-2566 (2007).

7. Wilber, A. et al. Efficient and Stable Transgene Expression in Human Embryonic Stem Cells Using Transposon-Mediated Gene Transfer. Stem Cells, 2007-0026 (2007).

8. Vallier, L., Alexander, M. & Pedersen, R. Conditional Gene Expression in Human Embryonic Stem Cells. Stem Cells 25, 1490-1497 (2007).

9. Denning, C. et al. Common culture conditions for maintenance and cardiomyocyte differentiation of the human embryonic stem cell lines, BG01 and HUES-7. Int J Dev Biol 50, 27-37 (2006).

10. Zwaka, T.P. & Thomson, J.A. Homologous recombination in human embryonic stem cells. Nat Biotech 21, 319-321 (2003).

11. Costa, M. et al. A method for genetic modification of human embryonic stem cells using electroporation. Nat. Protocols 2, 792-796 (2007).

12. Eiges, R. et al. Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. Current Biology 11, 514-518 (2001).

13. Draper, J.S. et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotech 22, 53-54 (2004).

14. Baker, D.E.C. et al. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotech 25, 207-215 (2007).

15. Passier, R. et al. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 23, 772-80 (2005).

CHAPTER THREE

Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic modification

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Abstract

Realizing the potential of human embryonic stem cells (hESC) in research and commercial applications requires generic protocols for culture, expansion and genetic modification that function between multiple lines. Here we describe a feeder-free hESC culture protocol that was tested in 13 independent hESC lines derived in 5 different laboratories. The procedure is based on Matrigel adaptation in mouse embryonic fiboblast conditioned medium followed by monolayer culture of hESC. When combined, these techniques provide a robust hESC culture platform, suitable for high efficiency genetic modification via plasmid transfection (using lipofection or electroporation), siRNA knockdown and viral transduction. In contrast to other available protocols, it does not require optimization for individual lines. hESC transiently expressing ectopic genes are obtained within 9 days and stable transgenic lines within 3 weeks.

Introduction

hESC hold great promise as models for human development and disease, as well as for drug discovery and cell replacement therapies. Progress towards these goals has been impeded by technical issues, exemplified by the lack of generic strategies to culture multiple hESC lines in a format that is permissive to high efficiency genetic manipulation. Most protocols are optimized on individual hESC lines and so do not readily translate effectively between independently-derived lines or between laboratories. It is not surprising that most optimization is restricted to specific hESC lines given the labor intensiveness of maintaining multiple lines and the desire to select lines with greater propensity to differentiate towards particular lineages that are relevant to the research goals of the laboratory. For example, expression of the endodermal marker, alpha fetoprotein (AFP), was up to 3,000-fold higher in differentiating HUES-8 cells than 16 other HUES lines derived, cultured and differentiated in parallel conditions by the same group[1].

Recently a variety of methods for genetic manipulation of hESC have been described including siRNA knockdown and transient- and stable over-expression. DNA delivery was often inefficient and largely dependent on viral vectors (reviewed in 2). To date only four labs have reported successful gene-targeting in hESC[3-7]. In general all of these methods have relied on the use of feeder cells to maintain the hESC in an undifferentiated state. Drug selection has consequently necessitated the use of either drug resistant feeders or re-supplementation of feeders during the procedure to compensate drug-induced feeder loss. Feeder-layers also limit the transfection efficiency[8] and are a major source of variability, as illustrated by a recent study where a single plasmid transfection protocol applied to several independently-derived lines resulted in transfection efficiencies ranging from 3 to 35%[9].

To develop highly efficient generic transfection in hESC, we tested protocols in 13 different lines (BG01, HES-2, ENVY, HUES-1, -5, -7, -15, HESC-NL1, -2, -3, -4, NOTT-1, -2). These lines were derived in 5 independent laboratories and grown under the most diverse conditions we had available: mechanical passage on MEFs in serum containing medium, mechanical passage on human feeders in KnockOut-serum replacement (K-SR) medium and enzymatic passage on mouse embryonic fibroblast (MEFs) in K-SR medium. hESC lines were temporarily transferred to feeder-free conditions at high density, where they adapted quickly in the absence of gross karyotypic changes (tested by G-banding for HUES-7, HESC-NL-1,-2 and NOTT-1,-2). The expression of high levels of stem cell markers was reproducible in all lines[8], making the cells particularly suitable for studying stemness and signal transduction. Furthermore we found hESC grown under these conditions particularly suitable for proteomics studies[10]. Replating hESC at lower densities resulted in a substantial increase in genetic modification efficiency, enabling efficiencies of up to 90% for chemical transfection and viral transduction and 50% for electroporation in all hESC lines tested. The culture conditions also supported clonal growth. Stably transfected cells could then be returned to their original growth conditions, if required, where they retained their differentiation capacity[8].

The protocols described here result in a robust, reproducible, simple and efficient platform for hESC culture that allows highly efficient transfection/transduction without altering self-renewal and pluripotency. The major difference from procedures described by others previously is the use of Matrigel in combination with feeder cell conditioned medium to culture the cells in a true monolayer rather than in tight colonies of multilayered cells. Although our protocol is not based on potentially clinically compliant, xenoreagent free growth media and substrates recently described by others[11,12], for all non-therapeutic uses of hESC, requiring transient or sustained gene expression, the method will be extremely useful. Applications include genetic lineage marking using tissue specific promoter-reporter constructs to select subpopulations of (differentiated) cells, introduction of gene constructs for targeting and knock-in strategies, ectopic overexpression and siRNA mediated knockdown, including high throughput approaches using siRNA or gene expression libraries. The protocol is applicable to any research requiring high efficiency introduction of genes or gene constructs into hESC.

Experimental design

DNA CONSTRUCT AND TRANSFECTION

Vectors for use in hESC can be generated using either conventional restriction enzyme based plasmid cloning or recombineering[13]. If a source of genomic DNA is required in the cloning process, bacterial artificial chromosome (BAC) DNA is recommended because of its high quality.

Successful expression of the transgenic cassette is particularly dependent on the heterologous promoter and the use of the phosphoglycerate kinase (PGK) or CAG (chicken b-actin / CMV hybrid) promoter is recommended. However even with these promoters, locus dependent silencing can occur. This is likely related high level expression of de novo DNA methyltransferases in hESC, causing methylation of CpG islands and rendering the promoter inactive[14]. This problem can be partially resolved by using bicistronic cassettes that enable continuous drug selection of the cells retaining transgene over expression. For example, we have successfully used the phosphoglycerate kinase-green fluorescent protein-internal ribosome entry site-neomycin phosphotransferase (pPGK-GFP-IRES-Neo^r) or pCAG-GFP-IRES-PAC (puromycin-N-acetyltransferase) cassettes[8,15], which allow selection by neomycin / G418 or puromycin, respectively These expression cassettes also provide validated controls for performing transient and stable transfections / transductions. Alternatively, for vector based micro-RNA gene knockdown, we have successfully used the pcDNA6.2-GW/EmGFP-miR from Invitrogen. Although GFP and the miRNA are both driven by a CMV promoter, this vector is suitable for transient (but not stable) transfections.

Materials

REAGENTS

- HUES1, -5, -7, 15; supplied by Harvard University[16] http://www.mcb.harvard.edu/melton/hues/
- NOTT1 and NOTT2; derived by the University of Nottingham [17] and available from the UK stem cell bank http://www.ukstemcellbank.org.uk/catalogue.html
- BG01; available from NSCB (National Stem Cell Bank) [18] http://www.nationalstemcellbank.org
- HES2[19], available from NSCB http://www.nationalstemcellbank.org
- Envy[20]; contact ES Cell International http://www.escellinternational.com
- HESC-NL LINES 1,-2,-3,-4 derived by the Hubrecht Institute, contact ES Cell International http://www.esellinternational.com
- HEK293T available from ATCC (CRL-11268) http://www.lgcpromochem-atcc.com/
- Mouse embryonic fibroblast (strain CD1; 13.5 days post coitum, (for protocol see 21)
- PBS (Invitrogen, Gibco, 14040)
- PBS-, without $MgCl_2$ and $CaCl_2$ (Invitrogen, Gibco, 14190)
- Opti-MEM I Reduced-Serum Medium (Invitrogen, Gibco, 31985)
- Genejammer (Stratagene, 204130)
- Lipofectamine 2000 (Invitrogen, 11668-019)
- AllStars Negative Control siRNA (20 nmol), Alexa488 conjugated (Qiagen, 1027292
- Matrigel growth factor reduced (BD, 354230)
- 0.05% Trypsin-EDTA (Invitrogen, Gibco, 25300)
- Geneticin (Invitrogen, Gibco, 11811)
- Puromycin (Invivogen, ant-pr-1)
- Plasmocin (Invivogen, ant-mpt)
- Mycoalert kit (Cambrex LT07-118)
- psPAX2; (Addgene 12260)
- pMD2G; (Addgene 12259)
- pWPI; (Addgene 12254)
- KaryoMAX® Colcemid® Solution, liquid (10 µg/ml), in PBS (Invitrogen, Gibco 15212) Caution wear safety glasses and protective gloves
- D-MEM/F-12 (1:1) (1X), liquid - with GlutaMAX™ (Invitrogen, Gibco, 31331)
- KnockOut™ Serum Replacement (Invitrogen, Gibco, 10828)
- Non-essential amino acids (Invitrogen, Gibco, 11140)
- Penicillin/streptomycin (Invitrogen, Gibco, 15070)
- β-mercaptoethanol (Invitrogen, Gibco, 31350-010)
- Basic fibroblast growth factor (bFGF; Peprotech, 100-18b) Critical: specific activity of bFGF may vary among companies.
- D-MEM (Invitrogen,Gibco, 11960)
- Fetal calf serum (FCS; Sigma, F7524)

- Glutamine (Invitrogen, Gibco, 25030)
- Hybrimax dimethylsulphoxide (DMSO; Sigma D2650) Caution: Keep away from sources of ignition. Take measures to prevent the build up of electrostatic charge. Wear safety glasses and protective gloves
- Mitomycin C (Sigma, M0503) Caution: Do not breathe dust. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU). Wear compatible chemical-resistant gloves and chemical safety goggles.
- Methanol, (Sigma 322415) Caution: Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. Keep away from sources of ignition. Take measures to prevent the build up of electrostatic charge. Work in a chemical hood, wearing safety glasses and gloves
- Glacial acetic acid, Sigma 695084 Caution Do not breath vapor. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure. Work in a fume hood wearing compatible chemical-resistant gloves and chemical safety goggles
- Leishman's stain, Sigma L6254
- Sodium Citrate, Fisher S/3380/53
- Di-sodium-hydrogen-ortho-phosphate Na_2HPO_4 Fisher P285-500
- Potassium-di-hydrogen-ortho-phosphate KH2HP04 Fisher BP332-500

EQUIPMENT

- Tissue culture incubator, humidified 5% CO_2 atmosphere
- Tissue culture hood
- Stereomicroscope (Leica, MZ7.5)
- IVF organ dishes (Falcon, 353037)
- 6 well culture plates (Greiner, 657160)
- 12 well culture plates (Greiner, 665120)
- 24 well culture plates (Greiner, 662160)
- 25 cm^2 tissue culture flask (Greiner, 690160)
- Cryo Ampoules (Greiner, 123263)
- Electroporator (Gene pulser, Bio-Rad)
- Electroporation cuvettes (Eurogentec ce-004-06)
- Nalgene "Mr. Frosty" Freezing Container (Fisher Scientific, Cat: 15-350-50)
- Amicon Ultra-15 Filter Unit (100NMWL from Millipore UCF910008)

REAGENT SET-UP

- **hESC medium:** D-MEM/F-12 (1:1) (1X), liquid - with GlutaMAX™, 15% (v/v) KnockOut™ Serum Replacement, 10mM non-essential amino acids, 0.1% (v/v) penicillin/streptomycin, 100 μM β-mercaptoethanol, 4 ng ml^{-1} basic fibroblast growth factor.
- **Medium for MEFs and HEK293T cells:** D-MEM, 10% (v/v) fetal calf serum, 0.5% (v/v)

penicillin/streptomycin, 1% (v/v) Glutamine, 10mM non-essential amino acids.

- **Freezing medium:** 20% Hybrimax dimethylsulphoxide, 80% (v/v) FCS.
- **MMC treatment MEFs:** Confluent mouse embryonic fibroblasts (MEFs) are mitotically inactivated for 2.5 hours with mitomycin C (10 µg ml^{-1} in MEF medium). Cells are washed with medium and then twice with PBS, trypsinized and seeded at 6.4 x 10^4 cells per cm^2 in MEF medium.
- **Karyotype fixative:** 5 parts methanol : 1 part glacial acetic acid
- **Leishman's Stain:** 1.5 g Leishman's stain added to 1 liter of methanol. The stain is left to "mature" for several days at room temperature (18-20 °C) before use. To produce a working solution, dilute Leishman's stain 1 in 5 with Sorenson's buffer immediately prior to use.
- **Sorenson's buffer:** 9.47g di-sodium-hydrogen-ortho-phosphate and 9.08g potassium-di-hydrogen-ortho phosphate made up to one liter with deionised water.
- **Trypsin for G-banding:** 1.2g of trypsin dissolved in 1 liter of Sorenson's buffer for 20 min. Decant solution into 20 ml aliquots and store at -20°C until use.

Procedure

MEF CONDITIONING: DAY 1-8

1. Allow mitomycin C inactivated MEFs (see REAGENT SETUP) to attach for a minimum of 4-hours, preferably 24-hours. Wash with PBS and replace medium for hESC medium. Use 25 ml for a T75 flask.

2. After 24 hours, harvest MEF conditioned medium (CM) and replace with fresh unconditioned hESC medium. Add 4 ng ml^{-1} bFGF to the fresh CM. Harvest the CM for up to seven consecutive days. Filtration is not essential but helps to remove any dead fibroblast cells. CM can be used fresh or stored frozen at -20°C or -80°C for up to 6 months.

ALIQUOTTING MATRIGEL: 15 MIN.

3. Thaw one bottle of Matrigel overnight at 4 °C in at least 500 gram ice.

4. Transfer the bottle on ice to a tissue culture hood.

5. Pippette 500 µl Matrigel to each sterilized pre-chilled Eppendorf tube using pre-chilled pipettes. Critical step; Keep Matrigel on ice since it naturally polymerizes as the temperature rises above 4°C).

6. Freeze aliquots immediately at -20°C or -80°C for up to 6 months.

MATRIGEL COATING: 1-HOUR

7. Thaw and dilute one aliquot of Matrigel (0.5 ml) by repetitive pipetting in 50 ml of cold DMEM/F12 (directly from the fridge).

8. Pipette the diluted Matrigel immediately into culture vessels and allow to polymerize for at least 45 min at room temperature. Note that the layer of polymerized Matrigel is only a few microns thick and should not be visible even under the microscope. Appearance of lumpy areas indicates premature polymerization. Use 0.5 ml for 24-well plates, 1 ml for IVF organ culture and 12-well plate, 2ml for 6-well plates, 5 ml for T25 and 12 ml for T75.

9. Plates can be used immediately or stored at 4°C. Before use, aspirate excess medium and un-polymerized Matrigel, and then rinse once with PBS. Critical step: Never let Matrigel dry out as this causes irreversible loss of extracellular matrix properties.

PAUSE POINT: For storage wrap plates and dishes with Parafilm to prevent contamination and drying of the Matrigel. Use the plates within 4 weeks of preparation.

TRANSFER OF HESC TO FEEDER-FREE CULTURE: DAY 2-7

10. Start with a high quality undifferentiated hESC culture (Figure 3.1A,B). Using a glass needle, slice 10 colonies from one IVF organ dish (Figure 3.1C) release the cells by vigorously pipetting with a P1000 Gilson pipette and transfer them to two Matrigel coated IVF organ dishes containing 1ml CM (from step 2). At this stage dispase may be used to release the cells from the feeders. In our experience dispase helps to release the cells but is not necessary if the cells are cultured for example on human foreskin fibroblast feeders.
CRITICAL STEP: Steps 10-15 are specifically for cells maintained by mechanical 'cut and paste' passaging. For optimal maintenance of genetic stability, hESC cultures should in our experience be routinely maintained by mechanical passaging[7,22] and scaled up for experimentation for up to 10 enzymatic passages under feeder-free conditions. For cells already adapted to enzymatic passage, go to step 12.

11. Refresh CM daily for 4-5 days while cells are spreading and growing (Figure 3.1D,E) until colonies start to touch each other. When confluent, remove 3D differentiated areas from the middle of a colony with a glass needle or P200 Gilson pipette (Figure 3.1F).

12. Wash remaining attached undifferentiated cells with PBS, add 200 µl trypsin and incubate for 2-3 min at 37°C. Critical step; short incubation will not release cells, too long will damage the cells, decrease cell survival and may result in premature karyotypic change.

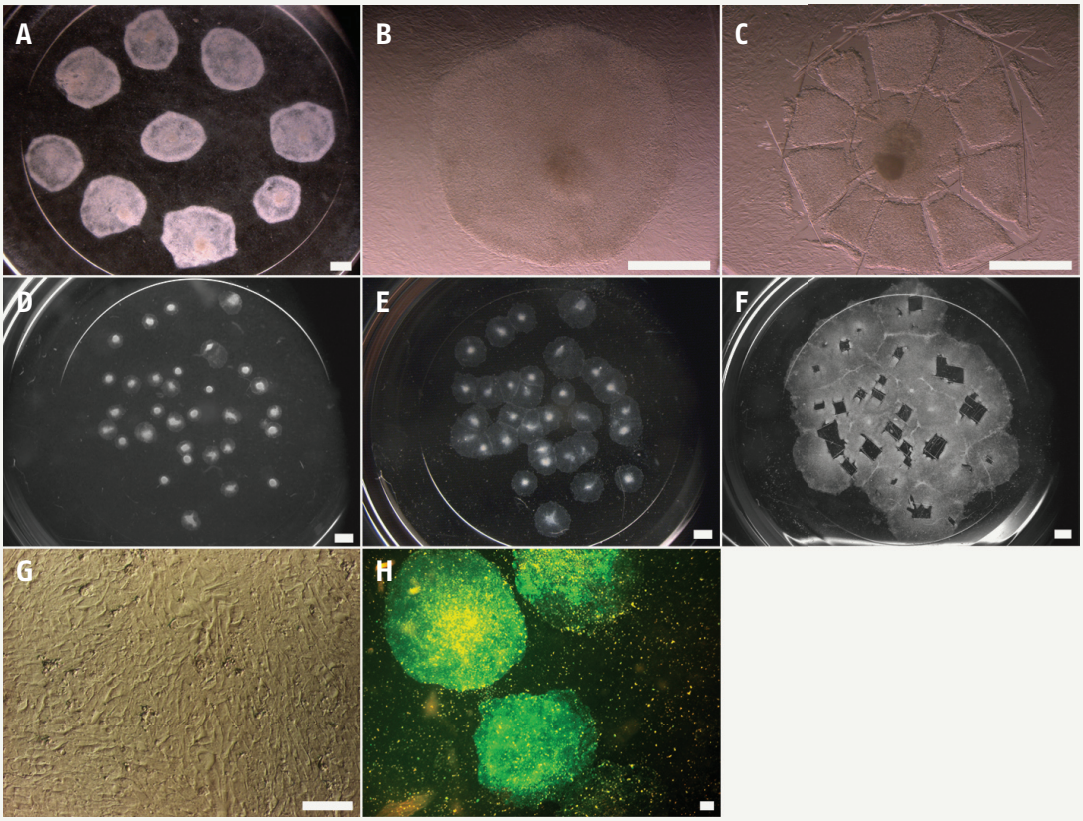

Figure 3.1

Photographs of hESC at different stages

(A,B) Day 7 hESC colonies grown on MEFs, (C) Day 7 hESC colony sliced before dislodgement and transfer to Matrigel, (D) hESC grown on Matrigel in conditioned medium at day 1, (E) hESC grown on Matrigel in conditioned medium at day 2 (F) hESC grown on Matrigel with the central area removed; these cultures are ready for trypsinization (G) hESC monolayer culture on Matrigel (H) GFP positive primary hESC colonies after Genejammer transfection with a GFP vector. Scale bars 1 mm (A–F+H) and 100 µm (G).

13. Add 1 ml of hESC medium and resuspend the cell suspension vigorously with a P1000 pipet to release the cells. Collect the cells into a 15 ml centrifuge tube, add another 4 ml of hESC medium to dilute the trypsin and pellet the cell suspension (180 x g, room temperature, 4 min.). Critical Step; Trypsinization and resuspension should yield a cell-suspension of smaller and larger hESC clumps.

14. Following centrifugation, resuspend the cells in 1ml CM and replate at very high density (approximately 1-2.10^5 per cm^2) i.e from two organ dishes to one Matrigel coated IVF organ dish. A small proportion of cells die during this first enzymatic passage.

15. The next day refresh the medium for 1 ml fresh CM and carefully check morphology off individual cells at 100-400x magnification, keep refreshing the medium daily until the cells form a confluent monolayer. Generally this takes 1-2 days. Confluent cells growing in a monolayer should be morphologically similar to individual cells in colonies (Figure 3.1G). Critical step; the medium may be very acidic (yellow). This is normal and does not influence the results. Troubleshooting

16. When confluent, wash cells with PBS and add 200 µl trypsin for exactly 1,5 min. Dilute cells / trypsin in at least 5 ml of hESC medium and centrifuge immediately to remove the trypsin (180 x g, room temperature, 4 min.). Resuspend in 2ml CM and seed at appropriate split ratio; depending on cell line, split ratios are usually 1 in 3 and should reach confluency after 48-72h. Some fast growing lines can be split occasionally in a 1 in 5 ratio. Critical step; cells split at low density which are cultured for at least 72h might require slightly longer trypsin incubations. In general; using a 1 in 3 split max. 1,5 min trypsin should be enough to yield a cell suspension with some single cells and clumps of 3-10 cells. Troubleshooting

17. Scale culture up to T25 flasks without exceeding a 1 in 3-5 split ratio and with daily replacement of CM. This culture method preserves genetic integrity for at least 10-20 passages for the lines tested in longer-term culture (HUES7, NOTT-1, -2, HESC-NL-1, -2). hESC cultured in this system have been shown to be highly positive (>90%) for all stem cell markers tested (OCT4, SOX2, GCTM2, Tra-1-60, SSEA4)8. The culture is now ready for further manipulation or cryopreservation (as described in Box 1). Troubleshooting

BOX 1. CRYOPRESERVING CELLS: 30-MIN

1. Trypsinize a confluent T25 culture flask as described in step 16, resuspend in hESC medium and pellet cells.
2. Following centrifugation, resuspend the cell pellet in 750 µl of 100% FCS on ice.
3. Add 750 µl freezing medium, mix gently and divide between three ampoules (final concentration of DMSO is 10%).
4. Immediately place ampoules in a Nalgene cryopreservation container containing propan-2-ol. Transfer to -80°C and after 24 hours to liquid nitrogen for long-term storage.

18. The following options can be used to transfect/transduce the cultured hESC with desired sequences to obtain stably transfected transgenic cells. Option A can be followed for si-RNA transfection, Option B for Genejammer plasmid transfection, option C for electroporation and option D for lentiviral transduction. Critical step; Some hESC clones will progressively silence stably transfected constructs even when an appropriate promoter is used. It is highly recommended to include a reporter construct (e.g. fluorescent protein or drug resistance marker) in the vector to allow visualization or continuous drug selection of transgene expression.

A. SI-RNA TRANSFECTION (3 DAYS)

I. The day before transfection, trypsinize cells as described in step 16 and plate in CM at a density of 1-2 x 10^5 cells per well onto a Matrigel-coated 12-well plate.

II. For each well to be transfected, prepare siRNA-Lipofectamine complexes as follows: Dilute 3 µl siRNA in 75 µl of Opti-MEM I medium and mix by flicking the tube. Note: the stock siRNA solution should be made at 20µM, according to the manufacturers' instructions

III. Mix Lipofectamine 2000 by inversion before use and then dilute 1,5 µl in 75 µl of Opti-MEM I Medium. Mix gently and incubate for 5 min. at room temperature.

IV. After the 5 min. incubation, combine the diluted siRNA with the diluted Lipo fectamine 2000; total volume is 154,5 µl. Mix gently and incubate for 20 min. at room temperature to allow the siRNA:lipid complexes to form.
Critical step: It's highly recommend to use a fluophore conjugated siRNA as control to monitor transfection efficiency.

V. Aspirate medium from target cells and replace with 450 µl of CM.

VI. Add the 154,5 µl of siRNA:Lipofectamine complexes dropwise to each well. This gives a final concentration of 100nM siRNA. Mix gently by rocking the plate back and forth.

VII. Add 1.5 ml CM after 4 hours.

VIII. Incubate the cells at 37°C for 48 hours changing CM daily and analyze 1-5x10^4 cells by FACS[23] or fluorescence microscopy[24]. › TROUBLESHOOTING

B. GENEJAMMER PLASMID TRANSFECTION (3 DAYS)

I. The day before transfection, trypsinize cells as described in step 16 and plate cells in CM at a density of 1–2 x 10^5 cells per well onto a Matrigel-coated 12-well plate.

II. For each transfection sample; prepare DNA-Genejammer complexes as follows
- Mix 5,25µl Genejammer to 75µl Opti-MEM
- Incubate at room temperature for 10 min.
- Add 1,75 µg target vector to the solution and mix gently, incubate at room temp for a further 10 min. Critical step: It is highly recommended that a vector encoding a fluorescent protein (e.g. pPGK-GFP-IRES-Neo or pCAG-GFP-IRES-PAC) be used as a control to monitor transfection efficiency

III. Replace medium for 400 µl CM

IV. Add the 82 µl of DNA:Genejammer complexes drop wise to each well. This gives a final concentration of 3.6ng μl^{-1} plasmid. Mix gently by rocking the plate back and forth.

V. Add 1.5 ml CM after 4-hour

VI. Incubate the cells at 37°C for 48 hours changing CM daily and analyze 1–5x10^4 cells by FACS[23] or fluorescence microscopy[24].
› TROUBLESHOOTING

C. ELECTROPORATION (2 DAYS)

I. Trypsinize a confluent T25 culture flask as described in step 16.

II. Following centrifugation (180 x g, room temperature, 4 min.) resuspend the cells in 800 µl CM containing 15–50 µg linearized DNA

III. Transfer the cell/DNA mix to an 800µl / 4mm gap electroporation cuvettes and incubate for 5 min at room temperature

IV. Flick the cuvette to ensure a homogeneous cell suspension and electroporate at 320 Volts (V) / 240 micro Faradays (µF). Critical step: It is highly recommended that a vector encoding a fluorescent protein (e.g. pPGK-GFP-IRES-Neo or pCAG-GFP-IRES-PAC) be used as a control to monitor transfection efficiency.

V. Incubate for 5 min at room temperate and resuspend the cells in at least 10–15 ml of CM. Critical step: If smaller numbers of cells are electroporated, resupend cells in at least 5 ml CM to prevent cell death arising due to debris and DNA toxicity.

VI. Plate cells on 2–3 Matrigel-coated 60 mm dishes. This is largely dependent on growth characteristics of a particular hESC line.

VII. Incubate the cells at 37°C for 48 hours changing CM daily and analyze 1–5x10^4 cells by FACS[23] or fluorescence microscopy[24].

D. HESC LENTIVIRAL TRANSDUCTION (6 DAYS)

I. To prepare lentiviral particles for hESC transduction, first seed a T175 culture flask with 5 x 10^6 HEK293T cells in 20 ml of medium and allow the cells to attach overnight in the incubator. HEK293T cells are used as a packaging cell line for the pro-

duction of a viral supernatant that contains infectious particles.

II. For each flask of HEK293T cells to be transfected, combine into 2ml of Opti-MEM I medium, 50µg of packaging plasmid (e.g. psPAX2), 20µg of envelope plasmid (e.g. pMD2G) and 67µg transfer plasmid (e.g. GFP expressing plasmid such as pWPI). Critical step: These plasmids will generate 2nd generation, replication deftive lentiviral particles. Ensure the transfer plasmid to be used is second generation as first and third generation systems will require different combinations of plasmid.

III. To a separate tube, add 40µl of Lipofectamine 2000 to 2ml of Opti-MEM I medium and mix gently. Incubate for 5 min. at room temperature.

IV. After the 5 min incubation, combine the diluted plasmid DNAs with the diluted Lipofectamine 2000. Mix gently and incubate for 20 min. at room temperature to allow the DNA:lipid complexes to form.

V. Aspirate medium from target HEK293T cells and replace with 16ml of Opti-MEM I medium.

VI. Add the DNA:lipid complexes (~4ml) to each T175 flask. Mix gently by rocking the plate back and forth and incubate for 4 hours.

VII. Aspirate the transfection medium and replace with 20ml HEK293T medium.

VIII. Incubate for 48 hours, during which time lentiviral particles will be produced and released into the medium.

IX. Trypsinize hESC as described in step 16 and plate cells in CM at a density of 5 x 10^4 cells per well onto a Matrigel-coated 6-well plate. Allow the cells to attach for 4-6 hours in the incubator.

X. To harvest lentiviral particles for the transduction of hESC, harvest the medium (20ml) from the HEK293T cells. This supernatant contains the replication defective lentiviral particles.

XI. Pass the lentiviral supernatant through a 0.2mm filter to eliminate any detached HEK293T cells.

XII. Concentrate the virus by transferring to an Amicon Ultra-15 Filter Unit by centrifug ing at 4000 x g for 20 min. at room temperature. This will provide 200µl of concentrated viral supernatant. Critical step: for best results, use the virus fresh. Storage at 4°C or -80°C can reduce the number of active transducing units in the superatant.

XIII. Aspirate medium from the hESC and replace with 4ml CM. Add increasing amounts of the concentrated virus (i.e. add no virus to well 1 of the 6-well plate of hESC, 1µl to well 2, 3.3µl to well 3, 10µl to well 4, 33µl to well 5 and 100µl to well 6).

XIV. Incubate the cells at 37°C for 72 hours changing CM daily and analyze 1-5x10^4 cells by FACS[23]. First, plot FACS-based transduction efficency on the Y-axis of a graph and volume of viral supernatant on the X-axis. Second, from a linear point on the curve, read off the transduction efficiency and volume of viral supernatant. Put these figures into the following equation:

$$\text{No. of transducing particles / µl} = \frac{\text{\% transduction efficiency x starting hESC number}}{\text{100 (converts percentage) x volume of viral supernatant}}$$

For example, if 20ml of viral supernatant resulted in 50% transduction efficiency of $5x10^4$ cells, then:

$$\text{No. of transducing particles / µl} = \frac{50 \times 5 \times 10^4}{100 \times 20}$$
$$= 1250 \text{ / µl}$$
$$= 1.25x10^6 \text{ / ml}$$

SELECTION OF STABLY TRANSFECTED CELLS

19. Apply selection 48 hours after electroporation, Genejammer transfection or viral transduction while changing CM daily. By this stage the dish should be ~60–90% confluent. Critical step: If cells are too confluent, the action of the antibiotics will be delayed or ineffectual and death of untransfected cells will not be observed. The recommended final concentration for the antibiotic G418 is 50 µg ml^{-1} whereas puromycin is 300 ng/ml^{-1}. Critical step; the antibiotic may need to be titrated and optimized for different hESC lines.

20. Suspend antibiotic selection when approximately 50–80% of the cells are killed. This slows the kill rate so that residual untransfected cells act as feeder cells to the stably transfected cells to reduce the stress of clonal growth. Critical step; when cells are growing extremely fast this step may not be necessary. However it greatly improves clonal growth especially with slower growing cells

21. Restart antibiotic selection after an additional 2 days, while changing CM daily. It is advisable to maintain selection thereafter to eliminate cells that may silence the transgene.

22. After approximately 14 days individual colonies should be visible (Figure 3.1H). Troubleshooting

COLONY TRANSFER

23. Let the colonies grow until they are approximately 400 µM in diameter or until they start to touch each other. Using a P200 pipette or glass needle, slice colonies into a grid motif and transfer to 12- or 24-well plate. RNA or DNA analyses can be performed directly on lysed colony fragments or later from the expanded cell

populations. Cells can be cultured further on Matrigel or feeders, as required.

24. Check clones for their karyotype, as below, and differentiation potential

KARYOTYPE PROCEDURE FOR G-BAND ANALYSIS

25. To hESC cultures in log phase growth, add Karyomax Colcemid to the medium at a 1:100 dilution to give a concentration of 100ng/ml (50µl into 5ml). Incubate at 37°C for 60 min.

26. Remove medium+karyomax+floating cells to a tube. Passage as in step 12, add cells to the same tube as before and collect by centrifugation.

27. Aspirate and resuspend the pellet in 0.3ml hESC medium with a P1000 pipette. Critical step: Pipetting ensures that a single cell suspension is produced to prevent clumping and to maximize swelling in the hypotonic solution.

28. Add 8ml of fresh hypotonic solution (0.6% sodium citrate in H_20) dropwise whilst vortexing. Critical step: adding the hypotonic solution dropwise reduces the risk of osmotic shock.

29. Incubate cells for 20 min at room temperature to swell.

30. Centrifuge the cell suspension at 300 x g for 5 min. Aspirate and resuspend in 0.3ml of hypotonic solution with a P1000 pipette. Critical step: Pipetting ensures that a single cell suspension is produced to prevent clumping in the fixative.

31. Add 8ml karyotype fixative dropwise whilst vortexing.

32. Spin the cell suspension (300 x g, room temperature, 4 min.) and resuspend the pellet in fixative. Then repeat this step twice more.

33. Centrifuge and resuspend the pellet in a 0.5-1ml of fix.

34. Drop cells from a height of ~30 cm onto polished slides to burst the cells and spread the metaphase chromosomes. Incubate slides at 75°C overnight prior to G-banding.

35. Treat the slides with trypsin for 3 min. and then with diluted Leishman's stain (diluted 1 in 5 with Sorenson's buffer immediately prior to use, as under Reagent Set up) for 2 min. Finally, rinse with water and air dry.

36. Analyze 30 metaphase spreads per sample line to determine karyotype to greater than 95% confidence limits. This highly specialized procedure is best performed by a clinical cytogenetics service division.

TIMING

See flowchart below (figure 3.2)

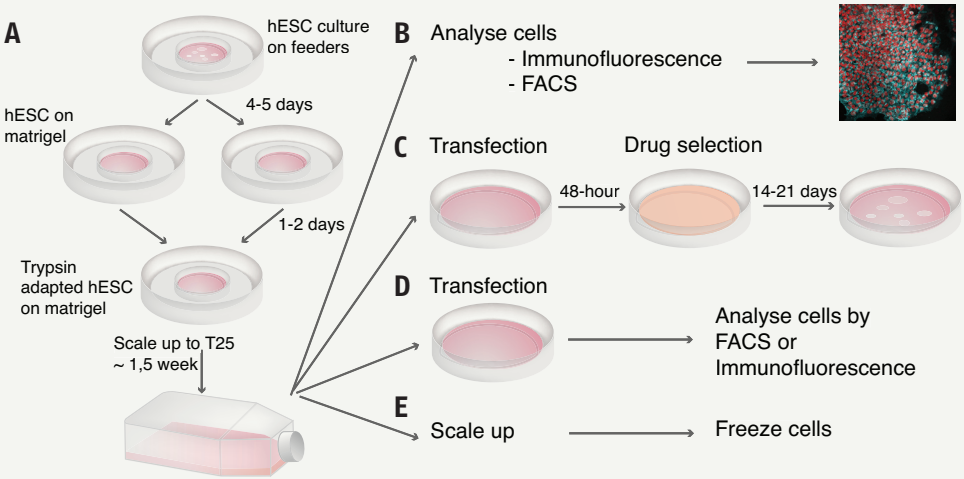

Figure 3.2

Flowchart for experimental procedures

(A) hESC grown on feeders are plated on Matrigel and grown for an additional 4–5 days. Differentiated three-dimensional structures are removed and hESC are trypsinized, replated in MEF-conditioned medium at high density by combining cells from two equivalent-size plates, grown for another 1–2 d and split several times to scale up to at least a T25 culture flask. These cells can now be used for (B) Immunofluorescence/FACS, (C) stable transfection, (D) transient transfection or (E) upscaling and freezing

TABLE 3.1
Troubleshooting table

STEP	PROBLEM	SOLUTION
15,17	(a) Cells dying or differentiating	Cell density too low. Culture cells until they are confluent and fully compacted. hESC interact with Matrigel, which leads to cell spreading and loss of their typical morphology in colonies. High density cultures should have a similar morphology as hESC in colonies. Split cells 1:1 or max 1:2 until they look healthy. At this point hESC may be split 1:3 – 1:5 max.
		Bad quality conditioned medium. In general when feeders are capable of supporting hESC they should be suitable for feeder conditioning
		Mycoplasm infection. Check cultures regularly using a mycoalert kit (Cambrex LT07-118). If positive it is best to dispose of cultures as the infection can rapidly spread to other culture flasks. However, if they culture is very precious, it may be possible to cure the infection with plasmocin
		Low specific activity of bFGF, It is recommended that new batches bFGF be compared side by side with the previous batch. If problem persists, increasing the bFGF concentrations to 10 ng/ml may help.
		Differentiating cultures can sometimes be rescued by stepwise trypsinization. Compacted hESC colonies are usually more difficult to trypsinize compared with differentiated (mesenchyme-like) cells. Trypsinize cells under a stereomicroscope and wash away differentiated cells using PBS. Use fresh trypsin to release the undifferentiated cells and seed them on a new Matrigel coated dish. A 1:1 split is recommended at this stage.
A,B,C,D	(b) low transfection efficiency	Check for Mycoplasm infection regularly using a mycoalert kit (Cambrex LT07-118). Treatment as indicated for steps 10-17.
		Cell density too high. Lower cell densities gives generally much higher transfection efficiencies[8]. Lower the cell density.
B	(c) transfection induced toxicity	Use pure plasmid DNA from either a midi-, or maxiprep. Endotoxin-free plasmid purification kits (e.g. from Qiagen) may also be beneficial.
19-22	(d) No drug resistant colonies	Confirm that the expression vector works in another cell type, for example HEK293 or COS
19-21	(e) Cells not dying during drug selection	Increase drug concentration. It is recommended to titrate the antibiotic dose for each cell line and every new drug batch
		Cell density at onset of drug treatment too high. Reduce to ~60% confluence in next experiment.
36	(f) Karyotypic abnormalities	If normal cells are present, single cell cloning can be considered. Otherwise restart the process, ensuring that the culture is karyotypically normal and growing exponentially.

TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.1.

ANTICIPATED RESULTS

Starting with any high quality undifferentiated culture of hESC cells and following steps 1–17, hESC should yield a robust growing monolayer culture within two weeks. These cells should express high levels of hESC markers (e.g. OCT4, SOX2, NANOG, SSEA4, TRA1–81, GCTM2) and be amenable for genetic manipulation.

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COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

References

1. Osafune, K., et al. Marked differences in differentiation propensity among human embryonic stem cell lines. Nat Biotech 26, 313–315 (2008).

2. Giudice, A. & Trounson, A. Genetic Modification of Human Embryonic Stem Cells for Derivation of Target Cells. Cell Stem Cell 2, 422–433 (2008).

3. Davis, R.P., et al. Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. Blood 111, 1876–1884 (2008).

4. Urbach, A., Schuldiner, M. & Benvenisty, N. Modeling for Lesch-Nyhan Disease by Gene Targeting in Human Embryonic Stem Cells. Stem Cells 22, 635–641 (2004).

5. Costa, M., et al. A method for genetic modification of human embryonic stem cells using electroporation. Nat. Protocols 2, 792–796 (2007).

6. Zwaka, T.P. & Thomson, J.A. Homologous recombination in human embryonic stem cells. Nat Biotech 21, 319–321 (2003).

7. Draper, J.S., et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotech 22, 53–54 (2004).

8. Braam, S.R., et al. Improved genetic manipulation of human embryonic stem cells. Nat Meth 5, 389–392 (2008).

9. Liew, C.-G., Draper, J.S., Walsh, J., Moore, H. & Andrews, P.W. Transient and Stable Transgene Expression in Human Embryonic Stem Cells. Stem Cells 25, 1521–1528 (2007).

10. Dormeyer, W., et al. Plasma Membrane Proteomics of Human Embryonic Stem Cells and

Human Embryonal Carcinoma Cells. J. Proteome Res. 7, 2936–51 (2008).

11. Ludwig, T.E., et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotech 24, 185–187 (2006).

12. Ng, E.S., Davis, R., Stanley, E.G. & Elefanty, A.G. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat. Protocols 3, 768–776 (2008).

13. Wu, S., Ying, G., Wu, Q. & Capecchi, M.R. A protocol for constructing gene targeting vectors: generating knockout mice for the cadherin family and beyond. Nat. Protocols 3, 1056–1076 (2008).

14. Kameda, T., Smuga-Otto, K. & Thomson, J.A. A severe de novo methylation of episomal vectors by human ES cells. Biochem Biophys Res Commun 349, 1269–1277 (2006).

15. Anderson, D., et al. Transgenic Enrichment of Cardiomyocytes From Human Embryonic Stem Cells. Mol Ther 15, 2027–2036 (2007).

16. Cowan, C.A., et al. Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 350, 1353–1356 (2004).

17. Burridge, P.W., et al. Improved Human Embryonic Stem Cell Embryoid Body Homogeneity and Cardiomyocyte Differentiation from a Novel V-96 Plate Aggregation System Highlights Interline Variability. Stem Cells 25, 929–938 (2007).

18. Mitalipova, M., et al. Human Embryonic Stem Cell Lines Derived from Discarded Embryos. Stem Cells 21, 521–526 (2003).

19. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18, 399–404 (2000).

20. Costa, M., et al. The hESC line Envy expresses high levels of GFP in all differentiated progeny. Nat Meth 2, 259–260 (2005).

21. Michaoska, A.E. Unit 1C.3 Isolation and Propagation of Mouse Embryonic Fibroblasts and Preparation of Mouse Embryonic Feeder Layer Cells. Current Protocols in Stem Cell Biology (2007).

22. Baker, D.E.C., et al. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotech 25, 207–215 (2007).

23. Shapiro, H.M. Practical Flow Cytometry, (Wiley Interscience, 2003).

24. Brown, C.M. Fluorescence microscopy – avoiding the pitfalls. J Cell Sci 120, 1703–1705 (2007).

CHAPTER FOUR

Feeder-free monolayer cultures of human embryonic stem cells

express an epithelial plasma membrane protein profile

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Abstract

Human embryonic stem cells (hESC) are often co-cultured on monolayers of mitotically-inactive fibroblast feeder cells to maintain their undifferentiated state. Under these growth conditions, hESC form multi-layered colonies of morphologically heterogeneous cells surrounded by flattened mesenchymal cells. In contrast, hESC grown in feeder cell-conditioned medium and maintained on Matrigel instead of feeder cells tend to grow as monolayers with a uniform morphological phenotype. Using mass spectrometry and immunofluorescence microscopy, we show that hESC under these conditions primarily express proteins belonging to epithelium-related cell-cell adhesion complexes, including adherens junctions, tight junctions, desmosomes and gap junctions. This indicates that monolayers of hESC cultured under feeder-free conditions retain a homogeneous epithelial phenotype similar to that of the upper central cell layer of colonies maintained on feeder cells. Notably, feeder-free hESC also coexpressed VIMENTIN, which is usually associated with mesenchyme, suggesting that these cells may have undergone epithelium-to-mesenchyme transitions, indicating differentiation. However, if grown on a "soft" substrate (Hydrogel), intracellular VIMENTIN levels were substantially reduced. Moreover, when hESC were transferred back to feeder cells, expression of VIMENTIN was again absent from the epithelial cell population. These results imply that on tissue culture substrates, VIMENTIN expression is most likely a stress-induced response, unrelated to differentiation.

Introduction

Human embryonic stem cells (hESC) are derived from the inner cell mass (ICM) of blastocyst-stage embryos and can self-renew indefinitely, as well as give rise to any adult cell type[1-3] Conventionally, hESC are grown as colonies on monolayers of mitotically inactive mouse embryonic fibroblast feeder cells (MEFs) in serum-containing medium. More recently, hESC have also been derived and maintained on human feeder cells and on feeder-free matrices in various growth factor-supplemented basal media[4-8].

hESC on feeder cells have been described as multilayered colonies with, on their periphery, epithelium-like polarized cells that are connected by desmosomes[9]. In addition to ESC-associated transcription factors, such as OCT4, SOX2, and NANOG, epithelial proteins have been reported to be expressed by hESC on feeder cells[10-14].

When transferred to feeder-free conditions, hESC were reported to grow initially as three-dimensional colonies composed of three distinct cell types[15]. At the core were ICM-like polygonal cells with large nuclei, relatively little cytosol, few desmosomes, and no gap junctions. On top was a single layer of polarized epithelium-like cells connected at their apical-lateral side by cell-cell adhesion complexes containing epithelial cadherin (E-cadherin) and gap junction protein a1 (connexin 43). At the periphery were mesenchyme-like cells with low nucleus-to-cytosol ratios, lacking polarity, and only a few were connected by cell junctions. It was suggested that cells at the upper layer go through an epithelium-to-mesenchyme transition (EMT) and move to the periphery[16]. On the other hand, hESC cultured for longer periods in the absence of feeder cells tend to grow as monolayers[5] coupled by functional gap junctions[12].

In a mass spectrometry (MS)-based plasma membrane proteome analysis of hESC cultured as monolayers on Matrigel (BD Biosciences, San Diego, http://www.bdbiosciences.com) in MEF-conditioned medium, we identified 237 plasma membrane proteins[17]. In contrast to the heterogeneous multilayered hESC colonies on feeder cells, monolayers exhibited an exceptionally uniform morphology. Here we show that these feeder-free hESC express an epithelial plasma membrane protein profile. Cell surface localization of proteins associated with epithelial cell-cell adhesion complexes (adherens junctions, tight junctions, desmosomes, and gap junctions) identified by MS was confirmed by immunofluorescence microscopy. Expression of VIMENTIN in these cells resulted most likely from mechanical stress rather than an EMT, since growth on mechanically soft substrates reduced intracellular VIMENTIN to levels similar to that of the epithelial cell population at the top of hESC colonies cultured on MEFs.

Materials and Methods

hESC culture

HUES-7 hESC[3] were cultured on Matrix Growth Factor Reduced Matrigel (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) as described previously[4], on a thick layer of Extracel-HP Hydrogel (Glycosan BioSystems, Salt Lake City, http://www.glycosan.com) containing 10 mg/ml fibronectin (Harbor Bioproducts, Norwood, MA), or on MEFs, such as HES-2 hESC[2] as described previously[13,18].

Plasma membrane protein extraction and MS analysis

Extraction and MS analysis of plasma membrane proteins from HUES-7 cells was performed as described previously[17].

Immunofluorescence microscopy

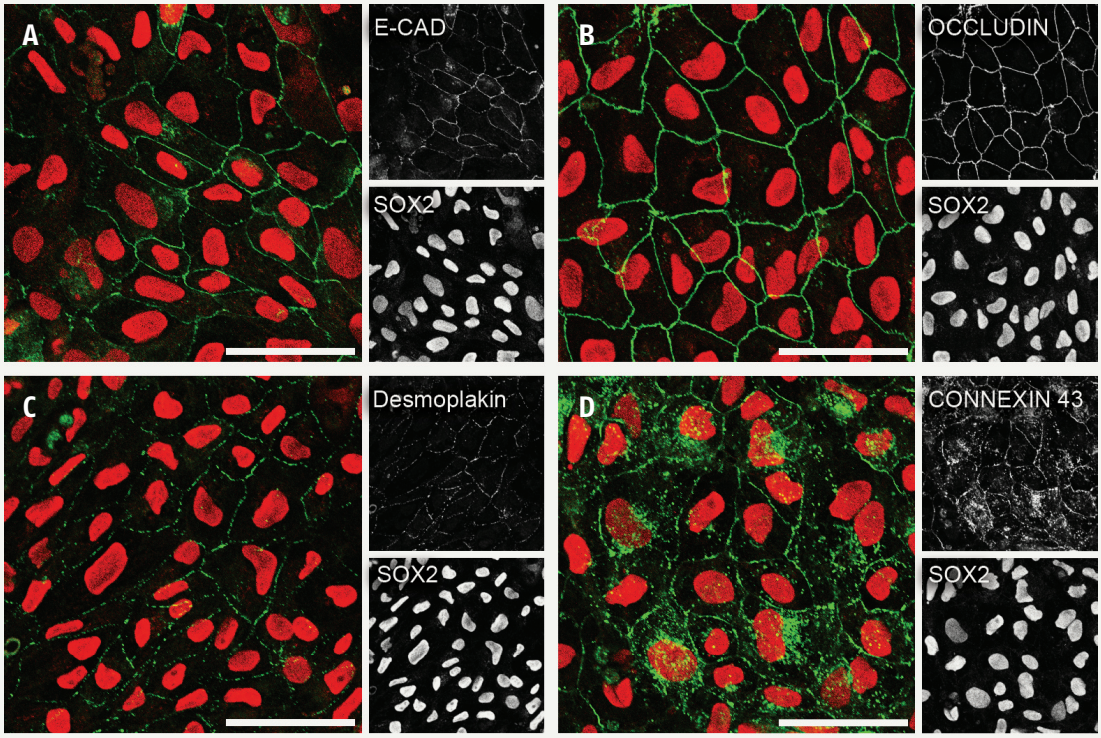
Immunofluorescence microscopy was conducted as described previously[13]. Primary rabbit anti-SOX2 antibody (Chemicon, Temecula, CA, http://www.chemicon.com) at 1:400 was used in combination with mouse anti-human E-CADHERIN (clone HECD-1; Zymed Laboratories Inc., San Francisco, http://www.invitrogen.com), 1:200; mouse anti-OCCLUDIN (clone OC-3F10; Invitrogen, Carlsbad, CA), 1:200; mouse anti-DESMOPLAKIN (mixture of clones DP-2.15, DP-2.17, and DP-2.20; Progen Biotechnik, Heidelberg, Germany, http://www.progen.de), undiluted; mouse anti-CONNEXIN 43 (clone 2; BD Transduction, BD Biosciences), 1:200; or mouse anti-human VIMENTIN (clone V9; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 1:200. Secondary antibodies were Cy3-conjugated goat anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) at 1:200 and fluorescein isothiocyanate-conjugated goat anti-mouse (Jackson Immunoresearch Laboratories) at 1:200. Cells were imaged with confocal laser microscopes, types SP2 and SPE (Leica, Heerbrugg, Switzerland, http://www.leica.com). Maximal projections were made using the accompanying Leica software, and z-stacks were merged using Paint Shop Pro 9 (Corel, Fremont, CA, http://www.corel.com).

Western blotting

Western blotting was conducted as described previously[13], using rabbit anti-DPI/DPII (NW6) at 1:5,000, and mouse anti-human VIMENTIN (clone V9; Sigma-Aldrich) at 1:1,000 in combination with mouse anti-β-actin (clone AC-15; Sigma-Aldrich) at 1:5,000.

Results

Enzymatically-passaged hESC (line HUES-7) cultured on Matrigel in MEF-conditioned medium grow as monolayers[19]. MS analysis of these cells[17] indicated that they express proteins associated with four cell-cell adhesion complexes that are characteristic of epithelial cells: (a) adherens junctions, (b) tight junctions, (c) desmosomes, and (d) gap junctions (Table 1).



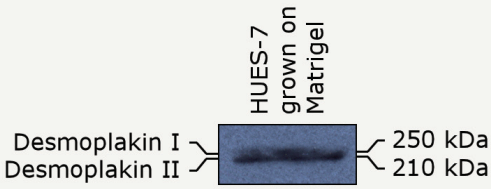


Figure 4.1

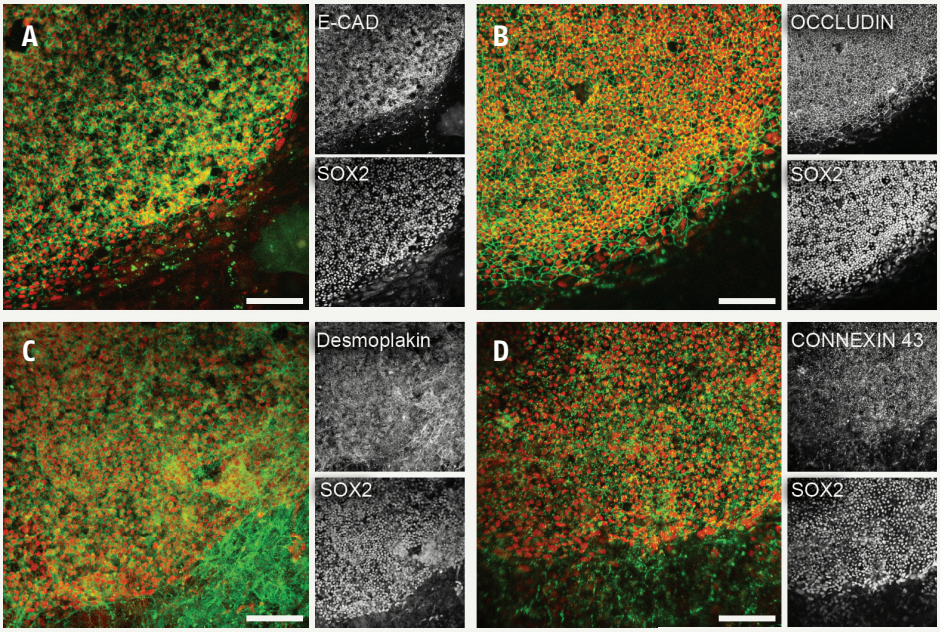
hESC express an epithelial plasma membrane profile

z-Stacks showing the plasma membrane localization of protein components belonging to cell-cell adhesion complexes that connect adjacent epithelium-like HUES-7 human embryonic stem cells (hESC), which grow as monolayers under feeder-free conditions. Undifferentiated hESC expressing SOX2 ((A-D), shown in red) were also positive for the adherens junction protein E-CADHERIN ((A), shown in green), the tight junction protein OCCLUDIN ((B), shown in green), the desmosomal protein DESMOPLAKIN ((C), shown in green), and gap junction protein CONNEXIN 43 ((D), shown in green). Western blot shows that both desmoplakin I and II were expressed by HUES-7 hESC grown on Matrigel (E). Scale bars = 50 µm (A-D).

Figure 4.2

Epithelial protein expression in hESC–MEF cultures

Maximal projections showing the expression and distribution of proteins associated with cell–cell adhesion complexes in HUES-7 human embryonic stem cell (hESC) colonies cultured for 1 week on mouse embryonic fibroblast feeder cells (MEFs). All cells in HUES-7 hESC colonies were positive for SOX2 ((A–D), shown in red). E-cadherin ((A), shown in green), OCCLUDIN ((B), shown in green), and CONNEXIN 43 ((D), shown in green) showed the highest levels on the plasma membranes of cells located in the center. Desmoplakin was expressed in hESC, as well as MEFs ((C), shown in green). Scale bars = 100 µm (A–D).



To confirm plasma membrane localization of these proteins, we used antibodies specifically recognizing single proteins of each of the different adhesion complexes. E-CADHERIN (Figure 4.1A) is present in adherens junctions[20,21], OCCLUDIN (Figure 4.1B) is specific for tight junctions[20,22], DESMOPLAKIN (Figure 4.1C) is located in desmosomes[23], and CONNEXIN 43 (Figure 4.1C) is one of the 18 gap junction proteins[24,25] that were detected in hESC[10,12]. Concomitant staining of SOX2 indicated that hESC positive for these plasma membrane–associated proteins were undifferentiated (Figure 4.1). Both DESMOPLAKIN I and II are expressed by hESC (Figure 4.1E). Immunostaining of HUES-7 colonies grown for 1 week on MEFs showed that these cells retain their epithelial characteristics (Figure 4.2A–D). Lower staining of cells at the periphery

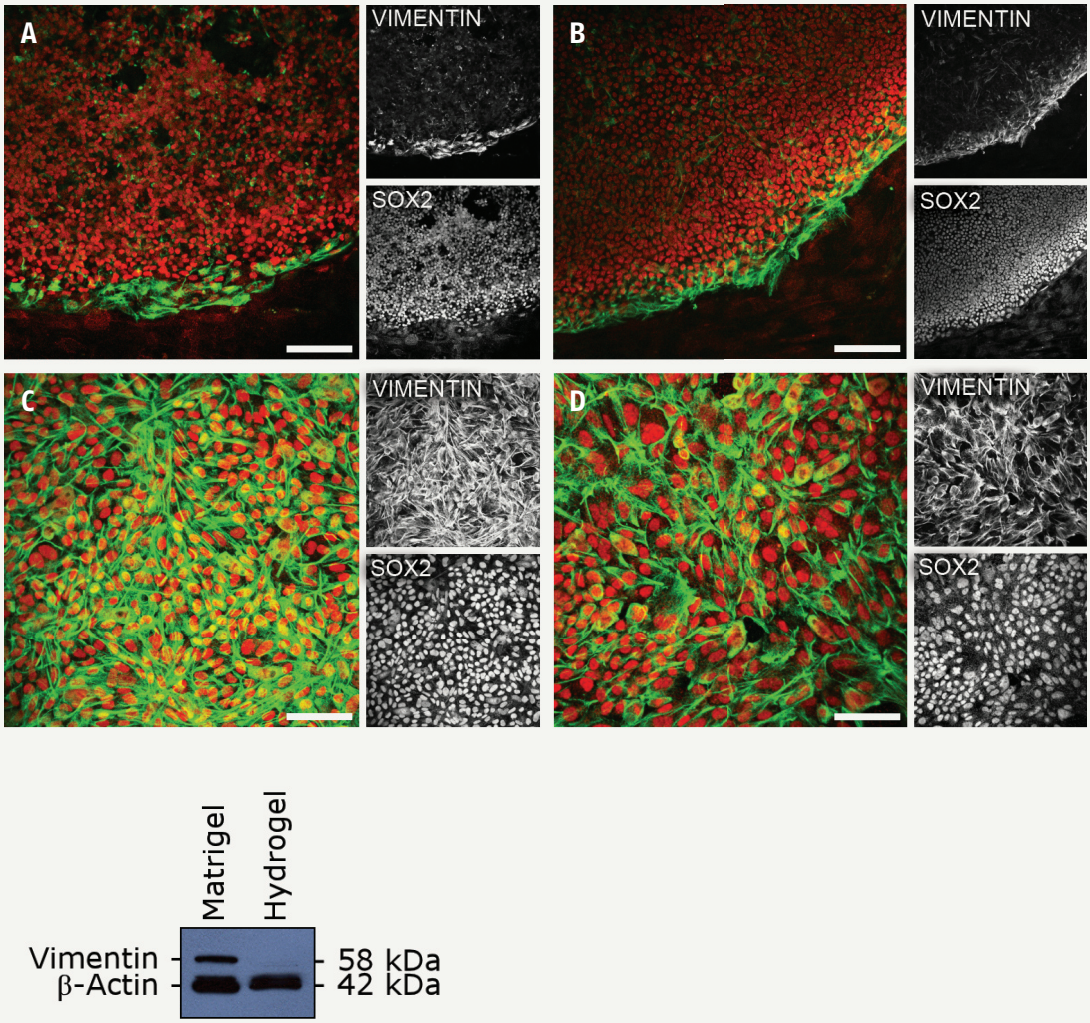


Figure 4.3

VIMENTIN expression is related to stress rather than differentiation

Differential expression of VIMENTIN in HUES-7 and HES-2 human embryonic stem cell (hESC) colonies and in HUES-7 hESC grown on rigid or soft substrates. All HUES-7 and HES-2 hESC grown as colonies on mouse embryonic fibroblast feeder cells and HUES-7 hESC grown on Matrigel or fibronectin-containing Hydrogel expressed SOX2 ((A-D), shown in red). VIMENTIN was almost exclusively expressed in cells at the periphery of the hESC colonies ((A, B), shown in green). Feeder-free HUES-7 hESC grown on a thin layer of rigid Matrigel expressed higher levels of VIMENTIN ((C), shown in green) than those on a thick layer of soft, flexible Hydrogel that was supplemented with fibronectin ((D), shown in green). These differences in VIMENTIN expression were confirmed by Western blot (E). Scale bars = 100 μm (A, B) and 50 μm (C, D).

of colonies supported the suggestion that these cells had undergone an EMT[16].

VIMENTIN was almost completely absent from the epithelial-like cells but was highly expressed in peripherally located cells (Figure 4.3A). A similar distribution of VIMENTIN was observed for HES-2 hESC colonies maintained on MEFs for 58 passages (Figure 4.3B). Remarkably, all hESC cultured on Matrigel also express VIMENTIN (Figure 4.3C). However, substantially less VIMENTIN was found when they were grown on soft Hydrogel (Figure 4.3D,E). This implied that VIMENTIN expression in hESC maintained as monolayers under feeder-free conditions is the result of mechanical stress rather than an EMT associated with differentiation.

Discussion

Transfer of multilayered hESC colonies from feeder cells to feeder-free conditions has been described as enhancing differentiation through an EMT[16]. However, our results indicate that hESC grown in the absence of feeder cells in fact retain their epithelial character, even though they express VIMENTIN. MS-based proteomics and immunofluorescence microscopic verification showed that feeder-free monolayers of hESC have a highly uniform morphology and express an epithelial plasma membrane protein profile. After nine passages (3 weeks) under feeder-free conditions, these cells collectively retained expression of SOX2 in addition to components of epithelium-associated cell-cell adhesion complexes. They thus resemble the central top layer of hESC colonies cultured on feeder cells[9,16].

Like cells located at the periphery of hESC colonies cultured on feeder cells (as described here for lines HUES-7 and HES-2, and previously for VUB01[16], feeder-free hESC also express VIMENTIN, which could indicate that they had undergone an EMT[16]. However, VIMENTIN is not specific for mesenchymal cells but is also found in migrating epithelial cells[26] and cells experiencing mechanical stress[27]. Since VIMENTIN levels were substantially reduced when feeder-free hESC were maintained on Hydrogel, it is more likely that VIMENTIN expression results from growth on a rigid substrate rather than the initiation of differentiation due to the absence of feeder cells.

Conclusion

Combined, our MS data and complementary immunofluorescence evidence show that hESC grown for extensive periods without feeder cells uniformly exhibit an epithelial phenotype. This suggests that direct contact and communication with adjacent cells via cell-cell adhesion complexes is vital, as evidenced by their poor clonal survival. Furthermore, since VIMENTIN is essential for cell integrity, its abundant expression in feeder-free hESC might explain why these cells are relatively easy to handle and less delicate than hESC in colonies[4].

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References

1. Thomson, J.A., et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147 (1998).

2. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18, 399-404 (2000).

3. Cowan, C.A., et al. Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 350, 1353-1356 (2004).

4. Braam, S.R., et al. Improved genetic manipulation of human embryonic stem cells. Nat Methods 5, 389-392 (2008).

5. Klimanskaya, I., et al. Human embryonic stem cells derived without feeder cells. Lancet 365, 1636-1641 (2005).

6. Ludwig, T.E., et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotech 24, 185-187 (2006).

7. Crook, J., et al. The Generation of Six Clinical-Grade Human Embryonic Stem Cell Lines. Cell Stem Cell 1, 490-494 (2007).

8. Rao, B.M. & Zandstra, P.W. Culture development for human embryonic stem cell propagation: molecular aspects and challenges. Curr Opin Biotechnol 16, 568-576 (2005).

9. Sathananthan, H., Pera, M. & Trounson, A. The fine structure of human embryonic stem cells. Reprod Biomed Online 4, 56-61 (2002).

10. Ginis, I., et al. Differences between human and mouse embryonic stem cells. Dev Biol 269, 360-380 (2004).

11. Raymond, C.B.W., Alice, P., Linh, T.V.N., Karen, L.L.K. & Martin, F.P. Presence of Functional Gap Junctions in Human Embryonic Stem Cells. Stem Cells 22, 883-889 (2004).

12. Huettner, J.E., et al. Gap junctions and connexon hemichannels in human embryonic stem cells. Stem Cells 24, 1654-1667 (2006).

13. Van Hoof, D., et al. A quest for human and mouse embryonic stem cell-specific proteins. Mol Cell Proteomics 5, 1261-1273 (2006).

14. Baharvand, H., Hajheidari, M., Ashtiani, S.K. & Salekdeh, G.H. Proteomic signature of human embryonic stem cells. Proteomics 6, 3544-3549 (2006).

15. Carpenter, M.K., et al. Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn 229, 243-258 (2004).

16. Ullmann, U., et al. Epithelial-mesenchymal transition process in human embryonic stem cells cultured in feeder-free conditions. Mol Hum Reprod 13, 21-32 (2007).

17. Dormeyer, W., et al. Plasma Membrane Proteomics of Human Embryonic Stem Cells and

Human Embryonal Carcinoma Cells. J Proteome Res 7, 2936-51 (2008).

18. Mummery, C., et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107, 2733-2740 (2003).

19. Denning, C., et al. Common culture conditions for maintenance and cardiomyocyte differentiation of the human embryonic stem cell lines, BG01 and HUES-7. Int J Dev Biol 50, 27-37 (2006).

20. Hartsock, A. & Nelson, W.J. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta 1778, 660-669 (2008).

21. Pokutta, S. & Weis, W.I. Structure and mechanism of cadherins and catenins in cell-cell contacts. Annu Rev Cell Dev Biol 23, 237-261 (2007).

22. Schneeberger, E.E. & Lynch, R.D. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 286, C1213-1228 (2004).

23. Stokes, D.L. Desmosomes from a structural perspective. Curr Opin Cell Biol 19, 565-571 (2007).

24. Giepmans, B.N. Gap junctions and connexin-interacting proteins. Cardiovasc Res 62, 233-245 (2004).

25. Willecke, K., et al. Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem 383, 725-737 (2002).

26. Gilles, C., et al. Vimentin contributes to human mammary epithelial cell migration. J Cell Sci 112 (Pt 24), 4615-4625 (1999).

27. Goldman, R.D., Khuon, S., Chou, Y.H., Opal, P. & Steinert, P.M. The function of intermediate filaments in cell shape and cytoskeletal integrity. J Cell Biol 134, 971-983 (1996).

CHAPTER FIVE

Recombinant vitronectin is a functionally defined substrate

that supports hESC self renewal via αVβ5 integrin

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Abstract

Defined growth conditions are essential for many applications of human embryonic stem cells (hESC). Most defined media are presently used in combination with Matrigel™, a partially defined extracellular matrix (ECM) extract from mouse sarcoma. Here, we defined ECM requirements of hESC by analyzing integrin expression and ECM production and determined integrin function using blocking antibodies. hESC expressed all major ECM proteins and corresponding integrins. We then systematically replaced Matrigel™ by defined media supplements and ECM proteins. Cells attached efficiently to natural human vitronectin, fibronectin and Matrigel™ but poorly to laminin+entactin and collagen IV. Integrin blocking antibodies demonstrated that $\alpha V\beta 5$ integrins mediated adhesion to vitronectin, $\alpha 5\beta 1$ to fibronectin and $\alpha 6\beta 1$ to laminin+entactin. Fibronectin in feeder cell-conditioned medium partially supported growth on all natural matrices but in defined, non-conditioned medium only Matrigel™ or (natural and recombinant) vitronectin were effective. Recombinant vitronectin was the only defined functional alternative to Matrigel™, supporting sustained self-renewal and pluripotency in three independent hESC lines.

Introduction

Human embryonic stem cells (hESC) are self-renewing, pluripotent cells with expected clinical applications in cell therapy, tissue repair and drug development because of their ability to differentiate to all cell types present in the adult body. Since the first derivation of hESC[1], many lines have been generated under a wide variety of culture conditions. Most have included mouse or human feeder cells to inhibit differentiation[2] but also to maintain karyotypic stability[3,4]. In addition, many studies have shown a consistent requirement for FGF2, either secreted by the feeders or added as recombinant protein[5,6]

Attempts to replace feeder cells have shown that hESC can be maintained on Matrigel™ [7], a laminin-111-rich, mouse sarcoma-derived commercial product also containing collagen IV, entactin, heparin sulfate proteoglycan and multiple other components including growth factors, but no vitronectin. More recently defined culture conditions that use a matrix of natural human collagen IV, fibronectin, laminin and vitronectin have been described[8] but whether a specific matrix integrin interaction is essential is unclear. In addition, protein products from natural sources tend to show batch-to-batch variability. In a recent comparison of several commercial human laminin preparations, for example, both compositional and functional differences were found between batches, with highly fragmented proteins, mixtures of isoforms and/or contaminating fibronectin detected in variable amounts[9]. This is an important concern in attempts to understand the role of matrix-integrin interactions in pluripotency and self renewal. Therefore we aimed to establish culture conditions based entirely on recombinant / synthetic substrates.

Integrins are the cell surface receptors that mediate cell-ECM adhesion and signaling[10]. Previously, a number of integrins were detected in hESC by RT-PCR. Among them were α6 and β1 which mediate binding to matrigel™ [7]. Integrins are heterodimeric transmembrane molecules with large extracellular domains and relatively small cytoplasmic domains formed by α and β subunits that can switch between inactive and active conformations. In the inactive state, integrins have a low affinity for ECM proteins. Intracellular signaling can prime the integrins, resulting in conformational changes that expose the ligand-binding site. Ligand binding activates signaling cascades that lead to the assembly of multiprotein focal adhesion signaling complexes at the site of cell adhesion to the ECM. This causes cells to assemble connections between the ECM and the actin cytoskeleton and induces many intracellular signaling pathways. The pathways that are activated depend on the particular ECM ligand-receptor interaction; they may give rise to cytoskeletal changes that result in different migratory behaviour or to changes in growth and/or differentiation[10].

In nearly all cell types, integrin binding activates focal adhesion kinase (FAK)[11]. FAK disseminates integrin signals by forming complexes with signaling proteins that have numerous effects on cell function like proliferation, differentiation and apoptosis[12].

While functional integrin-ECM interaction is clearly crucial for derivation, self renewal and maintenance of pluripotency by hESC, the ECM constituents and their role in signaling have not been systematically investigated. Here we have determined integrin and ECM protein expression profiles in three independently derived hESC lines (HES2, HUES1 and HESC-NL3), with a history of maintenance and culture under widely differing conditions. Functional analyses using integrin blocking antibodies showed that αVβ5 integrins were responsible for adhesion to vitronectin, α5β1 to fibronectin and α6β1 to laminin+entactin in all three cell lines. Attachment of cells to recombinant vitronectin through αVβ5 was demonstrated to be sufficient to support proliferation and self-renewal in chemically defined (mTeSR1) medium. Stem cell marker expression and pluripotency *in vitro* was retained over multiple passages without loss of karyotypic stability. Recombinant vitronectin matrix therefore represents a defined functional alternative for Matrigel™ in a chemically defined medium.

Material & Methods

CELL CULTURE AND MATRICES

HUES1 are routinely cultured[13] on CD1 mouse embryonic fibroblast feeders (MEFs) (kind gift of Dr. C. Denning) in KO-DMEM (Invitrogen) containing 10% Plasmanate and 10% Knock Out-serum replacement (KSR) (Invitrogen), HES2[14] on 129SV MEFs as described previously [15] and HESC-NL3 on human foreskin fibroblast feeders in Knockout DMEM/ KSR supplemented with bFGF[16].

Furthermore HUES1 cells were cultured feeder-free as a monolayer on Matrix Growth Factor Reduced Matrigel (BD Biosciences) in MEF conditioned medium. Trypsin/EDTA (Invitrogen) was used to release the cells which were then dissociated to a suspension of single cells and small clumps[17,18]. For the preparation of conditioned medium (CM), MEFs (mouse strain CD1) were mitotically inactivated by mitomycin C (MMC) (10 µg/ml, 2.5-3 hours) and seeded at 4,6 x 10^6 cells per 75 cm^2 flask. The next day, MEFs were incubated with 25 ml of hESC medium (Glutamax based DMEM-F12 supplemented with 15% KSR, 100 µM β-mercaptoethanol, 1% non-essential amino acids (all from Invitrogen) and 4ng/ml basic fibroblast growth factor (Peprotech). CM was harvested after 24 hours and supplemented with 4ng/ml bFGF. This procedure was repeated for 7 consecutive days by replacing CM on MEFs with fresh hESC medium.

The ECM protein concentrations for coating culture plates were determined in dose response curves (Figure 5.2A). ECM concentrations used for integrin blocking assays were: 10 ng/µl collagen IV (isolated from human placenta; Chemicon), 40 ng/µl fibronectin (isolated from human plasma; Harbor Bio-products, Norwood, MA), 10 ng/µl vitronectin (isolated from human plasma; Chemicon), 50 ng/µl laminin (laminin-111), 50 ng/µl mLaminin+entactin and Matrigel™ (84 ng/ul) (all isolated from Engelbreth-Holm-Swarm mouse tumor; BD Biosciences). Throughout the manuscript the new nomenclature for laminins is used, i.e. indicating the three chains of each isoform [19].

For feeder-free defined cultures all three lines were cultured in mTeSR1 (Stem Cell technologies) on recombinant vitronectin (5ng/µl) (R&D systems). Cells were grown in colonies as shown in Figure 5.4C-E. Once weekly, the colonies were dissected using glass needles into small pieces of 50-80 µM and transferred manually to new dishes without enzymes[20]. A minority of colonies contained some differentiated cells at the centre; these areas were omitted from transfer. Directed differentiations to meso- endo- and ectoderm were performed as described previously[21].

FACS ANALYSIS

hESC cells were trypsinized, and stained with specific integrin antibodies (supplementary data) and FITC-conjugated secondary antibody. Cells were analyzed on a FACSCalibur or a FACSCanto (BD Biosciences).

IMMUNOFLUORECENT STAINING

Cells on plastic coverslips (Sarstedt Inc, Newton, NC) were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were stained using specific primary antibodies (supplementary data) and fluorophore conjugated secondary antibodies; when applicable cells were co-stained with phalloidin-TRITC (Sigma). Nuclei were stained with TOPRO-3 (Invitrogen) or DAPI. Cells were mounted using Mowiol or using Vectashield hard set with Dapi (Vectalabs). Plastic coverslips were immobilized on glass microscope slides and sandwiched with glass coverslips. Pictures were taken using Laser Confocal Scanning Microscopes TCS-SP2 and TCS-SPE (Leica, Mannheim, Germany).

ADHESION ASSAY

Adhesion assays were essentially performed as described previously[22]. In brief, hESC were trypsinized, counted and incubated for 30 min. at 37°C in the presence or absence of integrin blocking antibodies in IMDM (Invitrogen) containing 0.35% bovine serum albumin (BSA). Purified antibodies were tested at 1 and 10 µg/ml concentrations and hybridoma supernatants at 1/5 and 1/50 dilutions. Dilutions appropriate for blocking were used in all subsequent assays.

They were subsequently seeded into 96-well flat-bottom plates, which were previously coated with various matrix components and blocked with 2% BSA, at 50,000-100,000 cells/well, depending on the substrate. They were left to adhere for 30 min at 37°C. The non-adherent cells were carefully washed away with IMDM/0.35% BSA three times, and finally once with PBS. The adherent cells were fixed with 100% ethanol for 5 min at RT and stained with 0.4% crystal violet in methanol for 5 min at RT. The wells were washed extensively with demi-water. The cells were solubilized by adding 40 µl 1% SDS and cell adhesion was determined by measuring OD_{570}. Results are expressed as relative adhesion normalized to one. Blank controls were without cells but otherwise treated identically to controls

INTEGRIN BLOCKING ASSAYS

HUES1 from Matrigel™ were trypsinized and replated on the indicated matrix in the presence or absence of integrin blocking antibodies. For αVβ5 antibody (Chemicon) a concentration of 2 µg/ml was used, for AIIb2 (anti-β1 integrin) the supernatant was diluted 50 times. The cells were allowed to grow in MEF-CM for 4 days and the medium containing the antibodies was refreshed every day. Coverslips were stained for Tra-1-60, human nuclei and fibronectin or Tra-1-60, phalloidin and OCT4 as described above

CELL EXPANSION ASSAYS

HUES1 from Matrigel™ were trypsinized and replated on the indicated matrix. 4 hours later GoH3 1 µg/ml and / or Alpha5 blocking antibody (Chemicon) 1 µg/ml was added. Cell proliferation was assayed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) at 24, 48, 72 and 96h post plating. MEF-CM was refreshed daily. As required, integrin blocking antibodies were added freshly, daily.

KARYOTYPE ANALYSES

hESC cultured on recombinant vitronectin in mTeSR1 were treated with 100 ng/ml colcemid (Karyomax) for 1 hr. hESC colonies were dissociated using TrypLE (Invitrogen). After hypotonic solution treatment (0,6 % citrate buffer), lysed cells were fixed in methanol/glacial acetic acid (5:1). G banding was performed for identification of chromosomes, 30 metaphase spreads were examined; full analysis involving band-by-band comparison between chromosome homologues was performed on 10 spreads, and the presence of gross abnormalities was visually examined in the remaining spreads.

Results

EXTRACELLULAR MATRIX EXPRESSION IN HESC CULTURES

Understanding the composition of ECM components in hESC cultures on feeder layers may provide clues on which substrates favor sustained undifferentiated proliferation of hESC. MEFs are most commonly used and produce a complex matrix of many structural ECM proteins including laminins, various collagens and fibronectin[23]. Detail is lacking on the exact composition of feeder derived matrix components making it difficult to predict which components are essential versus those that are redundant. In addition the exact interaction between hESC and feeder-derived ECM has not been investigated in detail. Therefore we first immuno-stained ECM proteins in three hESC lines that have all been derived and cultured under completely different culture conditions. HUES1[13], is cultured on MEFs in plasmanate/KO-SR containing medium and passaged by trypsinization. HES2[14] is cultured on MEFs in FCS-containing medium and passaged mechanically using dispase. HESC-NL3 was derived in our lab on human foreskin feeders (HF), and is cultured in serum replacement medium and passaged mechanically without the use of enzymes.

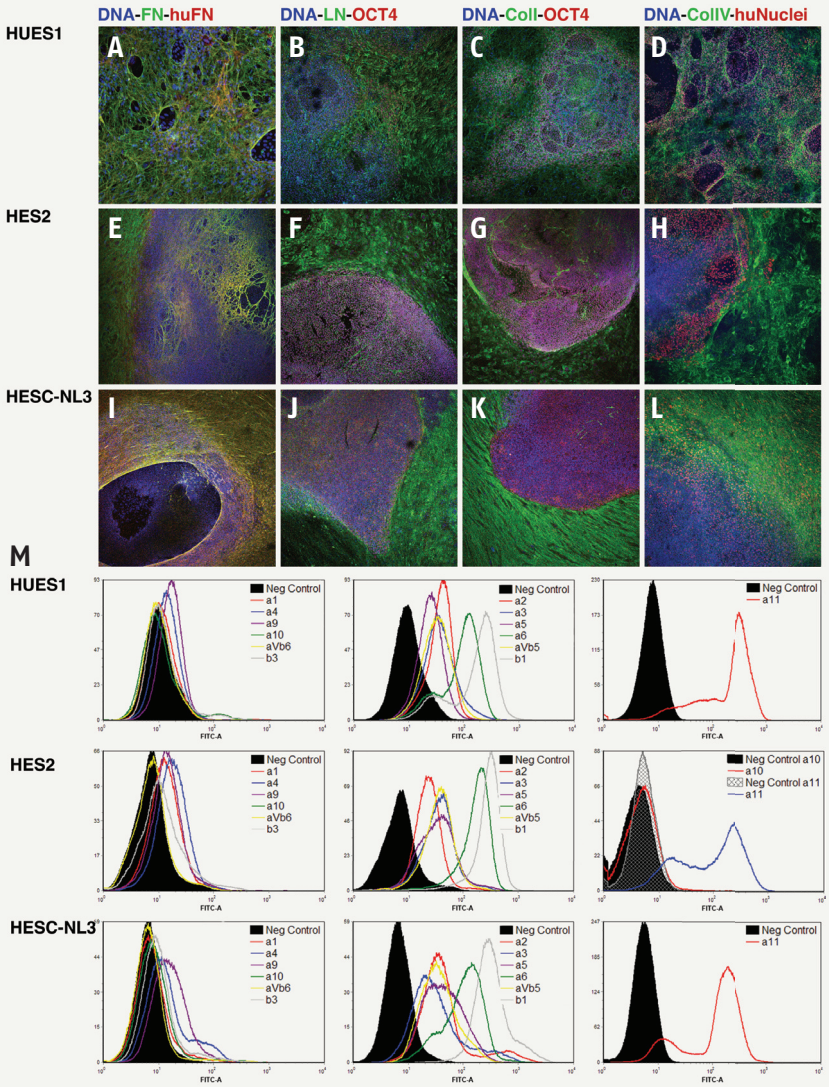

Figure 5.1

Extracellular matrix and integrin protein expression in hESC cultures

HUES1, HES2 and HESC-NL3 stained for the ECM components fibronectin, laminin-α1, collagenI and collagenIV. (A,E,I) polyclonal anti-fibronectin antibody recognizing both mouse and human fibronectin is shown in green, human fibronectin (mAb) is shown in red. (B,F,J) polyclonal anti laminin-α1 recognizing both mouse and human is shown in green, with OCT4 shown in red. (C,G,K) polyclonal anti-collagenI recognizing both mouse and human is shown in green, with OCT4 shown in red. (D,H,I) CollagenIV is shown in green, with human nuclei in red. In all pictures nuclei are counterstained in blue with TO-PRO (M) FACS analysis for the majority of integrin chains on the three hESC lines. All integrins tested were expressed at essentially the same level on the three lines.

These diverse hESC lines were selected to address the question whether culture conditions themselves caused hESC to adapt with respect to their molecular mechanisms for cell–cell or cell substrate interactions.

As shown in Figure 5.1A, E and I large quantities of fibronectin were present in all three cultures under native conditions. There is abundant staining of fibronectin in the feeder areas, which is not recognized by the antibody specific for human fibronectin, and therefore appears to be of mouse origin. (Figure 5.1E and not shown). However, human fibronectin was found at the edges and at the center of the hESC colonies (Figure 5.1E and not shown). These are the areas that usually contain mostly differentiated hESC. It is most likely, therefore, that fibronectin is mainly secreted and assembled into bundles by the feeders and differentiated hESC, although we cannot exclude secretion by undifferentiated hESC. Importantly the human specific fibronectin antibody stained the human fibroblast feeders (HFs) exclusively (Figure 5.1I) but not the MEFs (Figure 5.1A,E). Both Laminin and collagen I were also found predominantly in the feeder area, again suggesting mouse origin (Figure 5.1B,F,J and 5.1C,G,K). However there was some staining between hESC. Collagen IV was found exclusively in the feeder area, again suggesting mouse origin (Figure 5.1D,H,J). Immunolabeling with anti-vitronectin antibodies from multiple sources resulted in no obvious staining. Thus a number of major ECM components were present in the native cultures of three different hESC lines, primarily associated with the feeders or differentiated cells around the hESC, but independent of the type of feeder used and method of passage or culture medium.

INTEGRIN EXPRESSION IN HESC

Undifferentiated hESC need signaling factors from feeders for undifferentiated growth. This signaling may be mediated by growth factors but also ECM integrin interactions may contribute to signaling. Four major ECM components were present in hESC cultures, most of them secreted by the feeder cells, as described above. However, it is not known which ECM receptors on hESC mediate adhesion to the matrix present in the cultures. An important class of ECM receptors is the integrin family of proteins. In order to further investigate ECM–hESC interaction at the molecular level we determined cell surface expression of the majority of the α- and β-integrin chains by FACS analysis (Figure 5.1M). The three hESC lines tested exhibited comparable integrin expression profiles. Integrin chains α1, α4, α10, β3 and integrins α9β1 and αVβ6 were not detected or at very low levels (Figure 5.1M, left panels). Integrin chains α2, α3, α5, α6, α11, β1 and integrin αVβ5 were expressed (Figure 5.1M, middle and right panels). These integrin chains can form the combinations α2β1 (binding collagen, laminin), α3β1 (binding nidogen, laminin, collagen I, fibronectin), α5β1 (binding fibronectin), α6β1 (binding laminin-111, 211 and 411), α11β1 (binding collagen), αVβ5 (binding vitronectin, fibronectin) and αVβ1 (binding fibronectin). Thus hESC have cell surface receptors for all major ECM components, independent of their method of derivation or culture (Figure 5.1M). Even though α4 and α9β1, and sometimes α1 or β3, were detected at low levels by FACS analysis, immunofluorescent staining of these integrins showed only background levels (not shown) and are therefore unlikely to play a major functional role in adhesion. By contrast, integrin

chains α2, α3, α5, α6, α11, β1, and integrin αVβ5 all showed distinct plasma membrane localization in the three hESC lines (Figure S1). Furthermore independent mass-spectroscopy analyses of membrane proteins from a fourth hESC line, HUES7, confirmed the presence of α2, α3, α5, α6, αv, and β1 integrin peptides[24] (Table S1). The peptides identified were blasted against reference databases, confirming their human origin. In summary, it is evident that the collagen receptor α2β1 and α11β1, the fibronectin receptor α5β1, the vitronectin receptor αvβ5 and the laminin receptor α6β1 are expressed on all three hESC lines.

Next we characterized the ability of hESC to attach to various ECM coated substrates. Since we observed no major differences in integrin expression profiles among the three tested lines, HUES1 was chosen for a detailed dose-response curve of their adhesion to laminin-111, laminin-111+entactin, Matrigel™, collagen IV, fibronectin and vitronectin (Figure 5.2A). Monolayer HUES1 cultures using Matrigel™-coated substrates in KSR-supplemented MEF conditioned medium (CM) have already been shown to be beneficial for cell manipulation. These conditions support undifferentiated proliferation of various hESC lines and to maintain their karyotype stably for at least 10 passages[17]. Since this system is based on trypsin passage it was clearly beneficial for attachment assays. Attachment was very efficient on vitronectin and Matrigel™ but poorer on fibronectin and collagen IV. Attachment to pure laminin-111 was extremely weak. Since laminin-111 and Matrigel™ are both isolated from the EHS tumor this suggests that the rigorous chemical purification of the commercial laminin product destroys its functionality. Indeed the less pure laminin+entactin combination from the same source rescued the binding to some extent (Figure 5.2A). Therefore throughout the manuscript laminin+entactin will be used when referring to laminin-111.

FUNCTIONAL VALIDATION OF DETECTED INTEGRINS

To investigate the functionality of these integrins in HUES1 further, we performed short term adhesion assays but now in the presence of specific integrin blocking antibodies. hESC were first incubated with the antibodies, then allowed to adhere mouse laminin+entactin, fibronectin, vitronectin or collagen IV (Figure 5.2B).

Both anti-α6 and anti-β1 antibodies significantly reduced adhesion of hESC to laminin+entactin, indicating that α6β1 was indeed functional in binding its major ligand laminin-111 in hESC (Figure 5.2B). Blocking the integrin chains that make up the fibronectin receptor α5β1, as expected, significantly impaired adhesion to fibronectin, while blocking the vitronectin receptor αVβ5 reduced binding to vitronectin (Figure 5.2B). The functionality of α2β1 and α11β1 could not be tested, as there where no specific blocking antibodies available. Taken together α6β1 was shown to mediate adhesion of hESC to laminin-111, α5β1 to fibronectin and αVβ5 to vitronectin.

THE VITRONECTIN RECEPTOR αVβ5 RESCUES THE β1 INTEGRIN BLOCK

Since most functional integrin combinations rely on the β1 chain, we examined whether this chain was a specific signaling intermediate crucial for stem cell proliferation by adding

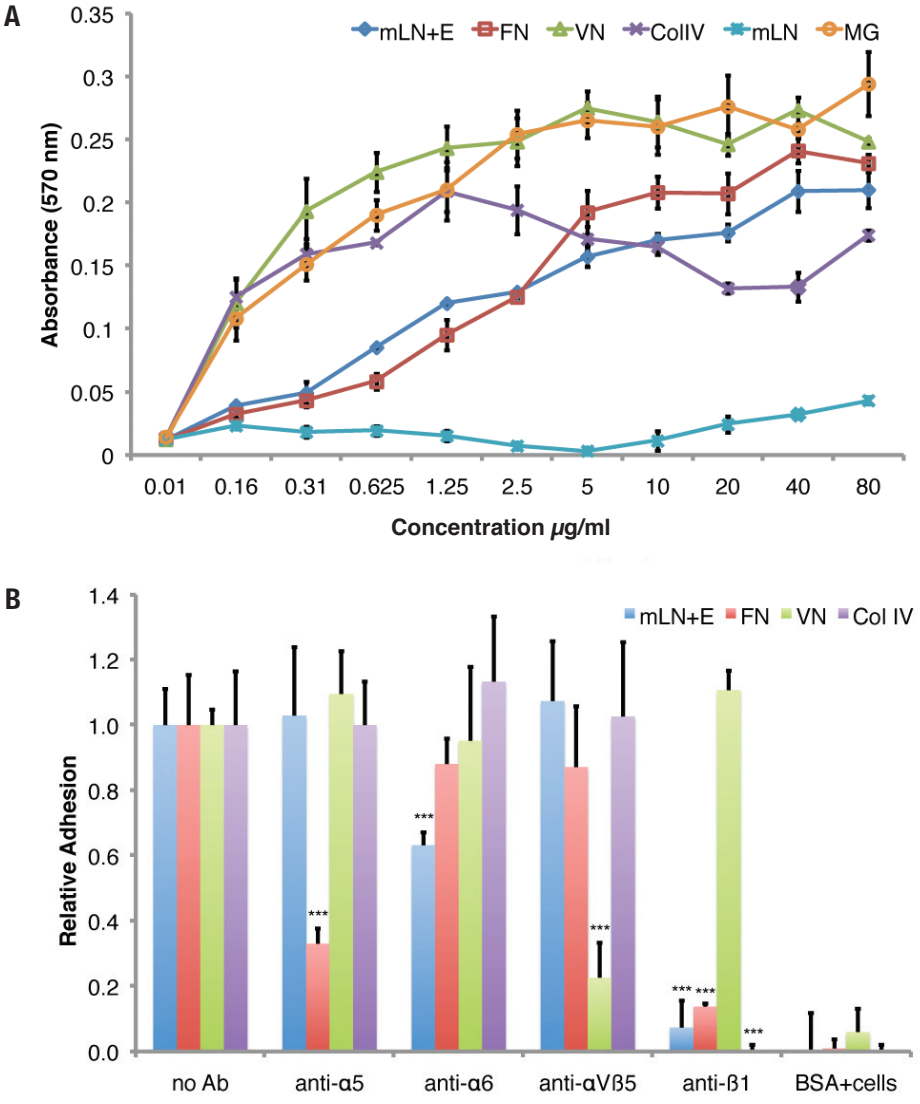

Figure 5.2

hESC Integrin function in initial substrate adhesion

(A) HUES1 cell attachment dose response binding curve to various natural matrices, (B) Functional validation of integrin binding capacity on various matrices using integrin specific blocking antibodies. *** p≤0.01. Data from at least three independent experiments were collected and displayed as average +/- SEM.

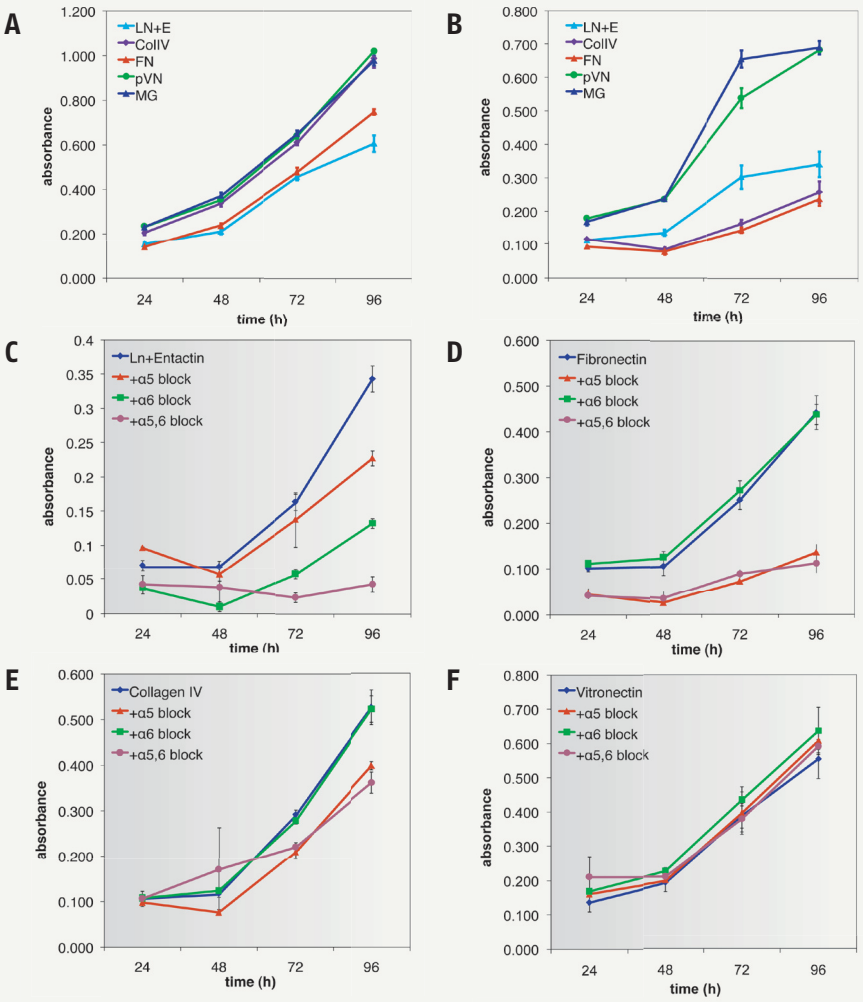

Figure 5.3

hESC expansion in various media is matrix dependent

HUES1 cells were trypsinized and replated in triplo in 96-well plates on various matrices. Cell proliferation was measured quantitatively at different time-points (A) HUES1 cell expansion on various substrates in MEF-CM, (B) HUES1 cell expansion on various substrates in mTeSR1, chemically defined medium. (C-F) HUES1 cell expansion assays in the presence or absence of integrin α5 and α6 blocking antibodies which were added 4-hours post-plating to circumvent differential attachment on (C), Ln+entactin (D), fibronectin (E) collagen IV and (F) vitronectin. Experiments were repeated at least three times, one representative experiment is shown here. Each timepoint represents the mean absorbance of a triplicate, +/- SEM.

blocking β1 antibodies to HUES1 cultured on Matrigel™. This completely inhibited attachment/proliferation, while blocking the vitronectin receptor αVβ5 had as expected no effect (Figure S2). The combination of Matrigel™ with vitronectin but not fibronectin however, rescued the β1 block, whereas the combination of Matrigel™ and fibronectin had no effect (Figure S2). The rescue of vitronectin was mediated by the αVβ5 integrin since a combination of β1 and αVβ5 blocking antibodies blocked the attachment and growth completely (Figure S2). These results indicated that the attachment and growth of hESC not necessarily require binding via the β1 chain but that ligation of αVβ5 to its ligand vitronectin could act as a functional substitute to mediate adhesion and support undifferentiated growth.

MATRIGEL™ AND VITRONECTIN SUPPORT ROBUST HESC PROLIFERATION IN DEFINED MEDIA

To test the functionality of hESC expressed integrins further, we examined the ability of purified ECM preparations to support proliferation of HUES1 in MEF conditioned media and mTeSR1. As expected, cells cultured in both media proliferated well when seeded on Matrigel™ coated plates, as reported previously[7,25]. However on all other matrices tested there was a clear difference between mTESR and MEF conditioned medium. Cells cultured in mTeSR1 supported hESC growth exclusively on purified vitronectin but not on fibronectin, laminin and collagen IV while MEF conditioned medium supported hESC growth on any tested substrate (Figure 5.3A,B). To test these effects further, cells cultured in MEF-CM on various substrates were stained for OCT4 expression (Figure 5.3) and cultured for extended periods on the same ECM coated substrates. All cells retained OCT4 expression although we observed higher culture expansion rates of hESC on Matrigel™ and vitronectin (passage 7), than on laminin, fibronectin and collagen IV (passage 5) after one month of continuous culture. A possible explanation for the observed differences between MEF-CM and mTeSR1 would be the presence of additional factors present in KSR or secreted by the feeders that mediate binding of hESC to their substrate. FIBRONECTIN and LAMININ were selected as candidate proteins mediating the observed difference in culture expansion rates between the two media. This was based on extrapolation of the data in Figure 5.1 and a published secretome analyses of human fetal, human neonatal and mouse embryonic feeder fibroblast respectively[26]. We therefore carried out culture expansion assays in MEF conditioned media in the presence or absence of α6β1 (laminin receptor) and α5β1 (fibronectin receptor) blocking antibodies on laminin+entactin, fibronectin, collagen IV and vitronectin (Figure 5.3C-F). Surprisingly, α5β1 reduced culture expansion on collagenIV and laminin+entactin, indicating that fibronectin secreted by the feeders is a major bioactive molecule in MEF conditioned medium. As expected, blocking α6β1 reduced culture expansion on laminin+entactin without a significant effect on the other matrices tested and blocking α5β1 reduced culture expansion on fibronectin as expected. Interestingly, culture expansion on vitronectin was not impaired by blocking fibronectin signaling. This confirmed the suitability of vitronectin as replacement for Matrigel™.

hESC culture on a completely defined substrate

The aim of this study was to find defined synthetic ECM substrates for sustained self renewal of hESC without loss of pluripotency or changes in karyotype. We identified purified vitronectin as a candidate ECM protein for this purpose. Since human recombinant vitronectin is commercially available, we next tested whether this would be as effective as purified human vitronectin from plasma, preferably in combination with defined medium rather than medium conditioned by MEFs or HFs. HUES1 grown on plasma vitronectin expanded at the same rate as cells grown on recombinant vitronectin (Figure 5.4A) and used integrin $\alpha V\beta 5$ for cell attachment (Figure 5.4B). Next we tested the ability of recombinant vitronectin to support long term self-renewal in defined (mTeSR1) medium. For this we used all three lines used at the outset (HUES1, HES2 and HESC-NL3) to examine the general applicability of this culture method. Each of the lines proliferated robustly in a 'cut and paste' culture in continuous culture for up to 12 weeks, the maximum presently tested. Under these culture conditions hESC had a similar growth rate to conventional cultures grown on feeders cells and were passaged once a week. After minimally 5 weeks of culture (5 passages) on recombinant vitronectin in mTeSR1 medium cells were analyzed in detail for karyotypic abnormalities and pluripotency in culture.

Under these defined conditions cells grew in compact colonies (Figure 5.4B-D) without obvious morphological differentiation. Karyotypic analyses of HESC-NL3 and cultured on recombinant vitronectin showed a normal 46xy karyotype in all cells analyzed (Figure 5.4E). Maintenance of stemness was demonstrated by staining for the stem cell markers E-CADHERIN, GCTM2, OCT4, SOX2, SSEA-3 and SSEA-4 (Figure 5.4F-K). To examine their differentiation potential, hESC cultures on recombinant vitronectin in mTeSR1 were treated with growth factor supplemented N2/B27 medium as described previously [21]. Cell types derived from each of the three germ layers were present for each of the line. Endoderm differentiation was confirmed using antibodies against alpha-fetoprotein (AFP), ectoderm using antibodies against betaIII-tublin, a neuronal protein and mesoderm differentiation was confirmed using antibodies recognizing alpha-actinin, a cardiac protein (Figure 5.4L-N). These results confirmed the potency of our fully defined hESC culture system.

Discussion

For both clinical applications as well as addressing fundamental questions regarding hESC biology and the molecular nature of `stemness'. it is clearly beneficial to culture with fully defined culture substrates and media based on recombinant (or clinical grade) proteins[27]. We demonstrated here that hESC express integrin receptors for laminin, fibronectin, collagen and vitronectin and that all of these receptors are functional in mediating adhesion. MEFs and HFs secrete large quantities of at least laminin, collagen I, collagen IV and fibronectin. Although all cell lines attached and proliferated well on the major ECM proteins if grown in MEF-CM. However if the fully defined mTeSR1 medium was used, only vitronectin supported

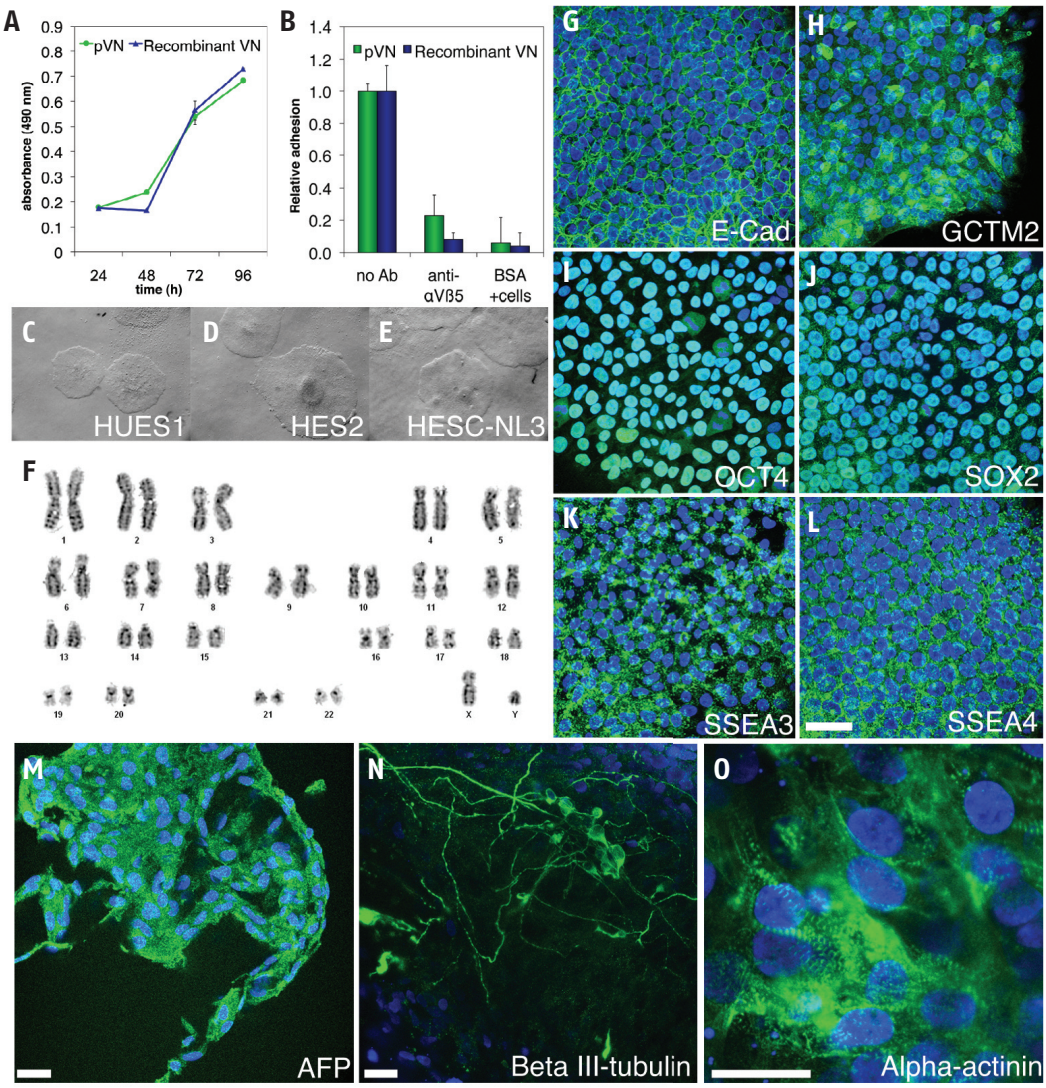

Figure 5.4

recombinant vitronectin supports hESC in mTeSR1

(A) HUES1 cell expansion assay on plasma Vitronectin (pVN) and recombinant vitronectin. (B) HUES1 cell attachment in the presence and absence of integrin αvβ5 blocking antibodies on both plasma vitronectin and recombinant vitronectin (C–E) representative brightfield pictures of HUES1, HES2 and HESC-NL3 cultured for 8 passages on recombinant vitronectin in mTeSR1 (F) Karyogram of HESC-NL3 cultured for 7 passages on recombinant vitronectin in mTeSR1 showing a normal diploid 46,xy karyotype (G–L) Immunostaining for stem cell markers and DNA for HESC-NL3 (overlay). (G) E-cadherin, (H) GCTM2, (I) OCT4, (J) SOX2, (K) SSEA3 (L) SSEA4. (M–O) Immunostaining for HESC-NL3 differentiation markers representing the three germ layers (M) AFP, endoderm (N) beta III tubulin, ectoderm and (O) α-actinin, mesoderm. Scale bars, 50 μm.

hESC growth as well as Matrigel™. We identified recombinant vitronectin as a suitable and functional alternative for Matrigel™. Three independently derived cell lines (HUES1, HES2 and HESC-NL3) were cultured long term on recombinant vitronectin in mTeSR1 medium and all maintained their characteristic compact morphology, expression of stem cell markers, normal karyotype and differentiation potential, demonstrating the general applicability of these conditions.

Most of the >400 hESC lines derived to date have been traditionally cultured on a feeder layer of MEFs to maintain self renewal and pluripotency. It was of interest to note in our study that MEF conditioned medium reduced the stringent ECM requirements for hESC i.e. fibronectin, collagen IV and laminin could replace Matrigel™ as well as vitronectin. We showed that feeder cells produce a highly complex matrix consistent of several ECM molecules and that secreted fibronectin is likely responsible for this loss of matrix stringency. However, it is of interest that FGF2 signaling, the primary signaling pathway for hESC self-renewal, is highly dependent upon the ECM. FGF family members bind to the extracellular matrix (ECM) becoming concentrated in specific regions where they are protected from proteolytic degradation[28]. FGF receptor activation is dependent upon heparan sulphate side chains on either ECM or cell surface proteoglycans[29]. Many of the growth factor signaling pathways are shared with integrin pathways[30]. ECM modulated signals may convergence with FGF signaling and be involved in the maintenance of "stemness".

Indeed, it has been suggested that integrin signaling in mouse ES cells converges with growth factor signaling to maintain stemness. Recently is has been shown that cells cultured on type I and IV collagen or poly-d-lysine remained undifferentiated while laminin or fibronectin induced differentiation[31]. Unfortunately vitronectin was not investigated in this study. However, as many studies have shown, results obtained in mouse ES cannot always easily be translated to hESC, particularly with respect to cell surface proteins and signal transduction. This is likely related to their different state of differentiation[32,33]. Nevertheless it might be interesting to study the relationship between integrin signaling in differentiation and self-renewal of hESC in more depth in the future.

Almost all of our knowledge related to hESC is based on studies carried out under the influence of feeder cells and/or serum. This might explain several inconsistencies in the literature, for example; the debated role of Activin A in hESC self-renewal[6,21]. Over recent years, a number of papers have described defined media for hESC but the majority made use of Matrigel™ [6,21,25,34-37] which is still incompletely defined. Apart from laminin, entactin, collagen and heparin sulfate proteoglycan Matrigel™ contains several growth factors, even in its growth factor reduced variant. Among these is TGF-β which might contribute to hESC self-renewal[38]. Therefore, under Matrigel™ culture conditions it is difficult to define growth factor requirements for undifferentiated growth or directed differentiation. Furthermore, Matrigel is derived from a tumour of mouse origin, a potential xenorisk, since it contains the non human sialic acid Neu5Gc, an immunogenic molecule that might be transferable to hESC[39].

Based on the work of Xu et al (ref 7) in which $\alpha 6\beta 1$ integrin was detected on the cell surface of hESC and feeder-free culture of hESC on Matrigel™ in MEF-CM was described, it has been generally assumed that laminin is the most important ECM protein for hESC in culture. Indeed hESC culture on purified laminin has been reported[7,40]. Both studies were however performed in MEF-CM. It is likely that these results were influenced by ECM proteins secreted by MEFs. Since feeders secrete fibronectin[26] and blocking the fibronectin receptor $\alpha 5\beta 1$ reduced hESC expansion on laminin and collagen IV it is likely that fibronectin is the major bioactive protein in MEF-CM in this respect. In fact, it has been reported that purified fibronectin supports hESC growth even in semi-defined hESC medium[41]. Here we observed even better cell growth and attachment on purified vitronectin. Furthermore, on vitronectin we observed no delay in cell expansion by blocking fibronectin signaling indicating that ligation of hESC through $\alpha v\beta 5$ is sufficient for hESC growth.

The recombinant vitronectin, used was produced in the mouse myeloma cell line, NS0 and therefore could potentially contain Neu5Gc. However, biotechnological innovations may and should overcome this problem. Previously, yeast was successfully genetically engineered to produce humanized glycosylated proteins[42]. Another possibility could be to use human protein expression platforms, such as the PER.C6 cell line[43]

Concurrent with the present study, two papers reported defined media for hESC culture. Gerecht et al. (2007) described a defined hydrogel based on hyaluronic acid. For routine use however, this approach has certain drawbacks: cells are difficult to access for subsequent analysis and use and encapsulation of cells depends on UV photoactivation. This might cause point mutations in the DNA. Furthermore the authors did not demonstrate serial passage, prolonged culture and karyotypic stability in this system[44]. Derda et al. used SAM surface arrays to detect peptides derived from laminin suitable for hESC-self-renewal[45]. The biological relevance of that study might be limited by the fact that surface arrays were coated with serum which contains high levels of fibronectin and the use of MEF-CM. As shown in our study fibronectin from MEF-CM mediated binding on several substrates that were not supportive in defined media. This illustrates the importance of media and substrate development progressing in parallel.

In conclusion; we defined a culture system for hESC based entirely on human/recombinant proteins that can be used for long-term self-renewal and selected differentiation of hESC. This will be essential for both basic research on hESC pluripotency and clinical applications.

SUPPLEMENTAL DATA

Supplemental Data contains three Supplemental Figures and two supplemental tables which can be found on-line at http://www3.interscience.wiley.com/journal/121587696/suppinfo

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References

1. Thomson, J.A., et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147 (1998).

2. Rao, B.M. & Zandstra, P.W. Culture development for human embryonic stem cell propagation: molecular aspects and challenges. Current Opinion in Biotechnology 16, 568-576 (2005).

3. Draper, J.S., et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotech 22, 53-54 (2004).

4. Baker, D.E.C., et al. Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotech 25, 207-215 (2007).

5. Xu, R.-H., et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Meth 2, 185-190 (2005).

6. Vallier, L., Alexander, M. & Pedersen, R.A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 118, 4495-4509 (2005).

7. Xu, C., et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotech 19, 971-974 (2001).

8. Ludwig, T.E., et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotech 24, 185-187 (2006).

9. Wondimu, Z., et al. Characterization of commercial laminin preparations from human placenta in comparison to recombinant laminins 2 ([alpha]2[beta]1[gamma]1), 8 ([alpha]4[beta]1[gamma]1), 10 ([alpha]5[beta]1[gamma]1). Matrix Biology 25, 89-93 (2006).

10. Hynes, R.O. Integrins: Bidirectional, Allosteric Signaling Machines. Cell 110, 673-687 (2002).

11. Guan, J.-L. Focal adhesion kinase in integrin signaling. Matrix Biology 16, 195-200 (1997).

12. Mitra, S.K. & Schlaepfer, D.D. Integrin-regulated FAK-Src signaling in normal and cancer cells. Current Opinion in Cell Biology 18, 516-523 (2006).

13. Cowan, C.A., et al. Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 350, 1353-1356 (2004).

14. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18, 399-404 (2000).

15. Mummery, C., et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107, 2733-2740 (2003).

16. Freund, C., et al. Insulin Redirects Differentiation from Cardiogenic Mesoderm and Endoderm to Neuroectoderm in Differentiating Human Embryonic Stem Cells. Stem Cells, 2007-0617 (2007).

17. Braam, S.R., et al. Improved genetic manipulation of human embryonic stem cells. Nat Meth 5, 389-392 (2008).

18. Braam, S.R., et al. Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic modification. Nature Protocols 26, 2257-2265 (2008).

19. Aumailley, M., et al. A simplified laminin nomenclature. Matrix Biol 24, 326-332 (2005).

20. Mummery, C., Ward-van Oostwaard, D. & Passier, R. UNIT 1F.2 Differentiation of Human Embryonic Stem Cells to Cardiomyocytes by Coculture with Endoderm in Serum-Free Medium. Current Protocols in Stem Cell Biology (2007).

21. Yao, S., et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proceedings of the National Academy of Sciences 103, 6907-6912 (2006).

22. Raymond, K., Kreft, M., Janssen, H., Calafat, J. & Sonnenberg, A. Keratinocytes display normal proliferation, survival and differentiation in conditional {beta}4-integrin knockout mice. J Cell Sci 118, 1045-1060 (2005).

23. Horák V, F.J. Immunocytochemical characterisation of rabbit and mouse embryonic fibroblasts. Reprod Nutr Dev 38, 683-695 (1998).

24. Dormeyer, W., et al. Plasma Membrane Proteomics of Human Embryonic Stem Cells and Human Embryonal Carcinoma Cells. J. Proteome Res. (2008).

25. Ludwig, T.E., et al. Feeder-independent culture of human embryonic stem cells. Nat Meth 3, 637-646 (2006).

26. Prowse, A.B.J., McQuade, L.R., Bryant, K.J., Marcal, H. & Gray, P.P. Identification of Potential Pluripotency Determinants for Human Embryonic Stem Cells Following Proteomic Analysis of Human and Mouse Fibroblast Conditioned Media. J. Proteome Res. 6, 3796-3807 (2007).

27. Ilic, D. Culture of human embryonic stem cells and the extracellular matrix microenvironment. Regenerative Medicine 1, 95-101 (2006).

28. Saksela, O., Moscatelli, D., Sommer, A. & Rifkin, D.B. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J. Cell Biol. 107, 743-751 (1988).

29. Schlessinger, J. Cell Signaling by Receptor Tyrosine Kinases. Cell 103, 211-225 (2000).

30. Yamada, K.M. & Even-Ram, S. Integrin regulation of growth factor receptors. Nat Cell Biol 4, E75-E76 (2002).

31. Hayashi, Y., et al. Integrins Regulate Mouse Embryonic Stem Cell Self-Renewal. Stem Cells 25, 3005-3015 (2007).

32. Brons, I.G.M., et al. Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature 448, 191-195 (2007).

33. Tesar, P.J., et al. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature 448, 196-199 (2007).

34. Yan Li, et al. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. Biotechnology and Bioengineering 91, 688-698 (2005).

35. Lu, J., Hou, R., Booth, C.J., Yang, S.-H. & Snyder, M. Defined culture conditions of human embryonic stem cells. Proceedings of the National Academy of Sciences 103, 5688-5693 (2006).

36. Wang, L., et al. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. Blood 110, 4111-4119 (2007).

37. Liu, Y., et al. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. Biochemical and Biophysical Research Communications 346, 131-139 (2006).

38. James, D., Levine, A.J., Besser, D. & Hemmati-Brivanlou, A. TGF{beta}/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 132, 1273-1282 (2005).

39. Lanctot, P.M., Gage, F.H. & Varki, A.P. The glycans of stem cells. Current Opinion in Chemical Biology 11, 373-380 (2007).

40. Humphrey, R.K., et al. Maintenance of Pluripotency in Human Embryonic Stem Cells Is STAT3 Independent. Stem Cells 22, 522-530 (2004).

41. Amit, M., Shariki, C., Margulets, V. & Itskovitz-Eldor, J. Feeder Layer- and Serum-Free Culture of Human Embryonic Stem Cells. Biol Reprod 70, 837-845 (2004).

42. Hamilton, S.R., et al. Humanization of Yeast to Produce Complex Terminally Sialylated Glycoproteins. Science 313, 1441-1443 (2006).

43. Jones, D., et al. High-Level Expression of Recombinant IgG in the Human Cell Line PER.C6. Biotechnol. Prog. 19, 163-168 (2003).

44. Gerecht, S., et al. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. Proceedings of the National Academy of Sciences 104, 11298-11303 (2007).

45. Derda, R., et al. Defined Substrates for Human Embryonic Stem Cell Growth Identified from Surface Arrays. ACS Chem. Biol. 2, 347-355 (2007).

CHAPTER SIX

Phosphorylation dynamics during early differentiation of human embryonic stem cells

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Abstract

Pluripotent stem cells self-renew indefinitely and possess characteristic protein-protein networks that remodel during differentiation. How this occurs is poorly understood. Using quantitative mass spectrometry, we analyzed the (phospho)proteome of human embryonic stem cells (hESC) during differentiation induced by bone morphogenetic protein (BMP) and removal of hESC growth factors. Of 5222 proteins identified, 1399 were phosphorylated on 3067 residues. Approximately 50% of these phosphosites were regulated within 1 hr of differentiation induction, revealing a complex interplay of phosphorylation networks spanning different signaling pathways and kinase activities. Among the phosphorylated proteins was the pluripotency-associated protein SOX2, which was SUMOylated as a result of phosphorylation. Using the data to predict kinase-substrate relationships, we reconstructed the hESC kinome; CDK1/2 emerged as central in controlling self-renewal and lineage specification. The findings provide new insights into how hESC exit the pluripotent state and present the hESC (phospho)proteome resource as a complement to existing pluripotency network databases.

Introduction

All future applications of pluripotent cells depend on exquisite control of their developmental fate. An essential first step for differentiation is to exit the pluripotent state but exactly how this is regulated is unclear. Recent evidence for a core transcriptional machinery regulating pluripotency[1] has indicated the most important pathways for functional interrogation, but information on the activation state of component proteins is largely unknown. Here, we analyzed the proteome and phosphoproteome of human embryonic stem cells (hESC), prototype pluripotent cells, for this purpose and compared this profile globally with that immediately after induction of differentiation.

ESC are derived from preimplantation embryos, self-renew indefinitely, and can undergo multilineage differentiation[2]. Although mouse ESC (mESC) and hESC have a similar core transcriptional regulatory network, involving OCT4[3], SOX2[4,5], and NANOG[6], they differ in their growth requirements. BMP and leukemia inhibitory factor sustain self-renewal of mESC[7], but hESC require basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β)/Activin A signaling[8,9]. In fact, BMPs rapidly induce differentiation of hESC[10]. In all pluripotent cells however, the pluripotency genes, OCT4, SOX2, and NANOG collaborate by co-occupying the promoters of many genes, including their own, thereby promoting expression of ESC-associated genes and repressing lineage specification genes[11,12].

Recent studies have shown that SOX2 can act synergistically with OCT4 *in vitro* to activate OCT-SOX enhancers, and that SOX2 is necessary for regulating multiple transcription factors affecting OCT4 expression[13]. Exactly how differentiating cells downregulate the transcription factors controlling self-renewal is unclear. Transcriptional regulation of NANOG by TGF-β/Activin A and BMP-responsive SMADs has been demonstrated recently. In undifferentiated hESC, SMAD2/3 dominates through TGF-β signaling, whereas SMAD1/5/8 becomes activated upon BMP-induced differentiation. These SMADs bind the proximal promoter of NANOG with opposing effects; SMAD2/3 promotes but SMAD1/5/8 inhibits its expression[14]. Furthermore, NANOG activity is regulated post-translationally by caspase-mediated proteolytic cleavage, which is associated with differentiation[15]. Although mESC lacking NANOG are prone to differentiate, they still self-renew, suggesting that downregulation of NANOG alone is not sufficient to induce differentiation[16].

Mass spectrometry (MS)-based proteomics is presently the most powerful tool for dissecting stimulus-dependent dynamics of phosphorylation events in living cells[17,18]. We used stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative MS[19] to study early phosphorylation and dephosphorylation events in hESC upon BMP-induced differentiation. Metabolically-labeled hESC were compared quantitatively with unlabeled differentiating cells exposed for various times to BMP. TiO_2-based phosphopeptide enrichment followed by MS identified a large set of proteins phosphorylated in hESC, including SOX2. Mutational analysis suggested that phosphorylation of one or more consecutive serine residues induced

SUMOylation. This post-translational modification (PTM) has been described as inhibiting its transcriptional activity[20]. Using the NetworKIN algorithm[21] we linked the phosphorylation sites to their cognate kinases. This showed that of the multiple active kinases, CDK1/2 had the largest number of substrates, including SOX2. Furthermore, comparison of cells before and after BMP exposure provided a global map of protein phosphorylation dynamics as hESC exit the pluripotent state.

Results

SILAC analysis of hESC and the effect of BMP-induced differentiation

Undifferentiated hESC were collected after 1 week of metabolic labeling with [$^{13}C_6$,$^{15}N_4$]-arginine and [$^{13}C_6$,$^{15}N_2$]-lysine (Figure 6.1). Rapid differentiation of unlabeled hESC was induced by BMP4 addition[10]; cells were harvested at various times thereafter (Figure 6.1). Western blot analysis shows that BMP4-induced differentiation, as previously, resulted in downregulation of OCT4 and SOX2 (Figure S1). Phosphorylation dynamics were studied using an automated approach to enrich for phosphopeptides based on SCX and TiO_2[22] combined with SILAC technology[19] for accurate relative quantitation. Proteins from undifferentiated hESC (0 min) and differentiated cells (30, 60, and 240 min after BMP4 addition) were extracted, combined, and processed for quantitative MS analysis (Figure 6.1). MS intensities of the "light" and "heavy" peaks reflect the relative changes in protein phosphorylation between the two samples analyzed. Collectively, the three SILAC mixtures provided a four-time-point profile of phosphorylation events during early differentiation of hESC (Figure 6.1).

The hESC proteome and phosphoproteome

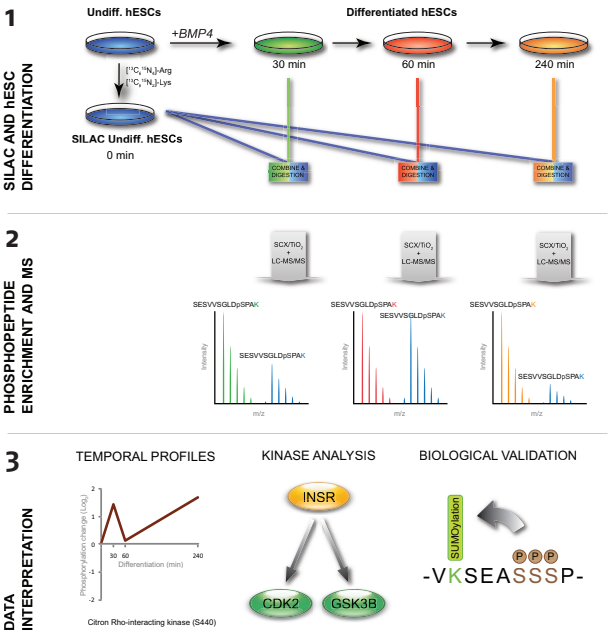
From 800,000 spectra collected over 144 LC-MS/MS runs, 5,222 proteins were identified with high confidence using a highly conservative Mascot Score of 35 at the peptide level (false discovery rate<1%) (Tables S1 and S2). Because protein abundance cannot be predicted accurately from mRNA levels [23], these data corroborated protein presence for many transcripts, and additionally demonstrated the existence of proteins for which there was no direct evidence at the transcriptional level. Of the proteins identified, 1399 (27%) were phosphorylated on one or multiple residues (Figure S2, Table S3 and File S1), making these proteome and phosphoproteome data sets the largest reported to date for hESC. In total, 3090 unique phosphopeptides were detected, carrying 3067 phosphorylation sites localized on 2431 serines, 582 threonines and 54 tyrosines. These proteome profiles represent a unique opportunity to gain insights into the functional protein content and identify active signaling pathways involving these proteins.

The proteins identified were first classified by molecular function, biological function, and subcellular localization (Figure S3). The two most abundant categories (molecular function) consisted of nucleic acid binding proteins (941 proteins, 18.0%) and transcription factors (343 proteins, 6.6%), suggesting that chromatin remodeling, modification, and transcription

Figure 6.1

Experimental design.

(1) Two populations of hESC were used, one labeled with SILAC amino acids [$^{13}C_6$,$^{15}N_4$]-arginine and [$^{13}C_6$,$^{15}N_2$]-lysine. Differentiation of unlabeled hESC was induced by BMP4 and samples collected at 30, 60 and 240 min. Differentiated cells and SILAC labeled-hESC were combined, lysed and enzymatically digested. (2) The three mixtures were subjected to phosphopeptide enrichment based on TiO_2 and SCX prior to high-resolution LC-MS/MS analysis. The peak intensities of the identified phosphopeptides are proportional to their relative abundance. (3) The three SILAC mixtures provide a profile of phosphorylation events over 4 time points after the onset of differentiation. Phosphorylation networks were reconstructed by predicting potential kinases for every phosphosite identified. SUMOylation of the pluripotency transcription factor SOX2 was found to be phosphorylation-dependent.



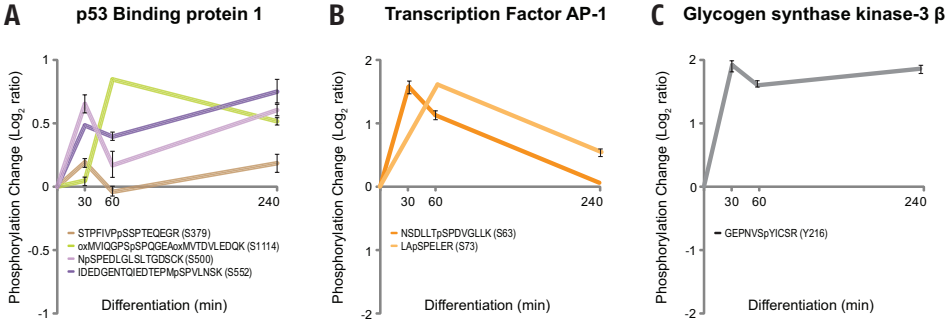


Figure 6.2

Temporal profiles of phosphorylation during differentiation.

Changes in phosphorylation levels are shown for those proteins cited in the text. (A) Specific regulation of four different phosphorylation sites from TP53BP1. S1114 shows a delayed phosphorylation whereas S500 and S552 belong to intermittent profile. S379 is not regulated during differentiation. (B) Two phosphopeptides were identified for JUN, only one (S73, temporal phosphorylation) was quantified in the three time points. (C) Y216 from GSK3β shows a sustained phosphorylation profile. Error bars are shown if the phosphopeptide was quantified more than two times. See Figure S10 for confirmatory western blot and immuno-fluorescence analyses.

are highly active in hESC. Gene ontology (GO) analyses confirmed enrichment of several GO terms related to these categories in our hESC proteome ($P<0.001$, Hypergeometric test) (Table S4) compared to the entire human genome. To evaluate whether this overrepresentation was specific for pluripotent cells, we also classified two proteomes previously reported by our group[24], hESC (HES-2) and their differentiated derivatives (12 days in the absence of feeder cells) (Figure S4). Interestingly, we found a reduction ($P<0.05$, Chi-square test) in the abundance of transcription factors and nucleic acid binding as ESC differentiated indicating that this class of transcriptional and translational proteins is highly represented in ESC. Next we assessed whether proteins identified originated from genomic regions predicted to be active, as indicated by K4 trimethylation of histone 3 (H3K4me3), or silent, marked by trimethylation on K27 (H3K27me3)[25,26]. Our proteome notably correlated with whole-genome analysis of histone 3 methylation in hESC[27] since 2848 proteins in our catalogue carried the activating H3K4me3 mark on their corresponding genes, 279 proteins had both H3K4me3 and H3K27me3 marks, 240 proteins with neither H3K4me3 nor H3K27me3, whereas only 9 were reported as silenced genes (Figure S5 and Table S5).

PHOSPHORYLATION DYNAMICS DURING DIFFERENTIATION

Of the 3090 unique phosphopeptides identified, 1987 were quantified in at least one of the three SILAC mixtures (Figure S6, Table S3 and File S1). We achieved a relative standard deviation (RSD) of 7% (data not shown) by measuring modified variants of the reported phosphopeptides resulting from trypsin missed cleavages or methionine oxidations. On this basis, we found that 1091 phosphopeptides (50% of the quantified phosphoproteome) were regulated during differentiation (Table S3 and Data Set S1). Next, we examined the relationship between protein abundance and its regulation status (i.e. differentially regulated or not), applying total peptide count (including multiple observations of the same peptide within a single experiment). We observed an inverse correlation between frequency and regulation status (Figure S6B), indicating that low-abundant peptides tended to be more regulated than high-abundant peptides. Thirty minutes after BMP4 addition, the level of phosphorylation of 407 proteins was increased, whereas only 25 decreased. Changes of similar magnitude were found at 60 min (506 upregulated, 51 downregulated), and 240 min (622 upregulated, 42 downregulated), suggesting an overall increase in kinase activity. The time course showed a trend in most of the phosphopeptide profiles, showing up- or downregulation at minimally two consecutive time points, suggesting the robust- ness of the data set. Second, we confirmed phosphorylation profiles for GSK3β and 4EIFEBP1 by immunofluorescence and western blotting, the method of choice being dictated by the properties of available antibodies (Figure S10).

To gain insight into temporal regulation during differentiation, phosphopeptides quantified at all time points and significantly differing in phosphorylation in at least one of the four time points were grouped by k-means clustering (Figure S7 and Table S6). Two phenomena become apparent from these data. First, the most dramatic phosphorylation changes took place during the first hour, coincident with SMAD1/5/8 phosphorylation in hESC[10] and no

phosphopeptides were regulated after 4 h only, indicating that events are initiated by BMP4 within this time frame. Second, apart from a small group of dephosphorylation events (cluster "dephosphorylation" in Figure S7), most changes represent an increase in phosphorylation level. In several cases, the same proteins occupied more than one cluster, indicating that regulation of phosphorylation is site-specific, and that individual sites may be affiliated with different functions. This is congruent with the idea that proteins can serve as platforms for integrating signals from various kinases/cascades[28]. One example is tumor suppressor p53-binding protein 1 (TP53BP1), a hyper-phosphorylated transcription activator engaged in the response to DNA damage[29], checkpoint signaling during mitosis (Swiss-Prot by similarity) and hESC differentiation[27]. Three phosphopeptides identified for TP53BP1 were regulated with different kinetics since they belonged to different profile clusters (delayed and intermittent) (Figure 6.2, Table S6).

SIGNALING PATHWAYS ACTIVATED BY BMP4

The coverage of signaling pathways was assessed by projecting protein identification and quantitative phosphorylation on 130 signaling pathways in Panther[30]. Components of nearly all pathways were represented in our data (Figure 6.3A and Figure S8), 86 of which contained between 1 and 79 phosphorylated proteins. Seventy pathways were represented by proteins with dynamic phosphorylation. (Table S7). This suggests the activation of a protein network composed of multiple signaling cascades. To investigate this in more detail, we considered key components of individual pathways and looked at whether observed phosphorylation sites were those known to be involved in protein activation. Activation of the BMP-pathway was indicated by phosphorylation of receptor-SMADs, SMAD5 and SMAD8 at S465 and S467, respectively, confirming activation of the BMP4-SMAD signaling cascade in hESC (Figure S9A,B) as previously[10].

Several components of PI3K signaling were identified (Figure 6.3 and Figure S10). Increased phosphorylation of PDPK1 at S241 indicated activation of this kinase for subsequent phosphorylate of its target AKT1 (PKB). Additionally, an increase in phosphorylation of various proteins with confirmed AKT1 target sites was observed, including S166 of MDM2, S280 of CHK1 (both of which lead to inhibition of p53-mediated apoptosis) and S341 and S588 of TBC1D4 (also known as AS160). These all point to activation of AKT1.

c-Jun, which forms the heterodimeric activator protein 1 transcription factor complex with c-Fos, was phosphorylated at S63 and S73, suggesting activation of the JNK pathway. Interestingly, the immediate phosphorylation of c-Jun after BMP activation at 30 min was followed by a decrease over the next hours (Figure 6.2B), indicated that JNK activation was transient. Wnt signaling is one of the best-covered pathways in our dataset and included multiple proteins with phosphorylation changes (Figure S9C). Amongst these was an activating phosphorylation at Y216 of GSK3B that was induced within 30 min of BMP addition (Figure 6.2B). Phosphorylation of this tyrosine over time was confirmed in a biological repeat on cell lysates using western blotting and at the single-cell level using immunofluorescent

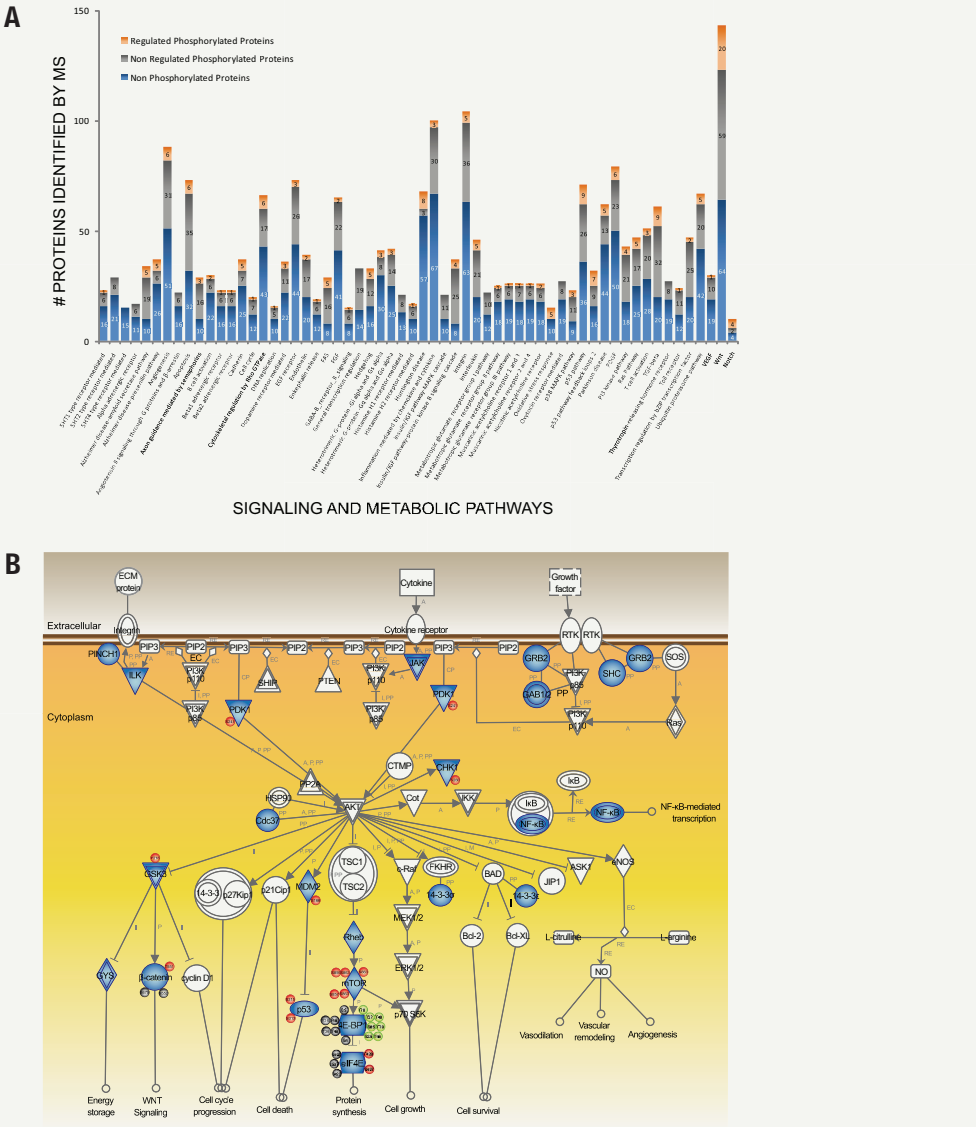


Figure 6.3

Signaling pathways analysis

(A) Nonphosphorylated proteins, nonregulated phosphoproteins, and regulated phosphoproteins were mapped to 130 pathways with Panther classification system [30]. Only those pathways with a relative high coverage are shown. The complete analysis can be found in Table S7 and Figure S8. (B) Graphical representation of PI3K/Akt signaling pathway. Identified proteins by MS are represented in blue color. Identified phosphosites are also shown when applicable (red, upregulated site; green, downregulated; grey, not regulated or not quantified). Some examples of other pathways can be found in Figure S9.

antibody staining (Figure S10). Furthermore, several peptides of the key Wnt signaling protein, β-catenin, were identified. However, the N-terminal peptide marking activity when phosphorylated was not detected so that activation status of β-catenin cannot be determined from our data directly. However, several co-activators (BCL9, BCL9L, PYGO2) and repressors (TLE1, TLE3)[31] of β-catenin were differentially phosphorylated at several positions, indicating some level of regulation of Wnt signaling. Moreover, phosphorylation of casein kinase I, which phosphorylates β-catenin, was increased on at least two sites. Combined, these data implied rapid activation of the Wnt pathway at the onset of BMP-induced differentiation.

Many other phosphorylation events were observed, but their effect on the activation of the proteins affected are largely unknown. Nonetheless, their multitude and diversity, as well as the fact that some of them are shared by several pathways (e.g. G3BP1 and 2)[32] indicated that induction of differentiation by BMP4 initiates signaling via an intricate network that vigorously alters the behavior of hESC.

ADDITIONAL EFFECTS OF BMP4

To investigate the effects of BMP4 induction beyond kinase signaling, proteins with altered phosphorylation levels (>0.5 fold, $\log_2$ scale) were mapped to GO terms with respect to molecular function (Table S8) and biological process (Table S9). These analyses showed the broad range of protein classes affected by BMP4, indicating that its effects extended well beyond those proteins participating in established signal transduction cascades. Some of the processes are related to differentiation (e.g. alteration of cell cycle kinetics or cell cycle exit and initiation of development); however, it was unclear exactly how the individual components were affected. To address this, the same set of phosphoproteins was subjected to an unbiased analysis using Anni[33], an ontology-based interface that applies text mining to retrieve associations between proteins by co-occurrence in the literature. This tool classifies proteins by a term or "concept" they have in common, which is not necessarily a GO term. Although some of the 39 clusters identified in this manner (Figure S11 and Table S10) were also defined by GO analysis (e.g. cell cycle, cytoskeleton), many additional "concepts" emerged, such as transcriptional repression, transcriptional elongation factors, translation initiation factors, and SWI/SNF chromatin remodeling[34]. This implies that established mechanisms associated with differentiation, like genome remodeling, silencing of the pluripotency network, and activation of differentiation inducing genes, are initiated rapidly after induction. Additionally, the presence of a protein SUMOylation cluster indicated active post-translational processing of proteins. Furthermore, clusters containing Rab11 family-interacting proteins, microtubule associated proteins, VIMENTIN, GTPase-activating proteins, tight junctions and focal adhesions strongly suggested remodeling of the cellular matrix.

PHOSPHORYLATION OF PLURIPOTENCY-ASSOCIATED PROTEINS

To determine the phosphorylation status of proteins associated with pluripotency, we mapped MS identification to ESC-associated genes defined by the International Stem Cell Initiative[35]. From the top 20 genes with a NANOG pairwise correlation coefficient >0.5, we detected the protein

TABLE 6.1 hESC MARKERS AND PHOSPHOSITES (regulated sites in bold)

IPI Number	Gene Symbol (Protein Name)	Phosphosites
IPI00002948	LIN28 (Lin-28 homolog A)	**S3**, S200, T202
IPI00012593	DNMT3B (Isoform 1 of DNA(cytosine-5)-methyltransferase 3B)	**S82**, S136, S202, **S209**, T383, S387
IPI00300620	IFITM1 (Interferon-induced transmembrane protein 1)	
IPI00219089	POU5F1 (Isoform A of POU domain, class 5, transcription factor 1; OCT4)	
IPI00009703	SOX2 (Transcription Factor SOX2)	S249, S250, S251
IPI00026637	GAL (Galanin precursor)	**S116**
IPI00020668	UTF (Undifferentiated embryonic cell transcription factor 1)	S18, **T35**, **S245**
IPI00007164	FOXD3 (Forkhead box protein D3)	
IPI00299659	GDF3 (Growth/differentiation factor 3 precursor)	
IPI00045497	NODAL (Nodal homolog precursor)	
IPI00299116	PDXL (Podocalyxin-like protein 1 precursor)	
IPI00434539	NANOG (Isoform 1 of Homeobox protein NANOG)	

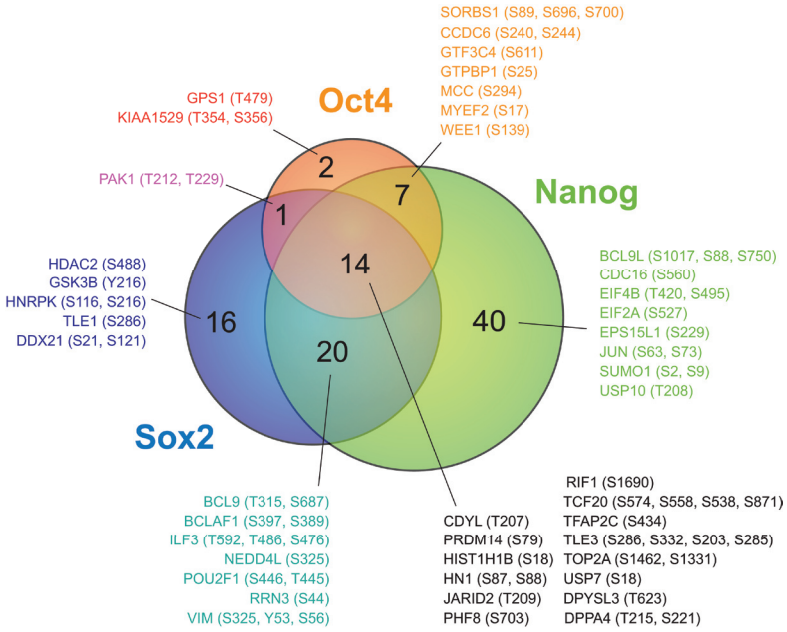


Figure 6.4

Downstream targets of the core transcriptional regulatory circuitry regulated by phosphorylation

Venn diagram representing the overlap of OCT4, SOX2 and NANOG target genes differentially phosphorylated during differentiation. Examples of regulated phosphosites are shown for every section of the diagram. The full list of proteins can be found in Table S11.

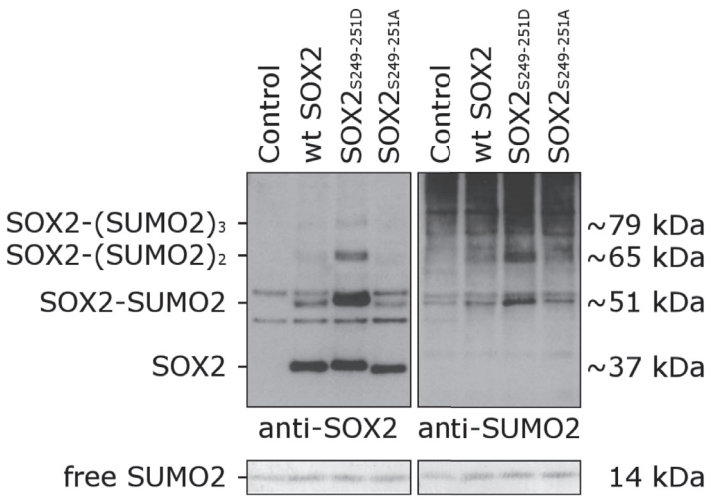


Figure 6.5

Phosphorylation-dependent SUMOylation of SOX2.

HeLa cells stably expressing His_6-SUMO2 ($HeLa^{SUMO2}$) were transfected with pCAG expression constructs harboring either GFP (Control), wt Sox2, $Sox2_{S249-251D}$, or $Sox2_{S249-251A}$. Cells were lysed and His_6-SUMO2 conjugates were enriched on nickel-nitrilotriacetic acid-agarose beads. Purified fractions were subjected to SDS-PAGE, and immunoblotted using antibodies to detect Sox2 or SUMO2/3. These results indicate that SUMOylation of SOX2 depends on phosphorylation at S249-251).

products of 12, including the three core transcription factors OCT4, SOX2, and NANOG (Table 1). Sixteen novel phosphosites were identified in five of these proteins; among these were three residues of UTF1, three residues of LIN28, seven residues of DNMT3B, and three consecutive residues of SOX2. During differentiation, the phosphorylation state changed for 6 of the sites identified, suggesting that the ESC-associated proteins to which they belong are regulated post-translationally. To find differential phosphorylation sites of downstream targets for OCT4, SOX2, and NANOG, we mapped the phosphoproteomes to chromatin immunoprecipitation data sets published for OCT4, SOX2 and NANOG[11]. Of the 2,260 genes reported, we found 586 products, 100 of which were phosphoproteins regulated during differentiation, including 19 transcriptional regulators (Figure 6.4 and Table S11). Interestingly, these phosphorylated proteins are predominantly components of the RNA post-transcriptional modification (e.g. SFPQ, DDX20, ADAR, ACIN1, SFRS7) and the gene expression machinery (e.g. POU2F1, SFPQ, PAK1, STAT3, SUMO1, SOX2, HIST1H1). These results suggested that many components of the hESC core transcriptional circuitry are regulated by phosphorylation, and that differentiation is accompanied by active modulation of these processes.

PHOSPHORYLATION-DEPENDENT SUMOYLATION OF SOX2

Our MS results showed that SOX2 can be phosphorylated at three consecutive serine residues: S249, S250, and S251 (Table S3 and File S1). This serine triplet flanks an upstream SUMOylation site of SOX2[20], the combination of which resembles the phosphorylation-dependent SUMOylation motif (PDSM), YKxExxSP, described in other SOX family members[36]. We hypothesized that SUMOylation of SOX2 depends on phosphorylation of one or more of these serine residues in a similar manner. To address whether crosstalk occurs between these phosphorylation and SUMOylation sites, a set of SOX2 mutants was created, and their SUMOylation state investigated using HeLa cells stably expressing SUMO2[37] (Figures 6.5, S12A and S12B). Expression of wt human SOX2 in these cells showed a low basal level of SUMOylated SOX2 compared with free SOX2 (Figure 6.5 and S12B). However, when the serine residues were replaced by aspartic acid ($SOX2_{S249-251D}$) to mimic constitutive phosphorylation, high levels of the SUMOylated SOX2 mutant were observed; including double and triple SUMOylated forms (Figure 6.5 and S11B). On the other hand, replacing the serine residues by alanine ($SOX2_{S249-251A}$) showed a ratio between the free and SUMOylated form similar to that of wt SOX2 (Figure 6.5 and S12B). Furthermore, replacing K245 of the PDSM by alanine ($SOX2_{K245A}$) completely prevented SUMOylation of the transcription factor (Figure S11B), indicating that this lysine residue is the only target for SUMOylation under these conditions. Notably, none of these mutations affected the nuclear localization of SOX2 in HUES-7 cells (Figure S11C), which suggested that phosphorylation of these serine residues did not initiate immediate export of SOX2 from the nucleus.

THE hESC KINOME NETWORK

Consensus motifs of phosphorylation sites are inappropriate for systematic matching with their corresponding kinases as specificity is largely determined by context[21]. Linding et al. addressed this issue by developing an algorithm (NetworKIN) which introduces the novel concept of combining probabilistic modeled network context with linear motifs recognized by the catalytic domain of kinases[21]. Relationships between kinases and all the phosphorylation sites defined here were predicted, creating for the first time a kinase-substrate database for hESC (Table S12). This was used to create an *in vivo* kinome for hESC (Figure 6.6A), comprising 107 kinases, representing most of the known kinase families[38]. Interestingly, CDK1/2 appeared to play a prominent role, since it was predicted to phosphorylate ~1,200 peptides in our assay. To investigate this further, all kinases were predicted for Phospho.ELM[39] and PhoshoSite (www.phosphosite.org), two publically available repositories of human phosphorylation sites, with >23,000 unique phosphosites. By comparing this hypothetical absolute kinome with the hESC-specific kinome, kinases that are under- or over-represented in hESC, based on the incidence of specific phosphorylations, could be identified (Figure S13). For instance, CDK1/2 was found to mediate 26% of the phosphorylation events in hESC, which is significantly more ($P<0.0001$, Chi-square test) than the 12% of all theoretical phosphorylation sites in humans. Additional kinases with activities over-represented in hESC were MAPK8, MAPK11, MAPK14, TGFBR2, GSK3B, and NEK2, the latter being a known modulator of the cell cycle, just as CDK1/2. Furthermore, CDK1/2, NEK2, and GSK3B are potential effectors of SOX2 phosphorylation (Table

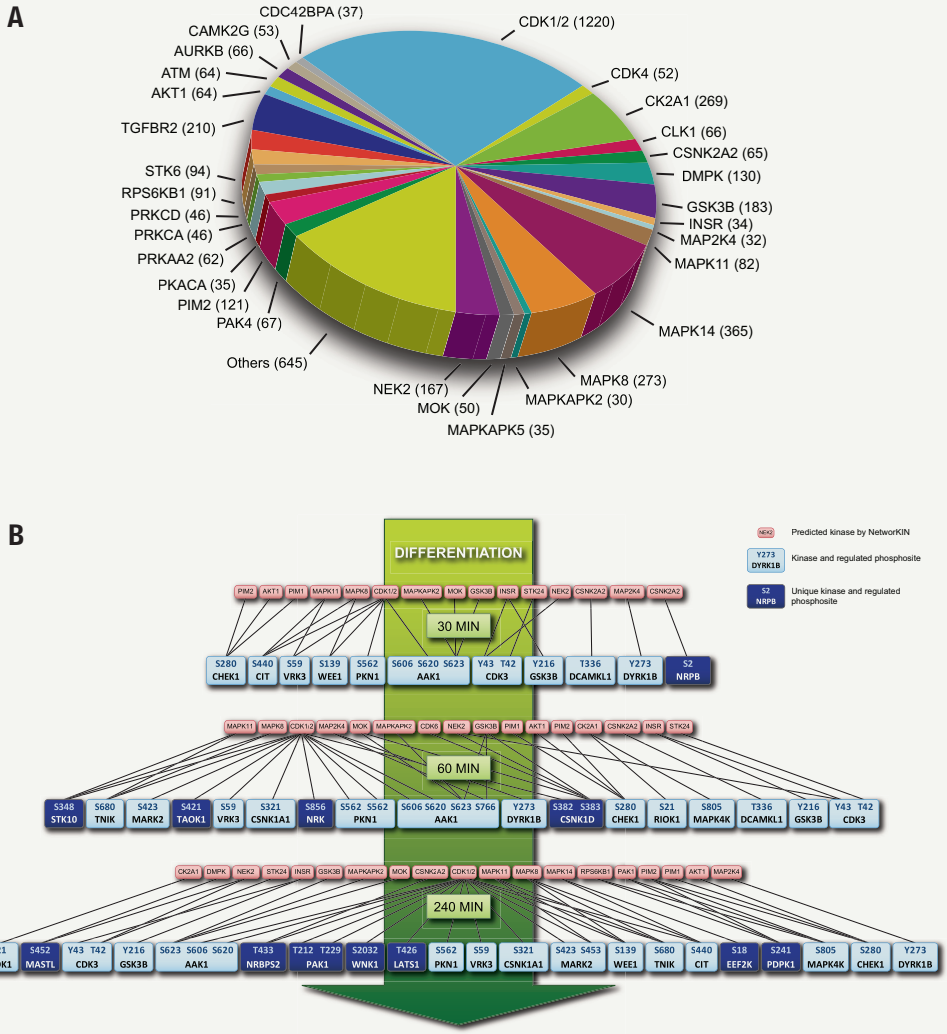


Figure 6.6

Phosphorylation networks during hESC differentiation

The NetworKIN algorithm was used to predict kinases for every phosphorylation site identified. (A) In total, 107 kinases were predicted to regulate the hESC phosphoproteome; the number of substrates is indicated in the pie chart. Kinases predicted to phosphorylate <30 substrates are shown combined in "others". (B) By linking regulated kinases (blue boxes) to their predicted upstream kinases (red boxes), kinase cascades during hESC differentiation were modeled. Kinases regulated at only one time point are depicted as dark blue boxes. This illustrates how a signal spreads over time.

S12). These findings suggest that prevalent kinases control cell cycle processes as well as the activity of pluripotency-associated transcription factors, both of which are characteristic of ESC. The MS data indicated that CDK2 is phosphorylated progressively at Y15, marking a decrease in its activity[40]. Linking site-specific kinases that were found regulated over the investigated time course with their corresponding upstream kinases, generated a temporal kinase-cascade model that illustrates the dynamics in kinase relationships during differentiation (Figure 6.6B). This overview suggested that the initial signal that disturbs the ESC-associated network expands over time by activating an increasing number of kinases, resembling the model proposed previously[41].

Discussion

Self-renewal in pluripotent hESC is highly sensitive to factors that trigger differentiation through transmission of signals to the nucleus. Activation and inactivation of intracellular proteins by phosphorylation and dephosphorylation are among the earliest events following binding of a ligand to its receptor. In addition to regulating protein activity, phosphorylation also contributes to controlling cell identity by fine-tuning protein expression. We used MS-based quantitative proteomics here to monitor dynamic changes in the phosphoproteome of hESC at the onset of BMP4-induced differentiation. We probed the hESC proteome to a depth of 5222 proteins, 586 of which are thought to be regulated by the core transcriptional network. Since this core transcriptional network was postulated on the basis of ChIP data and transcription factor binding does not necessarily mean that the particular gene is regulated by that factor; our dataset provides high quality confirmation of the involvement of many proposed players. GO annotation of the hESC proteome resulted in prominent subclasses consisting of proteins associated with epigenetic modification[27], transcription[11,42], and translation[43], suggesting that these processes are prominent in hESC. Among the 1399 proteins phosphorylated on a total of 3067 residues, more than 1200 sites had not been reported previously to our knowledge, 430 belonging to the class of proteins that participate in the core transcriptional regulatory circuitry[11]. This implies that the activity of many proteins involved in the pluripotency and self-renewal network is regulated by a selection of kinases and phosphatases.

Interruption of self-renewal by exogenous factors has major effects on signaling networks that sustain the undifferentiated state. Changing the identity of hESC by exposure to extracellular stimuli is immediately followed by a complete reconstitution of signaling pathways. This was exemplified by the observation that half of the phosphoproteome was modulated within the first hour of BMP4 exposure. In addition to the expected phosphorylation of receptor SMADs, increased phosphorylation of several substrates of AKT1 were detected, indicating activation of the PI3K/AKT pathway. This might have resulted from signaling through the insulin receptor[44], as hESC differentiated in medium containing insulin as a survival factor. In addition, S63 and S73 of c-Jun were phosphorylated, though temporarily, suggesting transient activity of JNKs. Although cross-talk between intracellular pathways cannot be deduced from the present data,

there is evidence for reciprocal interactions between JNKs and AKT. JNKs can phosphorylate AKT1 at T450, thus priming this protein for activation through phosphorylation by PDK1[45]. Conversely, AKT1 can inhibit the JNK pathway by phosphorylating ASK1 (MAPKKK5), which lies upstream of JNKs. This apparent dichotomy is in agreement with our data, as phosphorylation of c-Jun was early and transient, whereas phosphorylation of AKT substrates was sustained. This would tip the balance of pro-apoptotic (JNKs) and anti-apoptotic signaling (AKT) in favor of the latter.

In addition to changes in signaling pathways, we also found alterations in established and proposed regulators of pluripotency. For instance, a novel acetylation and three novel phosphorylation sites on LIN28 were identified. Ectopic expression of LIN28 contributes to formation of iPS cells, illustrating the importance of this pathway in regulating the pluripotent state[46]. Furthermore, LIN28 is known to block maturation of primary pri-Let-7g transcripts to mature Let-7g transcripts[47]. This led to the hypothesis that Let-7 and LIN28 participate in an incoherent feed-forward loop that contributes to rapid differentiation[48]. Another example is the detection of three novel phosphorylation sites on the transcription factor UTF1, a chromatin-associated transcriptional repressor crucial for differentiation of mESC[49]; two of the phosphorylation sites were regulated upon differentiation. For DNMT3B, six novel phosphorylation sites were identified, two of which were regulated. Upon differentiation, methyltransferases DNMT3A and DNMT3B are recruited to the OCT4 promoter, thereby repressing its transcription through CpG methylation[50]. We also identified three novel consecutive phosphosites on SOX2, which we investigated in more depth.

The transcription factor SOX2 is one of the three core transcription factors that play essential roles in embryonic development[4,5] and self-renewal of ESC[13,51]. However, little is known about how the activity of SOX2 itself is regulated. Whereas SUMO-conjugation was found to inhibit binding of mouse Sox2 to DNA[20], the PDSM defined initially in Sox3, Sox8, and Sox9 amongst other transcriptional regulators, had not been identified in Sox2[36]. This can be explained by a surplus residue between E247 and S251, which does not match the established consensus sequence[36]. Combined, our findings indicated that the consensus of this PDSM is rather flexible and suggested that the three adjacent serine residues immediately upstream of P252 have redundant functions. Moreover, they imply that transcriptional activity of SOX2 is regulated by SUMOylation that results from phosphorylation. Interestingly, all phosphorylated forms of SOX2 were identified in hESC, but none increased significantly upon differentiation, suggesting that transcriptional activity of SOX2 is controlled stringently both in differentiating and undifferentiated ESC. A critical level of functional SOX2, essential for self-renewal[52,53], is probably maintained by a fine balance between de novo synthesis, PTMs that alter its activity, and translocation from the nucleus followed by degradation. Of note is that all SOX2 mutants were localized predominantly in the nuclei of HUES-7 transfectants. This implies that phosphorylation of SOX2, as mimicked by the S249-251D mutation, and the consequent SUMOylation at K245, does not immediately lead to massive nuclear export and subsequent targeting for degradation. Presumably, the SUMOylation pathway is saturated

when SOX2 is overexpressed in HUES-7 cells, requiring concomitant overexpression of SUMO in order to detect SUMOylated forms of SOX2, as observed for SUMO2 overexpressing HeLa cells. Further investigation is needed to determine the balance between de novo synthesis versus degradation of SOX2, and the role of PTMs during its life span.

Using the NetworKIN algorithm to predict the kinases responsible for phosphorylation during differentiation, we reconstructed a hESC kinome composed of 107 kinases, 26 of which were regulated by phosphorylation upon BMP4 stimulation. Because of their low abundance, only 54 phosphotyrosine peptides were identified, consequently receptor Tyr Kinases (e.g. INSR, EPHA4 and IGFIR) seem under represented. In contrast, 2,431 phosphoserine peptides were identified, pointing to a central role for CDK1/2. In addition to CDK1/2 and GSK3B, MAPK8 (a JNK protein), as well as MAPK11 and MAPK14 (p38 MAPK proteins) of the CMGC Ser/Thr protein kinase family[38], have well-documented functions in differentiation. p38 MAPKs are inhibitory during cell commitment and are anti-apoptotic during late stages of differentiation, whereas the pro-apoptotic JNKs are involved in ectoderm and primitive endoderm differentiation[54]. However, protein phosphorylation is regulated by a precise control of protein kinase (PKs) and protein phosphatase (PPs) activities. The latter are regulated, in the same way as kinases, by an array of targeting and regulatory subunits, PTMs and by specific inhibitors. A total of 43 PPs (30% of all phosphatases reported in SwissProt) were identified in our data set, 4 of them presented differentially regulated phosphosites: PPMH1 (Ser/Thr PPs), DUSP19 (Dual Specificity PPs) and, PTPN13 and PTPN14 (Tyr PPs). Both PKs and PPs possess basal activities; therefore we should take into account that some of the up-regulated phosphopeptides observed in our study could result from an inhibition of their specific PPs, and not because of an increase of the kinase activity. The same applies for down-regulated phosphopeptides.

It is not surprising that most dynamic phosphorylation of signaling cascade components occurred within 30 min of BMP4 exposure since similar kinetics had been observed by others studying EGF stimulation in HeLa cells[28]. The multiplicity of components of the network in hESC, and their initiation upon differentiation is far too complex to be covered in entirety here. Nevertheless, the multitude and variety of proteins that undergo phosphorylation changes during the first hours of differentiation suggests rapid and dramatic reorganization of the proteome that extends far beyond signaling alone. Proteins affected were classified based on GO-annotation and contextual co-occurrence in literature. These included methylation, transcription initiation, and transcriptional elongation, indicating that silent developmental genes controlled by OCT4, SOX2, and NANOG experience complex transcriptional regulation[55]. These genes are occupied by nucleosomes with histone H3K4me3 and histone H3K27me3. Although transcription is initiated, there is no productive elongation because of repression by PcG proteins[56,57]. Transcriptional repression of ESC-associated genes and SWI/SNF chromatin remodeling are also concepts associated with differentiation. Furthermore, the emergence of a protein SUMOylation cluster (consisting of E3 ligases, hydrolase and SUMO-isopeptidase) suggests that this type of PTM is a concept linked to differentiation that extends beyond the effects on SOX2 described above. Finally, the presence of clusters containing Rab11 family-

interacting proteins, microtubule-associated proteins, VIMENTIN, GTPase-activating proteins, tight junctions, and focal adhesion proteins suggested that initiation of differentiation leads to remodeling of cell shape. We had shown previously that hESC grown as monolayer cultures under feeder-free conditions (i.e. identical to those used in this study) have an epithelial phenotype, evidenced by the expression of proteins belonging to adherence junctions, tight junctions, gap junctions, and desmosomes[58]. The observation that proteins forming these structures experience increased phosphorylation strongly suggests that their biogenesis changes [59] during a process that is initiated at the onset of differentiation.

Concluding remarks

In addition to an extensive profile of the hESC proteome, our approach generated a dynamic map of protein phosphorylation during differentiation of hESC showing concordance over time, which we used to define a wide spectrum of novel kinase substrates. These data provide a rich resource for further investigation of the function of individual proteins, as exemplified by the phosphorylation sites of SOX2 that regulate its transcriptional activity through SUMOylation. Linking genomic, epigenomic, transcriptomic, and proteomic approaches will improve our knowledge of stem cell biology and (human) development and, thereby, our ability to control self-renewal versus differentiation fate decisions by pluripotent cells.

Experimental Procedures

SILAC labeling of hESC and differentiation

HUES-7 hESC were cultured as previously[60] on Matrigel without MEFs and differentiation induced by 50 ng/ml of BMP4. For details see Supplementary Experimental Procedures.

Western blotting

Western blotting was carried out as previously[24]. For details see Supplemental Methods.

Phosphopeptide enrichment and mass spectrometric analysis

1 mg of protein was first reduced/ alkylated and digested with Lys-C. The mixture was then diluted 4-fold to 2 M urea and digested further with trypsin. Strong cation exchange was performed as before[22]; 24 fractions (1 min each, i.e. 50 µl elution volume) were collected by hand. The online TiO_2 chromatography was set using a triple stage precolumn and both TiO_2 eluate and Flow-Through fractions were chromatographically resolved using a 100 min linear gradient in the analytical column. The LTQ-Orbitrap was operated in data-dependent mode, automatically switching between MS and MS/MS. Full scan MS spectra (m/z 400-1,500) were acquired in the Orbitrap with a resolution of 60,000 while the two most intense ions were selected for MS/MS fragmentation in the linear ion trap. Further details can be found in Supplemental Experimental Procedures.

PEPTIDE IDENTIFICATION AND QUANTITATIVE ANALYSIS

Raw data were converted to single DTA files using Bioworks 3.2 and merged into Mascot Generic Format files which were searched using an in-house licensed Mascot v2.2 search engine against IPI human 3.28 database (containing 68,020 sequences). Carbamidomethyl cysteine was set as fixed modification; protein N-acetylation, oxidized methionines, serine, threonine and tyrosine phosphorylation, deamidation and SILAC-labels ([$^{13}C_6$,$^{15}N_4$]-arginine and [$^{13}C_6$,$^{15}N_2$]-lysine) were set as variable modifications. The mass tolerance of the precursor ion was 10 ppm, and 0.9 Da for fragments ions. The false discovery rate was determined as <1% (Mascot score threshold of 35) using the decoy database approach. MSQuant (http://msquant.sourceforge.net) was used to quantitate the levels of the identified phosphopeptides and determine the exact phosphorylation site within the peptide[28]. Every phosphopeptide quantitation was manually validated; peptides with low signal/noise ratios, low number of MS scans or overlapping peaks were not included for quantitative purposes. StatQuant, an in-house developed java software was used to correct for the arginine to proline conversion artifacts[61], statistical analysis, comparison and normalization.

CONSTRUCTION OF HUMAN SOX2 MUTANTS

Wild-type human SOX2 was amplified by PCR using 5′-GGAATTCTCGGCGGCCGCCGGCGG-3′ in combination with5′-GGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCCATGTGTGAGAGGGGCAGTGTGC-3′ (EcoRI site underlined; myc-tag sequence italicized), and pcDNA3.1/Zeo-hSOX2 (a gift from Dr. N.A. Hanley) as template. The myc-tagged human SOX2 PCR fragment was ligated into pGEM-T, and sequenced to check for errors. Mutations were introduced by digestion of this pGEM-hSOX2-myc plasmid with BpII, followed by ligation of synthetic double-stranded oligos outlined in Table S13, and sequencing to verify the mutations. Each human SOX2 construct was isolated from the different pGEM-hSOX2-myc variants by digestion with EcoRI, and ligated into pCAG-GFP-IRES-Puro^{r} [60] linearized using EcoRI, thus replacing the existing GFP open reading frame. The resulting pCAG-hSOX2-myc mutants were sequenced to confirm correct orientation behind the pCAG promoter.

TRANSFECTION AND IMMUNOFLUORESCENT ANALYSIS OF HUES-7 CELLS

HUES-7 cells were transfected as described previously[60] with the pCAG-hSOX2-myc variants, and fixed after 48 h. Fixed cells were stained using specific antibodies. For details see Supplementary Experimental Procedures.

Culture and transfection of HeLa cells, and purification of His_6-SUMO2-conjugated proteins HeLa cells, stably expressing His_6-SUMO2 were cultured as described previously[62], and transfected with the pCAG-hSOX2-myc variants using polyethyleneimine. The following day, cells were lysed and His_6-SUMO2 conjugates purified on nickel-nitrilotriacetic acid-agarose beads (Qiagen) as described previously[63].

SUPPLEMENTAL DATA

Supplemental Data contains Supplemental Experimental Procedures, 13 Supplemental Figures,

13 Supplemental Tables, one Supplemental File and Supplemental references which can be found on-line at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00230-6.

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References

1. Muller, F.J., et al. Regulatory networks define phenotypic classes of human stem cell lines. Nature 455, 401-405 (2008).
2. Thomson, J.A., et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147 (1998).
3. Niwa, H., Miyazaki, J. & Smith, A.G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 24, 372-376 (2000).
4. Avilion, A.A., et al. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17, 126-140 (2003).
5. Yuan, H., Corbi, N., Basilico, C. & Dailey, L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev 9, 2635-2645 (1995).
6. Mitsui, K., et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113, 631-642 (2003).
7. Ying, Q.L., Nichols, J., Chambers, I. & Smith, A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115, 281-292 (2003).
8. Vallier, L., Alexander, M. & Pedersen, R.A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 118, 4495-4509 (2005).
9. Xu, R.H., et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2, 185-190 (2005).
10. Pera, M.F., et al. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 117, 1269-1280 (2004).
11. Boyer, L.A., et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122, 947-956 (2005).
12. Loh, Y.H., et al. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38, 431-440 (2006).
13. Masui, S., et al. Pluripotency governed by Sox2 via regulation of OCT4 expression in mouse embryonic stem cells. Nat Cell Biol 9, 625-635 (2007).
14. Xu, R.H., et al. NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell 3, 196-206 (2008).

15. Fujita, J., et al. Caspase activity mediates the differentiation of embryonic stem cells. Cell Stem Cell 2, 595–601 (2008).

16. Chambers, I., et al. Nanog safeguards pluripotency and mediates germline development. Nature 450, 1230–1234 (2007).

17. Gruhler, A., et al. Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. Mol Cell Proteomics 4, 310–327 (2005).

18. Kratchmarova, I., Blagoev, B., Haack-Sorensen, M., Kassem, M. & Mann, M. Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science 308, 1472–1477 (2005).

19. Ong, S.E., et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1, 376–386 (2002).

20. Tsuruzoe, S., et al. Inhibition of DNA binding of Sox2 by the SUMO conjugation. Biochemical and Biophysical Research Communications 351, 920–926 (2006).

21. Linding, R., et al. Systematic discovery of in vivo phosphorylation networks. Cell 129, 1415–1426 (2007).

22. Pinkse, M.W., et al. Highly robust, automated, and sensitive online TiO2-based phosphoproteomics applied to study endogenous phosphorylation in Drosophila melanogaster. J Proteome Res 7, 687–697 (2008).

23. Gygi, S.P., Rochon, Y., Franza, B.R. & Aebersold, R. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19, 1720–1730 (1999).

24. Van Hoof, D., et al. A quest for human and mouse embryonic stem cell-specific proteins. Mol Cell Proteomics 5, 1261–1273 (2006).

25. Barski, A., et al. High-resolution profiling of histone methylations in the human genome. Cell 129, 823–837 (2007).

26. Mikkelsen, T.S., et al. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448, 553–560 (2007).

27. Pan, G., et al. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. Cell Stem Cell 1, 299–312 (2007).

28. Olsen, J.V., et al. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648 (2006).

29. Botuyan, M.V., et al. Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. Cell 127, 1361–1373 (2006).

30. Mi, H., Guo, N., Kejariwal, A. & Thomas, P.D. PANTHER version 6: protein sequence and function evolution data with expanded representation of biological pathways. Nucleic Acids Res 35, D247–252 (2007).

31. Chen, G. & Courey, A.J. Groucho/TLE family proteins and transcriptional repression. Gene 249, 1–16 (2000).

32. Irvine, K., Stirling, R., Hume, D. & Kennedy, D. Rasputin, more promiscuous than ever: a review of G3BP. Int J Dev Biol 48, 1065–1077 (2004).

33. 33. Jelier, R., et al. Anni 2.0: a multipurpose text-mining tool for the life sciences. Genome Biol 9, R96 (2008).

34. Jaenisch, R. & Young, R. Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132, 567–582 (2008).

35. Adewumi, O., et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol 25, 803–816 (2007).

36. Hietakangas, V., et al. PDSM, a motif for phosphorylation-dependent SUMO modification. Proc Natl Acad Sci U S A 103, 45–50 (2006).

37. Vertegaal, A.C., et al. Distinct and overlapping sets of SUMO-1 and SUMO-2 target proteins revealed by quantitative proteomics. Mol Cell Proteomics 5, 2298–2310 (2006).

38. Manning, G., Whyte, D.B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. Science

298, 1912-1934 (2002).

39. Diella, F., et al. Phospho.ELM: a database of experimentally verified phosphorylation sites in eukaryotic proteins. BMC Bioinformatics 5, 79 (2004).

40. Gu, Y., Rosenblatt, J. & Morgan, D.O. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J 11, 3995-4005 (1992).

41. Miller-Jensen, K., Janes, K.A., Brugge, J.S. & Lauffenburger, D.A. Common effector processing mediates cell-specific responses to stimuli. Nature 448, 604-608 (2007).

42. Efroni, S., et al. Global transcription in pluripotent embryonic stem cells. Cell Stem Cell 2, 437-447 (2008).

43. Sampath, P., et al. A hierarchical network controls protein translation during murine embryonic stem cell self-renewal and differentiation. Cell Stem Cell 2, 448-460 (2008).

44. Freund, C., et al. Insulin redirects differentiation from cardiogenic mesoderm and endoderm to neuroectoderm in differentiating human embryonic stem cells. Stem Cells 26, 724-733 (2008).

45. Shao, Z., et al. c-Jun N-terminal kinases mediate reactivation of Akt and cardiomyocyte survival after hypoxic injury in vitro and in vivo. Circ Res 98, 111-118 (2006).

46. Yu, J., et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917-1920 (2007).

47. Viswanathan, S.R., Daley, G.Q. & Gregory, R.I. Selective blockade of microRNA processing by Lin28. Science 320, 97-100 (2008).

48. Marson, A., et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell 134, 521-533 (2008).

49. van den Boom, V., et al. UTF1 is a chromatin-associated protein involved in ES cell differentiation. J Cell Biol 178, 913-924 (2007).

50. Feldman, N., et al. G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. Nat Cell Biol 8, 188-194 (2006).

51. Fong, H., Hohenstein, K.A. & Donovan, P.J. Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. Stem Cells 26, 1931-1938 (2008).

52. Chew, J.L., et al. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol Cell Biol 25, 6031-6046 (2005).

53. Kopp, J.L., Ormsbee, B.D., Desler, M. & Rizzino, A. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells 26, 903-911 (2008).

54. Binetruy, B., Heasley, L., Bost, F., Caron, L. & Aouadi, M. Concise review: regulation of embryonic stem cell lineage commitment by mitogen-activated protein kinases. Stem Cells 25, 1090-1095 (2007).

55. Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R. & Young, R.A. A chromatin landmark and transcription initiation at most promoters in human cells. Cell 130, 77-88 (2007).

56. Azuara, V., et al. Chromatin signatures of pluripotent cell lines. Nat Cell Biol 8, 532-538 (2006).

57. Bernstein, B.E., et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125, 315-326 (2006).

58. Van Hoof, D., et al. Feeder-free Monolayer Cultures of Human Embryonic Stem Cells Express an Epithelial Plasma Membrane Protein Profile. Stem Cells (2008).

59. Eckert, J.J. & Fleming, T.P. Tight junction biogenesis during early development. Biochim Biophys Acta 1778, 717-728 (2008).

60. Braam, S.R., et al. Improved genetic manipulation of human embryonic stem cells. Nat Methods 5, 389-392 (2008).

61. Van Hoof, D., et al. An experimental correction for arginine-to-proline conversion artifacts in SILAC-based quantitative proteomics. Nat Methods 4, 677-678 (2007).

62. Vertegaal, A.C., et al. A proteomic study of SUMO-2 target proteins. J Biol Chem 279, 33791-33798 (2004).

63. Schimmel, J., et al. The ubiquitin-proteasome system is a key component of the SUMO-2/3 cycle. Mol Cell Proteomics (2008).

CHAPTER SEVEN

Multipotent *NKX2-5*$^{+}$ cardiac progenitors derived from human embryonic stem cells

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Abstract

Cardiac progenitor cells give rise to the major cellular components of the heart. Understanding how they form and what they can become has important implications for elucidating the mechanisms underlying (abnormal) heart development. In addition, the ability to isolate these cells from pluripotent stem cells allows characterization of their properties and possibly expansion in culture, a crucial step for advancing cardiac translational medicine. Functional analysis of these cells has been hampered by the paucity of lineage specific markers and inefficient, undefined differentiation procedures. Here we describe the targeted modification of two independent human embryonic stem cell (hESC) lines in which EGFP was inserted into the locus of NKX2-5, one of the earliest transcription factors expressed in heart development. EGFP fluorescence driven by the endogenous NKX2-5 promoter faithfully reported cardiovascular lineage commitment of differentiating hESC under defined culture conditions. The early NKX2-5 positive cell population contained multipotent progenitor cells capable of directed differentiation to cardiomyocytes, endothelial and vascular smooth muscle cells. NKX2.5 overlapped only partially with previously reported selection markers for the cardiovascular lineage. These data are compatible with expectations from heart development and identify a progenitor that can give rise to the three major cell types of the heart or through fine temporal fine tuning, almost exclusively cardiomyocytes. Taken together, these experiments demonstrate the utility of hESC for analyzing the previously inaccessible events of human cardiac lineage specification.

Introduction

The ability to generate functional cardiovascular (progenitor) cells from human pluripotent stem cells is beginning to offer unprecedented opportunities for translational research in cardiac medicine. Applications range from the use of cells to study early human cardiac development, the development of disease models, predictive cardiotoxicity assays, drug development and ultimately the development of cell therapy for degenerative diseases[1]. Advance of all of these applications depends on robust differentiation procedures underpinned by detailed knowledge of the signaling mechanisms regulating cell fate.

Over the previous decade methods for expansion and directed differentiation of human embryonic stem cells (hESC) has improved considerably. One recurring theme is that most successful differentiation protocols recapitulate the regulatory circuits that control normal development. Initially, protocols supporting cardiomyocyte differentiation relied on poorly defined reagents and inefficient cell culture methods. This resulted in cultures with only a very small percentage of beating cells (<1%)[2]. Later it was shown that co-culture with endoderm-like cells, mimicking signaling from endoderm in normal development[3] resulted in a relatively efficient and robust cardiomyocyte differentiation[4]. More recently, several groups have shown that the cytokines Bone Morphogenetic Protein 4 (BMP4) and Activin A induce mesendoderm in differentiating hESC cultures[5,6,7]. However, the exact timing, concentration and spectrum of growth factor requirements for the initiation of cardiogenesis, formation of cardiac progenitors and further maturation remain to be defined.

In mouse the earliest cardiac progenitor identified, arises in the primitive streak and expresses Flk-1/KDR[8]. Later in development at least two progenitor populations are present. The first population expresses the transcription factor Nkx2-5 and is present in the cardiac crescent or the first heart field (FHF). Nkx2-5 is one of the earliest cardiac progenitor markers and continues to be expressed in the adult myocardium[9]. The committed FHF progenitors contribute to left and right ventricles, both atria and AV canal[10,11]. The second population, known as the second heart field (SHF) is characterized by the expression of the transcription factor Isl1 in addition to Nkx2-5. These cells give rise mainly to the outflow tract and right ventricle but also contribute to all other regions of the heart except the left ventricle[10]. In the developing SHF, multipotent $Isl1^{+}/Nkx2\text{-}5^{+}$ progenitors can differentiate to endothelial cells, vascular smooth muscle and cardiomyocytes[12].

Two studies to date have used a human pluripotent stem cell model for identification, selection and characterization of cardiac progenitors[6,13]. An antibody mediated cell sorting method developed in mice and based on cell surface expression of the VEGF-receptor FLK-1/KDR was instrumental in the first isolation human cardiovascular progenitors from hESC[6]. Later, genetic tagging of Isl1 allowed the isolation of another population of human cardiac progenitor cells from hESC. Although both methods worked effectively neither was an exclusive cardiac progenitor marker. Flk-1/KDR is not specific for cardiac mesoderm[14,15] and ISL1 is broadly

expressed in multiple non-cardiac lineages during early development.

Here we report targeted modification of NKX2-5 with EGFP in hESC. The activity of the reporter faithfully recapitulated the process of cardiac development and allowed purification of cardiomyocytes from a heterogeneous cell population. The transgenic line permitted development of a cytokine screening strategy in defined medium that ultimately resulted in an optimized cardiac differentiation protocol. FACS based purification of early NKX2-5 positive cells revealed a multipotent progenitor population capable of directed differentiation to the cardiac, endothelial and vascular smooth muscle cell lineages.

Materials and methods

hESC culture and differentiation

hESC culture and gene targeting were performed as previously described[7]. Differentiation was induced by forced aggregation in APEL media[16], with the following cytokines: 20 ng/ml of BMP4 (R&D) and Activin A (R&D), 30 ng/ml VEGF (R&D) and 40 ng/ml SCF (R&D). hESC-CMs were also derived using the END2 co-culture system, as previously described[4].

Flow cytometry

Embryoid bodies (EBs) were dissociated using TrypLE Selectä (Invitrogen), and intracellular flow cytometry carried out on cells fixed and stained with anti-ISL1 (DSHB, clone 39.4D5), anti-OCT4 (Santa Cruz Biotechnology) and anti-NKX2.5 (Abcam) antibodies as previously described[17]. Mouse anti-human primary antibodies reacting with cell surface antigens for live cell sorting included unconjugated anti-E-CADHERIN (Zymed), anti-TRA-1-60 (Chemicon), anti-SSEA4 (Chemicon), FITC-conjugated anti-CD9 (BD Biosciences), anti-PDGFRα (BD Biosciences), anti-VCAM (BD Biosciences) and phycoerythrin (PE) conjugated anti-CXCR4. Non-conjugated antibodies were detected with allophycocyanin (APC)-conjugated goat anti-mouse IgG (BD Biosciences). Flow cytometric gates were set using control cells labeled with the appropriate isotype control antibody. Single cell cloning was performed using the single cell deposition function of a FACSaria FACS station as previously described[7].

Imaging

Cells were cultured on glass coverslips, fixed in 4% PFA for 30 min, permeabilized for 5 min. with 0.1% Triton X-100 in PBS and blocked with 4% normal goat serum. Cells were incubated with primary antibodies and labeled with alexa405, Cy3, Cy5 and/or alexa647 conjugated secondary antibodies. Primary antibodies used in this study were against α-actinin (clone EA53, Sigma), PECAM (DAKO), smooth muscle actin (Abcam), NKX2-5 (Santa Cruz) MLC2V (Synaptic Systems), MLC2A (Synaptic Systems), MYH6 (Chemicon), GATA4 (Santa Cruz), c-KIT(Dako), and PDGRα BD) Nuclei were counter stained with DAPI. Confocal scanning was performed on a Leica SP5 confocal laser scanning microscope (40x and 63x oil immersion objectives). For live cell imaging, cells were cultured on MatTek dishes (MatTek Corp. Amsterdam) and imaged

on a Leica SP5 using the resonance scanner at a resolution of 512x256 (30fps). For calcium imaging, cells were loaded with Fura Red (Molecular Probes, Invitrogen). The 488nm laser was used to excite EGFP and Fura Red (calcium free). Two PMTs were used simultaneously for detecting EGFP and Fura Red specific photons respectively. ImageJ imaging processing software was used for normalization of Fura Red fluorescence to EGFP and for further data analyses.

ELECTROPHYSIOLOGY

Patch clamp electrophysiology was carried out essentially as described previously, with minor modifications[18]. Briefly, beating clumps of cardiomyocytes were dissociated using Tryp.Le, replated on gelatin-coated coverslips and measured in current clamp mode between 7 and 14 days after plating.

For MEA electrophysiology, hESC-CM clusters were microdissected and replated on plasma cleaned fibronectin coated 60 electrode MEAs. Extracellular recording was performed using a MEA1060INV MEA amplifier (Multi Channel Systems, Reutlingen, Germany) at 37°C. Output signals were digitized at 10 kHz by use of a PC equipped with a MC-card data acquisition board (Multi Channel Systems, Reutlingen, Germany).

FORCE MEASUREMENTS

A beating region within an embryoid body was selected and viewed through a Nikon Eclipse TS100 inverted microscope with Hoffman modulation contrast optics, coupled to a Basler (model A602f) camera. Quick Caliper (SDR Clinical Technologies) enabled the acquisition of a 10 second capture (80 frames/second). Prior to the addition of agonists/activators a 10 second capture was obtained to establish baseline conditions. Following this, either forskolin (1µM), endothelin-1 (ET-1; 10nM) or vehicle controls were added to the well, and the plate placed back in a humidified incubator (37°C, 5% CO_2). A second 10 second capture was then taken 2 and 5 minutes post forskolin or ET-1.

ET-1 was dissolved in physiological salt solution (PSS) buffer consisting of (in mM) NaCl 140, KCl 6, $CaCl_2$ 2, MgCl2 1, HEPES 20 and glucose 10, supplemented with 1.4 % (w/v) BSA (pH 7.4, 37ºC). Forskolin was dissolved in ethanol and the final ethanol concentration did not exceed 0.01% of the media volume in the well.

The video captures were analysed using the Metamorph® Imaging System (Molecular Devices Ltd). For each image stack constituting a 10 second capture, an intensity threshold was set for the beating image. The Metamorph® software was then used to track the intensity centroid of a selected particle throughout the 10 second image stack. The amplitude of contraction of the selected particle (in pixels) was plotted against time for each time point (See Figure 7.3C). The number of peaks within the 10 second capture was converted to beats per minute to represent the contraction frequency. The frequency recorded in response to an agonist was reported as a percentage of the basal spontaneous rate (i.e. prior to agonist addition).

As previously reported hESC-derived cardiomyocytes, not all beating aggregates responded to agonist stimulation. Responding and non-responding aggreagtes were identified using a repeated measures ANOVA test of the time interval between beats in each 10 second capture. Differences between means were tested using the Student's t-test and values of $p < 0.05$ were considered statistically significant.

Results

NKX2-5EGFP/W HESC LINE FACILITATES IDENTIFACTION AND PURIFACTION OF HUMAN CARDIAC PROGENITOR CELLS

To investigate early differentiation events in human cardiogenesis we introduced sequences encoding the green fluorescent protein (EGFP) into the NKX2-5 locus of hESC (Figure 7.1). The targeting vector contained a PGK promoter driving expression of neomycin acetyltransferase flanked by loxP sequences, the recognition sites of bacteriophage Cre recombinase. Correctly targeted hESC clones were identified by polymerase chain reaction (PCR) amplification (Figures 7.1A & data not shown) and sequencing of the resultant PCR products. The selection cassette was removed by transient transfection of hESC with a Cre recombinase expression vector and subsequent single cell deposition by FACS[7]. Cassette deletion was validated by PCR (data not shown). Southern blotting with an EGFP probe confirmed that the identified NKX2-5EGFP/w clones contained only a single integration event (Figure 7.1B). Two hESC lines, HES3[19] and MEL1 (Millipore) were targeted in this manner to generate H3 NKX2-5EGFP/w and M1 NKX2-5EGFP/w, respectively. All NKX2-5EGFP/w clones expressed markers of undifferentiated HESC, were karyotypically normal and formed teratomas (data not shown). To derive cardiomyocytes NKX2-5EGFP/w cells were initially differentiated[20] in the END-2 co-culture system. As expected, this protocol resulted in reproducible and robust cardiac differentiation. Importantly, eGPF driven by the NKX2-5 promoter was specifically activated in beating foci. Beating areas could be readily identified based on bright green fluorescence and all green beating foci stained positive for cardiac α-actinin (Figure 7.1C). To verify that EGFP expression recapitulates endogenous NKX2-5 expression, dissociated EGFP$^+$ cells were stained with anti-NKX2-5 antibody. All EGFP$^+$ cells showed prominent nuclear NKX2-5 staining, as well as expression of the cardiac protein a-actinin (Figure 7.1D). Furthermore, dissociated cardiomyocytes stained positive for the cardiac structural proteins MLC2a, MLC2v and beta myosin heavy chain as well as transcription factors NKX2-5, ISL1 and GATA4 (data not shown). EGFP cardiomyocytes displayed a similar fetal sarcomeric organization as cardiomyocytes from control hESC lines[21].

OPTIMIZED DIFFERENTIATION IN DEFINED MEDIA

Having validated the integrity of the GFP reporter cell lines and retention of the ability to form cardiomyocytes robustly, we designed a cytokine screening strategy based on the fluorescent reporter as a read-out. The aim was to develop a method for directed cardiovascular differentiation of hESC under chemically defined culture conditions. To screen and cross titrate these factors properly without interference from undefined components in co-cultures we

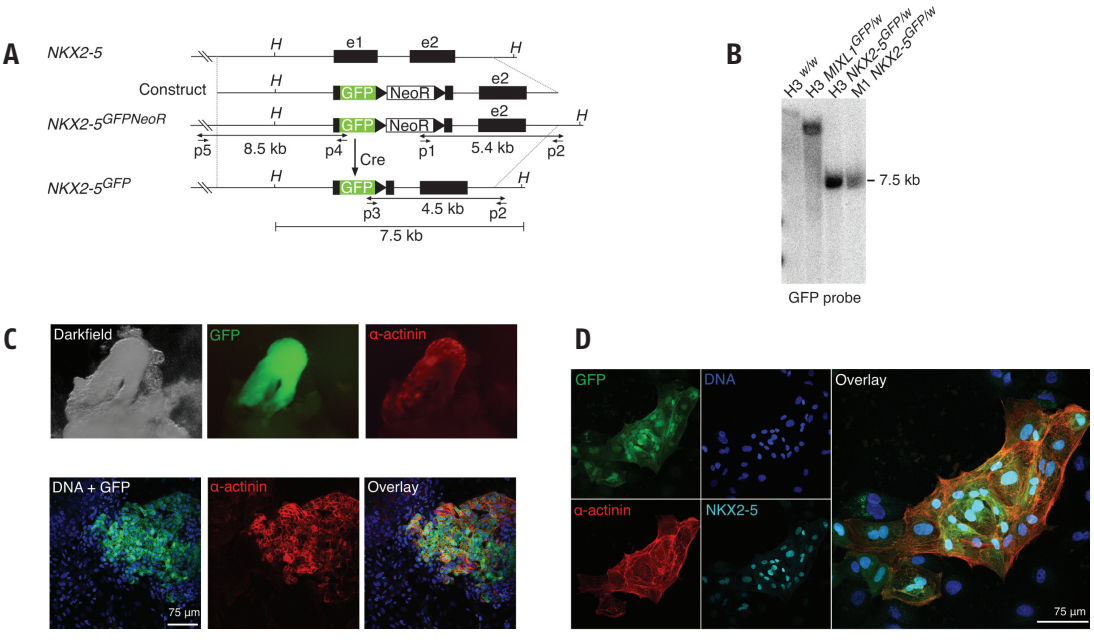

Figure 7.1

Generation and characterization of NKX2-5EGFP/w knock-in lines

(A) Design of the gene targeting vector to insert sequences encoding EGFP into exon 1 of the NKX2-5 locus. NeoR is the PGK-Neo cassette encoding neomycin acetyltransferase, flanked by LoxP sites (black triangles) (B) Southern blot with a GFP probe to confirm a single integration in the genome. (C) α-actinin staining on cardiac differentiated H3 NKX2-5EGFP/w (D) co-staining for NKX2-5 and α-actinin on dissociated H3 NKX2-5EGFP/w.

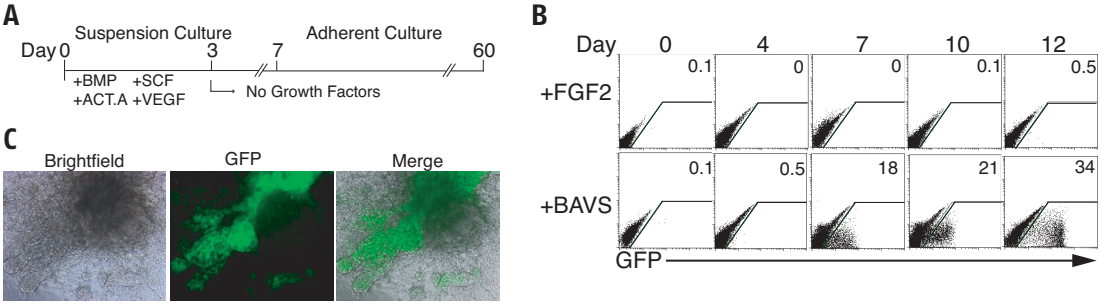

Figure 7.2

Cardiac differentiation in defined APEL medium

(A) Optimized differentiation protocol based on temporal addition of BMP, SCF, Activin A and SCF for three days. (B) Temporal expression pattern of NKX2-5 during EB differentiation by flow cytometry for EGFP+ cells (C) Immunofluorescence of NKX2-5 reporter gene expression

used a forced aggregation (or "spin EB") protocol in a chemically defined media (APEL)[16,17] supplemented with BMP 4 and Activin A[5,6]. Since cardiac mesoderm derives from a cell population expressing the VEGF receptor, KDR[6], and stem cell factor (SCF) has been shown to improve cell viability in spin EBs[22] both VEGF and SCF were included in the differentiation medium (Figure 7.2A).

Optimal cytokine concentrations were determined by cross titration of the growth factors mentioned above, examination of EBs for eGFP expression and scoring for the appearance of spontaneous contractile foci. The optimized protocol presented here consists of two initial steps performed in suspension culture; i) mesendoderm induction by a combination of BMP4 and Activin A until day 3 of differentiation and ii) further specification of cardiac mesoderm relying on endogenous signaling networks established by the primitive streak-like population generated in step I (Figures 7.2A & S7.2). Removal of growth factors on day 3 leads to a significant increase in the production of NKX2-5EGFP+ cells (Figure 7.2). This suggests that by day 3 EBs contain sufficient patterning information for cardiomyogenesis to proceed autonomously without the addition of exogenous factors.

The temporal expression pattern of NKX2-5 during EB differentiation was established by flow cytometry for EGFP+ cells. Control experiments using FGF2 demonstrated a requirement for mesendoderm induction to allow cardiac differentiation to proceed (Figure 7.2B). EGFP+ cells emerged on days 6-7 of differentiation and increased as a percentage of the culture until day 12 (Figure 7.2B). After transfer to adherent culture beating foci appeared within EBs. NKX2-5EGFP/w EBs maintained for 90 days continue to contract; however, after day 12 little increase was observed in percentage of EGFP+ cells. To confirm that EGFP expression recapitulated NKX2-5 expression, sorted EGFP+ cells were stained with anti-NKX2-5 antibody. All EGFP+ cells examined showed significant nuclear NKX2-5 staining, as well as expression of cardiac α-actinin (data not shown).

NKX2-5EGFP/W CELLS CAN DIFFERENTIATE TO FUNCTIONAL CARDIOMYOCYTES

For many future applications requiring human cardiomyocytes derived from stem or progenitor cells, it will be important to know their electrical and functional properties. We therefore analyzed the action potentials of cardiomyocytes generated from the H3 NKX2-5EGFP/w line. Cells were classified as atrial-like, ventricular-like or pacemaker-like based on the profiles of the action potentials, as previously. As in cardiomyocytes from the parent HES3 line induced by END2 cell co-culture, the majority of cardiomyocytes from the NKX2-5EGFP/w cell line displayed a ventricular-like action potential; only a minority of cells displayed characteristics of pacemaker- or atrial cells[18]. The average upstroke velocity was 6,4±2,3 V/s , the 90% repolarization 522,8±199,6 ms, the amplitude 88,8±6,6 mV and the resting potential was -43,5±5,7 mV in 18 independent measurements, A representative ventricular like action potential is shown in Figure 7.3A. Next the cells were analyzed using Multielectrode array (MEA) field potential analyses. As expected the cells showed a trace typical for hESC-CM (Figure 7.3B)[21]. In summary, the NKX2-5 heterozygous cells were indistinguishable from unmodified

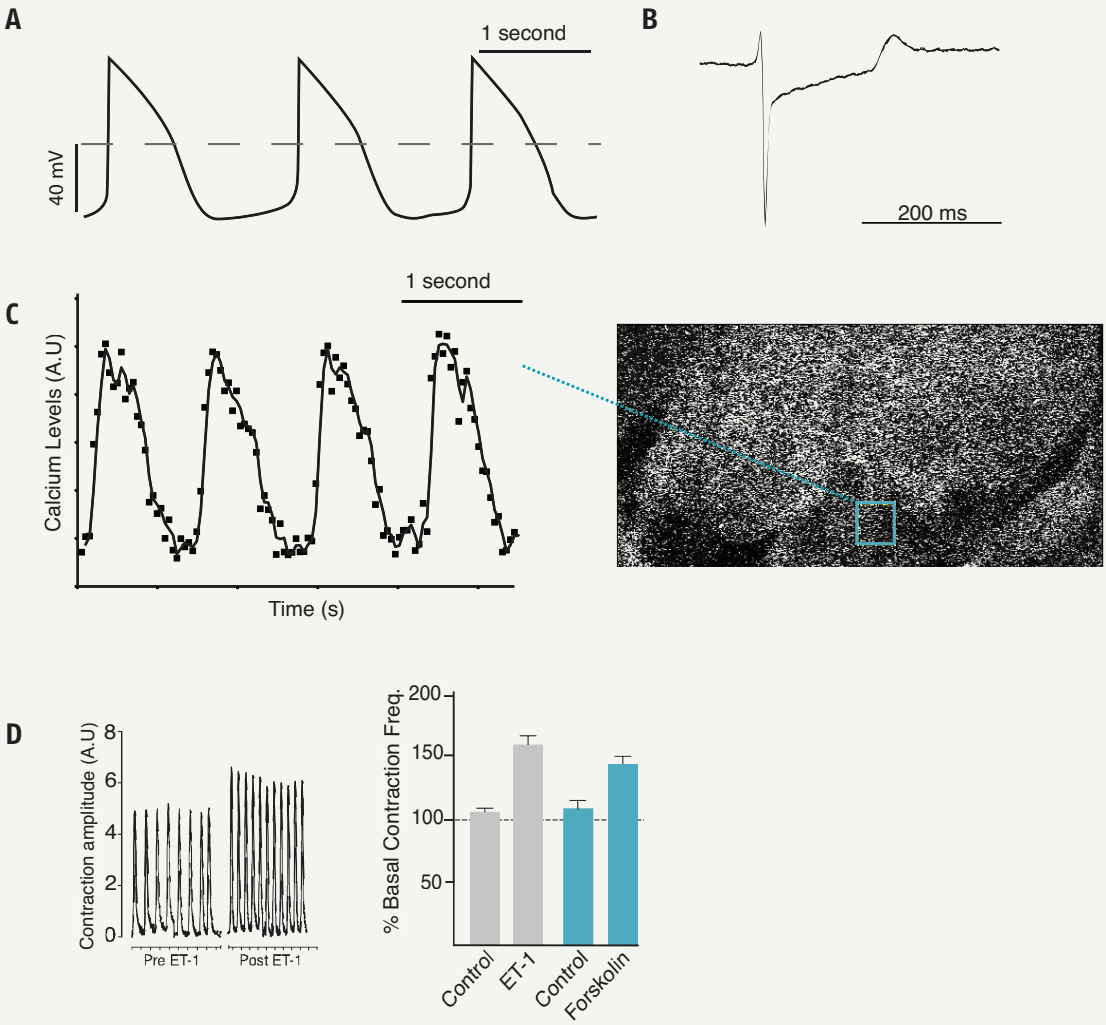

Figure 7.3

Characterization of NKX2-5EGFP/w cardiomyocytes

(A) Patch clamp electrophysiology showing a representative ventricular like action potential from H3 NKX2-5EGFP/w, (B) Field potential from a cluster contracting cardiomyocytes measured on a multi electrode array (recording from one electrode). (C) Fura red calcium imaging using confocal microscopy from a beating cluster. (D) Contraction force measurements of beating EBs in response to ET-1 and Forskolin

hESC-CM with respect to their electrical phenotype. To investigate properties of the cells in more detail, they were labeled with Fura Red to visualize the calcium homeostasis. As expected the cells showed typical calcium traces that accompanied contraction of the cells, indicating normal calcium homeostasis (Figure 7.3C, movie S7.2). Finally the cells were tested functionally by adding the ionotropic/chronotrophic agents endothelin-1 (ET-1) and Forskolin. Both compounds exert similar effects on cardiomyocytes but the mechanisms are different: ET-1 increases the concentration of cytosolic Ca^{2+} while Forskolin activates adenylyl cyclase resulting in elevated cAMP levels. Approximately 57% (4/7) of the spontaneously beating cardiomyocyte aggregates responded to ET-1 (10nM) addition, whereas 66% (2/3) responded to forskolin (1μM) addition. In the responding aggregates, ET-1 (10nM) increased contraction frequency by 58 ± 11% ($p<0.05$, students t-test, n=4). Preliminary investigations with forskolin indicated that it, also, increases contraction frequency (by 43±7%; n=2). Although the responses were not homogeneous, the hESC-CM do contain functional endothelin receptors, as well as intact cAMP signalling pathways, both of which are involved in the modulation of spontaneous contraction frequency.

ISOLATION AND CHARACTERIZATION OF MULTIPOTENT NKX2-5+ CARDIAC PROGENITOR CELLS (CPCs)

Having demonstrated the cardiomyogenic potential, the validity of the reporter and the functionality of cardiomyocytes generated from H3 NKX2-5EGFP/w we used the line to investigate the temporal dynamics of the appearance of NKX2-5 positive cells in greater detail. Studies in mouse embryos and mESC have shown that early Nkx2-5 positive cells co-express Flk-1 and c-kit. These cells have the potential to differentiate into cardiomyocytes, vascular smooth muscle and endothelial cells[23]. To determine whether this is also the case for early NKX2-5+ cells from hESC we analyzed the NKX2-5+ cells at day 7 of END-2 co-culture for the membrane markers c-Kit, PDGFRα and Flk-1/KDR by confocal imaging (Figure 7.4A). While PDGRα was expressed at low levels we could find no evidence for c-Kit expression or Flk-1/KDR (Fig 7.4A and data not shown). Many cells that expressed high levels of PDGFRα did not express NKX2-5 (Figure 7.4A, see insert), thus did not correspond with a cardiac progenitor population. Furthermore, it was clear that none of these markers segregated exclusively with the NKX2-5 population. Analyses of day7 cells derived from spin EB indicated the presence of a comparable population. PDGFRα was expressed at low levels and Flk-KDR expression did not exceeded background levels. We next sorted day 7 NKX2-5+ cells from H3 NKX2-5EGFP/w differentiated in End-2 co-culture using flow cytometry (Figure 7.4C). Cells were re-plated on fibronectin coated substrates in defined culture medium supplemented with selected recombinant growth factors. To date, most CPC differentiation protocols have not used defined media; as a result, the signaling pathways required for cardiovascular lineage specification have not been precisely defined. Addition of bFGF plus BMP4 resulted in the appearance of SMA and PECAM positive cells as well as a-actinin positive cells (Figure 7.4D), suggesting that the sorted NKX2-5+ cell population contained multi-lineage cardiovascular progenitors. The NKX2-5- cells did not form these cells under the same conditions indicating the NKX2-5 defined the cell population with cardiovascular differentiation potential more precisely than

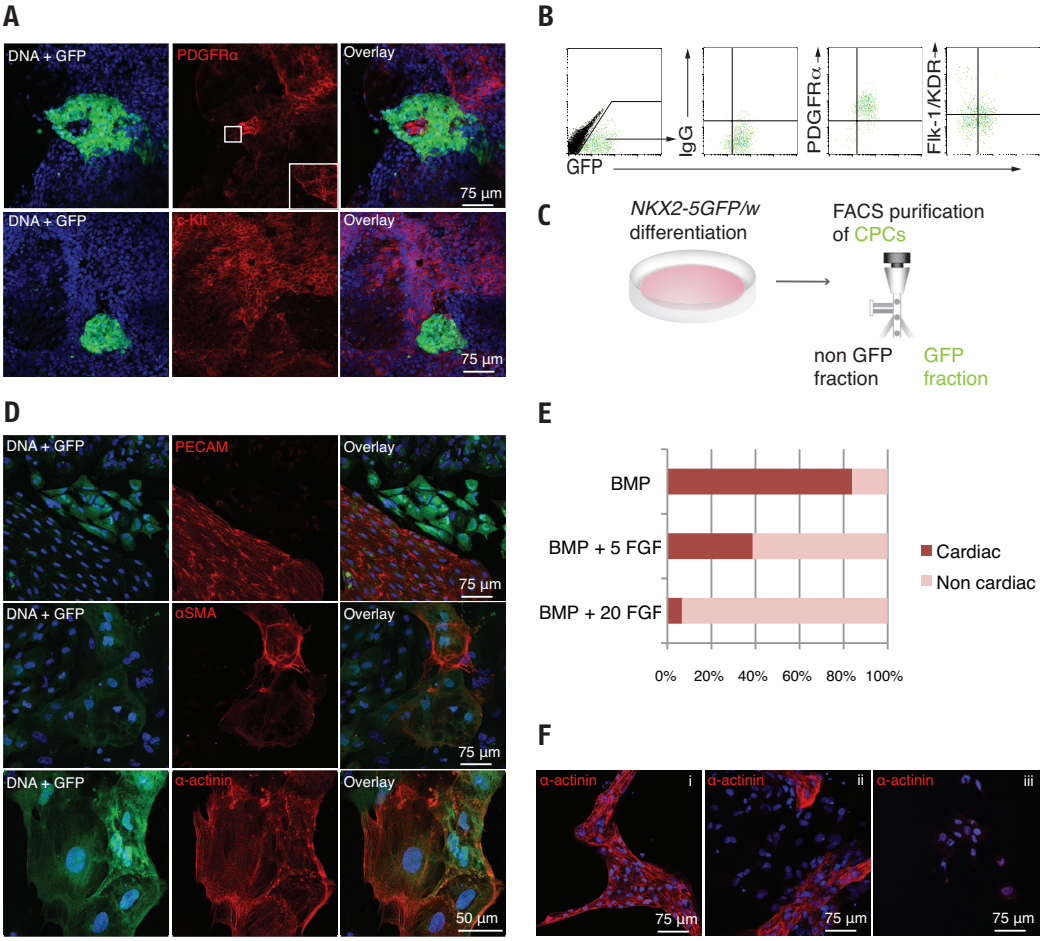

Figure 7.4

Isolation and directed differentiation of NKX2-5 progenitor cells

(A) Confocal microscopic imaging of END-2 co-cultures at day 7 of differentiation. NKX2-5^{+} areas stained for PDGFRα and c-Kit. (B) FACS analyses of day 7 spin-EB differentiations stained for PDGFRa and Flk-1/KDR (gated on NKX2-5^{+} cells) (C) experimental flowchart describing FACS sorting NKX2-5 progenitors (D) directed differentiation of END-2 derived CPCs to endothelial lineages (positive for PECAM), vascular smooth muscle (α-smooth muscle actin) and cardiomyocytes (α-actinin)

the other markers tested. Interestingly, the balance between FGF and BMP signaling has been described as a fate regulator of pericardial chick mesoderm, determining the choice between the epicardial and myocardial lineages[24,25]. We therefore cross-titrated these two growth factors in the sorted NKX2.5-GFP cells and determined their cardiac differentiation potential. Preliminary results indicated that BMP alone gives rise to cultures highly enriched for contracting cardiac a-actinin positive cells. Addition of bFGF led to a clear dose dependent lineage shift to non-myocardial cells, suggesting that the balance between BMP and FGF regulates the fate of the NKX2-5+ CPC population from hESC as in the chick.

Discussion

The origin and fates of human cardiovascular progenitor cells are still largely unknown. Here we show that the combined use of pluripotent stem cells and gene targeting allows dissection of previously inaccessible events during human cardiac progenitor development. Insertion of eGFP in the NKX2-5 gene resulted in two independent hESC lines that captured cells with cardiovascular developmental potential from the heterogenous mixtures of hESC derivatives. Beating foci were brightly fluorescent and eGFP positive areas stained positive for both cardiac a-actinin and NKX2-5 proteins. Electrophysiological analysis, calcium imaging and force measurements confirmed their phenotype as functional cardiomyocytes, indistinguishable from cardiomyocytes generated from wild type hESC. The bright eGFP reporter activity driven by the endogenous NKX2-5 promoter line permitted development of a cytokine screening strategy that ultimately resulted in an optimized cardiac differentiation protocol. This new protocol makes use of defined medium supplemented with the cytokines BMP, Activin A, SCF and VEGF. Notably, removal of the growth factors at day 3 led to a significant increase in the production of NKX2-5EGFP+ cells (up to 35%), suggesting that by day 3 the EB contains sufficient patterning information for cardiomyogenesis to proceed without further addition of exogenous factors. Recent work on mESC suggests that EBs can be polarized in an anterior-posterior direction and form a structure that resembles the primitive streak. Local activation of the Wnt pathway resulted in an epithelial-to-mesenchymal transition and differentiation into mesendodermal structures. This process required exogenous factors but was self-reinforcing after initiation[26]. A similar process seemed to occur in our protocol despite the absence of exogenous Wnt signals. Addition of fresh cytokines at later time-points never enhanced differentiation outcome. This suggested that the auto- and endocrine signaling between the different cell types is so balanced that alteration of exogenous signaling mainly disturbs their differentiation potential.

Isolation of human cardiac progenitors based on NKX2-5 expression could be a good approach to dissect cardiac progenitor lineage decisions without interference of cells not associated with this lineage. In the mouse, Nkx-2.5 transcripts are first detected at early headfold stages in myocardiogenic progenitor cells. Expression precedes the onset of myogenic differentiation, and continues during lifetime of a cardiomyocyte[9]. NKX2-5 is one the earliest

genes that control cardiac differentiation and is present in both the progenitors of the FHF and SHF. Transcripts are mainly expressed in the heart although expression is also detected in the cardiac associated pharyngeal endoderm, lingual muscle, spleen and stomach[9]. In the mouse several cardiac progenitor populations have been described and the majority of them expressed Nkx2-5. Only the Flk-1 progenitor described by Kattman et al emerges at such an early developmental stage that Nkx2-5 is not yet expressed[8]. However according to fate mapping experiments performed in mouse embryos, Flk1$^+$ progenitors contribute only to a subset of cardiac muscle cells[15]. Moreover Flk-1 is not expressed in all cells of the cardiac crescent during development, nor is it cardiac specific. Two independent groups have described the isolation of putative primordial / FHF specific progenitors in the mouse[23,27]. Both of these studies made use of a Nkx2-5 reporter construct for the isolation of CPCs. However there were differences between the cell populations. The cells identified by Wu and colleagues co-expressed c-Kit, were bi-potent, indicated by their differentiation to cardiomyocytes and smooth muscle cells[27]. The cells identified by Christoforou and colleagues co-expressed c-kit and Flk-1/KDR and were reported to be multipotent: besides cardiomyocytes and vascular smooth muscle cells they were also capable of forming endothelial cells[23]. Whether the cells from these two studies represent the same population remains to be investigated. In the developing SHF a similar multipotent cell can be identified. This progenitor expresses Isl1$^+$/ Nkx2-5$^+$ and is capable of directed differentiation to endothelial, vascular smooth muscle and cardiomyocyte lineages[12].

Isolation of NKX2-5$^+$ cells from the H3 NKX2-5EGFP line at day 7 of differentiation resulted in multi-lineage differentiation to cardiomyocytes, endothelial cells and vascular smooth muscle cells. These findings indicate a differentiation potential comparable to the mouse multipotent progenitors found in the SHF, and the Nkx2-5 progenitors described by Christoforou et al. However, in contrast to the mouse Nkx2-5 CPCs our cells do not express c-KIT, and FLK-1 is only expressed at low levels, which suggests a difference to the mouse cells described by Christoforou. Furthermore the relationship to ISL1 remains largely unclear. According to Bu et al (2009) the expression of ISL1 in human cardiac development precedes NKX2-5 expression, however this was not investigated in detail. Although some of our NKX2-5$^+$ cells do express ISL1 it is certainly not co-expressed in all cells. In general, from studies in the mouse it seems that ISL1 and NKX2-5 mark a distinct but overlapping population of cardiac progenitors. However it is of note that ISL1 is expressed in multiple organs and tissues in the developing embryo, including motor neurons and pancreas progenitors[28,29]. In accordance with this, only 4.2% of the human Isl1 clones gave rise to cells expressing cardiac genes, 3.1% to endothelial and 44.1% to smooth muscle[13].

Further follow up studies including clonal assays on human NKX2-5 positive cells, will be required to confirm the multipotency of our cells and their relationship with ISL1. Finally, it should be mentioned that analyses of the ISL1 family of cardiac progenitors suggests clear differences in mouse and human cardiac development. In the mouse, a rapid transition from an Isl1 progenitor to migration and conversion to cells of the SHF is observed.

Consequently, later in development relatively few Isl1-expressing cells can be found in the heart. Furthermore, Isl1-progenitors generally co-express the transcription factors Nkx2-5 and Flk-1/KDR; indicative of a rapid fate restriction. In the first trimester of cardiac human development relatively large numbers of ISL1 expressing cells are present that do not co-express KDR or Smooth muscle myosin heavy chain or cardiac Troponin, perhaps reflecting the fact that heart development in humans is a much longer process than rodents. It has been suggested that these cells represent the upstream precursor for the multipotent progenitors in the SHF lineage[13]. Therefore the presence of a family of ISl1 expressing intermediate, lineage-restricted progenitors could reflect the requirement for the more extensive cell expansion necessary to produce cell numbers orders of magnitude greater in adult human hearts than in mice[30].

In summary, the findings reported here demonstrate the combined potential of the pluripotent stem cell model with genetic manipulation of these cells. Targeted modification of NKX2-5 with EGFP resulted in a highly specific reporter line that can be used for the isolation and lineage dissection of human cardiac progenitors. Specifically we show that day 7 CPCs are capable of directed differentiation to cardiomyocytes, vascular smooth muscle and endothelial cells. Interestingly it seems that the ratio of exogenous bFGF to BMP determines the differentiation fate of the NKX2.5 marked CPCs. BMP2 is a known cardiogenic factor expressed in lateral plate mesoderm and cardiac-associated pharyngeal endoderm[31]. Our data suggests that this signaling might be a driver of CPC specification to cardiomyocytes. It is expected that further studies on CPCs will eventually lead to optimized culture conditions for self-renewal and directed differentiation to various cardiovascular lineages of the heart.

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References

1. Braam, S., Passier, R. & Mummery, C. Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery. Trends Pharmacol Sci (2009).

2. Passier, R. & Mummery, C. Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 16, 498–502 (2005).

3. Nijmeijer, R.M., Leeuwis, J.W., DeLisio, A., Mummery, C.L. & Chuva de Sousa Lopes, S.M. Visceral endoderm induces specification of cardiomyocytes in mice. Stem Cell Research 3, 170–178 (2009).

4. Passier, R., et al. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 23, 772–780 (2005).

5. Laflamme, M.A., et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25, 1015–1024 (2007).

6. Yang, L., et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 453, 524–528 (2008).

7. Davis, R.P., et al. Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. Blood 111, 1876–1884 (2008).

8. Kattman, S.J., Huber, T.L. & Keller, G.M. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. Dev Cell 11, 723–732 (2006).

9. Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I. & Harvey, R.P. Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119, 419–431 (1993).

10. Buckingham, M., Meilhac, S. & Zaffran, S. Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet 6, 826–835 (2005).

11. Wu, S.M., et al. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. Cell 127, 1137–1150 (2006).

12. Moretti, A., et al. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127, 1151–1165 (2006).

13. Bu, L., et al. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 460, 113–117 (2009).

14. Motoike, T., Markham, D.W., Rossant, J. & Sato, T.N. Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. Genesis 35, 153–159 (2003).

15. Ema, M., Takahashi, S. & Rossant, J. Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors. Blood 107, 111–117 (2006).

16. Ng, E.S., Davis, R., Stanley, E.G. & Elefanty, A.G. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. Nat Protoc 3, 768–776 (2008).

17. Ng, E.S., Davis, R.P., Azzola, L., Stanley, E.G. & Elefanty, A.G. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. Blood 106, 1601–1603 (2005).

18. Graichen, R., et al. Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation 76, 357–370 (2008).

19. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18, 399–404 (2000).

20. Bird, S.D., et al. The human adult cardiomyocyte phenotype. Cardiovasc Res 58, 423–434 (2003).

21. Mummery, C., et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107, 2733-2740 (2003).

22. Pick, M., Azzola, L., Mossman, A., Stanley, E.G. & Elefanty, A.G. Differentiation of human embryonic stem cells in serum-free medium reveals distinct roles for bone morphogenetic protein 4, vascular endothelial growth factor, stem cell factor, and fibroblast growth factor 2 in hematopoiesis. Stem Cells 25, 2206-2214 (2007).

23. Christoforou, N., et al. Mouse ES cell-derived cardiac precursor cells are multipotent and facilitate identification of novel cardiac genes. Journal of Clinical Investigation 118, 894-903 (2008).

24. Van Wijk, B., et al. Epicardium and Myocardium Separate From a Common Precursor Pool by Crosstalk Between Bone Morphogenetic Protein- and Fibroblast Growth Factor-Signaling Pathways. Circulation Research 105, 431-441 (2009).

25. Kruithof, B.P.T., et al. BMP and FGF regulate the differentiation of multipotential pericardial mesoderm into the myocardial or epicardial lineage. Dev Biol 295, 507-522 (2006).

26. ten Berge, D., et al. Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell 3, 508-518 (2008).

27. Wu, S.M., Chien, K.R. & Mummery, C. Origins and fates of cardiovascular progenitor cells. Cell 132, 537-543 (2008).

28. Pfaff, S.L., Mendelsohn, M., Stewart, C.L., Edlund, T. & Jessell, T.M. Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell 84, 309-320 (1996).

29. Ahlgren, U., Pfaff, S.L., Jessell, T.M., Edlund, T. & Edlund, H. Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature 385, 257-260 (1997).

30. Hansson, E.M., Lindsay, M.E. & Chien, K.R. Regeneration next: toward heart stem cell therapeutics. Cell Stem Cell 5, 364-377 (2009).

31. Brand, T. Heart development: molecular insights into cardiac specification and early morphogenesis. Dev Biol 258, 1-19 (2003).

CHAPTER EIGHT

Prediction of drug induced cardiotoxicity using hESC cell-derived cardiomyocytes

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Abstract

Recent withdrawals of prescription drugs from clinical use because of unexpected side effects on the heart have highlighted the need for more reliable cardiac safety pharmacology assays. Block of the human Ether-a-go go Related Gene (hERG) ion channel in particular is associated with life-threatening arrhythmias, such as Torsade de Pointes (TdP). Here we investigated human cardiomyocytes derived from pluripotent (embryonic) stem cells (hESC) as a renewable, scalable and reproducible system on which to base cardiac safety pharmacology assays. Analyses of extracellular field potentials in hESC-derived cardiomyocytes (hESC-CM) and generation of derivative field potential duration (FPD) values showed dose-dependent responses for 12 cardiac and non-cardiac drugs. Serum levels in patients of drugs with known effects on QT interval overlapped with prolonged FPD values derived from hESC-CM, as predicted. We thus propose hESC-CM FPD prolongation as a safety criterion for pre-clinical evaluation of new drugs in development. This is the first study in which dose responses of such a wide range of compounds on hESC-CM have been generated and shown to be predictive of clinical effects. We propose that assays based on hESC-CM could complement or potentially replace some of the preclinical cardiac toxicity screening tests currently used for lead optimization and further development of new drugs.

Introduction

The pharmaceutical industry presently generates large libraries of "new chemical entities" (NCEs) with the aim of selecting those most promising for clinical trials. Distinguishing specific from detrimental off-target effects at an early stage has the potential to accelerate clinical introduction, improve drug safety and reduce drug development costs. The heart has proven to be particularly sensitive to off-target, life threatening toxic effects of non-cardiac drugs. Over the last several years, reports of unexpected drug-induced ventricular arrhythmias associated with sudden cardiac death, have led to the withdrawal of a number of drugs from the market, while for many others, additional safety labels have been required to warn of potential risk[1].

An important determinant for development of Torsade de Pointes (TdP), is prolongation of the QT interval indicating delayed ventricular repolarization. This is defined as the period between the beginning of the QRS complex (depolarization of the ventricles) and the end of the T-wave (repolarization of the ventricles) on an electrocardiogram (ECG). In 1998 the Food and Drug Administration (FDA) defined prolongation of the QT interval on an ECG as a major drug safety issue. Currently, assessing risk for QT interval prolongation is part of the standard pre-clinical evaluation of NCEs as defined by the International Conference of Harmonization (ICH) Expert Working Group in topic S7B for all drugs in development[2,3].

Drug-induced long QT is most commonly induced by affecting the rapid potassium current I_{kr} by binding to the hERG ion channel[4,5]. Both for cardiac and non-cardiac drugs, primary canine or rabbit Purkinje fibers, or cell lines ectopically expressing the hERG ion channel are the preclinical *in vitro* test systems presently available. Species differences or the lack of complex ion channel interactions in transgenic cell lines reduce the predictive value of these systems. For example, determination of the IC_{50} value for a drug compound in transgenic cell lines over-simplifies a complex process that is state-, time- and voltage dependent. Some drugs like dofetilide require channel activation to reduce the hERG current[6] while others, like fluvoxamine, interact exclusively with a closed channel. Finally, not all drugs that block hERG channels in these cell systems carry an arrhythmogenic risk; one well known example is verapamil [1]. Also of note: many anti-arrhythmics can interact with multiple ion-channels simultaneously. In addition to potassium channels, the activity of a heart cell is largely dependent on sodium and calcium channels. Whereas activation of sodium channels is required for the generation of an action potential, inward Ca^{2+} currents through (L-type) calcium channels counterbalance outward potassium currents in the repolarization phase. To illustrate the complexity of predicting QT prolongation, a recent comparison of 10 drugs on dog Purkinje fibers demonstrated a wide range of effects on QT time, ranging from 158% prolongation to 16% shortening, while all compounds blocked hERG channels by more than 50%[7].

Together, these findings emphasize the importance of assessing pre-clinical cardiotoxic responses to drugs on cells that closely resemble functional cardiomyocytes of the human heart. Moreover, any alternatives to the current models must be scalable, reproducible and preferably from an inexhaustible source. Human embryonic stem cell-derived cardiomyocytes (hESC-CMs) have this potential[8]. Embryonic stem cells can differentiate to all somatic cell types including cardiomyocytes and in recent years, much effort has been put into developing efficient and reproducible differentiation protocols[9] and methods for cardiomyocyte enrichment from differentiated cell populations[10-12]. Here, we describe the use of hESC-CM in a medium-throughput screening system using microelectrode arrays (MEAs) to determine action potential duration or QT interval by measuring extracellular field potentials. We provide a first validation of the system, using a selection of drugs previously associated with QT prolongation and/or TdP during clinical use in humans. We show that hESC-CM, as used here, can recapitulate drug toxicity mediated through human ion-channels and therefore represent an opportunity for pre-clinical NCE evaluation.

Results

VALIDATION OF HESC-CM

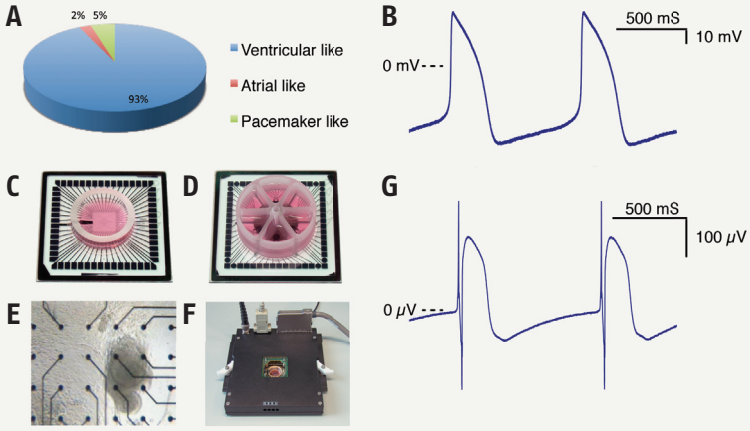
Co-culture of HES2 and HES3 cells with visceral endoderm-like END-2 cells is among the most efficient ways to produce enriched cultures of hESC-CM[9,13]. Using an optimized serum- and insulin free protocol, cultures containing ~25% cardiomyocytes can be reproducibly obtained within 12 days of initiating differentiation[14-16].

To test the cardioactivity of drugs, it is important that the electrical properties of the target cell are known. We therefore first analyzed action potentials of HES3 derived cardiomyocytes using patch clamp electrophysiology, as described previously[13]. Cells were designated as ventricle-like, atrial-like, or pacemaker-like based on the shape of the action potential (AP) defined by the following parameters: amplitude, maximal repolarization, maximum increase in AP (dV/dtmax), AP duration (APD90), and the overall pattern of the AP compared to those recorded in cells from 16 week human fetal hearts[13,17]. Ventricular-like APs characteristically show a plateau phase that results in longer repolarization compared to the more triangular shaped, atrial-like APs. Pacemaker-like cells are characterized by slow upstroke velocities and smaller amplitudes. As described previously for the HES2 cell line[13], cardiomyocytes generated from the HES3- END-2 co-cultures were almost exclusively ventricular-like (Figure 8.1A). An example of a typical action potential is shown in Figure 8.1B. We therefore concluded that these relatively homogeneous populations of cardiac ventricular cells may be particularly suitable for drug cardiotoxicity screening.

Traditional patch clamp electrophysiology, the most accurate measure of the properties of electrically excitable cells, requires precise positioning of the electrode on the plasma membrane to enable giga0hm seals to form between the electrode and the cell. However,

Figure 8.1

Experimental set-up



(A) Patch clamp analyses of cardiac differentiated HES3 cells. (B) Example of a ventricular-like action potential measured by patch clamp electrophysiology. (C) Multichannel electrode chip with culture chamber. (D) 6-Well multichannel electrode chip for higher throughput. (E) Example of hESC derived cardiomyocytes attached and cultured on a MEA chip. (F) Amplifier used for digitizing electrical signals recorded by one of the electrodes. (G) Example of a hESC-CM field potential

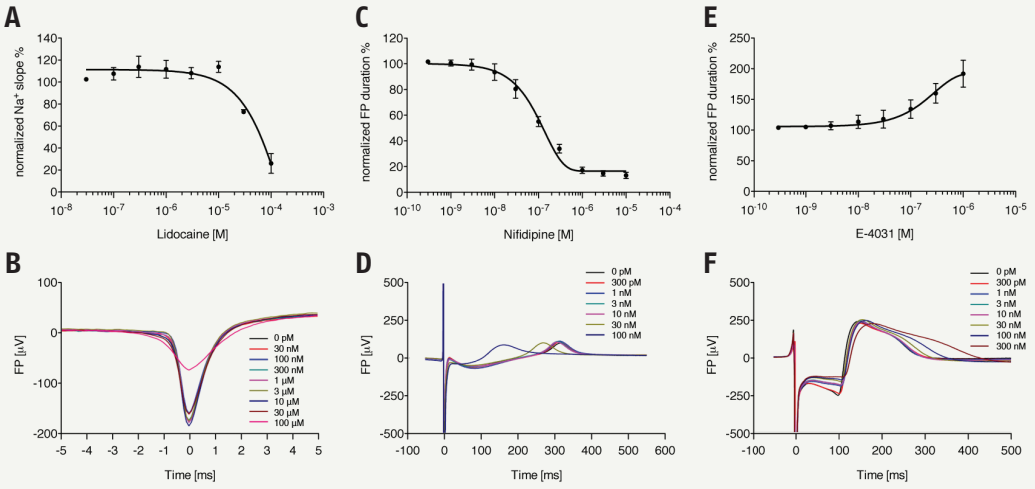


Figure 8.2

Validation of hESC-CM field potential responses

(A) Sodium peak amplitude-dose relationship for hESC-CM in the presence of increasing amounts of lidocaine. (B) Example of field potential raw data; note the reduction of amplitude at higher concentrations. (C) FPD-dose relationship for hESC-CM in the presence of increasing amounts of nifidipine. (D) Example of field potential raw data; note the reduction of field potential duration at higher concentrations of nifidipine. (E) FPD-dose relationship for hESC-CM in the presence of increasing amounts of E-4031. (F) Example of field potential raw data; note the prolongation of field potential duration in response to E-4031

this is labor intensive, limited to individual cells and therefore unsuitable for medium/high throughput drug screens. The more sophisticated planar patch clamp systems are potentially suitable but are difficult to implement since they require purified single cell suspensions. Measurement of external field potentials generated by clumps of beating cardiomyocytes derived from hESC, rather as an electrocardiogram on patients, represents an attractive alternative to conventional patch clamp analyses of APs that is amenable to medium throughput modalities. The signal recorded with one of the extracellular electrodes integrated into the substrate reflects local changes of the membrane potential in a cluster of electrically connected cardiomyocytes (Figure 8.1C–F). An example of a field potential is shown in Figure 8.1G. Parameters such as the field potential duration (FPD) are easy to define and measure and are directly linked to the action potential duration.

DRUG EFFECTS ON HESC-DERIVED CARDIOMYOCYTES

To determine whether hESC-CM would be a useful model to detect drug-induced QT elongation we first profiled field potential duration in response to selective ion channel blockers. Lidocaine was used to block I_{Na}, nifidipine to block I_{CaL} and E-4031 for I_{Kr}. As expected, Lidocaine did not affect the FPD in three independent experiments; however it caused cessation of beating in the 30 µM – 100 µM concentration range (data not shown). This is in agreement with a published I_{Na} IC_{50} for lidocaine of 61 µM[18] and with a study of Satin et al, who described initiation of the spontaneous action potentials in hESC-CM by the sodium current[19].

Nifidipine, a typical L-type calcium channel blocker, reproducibly induced a dose dependent shortening of the FPD, which was initiated at 10 nM and saturated at 1 µM in three independent experiments (Figure 8.2C,D). In 3 out of 4 independent experiments loss of spontaneous activity was observed between 300 nM and 1 µM, indicating the importance of a strong inward calcium current for cyclic contraction. Concomitant with decreased FPD, we observed a higher beating frequency but no arrhythmic behavior.

E-4031 is a specific blocker of the hERG channel. In four independent experiments E-4031 induced sustained prolongation of the FPD at concentrations of 30nM and higher (Figure 8.2E,F). At concentrations of 1–3 µM, early after-depolarizations (EADs), which indicate an increased tendency for arrhythmia, were observed in 3 out of 4 experiments (data not shown). Beating frequency was not affected at nanomolar concentrations; however, at higher concentrations it was reduced, indicating a concomitant non-specific ion-channel block.

RESPONSES TO REPOLARIZATION PROLONGING ANTI-ARRHYTHMIC DRUGS

A large class of cardioactive drugs is used clinically to treat arrhythmias. These drugs are classified in four categories and, except for category II (beta-adrenergic receptor-blocking), their mechanism of action is related to ion-channel block. We tested two compounds, quinidine (class Ia) and D,L-sotalol (Class II, III) for their effects on field potential profiles. Quinidine administration to patients is strongly associated with TdP (1–8.8%)[1]. In hESC-CM it induced a dose dependent FPD prolongation (Figure 8.3A). At the estimated unbound therapeutic

plasma concentration (ETPC unbound) which is 0.9–3.2 µM, we observed a 20–50% increase in QT time which was further prolonged at higher concentrations. D,L-sotalol administration to patients is also strongly associated with TdP (1.8–4.8%); it also induced a dose dependent FDP prolongation in hESC-CM. At the ETPC unbound (1.8 – 14 µM) we observed a 15 – 20% FPD prolongation, which increased at higher concentrations (Figure 8.3B). In summary, both drugs prolonged the FPD at physiologically relevant plasma concentrations, as expected.

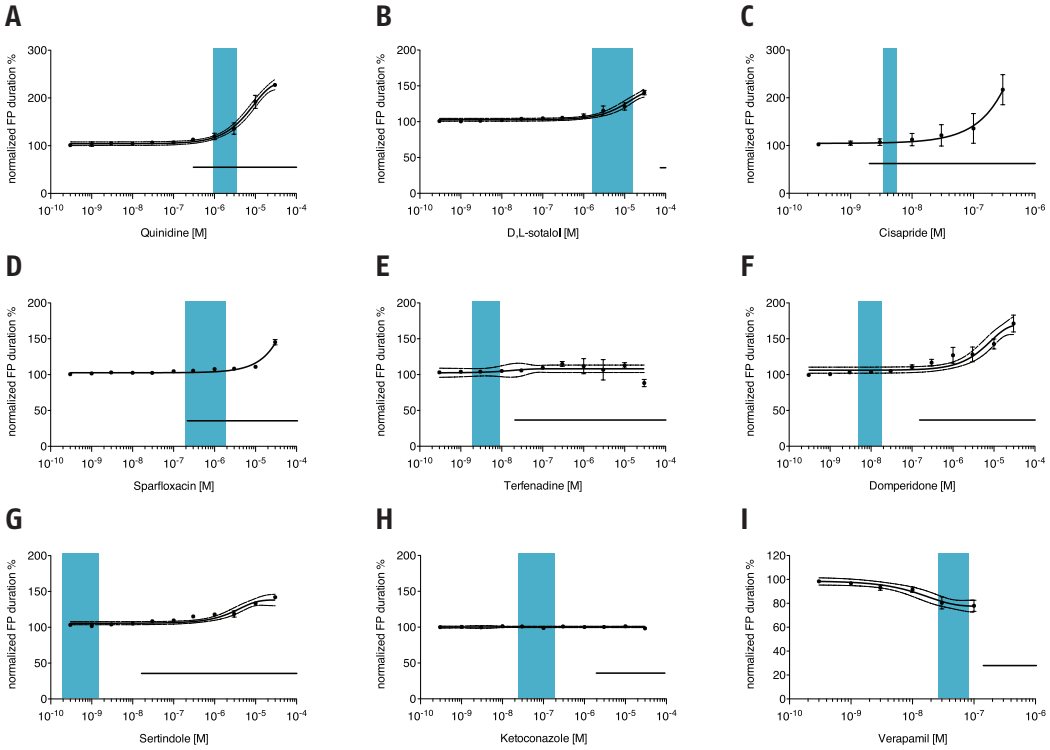


Figure 8.3

Drug responses of a various drugs

FPD–dose relationship for hESC-CM in the presence of increasing amounts of (A) Quinidine, (B) D,L-sotalol. (C) cisapride (D) sparfloxacin, (E) terfenadine, (F) domperidone, (G) sertindole. (H) ketoconazole (I) verapamil. The blue areas indicate the ETPC unbound measured in patients and the black line indicates hERG IC_{50}. Dotted lines indicate the 95% confidence interval of the fitted curve. ETPC unbound and hERG IC50 data are adapted from reference 1.

RESPONSES TO NON-CARDIAC DRUGS ASSOCIATED WITH TdP OR QT PROLONGATION

In addition to repolarization-prolonging anti-arrhythmic drugs, where QT prolongation is the desired effect, a large class of (non-cardiac) drugs prolongs the QT interval as a detrimental side effect. These drugs derive from various therapeutic areas and include but are not limited to antihistamines, antipsychotics and anti-microbial agents. Here we tested cisapride, sparfloxin, terfenadine, domperidone and sertindole, all of which are associated with QT prolongation and/or TdP as a side effect. Although the clinical incidence of TdP for all of these drugs is low, cisapride and terfenadine have been withdrawn from the market because of negative risk/benefit analyses. Cisapride is a serotonin 5-HT4 agonist which increases acetylcholine release in the enteric nervous system and was used for the treatment of a number of gastrointestinal disorders, particularly gastro-oesophageal reflux disease in adults and children. In a market span of 12 years, 384 cases of ventricular arrhythmias due to TdP were reported, giving a TdP incidence of 1/120,000[20]. The ETPC unbound is 2.6-4.9 nM [1]. At this concentration we observed no effects on the FPD (Figure 8.3C). However, at higher concentrations sustained prolongation of the FDP was observed (Figure 8.3C).

Sparfloxacin is an antibiotic used for the treatment of bacterial infections. The ETPC unbound is 0.19 - 1.76 µM which overlaps with published IC_{50} values (0.23 – 34.4 µM). This would classify sparfloxacin with repolarization prolonging anti-arrhythmics in a conventional hERG assay. However in integrated safety analysis of data from 6 multicenter phase III trials, QTc (heart rate corrected QT interval) prolongation (10 +/- 24 milliseconds) occurred in 1.1% of the patients. There were no ventricular arrhythmias related to QTc prolongation in any of these patients[21]. In hESC-CM we found no FDP prolongation at the ETPC unbound but only at higher concentrations (Figure 8.3D), thus supporting the toxicity screening potential of the hESC-CM model as used here. Terfenadine, an antihistamine used for the treatment of allergies, is a "prodrug" and generally metabolized to the active form Fexofenadine by intestinal CYP3A4. The association with TdP is low with an occurrence of once in 10,000 person years. Nevertheless the compound has been withdrawn from market due to perceived risks. The ETPC unbound is 0.1-0.29 nM. However in cases of genetic deficiencies or induced CYP3A4 inhibition (e.g grapefruit juice or erythromycin) Terfenadine concentrations in human plasma may reach 100 nM[22]. At this higher concentration, we observed 10% FPD prolongation (Figure 8.3E). At even higher concentrations, we observed a further increase, followed by a dose dependent reduction of FPD at micromolar concentrations. The shortening is most likely a result of calcium channel block[23]. Domperidone is an anti-dopaminergic drug administrated either orally, rectally and intravenously to suppress nausea and vomiting. TdP has been reported sporadically and solely with intravenous use[24,25]. The ETPC unbound is 5-19 nM; at this concentration there is only a minor prolongation of the FPD (Figure 8.3F). At higher concentrations, however, there is a biphasic dose dependent FPD prolongation that suggests block of multiple channels (Figure 8.3F).

Finally we tested the effects of sertindole. Sertindole is a drug used for the treatment of schizophrenia but was withdrawn from the market in 1998 after concern on the high rate of

proven or suspected TdP. After re-evaluation of existing data and new clinical data, Sertindole was reintroduced to the European market in 2002[26]. We did not observe FDP prolongation at the ETPC unbound (0.02-1.59 nM) and even at higher concentrations, only a relatively weak FPD prolongation was observed (Figure 8.3G). In summary, all of these compounds showed effects on the FPD interval, although in general, this was at concentrations higher than the ETPC unbound. This correlates well with the overall very low incidence of TdP induced by these drugs. In general, for these drugs a wide safety margin between plasma concentrations and side effects on cardiomyocytes would be advisable. Induction of TdP due to overdosing or disturbed metabolism (see for example terfenadine) is not acceptable and has led to numerous drug failures.

RESPONSES TO DRUGS NOT ASSOCIATED WITH TDP IN MAN

Next we investigated two drugs not clinically associated with QT prolongation or TdP (as negative controls). Ketoconazole is a drug used to treat a variety of fungal infections and there is no evidence that it causes TdP or QT prolongation. However ketoconazaole is a strong inhibitor of cyp34a and may therefore be involved in drug-drug interactions associated with TdP (for example terfenadine). We observed no FPD prolongation in hESC-CM in the concentration range of 0.3 nM- 30 μM, as expected (Figure 8.3H). Verapamil is a class IV anti-arrhythmic used clinically to treat atrial tachyarrhythmias. The compound exerts its action by blocking calcium channels but is also a potent hERG blocker. In fact the narrow safety margin between the ETPC unbound (25-81 nM) and the hERG IC_{50} (140 – 830 nM) would make this compound a false positive in conventional hERG assays (Redfern et al).

At the ETPC unbound concentration (25-81 nM) we observed a minor FPD shortening which was amplified at higher concentrations (Figure 8.3I). Neither of the negative controls in the assay, caused any FPD prolongation.

Discussion

Efficient, reproducible differentiation of hESC to ventricular cardiomyocytes, preferably phenotypically similar to adult human cardiomyocytes, is of critical importance for implementing hESC-CM technologies in drug cardiotoxicity screening. Previously we have shown that the hESC-END-2 co-culture system is a very efficient and reproducible method for cardiac lineage differentiation, in particular to ventricular-like cells. Here we show that these cardiomyocyte populations are phenotypically homogeneous, have strong electrophysiological responses and are suitable for drug safety pharmacology.

Previously, we and others have discussed the immaturity of hESC-CM compared to the human adult heart[8,13,19,27]. In a previous study we disaggregated beating areas that had formed during 12 days of differentiation and found by patch clamp electrophysiology that the cells were comparable to 16-week fetal ventricular cardiomyocytes[13]. One of the major

differences between hESC-CM and mature cardiomyocytes is their relatively low resting potential. This means that $Na_v1.5$ sodium channels can be spontaneously activated resulting in AP generation[19]. Finally, the initial upstroke velocity of the AP is relatively small due to a lower I_{Na} current compared to adult cardiomyocytes. Importantly, chronotropic responses of dissociated hESC-CM are comparable to human fetal ventricular cells. This was demonstrated by decreased beating frequency following treatment with the cholinergic agonist carbachol, whereas adrenergic agonists Phenylephrine and Isoprenaline increased beating frequency[13]. Interestingly, despite the relatively immature phenotype of hESC-CM, we found the drug responses highly predictive of clinically observed cardiotoxic effects. To validate hESC-CM as a potentially useful new *in vitro* test system for QT prolongation and TdP, we performed dose escalation studies for a number of drugs known to prolong the QT interval as a therapeutic- or side effect. Two previous studies also used hESC-CM in combination with the MEA system to measure drug responses[28,29] but none of them compared a number of compounds systematically over a wide concentration range in dose dependent manner. As a starting point, we used three drugs that block sodium, calcium and hERG potassium channels. As expected lidocaine, a classical sodium channel blocker, caused dose –dependent cessation of beating at 30 µM – 100 µM. Nifidipine, a classical calcium channel blocker, reduced the FPD in a dose dependent fashion and E-4031 increased the FPD by blocking the hERG channel. Interestingly we observed arrhythmic behavior in 3 of 4 independent beating areas in response to high E-4031 concentrations. E-4031 was developed as novel class III anti-arrhythmic but this development was stopped for reasons that have remained undisclosed. Most importantly, hESC-CM behavior was reproducible although a wide variety of field potential shapes were observed. This illustrates that hESC-CM as a model can be used to investigate the effects of drugs on various ion channels present in cardiomyocytes.

Having validated the different ion channel responses, we then profiled a series of drugs that fell into one of three categories: 1) repolarization prolonging anti-arrhythmics, 2) drugs associated with QT prolongation or with TdP as clinical side effect and 3) drugs not associated with QT prolongation or TdP (negative controls). Quinidine and D,L-sotalol are both used therapeutically to prolong the QT interval, but are also strongly associated with arrhythmias. Both D,L-sotalol and quinidine induced prolongation at concentrations relatively close to the ETPC unbound. D,L-sotalol is especially interesting since the hERG IC_{50} data (74 µM) is clearly distinct from the ETPC unbound (1.8 – 14 µM). Therefore, despite the strong association with QT prolongation and TdP, a conventional hERG assay would predict no effect QT effect of the compound at therapeutic plasma concentrations. Quinidine, showed a massive prolongation of the field potential at concentrations higher than the ETPC unbound. Quinidine ion channel interactions are known to be complex. In addition to its blocking effect on hERG, Quinidine blocks inward calcium current (I_{Ca}), the rapid (I_{Kr}) and slow (I_{Ks}) components of the delayed potassium rectifier current, the inward potassium rectifier current (I_{KI}), the ATP-sensitive potassium channel (I_{KATP}) and I_{to}[30]. Furthermore, quinidine-induced I_{Na} block has been shown to increase at higher beating frequencies, dependent on the ion channel state. It has a low affinity for the resting state, avidly blocks open channels but does not bind significantly

to inactivated sodium channels[31,32]. Nevertheless its net effect is prolongation of the AP in normal and diseased hearts, which was clearly shown in the hESC-CM.

Addition of drugs from the second category, domperidone, sparfloxacin and cisapride to hESC-CM had a clear effect on the FPD interval. Interestingly in contrast to the repolarization prolonging drugs tested here, the effective concentration triggering FDP prolongation was always higher than the ETPC unbound, which might explain the low incidence of TdP in patients taking these drugs. Although both sertindole and terfenadine, drugs from the same category, had only minor effects on the FPD interval this may nevertheless indicate functional interactions with ion channels. Terfenadine caused a minor prolongation of the FPD interval followed by a shortening at higher concentrations. This is in agreement with guinea-pig ventricular myocyte data where terfenadine has also been described as causing a small, but significant, prolongation of APD90 at 100 nM and 1 µM at 1 Hz stimulation frequency, while at higher concentrations, AP duration was shortened[33]. In the same study, dog Purkinje fibers showed no significant effects after Terfenadine administration[33]. In conclusion; we observed FPD changes for all tested drugs without exception but always at a higher concentration that the ETPC unbound. This was distinct from the repolarization prolonging drugs where we observed FPD changes at the ETPC unbound. We therefore hypothesize that drugs with a low pro-arrhythmic potential (ability to induce TdP) can act synergistically with known pro-arrhythmic risk factors such as silent mutations, drug-drug interactions, heart failure, ventricular hypertrophy, hypomagnesemia, hypokalemia, hypocalcemia, hypoxia or acidosis. The combination of any of these factors can induce TdP in patients taking these medicines[5,34]. Finally, addition of the negative control drugs verapamil and ketoconazole to hESC-CM did not result in FPD prolongation, as expected. The fact that verapamil did not prolong the FP interval is of interest since the close margin between the ETPC unbound (25-81 nM) and the hERG IC_{50} (140 – 830 nM) would suggest differently. Therefore verapamil is a classic "false positive" in a conventional hERG assay.

Previously, an association of 1.93 (95% CI: 1.89–1.98) between the anti-hERG activity of drugs, measured as log10 (ETCP unbound/IC_{50}), and reports of serious ventricular arrhythmias and sudden death to the WHO-UMC database was found[35]. In another study, a 30-fold safety margin (between the hERG IC_{50} and the ETPC unbound) was established as providing an acceptable degree of safety[1]. Using hERG IC_{50} as criteria, both verapamil and sparfloxacin would be classified incorrectly. Verapamil was introduced to the market in 1982 and has not been associated with TdP. This is likely the result of multiple channel blocks, which compensate the hERG blocking effect. In fact we observed a shortening of the FPD in hESC-CM, which is in agreement with clinical data[36]. Sparfloxacin has a low pro-arrhythmic potential. However, the hERG IC_{50} and the ETPC unbound values are in the same concentration range. This would suggest a strong association with QT prolongation and TdP, as anti-arrhythmic drugs prolonging repolarization. The hESC-CM model as used here would have accurately predicted the risk by showing drug induced QT prolongation but only at concentrations higher than the ETPC unbound. The data for both verapamil and sparfloxacin underscore the necessity of a

human model system that displays the full complexity of the human cardiomyocyte.

In conclusion, our results indicate that electrophysiological properties and drug responses of hESC-CM match clinical observations on QT prolongation/shortening and arrhythmia at similar concentrations. We showed here that by using FPD prolongation as a read out hESC-CM flag up adverse drug effects with greater confidence than standard hERG *in vitro* assays. Screening a wider variety of drugs under defined conditions would further validate and identify the strengths and weaknesses of the system. Ultimately this should lead to the definition of a safety margin which can be instrumental for improving pre-clinical decision making and reducing drug development costs.

Materials and Methods

CELL CULTURE AND DIFFERENTIATION

HES3 cells[37] were routinely cultured on 129SV Mouse embryonic fibroblast (MEFs) to maintain pluripotency and induced to differentiate to cardiomyocytes as described previously[13,14]. Large numbers of contracting areas are obtained within 12 days that routinely consist of 20-25% cardiomyocytes and 75-80% endodermal cells and other mesodermal derivatives but no neural cells.

PATCH CLAMP ELECTROPHYSIOLOGY

Patch clamp electrophysiology was essentially done as described previously[38,39]. Briefly, beating clumps of cardiomyocytes were dissociated using a collagenase-based method. Cardiomyocytes were plated on gelatin-coated glass and measured between 7 and 14 days after plating. Microelectrodes with a resistance between 1 and 3 MΩ were made from Borosilicate glass (Clark electrical instruments, GC-150 T) with Flaming/Brown Micropipette Puller Model 80 (Sutter Instruments, CA). During electrophysiological measurements were kept in a DMEM/F12 based medium without serum or Phenol red. The pipette contained buffer consisting of 145 mM KCl, 5 mM NaCl, 2 mM $CaCl_2$, 4 mM EGTA, 2 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.30 with KOH.

MEA ELECTROPHYSIOLOGY

MEA chips were plasma cleaned and coated with fibronectin for 1 hour at 37°C. Clumps of beating hESC-CM clusters were microdissected and replated on standard 60 electrode MEAs or alternatively 6-well MEAs. Extracellular recording was performed using a MEA1060INV MEA amplifier (Multi Channel Systems, Reutlingen, Germany). Output signals were digitized at 10 kHz by use of a PC equipped with a MC-card data acquisition board (Multi Channel Systems, Reutlingen, Germany). Standard measurements were performed in DMEM supplemented with 5% FCS. During recordings, temperature was kept at 37°C. Data were recorded using QT-screen (Multi Channel Systems, Reutlingen, Germany) and analyzed off-line with QT-analyzer (Multi Channel Systems, Reutlingen, Germany).

DATA PROCESSING

Field potential duration or sodium amplitude data from at least three independent beating areas were averaged using GraphPad Prism software. Datapoints are displayed as mean +/- SEM. Curves were fitted using the one phase decay algorithm, 95% confidence intervals are plotted as dashed lines.

COMPOUNDS

Lidocaine, nifedipine, E-4031, cisapride monohydrate, quinidine and verapamil hydrochloride, D-L-Sotalol, sparfloxacin, sertindole, terfenadine, domperidone and ketoconazole were obtained from Sigma-Aldrich. The substances were dissolved DMSO at 10 mM and serial dilutions were made in medium. The highest concentration of DMSO is reached at 30µM drug which represents 0.3% DMSO. Measurements were started after a wash-in time of 2 min.

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References

1. Redfern, W.S., et al. Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. Cardiovascular Research 58, 32-45 (2003).

2. Bode, G. & Olejniczak, K. ICH Topic: The draft ICH S7B step 2: Note for guidance on safety pharmacology studies for human pharmaceuticals. Fundamental & Clinical Pharmacology 16, 105-118 (2002).

3. Cavero, I. & Crumb, W. ICH S7B draft guideline on the non-clinical strategy for testing delayed cardiac repolarisation risk of drugs: a critical analysis. Expert Opinion on Drug Safety 4, 509-530 (2005).

4. Moss, A.J. & Kass, R.S. Long QT syndrome: from channels to cardiac arrhythmias. J Clin Invest 115, 2018-2024 (2005).

5. Roden, D.M., et al. Multiple Mechanisms in the Long-QT Syndrome: Current Knowledge, Gaps, and Future Directions. Circulation 94, 1996-2012 (1996).

6. Weerapura, M., Hébert, T. & Nattel, S. Dofetilide block involves interactions with open and inactivated states of HERG channels. Pflügers Archiv European Journal of Physiology 443, 520-531 (2002).

7. Martin, R.L., et al. The utility of hERG and repolarization assays in evaluating delayed cardiac repolarization: influence of multi-channel block. J Cardiovasc Pharmacol 43, 369-379 (2004).

8. Denning, C. & Anderson, D. Cardiomyocytes from human embryonic stem cells as predictors of cardiotoxicity. Drug Discovery Today: Therapeutic Strategies AOP doi:10.1016/j.ddstr.2008.08.002 (2008).

9. Passier, R. & Mummery, C. Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 16, 498-502 (2005).

10. Anderson, D., et al. Transgenic Enrichment of Cardiomyocytes From Human Embryonic Stem Cells. Mol Ther 15, 2027–2036 (2007).

11. Huber, I., et al. Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. FASEB J. 21, 2551–2563 (2007).

12. Xu, X.Q., et al. Highly enriched cardiomyocytes from human embryonic stem cells. Cytotherapy 10, 376 – 389 (2008).

13. Mummery, C., et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm–like cells. Circulation 107, 2733–2740 (2003).

14. Passier, R., et al. Increased cardiomyocyte differentiation from human embryonic stem cells in serum–free cultures. Stem Cells 23, 772–780 (2005).

15. Mummery, C.L., Ward, D. & Passier, R. Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum–free medium. Curr Protoc Stem Cell Biol Chapter 1, Unit 1F 2 (2007).

16. Freund, C., et al. Insulin Redirects Differentiation from Cardiogenic Mesoderm and Endoderm to Neuroectoderm in Differentiating Human Embryonic Stem Cells. Stem Cells, 2007–0617 (2007).

17. Chuva de Sousa Lopes SM, R.J.H., Alie Feijen, Marga A. van Rooijen, Pieter A. Doevendans, Leon Tertoolen, Aart Brutel de la Rivière, Christine L. Mummery. Patterning the heart, a template for human cardiomyocyte development. Developmental Dynamics 235, 1994–2002 (2006).

18. Hill, R.J., Duff, H.J. & Sheldon, R.S. Class I antiarrhythmic drug receptor: biochemical evidence for state– dependent interaction with quinidine and lidocaine [published erratum appears in Mol Pharmacol 1989 Nov;36(5):823]. Mol Pharmacol 36, 150–159 (1989).

19. Satin, J., et al. Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes. The Journal of physiology 559, 479–496 (2004).

20. Darpo, B. Spectrum of drugs prolonging QT interval and the incidence of torsades de pointes. Eur Heart J Suppl 3, K70–80 (2001).

21. Lipsky, B.A., Dorr, M.B., Magner, D.J. & Talbot, G.H. Safety profile of sparfloxacin, a new fluoroquinolone antibiotic. Clinical Therapeutics 21, 148–159 (1999).

22. Roy, M.–L., Dumaine, R. & Brown, A.M. HERG, a Primary Human Ventricular Target of the Nonsedating Antihistamine Terfenadine. Circulation 94, 817–823 (1996).

23. Liu, S., Melchert, R.B. & Kennedy, R.H. Inhibition of L–Type Ca2+ Channel Current in Rat Ventricular Myocytes by Terfenadine. Circ Res 81, 202–210 (1997).

24. Osborne, R.J., Slevin, M.L., Hunter, R.W. & Hamer, J. CARDIOTOXICITY OF INTRAVENOUS DOMPERIDONE. The Lancet 326, 385–385 (1985).

25. Drolet, B., Rousseau, G., Daleau, P., Cardinal, R. & Turgeon, J. Domperidone Should Not Be Considered a No–Risk Alternative to Cisapride in the Treatment of Gastrointestinal Motility Disorders. Circulation 102, 1883–1885 (2000).

26. Lindstrom, E., Farde, L., Eberhard, J. & Haverkamp, W. QTc interval prolongation and antipsychotic drug treatments: focus on sertindole. Int J Neuropsychopharmacol 8, 615–629 (2005).

27. Passier, R., van Laake, L.W. & Mummery, C.L. Stem–cell–based therapy and lessons from the heart. Nature 453, 322–329 (2008).

28. Reppel, M., et al. The electrocardiogram of human embryonic stem cell–derived cardiomyocytes. Journal of Electrocardiology 38, 166–170 (2005).

29. Caspi, O., et al. In Vitro Electrophysiological Drug Testing using Human Embryonic Stem Cell Derived Cardiomyocytes. Stem Cells and Development 0.

30. Nenov, N.I., Crumb, W.J., Jr., Pigott, J.D., Harrison, L.H., Jr. & Clarkson, C.W. Quinidine Interactions With Human Atrial Potassium Channels : Developmental Aspects. Circ Res 83, 1224–1231 (1998).

31. Hondeghem, L.M. & Matsubara, T. Quinidine blocks cardiac sodium channels during opening and slow inactivation in guinea-pig papillary muscle. Br J Pharmacol 93, 311-318 (1988).

32. Tsujimae, K., Suzuki, S., Yamada, M. & Kurachi, Y. Comparison of kinetic properties of quinidine and dofetilide block of HERG channels. European Journal of Pharmacology 493, 29-40 (2004).

33. Terrar, D.A., Wilson, C.M., Graham, S.G., Bryant, S.M. & Heath, B.M. Comparison of guinea-pig ventricular myocytes and dog Purkinje fibres for in vitro assessment of drug-induced delayed repolarization. Journal of Pharmacological and Toxicological Methods 56, 171-185 (2007).

34. Roden, D.M. & Viswanathan, P.C. Genetics of acquired long QT syndrome. J Clin Invest 115, 2025-2032 (2005).

35. De Bruin, M.L., Pettersson, M., Meyboom, R.H.B., Hoes, A.W. & Leufkens, H.G.M. Anti-HERG activity and the risk of drug-induced arrhythmias and sudden death. Eur Heart J 26, 590-597 (2005).

36. Fauchier, L., et al. Effect of verapamil on QT interval dynamicity. The American Journal of Cardiology 83, 807-808 (1999).

37. Costa, M., et al. The hESC line Envy expresses high levels of GFP in all differentiated progeny. Nat Meth 2, 259-260 (2005).

38. Moore, J.C., et al. Human embryonic stem cells: Genetic manipulation on the way to cardiac cell therapies. Reproductive Toxicology 20, 377-391 (2005).

39. Graichen, R., et al. Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation 76, 357-370 (2008).

CHAPTER NINE

Cardiomyocytes from human pluripotent stem cells in regenerative medicine and drug discovery

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Abstract

Stem cells derived from either preimplantation human embryos or from somatic cells by reprogramming are pluripotent and self-renew indefinitely in culture. Pluripotent stem cells are unique in being able to differentiate to any cell type of the human body. Differentiation towards the cardiac lineage has always attracted significant attention, initially with a strong focus on regenerative medicine. Although an important research area the heart has proven challenging to repair by cardiomyocyte replacement. However, the ability to reprogram adult cells to pluripotent stem cells and genetically manipulate stem cells presented opportunities to develop human disease models. The availability of human cardiomyocytes from stem cell sources is expected to accelerate cardiac drug discovery and safety pharmacology by offering more clinically relevant human culture models than presently available. Here we review the state of the art using stem cell-derived human cardiomyocytes in drug discovery, drug safety pharmacology and regenerative medicine.

Introduction

The first derivation of human embryonic stem cells (hESC) in 1998 led to great excitement in the field of regenerative medicine[1]. For the first time there were opportunities to treat a multiplicity of diseases with cell therapy. Restoration of heart function by replacing damaged cardiomyocytes by cardiomyocytes from stem cells was among the most appealing applications. However, issues like effective transplantation techniques, proper functional integration, safety related to introduction of electrically active cells in the heart and teratoma formation by residual undifferentiated cells as well as immune rejection of transplanted cells are now considered to represent significant hurdles to clinical introduction. For other tissues, these issues may be less significant obstacles for clinical trials. For example, Geron, a US stem-cell based company, recently received FDA approval for the first trial to evaluate the safety of transplanting hESC-derived oligodendrocytes into the spinal cords of patients suffering from acute crush lesions. In addition, clinical trials planned to transplant hESC-derived retinal epithelial cells intraoccularly in patients with macular degeneration are likely to receive regulatory approval shortly. However, apart from these exceptions, it is generally accepted that cell therapy based on pluripotent stem cell derivatives is a long-term perspective. By contrast, largely as a result of refinements to culture and differentiation protocols over the past 10 years, short-term applications of pluripotent human stem cells are expected in the area of drug discovery and safety pharmacology.

The pharmaceutical industry has been developing and marketing small molecule drugs to treat various diseases successfully for decades. Factors that have shaped the industry include the search for 'best in class' drugs with unquestionable benefit versus risk profiles and highest predicted return-on-investment. This research and development (R&D) strategy has resulted in multiple drugs with revenues exceeding $1 billion annually. However, this R&D strategy also led to a steady decline in R&D productivity from 53 new molecular entities (NMEs) approved in 1996 to only 21 in 2008[2]. In addition, the recent withdrawal of the anti-inflammatory drug Vioxx from the market due to unforeseen cardiotoxic effects has forced regulatory bodies to review their approval policy, which will most likely lead to tighter registration requirements and subsequently to higher costs of drug development. As a consequence, focus is on more personalized, predictive and preventive medicine[3].

Most R&D expenditures are presently incurred during late stages of clinical development, and therefore companies are focusing on cost reductions at these stages. Whilst difficult to speed up regulatory processes or reduce the size of clinical trials, historical perspectives indicate that clinical attrition can be minimized if the principle causes are identified. In 1990 >40% of drug attrition was the result of pharmacokinetic and bioavailability issues; in 2000 this was reduced to <10%[3]. Unforeseen (cardio)toxicity and lack of efficacy are at present the most common cause of clinical attrition[4,5]. This may in part be because much pre-clinical drug discovery-related R&D is carried out initially on cell lines with low clinical relevance and only much later in costly animal models. The tumour cell lines or primary cells used are not

always representative for the end point of interest. Implementation of model systems based on normal human cells could reduce the incidence of unexpected toxicology and lack of efficacy by offering specific human targets in a cost effective way rather than less physiological non-human biological environments. Pluripotent stem cells in combination with specific differentiation protocols offer the opportunity to create appropriate human test models with high biological relevance. Indeed, at least 70% of the top 20 pharmaceutical companies are working with stem cells, 64% of them now investigating the use of hESC[6]. In addition, 50% of the top 10 biotech companies make use of stem cells, 20% of them makes use of hESC[6].

In this review we will discuss the opportunities for cardiomyocytes derived from human pluripotent stem cells with respect to cell therapy, safety pharmacology and drug development (Figure 9.1). In addition, we will comment on the hurdles in bringing these cells from the research bench to the pharmaceutical R&D laboratory and clinical practice.

Human pluripotent stem cells

hESC were first derived from blastocyst stage embryos surplus to assisted reproduction requirement in 1998[1]. Since then it has been estimated that >800 normal and genetic disease-bearing lines have been generated under various culture conditions. Because of their ability to self-renew indefinitely and differentiate to all cell types of the body, hESC were initially of great interest for developing clinically relevant cell replacement therapies. In addition, they presented opportunities for studying differentiation in early human development, genetic disorders and screening new drug and compound libraries. However, progress has lagged behind expectations, in part because of: (1) labour intensive non-defined culture protocols for self-renewal, (2) few efficient directed differentiation protocols (see below), (3) poor transfection efficiencies which have limited functional analysis of signalling pathways controlling fate choices and cell selection, and (4) low single cell cloning efficiencies compared with, for example, mouse ESCs. The first hESC lines were derived on mouse embryonic feeder cells (MEFs) in medium containing fetal calf serum (FCS)[1,7] . In contrast to mouse ESCs, leukemia inhibitory factor (LIF) was unable to replace MEFs in supporting self-renewal of hESC. hESC instead required basic fibroblast growth factor (bFGF) for self-renewal under (semi) defined conditions[8]. Multiple "defined" culture condtions for hESC, based on slightly different basal media and supplements have since been described[9–13]. The International Stem Cell Initiative[14] is presently comparing these different conditions on different hESC lines with view to identifying the most robust and ubiquitously applicable. Both mechanical "cut-and-paste" and enzymatic (trysin, dispase, collagenase, Tryp.LE) passaging methods are being compared for their ability to sustain self renewal without loss of developmental potency and karyotypic stability. Defined growth conditions for hESC are not only important for therapeutic application but are also essential for developing robust and reproducible *in vitro* assays. The first fully defined medium for hESC was called mTeSR. Initially mTeSR only worked in combination with a complex mixture of purified human collagen IV, fibronectin, laminin and

vitronectin matrix components [11]. Later it was shown that Matrigel™ a laminin-111-rich, mouse sarcoma-derived commercial product also containing collagen IV, entactin, heparin sulfate proteoglycan and multiple other (growth factor) components also supported hESC self renewal [15]. Recently we showed that mTeSR1 in combination only with recombinant vitronectin was sufficient to support sustained self-renewal in three independently derived hESC lines, retaining karyotypic stability and pluripotency[16]. Recently the first hESC lines were derived under conditions compliant with Good Manufacturing Practice (GMP), although co-culture with human feeder cells pre-cultured in FCS-containing medium and bovine serum albumin supplement was still required. Whether these culture conditions will be generally accepted as best practice for GMP remains to be established[17]. Methods for efficient gene transfection using chemical, viral and electroporation based methods have now been developed for multiple cell lines using trypsin based monolayer culture[18-21] . The issue of low cloning efficiency (which limits selecting genetically modified clones and demonstrating pluripotency at the single cell rather than population level) has been partly solved by addition of a selective inhibitor of p160-Rho-associated coiled-coil kinase (ROCK) to the culture medium[22]. Finally, for scaling up stem cell production, it was recently shown that the CompacT SelecT™ robotics system could be used for serial propagation of hESC cells on Matrigel™ without loss of pluripotency or differentiation potential[23]. This widely used system can manage 90 T175 flasks simultaneously which facilitates scaling to $>10^9$ cells in one batch. Together, these improvements in hESC culture techniques have set the stage for medium to high throughput robotics and production scale up that would be essential for both *in vitro* and therapeutic use.

INDUCED PLUIPOTENT (iPS) CELLS

One of the most remarkable discoveries of the last few years is that adult somatic cells can be reprogrammed to a pluripotent stem cell state by the transient expression of just a few key transcription factors. In 2006, Yamanaka showed that four genes (c-Myc, Klf4, Oct4 and Sox2) could convert adult mouse fibroblasts to cells virtually indistinguishable from mouse ESCs[24]. In 2007 Yamanaka and Thomson independently described the ability to derive induced pluripotent stem (iPS) cells from human somatic cells[25,26]. Human iPS cells, like their mouse counterparts are very similar to hESC but do not require embryo use for derivation. In addition, they can be derived from patients with complex genetic defects to create disease models that would otherwise only be available through derivation of hESC lines from embryos. Today, iPS cell lines can be generated without integration of transgenes, as required in the original method[27,28]. Although there is accumulating evidence that iPS cells differ from ESCs based on analysis of methylation state and gene (mRNA) and miRNA expression[29,30] it seems most likely that the cells can be used to study disease processes. However, some disease-associated mutations might interfere with iPS cell generation as recently shown for mutations in the Fanconi Anaemia pathway[31]. Nevertheless, studies have shown that iPS cell lines from patients with neurodegenerative disorders like spinal muscular atrophy[32] and amyotrophic lateral sclerosis (ALS)[33] recapitulate the disease phenotype. IPS cells thus represent an opportunity to study disease development "in a dish" and hence can serve as models to test methods of delaying its advance or reversing its phenotype.

Cardiac differentiation of pluripotent stem cells

Various methods have been described to induce differentiation in pluripotent cell lines. Most widely used is the growth of undifferentiated cells as aggregates in suspension which causes them to form structures called embryoid bodies[34]. Within the embryoid body, derivatives of the three primary germ layers, ectoderm, endoderm and mesoderm develop spontaneously. Cardiomyogenic mesodermal progenitors are normally formed in the embryo during gastrulation as cells of the epiblast pass through the primitive streak[35]. Many although not all protocols for directed differentiation (to cardiomyocytes) are based on these developmental principles. In general two different directed differentiation strategies have been applied: (1) co-culture of hESC with cardio-inductive cell types and (2) induction of gastrulation by specific growth factors. Both approaches have made considerable progress over the last few years with respect to cardiomyocyte differentiation efficiencies.

CO-CULTURE OF HESC WITH CARDIO-INDUCTIVE CELL TYPES

The discovery of cardio-inductive signals originating from the visceral endoderm represents a key step in the directed differentation of pluripotent stem cells to cardiomyocytes. Co-culture of pluripotent stem cells with END-2 an endodermal cell line, is a very robust and efficient method for cardiac differentiation[36,37]. Although the exact mechanism of END-2 cardiac induction is still unclear, the transcriptome and secretome of END-2 cells have been described[38,39]. Cardiac induction by END-2 conditioned medium can be (partly) mimicked by insulin depletion[40], inhibition of p38 MAPK[41] and addition of prostaglandin E[42]. Interestingly, high concentrations of insulin appear to favor differentiation to neuroectoderm and blocking p38 MAPK further enhances cardiac differentiation. P38 MAPK signaling is highly active in neuroectoderm and blocking of this pathway may favor meso-/endoderm differentiation.

INDUCTION OF GASTRULATION AND CARDIAC COMMITMENT BY SPECIFIC GROWTH FACTORS

The heart is the first organ to develop in the embryo and its development has been studied extensively in various animal models. As gastrulation proceeds, cardiac progenitors form in the posterior primitive streak. These cells express genes like Brachyury T, MIXL and MESP1. MESP1 is thought to induce an epithelial-mesenchyme transition in the epiblast and to bind directly to regulatory DNA sequences located in the promoters of many members of the core cardiac regulatory network, including NKX2-5. This promotes development of mesoderm precursors of the cardiovascular lineage[43,44]. MESP1 also directly represses the expression of key genes regulating other early mesoderm derivates. These signaling pathways can be recapitulated in cell culture by the addition of specific recombinant growth factors like bFGFs, bone morphogenetic proteins (BMPs) and WNTs. Several studies have shown that various combinations of BMP4, WNT3a and ACTIVIN A induce gastrulation-like events and meso- / endoderm development in hESC[45-47]. Recently an efficient cardiac differentiation protocol

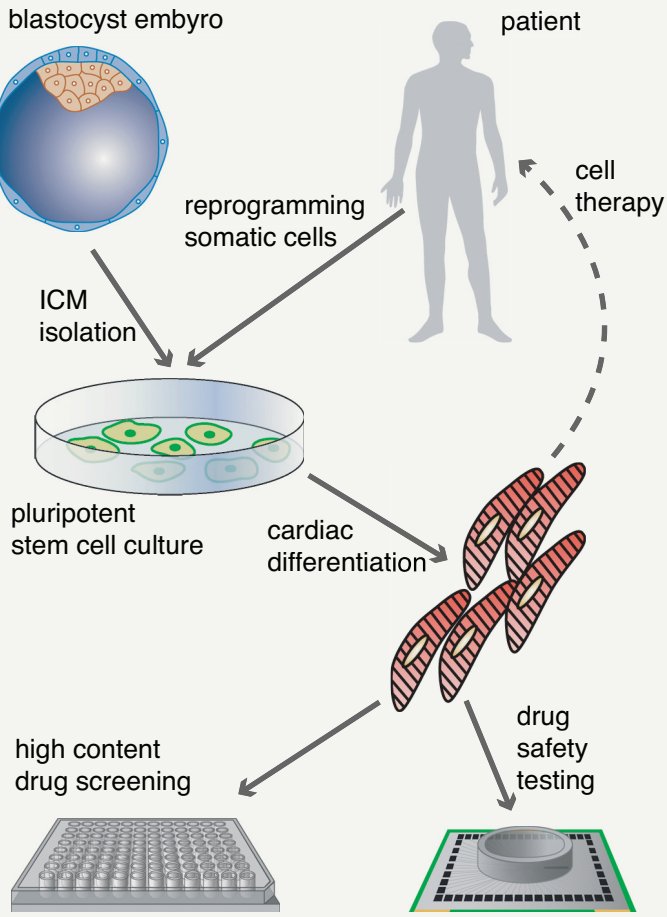

Figure 9.1

Applications of cardiomyocytes from pluripotent stem cells

Human pluripotent stem cells can be obtained from preimplantation human embryos or from somatic cells by reprogramming. These stem cells can be kept in culture indefinitely and can differentiate towards all cell types present in the human body. Differentiation towards the cardiac lineage has always been of great interest, initially with the focus on cell therapy. However, it is widely believed that cell therapy based on pluripotent stem cell derived cardiomyocytes is a long term perspective. Instead, focus is on applications in drug discovery and cardiac safety pharmacology.

based on temporal stimulation with BMP4, bFGF and Activin A, followed by VEGF and WNT inhibition through DKK was descibed. By day 6 of differentiation, a $KDR^{low}C\text{-}Kit^{neg}$ population had emerged which was primed for cardiac differentiation[48]. Recently it was shown that Isl1 heart progenitors are capable of self-renewal and expansion before differentiation to cardiomyocytes, endothelial or smooth muscle cells[49].

The availability of reporter cell lines expressing fluorescent proteins under the control of lineage specific promoters can be a highly instructive tool for identifying factors that improve the efficiency of directed differentiation. Recently eGFP was inserted downstream of the endogenous MIXL promoter in two different hESC lines, thereby marking cells undergoing gastrulation[50]. Similar strategies can now be used in high content differentiation screens whereby several growth factors can be cross-titrated in 96- or 384 well plates followed by automatic imaging. Screening for small molecules that enhance differentiation in a similar high content screen could directly activate appropriate signaling pathways in a ligand independent way. This concept has been successfully applied to cardiac differentiation of pluripotent mouse stem cells and recently on hESC differentiating to pancreas cells[51–53].

Functionality of hESC-derived cardiomyocytes

Differentiation of pluripotent stem cells to cardiomyocytes generally results in mixtures of ventricular-like, atrial-like and pacemaker-like cells defined by patch-clamp electrophysiology of action potentials (APs). Ventricular-like action potentials are defined by a relatively fast upstroke velocity and a plateau phase that results in longer repolarization compared with the more triangular shaped atrial-like APs (Figure 9.2). Relative slow upstroke velocities and much smaller amplitudes characterize pacemaker-like cells (Figure 9.2). Interestingly, specific differentiation protocols seem to affect the ratios of the different cardiac cell types formed in culture. While most differentiation protocols based on embryoid bodies/cell aggregates lead to equal numbers of ventricular and atrial like cells, cardiac induction by END-2 co-culture results largely in homogenous populations of ventricular-like cells[36]. Comparison of these cells with the human fetal heart shows that hESC-derived cardiomyocytes (hESC-CM) are morphologically still relatively immature with irregular sarcomere structures but contain the proper ion channels coupled to downstream signaling pathways, which have been shown to be modified by specific cardiac drugs[54] . Nevertheless the auto-arrhythmic behaviour and small sodium currents indicate electrical-, in addition to morphological, immaturity. During fetal development, the electrophysiology of the heart changes dramatically. For example, the resting potential of the ventricular cells in early embryos is low (e.g. -40/-50 mV) and this changes progressively during development towards that of adult cells (e.g. -75/-85 mV). The maximum rate of rise of the action potential upstroke velocity (dV/dtmax) also increases dramatically during development, from about 20 V/sec to about 200 V/sec in the late embryonic stage. This increase is the result of a much greater number (density) of TTX-sensitive fast

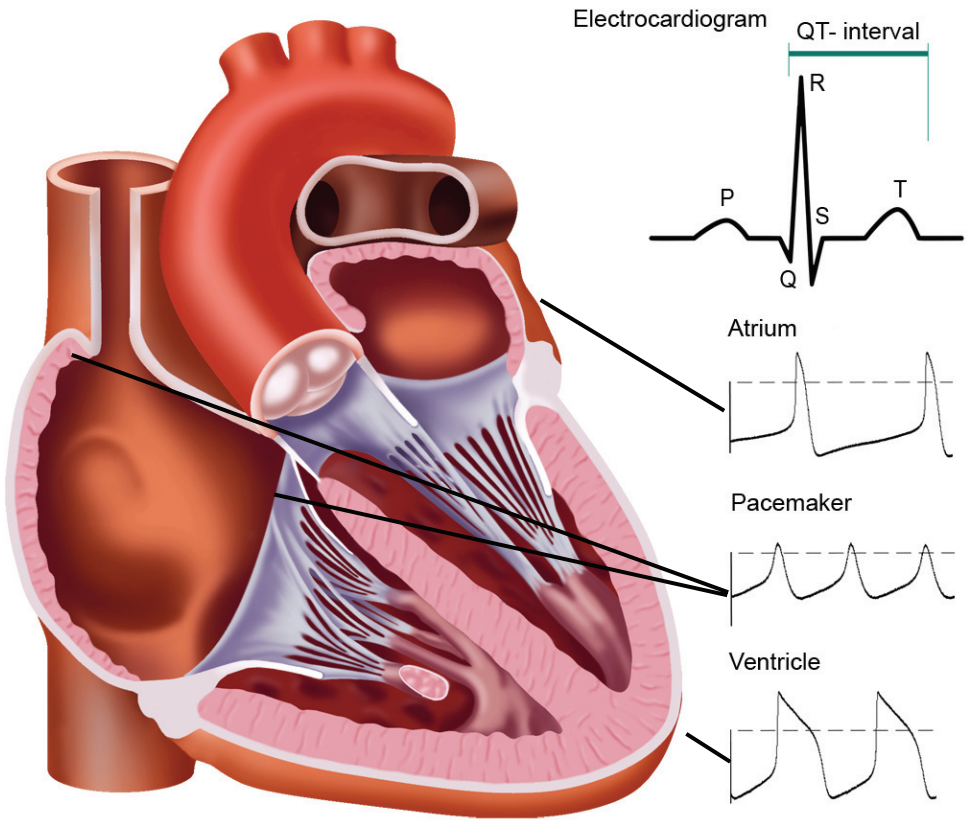

Figure 9.2

Electrical activity of the heart

The normal electrophysiological behaviour of the heart is determined by ordered propagation of excitatory stimuli that result in rapid depolarization and slow repolarization of various excitable cell types [51]. The electrical activity of the heart is initiated in the sinoatrial node (not visible in the picture) and initiates depolarization of atrial myocytes which can be seen as the P wave on an ECG. The electrical activity is then slowed down by the atrioventricular node (not visible in the picture) and spreads via the common bundle and bundle branches to the ventricular muscle. The QRS complex results from ventricular depolarization, while the T wave is indicative of ventricular repolarization. The human ventricular action potential can be subdivided into five phases. Phase 0 is the fast depolarization of the membrane potential due to sodium channel (SCN5a) activation and a rapid increase in membrane permeability to Na^+, followed by a rapid repolarization (phase 1). This is followed by a plateau phase (phase 2), where Ca^{2+} ions enter the cell through L-type calcium channels. The slow gating kinetics of the L-type Calcium channel results in long lasting inward flow of Ca^{2+} that supports optimal contraction of the ventricles. Repolarization, or phase 3, results from the inactivation of calcium channels and an increase in net outward potassium currents carried mainly by the slow (I_{Ks}) and rapid (I_{Kr}) components of the delayed rectifier potassium channels. Inward rectifying potassium channels (I_{Ki}) contribute to phase 3 repolarization and to the maintenance of the resting potential (phase 4).

Na^+ channels. In hESC-CM, membrane potentials and upstroke velocities are comparable to 16-week fetal hearts[36]. The major difference between hESC-CM and adult heart cells is the absence of I_{K1} in hESC-CM[55]. In adult heart cells this inward rectifier K^+ channels (Kir) clamps the membrane potential to a value near the K^+ reversal potential. At such hyperpolarized potentials the probability of the $Na_v1.5$ channels opening approaches 0. The absence of this current leads to a slightly depolarized membrane potential, which can lead to spontaneous activation of $Na_v1.5$ thereby driving the spontaneous action potential. Absence of physical work and the haemodynamics of blood flow[56] might underlie the relatively immature phenotype of hESC-CM. Advanced tissue engineering in combination with cyclic stretch and strain might enhance maturation of the cells in culture.

Cardiomyocytes from pluripotent stem cells to regenerate the heart

Restoring function of failing hearts by replacing damaged cardiomyocytes is straightforward in principle but probably among the most challenging paradigms of regenerative medicine. Any transplanted cells would have to be immunomatched, integrate into the (hypertrophic) host myocardium, receive a blood supply via the vasculature and cardiomyocytes should couple with residual host cardiomyocytes to contract synchronously in response to the cardiac conduction system.

Bone marrow cells (BMCs) were amongst the first non-cardiomyocyte sources of regenerative cells described for the heart. Initially transdifferentiation of BMCs to cardiomyocytes was thought to take place, explaining functional improvement, but this later turned out to be autofluorescence from scar tissue[57-59]. Nevertheless, these early results evoked an unprecedented progression to completion of the first randomized clinical trials within 5 years. Early reports of non-controlled pilot studies were unanimously positive but the results of recent randomized (placebo-) controlled trials were somewhat disappointing, especially after longer follow-up times. Most researchers now agree that if BMCs improve cardiac function, it is more likely to result from early salvage of ischemic myocardium by some kind of paracrine action from the transplanted cells (reviewed in 60).

Cell therapy for the heart would ideally require replacement of cardiomyocytes, vascular endothelial (EC) and smooth muscle cells (SMCs); hESC and hiPS cells are therefore promising candidates because of their ability to form not only bona fide cardiomyocytes but also supportive ECs and SMCs *in vitro* with high efficiency. Transplantation of hESC-derived cells would likely be heterologous and sensitive to immune rejection whereas iPS could theoretically be patient-derived and hence immunologically matched. However, aside from safety issues, it remains to be seen whether this degree of "personalized medicine" would be financially viable for health and insurance services.

The first transplantation of hESC-CM demonstrated their potential to act as biological pacemakers in electrically silenced or AV-blocked hearts[61,62]. This was rapidly extended to studies investigating their ability to regenerate the working myocardium. When hESC-CM were transplanted into the healthy myocardium of immunodeficient rats or mice, they survived[45, 63,64] and matured[63,64] for at least 12 weeks after transplantation[63]. In all studies, mixtures of cardiomyocytes and other differentiated hESC-derived cells were injected. All reported preferential survival of cardiomyocytes, while non-cardiac elements were lost over time. Although grafted human cells formed a syncytium with each other, they were largely separated from the rodent myocardium by a layer of fibrotic tissue. When transplanted into infarcted hearts in rodents, hESC-CM were the only cells that formed considerable grafts, although in one study, the addition of a pro-survival cocktail resulted in larger grafts[45]. Cardiac function in animals receiving hESC-CM was better than in animals receiving non-cardiomyocyte derivatives[45,63]. Notably, rodents receiving non-cardiac hESC-derived cells also showed some improvement compared the vehicle-injected animals. Hence, there were cardiomyocyte-specific benefits additional to an enhancement of cardiac function by hESC-derived cells in general. This was correlated in one study quantitatively with the degree of neovasculature derived from the host in the border zone of the infarct[65]. Only one study so far has, however, extended functional follow up to 12 weeks after transplantation[63]. In mice analyzed at this time-point the advantage of hESC-CM over hESC-non-CM was no longer present[63] even when the number of cells transplanted or the number of injections was increased[65, 66]. Of interest, the grafts in mice in these studies often contained human ECs and SMCs at later time points (6 months)[65] suggesting that either these cells or cardiac progenitors[67] were present in the cell populations injected[65]. "Priming" hESC with BMP to differentiate hESC to the cardiac progenitor stage only has been suggested as an alternative therapeutic option[68].

Primary fetal human cardiac progenitors, although difficult to obtain routinely, have also been described to differentiate in the rodent heart after MI and improve cardiac function, perhaps for even longer periods than hESC-CM[69]. Overall, though, functional enhancement by hESC-CM with the current strategies may generally be limited to mid-term at most. The therapeutic benefit of the cell therapy may be prolonged when using a pro-survival cocktail or different timing of injection, for example after the initial inflammatory phase when the environment may be hostile to the donor cells, but this remains to be proven. A fibrotic layer can develop between injected and host cells[65] that may or may not impede transduction of electrophysiological signals. The question rises whether rodents are the most useful model animals to address potential safety issues in humans. Rodent hearts beat at 400-600 times per minute, humans at 60-100 times per minute. Injection of human cardiomyocytes with intrinsic different electrical properties into rodent hearts is therefore unlikely to contribute to cardiac function. Secondly, transplantation in the rodent myocardium is less likely to create arrhythmic substrates than the same procedure in humans. Transplantation of new cardiomyocytes into the human heart is thus likely to be fraught with safety and efficacy issues at the outset. It might be better to use iPS cells or ESCs from larger animal species (pigs, goats, sheep, non-human primates) preferably from the same species. This would also

provide the opportunity to assess the risk of tumor/teratoma formation, a recognized risk of ESCs and iPS cells, without crossing xeno barriers. Grafts may also provide passive support only sufficient to prevent dilatation and retain cardiac function temporarily, but inadequate for sustained improvement. Similarly, if paracrine mechanisms were the main modus of action for hESC-CM, as thought to be the case for BMCs[70], these may provide a transient protection only. Finally, the host immune response to grafted cells is poorly understood. Unless a cell transplant is derived from the patient's own cells, which might be feasible using iPS cell technology, the cells will be targeted for rejection by the immune system. An emerging approach to address this issue is 're-educating' the immune system to induce tolerance to foreign cells[71]. Taken together, the clinical use of cardiovascular derivatives from pluripotent stem cells would appear challenging, since safety issues are likely to be a significant hurdle to the broader repair of damaged myocardium in the near future. Although beyond the scope of this review, options including tissue engineering may present better strategies for moving forward into clinical therapy[60] .

Applications of cardiac differentiated stem cells in safety toxicology

Cardiomyocytes, especially from the adult heart, have a very limited proliferation capacity and can not be propagated as a cell-line. Repeated isolation of primary tissue is therefore necessary for *in vitro* functional analyses. Primary canine cardiomyocytes are currently the most widely used preclinical model for cardiac safety pharmacology. The strongest arguments for using canine cardiomyocytes (or Purkinje fibres) are their physiological similarity to humans and availability in large numbers. However their use is costly and has ethical issues associated with euthanasia of hundreds of dogs annually. Alternatives are cell lines expressing specific ion channels ectopically but these have the disadvantage that over-expressed human single ion channels function in isolation from the complex ion channel interactions normally present in cardiomyocytes. Their advantage, however, is scalability that allows automatic screening in 384 wells for potential safety issues. The number of cases where unforeseen drug induced cardiotoxicity occurs and the number of drug withdrawals from the market clearly indicates the need for better reliable test systems.

One of the most recent and well-known cases of unexpected cardiotoxicity is Vioxx. Vioxx is a COX-2 inhibitor prescribed to patients with arthritis and other conditions causing acute and chronic pain. On September 30, 2004, Merck voluntarily withdrew Vioxx from the market because of concerns about increased risk of cardiotoxicity and stroke associated with long-term, high-dosage use. Vioxx was one of the most widely used drugs ever to be withdrawn from the market, over 80 million people were prescribed Vioxx at some time. In the year before withdrawal, Merck had sales revenues of US$2.5 billion from Vioxx Worldwide[72]. Although the exact mechanism is still unclear it has recently been shown that selective deletion of

TABLE 9.1
Drugs withdrawn from the market due to cardiotoxicity

DRUG	INDICATION	MARKET SPAN	REASON FOR WITHDRAWAL
Astemizole (Hismanal)	Antihistamine	1983–1999	Withdrawn from the market due to TdP
Cisapride (Propulsid)	Prokinetic	1988–2000	Withdrawn from the market due to TdP
Droperidol	Antipsychotic/antiemetic	1970–2001	Withdrawn from the market due to TdP
Grepafloxacin	Antibiotic	1997–1999	Withdrawn from the market due to TdP
Levomethadyl	Opioid agonist	1993–2001	Withdrawn from use in EU due to TdP; use restricted in US
Prenylamine	Anti-anginal	1960s–1988	Withdrawn from the market due to TdP
Rofecoxib (Vioxx)	Nonsterodial	1999–2004	Withdrawn because of risk of myocardial infarction
Sertindole	Antipsychotic	1996–1998	Withdrawn from the market due to TdP
Terodiline	Bladder incontinence	1986–1991	Withdrawn from the market due to TdP
Tegaserod (Zelnorm)	5-HT4 agonist	2002–2007	Withdrawn because of imbalance of cardiovascular ischemic events, including heart attack and stroke. Was available through a restricted access program until April 2008
Terfenadine (Seldane)	Antihistamine	1982–1997	Withdrawn because of risk of cardiac arrhythmias; superseded by fexofenadine

COX-2 in cardiomyocytes depressed cardiac output and enhanced susceptibility to induced arrhythmogenesis[73].

One of the most appealing successes of the biotechnology industry is the development of Herceptin (trastuzumab). Herceptin binds a membrane protein HER-2 which is overexpressed at 20–30% of early stage breast cancers and is used to treat these cancers. However, also in the case of herceptin heart failure is observed in 1 to 4% of patients treated with the antibody, while 10% have a decreased cardiac function[74]. The combination of herceptin with chemotherapy has been shown to increase both survival and response rate, in comparison to Trastuzumab alone. However, when herceptin is co-applied with anthracycline based chemotherapy cardiac failure is severely increased[75]. These effects were not known prior to clinical trials and today every single patient is screened for heart failure before herceptin treatment. The fact that drugs like herceptin, which are clearly not harmless, are still used in the clinic has to do with their risk/reward profile. In general, the safety evaluation of biologicals is further complicated by the fact they are designed to act on a human target. Traditionally widely used animal models might therefore be a less relevant choice. In order to detect potential safety issues associated with this class of drugs, human cell models have to be developed that detect parameters like cell viability and force of contraction.

TABLE 9.2
Drugs associated with cardiotoxicity

DRUG	INDICATION	BLACK BOX WARNING?
Herceptin	Breast cancer	Yes, cardiomyopathy
Doxorubicin (and other anthracyclins)	Chemotherapeutic	Yes, cardiotoxicity
Sunitinib	RTK inhibitor (anticancer drug)	None, but case reports of cardiotoxicity[81]
Rosiglitazone (avandia)	Anti-diabetic	Yes, congestive heart failure and myocardial infarction
Non- Selective NSAIDs	Anti-inflammatory	Yes, cardiovascular risk
Mitoxantrone	Anti-neoplastic agent	Yes, cardiotoxicity
Thioridazine	Anti-psychotic	Yes, QTc prolongation, TdP, sudden death
Mesoridazine	Anti-psychotic	Yes, QTc prolongation, TdP, sudden death
Muromonab	Immunosuppressant	Yes, cardiotoxicity, cardiac arrest
Nilotinib	BCR-ABL inhibitor, anticancer drug	Yes, QTc prolongation, TdP, sudden death
Itraconazole	Antifungal agent	Yes, congestive heart failure
Flecainide	Class Ic anti-arrhythmic agent	Yes, ventricular pro-arrhythmic effects
Cetuximab	EGFR inhibitor, metatatic colon cancer	Yes, cardiopulmonary arrest
Clozapine	Anti-psychotic	Yes, myocarditis
Alglucosidase alfa	Enzyme replacement therapy, pompe disease	Yes, cardiorespiratory failure
Amiodarone	Class III Anti-arrhythmic	Yes, pro arrhythmic affects
Arsenic trioxide	Chemotherapeutic	Yes, QTc prolongation, TdP, sudden death
Tocaininde	Class Ib anti-arrythmic agent	Yes, ventricular pro-arrhythmic effects
Imatinib	BCR/ABL inhibitor, anticancer drug	None, but case reports of cardiotoxicity[82]

Besides cardiac toxicity due to modulation of signaling pathways in cardiomyocytes there is actually a more profound reason for cardiotoxicity. The hERG channel, which produces the I_{Kr} current, is robustly blocked by a large class of drugs. Since this current has a major function in cardiac repolarization it affects the length of the action potential and the QT interval on a surface electrocardiogram. In 1998, the Food and Drug Administration (FDA) recognized that prolongation of the QT interval on a surface electrocardiogram formed a major drug safety issue. The QT interval represents the duration of ventricular depolarization and subsequent

repolarization, and is measured from the beginning of the QRS complex to the end of the T wave. A prolongation to more than 440 to 460 milliseconds may allow life threatening arythmias, e.g. torsade de pointes (TdP) to occur. Identification of QT prolongation and torsade de pointes has led to the removal of several drugs from the market in the United States, including terfenadine, cisapride and grepafloxacin (Table 1), while many others have been required by the FDA to carry additional black box safety labeling warning of the potential risk (table 2). Today, risk assessment for delayed ventricular repolarization and QT interval prolongation is part of the standard pre-clinical evaluation of novel drug candidates as defined by the International Conference of Harmonization (ICH) Expert Working Group (topic S7B) for drugs in development[76]. Several compounds with known hERG liability have already been tested on hESC cardiomyocytes[54].

In conclusion, both small molecules and biologic drugs may affect human cardiomyocyte biology thereby significantly reducing cardiac function (Table 1 and 2). In addition, a large class of small molecules can interfere with hERG channel function, thereby increasing the arrhythmogenic risk. Therefore there is a great need for novel model systems that recapitulate human biology, are scalable, reproducible and preferably from an inexhaustible source. Human pluripotent stem cell-derived cardiomyocytes may have this potential. However their relatively immature phenotype might affect their drug responses. It therefore remains to be seen if drug responses on these cells recapitulate drug responses of the adult myocardium. Given the recent interest of the pharmaceutical industry and advances in cell tissue engineering, it is highly likely that human pluripotent cardiac models will be implemented in standard safety evaluation of novel drug candidates. One of the most promising tools will be the creation of a library of stem cell lines of different genetic origin, so that drug response and toxicity can be specified against a specific genetic background, enabling personalized treatment solutions, in combination with the FDA-required companion diagnostics.

Applications of cardiomyocytes from pluripotent stem cells in drug discovery

Human pluripotent stem cell lines, either hESC or hiPS cells, are considered promising novel tools for drug target discovery, in part because they may be derived as "healthy controls" but also because of the opportunities they represent for creating cardiac disease models. Clinically relevant mutations can be introduced by gene targeting in hESC or hiPS cell lines can be derived from cells of patients with a particular genetic disease of choice. This should facilitate the creation of innovative human disease models caused by simple as well as complex genetic abnormalities. This could be dependent on selected genetic backgrounds with specific predisposition factors affected the phenotype. In addition, non-genetic organ diseases can be mimicked in disease models using advanced tissue engineering and culture conditions.

Although cardiac drug discovery using pluripotent stem cells is a relatively novel concept, there are a few encouraging reports in the literature to suggest that it might work. A screen with P19cl6 pluripotent stem cells for Nkx2.5 gene activation led to the identification of a family of sulfonylhydrazone small molecules can trigger cardiac differentiation in adult stem/progenitor cells. Human mobilized peripheral blood mononuclear cells treated with this small molecule engraft in an infarcted rat heart and improve cardiac recovery[51]. Another example is the isolation of mouse multipotent ISL1^{+}/NKX2-5^{+} cardiac progenitors[77]. These cells are present in the fetal heart and can give rise to cardiomyocytes, endothelial cells and vascular smooth muscle. Automatic high content screening with 15.000 compounds resulted in 25 compounds that increased ISL1^{+} self-renewal[78]. A secondary screen confirmed the activity of three compounds, two of these compounds were unknown the third 6-bromoindirubin-3′-oxime (BIO) has been previously described as an inhibitor of GSK-3β. Further experiments confirmed self-renewal of ISL1+ cardiovascular progenitors via the by the WNT/ß-catenin pathway. These experiments may be a starting point for further drug development.

Challenges to be overcome before hESC-CM can be successfully implemented in the drug discovery pipeline are scaled differentiation and purification of cell fractions of interest. High content screens usually require >10^{8} purified cells, which is still a significant challenge. Genetic enrichment at present seems to be the most promising strategy. At least two studies indicated that transgenic enrichment of hESC-CM by genetic selection is a feasible approach. In one study the [alpha]MHC promoter was used to drive expression of GFP-IRES-Puro^{r} while another used the same promoter driving expression of Neo^{r} [79, 80].

Conclusions and future perspectives

In recent years, it has become clear that transplantation of heart cells derived from pluripotent stem cells has faced several major obstacles. This makes it unlikely that these cells will be used in clinical trials for the treatment of heart disease in the near future. It is thought that a combination of different cell-types, such as endothelial cells, smooth muscle cells, fibroblasts and cardiomyocytes seeded on a (pre-formed) biodegradable matrix mimicking the *in vivo* environment (e.g. stretch or electrical fields) followed by transplantation will be necessary for long-term beneficial effects. Besides the common hurdles that are encountered when only one cell-type is transplanted in the heart, the diversity of disciplines needed for generating a multi-cellular tissue-engineered bio-construct capable of integrating and communicating with the host tissue, indicates the complexity of this issue and the need for further basic research.

Recent developments in defined culture conditions, genetic manipulation, directed differentiation and derivation of (disease-specific) pluripotent stem cells prompted academic researchers and pharmaceutical industry to change their focus to pre-clinical drug discovery. Human cardiomyocytes derived from pluripotent stem cells have unique properties and a

clear preference over animal-derived cardiomyocytes or "artificial" transgenic cell lines. This makes them exceptionally suitable for applications in drug discovery and safety pharmacology. Although it is expected that further advances in stem cell-based assay developments will lead to a higher throughput and predictability by combination of pure cardiomyocytes populations and functional three-dimensional multi-cellular tissue-engineered models, future studies are necessary to compare these assays with the current "gold standard" (depending on the type of assay) in order to resolve these issues. Any possibility to improve decision-making and reduce timelines and attrition rates would provide an enormous benefit to the process of drug discovery and development. The latest technologies developed in human pluripotent stem cell research hold great promise to improve this process.

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References

1. Thomson, J.A., et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145-1147 (1998).

2. Hughes, B. & Hughes, B. 2008 FDA drug approvals. Nature reviews Drug discovery 8, 93 (2009).

3. Woodcock, J. The Prospects for |[ldquo]|Personalized Medicine|[rdquo]| in Drug Development and Drug Therapy. Clinical Pharmacology & Therapeutics 81, 164 (2007).

4. Kola, I. & Landis, J. Can the pharmaceutical industry reduce attrition rates? Nat Rev Drug Discov 3, 711-716 (2004).

5. Bogdan, B. & Villiger, R. Valuation in Life Sciences: A Practical Guide. 334 (2008).

6. Jensen, J., Hyllner, J. & Björquist, P. Human embryonic stem cell technologies and drug discovery. J Cell Physiol 219, 513-519 (2009).

7. Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18, 399-404 (2000).

8. Xu, R., et al. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Meth 2, 185-190 (2005).

9. Lu, J., Hou, R., Booth, C.J., Yang, S. & Snyder, M. Defined culture conditions of human embryonic stem cells. Proceedings of the National Academy of Sciences 103, 5688-5693 (2006).

10. Liu, Y., et al. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human embryonic stem cells. Biochemical and Biophysical Research Communications 346, 131-139 (2006).

11. Ludwig, T.E., et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotech 24, 185-187 (2006).

12. Yao, S., et al. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proceedings of the National Academy of Sciences 103, 6907-6912 (2006).

13. Vallier, L., Alexander, M. & Pedersen, R.A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 118, 4495-4509 (2005).

14. Initiative, I.S.C., et al. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nat Biotechnol

25, 803–816 (2007).

15. Ludwig, T.E., et al. Feeder-independent culture of human embryonic stem cells. Nat Meth 3, 637–646 (2006).

16. Braam, S.R., et al. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells 26, 2257–2265 (2008).

17. Hewitt, Z.A., Amps, K.J. & Moore, H.D. Derivation of GMP Raw Materials for Use in Regenerative Medicine: hESC-based Therapies, Progress Toward Clinical Application. Clin Pharmacol Ther 82, 448–452 (2007).

18. Denning, C., et al. Common culture conditions for maintenance and cardiomyocyte differentiation of the human embryonic stem cell lines, BG01 and HUES-7. Int J Dev Biol 50, 27–37 (2006).

19. Costa, M., et al. A method for genetic modification of human embryonic stem cells using electroporation. Nat Protoc 2, 792–796 (2007).

20. Braam, S., et al. Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic modification. Nat Protoc 3, 1435–1443 (2008).

21. Braam, S.R., et al. Improved genetic manipulation of human embryonic stem cells. Nat Methods 5, 389–392 (2008).

22. Watanabe, K., et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 25, 681–686 (2007).

23. Thomas, R.J., et al. Automated, scalable culture of human embryonic stem cells in feeder-free conditions. Biotechnology and Bioengineering 102, 1636–1644 (2009).

24. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676 (2006).

25. Yu, J., et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917–1920 (2007).

26. Takahashi, K., et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872 (2007).

27. Kaji, K., et al. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458, 771 (2009).

28. Yu, J., et al. Human induced pluripotent stem cells free of vector and transgene sequences. Science 324, 797–801 (2009).

29. Chin, M.H., et al. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell 5, 111–123 (2009).

30. Deng, J., et al. Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. Nat Biotechnol 27, 353 (2009).

31. Raya, A., et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. Nature 460, 53–59 (2009).

32. Ebert, A.D., et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 457, 277–280 (2009).

33. Dimos, J.T., et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321, 1218–1221 (2008).

34. Itskovitz-Eldor, J., et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6, 88–95 (2000).

35. Nury, D., Neri, T. & Pucéat, M. Human embryonic stem cells and cardiac cell fate. J Cell Physiol 218, 455–459 (2009).

36. Mummery, C., et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 107, 2733–2740 (2003).

37. Passier, R., et al. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 23, 772–780 (2005).

38. Kang, Y., Nagy, J.M., Polak, J.M. & Mantalaris, A. Proteomic characterisation of the conditioned media produced by the visceral endoderm-like cell lines HepG2 and End2: Towards a defined medium for the osteogenic/chondrogenic differentiation of embryonic stem cells. Stem Cells Dev 18, 77-92 (2008).

39. Arrell, D.K., Niederländer, N.J., Faustino, R.S., Behfar, A. & Terzic, A. Cardioinductive network guiding stem cell differentiation revealed by proteomic cartography of tumor necrosis factor alpha-primed endodermal secretome. Stem Cells 26, 387-400 (2008).

40. Freund, C., et al. Insulin Redirects Differentiation from Cardiogenic Mesoderm and Endoderm to Neuroectoderm in Differentiating Human Embryonic Stem Cells. Stem Cells 26, 724-733 (2007).

41. Graichen, R., et al. Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation 76, 357-370 (2008).

42. Xu, X.Q., et al. Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells. Differentiation 76, 958-970 (2008).

43. Bondue, A., et al. Mesp1 Acts as a Master Regulator of Multipotent Cardiovascular Progenitor Specification. Cell Stem Cell 3, 69-84 (2008).

44. Lindsley, R., et al. Mesp1 Coordinately Regulates Cardiovascular Fate Restriction and Epithelial-Mesenchymal Transition in Differentiating ESCs. Cell Stem Cell 3, 55-68 (2008).

45. Laflamme, M.A., et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25, 1015-1024 (2007).

46. Sumi, T., Tsuneyoshi, N., Nakatsuji, N. & Suemori, H. Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/{beta}-catenin, Activin/Nodal and BMP signaling. Development 135, 2969 (2008).

47. Vijayaragavan, K., et al. Noncanonical Wnt Signaling Orchestrates Early Developmental Events toward Hematopoietic Cell Fate from Human Embryonic Stem Cells. Cell Stem Cell 4, 248-262 (2009).

48. Yang, L., et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature 453, 524-528 (2008).

49. Bu, L., et al. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 460, 113-117 (2009).

50. Davis, R.P., et al. Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. Blood 111, 1876-1884 (2008).

51. Sadek, H., et al. Cardiogenic small molecules that enhance myocardial repair by stem cells. Proc Natl Acad Sci USA 105, 6063-6068 (2008).

52. Chen, S., et al. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat Chem Biol 5, 258-265 (2009).

53. Borowiak, M., et al. Small Molecules Efficiently Direct Endodermal Differentiation of Mouse and Human Embryonic Stem Cells. Cell Stem Cell 4, 348-358 (2009).

54. Denning, C. & Anderson, D. Cardiomyocytes from human embryonic stem cells as predictors of cardiotoxicity. Drug Discovery Today: Therapeutic Strategies, 10 (2008).

55. Satin, J., et al. Mechanism of spontaneous excitability in human embryonic stem cell derived cardiomyocytes. The Journal of Physiology 559, 479-496 (2004).

56. Hove, J.R., et al. Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. Nature 421, 172 (2003).

57. Murry, C.E., et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428, 664 (2004).

58. Balsam, L.B., et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428, 668 (2004).

59. Nygren, J.M., et al. Bone marrow|[ndash]|derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nature Medicine 10, 494 (2004).

60. Passier, R., van Laake, L.W. & Mummery, C.L. Stem-cell-based therapy and lessons from the heart. Nature 453, 322-329 (2008).

61. Kehat, I., et al. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nat Biotechnol 22, 1282-1289 (2004).

62. Xue, T., et al. Functional integration of electrically active cardiac derivatives from genetically engineered human embryonic stem cells with quiescent recipient ventricular cardiomyocytes: insights into the development of cell-based pacemakers. Circulation 111, 11-20 (2005).

63. van Laake, L.W., et al. Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. Stem Cell Research 1, 9-24 (2007).

64. Dai, W., et al. Survival and maturation of human embryonic stem cell-derived cardiomyocytes in rat hearts. J Mol Cell Cardiol 43, 504-516 (2007).

65. van Laake, L.W., et al. Improvement of mouse cardiac function by hESC-derived cardiomyocytes correlates with vascularity but not graft size. Stem Cell Research (2009).

66. van Laake, L.W., Passier, R., Doevendans, P.A. & Mummery, C.L. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. Circulation Research 102, 1008-1010 (2008).

67. Wu, S.M., Chien, K.R. & Mummery, C. Origins and fates of cardiovascular progenitor cells. Cell 132, 537-543 (2008).

68. Tomescot, A., et al. Differentiation In Vivo of Cardiac Committed Human Embryonic Stem Cells in Postmyocardial Infarcted Rats. Stem Cells 25, 2200 (2007).

69. Smits, A.M., et al. Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium. Cardiovascular Research 83, 527-35 (2009).

70. Uemura, R., Xu, M., Ahmad, N. & Ashraf, M. Bone Marrow Stem Cells Prevent Left Ventricular Remodeling of Ischemic Heart Through Paracrine Signaling. Circulation Research 98, 1414 (2006).

71. Chidgey, A.P., Layton, D., Trounson, A. & Boyd, R.L. Tolerance strategies for stem-cell-based therapies. Nature 453, 330-337 (2008).

72. Couzin, J. DRUG SAFETY: Withdrawal of Vioxx Casts a Shadow Over COX-2 Inhibitors. Science 306, 384-385 (2004).

73. Wang, D., et al. Cardiomyocyte cyclooxygenase-2 influences cardiac rhythm and function. Proc Natl Acad Sci USA 106, 7548 (2009).

74. Chien, K.R. Herceptin and the Heart -- A Molecular Modifier of Cardiac Failure. N Engl J Med 354, 789-790 (2006).

75. Sawyer, D. Modulation of Anthracycline-Induced Myofibrillar Disarray in Rat Ventricular Myocytes by Neuregulin-1beta and Anti-erbB2: Potential Mechanism for Trastuzumab-Induced Cardiotoxicity. Circulation 105, 1551-1554 (2002).

76. Cavero, I. & Crumb, W. ICH S7B draft guideline on the non-clinical strategy for testing delayed cardiac repolarisation risk of drugs: a critical analysis. Expert Opinion on Drug Safety 4, 509-530 (2005).

77. Moretti, A., et al. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 127, 1151-1165 (2006).

78. Qyang, Y., et al. The Renewal and Differentiation of Isl1+ Cardiovascular Progenitors Are Controlled by a Wnt/β-Catenin Pathway. Cell Stem Cell 1, 165-179 (2007).

79. Xu, X.Q., et al. Highly enriched cardiomyocytes from human embryonic stem cells. Cytotherapy 10, 376-389 (2008).

80. Anderson, D., et al. Transgenic enrichment of cardiomyocytes from human embryonic stem cells. Mol Ther 15, 2027-2036 (2007).

CHAPTER TEN

General Discussion

Abstract

In this thesis various aspects of human embryonic stem cell (hESC) biology and technology development were studied with the aim of enhancing reproducibility and efficiency of their growth, differentiation and genetic manipulation. All future applications of human embryonic stem cells depend on the robustness of these procedures, exquisite knowledge of their biology and control of their signaling and fate choices.

Culture methods for efficient genetic manipulation and completely defined conditions for expansion on a defined substrate were developed. Quantitative phosphoproteomics provided new insights on maintenance of pluripotency but more importantly exit from the pluripotent state, an essential first step in differentiation but rather neglected compared with the focus on fate control. Genetic manipulation of hESC and targeting of the cardiac transcription factor Nkx2.5 with EGFP, resulted in a cardiac reporter cell line that was used to optimize cardiac differentiation in defined conditions, isolate progenitor cells and study human cardiac fate choices. Finally hESC cardiomyocytes were used to develop and validate an assay for cardiac safety pharmacology of QT prolonging drugs. The major implications of these findings are discussed in this chapter. This includes future perspectives of hESC in safety pharmacology, disease modeling, drug discovery and personalized medicine.

The human embryonic stem cell model

For decades (bio) medical researchers have used model systems to study human biology, development and disease. In particular, model organisms are widely used to investigate treatments and potential causes of human disease when experiments directly on humans are not feasible or become unsafe and thus unethical. Examples of commonly used laboratory model systems include the yeast *Schizosaccharomyces pompe*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the zebrafish *Danio rerio* and the mouse *Mus musculus*. Largely because of the ability to perform reverse genetics, the mouse has evolved as the favorite model to study human genes and disease processes[1]. Although all of these model organisms have their own specific advantages and disadvantages, they have one major disadvantage in common, namely they are not human. Care must always be taken when extrapolating from any model system to humans.

hESC have significant potential to be developed into an acceptable alternative to the model systems mentioned previously provided they are fully validated. The main reason for this is their capacity for both self renewal and differentiation to specialized cells, such as contracting cardiomyocytes, that share many common features with human adult cardiomyocytes. hESC technology is expected to contribute to basic studies on human development, drug target discovery and drug safety pharmacology.

In general, model systems are simpler and idealized and are readily accessible and easily manipulated[2]. It needs few arguments to convince that a cell model is much simpler than a complete human being. This has clear advantages but also major disadvantages since multi-cellular or multi-organ processes like cognition and metabolic syndromes are difficult to study in a cell model. However processes "at the cellular level" like transcriptional regulation, proliferation, differentiation, cell migration and specific cellular functions (e.g. contractility) are very well suited to being studied in an *in vitro* cell model[3]. Furthermore, it might be possible to use advanced tissue engineering and culture conditions to mimic processes that can normally only be studied in an animal model[4]. For example; patterning and alignment of cardiomyocytes on thin films results in sheets of cardiomyocytes that can be used to study force of contraction[5]. The aim here has been to address several criteria for translational use of pluripotent stem cells, namely their availability and ease of manipulation and the ability to direct differentiation into the required cell types for the particular assay in development.

PLURIPOTENT STEM CELL CULTURE TECHNOLOGY

Although discovered more than a decade ago, hESC have been difficult to expand in a controlled manner that maintains their undifferentiated status. Furthermore, it has proven to be even more difficult to differentiate them with high efficiency in culture to any of the possible 220 cell types they might be able to make. Despite great interest, some cell types like skeletal muscle cells, germ cells, liver cells and lung cells are still very difficult / impossible to generate in culture. For robust efficient differentiation, it is preferable to start with a highly homogeneous

defined undifferentiated cell population, which is not available easily in the traditional feeder and serum based culture systems. We first decided to focus on developing common culture conditions (Chapter 2,3) that would robustly support pluripotency in a wide variety of lines, independent of whether these are mechanically (cut-and-paste) or enzymatically passaged, cultured using feeder- and serum or one of the (semi-)defined commercial media. It was envisaged that this would be particularly useful for genetic modification of hESC lines since their derivation and maintenance under various culture conditions before the present work required optimization of gene transfer protocols for every individual cell line and growth condition[6]. We found that MEF conditioned medium in combination with Matrigel supported all of every 12 hESC lines tested. We then showed that under these conditions hESC could be passaged using trypsin and cultured at low density. This allowed us to achieve very high transfection efficiencies for multiple types of gene constructs using several of the standard gene delivery systems and vectors, like bacterial plasmids, double stranded RNA, adenovirus and lentivirus.

The importance of defining conditions for hESC or stem-/progenitor cell culture in general is very evident from studies on signaling pathways in these cells[7]. Initially, when hESC were cultured on poorly defined mouse or human feeder cells and in serum there were significant discrepancies in the results reported by independent laboratories. Examples include the debated role of LIF and Activin A in hESC self-renewal[8,9,10] and chromosomal stability[11,12]. Reverse engineering of the stem cell niche by micro-array and proteomic analyses on feeder cells and stem cells have also proven to be difficult/impossible[13,14]. From the first derivation of hESC it took 8 years of research until the Thomson laboratory showed that they could be cultured feeder-free in defined medium supplemented with bFGF and TGF-β/Activin A[10]. We have built on these findings and investigated integrin and matrix expression in hESC, on the premise that knowledge of extracellular matrix receptor (integrin) expression, would allow the appropriate matrix substrate to be defined and the matrix function (cell attachment, survival and proliferation) elucidated. Using blocking antibodies and functional assessment of binding capacity we demonstrated that recombinant vitronectin, signaling through integrin αVβ5, was a robust alternative to Matrigel™ (Chapter 5) and the only factor tested that supported long term self renewal. This has recently been validated independently by Stem Cell Technologies and Ludwig et al (personal communication) and the latter are now investigating whether recombinant vitronectin will support derivation of new hESC lines. Furthermore, recently it has been shown that vitronectin supports hiPSC derivation and growth as well[15]. Although promising, however, the high cost of recombinant vitronectin and the incompatibility with enzyme based bulk cultures likely limits its further development.

REGULATION OF PLURIPOTENCY

Starting from the question 'how is pluripotency regulated?' we characterized hESC as *bona fide* epithelial cells and confirmed the presence of all epithelial junctions at the protein level with protein mass-spectroscopy and immuno-fluorescence microscopy (Chapter 4). Since epithelial cells are known to require a basement matrix we reasoned that integrin and matrix

expression could be an important determinant of hESC pluripotency (Chapter 5). We showed that hESC have the potential to attach to a wide variety of natural substrates, but that only recombinant vitronectin by itself was capable of supporting sustained hESC proliferation. To investigate hESC further we developed methods for scalable culture of hESC, stable isotope labeling of cells in culture (SILAC) and very efficient differentiation from the pluripotent state to accommodate proteomic screens (Chapter 6). We induced differentiation with BMP4 because it induced the fastest differentiation evidenced by rapid loss of pluripotent markers SOX2 and OCT4 of any growth factor tested and therefore allowed us to investigate the earliest events following induction of differentiation, before overt gene expression patterns had changed[16]. This proteomic approach resulted in an extensive hESC proteome profile, which confirmed the predicted protein expression based on histone 3 tri-methylation[17]. More importantly our approach generated a dynamic map of protein phosphorylation during the first 4 hours of hESC differentiation. Linkage analyses of kinases to the identified phosphorylation suggest an important role for CDK1/2 in hESC pluripotency. Interestingly, we found a novel phosphorylation motif on SOX2, one of the core pluripotency factors. In depth analyses of this site confirmed that phosphorylation of SOX2 results in sumoylation. Sumoylaton of SOX2 is known to inhibit its DNA binding activity as a transcription factor[18]. SOX2 phosphorylation could therefore be a novel mechanism used by the cell to exit the pluripotent state and initiate a differentiation program. We propose that linkage of protein data with genomic, epigenomic and transcriptomic approaches will improve our knowledge of cell biology in general and of stem cell fate choices in particular.

DIFFERENTIATION

Differentiation of hESC can occur spontaneously but is usually induced with specific growth factors to specify development to a certain lineage. These growth factors are the same or similar to the ones active in the embryo during development. A pool of differentiating hESC induced towards cardiac differentiation follows a pattern of gene expression changes as expected. Differentiation is initiated with down-regulation of stem cell genes, transient up-regulation of primitive streak associated genes, transient up-regulation of early mesoderm/mesendoderm markers followed by markers associated with cardiac lineage commitment[19]. Although there is similarity with normal embryonic development described in other vertebrates like mouse, neither temporal nor 3-D spatial signaling are entirely recapitulated in a culture dish.

Differentiation of stem cells to any lineage can be best explained using the 'chaos theory'[20]. In a highly undifferentiated culture human embryonic stem cells themselves are essentially similar to each other, although there may be subtle differences between them. During differentiation the number of different cell types increases and cells influence each other in both auto- and endocrine fashions as differentiation proceeds. Directed differentiation by exogenous growth factors at later stages of the process is therefore problematic and subject to interference.

Rosenblueth & Wiener already stated in 1945 that an ideal model system must be accessible and easily manipulated. In order to study the cardiomyocytes from hESC, it is necessary to make them accessible experimentally in large numbers, to purify them and ideally to be able to expand their lineage committed progenitors in culture. To be able to understand the cardiac differentiation hierarchy being able to select cardiac progenitors and re-plate these in defined media allows study of their behavior in response to known signaling cues and activators.

OPTIMIZED DIFFERENTIATION

The increasing availability of accurate reporter hESC lines, for example like the knock-in of EGFP downstream the endogenous promoter of Nkx2-5 (Chapter 7), is expected to contribute strongly to the utility of hESC as a model system. Expression of fluorescent proteins from lineage promoters can be used in high content differentiation screens whereby the effects of growth factors or small molecules can be investigated in 96- or 384 well plates by automatically imaging. Although growth factors and other forms of extracellular signaling mainly regulate fate choices *in vivo*, it will certainly be more interesting to screen small molecules for their ability to achieve the same effect *in vitro*. Small molecules can be specfically optimized to activate or deactivate members of the entire 'druggable genome'[21]. By working with the druggable genome it is expected that higher specificity for signaling pathways can be achieved since it is not limited to receptors but can include kinases, phosphatases, proteases, transporters, nuclear hormone receptors, GPCRs and ion channels. This concept has already been applied to cardiac differentiated mouse pluripotent stem cells and recently on hESC differentiating to pancreas cells[22,23]. Furthermore, continuous refinement of the fluorescent toolbox[24,25] makes it possible to engineer stem cell fate reporter technology to monitor the whole process of differentiation using a multicolor differentiation system. A combination of reporters for mesoderm induction, cardiac specification and cardiac maturation would be very interesting to optimize growth factor timing. As showed in chapter 7, removal of BMP4/Activin-A/FGF/VEGF exactly at day 3 of differentiation is crucial for efficient cardiac differentiation.

Translational cardiac medicine

hESC technology is often associated with translational medicine. Translational medicine is the workflow – often referred to as "from bench to bedside" – by which the academic decision maker takes a focused point of view to move research discoveries from the academic laboratory into clinical practice for diagnosis and treatment of patients. One can think of various scenarios on how stem cells can be incorporated in the process of translational cardiac medicine. The most obvious way is to produce cell types to repair an injured heart. However, despite the huge interest relatively little robust progress has been made to achieve this goal for the heart (reviewed in Chapter 9). This and the availability of induced pluripotency stem cells from humans (hiPSC) and gene targeting to create human disease models are causing a shift in focus from cell therapy to drug safety pharmacology and target discovery (Chapter 9).

DRUG DISCOVERY

Drug discovery using differentiated stem cells is an exciting new application of stem cell technology (Chapter 9). Discovery of novel relevant drug targets is hampered by the fact that current disease model systems do not always sufficiently represent or reflect human disease and are therefore not ideal for target and subsequent drug discovery. Human evolution diverged approximately 65 million ago from small rodents as the mouse[26]. Therefore it is not very surprising that there are clear differences between the species. For example cardiac repolarization in mice differs from humans since the primary ion current controlling repolarization in these animals is I_{to} rather than I_{kr}[27]. A large class of drugs avidly blocks I_{kr}, which is a very important risk factor for Torsade de Pointes (TdP) in humans. Due to species differences mice are unsuitable to evaluate this risk. The sequencing of the human genome and the genome of lower organisms has revealed the presence of a number of human- or human-lineage specific genes that have no orthologs in lower species[28]. The functional relevance of at least one of these genes was recently proven. CHC22 turned out to be an important regulator of glucose metabolism and the fact that this gene is not present in mouse calls into question the use of mice to study some aspects of diabetes[29]. Finally, chromosomal genetic differences between lower species and human makes it very difficult to design a model system for karyotypic abnormalities like Down's syndrome since for example mice do not have a chromosome 21. Therefore any possibility to improve model systems for decision-making, reduction of attrition rates and timelines into clinical practice would provide an enormous benefit to the process of drug discovery and development. hESC and hiPSC technology together hold great promise to achieve this goal. The latest differentiation and selection procedures, encourages optimism on implementation of these cells in the drug discovery pipeline. The possibility to introduce clinically relevant mutations stably by gene targeting (Chapter 3,4), or to derive hiPSC cell lines from skin cells of patients with the disease of choice, clears the road to creating innovative human disease models, caused by both simple as well as complex genetics abnormalities. In addition, non-genetic organ diseases could be mimicked in disease models using advanced tissue engineering and culture conditions. For example, cardiac hypertrophy, a major determinant of heart failure, can be induced using chemicals as angiotensin II and phenylephrine. Examples of heart diseases that could be modeled include mutations in ion channel associated with arrhythmia and mutations in structural proteins associated with impaired contractility. However, successful implementation of stem cells will depend on functional responses of the cells. In general, differentiated cells derived from stem cells are immature compared to the cells present in the adult human body. Cardiomyocytes, for example, display both an immature electrical phenotype as well as an immature sarcomeric organization. It is thought that advanced tissue engineering, which could be based on progenitor cells (Chapter 7) and exquisite knowledge of cell maturation is required to develop an effective maturation protocol. Furthermore as mentioned before, a stem cell model is simpler than a complete animal model, which has the disadvantage that the cell type of interest is isolated from other cells/organs. Metabolism of compounds by the liver and the effects of these metabolites are difficult to test *in vitro*, unless co-culture systems are developed specifically aimed at these kinds of effects.

CARDIAC SAFETY PHARMACOLOGY

Proper heart function is one of the essential elements of life and any drug that interferes with this process is potentially life threatening. Besides that, the myocardium has a very small regenerative capacity[30,31]. Therefore it is of crucial importance to design safe drugs that have no side effects on the heart.

In 1998 it was widely acknowledged by regulatory bodies that a large class of chemical structures interfere with the human Ether-a-go-go Related Gene (hERG) channel[32]. The hERG channel is essential for proper cardiac repolarization and if blocked this can result in life threatening ventricular tachy-arrhythmias. We differentiated hESC to cardiomyocytes (hESC-CM), plated them on multi-electrode arrays and described systematic generation of field potential dose response curves for cardiac and non-cardiac drugs (Chapter 8). Although the field potential is a direct reflection of the action potential, it is less defined and more difficult to interpret as the action potential[33]. Despite this, field potential duration prolongation turned out to be a useful criterion for drugs affecting QT prolongation in the human heart (Chapter 9). Assays based on hESC-CM could therefore complement or potentially replace some of the preclinical tests currently used to select chemical compounds for development as new cardiac drugs and improve safety testing of drugs prior to clinical trials. The assays described here are aimed to evaluate drug-ion channel interactions directly and might be limited to some extend. Some drugs like arsenic trioxide and geldanamycin deviations are reported to affect hERG channel trafficking to the plasma membrane, and should be evaluated in long-term assays[34]. Furthermore the downside of a cell model is illustrated by the fact that drug-induced TdP is thought to arise from dispersion of cardiac repolarizaton across the transmural ventricular myocardium[35]. Dispersion of repolarization could be the trigger to ventricular arrhythmias, which is something difficult to model in an *in vitro* system.

Finally, cardiotoxic effects of certain drugs may only become evident under conditions that simulate a normal beating heart, in either a physiological (stretch-contraction cycle) or pathophysiological situation (e.g. excessive stretch or compensatory mechanisms associated with cardiac disease). Through combination of microfabrication and stem cell technology we expect to be able to develop flexible stretchable cardiac model systems with integrated passive electrodes for electrical and contractility read outs. These kinds of systems will likely make major contributions to the development of novel safe effective drugs.

PERSONALIZED MEDICINE

The option to use a large number of cell lines with different genetic background in parallel for drug target validation and safety pharmacology, allows the actual development of a tissue-directed *in vitro* clinical trial, prior to any clinical trial involving patients. Despite the limitations of an isolated tissue system, instead of a whole organism, this represents and unprecedented step towards personalized medicine. In general, not all individuals receiving a drug for a specific indication will exhibit therapeutic responses or toxic side–effects, which is, in part, determined by the genetic constitution of the patient. The use of a series of human

pluripotent stem cell lines with different genotypes will allow *in vitro* analyses, predicting both drug effectiveness and toxicity at and individual level against a specific genetic background, as well as identifying biomarkers for the development of companion diagnostic assays to predict individual therapy response and toxicity in the clinic.

Future prospects and conclusions

Over the last years knowledge of human embryonic stem cells has evolved considerably. Technology has been developed to grow stem cells in large quantities, manipulate them and differentiate them efficiently. It is expected that further developments eventually results in high throughput assays for analyses of differentiation, drug safety pharmacology and drug discovery. Future studies are required to compare the human stem cell model with the current gold standards in the industry eventually leading to broad acceptance of the pluripotent stem cell model.

References

1. Mak, T.W. Gene targeting in embryonic stem cells scores a knockout in Stockholm. Cell 131, 1027–1031 (2007).

2. Rosenblueth, A., Wiener, N., The Role of Models in Science. Philosophy of Science 12, 316–321 (1945).

3. Pouton, C. & Haynes, J. Embryonic stem cells as a source of models for drug discovery. Nat Rev Drug Discov 6, 605–616 (2007).

4. Nirmalanandhan, V.S. & Sittampalam, G.S. Stem cells in drug discovery, tissue engineering, and regenerative medicine: emerging opportunities and challenges. Journal of biomolecular screening : the official journal of the Society for Biomolecular Screening 14, 755–768 (2009).

5. Feinberg, A.W., et al. Muscular thin films for building actuators and powering devices. Science 317, 1366–1370 (2007).

6. Liew, C.-G., Draper, J., Walsh, J., Moore, H. & Andrews, P. Transient and Stable Transgene Expression in Human Embryonic Stem Cells. Stem Cells 25, 1521–1528 (2007).

7. Moore, H. The medium is the message. Nat Biotechnol 24, 160 (2006).

8. Dahéron, L., et al. LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. Stem Cells 22, 770–778 (2004).

9. Vallier, L., Alexander, M. & Pedersen, R.A. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J Cell Sci 118, 4495–4509 (2005).

10. Ludwig, T.E., et al. Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24, 185–187 (2006).

11. Draper, J.S., et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22, 53–54 (2004).

12. Mitalipova, M.M., et al. Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol 23, 19–20 (2005).

13. Prowse, A., McQuade, L., Bryant, K., Marcal, H. & Gray, P. Identification of Potential Pluripotency Determinants for Human Embryonic Stem Cells Following Proteomic Analysis of Human and Mouse Fibroblast Conditioned Media. J. Proteome Res. 6, 3796–3807 (2007).

14. Suárez-Fariñas, M., Noggle, S., Heke, M., Hemmati-Brivanlou, A. & Magnasco, M.O. Comparing independent microarray studies: the case of human embryonic stem cells. BMC Genomics 6, 99 (2005).

15. Rowland, T., et al. Roles of Integrins in Human Induced Pluripotent Stem Cell Growth on Matrigel and Vitronectin. Stem Cells Dev (2009).

16. Van Hoof, D., et al. Phosphorylation dynamics during early differentiation of human embryonic stem cells. Cell Stem Cell 5, 214-226 (2009).

17. Pan, G., et al. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. Cell Stem Cell 1, 299-312 (2007).

18. Tsuruzoe, S., et al. Inhibition of DNA binding of Sox2 by the SUMO conjugation. Biochemical and Biophysical Research Communications 351, 920-926 (2006).

19. Beqqali, A., Kloots, J., Ward-van Oostwaard, D., Mummery, C. & Passier, R. Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. Stem Cells 24, 1956-1967 (2006).

20. Furusawa, C. & Kaneko, K. Theory of robustness of irreversible differentiation in a stem cell system: chaos hypothesis. J Theor Biol 209, 395-416 (2001).

21. Hopkins, A.L., Hopkins, A.L., Groom, C.R. & Groom, C.R. The druggable genome. Nature reviews Drug discovery 1, 727 (2002).

22. Chen, S., et al. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat Chem Biol 5, 258-265 (2009).

23. Borowiak, M., et al. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. Cell Stem Cell 4, 348-358 (2009).

24. Giepmans, B.N.G., Adams, S.R., Ellisman, M.H. & Tsien, R.Y. The fluorescent toolbox for assessing protein location and function. Science 312, 217-224 (2006).

25. Shaner, N.C., et al. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat Biotechnol 22, 1567-1572 (2004).

26. Foote, M., Hunter, J., Janis, C. & Jr, S., J John. Evolutionary and Preservational Constraints on Origins of Biologic Groups: Divergence Times of Eutherian Mammals. Science 283, 1310 (1999).

27. Salama, G. & London, B. Mouse models of long QT syndrome. The Journal of Physiology 578, 43-53 (2007).

28. Stahl, P.D. & Wainszelbaum, M.J. Human-specific genes may offer a unique window into human cell signaling. Science signaling 2, pe59 (2009).

29. Vassilopoulos, S., et al. A Role for the CHC22 Clathrin Heavy-Chain Isoform in Human Glucose Metabolism. Science 324, 1192 (2009).

30. Bersell, K., Arab, S., Haring, B. & Kühn, B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell 138, 257-270 (2009).

31. Bergmann, O., et al. Evidence for cardiomyocyte renewal in humans. Science 324, 98-102 (2009).

32. Curran, M.E., et al. A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. Cell 80, 795-803 (1995).

33. Reppel, M., et al. The electrocardiogram of human embryonic stem cell–derived cardiomyocytes. Journal of Electrocardiology 38, 166-170 (2005).

34. Wible, B., et al. HERG-Lite®: A novel comprehensive high-throughput screen for drug-induced hERG risk. Journal of pharmacological and toxicological methods 52, 136-145 (2005).

35. Antzelevitch, C. Role of transmural dispersion of repolarization in the genesis of drug-induced torsades de pointes. Heart rhythm : the official journal of the Heart Rhythm Society 2, S9 (2005).

Summary

Human embryonic stem cells (hESC) hold great potential as a model for human development, disease pathology, drug discovery and safety pharmacology. All these applications will depend on comprehensive knowledge of their biology and control of their signaling mechanisms and fate choices.

To begin to address this, we developed a standardized feeder-free hESC culture protocol. This system is optimized and tested for 12 independently derived stem cell lines, and optimal for clonal growth and efficient gene transfer without loss of pluripotency (Chapter 2,3). Using these protocols we created stem cells ubiquitously expressing EGFP, showed efficient SOX2 knockdown and created a fluorescent reporter stem cell line for the stem cell regulator OCT4. Next we used mass spectroscopy to investigate the plasma membrane of hESC (Chapter 4). We were able to show that these cells express a uniform epithelial plasma membrane profile and that VIMENTIN, normally associated with mesenchymal cells is also expressed. This expression turned out to be related to stress and associated with hardness of the tissue culture plastic substrate rather than differentiation. We continued to investigate the plasma membrane of hESC and decided to focus on integrins, the cell surface receptors that bind extracellular matrix proteins. Functional analyses of their function showed human embryonic stem cells have the capacity to bind to a wide range of extracellular matrix proteins via specific integrin receptors. We were able to show that recombinant vitronectin robustly supports the maintenance of hESC in an undifferentiated state in completely defined culture medium. Having validated a completely defined culture protocol we began to investigate differentiation mechanisms under defined conditions (Chapter 5). We used Stable Isotope Labeling in Cell Culture (SILAC) and quantitative phopspho-proteomics to investigate how human embryonic stem cells exit the pluripotent state. BMP4 was used to trigger differentiation and protein samples were analyzed at 30 min, 60 min and 240 min. We showed that approximately 50% of the 3067 identified phosphosites were regulated within 1 hr of differentiation induction, revealing a complex interplay of phosphorylation networks spanning different signaling pathways. Among the phosphorylated proteins was the pluripotency-associated protein SOX2, which was SUMOylated as a result of phosphorylation. Using the data to predict kinase-substrate relationships we reconstructed the hESC kinome, with CDK1/2 emerging as a key kinase in controlling self-renewal and lineage specification.

Next we used gene targeting to create a fluorescent cardiac reporter cell line. EGFP was targeted into one allele of the NKX2-5 gene. EGFP fluorescence driven by the endogenous Nkx2-5 promoter faithfully reported cardiovascular lineage commitment of differentiating hESC under defined culture conditions. Using fluorescence activated cell sorting we showed that the early NKX2-5 positive cell population contained multipotent progenitor cells capable of directed differentiation to cardiomyocytes, endothelial and vascular smooth muscle cells (Chapter 7). Finally, we used the cardiomcyocytes from hESC to develop a system for cardiac safety pharmacology (Chapter 8). Recent withdrawals of prescription drugs from clinical use because

of unexpected side effects on the heart have highlighted the need for more reliable cardiac safety pharmacology assays. In particular, blocking of the human Ether-a-go go Related Gene ion channel is associated with life-threatening arrhythmias, such as Torsade de Pointes. We demonstrated that, as predicted, patient serum levels of drugs and known responses on QT interval overlapped with field potential duration values derived from hESC-CM,. On this basis, we propose field potential duration prolongation as a directly applicable safety criterion for pre-clinical evaluation of new drugs in development. In conclusion, the availability of human cardiomyocytes from stem cell sources is now expected to accelerate cardiac drug discovery and safety pharmacology by offering more clinically relevant human culture models than presently available (Chapter 9,10).

Nederlandse samenvatting

Een stamcel is een cel die zichzelf kan vermenigvuldigen en zich kan specialiseren tot tenminste één andere celsoort. Er zijn twee soorten stamcellen; embryonale stamcellen en volwassen stamcellen. Volwassen stamcellen zijn te vinden in een groot aantal organen en kunnen zich ontwikkelen tot een beperkt aantal celtypes, terwijl embryonale stamcellen pluripotent zijn. Dat wil zeggen dat de stamcellen zich kunnen specialiseren tot alle 220 celtypes die voorkomen in het menselijke lichaam. Humane embryonale stamcellen worden geïsoleerd uit een celklompje, blastocyst genaamd, wat een overblijfsel is van IVF behandelingen. Humane embryonale stamcellen kunnen in een kweekschaaltje eeuwig doorgroeien. Deze unieke eigenschappen maken humane embryonale stamcellen zeer aantrekkelijk als model om menselijke ontwikkelingsbiologie te onderzoeken, ziekten te bestuderen, medicijnen te ontwikkelen en deze op veiligheid te testen.

Om aan deze toepassingen te kunnen werken moeten er eerst een aantal problemen worden opgelost (hoofdstuk 1). Zo is het belangrijk dat de stamcellen efficiënt blijven delen. Daarnaast moeten de stamcellen hun stamcelidentiteit houden tot het moment dat de onderzoeker besluit dat ze zich mogen differentiëren (specialiseren tot een ander celtype). Verder is het erg belangrijk dat dit proces goed gestuurd kan worden. Het is nou eenmaal niet handig als de onderzoeker in hartspiercellen geïnteresseerd is, terwijl de stamcellen zich differentiëren in botcellen. Ten slotte is het van belang dat het DNA van de stamcellen gericht aangepast kan worden. Als we geïnteresseerd zijn in een ziekte die door een foutje in het DNA veroorzaakt wordt, willen we die fout kunnen aanbrengen in de stamcel om zo de ziekte te kunnen bestuderen.

Humane embryonale stamcellen groeien in grote kolonies op een laag van andere cellen die ervoor zorgen dat de stamcellen niet differentiëren. Omdat het kweekschaaltje regelmatig te klein wordt, moet de kolonie als een soort taart in stukjes verdeeld worden. Deze stukjes worden dan over gezet naar een nieuw schaaltje, zodat de cellen weer door kunnen groeien. Dit proces is zeer arbeidsintensief en dus inefficiënt. Daarnaast is het zo dat de cellen in de kolonies erg dicht op elkaar gepakt zijn, wat het erg moeilijk maakt om het DNA van de stamcellen gericht te veranderen. In hoofdstuk 2,3 laten we zien dat we de stamcellen kunnen laten groeien op een gel van speciale eiwitten. Door het gebruik van een enzym wat eiwitten aan de buitenkant van de cel kapot knipt, kunnen we de cellen van elkaar scheiden. De suspensie van cellen kan vervolgens verdeeld worden over een aantal nieuwe kweekschaaltjes. Op basis van deze nieuwe kweekmethode hebben we zeer efficiënte technieken ontwikkeld om de cellen genetisch aan te passen. Zo hebben we bijvoorbeeld een gen van een kwal toegevoegd; dit maakt de cellen groen fluorescent als je er met blauw licht op schijnt.

In hoofdstuk 4 zijn we begonnen met de verdere karakterisering van de stamcellen met behulp van massaspectrometrie, een zeer geavanceerde techniek om de aanwezigheid van

eiwitten te meten. Hieruit bleek dat stamcellen veel overeenkomsten met epitheelcellen vertonen, maar dat ze ook vimentine produceren. Dit zou erop wijzen dat ze een epitheel-tot mesenchymale transformatie hebben ondergaan. We hebben kunnen aantonen dat de aanwezigheid van vimentine eigenlijk een gevolg van stress is en in dit geval niets te maken had met epitheel tot mesenchymale transformatie. Humane embryonale stamcellen hebben dus een epitheel fenotype.

Epitheelcellen hechten in het lichaam aan een extracellulaire matrix. We bepaalden daarom de samenstelling en oorsprong van de extracellulaire matrix van stamcellen die groeien in kolonies op een laag van andere cellen en vonden dat alle bekende matrix eiwitten aanwezig waren (hoofdstuk 5). Daarnaast karakteriseerden we ook de receptoren voor extracellulaire matrix (integrines) op de stamcellen en concludeerden dat de stamcellen theoretisch aan alle extracellulaire matrix moleculen zouden moeten kunnen binden. Functionele experimenten wezen uit welke integrines belangrijk waren voor de verschillende matrices en dat stamcellen kunnen groeien op recombinant vitronectine. Deze vinding vormde de basis voor het eerste stamcelkweeksysteem waarvan alle componenten exact bekend zijn.

Vervolgens besloten we onze aandacht te richten op de vraag hoe stamcellen zich differentiëren. Stamcellen werden tot differentiatie gestimuleerd door het toevoegen van de groeifactor BMP4. Stamcel eiwitten werden vervolgens geïsoleerd en kwantitatief gemeten op tijdstip 0, op 30, 60 en 240 minuten na toevoeging van BMP4 (hoofdstuk 6). Naast de eiwitten zelf hebben we ook specifiek gekeken naar kinases, eiwitten die fosfaatgroepen toevoegen aan eiwitten, en naar gefosforyleerde eiwitten (eiwitten met fosfaat-groepen). Met deze methode hebben we 3067 fosfaatgroepen gevonden op een totaal van 5222 eiwitten. Ongeveer 50% van deze fosfaatgroepen werden gereguleerd binnen een uur na toevoeging van BMP4, wat op een zeer complex netwerk van signaleringsroutes duidt. Een van deze gereguleerde eiwitten was SOX2, een belangrijke stamcel eiwit. Dit laat zien dat het reguleren van fosfaatgroepen een belangrijk mechanisme voor differentiatie kan zijn. Na bio-informatische analyse vonden we de kinase CDK1/2 als een belangrijke regulator van stamcellen.

In hoofdstuk 7 beschrijven we een nieuwe cellijn die groen fluorescent kleurt zodra de cellen zich specialiseren tot hartspiercellen. Deze cellijn hebben we gebruikt om exact uit te zoeken welke groeifactoren er nodig zijn om hartvoorlopercellen en hartspiercellen te maken. We onderzochten deze hartvoorlopercellen verder en ontdekten dat deze cellen naast hartspiercellen ook gladde spiercellen en endotheelcellen kunnen worden. Dit laat zien dat stamcellen gebruikt kunnen worden om menselijke hartontwikkeling te bestuderen.

Ten slotte ontwikkelden we een methode om medicijnen te testen op bijwerkingen aan het hart (hoofdstuk 8). We kunnen menselijke embryonale stamcellen nu gecontroleerd laten uitgroeien tot hartspiercellen. Deze hartspiercellen lijken in veel opzichten op de cellen in het menselijke hart. Zo vertonen ze elektrische activiteit, waardoor de klompjes hartspiercellen ritmisch samentrekken. Medicijnen konden getest worden door deze simpel toe te voegen aan

de celkweken en door de elektrische activiteit van deze cellen te meten. Op basis van deze metingen hebben we nu voor het eerst laten zien dat we hartspiercellen kunnen gebruiken om het effect van medicijnen op het menselijke hart te voorspellen.

In hoofdstuk 9 en 10 bediscussiëren we de voorgaande hoofdstukken en bespreken we de grootste hindernissen en kansen voor stamcelonderzoek. We concluderen dat de mogelijkheid om hartspiercellen uit stamcellen te maken enorme mogelijkheden biedt voor fundamenteel en toegepast wetenschappelijk onderzoek. De komende jaren zullen ontwikkelingsbiologen op basis van stamcellen nieuwe ontdekkingen doen, zullen er menselijke ziektemodellen gemaakt worden voor geneesmiddelenonderzoek en zullen de testsystemen voor medicijn veiligheidstesten verder verfijnd worden. Stamcelonderzoek zal dus bijdragen aan de ontwikkeling van nieuwe generaties veilige geneesmiddelen.

Curriculum vitae

Stefan Braam was born on June 15th, 1983 in Dalfsen, the Netherlands. After graduating from secondary school in 2000 (Thomas a Kempis College, Zwolle) he started his bachelor study in Biochemistry at Saxion University, followed by a masters degree in biomedical sciences at Utrecht University. During his bachelor study he performed his first internship under the supervision of Prof. J Galama in the Department of Virology, UMC St Radboud. For his second internship, he moved to the laboratory of Dr. FAE Kruyt, VUMC Amsterdam, to work on adenoviruses for cancer gene therapy.

During his master study (Sept. 2004) he became interested in human embryonic stem cells and he moved to the Hubrecht Institute to work with Prof. Dr Christine L. Mummery and Dr. Leon G.J. Tertoolen on cardiomyoycte differentiation and electrophysiology. In 2005 he was awarded a WSC award and an Erasmus scholarship to work with Dr C. Denning (Nottingham UK) on human embryonic stem cell technology development. He received his Masters degree cum laude in April 2006.

Subsequently, he started as a PhD candidate supervised by Prof. Dr. C. Mummery at the Hubrecht Institute Utrecht. In 2008 he was awarded a EU Heart Repair travel award to work with Prof. Dr A Elefanty and Prof. Dr. E Stanley at the Monash Immunology and Stem Cell laboratories, Monash University, in Melbourne, Australia. After four months in Australia he continued his research work with Prof. Dr. C Mummery at the Leiden University Medical Centre. The results of these studies are presented in this thesis.

In 2010 he started working on Pluriomics together with Dr. R. Passier, Dr. Anja van de Stolpe and Prof. Dr. C Mummery. Pluriomics, is a biotech start-up initiative focused on cardiac safety pharmacology and drug discovery and was awarded the first prize in the NGI venture challenge December 2009

List of Publications

- **S.R. Braam**, R Nauw, D Ward-van Oostwaard , C Mummery, R Passier - Inhibition of ROCK improves survival of human embryonic stem cell-derived cardiomyocytes after dissociation - Ann N Y Acad Sci. 2010 Feb;1188:52-7.

- Van Hoof D, Dormeyer W, **Braam SR**, Passier R, Monshouwer-Kloots J, Ward-van Oostwaard D, Heck AJ, Krijgsveld J, Mummery CL – Identification of Cell Surface Proteins for Antibody-Based Selection of Human Embryonic Stem Cell-Derived Cardiomyocytes - J Proteome Res. 2010 Mar 5;9(3):1610-8

- **S.R. Braam**, L Tertoolen, A van de Stolpe, T Meyer, R Passier, C.L Mummery - Prediction of drug induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes - Stem Cell Research 2010 2010 Mar;4(2):107-16

- **S.R Braam**, C. Denning, CL Mummery – Genetic manipulation of human embryonic stem cells in serum and feeder-free media Methods Mol Biol. 2010;584:413-23

- **S.R Braam**, R. Passier , C.L Mummery– Cardiomyocytes from pluripotent stem cells in regenerative medicine and drug discovery Trends in Pharmacological sciences 2009 Oct;30(10):536-45. Epub 2009 Sep 15. Cover article

- **S.R Braam** (*), D van Hoof (*), J Munoz (*), M.W. Pinkse (*), R. Linding, A.J.R Heck, C.L Mummery, J Krijgsveld – Phosphoryalation dynamics during early differentiation of human embryonic stem cells Cell Stem Cell 2009 Aug 7

- **S.R Braam** (*), D van Hoof (*),W Dormeyer, A.J.R Heck, J Krijgsveld, C.L Mummery - Feeder-free human embryonic monolayer cultures express an epithelial plasma membrane protein profile Stem Cells 2008 Nov;26(11):2777-81. Epub 2008 Aug 14.

- **S.R Braam** (*), C Denning (*), E Matsa, L.E Young, R Passier, C.L Mummery - Feeder-free culture of human embryonic stem cells in conditioned medium for efficient genetic manipulation, Nature Protocols 2008;3(9):1435-43

- **S.R Braam**, L.M Zeinstra, S.H.M Litjens, D Ward-van Oostwaard, S van den Brink, L.W van Laake, F Lebrin, R Passier, A Sonnenberg, C.L Mummery - Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self renewal via $\alpha V\beta 5$ integrin, Stem Cells Sep;26(9):2257-65. Epub 2008 Jul 3. Cover article

- W Dormeyer, D van Hoof, **S.R Braam**, A.J.R Heck, C.L Mummery, J Krijgsveld - Plasma membrane proteomics of human embryonic stem cells and human embryonal carcinoma cells J. Proteome Research 2008 Jul;7(7):2936-51. Epub 2008 May 20.

- **S.R Braam**, C. Denning, S van den Brink, P Kats, R Hochstenbach, R Passier, C Mummery – Improved genetic manipulation of human embryonic stem cells Nat. Methods 2008 May; 5(5):389-92 Epub 2008 April 6

- R. Graichen, X Xu, **S.R Braam**, T. Balakrishnan, S. Norfiza, S. Sieh, S.Y. Yoo, S.C Tham, C.Mummery, A. Colman, R. Zweigerdt, B.P Davidson – Enhanced cardiomyogenesis of human embryonic stem cells by a small molecule inhibitor of p38 MAPK Differentiation 2008 Apr; 76(4):357-70

- J.C. Moore, L.W. van Laake, **S.R Braam**, T. Xue, S.Y. Tsang, D. Ward, R. Passier, L. Tertoolen, R.A Li, C.L. Mummery – Human embryonic stem cells: Genetic Manipulation on the Way to Cardiac Cell Therapies, Reproductive Toxicology 2005 Sep-Oct

- M.A.I Abou El Hassan, **S.R Braam**, F.A.E Kruyt – Paclitaxel enhances adenoviral gene expression and assembly in a cell-cycle-independent manner in NCI-H460 cells Cancer Gene Therapy 2006 Dec;13(12):1105-14. Epub 2006 Jul 14

- M.A.I. Abou El Hassan, **S.R Braam**, F.A.E Kruyt – A real time PCR assay for the quantitative determination of adenoviral gene expression in mammalian cells. J Virol. Methods 2006 Apr;133(1):53-61. Epub 2005 Nov 21

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Scientific Awards

- Recipient Bolk prize – Dutch society for Anatomy, January 2010
- Winner NGI venture challenge - Pluriomics , December 2009
- Heartrepair FP6 travel grant to Monash University, Melbourne Australia, April 2008
- Recipient Wijck-Stam-Caspers award, January 2007
- Erasmus grant for internship University of Nottingham, September 2006