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The Generation of Six Clinical-Grade Human Embryonic Stem Cell Lines

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The edict for producing clinically compliant human embryonic stem cells (hESCs) necessitates adherence to global ethical standards for egg procurement and embryo donation, conformity to regulations controlling clinical-grade cell and tissue product development, and compliance with current good tissue and manufacturing practices (cGTPs and cGMPs, respectively). For example, the U.S. FDA Center for Biologics Evaluation and Research recently promulgated regulations regarding human cells and cellular-based products (HCT/Ps) intended for tissue repair or replacement. Issued under Code of Federal Regulations parts 1270 and 1271 (Code of Federal Regulations, 2006a, 2006b), the rules are broadened by requirements for donor selection and cGMPs for HCT/Ps. By adhering to regulations and in anticipation of future standards, we have generated six clinical-grade hESC lines. Here we describe their manufacture, from embryo procurement to line characterization, including sterility

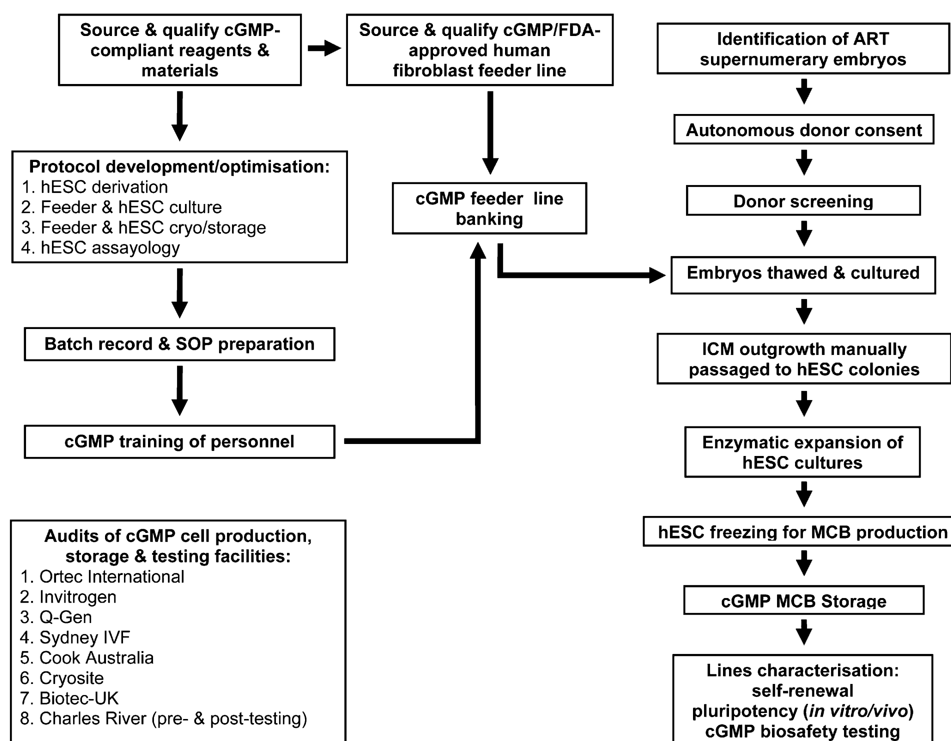

Figure 1. Overview of Activities and Requirements for cGMP hESC Line Derivation, Banking, and Characterization
The project commenced with the procurement of suitable embryos, paralleled by the selection, optimization, and verification of protocols inclusive of cGMP-compliant reagents and materials for hESC derivation, culture, cryopreservation, storage, and biosafety testing. Once validated, all protocols were translated to batch record and/or standard operating procedure (SOP) format for cGMP application. Qualified personnel received instruction for protocol execution according to principles of cGMP. On-site audits of selected key organizations/facilities for cGMP compliance were performed. Appropriate documentation confirming cGMP compliance was attained for all other supporting services (e.g., reagent production). cGMP activities were subsequently undertaken within an Australian Therapeutic Goods Administration (TGA) accredited cGMP facility (Q-GEN Pty Ltd) for fibroblast feeder banking followed by hESC line derivation, expansion, and cryopreservation for MCB production. All MCBs were transferred to an accredited facility for cGMP cryogenic storage. After characterization of each line, including biosafety testing, all documentation (exceeding 1000 pages per hESC line) was collated for final review and confirmation of successful cGMP line and bank production.

and pathogen testing (Figure 1). To our knowledge, the lines represent the first to have been produced in compliance with international regulatory requirements, suitable for therapeutic use.

Commencement of the project was contingent on the ethical procurement of quality embryos. Principles dictate that donors of blastocysts act voluntarily without coercion and are conversant of procedure and the option of "informed refusal" (Committee on Guidelines for Human Embryonic Stem Cell Research, National Research Council, 2005; International Society of Stem Cell Research, 2006; Code of Federal Regulations, 2006a, 2006b). Accordingly, we applied a comprehensive informed consent process (Table S1 in the Supplemental Data available with this article online), regulated by the Australian Commonwealth Research Involving Human Embryos Act 2002. Supernumerary embryos were procured after autonomous consent by suitable informed donors and with the approval of an Institutional Review Board (IRB) and the Australian National Health and Medical Research Council (NH&MRC) Embryo Research Licensing Committee. Approved donors satisfied requirements for lifestyle and blood testing (Table S2). Based on donor screening, 43 embryos frozen at blastocyst stage and stored for a minimum of 4 years were identified for the project.

The rigorous standards for obtaining embryos were matched by meticulously adhering to optimized protocols for embryo handling and tissue culture to maximize success within the constraints of cGMP. This assured the efficiency of hESC line derivation was similar to or higher than earlier reports of conventional research-grade line production (Cowan et al., 2004; Mitalipova et al., 2003; Klimanskaya et al., 2005; Stephenson et al., 2006). Thirty-six embryos were plated after thawing, with eight developing to putative hESC lines (22%; Table S3).

The "gold-standard" hESC line would be derived, cultured, and banked under defined conditions, independent of feeder cells and animal biologics. Although there have been many attempts to achieve this goal, including the development of xeno-free platforms, their utility is contentious (Rajala et al., 2007). Potential limitations relate to the cost of implementation, efficacy for hESC derivation and expansion, and impact on hESC differentiation and stability during prolonged culture. Accordingly, the application of current putative fully defined xeno-free methods for translational application and clinical product development is uncertain. Xeno-free methods are also unnecessary because the use of suitably qualified nonhuman animal-derived products such as bovine-sourced serum or albumin in hESC culture medium guarantees a robust and quality product and is acceptable from a regulatory perspective (Rajala et al., 2007; Fink, 2004). In support, the U.S. FDA has approved the therapeutic use of at least three live-cell products (Apligraf, Dermograft, and OrCel) where fetal bovine serum (FBS) and other bovine materials have been used in manufacture. Moreover, previous concerns about a potentially immunogenic nonhuman sialic acid, Neu5Gc, arising on the surface of hESCs grown in the presence of animal-derived components (Martin et al., 2005) are allayed by a recent finding that this is reversible by subsequent growth under animal component-free conditions (Heiskanen et al., 2007). Whether animal derived or synthetic, all reagents must be screened for unwanted adventitious agents such as microbial, viral, exogenous pyrogen, or chemical contaminants.

Given the limited availability of quality supernumary embryos and the cost burden of cGMP, we employed embryo and cell culture platforms that would guarantee production of bona fide hESC lines that are clinically compliant. Thus, all lines were generated from embryos initially plated and cultured in human serum albumin (HSA)-based blastocyst thaw and culture media, respectively (Table S4). Once isolated, hESCs were maintained on FDA-approved clinical-grade human foreskin fibroblasts in cGMP-manufactured bovine serum albumin (BSA)-based serum-replacement (SR) containing medium (Table S4). Prior to use, fibroblast feeders were derived and maintained in customized cGMP-grade FBS-based medium. Antibiotics were omitted from all media to avoid masking potential low-level bacterial contamination during culture. Their exclusion also forestalls regulatory concerns for clinical safety due to risk of hypersensitivity reactions in patients (Guidance for Human Somatic Therapy and Gene Therapy, 1998). Significantly, all hESC lines were produced under conditions free of live xeno-biologics, which qualifies them as nonxenogeneic for cell transplantation purposes (Code of Federal Regulations, 2006a, 2006b).

Embryos were hatched from the zona pellucida and replated whole (17 embryos) or bisected (19 embryos) for isolation and replating of the inner cell mass (ICM) alone. Excision of the ICM was performed manually with a splitting blade, avoiding the potential regulatory hurdles associated with immunosurgery as well as costly development of cGMP-grade antibodies and complement (Peura et al., 2007; Ellerstrom et al., 2006). ICM outgrowths formed 1–2 days after replating, with putative hESC lines being visible 10–20 days thereafter. Similar to ICM excision, hESC outgrowths were isolated manually from the ICM and cultured on fibroblasts in the absence of gelatin substrate. Of the lines that were successfully derived, four were from bisected embryos (21%; ESI-013, ESI-014, ESI-017, and ESI-051) and four were not (25%; ESI-027, ESI-035, ESI-049, and ESI-053). Hence the efficiency of derivation was similar for whole and bisected embryos. Applying the grading system stipulated by Stephenson et al. (2006), embryos were evaluated for expansion status, ICM appearance, and trophectoderm appearance (Table S3).

After manual dissection for the first two to three passages, bulk passaging or final harvesting of cultures was performed by enzymatic dissociation using cGMP-grade collagenase. This method resulted in the replating or harvest of hESC aggregates, with less than 10% fibroblast contamination for ongoing subculture or master cell bank (MCB) production. Our assessment of fibroblast contamination was based on flow cytometry analysis of fibroblasts within ESI-017 cultures

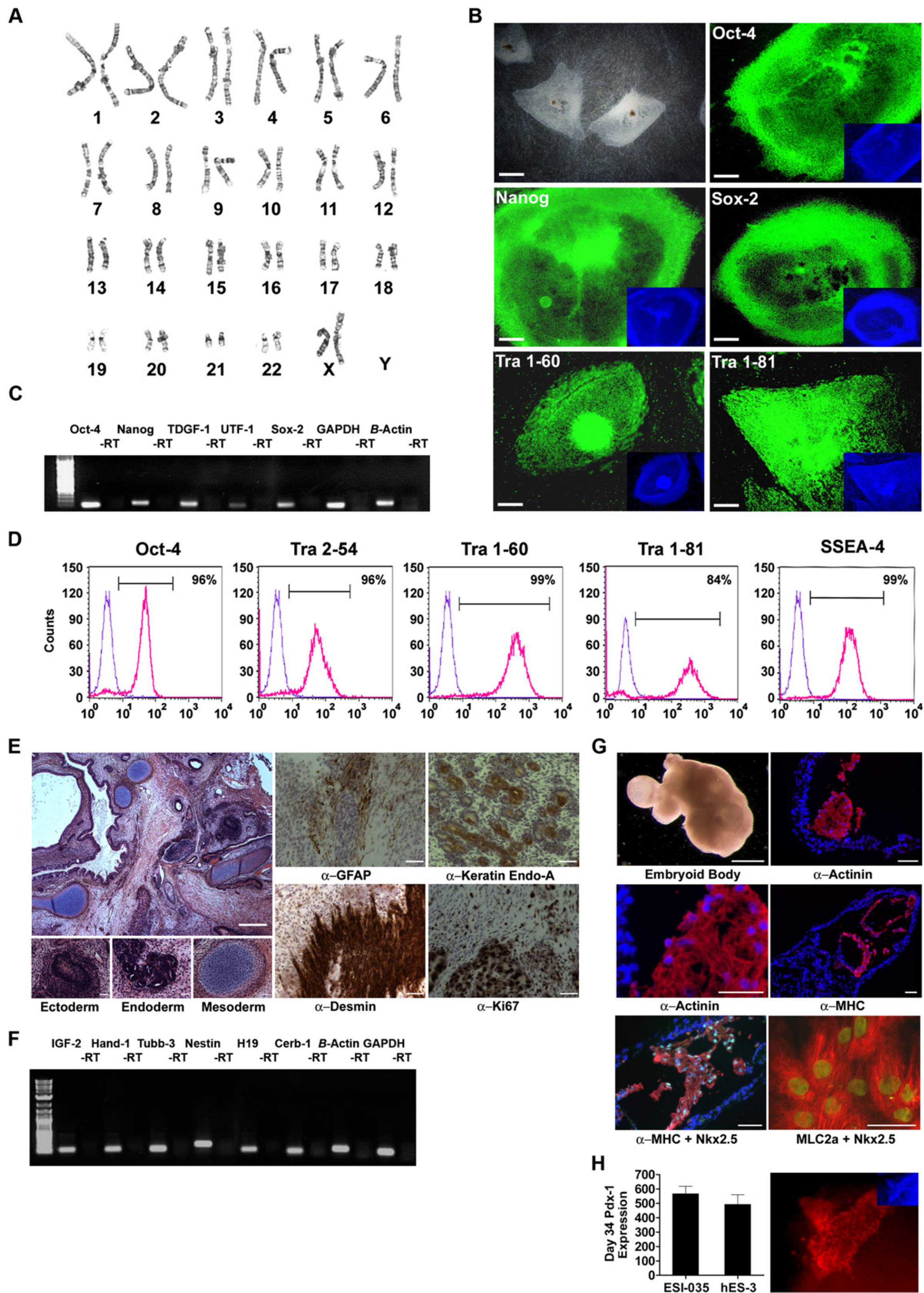

Figure 2. Characterization of Representative cGMP hESC Line ESI-035 Indicated Undifferentiated and Pluripotent hESC Status after Derivation, Culture through Eight Passages, Cryopreservation, Thawing, and Reculture through More Than Five Passages

(data not shown). Aggregate-based subculture allays concerns for putative karyotype changes associated with single-cell subculture (Buzzard et al., 2004; Mitalipova et al., 2005).

MCBs were successfully preserved after seven to nine passages by control rate freezing incorporating a cGMP-manufactured nonxeno and protein-free medium and hermetically sealed straws. MCBs comprised between 40 and 60 straws, with 3×10^6 cells per straw. All straws of an individual MCB were prepared from the same single pool/batch of hESCs. All banks were confirmed microbe free, including mycoplasma (Table S5).

Immediately after banking, hESC lines were karyotyped prior to further characterization (Table S3). Of the eight lines derived, six are karyotypically normal (ESI-014, ESI-017, ESI-035, ESI-049, ESI-051, and ESI-053) and two are trisomic mosaic for chromosome 16 (ESI-013 and ESI-027). The normal lines (Figure 2A and Table S3) comprise hESCs that can be banked, thawed, and recovered for prolonged subculture. Cells show normal growth kinetics (Table S3), express markers for undifferentiated status (Figures 2B–2D, Figures S1A and S1B, and Table S3), and form colonies exhibiting undifferentiated gross morphology (Figure 2B).

DNA fingerprinting of genetically aberrant lines confirmed the presence of triallelic profiles indicative of meiotic errors of parental origin. Hence the original embryos were trisomic rather than trisomy arising during or after hESC line derivation and culture. Fluorescent in situ hybridization indicated trisomy in 53% of ESI-013 cells, whereas ESI-027 exhibited trisomy in 63% of cells. Nontrisomic cells were diploid. Trisomy 16 is estimated to occur in more than 1% of clinically recognized pregnancies, making it the most commonly occurring trisomy in humans (Benn, 1998). Nonmosaic trisomy 16 is prevalent in males and is associated with a high probability of fetal death, preterm delivery, and intrauterine growth retardation. Conversely, trisomy 16 mosaicism is elevated in female karyotypes, exhibits a less severe nonlethal phenotype, and is often difficult to prove by standard chromosomal analysis. Although both mosaic lines showed normal growth behavior during cGMP derivation and expansion for banking, for the present each has been excluded from further characterization and consideration for clinical use.

Consistent with being undifferentiated, all the diploid, characterized lines are pluripotent. hESCs formed teratomas in SCID mice comprising endoderm, ectoderm, and mesoderm derivatives (Figure 2E and Figure S1C). RT-PCR of hESC-derived embryoid bodies (EBs) identified mRNA for *IGF-2* and *Hand-1* (mesodermal markers), *Tubb-3* and *Nestin* (ectodermal markers), and *H19* and *Cerberus-1* (endodermal markers) (Figure 2F and Figure S1D). In addition, each line has been induced to cardiomyogenic and pancreatic progenitor cells by in vitro methods for directed hESC differentiation (Table S3). For cardiomyocyte induction, floating EBs display robust cardiogenic contraction after 9–21 days in END2 cell-conditioned serum-free medium (Graichen et al., 2007; Dai et al., 2007). Specific immunolabeling of cardiac proteins Nkx2.5, myosin light chain 2a (MLC2a), cardiac α-myosin heavy chain (α-MHC), and the sarcomere marker α-actinin confirms the formation of extensive cardiomyocyte clusters within EBs and among seeded cells derived from EBs (Figure 2G). Cross-striations characteristic of sarcomeric structures were observed after α-actinin and MLC2a labeling (Figure 2G). Differentiation to pancreatic progenitors was supported by Pdx-1 induction (D'Alessandro et al., 2007; Phillips et al., 2007). Pdx-1 expression identifies early pancreatic progenitors in the vertebrate embryo, and its activation in vitro is considered a prerequisite for pancreatic differentiation and ultimately the formation of endocrine cell types from differentiating hESCs (Ashizawa et al., 2004). *Pdx-1* expression was first detected around day 12 of culture (data not shown) and persisted to day 34 (Figure 2H). Whole-mount immunohistochemistry revealed Pdx-1-positive cells to be confined to discrete domains within individual EBs, often forming clusters of ribbons (Figure 2H).

Having confirmed the stemness of each line, four of the six hESC banks

(A) A Giemsa band karyogram showing normal female karyotype.
(B) Bright-field (scale bar, 1 mm) and fluorescent (scale bar, 400 μm) microscopic images showing undifferentiated (non-cystic) colony morphology and qualitative immunocytochemistry of cells positive for transcription factors Oct-4, Nanog, and Sox-2 and extracellular matrix markers Tra 1-60 and Tra 1-81. Insets represent DAPI staining.
(C) RT-PCR showing transcript expression of marker genes *Oct-4*, *Nanog*, *TDGF-1*, *UTF-1*, and *Sox-2*. Lane 1, 100 bp DNA ladder.
(D) Quantitative flow cytometry indicating robust expression of Oct-4, alkaline phosphatase (Tra 2-54), Tra 1-60, Tra 1-81, and surface-antigen SSEA-4.
(E) Histology of teratomas. Left, large (scale bar, 400 μm) and small (scale bars, 50 μm) panels illustrating H&E staining. Teratomas comprised ectoderm (neuroepithelium), endoderm (gut-like epithelium), and mesoderm (cartilage). Right, immunolabeling of ectoderm (α-GFAP), endoderm (α-Keratin Endo-A), mesoderm (α-Desmin), and proliferating cells (α-Ki67).
(F) RT-PCR of in vitro-derived EBs showing transcript for *IGF-2* and *Hand-1* (mesodermal markers), *Tubb-3* and *Nestin* (ectodermal markers), and *H19* and *Cerberus-1* (endodermal markers). Lane 1, 100 bp DNA ladder.
(G) Cardiomyocyte induction. Contracting foci indicative of cardiomyocytes were typically observed proximal to or within cystic areas of an EB. Scale bar, 500 μm. Immunoreactivity (red labeling) to α-actinin and α-MHC on serial EB sections and MLC2a on seeded cells indicated the formation of cardiomyocyte clusters. Immunoreactivity to Nkx2.5 localized in the nucleus indicated that most if not all α-MHC- and MLC2a-positive cardiomyocytes costained with this cardiomyocyte-specific transcription factor. Note cross-striations typical of sarcomere structures in the middle-left and lower-right panels. Hoechst staining of cell nuclei is shown in blue. Scale bars, 50 μm. Cell nuclei in seeded cells appeared larger than EB-embedded cells at the same magnification.
(H) Expression of *Pdx-1* was determined by Q-PCR. On day 35, *Pdx-1* levels of ESI-035 and NIH-registered reference line hES-3 are equivalent. Pdx-1-positive β-like cells (red labeling) reside within discrete regions in individual day 34 ESI-035 EBs. Inset represents DAPI staining. Error bars represent standard error of the mean.

characterized were subsequently tested free of a variety of human and nonhuman pathogens by standard methods for mammalian cell bank and tissue screening (Table S5). The remaining two banks will be similarly screened.

In summary, the hESC lines described were manufactured according to cGTP and cGMP for clinical compliance and therapeutic application. We have developed and applied a model process comprising standardized protocols with validated reagents, materials, and procedures verified by documentation for regulatory approval. All lines remain pathogen free, karyotypically stable, and undifferentiated after derivation, expansion, cryopreservation for banking, thawing, and prolonged subculture. Surface antigens and gene expression signatures of undifferentiated hESCs correlate with in vitro EB and in vivo teratoma formation as consummate measures of developmental potency for hESCs. Directed differentiation to cardiomyogenic and pancreatic progenitor cells confirms the capacity for each line to be converted to specialized somatic cell types. As a source of clinical-grade stem cells, we anticipate the cell banks will facilitate the translation of hESC research and development to cell-based therapies. In addition, as fully characterized cell lines, they are attractive candidates for drug screening relevant to pharmacotherapeutics. The lines will be available for research at low cost and with no intellectual property reach-through claims from the A*Star (http://www.a-star.edu.sg) Singapore Stem Cell Consortium Bank. ESI-017, ESI-035, ESI-049, and ESI-053 are immediately available, and ESI-014 and ESI-051 will be available after completion of biosafety testing. Clinical grade stocks will be accessible under licensed agreement with ES Cell International Pte Ltd (http://www.escellinternational.com).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, one figure, and seven tables and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/1/5/490/DC1/.

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REFERENCES

Ashizawa, S., Brunicardi, F.C., and Wang, X.P. (2004). Pancreas *28*, 109–120.

Benn, P. (1998). Am. J. Med. Genet. *79*, 121–133.

Buzzard, J.J., Gough, N.M., Crook, J.M., and Colman, A. (2004). Nat. Biotechnol. *22*, 381–382.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D., and Melton, D.A. (2004). N. Engl. J. Med. *350*, 1353–1356.

Dai, W., Field, L.J., Rubart, M., Reuter, S., Hale, S.L., Zweigerdt, R., Graichen, R.E., Kay, G.L., Jyrala, A., Colman, A., et al. (2007). J. Mol. Cell. Cardiol. *43*, 504–516.

D'Alessandro, J.S., Lu, K., Fung, B.P., Colman, A., and Clarke, D.L. (2007). Stem Cells Dev. *16*, 75–89.

Ellerstrom, C., Strehl, R., Moya, K., Andersson, K., Bergh, C., Lundin, K., Hyllner, J., and Semb, H. (2006). Stem Cells *24*, 2170–2176.

Fink, D.W. (2004). Human embryonic stem cells: regulatory considerations. In Handbook of stem cells, R.P. Lanza, ed. (Boston: Elsevier Academic), pp. 775–786.

U.S. Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research (1998). Guidance for Human Somatic Therapy and Gene Therapy.

Graichen, R.E., Xu, X.Q., Braam, S.R., Balakrishnan, T., Norfiza, S., Sieh, S., Soo, S.Y., Tham, S.C., Mummery, C.L., Colman, A., et al. (2007). Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation, in press.

Heiskanen, A., Satomaa, T., Tiitinen, S., Laitinen, A., Mannelin, S., Impola, U., Mikkola, M., Olsson, C., Miller-Podraza, H., Blomqvist, M., et al. (2007). N-glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. Stem Cells *25*, 197–202.

International Society of Stem Cell Research (2006). Guidelines for the conduct of human embryonic stem cell research. Version 1.

Klimanskaya, I., Chung, Y., Meisner, L., Johnson, J., West, M.D., and Lanza, R. (2005). Lancet *365*, 1636–1641.

Martin, M.J., Muotri, A., Gage, F., and Varki, A. (2005). Nat. Med. *11*, 228–232.

Mitalipova, M.M., Calhoun, J., Shin, S., Wininger, D., Schulz, T., Noggle, S., Venable, A., Lyons, I., Robins, A., and Stice, S. (2003). Stem Cells *21*, 521–526.

Mitalipova, M.M., Rao, R.R., Hoyer, D.M., Johnson, J.A., Meisner, L.F., Jones, K.L., Dalton, S., and Stice, S.L. (2005). Nat. Biotechnol. *23*, 19–20.

Peura, T.T., Bosman, A., and Stojanov, T. (2007). Theriogenology *67*, 32–42.

Phillips, B.W., Hentze, H., Rust, W.L., Chen, Q.P., Chipperfield, H., Tan, E.K., Abraham, S., Sadasivam, A., Soong, P.L., Wang, S.T., et al. (2007). Stem Cells Dev. *16*, 561–578.

Rajala, K., Hakala, H., Panula, S., Aivio, S., Pihlajamäki, H., Suuronen, R., Hovatta, O., and Skottman, H. (2007). Hum. Reprod. *22*, 1231–1238.

Stephenson, E.L., Braude, P.R., and Mason, C. (2006). Regen. Med. *1*, 739–750.

Committee on Guidelines for Human Embryonic Stem Cell Research National Research Council (2005). Guidelines for Human Embryonic Stem Cell Research (Washington, D.C.: The National Academies Press).

Code of Federal Regulations (2006a). Food and Drugs, title 21, part 1270.

Code of Federal Regulations (2006b). Food and Drugs, title 21, part 1271.