In Vitro Cell.Dev.Biol.—Animal (2010) 46:294–299
DOI 10.1007/s11626-010-9298-y

Derivation of three new human embryonic stem cell lines

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Received: 31 March 2009 / Accepted: 16 January 2010 / Published online: 3 March 2010 / Editor: P. Andrews

Abstract Human embryonic stem cells are pluripotent cells capable of extensive self-renewal and differentiation to all cells of the embryo proper. Here, we describe the derivation and characterization of three Sydney IVF human embryonic stem cell lines not already reported elsewhere, designated SIVF001, SIVF002, and SIVF014. The cell lines display typical compact colony morphology of embryonic stem cells, have stable growth rates over more than 40 passages and are cytogenetically normal. Furthermore, the cell lines express pluripotency markers including Nanog, Oct4, SSEA3 and Tra-1-81, and are capable of generating teratoma cells derived from each of the three germ layers in immunodeficient mice. These experiments show that the cell lines constitute pluripotent stem cell lines.

Keywords Derivation · Human · Embryonic stem cells · Pluripotent

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Introduction

Human embryonic stem cells are pluripotent cells derived from the presumptive inner cell mass (ICM) of morula and blastocyst stage embryos. These stem cells have a high self-renewal capacity and are capable of differentiating into cells representative of the three germ layers. As such, they have been suggested to be a valuable cell source for future cell replacement therapies and regenerative medicine (Doss et al. 2004; Gerecht-Nir and Itskovitz-Eldor 2004). Besides direct therapeutic applications, it is believed that human embryonic stem cells and their differentiated products will revolutionize drug discovery and development (Pouton and Haynes 2007; Sartipy et al. 2007). This might include, for example, stem-cell-derived hepatocytes for metabolic profiling and neurons for neurodegenerative disease drug discovery. The embryonic nature of the cells also foreshadows a model for screening drug candidates for developmental toxicity.

The derivation of human embryonic stem cells was first reported in 1998 and it is estimated that over 300 lines have since been derived (Thomson et al. 1998; Abbott et al. 2006). However, the majority of these cell lines are insufficiently characterized, unpublished or generally unavailable to the research community. For embryo outgrowths to be considered pluripotent stem cell lines, several criteria that typify these cells are usually investigated (Hoffman and Carpenter 2005; Loring and Rao 2006). Firstly, as the self-renewal capacity of pluripotent stem cells is theoretically limitless, one criterion is the ability to maintain stable growth (that is, without cytogenetic change)

in culture, with a benchmark set at beyond ten passages. Secondly, the embryo outgrowths should express a panel of marker genes that identify human embryonic stem cells, including transcription factors Nanog and Oct4 (POU5F1), glycolipid antigens SSEA3 and SSEA4, and keratan sulfate antigens Tra-1-60 and Tra-1-81 (Adewumi et al. 2007). Lastly, as embryonic stem cells are pluripotent, they should

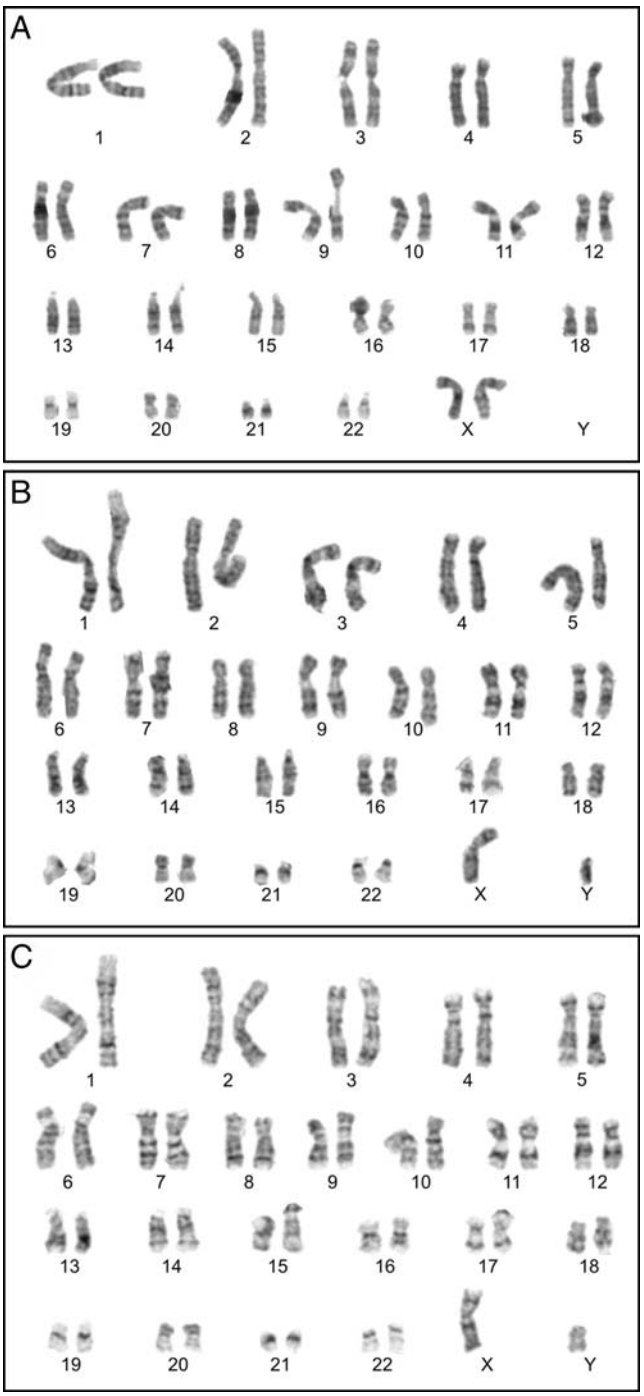

Figure 1. Cytogenetic analysis of early passage stem cell lines. Karyotyping of SIVF001 at p3 (*A*), SIVF002 at p7 (*B*) and SIVF014 at p7 (*C*).

Table 1. DNA profiling of stem cell lines. Data represents the number of tetranucleotide tandem repeats in the examined loci of stem cell lines SIVF001 at p4 and p33, SIVF002 at p21 and p58, and SIVF014 at p11

Cell line	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	D5S818	FGA
SIVF001	14	29, 32.2	10	10, 11	18	6, 9.3	11, 12	10, 12	17, 20	13, 14	18	8, 11	14, 19	9, 13	21, 22
SIVF002	12, 14	28, 30	8, 10	10, 11	14, 17	6	11	11, 12	23	13, 14	14, 16	8	13, 16	11, 12	19, 21
SIVF014	12, 14	28, 31.2	10, 12	10, 11	16	6, 9	8, 9	11	17, 24	14, 15	16, 17	8, 11	12, 15	12	21, 23

retain the ability to differentiate into cells derived from mesodermal, ectodermal, and endodermal tissues. These experiments can be performed either in vitro, typically by embryoid body formation, or in vivo, by teratoma studies in immunodeficient mice.

In this paper, we describe the derivation and characterization of three new human embryonic stem cell lines, designated SIVF001, SIVF002, and SIVF014.

Materials and Methods

Embryo donation. All embryos were produced in a clinical in vitro fertilization (IVF) program for the purpose of procreation and cultured to the blastocyst stage, as described elsewhere (Henman et al. 2005). The embryos were deemed excess to reproductive needs and donated by an informed consent process as outlined in detail previously (Peura et al. 2008). With the exception of SIVF014, which was derived in the Czech Republic, the stem cell lines were derived in Australia. All procedures were approved by Sydney IVF's independent human research ethics committee (or institutional review board), and where relevant by the Embryo Research Licensing Committee of Australia's National Health and Medical Research Council (NHMRC). SIVF001 and SIVF002 were derived under Australian NHMRC license 309703. SIVF014 was derived under the same ethical requirements as the other two cell lines and also conformed with all contemporary Czech ethical and clinical requirements. All stem cell lines were fully consented by donating couples and the couples have placed no restrictions on the use of the cells. Sydney IVF has agreed with donating families to retain a sample of early-passage cells in case, at some point in the future, they might be medically useful to a member of the donating family.

Derivation and culture of stem cells. Derivation and culture of embryonic stem cells was essentially performed as described previously (Peura et al. 2008, 2009). In brief, thawed embryos were plated, either whole or bisected into two parts with only the ICM-containing part

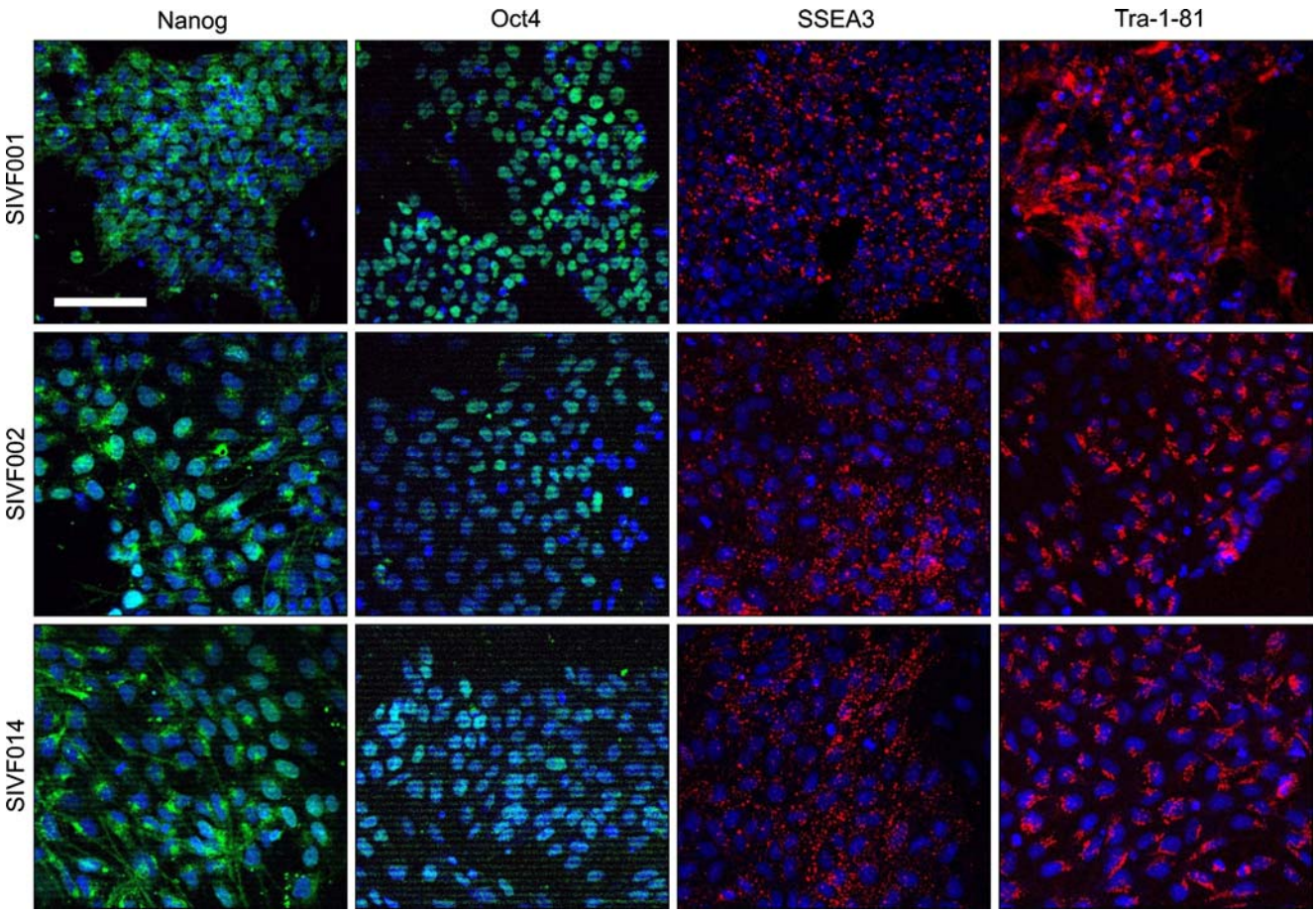

Figure 2. Expression of pluripotency markers in stem cell lines. Immuno-fluorescence staining of stem cell lines SIVF001 at p11, SIVF002 at p40, and SIVF014 at p38. *Blue staining* represents nuclear DRAQ5. The *scale bar* represents 100 μM.

being used, on mitomycin-inactivated human fetal fibroblasts (produced in-house or commercially available CCL-110/Detroit 551; ATCC) in KO-DMEM with 20% knockout-serum replacement (KSR), 2 mM glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 1× MEM-amino acids, 0.1 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA) and 4–50 ng/ml bFGF (Sigma, St. Louis, MO), and incubated at 37°C in 6% CO_2/5% O_2. Note that SIVF001 was derived in medium containing FCS in place of KSR, with the cell line being cultured in KSR medium from passage 5 onwards. Outgrowths from the ICM were expanded by manual passaging and cultured as described above with the exception that 4 ng/ml bFGF was used in the culture medium. Presumptive stem cell lines at early passages were cryopreserved using a modified vitrification procedure. The cell lines were negative for mycoplasma, as tested both by in-house testing using the MycoAlert Mycoplasma Kit (Lonza, Basel, Switzerland) and independently by the Victorian Infectious Disease Reference Laboratory (Melbourne, Australia).

Karyotyping and DNA profiling. Karyotyping was performed by incubation of stem cell colonies with 0.22 ng/ml colcemid and 37.5 μg/ml BrdU for 17–19 h or 5 ng/ml colcemid for 2.5 h, followed by dissociation using non-enzymatic cell dissociation solution (Sigma). Metaphase spreads with G-banding were prepared and analyzed by Sydney Genetics (Sydney, Australia, a subsidiary of Sydney IVF). DNA profiling was performed by DNA Labs (Sydney, Australia, also a subsidiary) using the AmpF/STR Identifiler PCR Amplification Kit (Applied Biosystems) which examines 15 tetranucleotide short-tandem repeat loci.

Teratoma experiments. Teratoma experiments were performed as described previously (Peura et al. 2008). In brief, manually or collagenase-passaged stem cell clumps (~1×10^6 cells) were injected into the upper thigh of 6-wk-old SCID mice (ARC; Perth, Australia). Tumors were harvested once visible, typically at 2–3 mo, and histopathology conducted by Symbion Pathology (Sydney, Australia) to identify cell lineages.

Immunostaining. Immunostaining for pluripotency markers was performed on feeder-free stem cell cultures using the following antibodies: Oct4 Alexa Fluor 488, SSEA4 Alexa Fluor 488, SSEA3 PE, Tra-1-60 Alexa Fluor 555, Tra-1-81 Alexa Fluor 555 (all BD Bioscience, Franklin Lakes, NJ) or Nanog (Santa Cruz Biotechnology, Santa Cruz, CA) combined with donkey anti-goat IgG Alexa Fluor 488 (Invitrogen) as the secondary antibody. Cells were counterstained with DRAQ5 (Biostatus, Leicestershire, UK) and analyzed using the IN Cell Analyzer 1000 and IN Cell Analyser 1000 Workstation 3.5 software (GE Healthcare, Buckinghamshire, UK). Alkaline phosphatase staining of stem cell colonies was performed using the Alkaline Phosphate Substrate Kit (Vector Laboratories, Burlingame, CA).

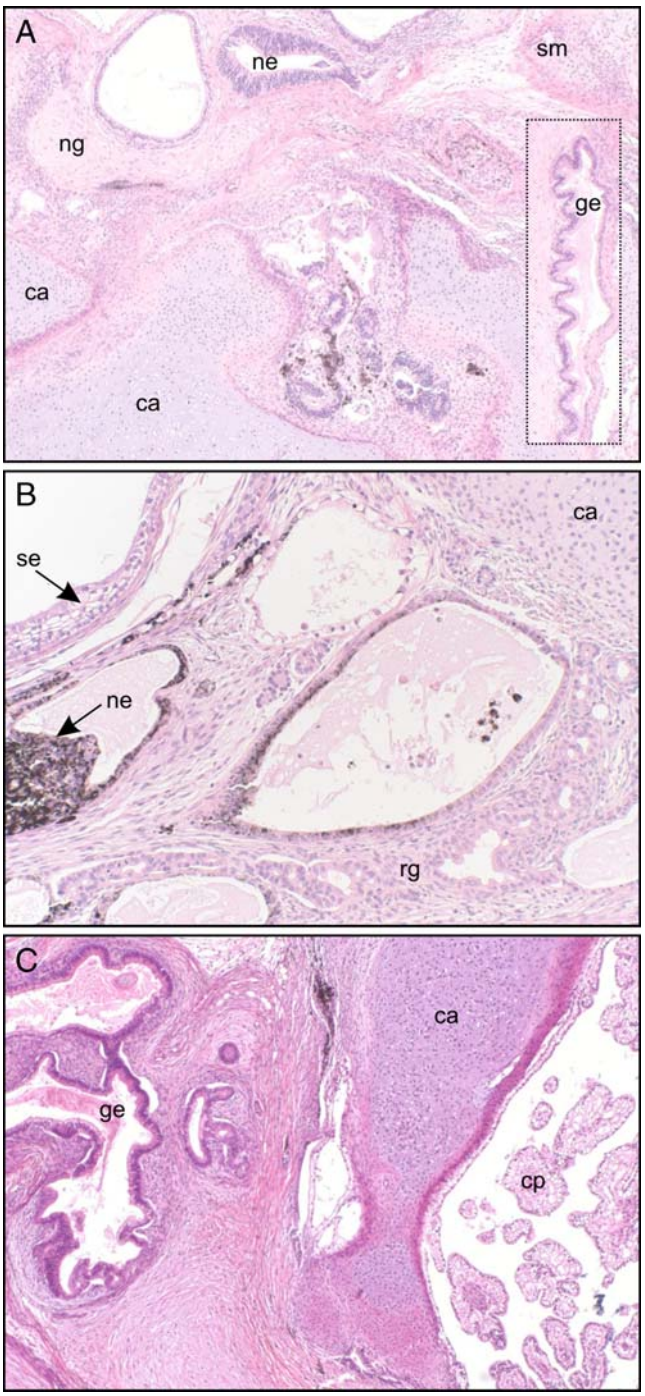

Figure 3. Formation of teratomas from stem cell lines. Hematoxylin and eosin stained teratoma sections from SIVF001 at p6 (*A*), SIVF002 at p9 (*B*) and SIVF014 at p12 (*C*). Ectodermal lineages include neuro-epithelium (*ne*; some of which was pigmented), neuroglia (*ng*), choroid plexus (*cp*), and squamous epithelium (*se*). Mesodermal lineages include cartilage (*ca*) and smooth muscle (*sm*). Endodermal lineages include gastrointestinal epithelium (*ge*) and respiratory glands (*rg*).

Results

Derivation and culture of stem cell lines. Stem cell lines SIVF001, SIVF002, and SIVF014 were derived from good-quality slow-frozen blastocysts which had been thawed and plated whole (SIVF001) or bisected (cut into two parts with only the ICM-containing part being plated; SIVF002 and SIVF014) onto mitotically inactive human fetal fibroblasts. The embryo outgrowths, or presumptive stem cell lines, displayed the typical morphology of human embryonic stem cells, including compact colony formation composed of cells with a high nuclear to cytoplasmic ratio (data not shown). Furthermore, the stem cell lines have been cultured for >40 passages ('p') and have maintained consistent growth rates and low levels of differentiation. At the time of writing, the highest manual passage conducted for each cell line is SIVF001 at p142, SIVF002 at p62 and SIVF014 at p44. All lines were cryopreserved by vitrification, including early passages, and post-thaw viability has been confirmed.

Cytogenetic and molecular characterization of stem cell lines. Outgrowths from the embryos were karyotyped at early passages and determined to be cytogenetically normal (Fig. 1). The karyotype of the cell lines was stable at later passages, with the highest manual passage examined being SIVF001 at p140, SIVF002 at p40, and SIVF014 at p35 (data not shown). In order to provide a permanent genetic signature for monitoring the integrity and use of the stem cells, the cell lines were DNA fingerprinted using tetranucleotide tandem repeat loci (Table 1). Furthermore, testing for mycoplasma has persistently been negative; the most recent passage tested independently comprise SIVF001 at p71, SIVF002 at p34, and SIVF014 at p38 (data not shown).

Evaluation of pluripotency of stem cell lines. The expression of pluripotency markers in the presumptive stem cell lines was evaluated by immuno-fluorescence staining. The cell lines were shown to express nuclear transcription factors Oct4 and Nanog, and cell surface markers SSEA4 and Tra-1-60 (Fig. 2). Additionally, the cell lines also expressed

Table 2. Summary of Sydney IVF human embryonic stem cell lines

Stem cell line	Karyotype	Disease status	>10 passages	Stem cell marker expression	Teratoma formation (3 germ layers)	Published
SIVF001	46, XX	–	Yes	Yes	Yes	
SIVF002	46, XY	–	Yes	Yes	Yes	
SIVF003	47, XX, +16	–	Yes	Yes	No	Peura et al. (2008)
SIVF004	46, XX	–	Yes	Yes	Yes	Peura et al. (2008)
SIVF005	46, XY	–	Yes	Yes	No	Peura et al. (2008)
SIVF006	46, XX	–	Yes	Yes	Yes	Peura et al. (2008)
SIVF007	46, XX	–	Yes	Yes	No	Peura et al. (2008)
SIVF008	46, XX	–	Yes	Yes	Yes	Peura et al. (2008)
SIVF009	Mosaic [46, XX, i(13)(q10)/46,XX]	–	Yes	Yes	Yes	Peura et al. (2008)
SIVF010	46, XX	–	Yes	Yes	Yes	Peura et al. (2008)
SIVF011	47, XY, +5	–	Yes	Yes	In progress	Peura et al. (2008)
SIVF012	46, XX	–	Yes	Yes	Yes	Peura et al. (2008)
SIVF013	46, XY	–	Yes	Yes	Yes	Peura et al. (2008)
SIVF014	46, XY	–	Yes	Yes	Yes	
SIVF015	46, XY	–	Yes	Yes	In progress	
SIVF016	46, XX	–	Yes	Yes	In progress	
SIVF017	46, XY	HD	Yes	Yes	Yes	[a]
SIVF018	46, XX	HD	Yes	Yes	In progress	[a]
SIVF019	46, XX	–	Yes	Yes	Yes	[a]
SIVF020	46, XX	HD	Yes	Yes	Yes	[a]

Note that cell lines SIVF003–SIVF013 were derived from biopsied blastocysts which had been found by pre-implantation genetic screening to be unsuitable for clinical use due to abnormal FISH signal patterns consistent with chromosomal abnormalities. Teratoma studies for stem cell lines that did not produce cells representative of all three germ layers (SIVF003; two germ layers only) or failed to produce teratoma growths (SIVF005 and SIVF007) are scheduled for repetition. For an up-to-date list of SIVF stem cell lines visit www.sydneyivfstemcells.com

HD Huntington's disease

[a] Manuscript in preparation

SSEA3, Tra-1-81 and alkaline phosphatase (data not shown). Moreover, teratoma induction experiments revealed the presence of cells representative of the three germ layers for each of the cell lines. This included ectodermal tissues such as neuroepithelium and squamous epithelium, mesodermal tissues such as cartilage and smooth muscle, and endodermal tissues such as gastrointestinal epithelium and respiratory epithelium (Fig. 3). Combined, these data confirm that the embryo outgrowths represent pluripotent stem cell lines.

Discussion

In this study, we describe the derivation of three new human embryonic stem cell lines, SIVF001, SIVF002, and SIVF014. For the characterization of the cell lines reported here, we largely followed the guidelines recommended by the ISCF International Stem Cell Banking Initiative (2008). These guidelines aim to ensure a minimum set of data is available for all human embryonic stem cell lines accessible to the research community, including characterization of the cells phenotype, genotype, cell line stability, and pluripotency. Accordingly, our embryo outgrowths displayed the hallmark characteristics of pluripotent stem cells including compact colony morphology, expression of pluripotency markers (phenotype), retention of a cytogenetically normal karyotype (genotype), stable growth rates beyond 40 passages (cell line stability), and formation of teratomas that contain cells representative of the three germ layers (pluripotency). In addition to these tests, we performed DNA fingerprinting to determine each cell lines unique genetic signature. We believe that this DNA profile is a useful approach to ensure the identity of the stem cell lines and to prevent or identify mix-up or contamination with other cell lines.

For all three cell lines, we maintain master stocks at low passage numbers as well as a working bank. The complete history of all stocks in terms of culture and subculturing methods, media and other conditions is documented. The stem cell lines have been fully consented by donating couples, with no restrictions placed on the use of the cell lines. They have been added to the repository of SIVF stem cell lines (Table 2; www.sydneyivfstemcells.com) and are available to the research community.

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