REPORT

Analysis of long-term culture properties and pluripotent character of two sibling human embryonic stem cell lines derived from discarded embryos

Parvathy Venu · Sanjukta Chakraborty ·
Maneesha S. Inamdar

Abstract We had earlier reported the derivation and characterization of two new sibling human embryonic stem cell lines BJNhem19 and BJNhem20, from discarded grade III embryos of Indian origin. We report here the characteristics of the two sibling cell lines after long-term continuous culture for over 2 yr during which they have been passaged over 200 times. We show that both cell lines adapt well to culture on various mouse and human feeders as well as in feeder-free conditions. The cells show normal diploid karyotype and continue to express all pluripotency markers. Both cell lines differentiate to derivatives of all three germ layers in vitro. However as reported earlier, BJNhem19 is unable to generate teratomas in nude or SCID mice or differentiate to beating cardiomyocytes when tested over several passages during long-term stable culture. On the other hand, the cardiac differentiation capacity of BJNhem20 is greatly increased, and it can generate beating cardiomyocytes that proliferate when isolated and cultured further. In conclusion, the two cell lines have maintained a stable phenotype for over 2 yr and are indeed immortal. Their derivation from grade III embryos does not seem to have any adverse effect on their long-term phenotype. The cells can be obtained for research purposes from the UK Stem Cell Bank and from the authors.

Keywords BJNhem19 · BJNhem20 · Sibling · Human embryonic stem cell · Long-term culture

P. Venu · S. Chakraborty · M. S. Inamdar (✉)
Jawaharlal Nehru Centre for Advanced Scientific Research,
Jakkur P.O.,
Bangalore 560064, India
e-mail: inamdar@jncasr.ac.in

Introduction

Human embryonic stem (hES) cells potentially provide an unlimited resource for regenerative medicine. Since the isolation of the first hES cell lines over 10 yr ago (Thomson et al. 1998), several groups have successfully derived hES cell lines using various techniques and from various genetic backgrounds (http://www.stemcellforum.org/isci_project/the_registry.cfm; http://www.mrc.ac.uk/Utilities/Documentrecord/index.htm?d=MRC003259; http://stemcells.nih.gov/research/registry/). The common theme in these has been the demonstration that hES cell lines can maintain their pluripotent characteristics in culture and can differentiate to derivatives of all three germ layers. It is generally acknowledged that hES cell lines derived from different genetic backgrounds differ in their characteristics and behavior (Enver et al. 2005) yet express common markers of pluripotency (Adewumi et al. 2007). However, little information exists about the effects of long-term culture on most hES cell lines in use. A couple of studies encouragingly showed that a set of cell lines in continuous culture for over a year maintained a stable phenotype (Rosler et al. 2004; Suemori et al. 2006). It is hoped that these features will be common to all hES cell lines derived thus far, as scale-up and long-term culture of hES cells under defined conditions will be essential for their therapeutic application. If this is not a dependable property of hES cells regardless of genetic background, then it limits their potential applications in therapy. We earlier reported derivation and characterization of two sibling hES cell lines BJNhem19 and BJNhem20, from India (Inamdar et al. 2009). We report here a preliminary characterization of their stability and pluripotency after long-term culture.

Methods

hES cell culture and differentiation. Stocks for long-term culture were maintained in antibiotic-free media on mouse embryonic fibroblasts (MEFs) and were passaged mechanically. Culture of hES cells on MEFs and differentiation to embryoid bodies was as described earlier (Inamdar et al. 2009). For culture on human feeders, human foreskin fibroblasts (HFF; ATCC cat. no. SCRC1041) were mitotically inactivated by gamma irradiation (60 Gy) and used as for MEFs. Other substrates used were gelatin (0.1% in phosphate buffered saline; Sigma cat. no. G1393), fibronectin (5 μg/cm^2, Sigma Cat. no. F2518), or Matrigel (1:100 dilution, Becton Dickinson cat. no. 354277). Culture on gelatin or fibronectin was in hES media conditioned by incubation for 24 h with mitotically inactivated MEFs. Culture on Matrigel was in mTeSR1 media (Stem Cell Technologies, Palo Alto, CA) according to the manufacturer's instructions.

Characterization of hES cells. Karyotyping, reverse transcription-polymerase chain reaction (RT-PCR) analysis, and immunostaining for pluripotency markers were done as described before (Inamdar et al. 2009). hES cells grown on feeders or appropriate matrix were fixed in 2.5% paraformaldehyde in phosphate buffer solution and stained with appropriate antibodies as described before (Siva et al. 2007). Primary antibodies used were against SSEA4, TRA-1-60, and TRA-1-81 (kind gift of Peter Andrews, Sheffield), OCT4, VEGFRII, (BD Pharmingen, San Diego, CA), vimentin, alpha-fetoprotein, α-actinin (Sigma Chemical Co., St. Louis, MO), Brachyury, Tbx-5 (Santa Cruz Biotechnology, Santa Cruz, CA), and nestin (Chemicon, Temecula, CA). Alexa Fluor-conjugated secondary antibodies were from Molecular Probes, Eugene, OR.

Cardiomyocyte culture and proliferation. Undifferentiated BJNhem20 colonies were removed manually and cultured in 0.75% dimethyl sulfoxide (DMSO) embryoid body (EB) media in a low attachment dish for 3 d as described before (Inamdar et al. 2009). EBs were transferred to gelatin-coated dishes on day 4 and fed with the same media for 10 d, after which, they were fed with normal EB media. Beating areas were cut with finely pulled glass microneedles after day 2 of beating, dissociated in microdrops of 0.05% trypsin-EDTA, and plated at low cell density on gelatin-coated dishes. Cultures were imaged each d in phase contrast, fixed after 8 d, and immunostained with Tbx-5 or α-actinin.

Teratoma analysis. Teratoma formation was tested in nude (N) mice as well as SCID (S) mice. A total of eight mice (4N+4S) for BJNhem19 and five mice (4N+1S) for BJNhem20 were injected with about 10 million cells at one subcutaneous site on the right flank. Teratomas were obtained only with BJNhem20 in three nude mice. Teratomas were excised after 8–10 wk, fixed, cryosectioned, and stained with hematoxylin and eosin. Representative images are shown in Fig. 1S–U.

Results and Discussion

We previously reported culture of BJNhem19 and BJNhem20 on MEFs. These were derived from poor quality grade III embryos generated during in vitro fertilization. Out of 21 discarded embryos obtained, one was lost during manipulation, two were cultured whole (group A), and 18 were subjected to immunosurgery (group B), of which, six were further dissected to isolate the inner cell mass (ICM; subset B1). Of 13 group B embryos that showed ICM-like outgrowths, five belonged to subset B1, of which, two sibling embryos gave hES cell lines. This indicates that mechanical dissection of the ICM greatly improved the success rate, probably due to complete elimination of trophectoderm. Hence, though the ICM is reduced or damaged in most discarded embryos, the efficiency of hES cell derivation can be greatly increased by isolating the ICM from the trophectoderm and dead cells.

We have since cultured the cells on MEFs derived from CF-1 mouse strain and obtained from different sources (ATCC, Chemicon, and derived in-house). The cells can be cultured successfully on MEFs from all three sources (data not shown). The density of MEFs consistently determined the uniform appearance of colonies in culture from early to late passages. For our hES cells, high density of MEFs promotes differentiation, whereas very low densities of MEFs are conducive to maintaining the undifferentiated phenotype. Further, colony remnants left to re-grow in the original dish grow better as there are almost no MEFs adhering to the dish (Fig. 1A). The MEFs that are sloughed-off during the course of culture may have laid down a matrix that supports robust growth of pluripotent hES cells. We also cultured the hES cells on HFF and in feeder-free conditions on dishes coated with various substrates such as gelatin, fibronectin, or Matrigel. HFF supported growth of BJNhem19 and BJNhem20, but the colony morphology was different from that on MEFs (Fig. 1B), while pluripotency marker expression was normal (data not shown). Gelatin did not support growth of the hES cells, whereas culture on fibronectin supported growth of undifferentiated colonies (Fig. 1C). BJNhem19 and BJNhem20 were also successfully cultured on Matrigel-coated tissue culture dishes for over ten passages with defined media (Fig. 1D). No adaptation was required for the cells. Such cultures could be passaged mechanically as well as enzymatically without
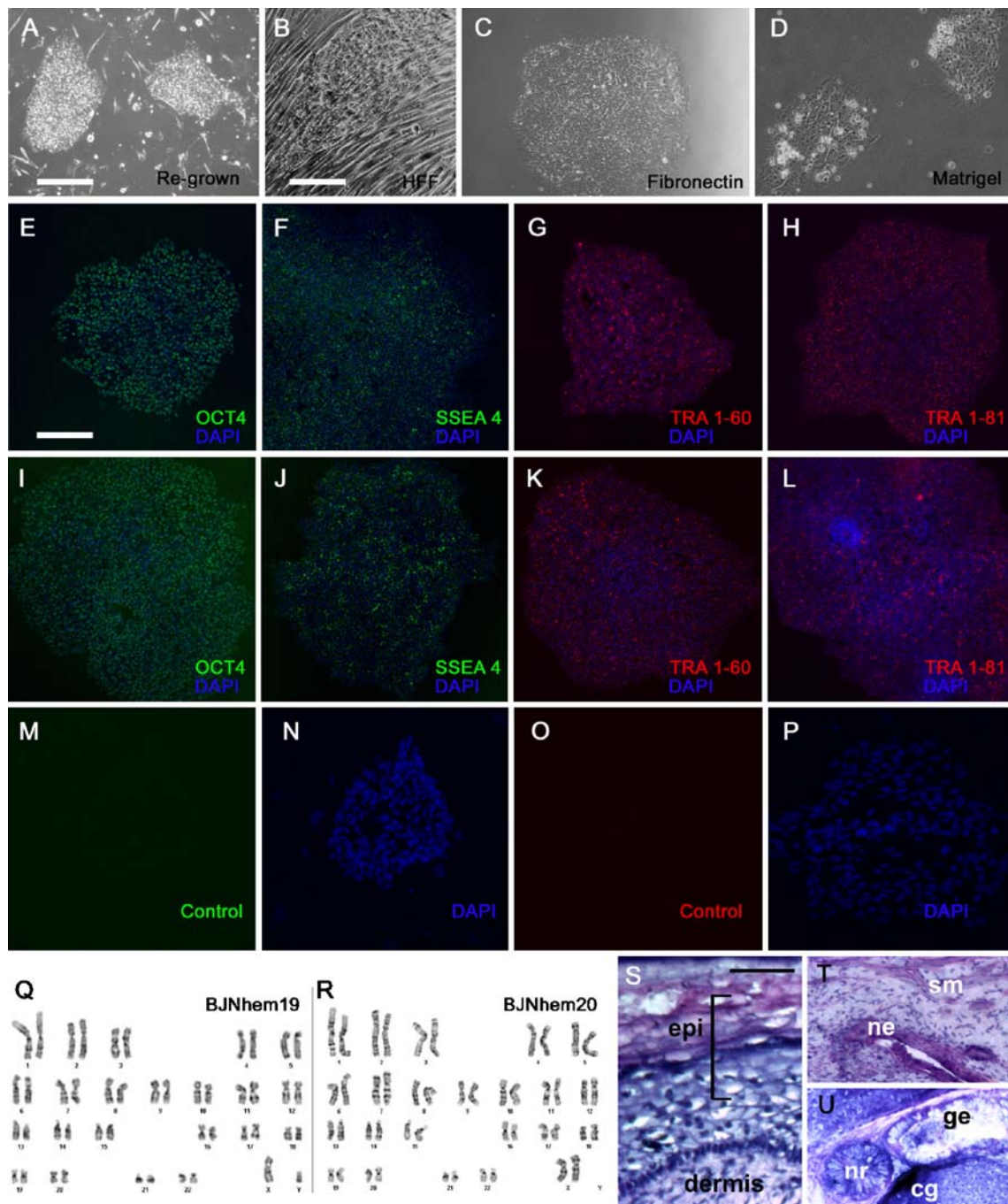
Figure. 1 Characterization of human embryonic stem (hES) cells. *A–P*, Morphology and marker analysis of hES colonies on various substrates. *A–D*, Phase contrast micrographs showing BJNhem20 cells growing on (*A*) ultra-low mouse embryonic fibroblasts density in a dish passaged by mechanical cutting and re-incubated for colony fragments to re-grow (*B*) human foreskin fibroblasts, (*C*) fibronectin, and (*D*) Matrigel. *E–L*, Fluorescent immunostaining of (*E–H*) BJNhem19 and (*I–L*) BJNhem20 cells grown on Matrigel and stained with antibodies recognizing (*E, I*) OCT4 (*green*), (*F, J*) SSEA4 (*green*), (*G, K*) TRA-1-60 (*red*), and (*H, L*) TRA-1-81 (*red*). *M–P*, No primary antibody controls showing (*M*) *green* secondary and (*N*) corresponding DAPI image and (*O*) *red* secondary and (*P*) corresponding DAPI image. In (*E–P*), nuclei are *blue* (DAPI). *Q–R*, Karyotype of (*Q*) BJNhem19 showing 46,XY and (*R*) BJNhem20 showing 46,XX chromosomes. *S–U*, Representative bright field photomicrographs of H&E stained teratoma sections of BJNhem20 teratoma showing differentiation to (*S*) ectodermal, (*T*) mesodermal, and (*U*) endodermal lineages. *cg* cartilage, *epi* epidermis, *ge* gut epithelium, *ne* neural epithelium, *nr* neural rosette, *sm* smooth muscle. *Scale bar*: (*A*) 200 μm, (*B–P, U*) 100 μm, (*S*) 25 μm, (*T*) 50 μm.
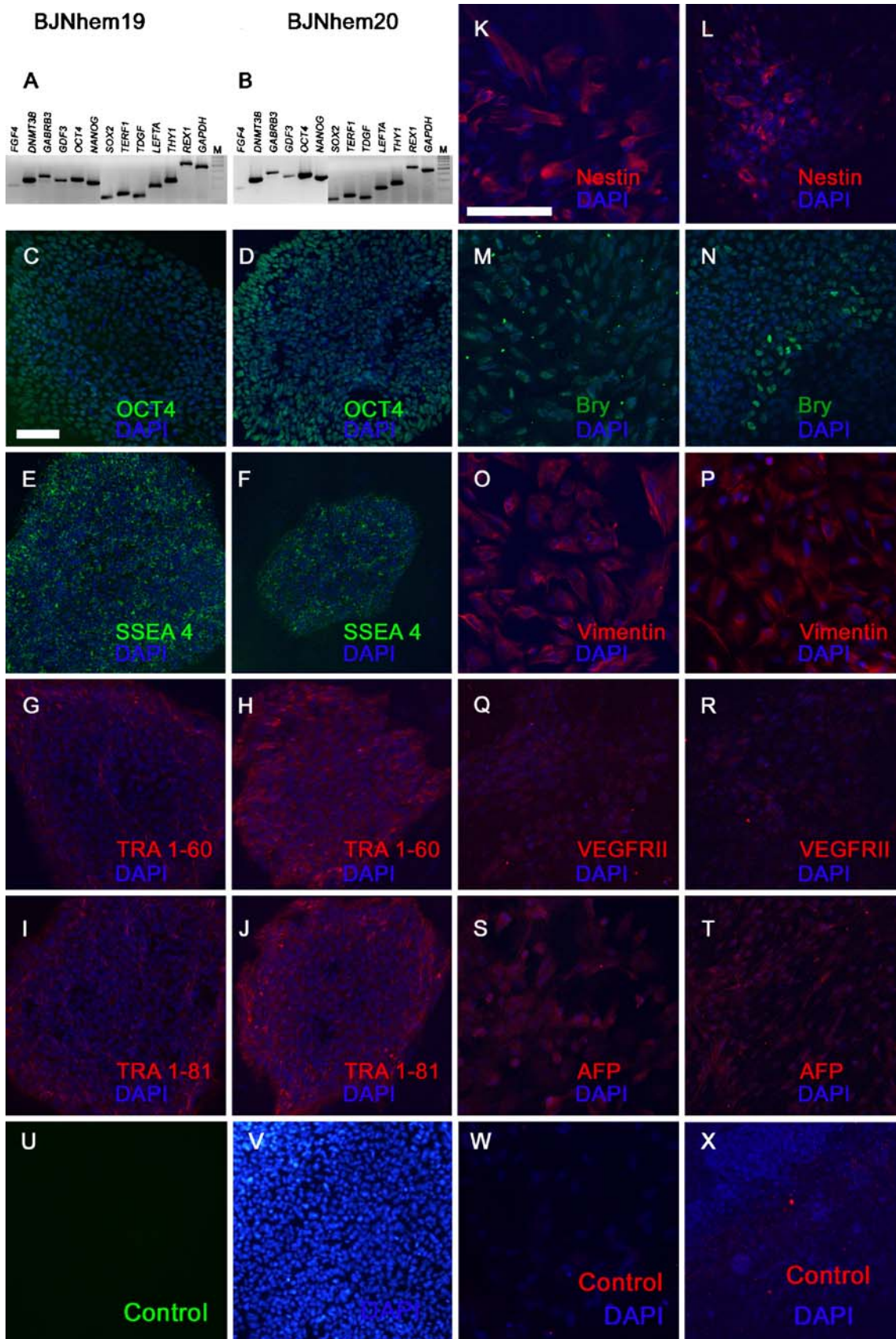
BJNhem19
BJNhem20

A
B

FGF4 DNMT3B GABRB3 GDF3 OCT4 NANOG SOX2 TERF1 TDGF LEFTA THY1 REX1 GAPDH M

K Nestin DAPI
L Nestin DAPI

C OCT4 DAPI
D OCT4 DAPI
M Bry DAPI
N Bry DAPI

E SSEA 4 DAPI
F SSEA 4 DAPI
O Vimentin DAPI
P Vimentin DAPI

G TRA 1-60 DAPI
H TRA 1-60 DAPI
Q VEGFRII DAPI
R VEGFRII DAPI

I TRA 1-81 DAPI
J TRA 1-81 DAPI
S AFP DAPI
T AFP DAPI

U Control
V DAPI
W Control DAPI
X Control DAPI

◀ **Figure. 2** Analysis of pluripotency and differentiation marker gene expression in BJNhem19 at p143 and BJNhem20 at p161. *A–B*, Reverse transcription-polymerase chain reaction (RT-PCR) analysis of undifferentiated BJNhem19 (*A*) and BJNhem20 (*B*) cells. PCR products were obtained using primers specific for *FGF4*, *DNMT3B*, *GABRB3*, *GDF3*, *OCT4*, *NANOG*, *SOX2*, *TERF1*, *TDGF*, *LEFTA*, *THY1*, and *REX1* as indicated. *M* molecular weight marker. *C–T*, Fluorescent immunostaining of undifferentiated (*C–J*) and differentiated EBs (*K–T*) of BJNhem19 (*C*, *E*, *G*, *I*, *K*, *M*, *O*, *Q*, *S*) and BJNhem20 (*D*, *F*, *H*, *J*, *L*, *N*, *P*, *R*, *T*) cells stained with antibodies recognizing various markers as indicated. *C*, *D* OCT4 (*green*), (*E*, *F*) SSEA4 (*green*), (*G*, *H*) Tra-1-60 (*red*), (*I*, *J*) Tra-1-81 (*red*), (*K*, *L*) ectodermal (nestin), (*M–R*) mesodermal (Brachyury, vimentin, and VEGFRII), and (*S*, *T*) endodermal (alpha-fetoprotein). In (*C–X*), nuclei are *blue* (DAPI). *U–X*, No primary antibody controls showing (*U*) green anti-mouse secondary and (*V*) corresponding DAPI image and (*W*) red anti-mouse secondary and (*P*) red anti-rat secondary. Magnification is same for (*C–J*, *U*, *V*) and for (*K–T*, *W*, *X*). *Scale bar*: 100 μm.

any adverse effect. Cultures grown on Matrigel were stained for pluripotency marker gene expression (Fig. 1*E–P*). BJNhem19 and BJNhem20 showed maintenance of pluripotent characteristics on Matrigel in defined media.

Both BJNhem19 and BJNhem20 have now been in continuous culture for 2 yr, and the stocks have been passaged mechanically. BJNhem19 is at passage 212 and BJNhem20 at passage 230, both showing normal diploid karyotype (Fig 1*Q*, *R*). A difference of about 15–20 passages has been maintained consistently between the two cell lines since around passage 15, as BJNhem19 was initially slow growing. However, BJNhem19 cultures showed marked improvement in colony morphology and growth characteristics from about passage 80, suggesting genetic or epigenetic adaptation to culture. The two cell lines have been analyzed at multiple passages for karyotyping and for expression of pluripotency markers, and data for the latest analysis are shown in Fig. 1*Q*, *R* and Fig. 2. RT-PCR analysis shows expression of *DNMT3B*, *GABRB3*,

GDF3, *OCT4*, *NANOG*, *SOX2*, *TERF1*, *TDGF*, *LEFTA*, *THY1*, *REX1*, and *FGF4* (Fig. 2*A*, *B*). Interestingly *FGF4* expression was not detected at early passages (Inamdar et al. 2009) for both lines suggesting that this late expression could be a result of adaptation to culture. Both BJNhem19 and BJNhem20 express OCT4, SSEA4, TRA1-60, and TRA1-81 uniformly in all the colonies (Fig. 2*C–J*).

BJNhem19 and BJNhem20 cells are capable of differentiating into derivatives of all three germ layers in vitro even after long-term culture (Fig. 2*K–T*). Cultures were stained at various days of differentiation as described before (Inamdar et al. 2009) and showed cells expressing the neuronal marker nestin (Fig. 2*K*, *L*), mesodermal markers Brachyury (Fig. 2*M*, *N*) and vimentin (Fig. 2*O*, *P*), endothelial marker VEGFRII (Fig. 2*Q*, *R*), and endodermal marker alpha-fetoprotein (Fig. 2*S*,*T*). However, no beating cardiomyocytes or teratomas were obtained from early or late passage BJNhem19 cells indicating that adaptation had not changed the differentiation capacity. BJNhem20 cells at late passages could be differentiated to cardiomyocytes spontaneously as well as with DMSO induction with a higher efficiency than for early passages. While only 5% of early passage (p) EBs showed spontaneous appearance of beating cardiomyocytes, later passages had 45.5% (p101), 58.3% (p115), and 62.5% (p135) beating EBs. We also tested whether the BJNhem20-derived cardiomyocytes proliferated in culture. Beating areas were observed by day 6 or 7 of differentiation as opposed to day 12 seen at earlier passages. The early differentiation was first observed at p91. Small groups of cells as well as isolated cells showed beating within 24 h after dissociation by trypsin. Cultures were monitored for 8 d for beating and proliferation by imaging in phase contrast (Fig. 3*A–D*, *H–K*) as well as counting DAPI-stained nuclei (not shown). Analysis of two representative cultures is shown in Fig. 3. Cultures
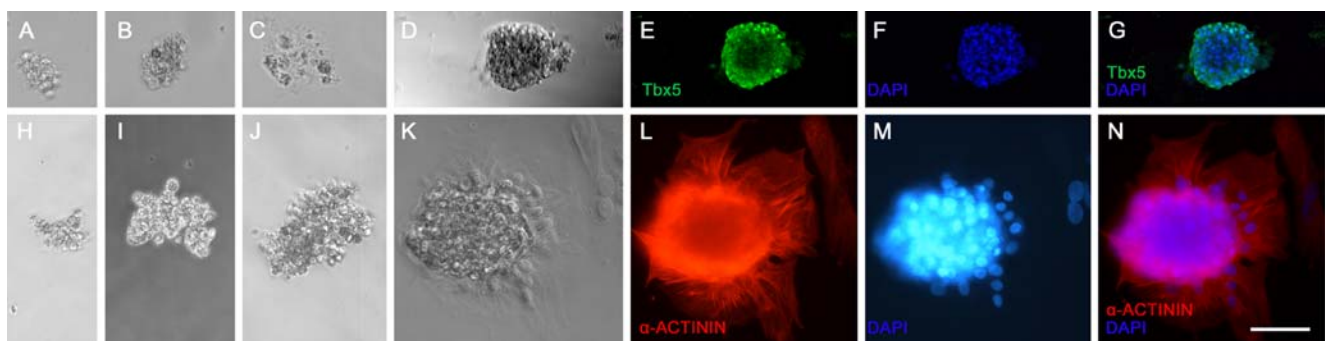

Figure. 3 Analysis of contracting cells obtained from BJNhem20 human embryonic stem cells. Beating clumps from trypsinized EBs were plated on gelatin and analyzed for proliferation and marker expression. Two representative cell clusters are shown here. *A–G* cells from EB1 imaged in (*A–D*) phase contrast at day 2 (*A*), day 4 (*B*), day 6 (*C*), and day 8 (*D*) and (*E–G*) immunofluorescence to show Tbx5 expression (*green*) at day 8. *H–N* cells from EB2 imaged in (*H–K*) phase contrast at day 2 (*H*), day 4 (*I*), day 6 (*J*), and day 8 (*K*) and (*L–N*) immunofluorescence to show α-actinin expression (*red*) at day 8. In (*F*, *G*, *M*, *N*), nuclei are in *blue* (DAPI). *G*, *N* show merged images of marker and nuclear staining. Magnification is the same for all images. *Scale bar*: 50 μm.

were also stained for the cardiac progenitor marker Tbx5 (Fig. 3*E*–*G*) and the cardiomyocyte marker α-actinin (Fig. 3*L*–*N*). While only a subset of cultured cells expressed Tbx5, all cells analyzed expressed α-actinin. These data show that hES cell-derived cardiac cells can be isolated and expanded in culture. Cultured cells showed increase in size and number (Fig.3*A*–*D*, *H*–*K*).

In conclusion, our data support and add to previous reports (Rosler et al. 2004; Suemori et al. 2006) indicating that hES cells are indeed immortal and stable over long-term culture. Also, hES cell lines derived from defective embryos can be cultured long-term and can maintain their diploid karyotype and pluripotent characteristics. Further, this is the first report of long-term culture of genetically related cell lines representing the Indian ethnic background. Thus, the cell lines BJNhem19 and BJNhem20 can be used reliably for studies on hES cell pluripotency and differentiation as well as for various drug screens that require testing on different genetic backgrounds. BJNhem20 is especially suitable for studies on cardiomyocyte differentiation. The two cell lines have also been deposited in the UK Stem Cell Bank (http://www.ukstemcellbank.org.uk/accessioned.html; UKSCB accession nos. R-08-021 and R-08-022) and are listed on the European hESC Registry (http://www.hescreg.eu/). Early passage stocks of the cells are also available for distribution.

Author disclosure statement The authors declare no competing financial interests.

References

Adewumi O.; Aflatoonian B.; Ahrlund-Richter L.; Amit M.; Andrews P. W.; Beighton G.; Bello P. A.; Benvenisty N.; Berry L. S.; Bevan S.; Blum B.; Brooking J.; Chen K. G.; Choo A. B.; Churchill G. A.; Corbel M.; Damjanov I.; Draper J. S.; Dvorak P.; Emanuelsson K.; Fleck R. A.; Ford A.; Gertow K.; Gertsenstein M.; Gokhale P. J.; Hamilton R. S.; Hampl A.; Healy L. E.; Hovatta O.; Hyllner J.; Imreh M. P.; Itskovitz-Eldor J.; Jackson J.; Johnson J. L.; Jones M.; Kee K.; King B. L.; Knowles B. B.; Lako M.; Lebrin F.; Mallon B. S.; Manning D.; Mayshar Y.; McKay R. D.; Michalska A. E.; Mikkola M.; Mileikovsky M.; Minger S. L.; Moore H. D.; Mummery C. L.; Nagy A.; Nakatsuji N.; O'Brien C. M.; Oh S. K.; Olsson C.; Otonkoski T.; Park K. Y.; Passier R.; Patel H.; Patel M.; Pedersen R.; Pera M. F.; Piekarczyk M. S.; Pera R. A.; Reubinoff B. E.; Robins A. J.; Rossant J.; Rugg-Gunn P.; Schulz T. C.; Semb H.; Sherrer E. S.; Siemen H.; Stacey G. N.; Stojkovic M.; Suemori H.; Szatkiewicz J.; Turetsky T.; Tuuri T.; van den Brink S.; Vintersten K.; Vuoristo S.; Ward D.; Weaver T. A.; Young L. A.; Zhang W. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat. Biotechnol.* 25: 803–816; 2007.

Enver T.; Soneji S.; Joshi C.; Brown J.; Iborra F.; Orntoft T.; Thykjaer T.; Maltby E.; Smith K.; Dawud R. A.; Jones M.; Matin M.; Gokhale P.; Draper J.; Andrews P. W. Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. *Hum. Mol. Genet.* 14: 3129–3140; 2005.

Inamdar M. S.; Venu P.; Srinivas M. S.; Rao K.; Vijayraghavan K. Derivation and characterization of two sibling human embryonic stem cell lines from discarded grade III embryos. *Stem. Cells Dev.* 18: 423–433; 2009.

Rosler E. S.; Fisk G. J.; Ares X.; Irving J.; Miura T.; Rao M. S.; Carpenter M. K. Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev. Dyn.* 229: 259–274; 2004.

Siva K.; Venu P.; Mahadevan A.; Shankar K. S.; Inamdar M. S. Human BCAS3 Expression in Embryonic Stem Cells and Vascular Precursors Suggests a Role in Human Embryogenesis and Tumor Angiogenesis. *PLoS ONE* 2(11): e1202; 2007.

Suemori H.; Yasuchika K.; Hasegawa K.; Fujioka T.; Tsuneyoshi N.; Nakatsuji N. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochem. Biophys. Res. Commun.* 345: 926–932; 2006.

Thomson J. A.; Itskovitz-Eldor J.; Shapiro S. S.; Waknitz M. A.; Swiergiel J. J.; Marshall V. S.; Jones J. M. Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145–1147; 1998.