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In Vitro Culture Conditions Favoring Selection of Chromosomal Abnormalities in Human ES Cells

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Abstract Previous studies in several laboratories have demonstrated inadvertent chromosomal abnormalities in long-term cultured human embryonic stem cells (HESC). Here, using a two-step selection process we report a functional adaptation of a HESC line, HS181, towards a decreased dependence of extra cellular matrix (ECM) for in vitro survival, that is for growth directly onto a plastic surface. Successful adaptation was paralleled with a karyotype change in 100% of the cells to 47,XX,del(7)(q11.2),+i(12)(p10). The resulting adapted population showed increased survival and growth on plastic and also maintained expression of HESC markers, but showed a decreased pluripotency, as demonstrated by results from embryoid body (EB) formation in vitro. The finding of reduced pluripotency may not be totally unexpected since the variant cells were selected for self-renewal and proliferation, not differentiation during the adaptation to growth on plastic. In the light of recent models of a germ cell origin of HESC it is of particular interest that similar to many of the reported spontaneous HESC mutants, one of the identified specific chromosome abnormalities, i(12p), has also been strongly implicated for human germ cell cancer. However, the mutated HESC variant carrying this mutation failed to grow as a xeno-graft in a mouse model in vivo. This is surprising and needs a further mechanistic analysis for its explanation. Increased knowledge of genetic integrity of HESC may have significance on the understanding of mechanisms for tumor progression and thus strategy for treatments, particularly for tumors occurring in early life. J. Cell. Biochem. 99: 508–516, 2006.

Key words: human embryonic stem cells; culture conditions; extra cellular matrix; chromosomal abnormalities; tumor progression

Since the first descriptions of cultures from human blastocysts [Fishel et al., 1984; Bongso et al., 1994; see also Edwards, 2001, 2002] and the first successful in vitro culture of human embryonic stem cells—HESC [Thomson et al., 1998], the use of in vitro expansion of pluripotent cells has been recognized as both a potential source for cell transplantations and a possibility to provide a unique resource for studies of early human development, not otherwise accessible for analysis [Pedersen, 1999]. Though optimal in vitro culture conditions of HESC are yet to be fully developed, it is today well established that HESC cultured under stringent conditions can exhibit a surprisingly stable chromosomal constitution also during extended in vitro culture, contrary to the experience from most all other cell culture systems [Brimble et al., 2004; Carpenter et al., 2004; Rosler et al., 2004]. However, it is also well established from several laboratories, including our own, that inadvertent genetic HESC

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variants can be found, including changes in several separate chromosomes, for example, 1, 6, 8, 5, 12, 13, 17, 18, 20 or X [Buzzard et al., 2004; Draper et al., 2004; Inzunza et al., 2004; Pera, 2004; Hoffman and Carpenter, 2005; Mitalipova et al., 2005; Plaia et al., 2005]. Apparently, any adaptation to in vitro growth would depending on the applied conditions potentially fix a variety of both genetic and epigenetic changes. Long-term culture per se would also seem to enhance appearance of mutations [Maitra et al., 2005] and the possibility of mutations is thus a concern particularly during bulk expansion of HESC. Conversely, the occurrence of growth adapted mutants offer the advantage of HESC being particularly well suited also as potential model for tumor progression, extending the preceding models of human EC cell lines.

HESC are dependent on support of extra cellular matrix (ECM) and, in general, exhibit a very low capacity to attach and survive directly on a plastic surface. The HS181 line was originally derived and adapted for undifferentiated growth on support from human foreskin fibroblasts. In the present report a high passage culture from the HESC line HS181 was adapted for survival and proliferation without physical support of feeder cells. The growth behavior, marker expression, and pluripotency of the adapted variant cells, referred to as $HS181^{7q-,i(12p)}$, were investigated in the present report.

MATERIALS AND METHODS

Cells

The HESC line HS181 [Hovatta et al., 2003] was cultured at indicated passage numbers as described previously [Imreh et al., 2004]. In brief; cells were kept at 37°C, 6.8% CO_2, high humidity, in HESC medium (=80% KO DMEM + 20% SR + 2 mM L-glutamine + 1% non-essential amino acids + 0.1 mM β-mercapoethanol + 4 ng/ml bFGF). For feeder cells, human foreskin fibroblasts (hFS) (CRL-2429) were cultured in Iscove's Medium + 10% FCS, irradiated with 35 Gy and seeded at a concentration of 2.1×10^4 cells/cm^2. The HS181 cells and the variant cells were passaged using Dispase enzyme (10 mg/ml) and mild mechanical splitting.

Extra Cellular Matrix (ECM)

A 'post-feeder ECM' was derived from irradiated hFS feeders cultured on plastic dishes (non-coated). Confluent hFS cell cultures were lysed using hypotonic shock in distilled water and the culture dishes were carefully rinsed with HESC medium in order to retain the underlying ECM.

Cytogenetic Studies

Q-banding. Samples of cells were treated with colcemid KaryoMAX, 0.07 μg/ml (Gibco, Paisley, Scotland) overnight. After washing, the cells were incubated in 0.4% trypsin solution (Gibco) for 2–3 min. Cells were treated with 1,400 IU/ml collagenase (Worthington, Lakewood, NJ) at 37°C for 20 min, and harvested using standard procedures. The metaphases were analyzed after Q-banding.

Fluorescent in situ hybridization (FISH). The commercially available probe Vysis TM TEL/AML1 ES was used to verify the copy number of 12p. The probe mixture was treated according to the manufacturer's instructions, added to the denatured chromosome preparations and covered by a 22 × 22 mm cover slip. Hybridization was performed in a moist chamber at 37°C overnight. The chromosome 12 centromere-specific probe D12Z1 was directly labeled with FluoroRed-dUTP (Amersham Buckinghamshire, England) using nick translation. The repetitive probe was hybridized in 60% formamide, 2× SSC, 50 mM phosphate buffer pH 7.0 at a probe concentration of 1–2 ng/μl. The probe mixture was added to the chromosome slide and was then denatured simultaneously with the slide at 75°C for 2 min. Hybridization was performed as above. After hybridization, the slides were washed for 5 min in 2× SSC at 72°C, dehydrated through an alcohol series and air-dried. The slides were mounted in glycerol containing 2.3% DABCO (1,4-diazabicyclo-(2,2,2) octane) as antifade, and DAPI (4,6-diamino-2-phenyl-indole) at 0.5 μg/ml as counterstain. The signals were visualized using a Zeiss Axiophot fluorescence microscope equipped with cooled CCD-camera (Sensys; Photometrics Ltd, Tuscon, AZ and processed with the SmartCapture software (Vysis).

Spectral karyotyping (SKY). Slides were freshly prepared and treated as described previously [Nordgren et al., 2002]. The multi-chromosome paint probe was applied according to the manufacturer's instructions (Applied Spectral Imaging, ASI, Migdal Ha'Emek, Israel), denatured in 75°C for 7 min and preannealed at 37°C for 1 h. Ten microliter of the denatured SKYTM mixture was added to

the denatured chromosome slides, covered by a 22×22 mm cover slip, and sealed with rubber cement. The slides were then incubated for 48 h at 37°C in a humidified chamber. The slides were washed 3×5 min in 50% formamide/2× SSC pH 7 at 45°C and 2×5 min in 1× SSC at 45°C. Detection was carried out according to the ASI protocol, after which the slides were mounted and counterstained with DAPI/ antifade solution. For each case, between 9 and 14 metaphase cells were captured with an SD200 Spectra cube system (ASI) connected to a Zeiss Axioskop fluorescence microscope with a triple bandpass optical filter and analyzed using the SKY view software (ASI).

Markers

Immunocytochemistry. Immunocytochemistry was performed as described previously [Gertow et al., 2004; Imreh et al., 2004] Briefly, cells were fixed in 4% formaldehyde and immunostained with primary antibodies specific for TRA-1-60, TRA-1-81, SSEA-4, and SSEA-1 (Chemicon, Temecula, CA), Oct-4 and CD9 (Santa Cruz Biotech, Inc., Santa Cruz, CA), and Vimentin (Sigma-Aldrich, St. Louis, MO). After blocking 40 min at room temperature in 3% BSA (fraction V, Sigma-Aldrich) + 0.1% BSA-c (Aurion, 900.022) incubation with primary antibody was performed (1 h at room temperature) followed by secondary fluorescent labeled antibody, AlexaFluor 488 (Molecular Probes, Inc., Eugene, OR) for 40 min at room temperature. The wells were mounted with Vectashield containing DAPI (Vector labs, Inc., Burlingame, CA).

The TERA-2 cell line and human foreskin fibroblasts were used as controls and non-immune serum as isotype control.

RT-PCR. RT-PCR was performed by extraction of total RNA using Micro-to-Midi Total RNA Purification System (Invitrogen Corporation). The RNA was DNase-treated (Invitrogen Corporation) to avoid DNA contamination in the following RT-PCR. After precipitation RNA was resolved to a final concentration of 0.1 μg/μl. Complementary DNA was synthesized using Superscript III First Strand Synthesis System (Invitrogen Corporation) according to the recommended protocol. Platinum Taq-polymerase was used in the following PCR reaction containing cDNA synthesized from 20 ng total RNA. Primers used are indicated in Table I.

FACS. Flow Cytometry using the above described protocol for immunocytochemistry using with the SSEA-4, TRA-1-60 antibodies and a secondary FITC labeled F(ab′)2 antibody (Caltag Laboratories). The analysis was performed on a FACSCalibur using CellQuestPro as software.

Xeno-Graft Model in Immunodeficient Mice

This was performed as described previously [Gertow et al., 2004]. Briefly, 10^4 HESC were implanted beneath the testicular capsule of a young (6 weeks) SCID/Beige male mice (C.B.-17/GbmsTac-scid-bgDF N7, M&B, Denmark) anesthetized using Isoflurane gas. Teratoma growth was determined by palpation and the mice were sacrificed (cervical dislocation) 5 or 10 weeks after implantation.

RESULTS

Chromosome Stability of HS181 Cells in Culture

The HESC line 'HS181' has previously been repeatedly reported to have the karyotype 46,XX; at passage 22 by Q-banding [Hovatta

TABLE I. Primer Sequences and PCR Conditions for Expression Analysis of the Listed Genes

Gene	Primer sequences	Annealing temperature (°C)	$[MgCl_2]$ mM
Oct-4	F:5′-AGGATCACCCTGGGATATACACA-3′; R:5′-AAGCTAAGCTGCAGAGCCTCA-3′	55	1.5
Nanog	F:5′-CGGCTTCCTCCTCTTCCTCTATAC-3′; R:5′-ATCGATTTCACTCATCTTCACACGTC-3′	60	1.5
hTERT	F:5′-CGGAAGAGTGTCTGGAGCAA-3′; R:5′-GGATGAAGCGGAGTCTGGA-3′	51	1.0
CD90	F:5′-ATGAACCTGGCCATCAGCATCGC-3′; R:5′-TCACAGGGACATGAAATCCGTGG-3′	55	2.0
CD31	F:5′-CAACGAGAAAATGTCAGA-3′; R:5′-GGAGCCTTCCGTTCTAGAGT-3′	49	4.0
CD34	F:5′-TGAAGCCTAGCCTGTCACCT-3′; R:5′-CGCACAGCTGGAGGTCTTAT-3′	51	4.0
CD45	F:5′-GGAATTCCAAAGCCCAACACCTTCC-3′; R:5′-GCGGATCCACTTGTGTACAATCATGTAA-3′	59	3.0
Brachyury	F:5′-GTGACCAAGAACGGCAGGAGG-3′; R:5′-TGTTCCGATAGCATAGGGGC-3′	52	1.0
VE-cadherin	F:5′-ACGGGATGACCAAGTACAGC-3′; R:5′-ACACACTTTGGGCTGGTAGG-3′	51	1.0
AFP	F:5′-CCATGTACATGAGCACTGTTG-3′; R:5′-CTCCAATAACTCCTGGTATCC-3′	52	4.0
Flt-1	F:5′-ATCAGAGATCAGGAAGCACC-3′; R:5′-GGAACTTCATCTGGGTCCAT-3′	51	3.0
Nestin	F:5′-CAGCTGGCGCACCTCAAGATG-3′; R:5′-AGGGAAGTTGGGCTCAGGACTGG-3′	55	2.0
GAPDH	F:5′-GCTCAGACACCATGGGGAAGGT-3′; R:5′-GTGGTGCAGGAGGCATTGCTGA-3′	54	3.0

et al., 2003] and at passage 39 by CGH [Inzunza et al., 2004]. This was confirmed in the current study at passage 45 and 54 using G-banding (data not shown). However, following two separate bulk expansions up to $>10^7$ cells, a selective amplification of the whole chromosome 12 was revealed (expansion at passage 33 gave a mean of 30% trisomy using FISH analysis, and the second separate expansion at passage 72 resulted in a mean of 68% trisomy using Q-banding, respectively). In an attempt to analyze the frequency of a potential contamination of trisomy 12 in HS181 cultures kept in undifferentiated state using our described culture conditions (19), we performed a targeted analysis of chromosome 12. When 1,400 single cells (passage 57) were analyzed using FISH, no triploid signal could be detected (0/1,400 cells; data not shown).

Culture Conditions

Undifferentiated HS181 cells seeded on hFS cells attach and proliferate with typical HESC morphology (Fig. 1a). Seeding directly to plastic dishes without feeder cell support yield few or no attaching colonies (Fig. 1b). After addition of hESC media conditioned by irradiated hFS cultures, attachment was improved, but in three independent experiments, we did not see continued proliferation of HS181 cells under

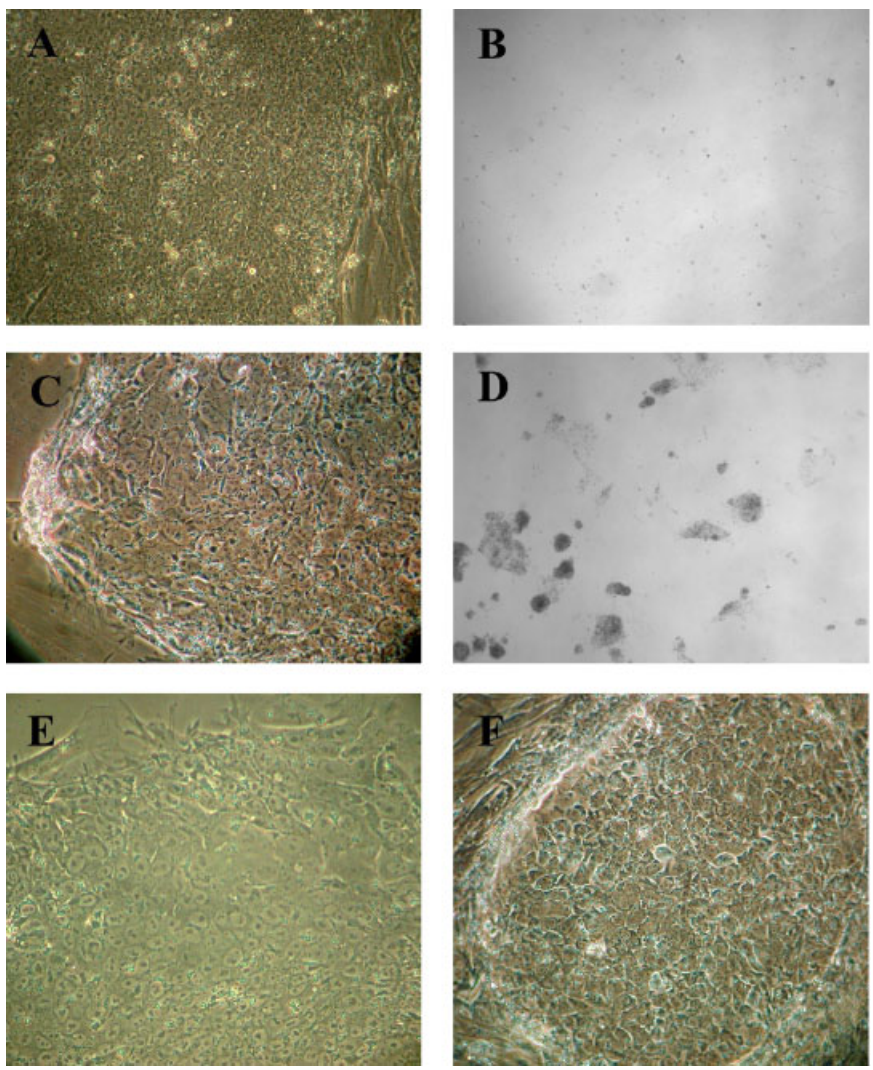

Fig. 1. Morphology of in vitro cultures. HS181 wild-type cells grown on feeder layer (**A**) (10×) and attachment when seeded on plastic (**B**) (5×). HS181$^{7q-,i(12p)}$ adapted to growth on ECM (**C**) (20×) followed by culture on plastic (**D**, **E**) (5× and 20×). HS181$^{7q-,i(12p)}$ cells replated on feeder cells (**F**) (20×).

these conditions. The same result was found with gelatin coated plastic culture dishes (data not shown).

As an alternative approach, we adapted HS181 cells for growth on a 'post-feeder ECM' similar to previously described for mouse ESC [Klimanskaya et al., 2005]. HS181p75 cells were seeded on post-feeder ECM using conditioned media. Although a 50% mixture of conditioned medium and non-conditioned medium supported some attachment and growth of HS181p75 cells, optimal attachment was obtained only by using 100% conditioned medium, suggesting that components in the conditioned medium were required for attachment. HS181 cells grown on post-feeder ECM showed growth characteristics and morphology (Fig. 1c) very similar to HS181 grown on hFS feeder cells (Fig. 1a).

After six passages on post-feeder matrix some of the HS181 cells were transferred to untreated plastic culture dishes (Falcon; 353046). Again, conditioned medium was found to be necessary for the attachment. Using 100% conditioned medium resulted in attachment and continued growth of a small (<1%) number of cells. Such proliferating variant cells have, so far, been successfully kept for up to 28 passages (using conditioned HESC medium) with stable growth characteristics and morphology (Fig. 1d,e). The HS181$^{7q-,i(12p)}$ cells grown on plastic showed a heterogeneous cell morphology. However, the growth rate (doubling time) of HS181$^{7q-,i(12p)}$ cells on plastic was reduced by half, compared to the HS181 cells when cultured at our regular conditions using feeder cell support [Imreh et al., 2004]. Interestingly, the morphology and also the doubling time of HS181$^{7q-,i(12p)}$ cells could be reversed back to the original HS181 state, after only one passage on hFS (Fig. 1f).

The cells could be cryopreserved in repeated freeze thaw cycles (DMSO, 'slow freezing') with maintained phenotype after thawing, though attachment immediately after thawing was not accomplished unless plated on feeder cells. After this initial thawing step, the variant could be transferred to growth on plastics with similar efficacy as before freezing.

Karyotyping of the Adapted Cells

After 11 passages on post-feeder ECM (with conditioned medium) HS181 cells showed a changed karyotype. When 26 metaphases were analyzed using Q-banding, 5/26 cells had a normal female chromosome constitution; 46,XX and 21/26 metaphases showed a deletion of 7q plus an isochromosome for the short arm of chromosome 12; 47,XX,del(7)(q11.2),+i(12)(p10) (Fig. 2a).

This karyotype was further endorsed by the culture directly on plastic surface. After four passages on plastic the 47,XX,del(7)(q11.2), +i(12)(p10) genotype was present in 25 of 25 metaphases.

The presence of the 7q deletion and the isochromosome 12p were both positively confirmed using SKY analysis (Fig. 2b) and FISH (data not shown). No other gross chromosomal abnormalities were detected using Q banding (Fig. 2a).

Markers

The HS181$^{7q-,i(12p)}$ cells grown on plastic were found to exhibit the following marker profile by using IHC; Oct4+, SSEA-4+, Tra 1-60+, Tra 1-81+, SSEA1+,Vimentin+, CD9+ (Table II), and by using RT-PCR; Nanog+, hTERT+, CD90+, CD31−, CD34+, Brachyury+, AFP−, VE-cadherin+, Flt-1+, Nestin+ (Table II). Thus, in summary the HS181$^{7q-,i(12p)}$ population showed markers associated with undifferentiated pluripotent cells (i.e., Oct4, Nanog, SSEA-4, Tra 1-60, Tra 1-81, hTERT), as well as a marker profile indicating differentiation towards all three germ layers.

Flow cytometry analysis on mutant cells grown on feeder cells revealed 89% SSEA-4 and 88% TRA-1-60 positive cells, while the same analysis on cells grown on plastic showed 81% SSEA-4 and 80% TRA-1-60 positive cells. These findings suggest a low frequency of spontaneous differentiation on feeder cells, being slightly higher on plastic.

Pluripotency

Pluripotency was tested in vitro by formation of EBs. While EBs from the original HS181 cells exhibited expression of all tested markers representing all three germ layers, the HS181$^{7q-,i(12p)}$ population showed no detectable expression of Brachyury, VE-Cadherin or AFP (Table II).

In vivo growth was tested by xeno-grafting. HS181$^{7q-,i(12p)}$ and HS181 cells were injected under the testes capsule in immunodeficient mice as described previously [Gertow et al., 2004]. While HS181 gave 100% teratoma

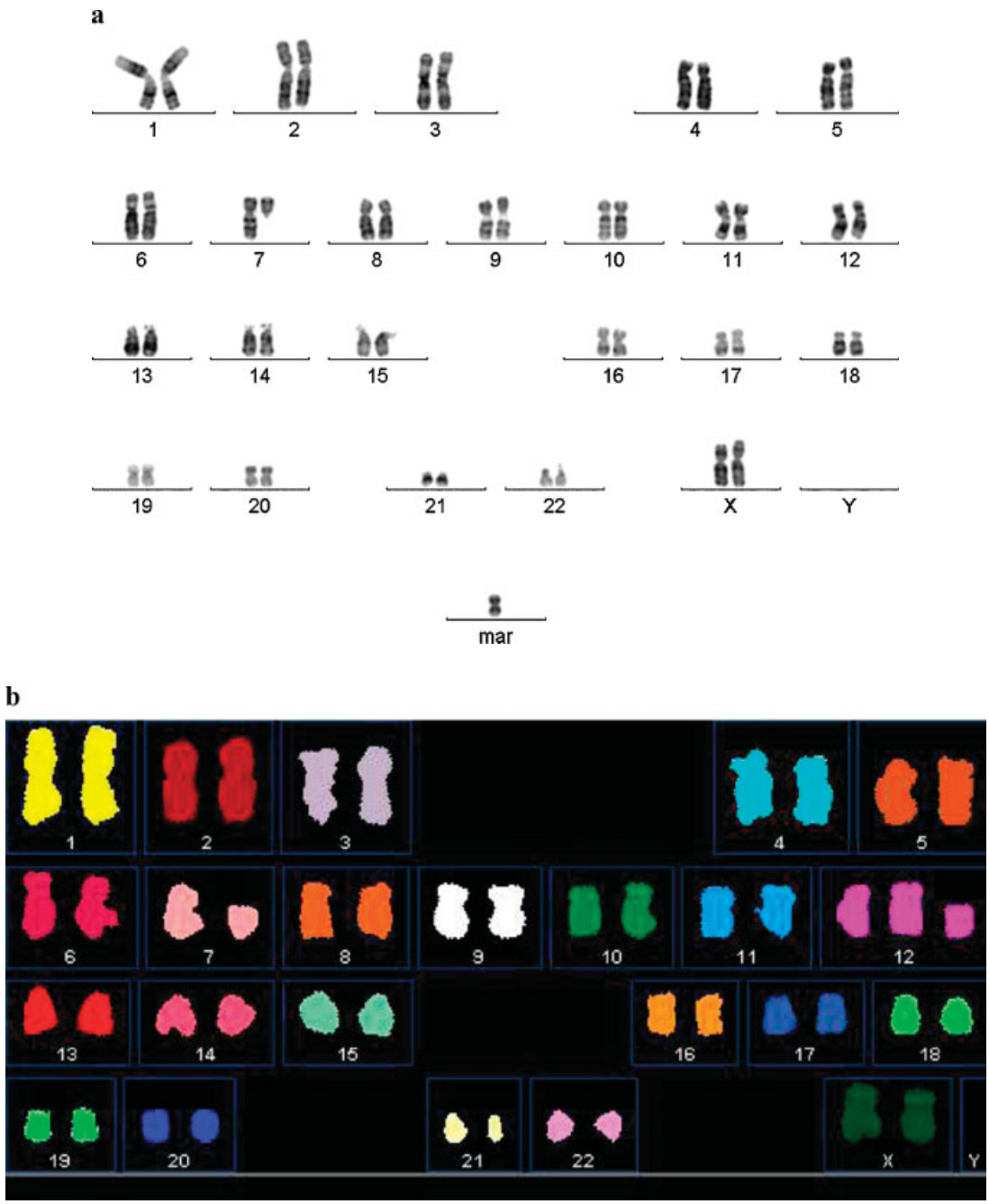

Fig. 2. Karyotyp of HS181$^{7q-,i(12p)}$ cells on post-feeder-ECM: (**A**) Q-banding showing a 47,XX,del(7)(q11.2),+i(12)(p10) karyotype. **B**: SKY analysis confirming the i12p and 7q deletion.

growth, the HS181$^{7q-,i(12p)}$ yielded no in vivo growth (0/12 mice) in three separate experiments.

DISCUSSION

In this study, we found that an adaptation towards independence of ECM for the survival and growth of HESC was paralleled by specific changes on chromosomes 7 and 12. The altered phenotype was preserved after freeze thawing and after shifts of growth conditions, that is cycles of alternate growth on feeder cells and plastic. There was altered cell morphology when grown on plastic (Fig. 2), similar to cells grown on ECM or Matrigel. However, the morphology was fully reversible when the cells were returned back to feeder cell support.

TABLE II. Marker Analysis

Marker	HS181[a]	HS181 7q-,i(12p)[b]	HS181 7q-,i(12p)[c]	HS181 EB[d]	HS181 7q-,i (12p) EB[e]
Using IHC					
Oct-4	+	+	+	Nt	Nt
Tra 1-60	+	+	+	Nt	Nt
Tra 1-81	+	+	+	Nt	Nt
SSEA-4	+	+	+	Nt	Nt
SSEA1	−	+	−	Nt	Nt
CD9	+	+	+	Nt	Nt
Vimentin	−	+	+	Nt	Nt
Using RT-PCR					
Oct-4	+	+	+	+	+
hTERT	+	+	+	+	+
Nanog	+	+	+	+	+
CD90	+	+	+	+	+
CD31	−	−	−	+	+
CD34	−	+	+	+	+
Brachyury	−	+	+	+	−
VE-cadherin	−	+	−	+	−
AFP	−	−	−	+	−
Flt-1	−	+	+	+	+
Nestin	+	+	+	+	+
GAPDH	+	+	+	+	+

Tested with immunohistochemistry (IHC) or RT-PCR.
Nt, not tested.
[a]HS181p48.
[b]HS181$^{7q-,i(12p)}$ p91 cultured on plastic surface.
[c]HS181$^{7q-,i(12p)}$ p91 cultured on feeder cells.
[d]EB from HS181 p49.
[e]EB from HS181$^{7q-,i(12p)}$ p92.

The HS181$^{7q-,i(12p)}$ variant cells maintained expression of HESC markers when grown on plastic. The RNA marker profile of the mutant culture, indicated presence of cells differentiated towards all three germ layers (Table II), and FACS analysis indicated the proportion of differentiated cells to be 10%–20%, which is slightly higher than the parental HS181 cells (data not shown). A possible de-differentiation could be observed after returning the variant cells to feeder cell support, in that the HS181$^{7q-,i(12p)}$ cells lost the expression of VE-cadherin (RT-PCR) and SSEA-1 (IHC).

It has been suggested that when cells of the inner cell mass of the blastocyst adapt to in vitro growth during the HESC derivation process [Thomson et al., 1998], they transition to a cell type with the closest in vivo equivalent of an early germ cell [Zwaka and Thomson, 2005]. This concept finds strong support from marker studies performed by Clark et al. (29). In this context, it is of interest that the specific chromosome 12 change observed in the present study, i(12p), has been repeatedly linked to clinical findings in germ cell tumors (GCT) [Looijenga et al., 2003]. Gain of 12p is a consistent finding in invasive GCT of type II (seminomas and non-seminomas) and up to 80% of such tumors have i(12p) (reviewed in [Sandberg et al., 1996; van Echten et al., 1998; Looijenga et al., 2003]). Non-seminomas are the only known pluripotent cancers and include embryonal carcinoma, yolk sac tumors, choriocarcinomas, and teratomas. Carcinoma in situ (CIS) is a precursor of such tumors but lack gain of 12p, leading to a suggested model where gain of 12p is associated with invasiveness (reviewed in [Looijenga et al., 2003]). In this model, loss of dependence of CIS cells from Sertoli cells (i.e., cell growth independent of ECM) is related to resistance of a phenomenon known as anoikis and is crucial for invasiveness of GCT. Sertoli cell independence and suppression of apoptosis is associated with gain of 12p. When escaping anoikis, cells escape their dependence of microenvironment without going into the spontaneous apoptotic suicide of anoikis. This apoptosis occurs in part because of deprivation of necessary integrin and cadherin mediated survival signals (reviewed in [Smalley et al., 2005]. Several candidate genes on 12p have been proposed as responsible [Clark et al., 2004].

Our data suggest that under routine passage of HS181 cells, using our described culture

conditions [Imreh et al., 2004] amplification of chromosome 12 is not detected (frequency of $<1.4 \times 10^{-3}$). However, following bulk expansion of HS181 cells with the aim to achieve larger numbers of cells ($>10^7$ cells) we were able to detect cells with trisomy of chromosome 12, in two separate experiments. These results could indicate the presence of a very low percentage of trisomy latently present but not detected by our analysis at earlier passages. Alternatively, in line with the above findings it is also possible that a more expansive growth favor de novo generation of trisomy 12. It should be noted, however, that the i(12p) mutation detected in the plastic growth adapted variant was not identical to the trisomy 12 detected in cultures aimed for maximal expansion, though it is possible that the isochromosome arises from the trisomy.

The HS181$^{7q-,i(12p)}$ variant showed a reduced pluripotency compared to the original HS181 cells. This may not be totally unexpected since the variant cells during the adaptation to growth on plastic were selected for self-renewal and proliferation, not differentiation. Interestingly, the selective growth advantage of HS181$^{7q-,i(12p)}$ cells when propagated on plastic in vitro did not seem to have an effect when the cells were transplanted in vivo. When the HS181$^{7q-,i(12p)}$ cells injected in Beige/SCID mice at a dose giving 100% take using the original HS181 cells, no growth of the variant was observed. This was of particular interest since chromosome 12 trisomic HS181 cells exhibits pluripotent in vivo growth under identical conditions (Gertow et al. unpublished). The fact that HS181$^{7q-,i(12p)}$ cells in addition to amplified 12p also have a deletion of 7q makes the situation even more intriguing since it has been suggested that a tumor-suppressor gene involved in cell–cell contact and adhesion reside on 7q31 [Zenklusen et al., 2001; Chene et al., 2004].

Deletions of 7q are found in a broad range of neoplasias such as ovarian cancers, prostate cancers and malignant myeloid tumors, and they are suggestive of a TSG on chromosome 7q (reviewed in [Zenklusen et al., 2001]. The loss of heterozygosity (LOH) of a putative TSG on chromosome 7q, may be part of an explanation for the enhanced in vitro growth potential of the HS181 variant, but is not in line with the in vivo findings.

Genes important for proliferation in the canonical Wnt-pathway (Wnt2) [Kopper and Hajdu, 2004] and survival pathways (P13K, MEK2, CAV1, CAV2) [Zenklusen et al., 2001], or potentially important to invasive growth (c-Met) [Gao and Vande Woude, 2005] can all be found on 7q. One could speculate that the findings with HS181$^{7q-,i(12p)}$ in vivo might reflect LOH involving, for example, lower activity of c-Met and its downstream effectors such as PI3K and that this effect overcomes the effect of loss of a putative TSG. Additionally there is a link between i(12p) and the 7q deletion considering cell cycle regulation. CCND2 and the CDK inhibitor, p27Kip1 lies on 12p while a catalytical partner, CDK6, reside on 7q [Bartkova et al., 2003].

This work highlights the possibility to study the importance of specific genetic anomalies in tumor progression, by production of HESC variants. Characterization of variants could reveal important knowledge on the process of tumor transformation and progression.

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