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Cell Lines Derived from Human Parthenogenetic Embryos Can Display Aberrant Centriole Distribution and Altered Expression Levels of Mitotic Spindle Check-point Transcripts

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Abstract Human parthenogenetic embryos have recently been proposed as an alternative, less controversial source of embryonic stem cell (ESC) lines; however many aspects related to the biology of parthenogenetic embryos and parthenogenetic derived cell lines still need to be elucidated. We present here results on human cell lines (HP1 and HP3) derived from blastocysts obtained by oocyte parthenogenetic activation. Cell lines showed typical ESC morphology, expressed Oct-4, Nanog, Sox-2, Rex-1, alkaline phosphatase, SSEA-4, TRA 1-81 and had high telomerase activity. Expression of genes specific for different embryonic germ layers was detected from HP cells differentiated upon embryoid body (EBs) formation. Furthermore, when cultured in appropriate conditions, HP cell lines were able to differentiate into mature cell types of the neural and hematopoietic lineages. However, the injection of undifferentiated HP cells in immunodeficient mice resulted either in poor differentiation or in tumour formation with the morphological characteristics of myofibrosarcomas. Further analysis of HP cells indicated aberrant levels of molecules related to spindle formation as well as the presence of an abnormal number of centrioles and autophagic activity. Our results confirm and extend the notion that human parthenogenetic stem cells can be derived and can differentiate in mature cell types, but

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also highlight the possibility that, alteration of the proliferation mechanisms may occur in these cells, suggesting great caution if a therapeutic use of this kind of stem cells is considered.

Keywords Human · Parthenogenetic cell lines · Centriole · Mitotic check-point transcripts

Introduction

Parthenogenesis is the process by which a single egg can develop without the presence of the male counterpart and is a form of reproduction common to a variety of organisms such as fish, ants, flies, honeybees, amphibians, lizards and snakes, that may routinely reproduce in this manner [1].

Mammals are not spontaneously capable of this form of reproduction, however, mammalian oocytes can successfully be activated *in vitro*, by mimicking the calcium wave induced by sperm at fertilization and stimulated to divide [2]. Mammalian parthenotes, however, are unable to develop to term and arrest their development at different stages after activation, depending on the species [3]. The reason for this arrest is believed to be due to genomic imprinting which causes the repression of certain maternally inherited imprinted genes [4].

Despite these limitations, following the success reported in mouse [5, 6] and non-human primate models [7, 8], human parthenogenetic embryos have recently been proposed as an alternative, less controversial source of embryonic stem cell lines [2, 9–12]. Parthenotes may also represent a possible tool for studies on the mechanisms driving early human embryogenesis and for the pre-clinical test of experimental protocols in human assisted reproduction (i.e. different oocytes cryopreservation procedures, oocyte *in vitro* maturation or polar body genetic screening) [13].

However many aspects related to the biology of parthenogenetic embryos and parthenogenetic derived cell lines still need to be elucidated. The decreased extent of heterozygosis may amplify any negative genetic component potentially present in the genotype [14, 15] and has recently been postulated as a major limitation in parthenogenetic lines derived from primates [7]. The very high incidence of chromosome instability and aberrant chromatid separation in oocytes retrieved from IVF patients, especially when over 34 year old [16–18] also represents a concern, given the fact that these correspond to a large part of the population accessing assisted reproductive therapy and, hence, are a major potential source of oocytes for parthenote derivation.

In an attempt to better elucidate some of these aspects, we present here results on human parthenogenetic cell lines recently derived in our laboratory; we characterize their pluripotency and differentiation plasticity, both *in vitro* and *in vivo*, showing many characteristics common to bi-parental embryonic stem cells; however we report the presence of abnormal centrosomes and altered expression levels of specific mitotic spindle check-point proteins, possibly related to their uniparental origin.

Materials and Methods

Human Oocytes Collection, Activation and Culture

Oocytes were collected at the Infertility Unit of the Department of Obstetrics and Gynecology of the "Ospedale Maggiore Policlinico Mangiagalli e Regina Elena". In Italy no more than three embryos per cycle can be obtained [19, 20] therefore, in our Unit, patients from whom more than three good quality oocytes are retrieved, are routinely offered the opportunity to cryopreserve supernumerary eggs. Patients refusing this possibility were proposed to participate to the present study. Approval for the study was obtained by the local institution review board and all participating women gave informed consent.

Fresh oocytes were obtained following controlled ovarian hyperstimulation and transvaginal follicular aspiration for oocyte retrieval was performed 36 h post-hCG as previously described [13]. The cell lines described in these experiments were obtained from twenty oocytes retrieved from four patients (age range 32–39 years) and activated according to Paffoni et al. [13].

Isolation of ICM, Establishment and Culture of Cell Lines from Human Parthenogenetic Embryos

Inner cell masses (ICM) were microsurgically removed from blastocysts, singly plated and cultured in Dulbecco's modified Eagle's medium, without pyruvate, high glucose formulation (Gibco, Italy) supplemented with 10% Knock-out serum replacer (Gibco, Italy), 5% fetal bovine serum (Gibco, Italy), 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma, Italy), 5 ng/ml human recombinant basic Fibroblast Growth Factor (R&D System, USA) and 1% nonessential amino acid stock (Gibco, Italy). Within 3 days, circular colonies with distinct margins of small, round cells were observed. When a colony enlarged enough to cover half or more of the well surface, cells were mechanically removed using a sterile microloop (Nunc, DK), they were transferred to a 50 μl drop of fresh medium and pipetted to small cell clumps, avoiding to obtain single cell suspension. Cells were then passaged on freshly prepared feeder-layers. Culture medium was changed every day.

Gene Expression in HP Cell Lines

Reverse Transcription-Polimerase Chain Reaction

All chemicals were purchased from Invitrogen (Milan, Italy) unless otherwise indicated.

RNA was extracted using the acid-phenol method according to Chomczynski and Sacchi and included a DNase I (1 U/μl) incubation. RNA was then immediately reverse transcribed, using Superscript-™ II Reverse Transcriptase and following the manufacturer's instruction. RNA from bi-parental embryonic stem cells was used as positive control. Amplifications were carried out in an automated thermal cycler (iCycler, Biorad), using the conditions appropriate for each set of primers. In particular, depending on the different experiments, we screened for the expression of pluripotency related transcripts (Oct-4, Nanog, Sox-2, Rex-1) and differentiation markers (Bone Morphogenetic Protein-4, BMP-4; Neurofilament-H, NF-H; α-amilase). Expression of β-actin was always examined as an internal control of the sample quality. Amplification products were purified in Spin-X centrifuge tube filters (Corning, the Netherlands), sequenced (SEQLAB, Gottingen, Germany) and aligned using Clustal W 1.82 (EMBL-EBI service).

Immunocytochemistry

Markers of stem cells and stem cell differentiation were assessed by immunocytochemistry using the following primary antibodies: Oct-4 (1:50, Chemicon, USA); Nanog (1:20, R&D System, USA); SSEA-4 (1:100, Chemicon, USA); Alcaline phosphatase (1:50, R&D System, USA); TRA-1-81 (1:100, R&D System, USA); Desmin (1:200, Chemicon, USA); Keratin 17 (1:200, Chemicon, USA); Vimentin (1:200, Chemicon, USA); Nestin (1:200, Abcam, UK); Map2 (1:200, Abcam, UK); CNPase (1:200, Abcam, UK); beta-tubulin III (1:250, Chemicon, USA). Staining conditions were as indicated by manufacturers. Incubation with suitable secondary antibodies (Alexafluor, Invitrogen, Italy) was carried out for 30 min and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, Italy). Samples were observed either under a TCS-NT laser confocal microscope (Leica Microsystems, Germany) or a Eclipse E600 microscope (Nikon, Japan).

Telomerase Activity

Telomerase activity measurement was performed in undifferentiated and differentiated HP cells respectively, using the TRAPeze® Telomerase Detection Kit (Chemicon, USA), following the manufacturer's instruction. Reactions were separated on non-denaturing TBE-based 12% polyacrylamide gel electrophoresis and visualized with SYBER Green staining.

Derivation of Embryoid Bodies

To induce the formation of EBs, HP cells were cultured in 30 μl hanging droplets, as previously described [21]. The medium was refreshed every day and after 7–9 days, EBs were detectable. Differentiation of EBs was confirmed by morphological examination and molecular analysis that demonstrated the expression of markers related to mesoderm (BMP-4), ectoderm (NF-H) and endoderm (α-amilase). Human genomic DNA was used as a positive control.

Spontaneous Differentiation of HP Cell Lines

Embryoid bodies were mechanically dissociated and cells were plated directly onto CultureWell Chambered Coverglass 16-well dishes (Molecular Probes, Italy) to encourage adherent culture conditions and spontaneous differentiation as previously described [22]. After 1 week cells were processed for RT-PCR amplification or immunocytochemistry.

Neural Differentiation of HP Cell Lines

EBs were prepared as described above and exposed to 10 μM retinoic acid (Sigma, Italy) and 10 ng/ml Sonic Hedgehog (R&D System, USA). They were kept 48 h in hanging drops culture conditions and then they were dissociated and plated on 0.1% gelatin coated CultureWell Chambered Coverglass 16-well dishes. Differentiation was carried out in Neural Progenitor Cell Basal Medium (Cambrex Bioscience, USA), supplemented with Neural Cell Survival Factor-1 (Cambrex Bioscience, USA) and 25 ng/ml Brain Derived Neurotrophic Factor (R&D System, USA). After a period from 9 days to 21 days of culture, cells were fixed and stained with specific antibodies.

Hematopoietic Differentiation of HP Cell Lines

Single-cell suspension was obtained by passaging EBs through a 21-gauge needle. Cell suspensions were plated in a serum-free medium (CellGro Medium, Cambrex Bioscience, USA) supplemented with 10% FBS (Biochrom, Germany) and with the following human recombinant cytokines: thrombopoietin (TPO, 10 ng/ml), Flt-3 ligand (FL, 50 ng/ml), stem cell factor (SCF, 50 ng/ml), interleukin-(IL)-6 (10 ng/ml), (Peprotech EC Ltd., UK). After 2 weeks cells were harvested and assayed for the evaluation of colony-forming cells (CFCs) in 2 ml of complete methylcellulose medium (H4434; StemCell

Technologies, USA). The medium contained 1% methylcellulose, 30% FBS, 1% Bovine Serum Albumine, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 3 IU/ml erythropoietin, 50 ng/ml SCF, 10 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF), 10 ng/ml Interleukin-3 (IL-3). After further 21 days of culture, colonies were scored and then picked up for morphological or flow cytometric analysis.

Colonies were spotted on Poly-D-lysine coated slides with a cytocentrifuge (Shandon Cytospin 4, Thermo Electron Corporation, USA) for 7 min at 200 rpm. Samples were then fixed and stained with May-Grunwald-Giemsa for evaluating their hematopoietic differentiation.

Flow Cytometry

To perform flow cytometry analysis on colonies, 2×10^5 cells were incubated with the following conjugated mouse-anti human antibodies: CD45 PE (Beckman Coulter, USA), CD34 FITC (Becton Dickinson, USA). Isotype immunoglobulins IgG1 PE (Chemicon, USA), IgG1 FITC (Beckman Coulter, USA) were used as negative controls. For each sample, at least 50.000 events were acquired with Cytomics FC500 (Beckman Coulter, USA) and analyzed using the CXP-analysis software.

"*In Vivo*" Differentiation

Undifferentiated HP cells of each line were harvested and injected in the hind limb of 3 Fox Chase SCID (C.B-17/IcrCrl-scid-BR) and 3 SCID Beige (C.B-17/IcrCrl-scid-bgBR) mice (Charles Rivers Laboratories, Italy). Each animal received 3.5×10^6 cells. The same amount of feeder cells were injected as a negative control. Tumor formations were palpable and were retrieved 7–10 weeks after the injection. They were fixed for 24 h in 10% neutral buffered formalin, dehydrated and processed using a routine wax-embedding procedure for histological examination after hematoxylin/eosin staining. Expression of specific antigens was determined on serial tissue sections by immunohistochemistry using the indirect avidin-biotin peroxidase technique (Vector Labs: VECTASTAIN Elite ABC Kit, Universal; DAB Substrate Kit, 3,3′-diaminobenzidine). Details about primary antibodies and immunohistochemical procedures employed are reported in Table 1.

Centrosome Localization

Undifferentiated cells were plated directly on CultureWell Chambered Coverglass 16-well dishes (Molecular Probes Europe, Italy) and cultured for 24 h. They were fixed in 100% methanol at −20°C and stained with a primary antibody specific for human Centrin 1 (1:100, Abcam, UK). Secondary detection was carried out with the appropriate Alexa Fluor antibody (Invitrogen, Italy). The results obtained were observed under a Nikon Eclipse E600 microscope at 100× magnification.

Electron Microscopy

Samples were fixed for 2 h in 0.1 M cacodylate buffer pH 7.2, containing 2% glutaraldehyde. Specimens were then washed in the same buffer and post-fixed for 2 h with 1% osmic acid in cacodylate buffer. After standard serial ethanol dehydration, specimens were embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica, Austria). Semi-thin sections were stained by conventional methods (crystal violet and basic fuchsin) and subsequently observed under a light microscope (Olympus, Japan). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol, Japan).

Mitotic Spindle Check-Point Molecules

RNA was extracted from HP cells and from three bi-parental embryonic stem cell lines, HES 7, HES I-3 and HES I-6 with the TaqMan®Gene Expression Cells to Ct kit (Applied Biosystem, CA, USA). Expression of mitotic checkpoint genes was evaluated using pre-designed gene-specific primer and probe sets from TaqMan®Gene Expression Assays (Applied Biosystem, USA) for the following human transcripts: Mitotic arrest deficient 1 (MAD1); Budding uninhibited by benzimidazoles 1 (BUB1); Centromere protein E (CENPE); TTK kinase (human homologue of the yeast monopolar spindle 1 kinase); Aurora A Kinase; Myc-associated factor X (MAX); *S*WI-Independent 3 (SIN3); β-actin. Gene expression level was reported as ΔCt value. For each individual gene the number of amplification cycles for the fluorescent reporter signal to reach a common threshold value (Ct) was estimated and then normalized by subtracting the Ct value obtained for the same sample for a positive control transcript (Δ-actin), to give ΔCt value.

Results

Three parthenogenetic cell lines (HP1, HP2 and HP3) were obtained from 20 oocytes. Unfortunately one of the cell lines was accidentally lost before its full characterization therefore results for only two lines are reported. These cell lines could be propagated extensively *in vitro* and constantly (67 passages) expressed cell markers that characterize human embryonic stem cells: Oct-4, Nanog, Rex-1, Sox-2, alkaline phosphatase, SSEA-4, TRA 1-81 (Fig. 1, panel **a** and **b**). Undifferentiated HP cells expressed high telomerase activity, while no telomerase activity could be detected once cells

Table 1 Primary antibodies and procedures used for immunohistochemistry

Antibody	Host species and clonality	Company	Clone or company code	Antigen retrieval	Working dilution	Incubation time	Secondary antibody
VIMENTIN	Mouse monoclonal	DAKO	3B4	ED[a]	1:1000	40 min at 37°C	Biotynilated anti-mouse
SMA	Rabbit monoclonal	EPITOMICS	E184	HIAR[b]	1:1200	40 min at 37°C	Biotinylated anti-rabbit
DESMIN	Rabbit monoclonal	EPITOMICS	1184-1	HIAR	1:2000	40 min at 37°C	Biotinylated anti-rabbit
MYOGLOBIN	Rabbit polyclonal	DAKO	L1860	NA	1:10	40 min at 37°C	Biotinylated anti-rabbit
GFAP	Rabbit polyclonal	DAKO	Z334	HIAR	1:10000	40 min at 37°C	Biotinylated anti-rabbit
S100	Rabbit polyclonal	DAKO	Z311	HIAR	1:15000	40 min at 37°C	Biotinylated anti-rabbit
SYNAPTOPHYSIN	Rabbit monoclonal	EPITOMICS	EP1098Y	HIAR	1:500	1 h at room temperature	Biotinylated anti-rabbit
CYTOKERATIN	Mouse monoclonal	ZYMED	AE1/AE3	ED	1:1000	40 min at 37°C	Biotynilated anti-mouse
FVIII	Rabbit polyclonal	DAKO	N1505	ED	1:80	40 min at 37°C	Biotinylated anti-rabbit
LYSOZYME	Rabbit polyclonal	DAKO	A 0099	ED	1:13000	40 min at 37°C	Biotinylated anti-rabbit

[a] ED: enzymatic digestion with pepsin solution (Digest all™ 3, Zymed)

[b] HIAR: heat-induced antigen retrieval, pressure cocker, sodium citrate solution pH 6

were induced to differentiate (Fig. 1, panel **c**), indicating a physiologically normal control of telomerase activity in these cells.

When cultured with the hanging drop method, the formation of EBs was observed regularly after 10–12 days. Embryoid bodies expressed several tissue-specific markers including BMP-4, NF-H, α-amilase (Fig. 2, panel **a**), keratin 17, vimentin, desmin (Fig. 2, panel **b**), indicating that derivatives representative of all three germ layers could be obtained.

Moreover, when cells were cultured in NPDM Bullet Kit (Cambrex Bioscience, USA), expression of nestin, β-tubulin III, CNPase and MAP-2 was observed, demonstrating the formation of more mature cell types of the neural lineage (Fig. 3, panel **a**).

Similarly, exposure of HP cells to specific cytokines and adequate culture conditions allowed for differentiation of these cells towards the hematopoietic lineage, with the generation of CD34/CD45 positive cells (Fig. 3, panel **b**) that were able to form colonies in methylcellulose-medium after a period of 3 weeks (Fig. 3, panel **c**). Staining of the differentiated cells with May-Grunwald–Giemsa demonstrated the presence of lymphoid, erythroid and myeloid sub-populations (Fig. 3, panel **d**).

In vivo differentiation ability of HP cells was tested through intramuscular injection of undifferentiated HP cells in immunodeficient mice and resulted, for both HP lines, either in poor differentiation or in tumours that were classified as myofibrosarcomas (Fig. 4).

Real-time PCR experiments demonstrated aberrant levels of molecules related to spindle formation in HP cells, when compared to those of three bi-parental embryonic stem cell lines (HES 7, HES I-3 and HES I-6). In particular higher levels of MAD1, MAX and SIN3 were detected, pointing to the possibility of a deregulation in the MAD1 dependent pathway. Furthermore, negligible transcription levels of CENP-E, TTK and Aurora A kinase, indicated abnormalities at different spindle check points in HP cells (Fig. 5). Immunohistochemical (Fig. 6, panel **a**) and ultrastructural (Fig. 6, panel **b**-**e**) analysis of HP cells demonstrated the presence of groups of multiple centrosomes showing abnormal shape. These cells were always accompanied by massive autophagic process (Fig. 6, panel **d**). The autophagic cargoes could include damaged centrioles (Fig. 6, panel **e**).

Discussion

The development of human parthenotes to the blastocyst stage was reported only recently [23–25] and not many data are available because of the limited accessibility of

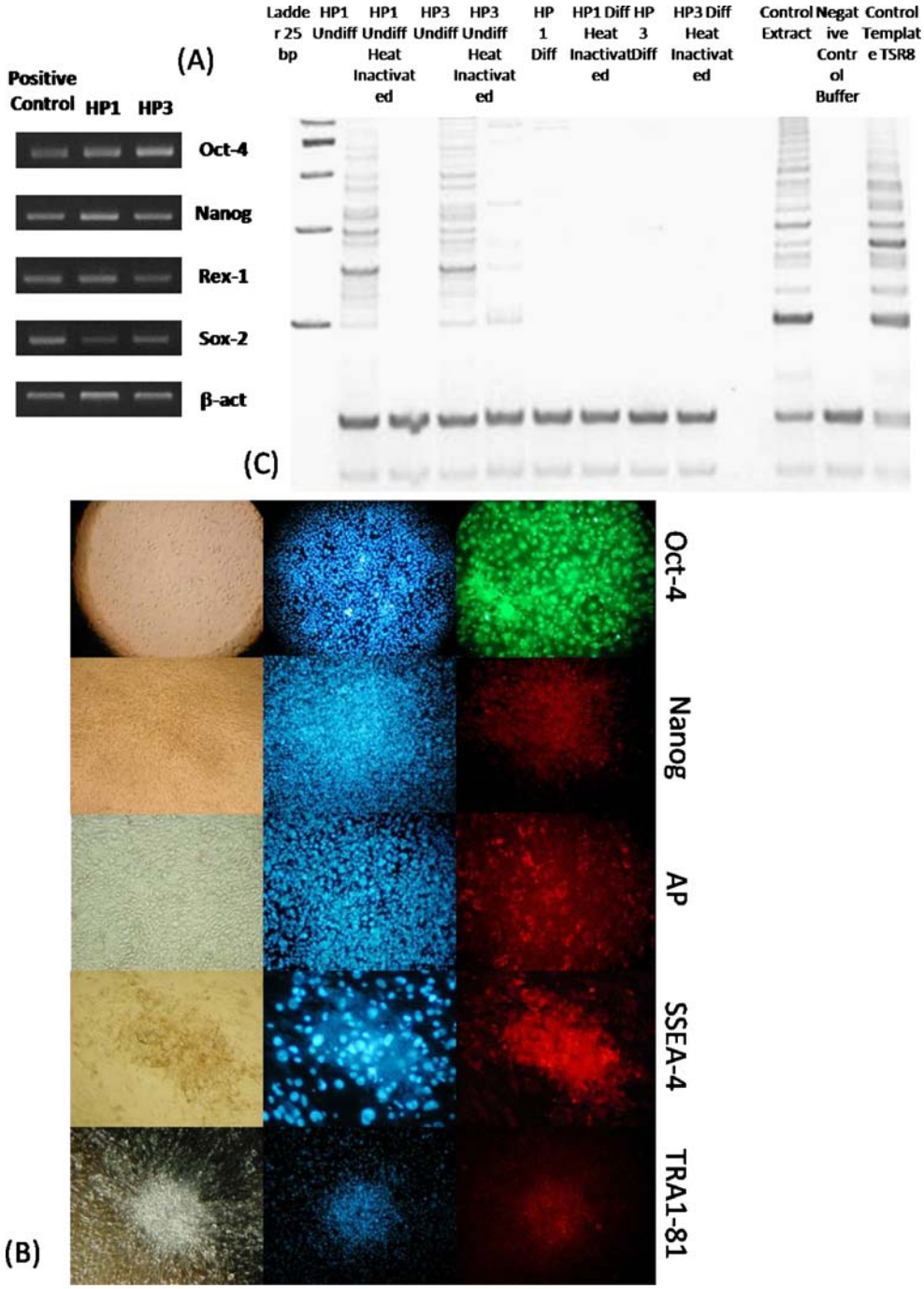

Fig. 1 Pluripotency of HP cells is demonstrated by their positivity to several known pluripotency-related markers and by their telomerase activity. The expression of Oct-4, Nanog, Rex-1, Sox-2 is shown by RT-PCR screening of RNA extracted from HP cells. Beta actin and RNA from bi-parental embryonic stem cells were used as positive control (panel **a**). Cytochemical analysis with specific antibodies demonstrated immunopositivity of HP cells for Oct-4, Nanog, alkaline phosphatase, SSEA-4 and TRA 1-81. Cell nuclei, stained with DAPI, are coloured in blue (panel **b**). Undifferentiated (Undiff) HP cells displayed high levels of telomerase activity, while no telomerase activity could be detected in differentiated (Diff) progeny, indicating a physiologically normal control of telomerase activity in these cells (panel **c**)

unfertilized human oocytes. Even more limited are the information related to the potential plasticity of the lines that can be derived from parthenogenetic human embryos, with specific regards to the potential abnormalities associated with their origin. The results presented in this manuscript describe the properties and limits of the cell lines derived from human parthenogenetic embryos in our laboratory.

These lines have been growing for over 67 passages and, in agreement with previous reports on human parthenogenetic stem cells [9–12], possess the main features of bi-parental stem cells, showing stable expression of pluripotency-related markers, high *in vitro* differentiation plasticity and the capability to respond to specific stimuli in order to give rise to high specification tissue differentiation. Consistent with these data is their high telomerase activity. Telomerase activity is indeed correlated with regeneration and immortality and is typically expressed in germ cells and embryonic stem cells, while it is absent in most somatic cell types [26, 27]. In our experiments, undifferentiated HP cells

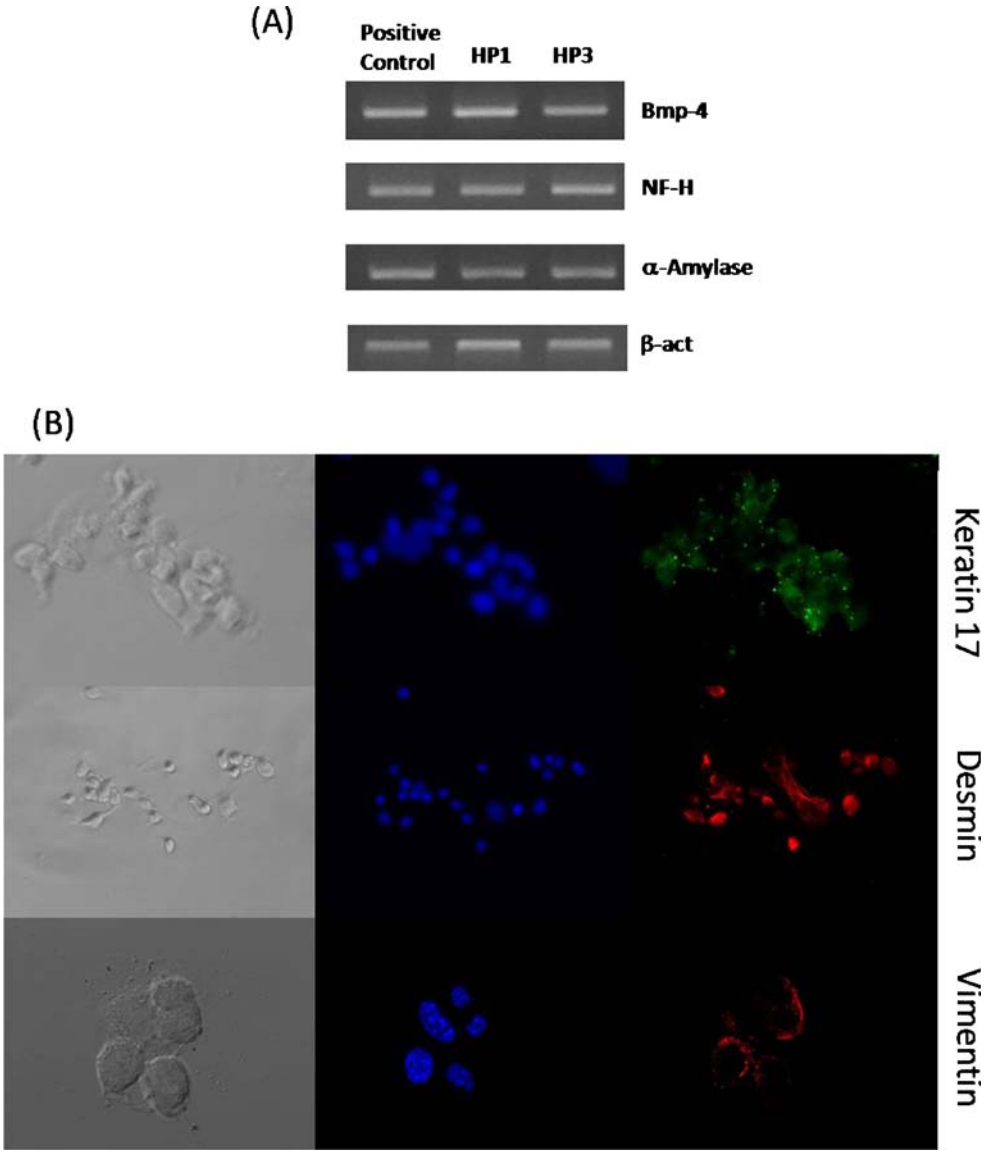

Fig. 2 Differentiation ability of human parthenogenetic cells after induction of EB formation. RT-PCR analysis of RNA extracted from EBs consistently demonstrated expression of markers specific for the three germ layers and, more in details bone morphogenetic protein-4 (Bmp-4, mesoderm), neurofilament H (NF-H, ectoderm) and α-amylase (endoderm). Beta actin and genomic DNA were used as a positive control (panel **a**). Disaggregation and plating of EBs confirmed HP cell plasticity, with consistent presence in the monolayer of cells displaying immunopositivity for keratin 17 (endoderm), desmin (mesoderm) and vimentin (ectoderm). Cell nuclei, stained with DAPI, are coloured in blue (panel **b**)

displayed high levels of telomerase activity, while no activity could be appreciated when cells were differentiated *in vitro* through the preparation of EBs. These data indicate that a physiologically normal control of telomerase activity is present in HP cells and that, if subjected to differentiation culture conditions, they respond turning down telomerase activity, as expected in normal somatic cells. Indeed an average of 10 days differentiation culture allowed us to obtain parthenogenetic EBs that actively transcribed RNAs involved in specification of the three embryonic germ layers, demonstrating HP cell potential to differentiate in the main tissue types of the body. HP cell plasticity was further demonstrated by immunostaining of the monolayers obtained after EB disaggregation and plating , with consistent presence of cells belonging to ectoderm, mesoderm and endoderm lineages.

A crucial point is to assess the possibility to drive differentiation of human parthenogenetic cells towards a specific lineage, in controlled culture conditions. In this line we carried out the sets of experiments presented in this manuscript aimed at the derivation of mature forms of neural and hematopoietic cell populations. HP cells were able to form different cell subtypes belonging to the neural lineage as well as to differentiate in the complex array of hematopoietic cells. Although further assays to test the real extent of cellular functionality are needed, these results further confirm human parthenogenetic cell lines differentiation potential. In particular our results showed that HP cells were able not only to give rise to early neural lineages as previously described [9–12] but also to more mature cell types expressing nestin, CNPase and MAP2. This is consistent with the observation that murine androgenetic

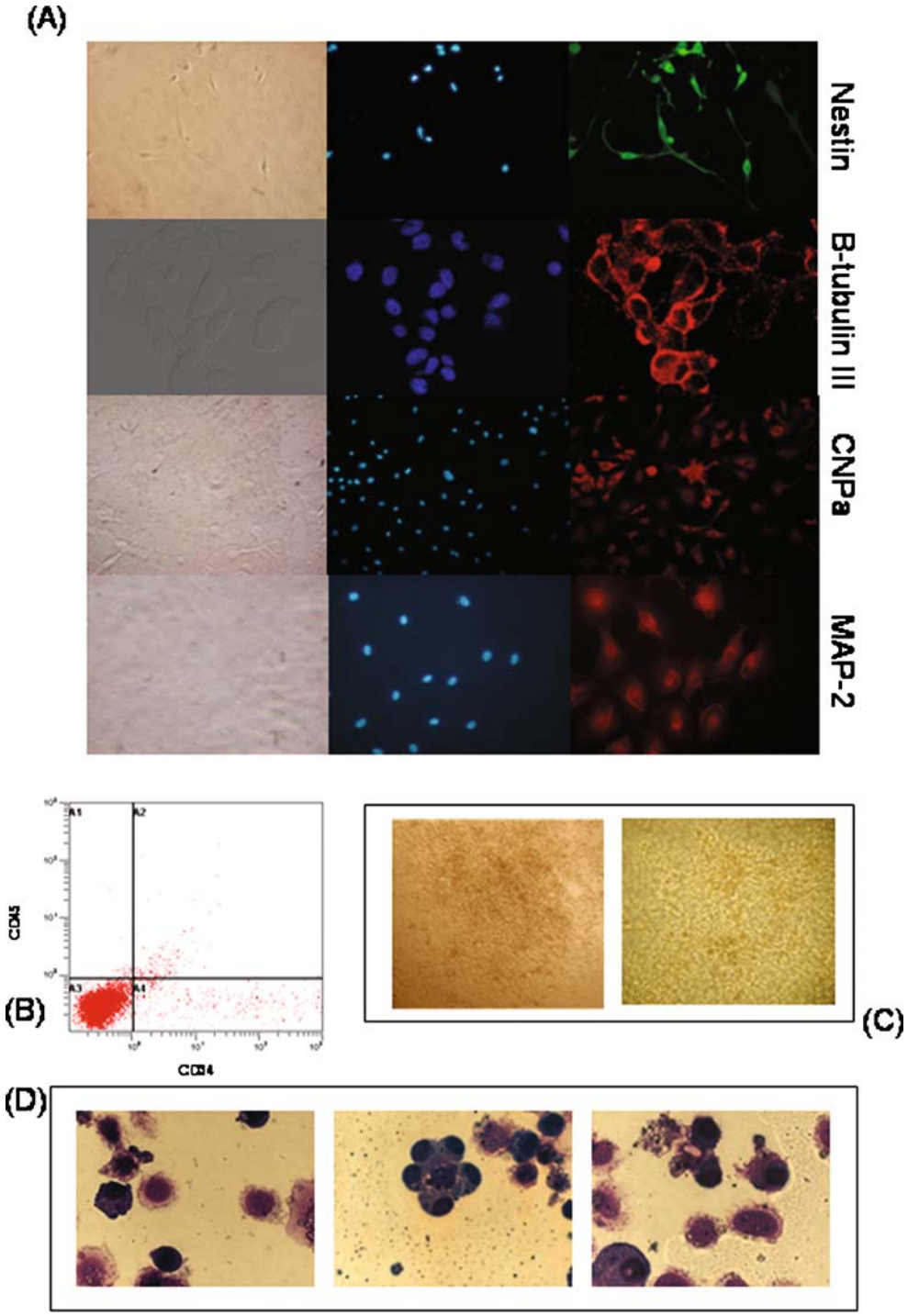

Fig. 3 Neural and hematopoietic differentiation of HP cells. Culture conditions routinely used to address bi-parental embryonic stem cells towards neural differentiation successfully drove HP cells to form different cell subtypes belonging to the neural lineage, with cells displaying immune-positivity for nestin, β-tubulin III, CNPase and MAP-2. Cell nuclei, stained with DAPI, are coloured in blue (panel **a**). Differentiation towards the hematopoietic lineage was obtained exposing HP cells to specific cytokines and adequate culture conditions. CD34/CD45 positive cells were demonstrated and separated by cell sorting (panel **b**) These cells were able to form colonies when cultured in methycellulose-medium (panel **c**) and to generate lymphoid, erythroid and myeloid subpopulations (panel **d**, *left* to *right*)

embryonic stem cells are able to differentiate into neuronal and glial cells [28]. Our results further expand current knowledge on human parthenogenetic cell *in vitro* differentiation plasticity, showing the formation of mature hemopoietic cell lineages. This confirms what has been previously reported by Mann et al. [31] in mouse androgenetic and gynogenetic stem cells, that where shown to be able to generate adult-transplantable hematopoietic stem cells, that can repopulate the hematopoietic system of adult transplant recipients [29]. Altogether these findings indicate that, outside the normal developmental paradigm, the differentiation potential of uniparental cells may be much less restricted than that of parthenogenetic cells in chimeras and that these cells can be an interesting and relevant model for the study of fundamental mechanisms involved in human lineage determination.

However, injection of HP cells in immunodeficient mice gave rise to poor differentiation or in the formation

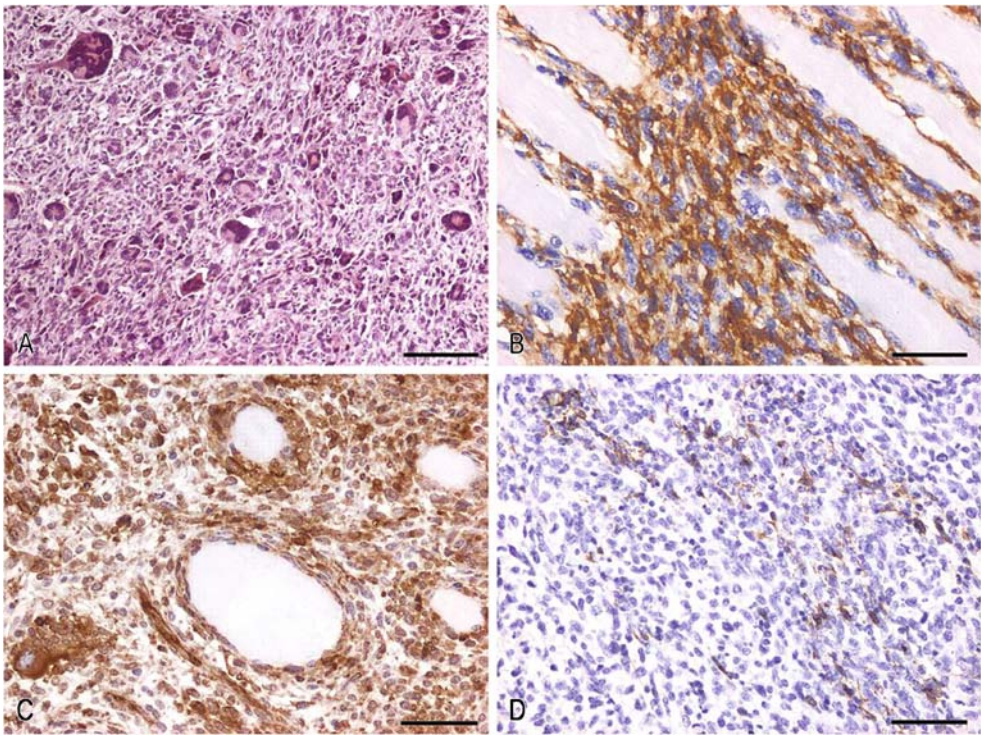

Fig. 4 Formation of myofibrosarcoma-like tumors following injection of undifferentiated HP cells in the hind leg of SCID Beige and Fox Chase SCID mice. Numerous multinucleated cells showing aberrant mitotic figures (**a**). Vimentin-positive (**b**) and Smooth Muscle Actin-positive (**c**) cells dissecting and separating residual skeletal myofibres. Scattered, isolated Desmin-positive (**d**) cells are also detectable. HE staining (**a**) and indirect avidin-biotin immunoperoxidase staining with 3,3′-diaminobenzidine chromogen reaction (**c**, **d**, **e**). Scale bar 100 μm (A) and 50 μm (**b**, **c**, **d**)

of myofibrosarcomas [30], depending on animal injected. This is consistent with the observation that the subcutaneous injection of androgenetic mouse embryonic stem cells in immunodeficient mice generates sarcomas with muscular differentiation [31] and suggests the possibility of an intrinsic deregulation of the mechanisms controlling the choice between proliferation and differentiation in embryonic stem cells obtained through parthenogenesis and androgenesis. Interestingly, this deregulated differentiation appears to be modulated by the microenvironment and, while undetectable, or repressed, when cells were differentiated *in vitro*, it became evident once cells were exposed to the less restrained *in vivo* milieu.

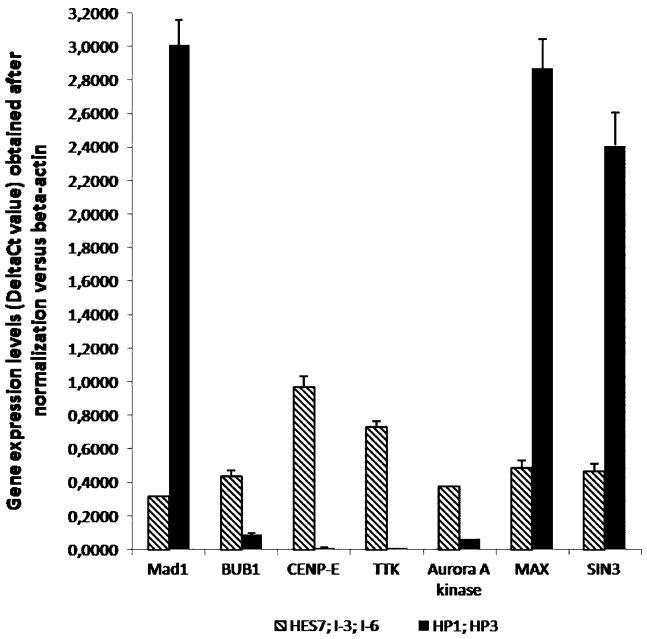

Fig. 5 Expression level of molecules related to spindle formation and chromosome segregation in HP cells and bi-parental cell lines HES 7, HES I-3 and HES I-6. Bars represent the average ΔCt of HP cells (*solid bars*) and bi-parental cells (*striped bars*) related to the genes examined. ΔCt value was obtained from the Ct of the target gene normalized with the Ct value for β-actin of the same sample

These results are in contrast to what previously described in other human parthenogenetic cell lines. We have no explanation for this differences and we cannot rule out the possibility that these anomalies simply derive from differences in the procedures used for the derivation of the cell lines. In particular while our activation protocol is almost identical to that described by Revazova et al. [11, 12] it did not include an electrical activation step as performed by Mai et al. [10] whereas Lin et al. used spontaneously activated oocytes [9]. Our cell lines were cultured on immortalized mouse fibroblasts similarly to Mai et al. [10] who used mouse embryonic fibroblasts, while in all other cases human fibroblast feeder layers where used [9, 11, 12]. However, since normal cell lines were obtained with protocols, in many aspects similar to the one used in our experiments which, in turn, are described in the literature for the culture of bi-parental cell lines, we think it is unlikely that the specific protocol used in our experiment can be the cause of the observed abnormalities.

Even if specific activation and culture conditions used in the current experiments cannot be excluded as main cause

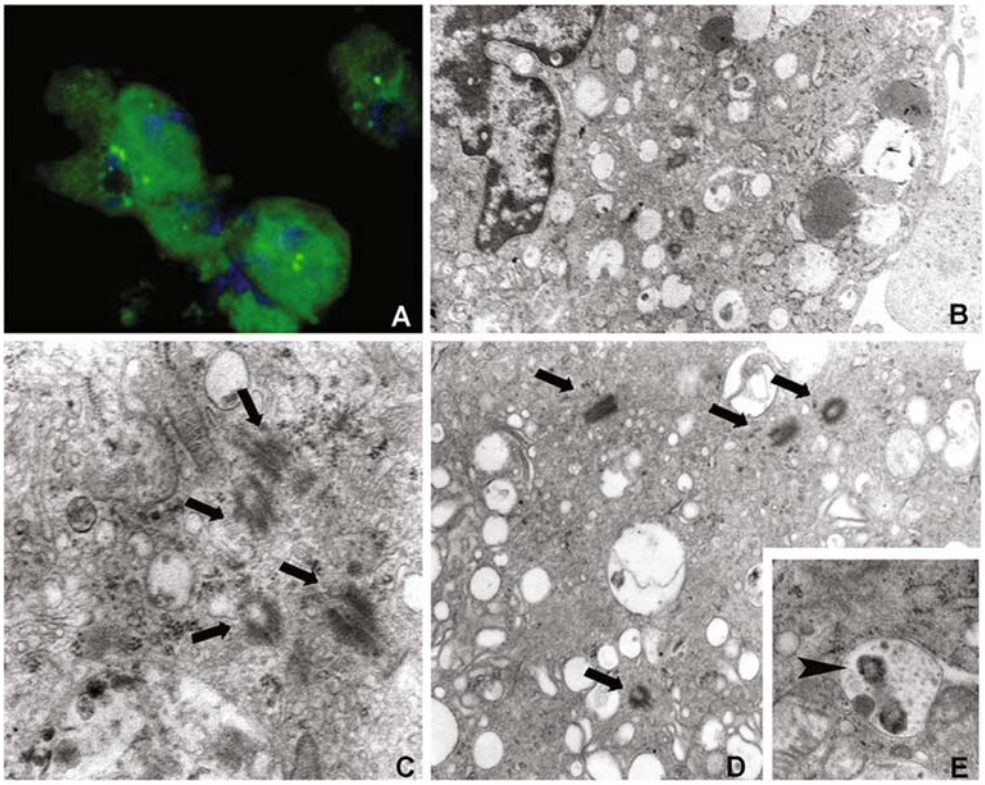

Fig. 6 Immunohistochemical (**a**) and ultrastructural (**b-c-d-e**) analysis of human parthenogenetic cells. The presence of amplified centrosomes loosely dispersed in the cytoplasm can be appreciated. Supernumerary centrioles (*arrows*) and massive autophagic processes generally coexist (**d**). Often the cargo of autophagic compartments resemble centrioles partially damaged (*arrowheads*)

of the altered behaviour of our cell lines upon *in vivo* transplantation, we hypothesize that the uniparental origin of HP cells and, in particular, the lack of the centrioles supplied by the male counterpart, may be a possible explanation with the use of relatively old donors as a potential aggravating factor. It has been demonstrated that centrioles degenerate and are lost during human oogenesis and while oogonia and growing oocytes display normal centrioles until pachytene stage, they are absent in the mature oocytes [32]. With the notable exception of mice [33] this degenerative process has been described in rhesus monkeys [34], rabbits [35], cows [36], sea urchins [37], *Xenopus* [38], and several other species [39]. Due to the absence of centrioles, the oocyte centrosomal material does not aggregate into unified foci and is unable to form astral microtubules and a correctly oriented spindle, unless rescued by a spermatozoon. The consequences of the lack of centrioles on parthenogenetic development have been studied in detail in lower species, where successful parthenogenesis largely depends upon the oocyte ability to generate complete and functional centrosomes in the absence of the material supplied by a male gamete. In particular, parthenogenetically activated sea urchin and insect eggs have been described to form multiple centrioles, possibly as the result of the lack of a correct control on the process of spindle formation [40–42]. Indeed, the inability of parthenogenetic oocytes to organize normal spindles due to the lack of a functional centrosome has been suggested as a strict checkpoint control to suppress parthenogenetic development [43] since it is not an evolutionarily preferred pathway even in species that are facultative parthenotes because it leads to genomic homogeneity that, in turn, results in the accumulation of genetic anomalies in the population. These observations on the parthenogenetic process in lower species are in agreement with our findings in HP cells. Similarly to what reported in sea urchins and many insects, for instance, we also found that HP cells display multiple centrioles, suggesting that a decreased ability to rearrange functional centrosomes is present in these cells. This is also consistent with the observations by Marshall [44], indicating that centriole de novo assembly is normally turned off when a centriole is present. The absence of sperm centriole in parthenotes may therefore lead to the lack of a negative regulatory mechanism that suppress *de novo* centriole assembly and may explain the presence of multicentriolar structures as the ones described in parthenotes and as the ones we detect in HP cells. Interestingly enough, HP cells can proliferate and divide and most importantly, they can correctly differentiate, into a variety of tissues, responding to experimental conditions that are able to induce differentiation in bi-parental cells. This ability does not seem to be limited by the abnormalities described above and suggests that the requirement of a paternal centrosome described in lower animals appears to be less stringent in human cells, at least in *in vitro* controlled conditions. Indeed, in higher

mammals, genomic imprinting is thought to be the main mechanism to ensure bi-parental fertilization [45]. However these abnormalities in spindle rearrangement may explain the presence of cells showing misshaped chromosomes in our cell lines. On the other hand, these abnormalities do not seem to be specific of HP cells, and do not seem to be related to the derivation protocol and/or the culture conditions used in our experiments, since spindle rearrangement and multiple chromosome mal-segregations have been previously described in human parthenogenetic embryos, that were obtained from oocytes spontaneously activated and/or induced with puromycin [46]. Furthermore they do not seem to be confined to human parthenotes and appear to be common to other mammalian species. Indeed a high incidence of abnormal spindle and misshaped chromosomal complements has been reported in parthenotes derived from bovine as well as porcine activated oocytes, with abnormalities occurring as early as completion of the first cell cycle. Each of these reports linked these phenomena to the absence of a paternally supplied centrosome [47, 48].

Altered expression levels of mitotic check point molecules were found when *in vitro* cultured HP cells were examined by real-time PCR. In particular, the comparison of HP cells with bi-parental embryonic stem cell lines indicated a much higher level of expression of Mad-1, and the related molecules MAX and SIN3 in parthenogenetic cells. Mad-1 is a central component of the spindle assembly checkpoint and recruitment of kinetochores [49–51]. The altered levels of such molecules present in HP cells may be related to the lack of paternal contribution in spindle assembly. A similar explanation could account for the very low transcription for TTK and CENP-E detected in these cells.

Conclusions

Cell lines derived from human parthenogenetic embryos have great potentials since these cells possess most of the main features of bi-parental stem cells, show high plasticity and give rise to high specification tissue differentiation. Whereas human parthenogenetic cell lines capable of normal differentiation, not only *in vitro* but also *in vivo* have been described, we observed malignant *in vivo* differentiation accompanied by aberrant centriole distribution and abnormalities in the control of the mitotic spindle check point. A series of experimental data, from our and other laboratories, suggest that these phenomena may be related to their uniparental origin but do not explain why these alterations have not been observed in other cell lines. We have no clear explanation for this but it is interesting to note that experiments with bovine parthenotes showed that the ability of maternal centrosomes to organize microtubules differs from oocyte to oocyte, and this may determine the developmental fate of each parthenote [52] and, presumably of the resulting cell lines. Indeed differences in the *in vivo* differentiation potential between their two human parthenogenetic cell lines have been described also by Mai et al. [10]. Further investigations are required in order to understand how individual variations can impact the derivation of stable lines from human parthenotes and how undesirable abnormalities can be prevented, especially if a therapeutic use of this kind of stem cells is considered.

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