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Department of Animal Science

Doctoral Course in Biotechnology Applied
to Veterinary and Animal Sciences

CENTROSOME BIOGENESIS AND ADAPTIVE RESPONSE IN MAMMAL PARTHENOGENETIC CELLS

Doctoral Thesis

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To my Family,
my Parents,
Trilly and Paco,
With Love

The purpose of each activity of the intellect is to reduce the mystery of something comprehensible.
(A. Einstein)

Stay hungry, stay foolish!
(Steve Jobs)

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ABSTRACT

Human parthenotes have been proposed as a source of embryonic stem cells despite the high incidence of aneuploidy described in parthenotes of most mammalian species. Through a comparative analysis between parthenogenetic and bi-parental cells lines we found that parthenogenetic cells are affected by chromosomal instability and centrosome amplification. We provide evidence that both alterations are determined by the lack of paternal centriole, normally contributed by the sperm at the time of fertilization, but parthenogenetic cell lines activate a series of adaptive mechanisms that allow them to proliferate and differentiate. These include down-regulation of the p53/p21 pathway, massive increase of autophagic activity and formation of a wide network of intercellular bridges with the morphological and molecular characters of blocked cell abscissions. These processes are commonly observed in transformed cells therefore parthenogenesis may be used to explore the mechanisms regulating oncogenesis and their link with self-renewal and pluripotency in human cell lines.

1. INTRODUCTION

1.1 Stem cells

Stem cells are a special kind of cell that have the unique capability to make identical copies of themselves (this property is referred to as "self-renewal") for the lifetime of the organism and, under appropriate conditions, to give rise to several specialized cell types [1,2] (Figure 1).

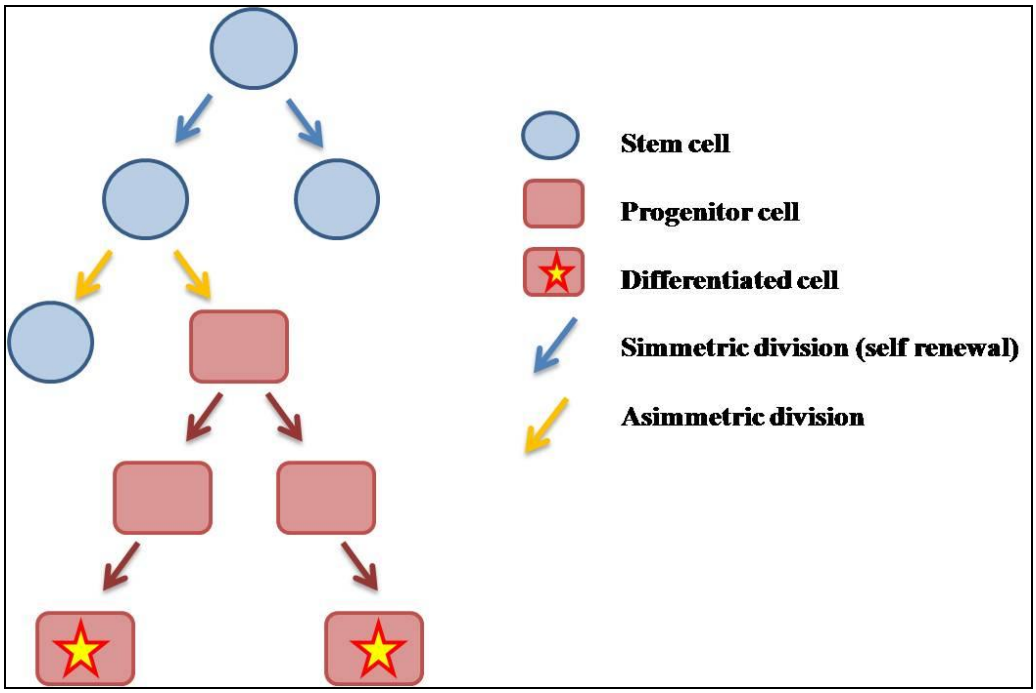


Figure 1. Symmetric and asymmetric division of stem cells.

Although most cells of the body, such as heart cells or skin cells, are committed to conduct a specific function; stem cells are uncommitted and remain uncommitted, until they receive a signal to develop into a specialized cell. Their proliferative capacity combined with the ability to become specialized makes stem cells unique. Recently, stem cells have received much attention following their possible use in regenerative medicine. This is a new concept in contemporary medical science based on the possibility to produce new cells that may be utilized to repair or renovate degenerated and damaged tissues.

Three different types of cells exist, based on their differentiation capability:

1. Totipotent cells: zygote;
2. Pluripotent cells: embryonic stem/germ/carcinoma cells;
3. Multipotent/unipotent: adult stem cells.

1.1.1 Totipotent cells

Totipotent cell —from the Latin "totus", meaning entire— has the potential to generate all the cells and tissues that make up an embryo and that support its development in utero (fetal membranes). Totipotent cells formed during sexual and asexual reproduction include spores and zygotes. In some organisms, cells

can dedifferentiate and regain totipotency [3]. For example, a plant cutting or callus can be used to grow an entire plant. Mammalian development commences when an oocyte is fertilized by a sperm forming a single celled embryo, the zygote. Consistent with the definition, zygote is considered the only existing totipotent cell. This single cell has the potential to develop into an embryo with all the specialized cells that make up a living being, as well as into the placental support structure necessary for fetal development. Thus, each totipotent cell is a self-contained entity that can give rise to the whole organism. This is said to be true for the zygote and for early embryonic blastomeres up to at least the 4-cell stage embryo (Figure 2). Experimentally, totipotency can be demonstrated by the isolation of a single blastomere from a preimplantation embryo and subsequently monitoring its ability to support a pregnancy following transfer into a suitable recipient. This approach was pioneered in rats and has been realized in several mammalian species including non-human primates [4-6]. The results obtained in rhesus monkey confirm the ability of isolated blastomeres from 2- and 4-cell stage IVF embryos to support pregnancies and to produce live animals, demonstrating the totipotency of these cells [7]. As embryo development progresses to the 8-cell stage and beyond depending on the species, the individual blastomeres that comprise the embryo gradually lose their totipotency (Figure 2). It is generally

believed that this restriction in developmental potential indicates irreversible differentiation and specialization of early embryonic cells into the first two lineages, the inner cell mass (ICM) that includes cells that will give rise to the fetus and the trophectoderm (TE), an outer layer of cells that is destined to an extraembryonic fate.

However it is important to note that totipotent cells are not stem cells because they lack the property of symmentric division, therefore they can indeed differentiate but are unable to self-renewal.

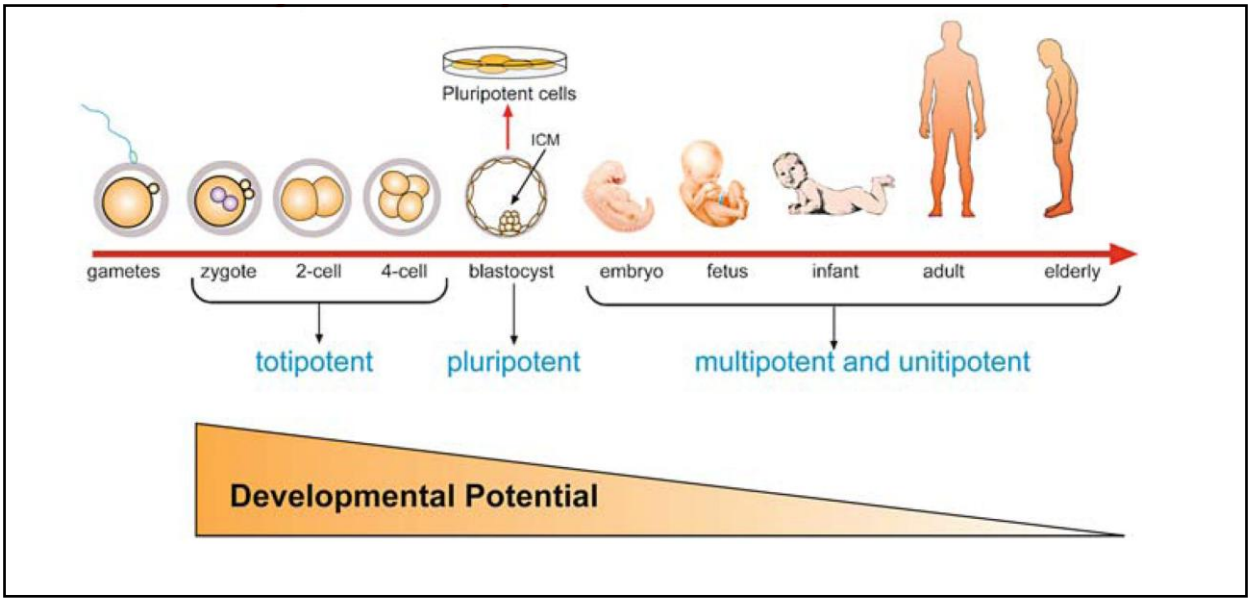


Figure 2. Development. Ontogeny begins from a single cell, the zygote. The zygote and each blastomere of the early embryo are totipotent with the potential to develop into the whole organism. As development unfolds, the developmental potential of individual blastomeres gradually declines resulting subsequently in pluripotent, multipotent, unipotent and terminally differentiated somatic cells. (Modified from Mitalipov S. and Wolf D., 2009).

1.1.2 Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent cells — from the Latin "plures", meaning several or many- derived from the inner cell mass (ICM) of preimplantation blastocysts. *In vitro*, these cells are capable of undergoing an unlimited number of symmetrical divisions without differentiating (long-term self-renewal) and, when exposed to specific conditions, can give rise to cells derived

from all three embryonic germ layers: ectoderm, endoderm and mesoderm (Table 1).

Embryonic Germ Layer	Differentiated Tissue
Endoderm	Thymus Thyroid, parathyroid glands Larynx, trachea, lung Urinary bladder, vagina, urethra Gastrointestinal (GI) organs (liver, pancreas) Lining of the GI tract Lining of the respiratory tract
Mesoderm	Bone marrow (blood) Adrenal cortex Lymphatic tissue Skeletal, smooth, and cardiac muscle Connective tissues (including bone, cartilage) Urogenital system Heart and blood vessels (vascular system
Ectoderm	Skin Neural tissue (neuroectoderm) Adrenal medulla Pituitary gland Connective tissue of the head and face Eyes, ears

Table 1. Embryonic germ layers from which differentiated tissues develop

Pluripotent stem cells can give rise to any fetal or adult cell type. These include more than 200 kinds of cells, such as nerve cells (neurons), muscle cells (myocytes), skin (epithelial) cells, blood cells (erythrocytes, monocytes, lymphocytes, etc.), bone cells (osteocytes), and cartilage cells (chondrocytes) (Figure 3). Consequently ESCs are considered a potential unlimited source of transplantation materials for replacement cell therapy.

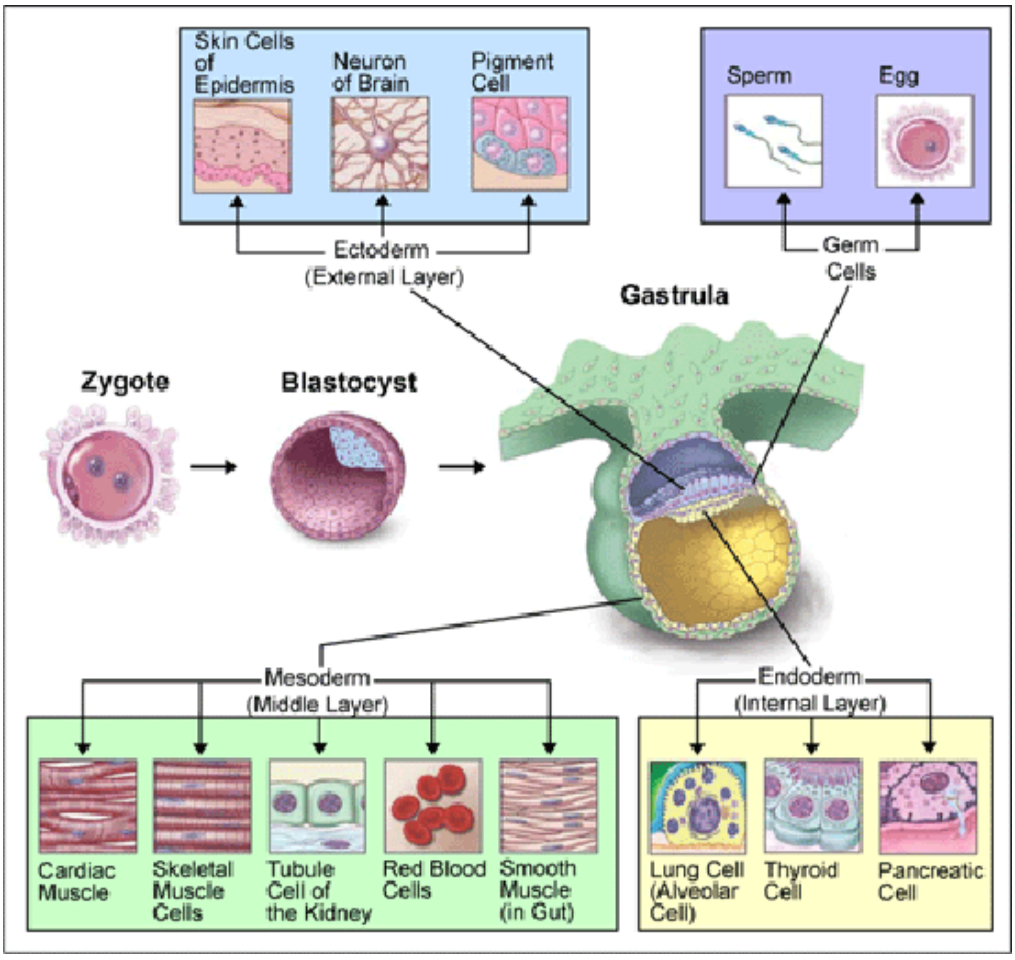


Figure 3. Pluripotent stem cells can give rise to different kind of adult cells.

It is fundamental to remember that a formally correct definition of embryonic stem cells implies the satisfaction of several criteria including: derivation without transformation or immortalization; stable diploid karyotype, clonogenic ability, unlimited self-renewal capacity, ability to generate all cell types *in vitro* and in teratomas, incorporation into embryonic development and contribution to all germ layers in chimera, germ-line colonization and transmission [8]. These definitions have been used to qualify cell lines derived from ICM of all mammalian embryos as ESCs. Any cell line which fails to satisfy all these requirements should be defined as stem cell-like or other dubitative descriptions.

Marker **Name**	**Mouse ECC/ ESC/EGCs**	**Monkey ESCs**	**Human ESCs**	**Human EGCs**	**Human ECCs**
SSEA-1	+	–	–	+	–
SSEA-3	–	+	+	+	+
SEA-4	–	+	+	+	+
TRA-1–60	–	+	+	+	+
TRA-1–81	–	+	+	+	+
Alkaline phosphatase	+	+	+	+	+
Oct-4	+	+	+	Unknown	+
Telomerase **activity**	+ ES, EC	Unknown	+	Unknown	+
Feeder-cell dependent	ES, EG, some EC	Yes	Yes	Yes	Some; relatively low clonal efficiency
Factors which aid in stem cell self-renewal	LIF and other factors that act through gp130 receptor and can substitute for feeder layer	Co-culture with feeder cells; other promoting factors have not been identified	Feeder cells + serum; feeder layer + serum-free medium + bFGF	LIF, bFGF, forskolin	Unknown; low proliferative capacity
Growth characteristics *in vitro*	Form tight, rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs	Form flat, loose aggregates; can form EBs	Form rounded, multi-layer clumps; can form EBs	Form flat, loose aggregates; can form EBs
Teratoma **formation *in vivo***	+	+	+	–	+
Chimera **formation**	+	Unknown	+	–	+

Table 2. Comparison of mouse, monkey and human pluripotent stem cells and their essential characteristics.

1.1.3 Adult stem cells

Multi/unipotent stem cell - derived from the Latin “multi”, which means few and “unus” meaning one- is a term that is usually applied to a cell in adult organisms: the adult stem cells. These are undifferentiated cell that are present in a differentiated (specialized) adult tissue and are capable of differentiating along a few or only one lineage. Adult stem cells, in fact, can yield all specialized cell types of the tissue from which it originated (Figure 4) and ensure tissue homeostasis and repair during the individual life. This characteristic is an advantage because it makes controlled differentiation easier than pluripotent cells. Moreover these kind of cells are also capable of self-renewal for the lifetime of the organism.

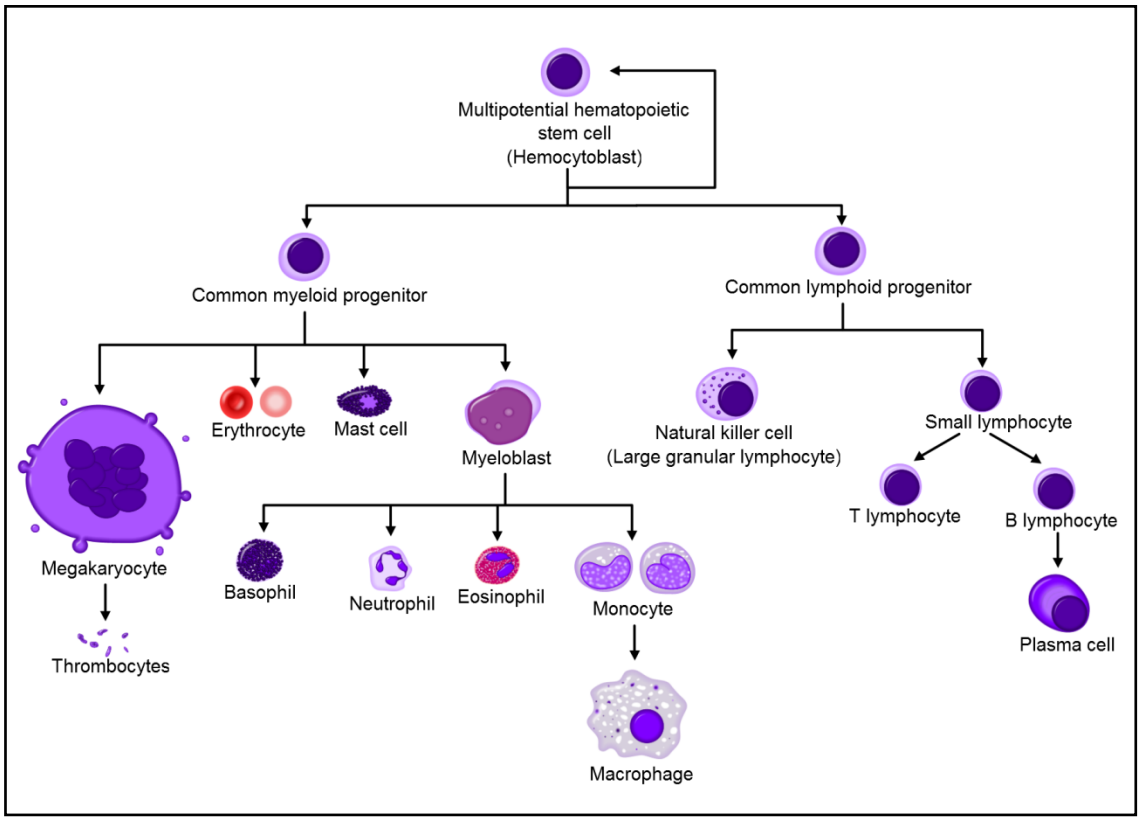


Figure 4. Hematopoietic stem cell differentiation. Representative picture of multipotent stem cells that are able to differentiate in all specialized cell types of the tissue from which it originated.

During the past decade, scientists discovered adult stem cells in tissues, such as in the bone marrow [9], brain, blood stream, cornea and retina of the eye [10,11], the dental pulp of the tooth, liver, skin [12], gastrointestinal tract and pancreas. Some of these cells are already being used for therapeutic applications, thanks to their easy isolation and progation *in vitro*. Unfortunately, not all adult stem cells, can be used for therapeutic application. Many of them, in

fact, are difficult to access (i.e. central nervous system cells) and/or show a limited proliferating ability (i.e. muscle cells) [13].

1.2 Embryonic stem cells

During the last decades the field of stem cell biology has received increasing amounts of attention from scientists, ethicists, industrialists, politicians and the general public. In particular, ESCs are considered of great interest because they are recognized as a potential renewable source of specialized cells that can be used for therapeutic purposes, replacing diseased or damaged cells.

Here we describe in more detail their characteristics in some species relevant for the subjects reported in this thesis.

1.2.1 Embryonic stem cells in mouse

ESCs were first derived by Martin [14] and Evans and Kaufman in 1981 [15]. They successfully isolated and characterized ICM-derived cells from *in vivo*-derived mouse blastocyst-stage embryos obtained from random bred ICR female mice with SWR/J males [14-16]. Undifferentiated cell colonies show a compact morphology with a high nucleus- cytoplasm ratio and prominent nucleoli (Figure 5).

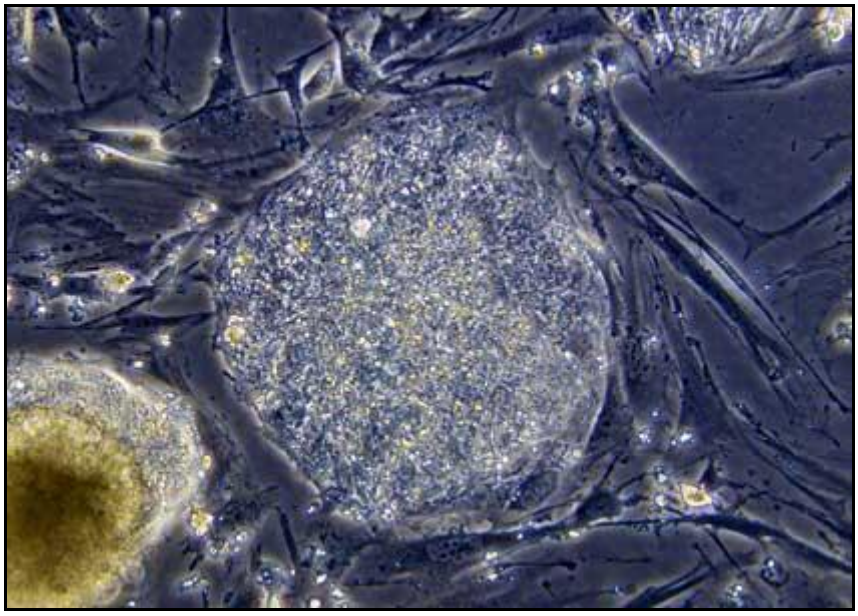


Figure 5. Representative picture of ESCs colony.

They express specific markers or characteristics similar but not identical to the transient pluripotent cells of an embryo. This includes stage specific embryonic antigens, enzymatic activities such as alkaline phosphatase and telomerase, and "stemness" genes that are rapidly down-regulated upon differentiation, including OCT4 and NANOG [17]. Furthermore mouse ESCs showed their ability to proliferate indefinitely in an undifferentiated state [15], when cultured in specific conditions, suggesting that their transcriptional activity and epigenetic regulators were capable to support pluripotency also *in vitro* [3]. However, when released from the influence of these culture conditions or following their introduction back into a host

embryo, ESCs retain their ability to differentiate into any cell-type, just like ICM cells. Alternatively, when injected in immune-deficient mice, they can differentiate *in vivo* in teratomas containing cells representing the three major germ layers: endoderm, mesoderm and ectoderm [18] or they can be directed to differentiate *in vitro* into any of the 200 cell types present in the adult body [19-22].

All these data obtained in mouse resulted in efforts aimed at establishing the same kind of cells in other species, including rabbit, hamster, cattle, sheep, goat, in no-human primate and human too. Actually human and mouse embryonic stem cell (ESC) lines have been the two predominant animal models investigated by researchers over the past one and two decades respectively.

1.2.2 Embryonic stem cells in human

In 1994 human ICMs, isolated from blastocysts created for reproductive purposes using IVF and donated by patients for research, were maintained in culture and generated aggregates with trophoblast-like cells at the periphery and ES-like cells in the center. These cells retained a complete set of chromosomes (normal karyotype) and showed a stem cell-like morphology,

although some ICM clumps differentiate into fibroblasts. These cultures were maintained only for two passages [23].

The first research paper that reported ESCs maintenance in undifferentiated state for more than 4 or 5 months was published in 1998 by James Thomson and colleagues [18]. They described methods for deriving and maintaining human ESCs and demonstrated differentiation their ability producing endoderm (gut epithelium), mesoderm (cartilage, bone, smooth muscle and striated muscle) and ectoderm (neural epithelium, embryonic ganglia, and stratified squamous epithelium) derived cells [18].

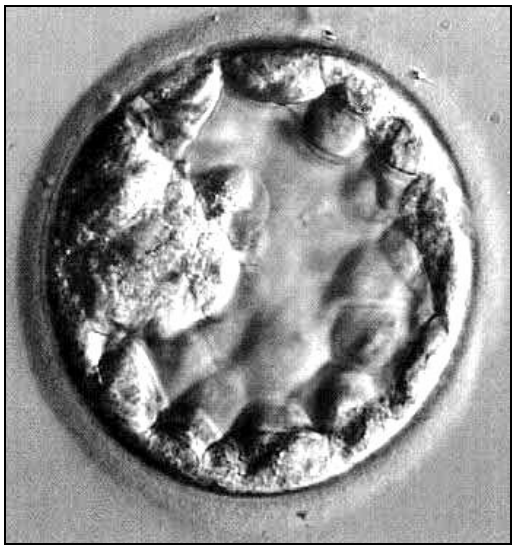


Figure 6. Human blastocyst showing distinct ICM and trophectoderm.

To date, several laboratories have demonstrated pluripotent characteristics of human ESCs, like expression of pluripotency markers, high telomerase activity, *in vitro* differentiation ability and, when injected into immune-deficient mice, formation of teratomas [24,25].

Obviously, two aspects of *in vivo* pluripotency typically tested in mouse have not been met by human cells: evidence that cells have the capacity to be injected into a human embryo and form an organism made up of cells from two genetic lineages; and evidence that they have the ability to generate germ cells, the precursors to eggs and sperm in a developing organism.

Furthermore it is important to underline that differences are emerged comparing mouse and human ESCs. Despite the apparent common origin and the similar pluripotency characteristics of cell lines obtained from the two species, recent studies have revealed that the two cell types use different signalling pathways to maintain their pluripotent status. Indeed mouse ESCs depend on leukaemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), whereas their human counterparts rely on activin (INHBA)/nodal (NODAL) and fibroblast growth factor (FGF). Moreover, despite having similar core transcription factor circuitry, human and mouse ESCs have substantially different target genes for Oct-4 and Nanog [17,26]. Until the last few years these differences between the two cell types had

not explanation. Only recent studies carried out on mouse epiblast stem cells (EpiSCs) [27,28] explained that these divergences are developmental and represented two different stages of embryonic development with human ESCs recapitulating the epiblast state [29]. Interestingly, EpiSCs share with human ESCs not only a dependence on activin/Nodal signaling, but also other key differences from mouse ESCs that have previously been attributed to species divergence. EpiSCs and human ESCs, in fact, grow in large, flat, epithelial colonies, they are not able to clonally propagated, show a limited capacity for colonising pre-implantation embryos and directly respond to known gastrulation signals *in vitro* [30-33]. Finally, EpiSCs (like human ESCs) have been shown to differentiate into trophectoderm in the presence of BMP4 [34], whereas mouse ESCs have little or no capacity for contribution to either primitive endoderm or trophectoderm lineages in chimaeric embryos [35] and differentiate into trophectoderm only when their Oct-4 gene is mutated by homologous recombination [36].

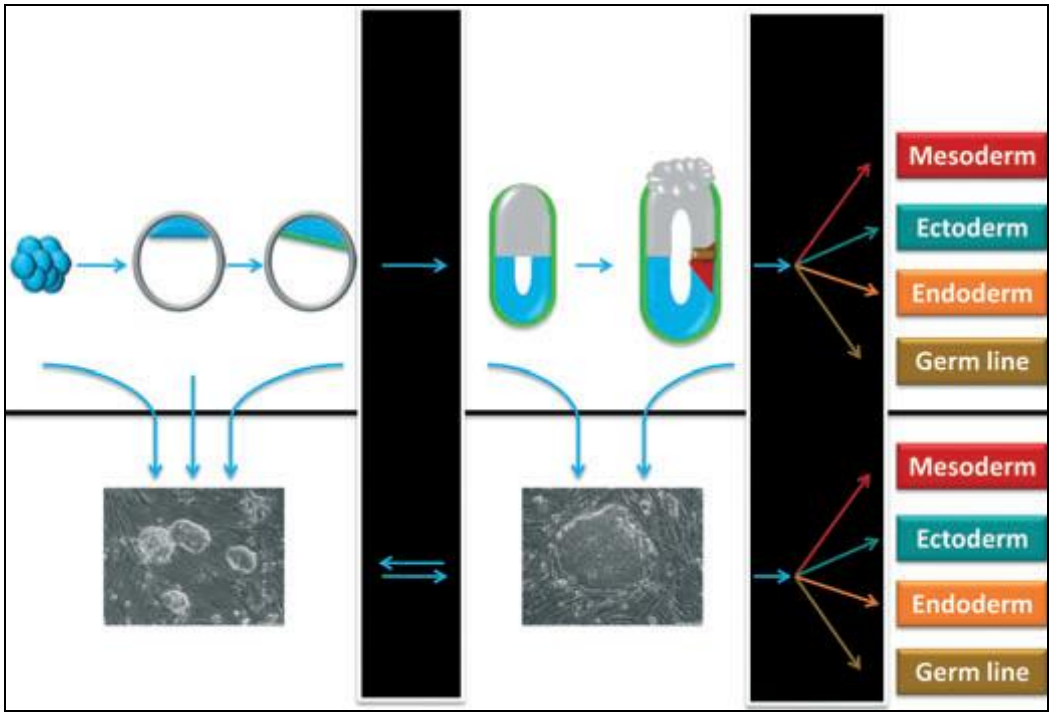


Figure 7. Early development. Pluripotent cells from a developmental window in the pre-implantation embryo grow as mouse embryonic stem (ES) cells, and those from the post-implantation epiblast grow as epiblast stem cells (EpiSCs). The ability to grow cells *in vitro*, and move between distinct pluripotent states will provide insight into the undefined molecular mechanisms (''black boxes'') that establish the early cell fates within the embryo. (From Chenoweth et al., 2010).

1.2.3 Embryonic stem cells in ungulates

Overall the ESC characteristics described above made them an invaluable genetic engineering tool for studying functional mammalian genetics, mammalian developmental biology and for producing animal models of human diseases. At present human

and mouse ESCs have been the two predominant animal models investigated by researchers. However, as described above, cells derived from mice and humans differ considerably. Furthermore because of legal/ethical problems tied to the use of blastocysts, the development of alternative, non-human embryonic stem cell lines is crucial for continued extension of research and for development of stem cell therapies.

In this scenario the establishment of ESC lines in large mammal is of great interest for basic research (comparative embryology and the cell biology of ungulate stem cell maintenance and differentiation) and represent an advantage due to their physiological similarity to the human and perhaps more relevant for clinical translation studies compared with mouse ESCs.

Over the past 20 years many reports of sheep [37], hamster [38], dog [39], cat [40], mink [41], rabbit [42], horse [43], cattle [44] and pig [45-47] ESC lines, or what are often presented as "ESC-like cell" lines, have been published. Despite this, the primary problem for ESCs in domestic animal is that no standard protocol for their isolation and culture currently exists. Indeed, based on previously definition of ESC, none of the ungulate cell cultures or cell lines described have been definitively proven to be ESCs, and none have been successfully used as biological reagents in a manner similar to that of human, monkey, or mouse ESCs, i.e., directed pluripotent *in vitro*

differentiation [48,49] or as a means of genetically engineering a mammal through embryonic chimera formation [50].

1.2.4 Embryonic stem cells in pig

The experiments described in this thesis were carried out in the pig. The choice of using the porcine model is based on the well known similarities in term of anatomy, physiology, metabolism and organ development with primates that make this large animal the ideal link between the classical rodent models and the human.
Peer-reviewed reports of porcine ESCs, ES-like or ICM cell lines have been published all of which used *in vivo*-derived blastocysts as their primary culture material [37,45,51-54]. This occurred because of the well known difficulties in the production of porcine embryos *in vitro* that is still challenging with low efficiency and quality. On the contrary, generally, primate ESC lines have been established from *in vitro*-fertilized (IVF) *in vitro*-cultured (IVC) blastocysts of humans [55,56]. Furthermore given the high cost and the low efficiency of ESC derivation from *in vivo*-derived embryos [57], more researchers are working with domestic species to produce *in vitro*-embryos and to use them for ESC isolation.
Only in the last period few studies have described the isolation of putative pig ESCs using *in vitro* produced blastocysts [58-61]. Furthermore putative porcine ESCs established by Notarianni et al. were poorly defined [45]. In other studies no ESC-like cells

survived passage 10, while epithelial-like lines survived up to passage 42, but failed to differentiate [47]. Talbot et al. reported that pluripotency was difficult to maintain in ESC-like and epithelial-like cell cultures for more than a few passages [62]. Further studies have been conducted by other authors [53,63], however, the general consensus is that none of these lines were truly ESCs and pluripotent [64,65] and a number of technical questions are still to be answered. The use of conventional protocols for culture of mouse and human ESC does not appear to sustain extended growth nor pluripotency of cultured porcine cells. Recently it has been demonstrated that medium components play a pivotal role in regulating intrinsic and extrinsic factors involved in the control of pluripotency and that specific pathways may be up-regulated, or down-regulated, in response to the addition of specific molecules [66]. It is evident that many important aspects need to be elucited in order to be able to establish homogeneous porcine outgrowths and ensure a favorable environment for porcine ESC maintenance[67].

1.2.5 Embryonic stem cells and ethical concern

Isolation of embryonic stem cells cannot be obtained without destroying a viable embryo and for this reason, despite the potential therapeutic usefulness, their derivation in the human

raises substantial religious, ethical as well as legal and political concerns. In this scenario a number of scientific attempts that do not require the generation and subsequent destruction of human embryos have been proposed in order to fill the gap between ethical questions and potential scientific and medical benefits [68]. These different approaches involves obtaining stem cells from embryos that are clinically dead or developmentally arrested [69,70], or removing single blastomeres from less developed embryos (morulae), using an approach similar to that used for preimplantation genetic diagnosis during *in vitro* fertilization (IVF), in which a single cell is extracted from an embryo and tested for genetic disorders [71,72]. The possibility to create genetically modified biological artifacts obtained through altered nuclear transfer (ANT) that create abnormal blastocysts unable to implant into the uterus but capable of generating customized ESCs has also been proposed as an alternative possibility to produce ethically acceptable cell lines [73-77]. Epigenetic reprogramming of adult stem cells is a further and very promising approach for the derivation of stem cells [78-80]. In particular, the recent identification of 4 factors that seem to play a crucial role for the induction and maintenance of pluripotency, namely Oct-3/4, Sox2, c-Myc and KLF4, have allowed for the establishment of new cell lines called induced pluripotent stem (iPS) cells that are similar to ESCs in morphology, proliferation and pluripotency [81-83]

but derived from terminally differentiated cells. The use of artificial parthenogenesis in order to create entities, namely parthenotes, which may represent an alternative ethical source for pluripotent cell lines is also a promising possibility [84]. Such embryos constitute a potential source of pluripotent cells that would be isogenic with the oocyte donor [85,86] and therefore suitable for use in cell or tissue replacement therapy.

Since many features of parthenogenetic cells are actually unknown and several biological aspects need to be elucidated, it is not possible to consider this cells suitable for therapy. In this thesis different cell lines isolated from human and porcine parthenogenetic embryos and from sheep parthenogenetic fetuses will be studied and analyzed in detail.

1.3 Parthenogenesis

1.3.1 Parthenogenesis in mammals

Parthenogenesis is a form of asexual reproduction, by which an oocyte can develop without the intervention of the male counterpart. This process may routinely occur naturally in some lower species such as fish, ants, flies, honeybees, amphibians, lizards and snakes.

By contrast, mammals are not spontaneously capable to use this form of reproduction. However, mammalian oocytes can be

successfully activated *in vitro*, using a variety of stimulations, such as ethanol [87-93], electric shock [94-96], strontium chloride treatment [97,98], and ionomycin [99,100], which mimic the intracellular calcium wave induced by sperm at normal fertilization [101,102]. This phenomenon causes cleavage divisions and embryonic development. However, mammalian parthenotes are unable to develop to term due to genomic imprinting alteration that result in the repression of paternally expressed genes [103]. The consequent developmental abnormalities cause an arrest of parthenote development at different stages after activation - depending on the species [104] - giving embryo-like structure unable to form a new individual.

Parthenotes can be obtained in two different ways. The most common consists of combining the activation of metaphase-2 oocytes with exposure to an actin polymerization inhibitor, usually cytochalasin D and B [89-91,105], cycloheximide [92,93] or 6-dimethylaminopurine (DMAP) [99,100,106,107]. Alternatively a diploid parthenote can be generated by preventing the extrusion of the first polar body. This protocol leads to the formation of tetraploid oocytes [89] and the diploid status is then re-established at the end of oocyte maturation with the extrusion of the second polar body.

1.3.2 Parthenogenesis and zygosity

An important aspect related to parthenogenesis is zygosity. It refers to the similarity of alleles for a trait in an organism and is used to describe the genotype of a diploid organism at a single locus on the DNA. If both alleles are the same, the organism is homozygous for the trait, while if alleles are different, the organism is heterozygous (Figure 8).

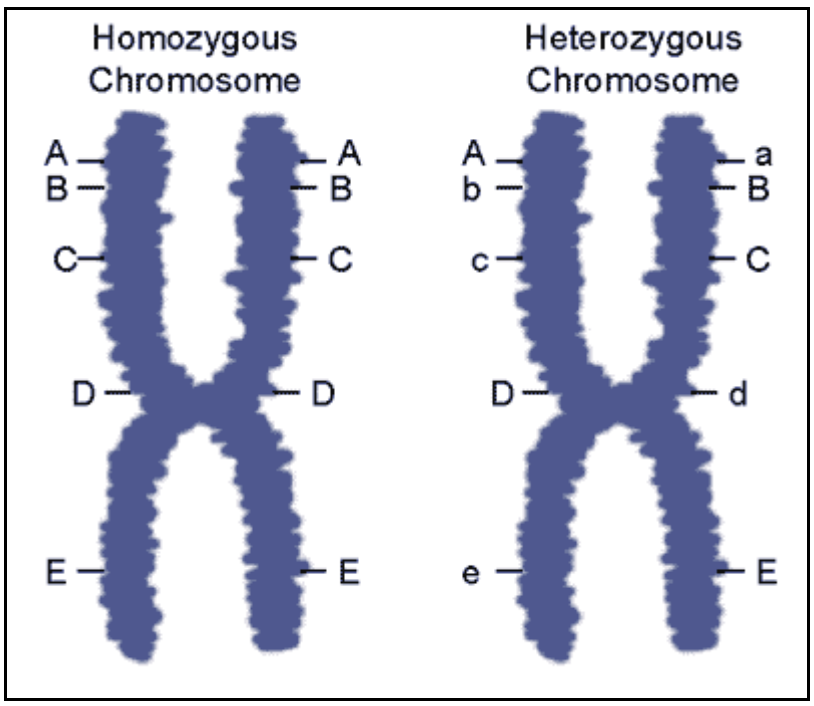


Figure 8. Representative figure of homozygous (left) and heterozygous (right) chromosome for different alleles.

Using one or the other method described above to obtain parthenogenesis in mammals (paragraph 1.3.1) has important consequences for the genetic make-up of the parthenote in question [108]. Performing oocyte activation before inhibition of the second polar body extrusion determines the formation of highly homozygous parthenotes, since diploid status of the parthenote is obtained after segregation of sister chromatids (Figure 6). In contrast, when the first polar body extrusion is inhibited, parthenotes are genetically identical to each other and have the same heterozygosity as their mother [89] (Figure 9).

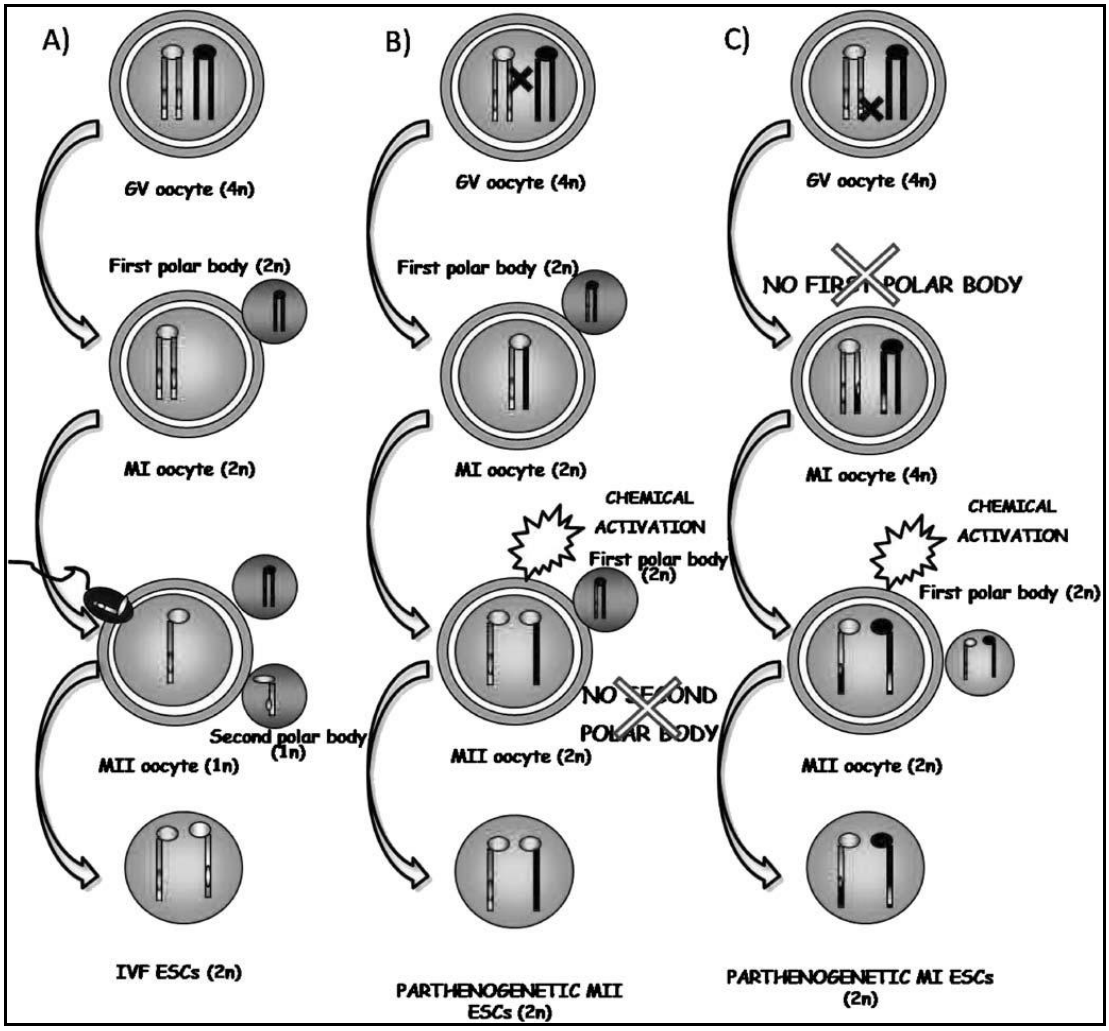


Figure 9. Genetic composition of *in vitro* fertilized embryos (A) and parthenotes where extrusion of the II polar body (A) or I polar body(C) is inhibited. (From Brevini et al., 2008).

The occurrence of a high degree of homozygosity in parthenotes has been evaluated in contrasting ways in the perspective of using these entities as a source of embryonic stem cells. Homozygosity can be seen as a potential benefit when reduction of immunogenicity of a stem cell derivative is considered. The

possibility of generating stem cells that are homozygous for all three sets of HLA (A, B and DR) would exponentially increase the number of phenotypes a graft fully matches. Furthermore, homozygosity has also been suggested to be an advantage to be exploited for selecting cell lines carrying drug response genes, or a disease gene or cancer gene correction, providing a useful research tool for drug testing and development [109]. At the same time, it must be remembered that homozygosity can represent a severe risk. Loss of heterozygosity could amplify any negative genetic component potentially present in the genotype.

1.3.3 Parthenogenesis and asymmetric imprinting

The term 'genomic imprinting' is used to describe the functional differences between parental genomes during mammalian development, but was not described in animals, other than mammals [110]. In particular, the imprinting can be consider an epigenetic mechanisms which regulates hereditary changes in gene expression not related to disturbed sequences of DNA nucleotides. It is involved in many processes of normal and pathological development of humans and animals [111]. Interestingly, a number of studies demonstrated that various anomalies and mortality exhibited by mammalian clones may be due to genomic imprinting [112,113]. In cloned mice and sheeps, for

example, uncontrolled growth of embryonic tissues and organs and an increase of 8 to 50% in embryo weight of embryos may reach 8 to 50% were observed [114]. These problems seems to be caused by disturbed expression of imprinted genes due to manipulations of gametes and early embryos [115-117].

This observation suggested that the parental genomes are complementary but functionally non-equivalent, despite the fact that they have equivalent genetic information [8]. This explains why both genomes are needed in mammalian development. Indeed as described by Surani, although oocytes are potentially totipotent in many organisms, this is not so in mammals [103]. This is because the maternal genome is epigenetically modified in the germ line to contain only maternal 'imprints', which normally results in repression of certain maternally inherited imprinted genes (Figure 10). A paternal genome is therefore essential to 'rescue' the oocyte, as the maternal genes are imprinted reciprocally to paternal imprints.

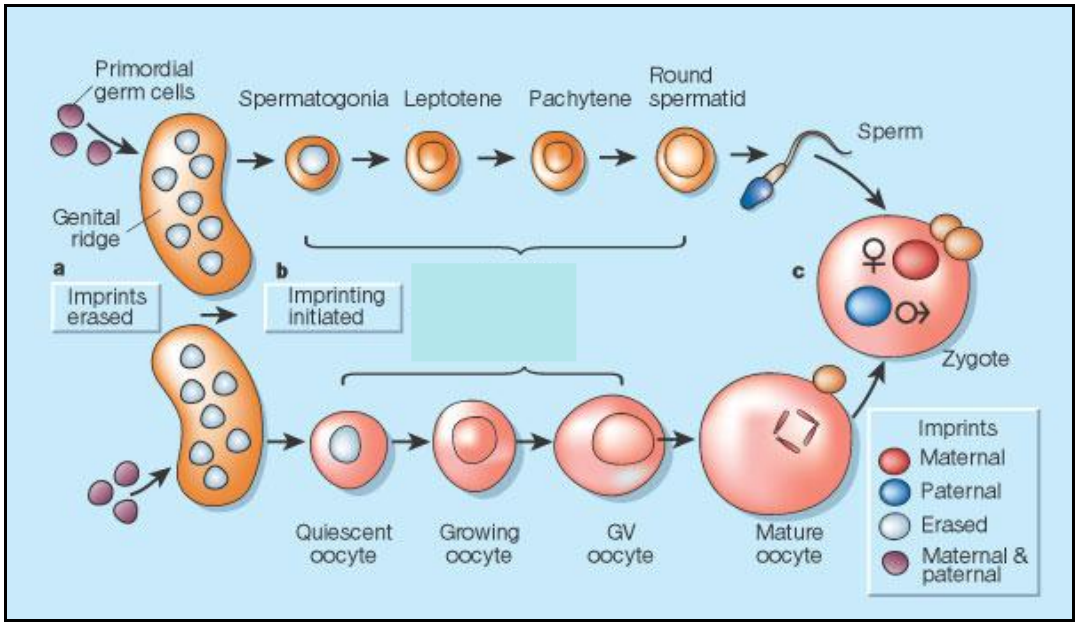


Figure 10. Imprinted genes are 'marked' in a parental-specific way. Primordial germ cells enter the genital ridge of developing male and female embryos and all imprinting marks are erased (a). Male (top) and female (botton) germ cells grow and develop into sperm/oocytes and reintroduction of paternal and maternal-specific imprints take plays (b). After fertilization, a zygote is formed with both genetic and epigenetic (imprinting) contributions from the parental genomes (c). (From Surani M.A., 2002).

Convincing evidence of parental imprinting was obtained in mice only in the 1980s, when two groups, Surani and his coworkers [118,119] and McGrath and Solter [120], demonstrated that both chromosome sets, maternal and paternal, are essential for the normal development of mammals. This explains why in none of

more than 4500 known species of mammals, parthenogenesis has been described.

However, the possibility of artificial parthenogenetic activation of mammalian oocytes lead numerous investigations of genomic imprinting using parthenotes as model. These studies began in the 1930s [121], but only in the 1980s effective methods were found for activation [122] and development of parthenogenetic embryos to the somite stages [123].

At present it is well known that imprinting has two major effects on parthenotes. Firstly, imprinting alteration affects parthenogenetic cell growth in the fetus and post-natally [124]. Secondly, it causes a restriction of cell lineages in which parthenogenetic cells can participate. During embryo development, in fact, the dramatic consequences of genomic imprinting have been clearly demonstrated by failure of parthenotes to give raise to their trophectoderm and primitive endoderm, which results in failure of the extra-embryonic tissues (especially placental tissue, [125]) in parthenogenetic conceptuses [118]. Indeed mammalian parthenogenetic embryos can develop normally up to the 40-somite stage [126,127], but they are unable to develop to term and die shortly after this stage, as showed in Table 3. In the mouse, for example, the most advanced parthenotes survive to the early limb bud stage, have little extra-embryonic tissue and almost no trophoblast [128].

Species	Maximum development (days)	Pregnancy length (days)	Reference
Mouse	10	21	Surani et al. 1986
Rabbit	10-11	31	Ozil 1990
Pig	29	114	Kure-bayashi et al. 2000
Sheep	25	150	Loi et al. 1998
Bovine	48	280	Fukui et al. 1992
Marmoset monkey	10-12	144	Marshall et al. 1998

Table 3. Summary of maximum development of mammalian pathenotes and length of normal pregnancy.

At present, approximately 50–100 imprinted genes have been identified in the mouse and human genomes. These display a vast range of functions, ranging from splicing factors, such as Snrpn [129], to growth factors, such as insulin (Ins1 and Ins2), Igf-2 [130,131], Igf2r [132], to genes that are functional as RNAs, such as H-19 [133] and Xist (reviewed by Bartolomei [134]).

Since both parental genomes are needed for generating a functional genome, it comes as a consequence that parthenogenetic blastocyst-like structures do not possess a functional genome that can be considered distinctive of a human embryo. Their genome is in reality constituted by a double set of an epigenetically imprinted female gamete genome and should be more correctly considered unfertilized eggs that have been

activated to initiate cell division. Since they are able to undergo a few cycles of cell division during the last years parthenogenetic blastocysts have been considered as a possible ethically acceptable source for human pluripotent cells.

1.3.4 *In vitro* animal parthenogenesis

The first paper describing the obtainment of a mammalian parthenote was published in 1936 by Gregory Goodwin Pincus, who successfully induced parthenogenesis in a rabbit [121]. During the following years this approach was applied to other mammalian species such as rat [135], mouse [136], ferret [137,138] and hamster [139]. A significant milestone in reproductive science has been achieved by Kono et al. with the birth of the first viable parthenogenetic mouse [140]. Using gene targeting, they were able to manipulate two imprinted loci H19/IGF2 and DLK1/MEG3 to produce bi-maternal mice at high frequency [141]. Pups were obtained at day 19.5 of gestation, showing that fatherless mice have enhanced longevity [142]. This work further expands what is achievable in artificial reproduction and may have important implications for understanding aspects of embryonic development and gene regulation.

At present parthenogenetic activation in no-rodent species is used for the derivation of embryonic stem cell lines (Table 4) or for biological studies.

Specie	Refecences
No-human primates	Vrana KE et al., 2003; Wei Q et al., 2011.
Pig	Brevini TA et al., 2007; Brevini TA et al., 2010.
Cow	Talbot NC et al., 2004; Talbot NC et al., 2007; Pashaiasl M et al., 2010.
Buffalo	Sritanaudomchai H et al., 2007.
Rabbit	Fang ZF, et al., 2006; Wang S et al., 2007; Hsieh YC et al., 2011.

Table 4. Parthenogenetic embryonic stem cell lines derived in non-rodent species.

1.3.5 *In vitro* human parthenogenesis

Data from the literature show that human oocytes can be successfully activated but often parthenogenetic development does not proceed beyond the eight-cell stage [143-148] [149-152]. Development of human parthenotes to the blastocyst stage has been reported only recently [109,153,154]. This achievement, together with the establishment of parthenogenetic stem cells in non-

human primates [155], stimulated new interest in human parthenogenesis because it could eliminate the requirement to produce or disaggregate normal competent human embryos for deriving pluripotent cell lines *in vitro* [153,156,157].

Data reported in the literature showed that a combination of ionomycin and 6-DMAP constantly enabled the development of parthenotes to the blastocyst stage [108]. This Ca^{2+} ionophore is able to induce one prolonged calcium peak that releases oocytes from metaphase arrest and then maturation-promoting factor quickly rises again and further development is arrested. The subsequent addition of 6-DMAP, which inhibits the reactivation of maturation-promoting factor following kinetics similar to those occurring after fertilization, leads to high *in vitro* development rates also in the human, confirming the results obtained in several other species including cattle [158], sheep [159], rhesus monkeys [160], rabbits [161] and pigs [162].

1.3.6 Ploidy of parthenogenetic embryos

Karyotype analysis has often been utilized to determine the normality of *in vitro*-produced embryos [163,164]. This investigation was carried out on parthenogenetic embryos in order to verify their ploidy. First studies performed in mice demonstrated that most of parthenotes displayed diploid chromosomal phenotypes

[87,105,123,165]. This high frequency of diploid chromosomal complements may be related to the maternal inheritance of centrosomes in this specie (see below paragraphs 1.4.3 and 1.4.4). Indeed, when parthenogenesis were applied on other mammalian specie, many chromosomal alterations were identified.

Winger et al. demonstrated that diploid bovine parthenotes arising from the application of standard procedures (ethanol or ionomycin activations following by cytochalasin D or 6-DMAP treatements) were a minority, while the majority of parthenogenetic embryo displayed polyploid and mixoploid chromosomal complements [166]. These results are in agreement with other studies revealing a greater incidence of chromosomal abnormalities as assessed by haploidy, mixoploidy and polyploidy in *in vitro* [167-169] or in spontaneously activated cattle embryos [170]. The same data were reported in porcine species activated by electric stimulation followed by cytochalasin B treatment [171] and in human puromycin or spontaneously activated oocytes [172-174].

The events contributing to these abnormal chromosomal complements seems to occur as early as completion of the first cell cycle [166]. As reported below (paragraphs 1.4.3 and 1.4.4), several studies demonstrated that the spermatozoa introduce the centrosome during fertilization in both bovine and human [99,175-177] species as well as in porcine one [178]. This complex mechanisms

may be the possible link between ploidy alterations and parthenogenesis.

1.4 Centrosome

One important aspect that may lead to abnormalities in parthenogenesis is the centrosome organization.

1.4.1 Centrosome and its function

The centrosome, approximately 1 μm in size, is a non membrane-bound cytoplasmatic organelle composed by a protein complexes in animal cells. Its duplication occurs in a semi-conservative manner with each centrosome receiving one old preexisting "maternal" centrioles and one new. It is a no static structure, since it changes shape and components throughout the cell cycle [179] and has several functions summarized in Table 5. In particular, the main function of centrosome is the organization of both interphase microtubule arrays responsible for cell polarity and of the mitotic spindle, which mediates bipolar separation of chromosomes. Indeed centrosomes are closely associated with the nucleus during interphase and undergo cell cycle-specific reorganizations during mitosis. In addition it is also implicated in numerous other cellular functions such as reported in table 6 (reviewed by Kimble M. and Kuriyama R. [180] and Rose M.D.[181])

and impacts cellular processes such as entry into mitosis, cytokinesis, Gl/S transition and monitoring of DNA damage.

Function	Responsible element
Intrinsic	
Reproducing element	Unknown
Microtubule organizer	Unknown γ-tubulin binding protein
Microtubule nucleation peripheral	γ-tubulin
Peripheral	
Nuclear association	Gene products
Configurational alterations	Affected and effected by MTs and MFs
Centrosome destruction	Absence of duplication of reproducing elements
Cell's pacemaker: orchestrator of the cell cycle's regulation	Arrangement of kinase, cyclins and phosphatises
Centriole assembly and axoneme excision	Involves centrin and microtubule assembly
Cell body organizer	Perhaps derived from microtubules

Table 5. Summary of centrosome functions.

Each mammalian somatic cell typically contains one centrosome, which must double at each cell cycle and divide between the two daughter cell at each cell division, like chromosomes and cytoplasm (Figure 11). The centrosome duplicate during interphase, at S phase, in coordination with maximum DNA decondensetion and subsequent replication (Figure 11). After duplication the two separated centrosomes undergo a maturation process and move to opposite sides of the nucleus. During cell division, centrosomes form the poles of the bipolar mitotic spindle. After anaphase and telophase, centrosomes become separated into two daughter cells (Figure 11).

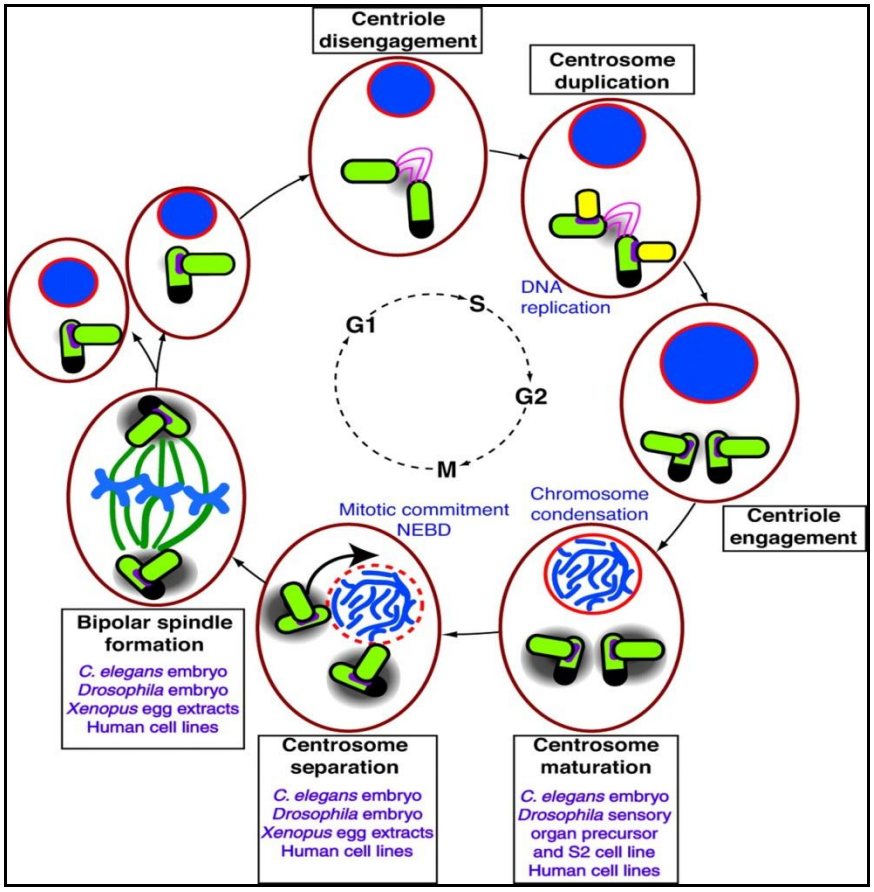


Figure 11. The centrosome cycle. At the beginning of G1 phase, cells contain a single centrosome with two perpendicularly aligned, closely associated centrioles. During G1 phase, the tight link (purple bar) between the centrioles is dissolved (centriole disengagement), but centrioles remain connected by a loose fibrous structure. In S phase, the centrosome duplicates simultaneously with DNA replication. Duplication involves the assembly of two new centrioles perpendicular to the existing centrioles. In late G2 phase, the two centrosomes undergo maturation by recruiting additional PCM (grey circle) components to prepare for their role as spindle poles. The centrosomes then separate and move to the opposite side of the nucleus. After chromosome segregation in anaphase and telophase, two new daughter cells are produced, each with one centrosome associated with the nucleus. (From Barr A.R. and Gergely F., 2007).

1.4.2 Centrosome molecular composition

Centrosome consists of a pair of orthogonally arranged cylinder-shaped centrioles surrounded by an amorphous matrix of electron dense proteins referred to as pericentriolar material (PCM Figure 12).

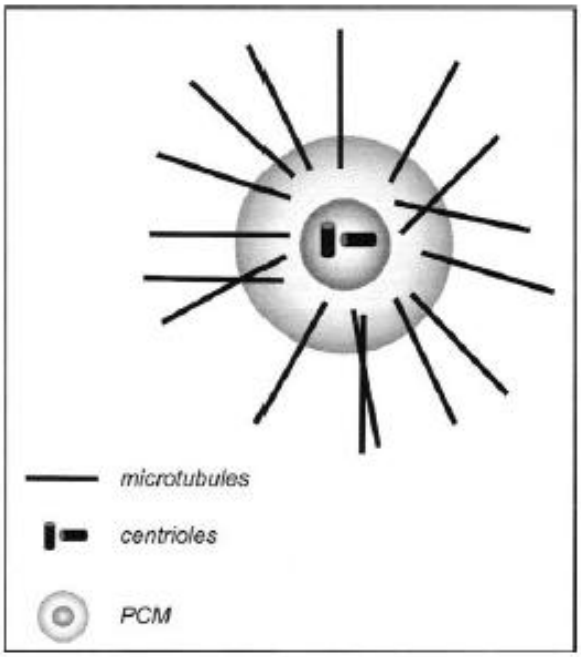


Figure 12. Centrosome structure. The centrosome is composed of two centrioles surrounded by a protein matrix called the pericentriolar material (PCM). (From Sun Q.Y and Schatten H., 2007).

Centrioles show the classic 9 + 0 pattern of nine triplet microtubule and no central pair of microtubule (Figure 13) and contains centrin, cenexin and tektin [182]. "The centrosome in vertebrates: more than a microtubule-organizing center). In particular, centrin is a calcium-sensitive 20-kDa protein with contractile properties [183,184] that bind calcium ion transients and cause a centrosome shape changes. Centrioles duplication is semi-conservative, with each daughter cell retaining one of the mother's centrioles and a newly formed daughter centrioles [185].

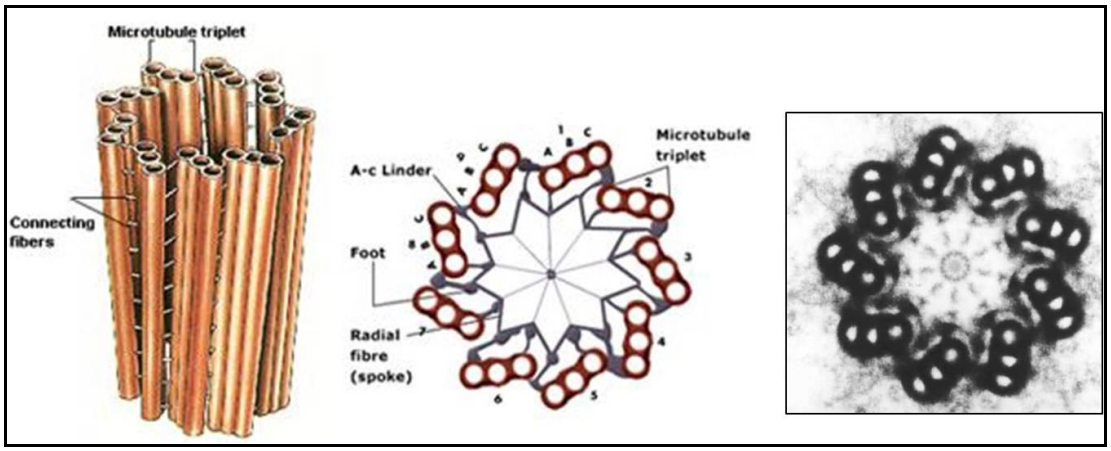


Figure 13. Centriole structure. Each centriole is made up of nine microtubule triplets, which lie evenly spaced in a ring. There are no microtubules in the center (9+0 arrangement). Transmission electron microscopy image of centrioles transverse section (right).

The PCM contains a complex meshwork of proteins responsible for microtubule nucleation and anchoring [186] including γ-tubulin [187,188] [189,190], pericentrin and ninein. Usually γ-tubulin and centrin are two classical markers of the centrosome with specific localizations, but the majority of these two proteins (80-90%) are not centrosome-associated, while matrix proteins are only concentrated at the centrosome with specific localizations [191]. Pericentrin anchors γ-tubulin complexes at centrosomes in mitotic cells, which is required for proper spindle organization. Another essential cell cycle-dependent centrosome-associated proteins is the nuclear mitotic apparatus protein (NuMA) that is distributed to the separating centrosomes during early mitosis. This protein ensures the cross-linking of spindle microtubules on the centrosome side facing the chromosomes, which is essential for the organization and stabilization of spindle poles from early mitosis until at least the onset of anaphase.

1.4.3 Centrosome reduction in gametes

Originally spermatids and primary oocytes display a typical centrosome organization with a pair of centrioles surrounded by pericentriolar material, in common with somatic cells. This somatic cell-like centrosomes undergoes profound modification and/or degeneration during the final stages of gametogenesis to

meet the specific needs of gamete functions and fertilization. Indeed in mammals, with the exception of mouse, spermatozoa retain centrioles but lose most of the pericentriolar centrosomal proteins, whereas oocytes lose centrioles while retaining a stockpile of centrosomal proteins [192]. This reciprocal reduction of centrosomal constituents makes sperm and oocyte complementary to each other and they became able to form a functional centrosome in the zygote only after fertilization.

In rhesus monkey and human, centrosomes are reduced during spermiogenesis, but not as completely as in mice (see above). Their spermatozoa have proximal centrioles intact (Figure 14), whereas the distal centrioles are mostly disorganized or highly degenerated together with γ-tubulin and centrosomal proteins [193,194] (Figure 15).

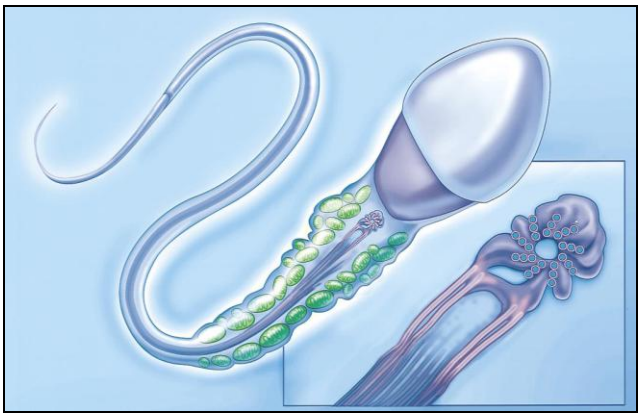


Figure 14. Proximal and distal centrioles of the human spermatozoon. (From Palermo G.D. et al., 1997).

In agreement with data reported in primates, previous studies demonstrated that boar ejaculated spermatozoa show residual centrioles in sperm tail [178] as well as proximal centrioles were present in sheep mature male gametes [195,196].

On the contrary, in mouse, gamete centrosome modifications and degenerations is completely different. Centrosome reduction takes place during spermatogenesis when it loses the microtubule nucleating function, then lose centrosomal proteins and finally lose centrioles (Figure 15). In particular, murine microtubule and centrosomal proteins are discarded during spermiation, and the distal centriole degenerates during the testicular stage of spermiogenesis, while the proximal centriole is lost during the epididymal stage [197,198].

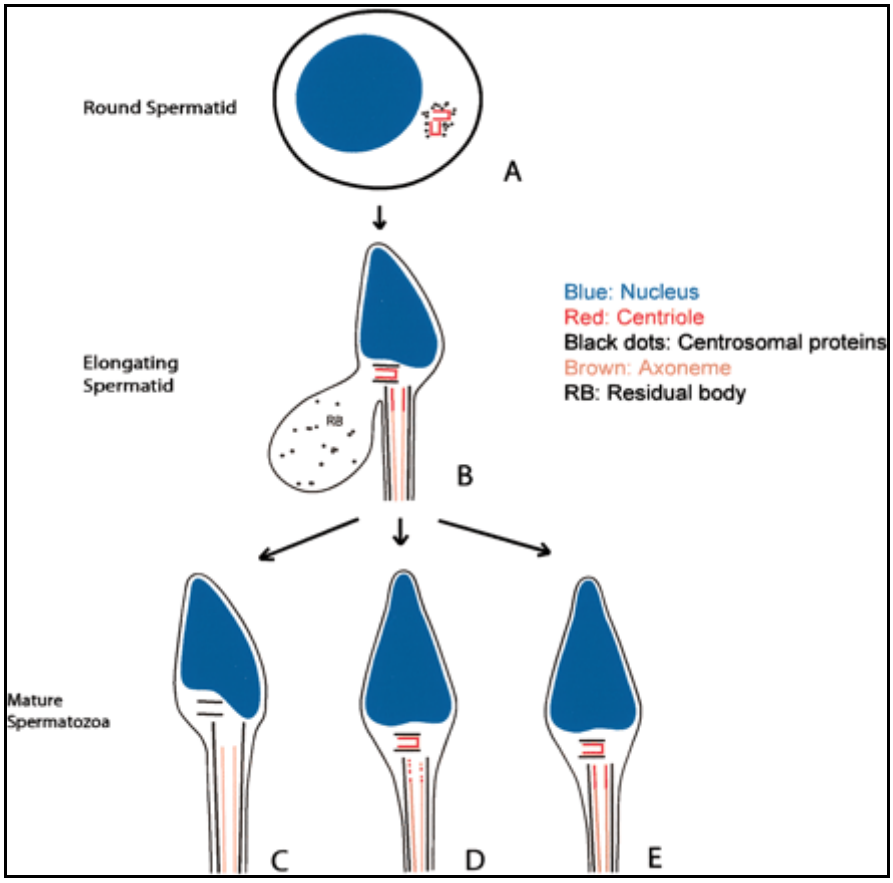


Figure 15. Centrosome reduction during spermiogenesis. The male germ cells possess intact centrosomes containing centrioles and centrosomal proteins until the round spermatid stage (A). The microtubules of the distal centriole extend as axoneme of the spermatid tail (B). During spermiation, γ-tubulin and other centrosomal proteins are disjuncted from the centrioles and discarded with the residual bodies (B). The centrioles are degenerated to various extents in spermatozoa of different species. Rodent and snail spermatozoa lose both centrioles completely (C), whereas primate spermatozoa retain proximal centrioles intact but degenerate distal centrioles partially (D). Xenopus and Drosophila spermatozoa possess both centrioles intact (E). (From Manandhar G. et al., 2005).

Contrary to spermatogenesis, centrioles disappear from the oocyte during early oogenesis. In particular, oogonia and fetal oocytes display normal centrioles until pachytene stage, while these organelles are absent in the mature oocytes [199]. This degenerative process has been demonstrated in rhesus monkeys [200], rabbits [201], sea urchins [202], Xenopus [203] and many other species [204] including sheep [195,196], cow [99,205] and pig [178], while there is no experimental evidence of centriole degeneration in mouse oogenesis [206] (Figure 16).

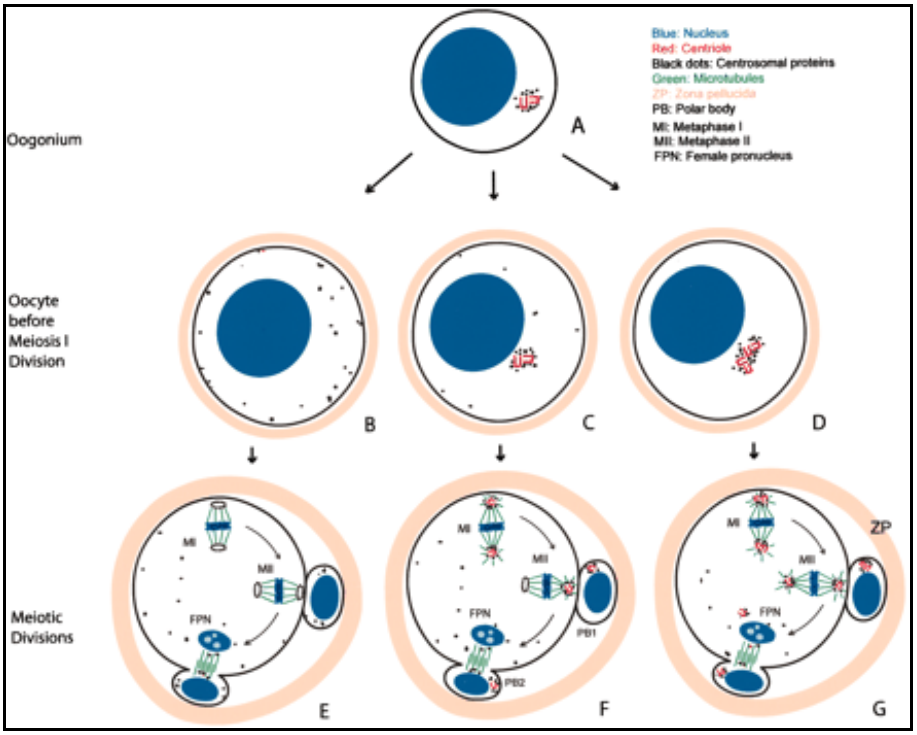


Figure 16. Centrosome reduction during oogenesis. The oogonia possess standard centrosomes containing centrioles and centrosomal proteins (A). The centrioles are either retained or degenerated during meiotic arrest in different animal oocytes. Mammalian primary oocytes lose both centrioles completely (B) resulting in acentriolar and anastral poles during meiotic I and II divisions (E). The pericentriolar centrosomal proteins are dispersed in the oocyte cytoplasm during non-dividing stage (B) or distributed as concentric poles of the barrel-shaped spindles during dividing stages (E). In snail primary oocytes, the centrioles are retained, but they do not replicate during the meiotic arrest (C) and the mature eggs are without centriole (F). In starfish primary oocytes the centrioles duplicate only one time during the dictyate stage producing four centrioles before beginning the dividing stage (D) and at the end of meiosis the mature eggs retain one centriole from the inner pole (G). (From Manandhar G. et al., 2005).

1.4.4 Centrosome restoration after fertilization

At fertilization, sperm and egg equally contribute haploid genomes as well as the relative centrosome components. Since in mammals, with the exception of mouse, centrosome of MII oocyte is greatly reduced/inactivated and centrioles are absent, early embryo development requires maternal and paternal contribution and, in particular, needs their elements to restore normal and functional centrosomal structure.

In fact, while in mouse there is no evidence of a functional centrioles in the sperm and they are maternally inherited, in the human and other mammalian species, the spermatozoa contribute the proximal centriole during fertilization (Figure 17).

In agreement with this, in mammalian species, whit the exception of mouse, sperm tail and its centriole-harboring connecting piece are incorporated into the ooplasm together with the sperm head. In the human after insemination, sperm head decondenses in the ooplasm and the proximal centriole remains intact forming the sperm aster sited around the male pronucleous subjacent to oocyte cortex. On the contrary, most sperm cytoplasmic structures including mitochondria, fibrous sheath, microtubule doublets, outer dense fibers and the striated columns of the connecting piece are discarded in a programmed order [207]. Sperm aster enlarges as male pronucleous decondenses and moves in

cytoplasm, ensuring male and female pronuclei apposition and formation of a single mitotic metaphase plate with a bipolar spindle. The human sperm centriole duplicates during the pronuclear stage, and at syngamy, one or two centrioles are located at opposite poles of the first mitotic spindle [208,209] together with a surrounding halo of electron-dense PCM that nucleates microtubules [207]. The mitotic spindle is fusiform and is generally almost centrally located [209,210], having never been identified in the cytocortex. In human embryos, centrioles were detected from the one-cell to the eight-cell stages of embryonic cleavage, and even in the hatching blastocyst [209]. Thus, the zygote centrosomes are ancestors of centrosomes in embryonic, fetal and adult somatic cells [209,211]. The same process were described in sheep, where 20 hours after insemination are detectable one bipolar spindle displaying two centrioles at the opposite pole (first embryo mitosis) [195].

On the contrary, recent study by Manandhar et al. revealed that in the pig sperm centrioles are lost in zygote after *in vitro* fertilization and they are not detectable until the late blastocysts stage. Accordingly, the early pre-implantation cleavages show broad and anastral spindle poles, while only blastomers of the hached blastocysts developed centrioles comparable with those of culture cells [178].

Figure 17. Centrosome inheritance in non-rodent mammalian specie. Meiotic spindle during the extrusion of the first polar body (a). Meiotic spindle for the extrusion of the second polar body (b). Sperm before fertilization contains a proximal centriole, while MII oocyte show a meiotic spindle with acentriolar centrosomes. Sperm proximal centriole–centrosome complex form the aster and, after pronuclear apposition, centriole replicate. At syngamy, duplicated centriole–centrosome complex migrates around the zygote nucleus, it relocates to opposite poles to form the centers of the mitotic spindle poles and drives first embryo mitotis (b). (From Palermo G.D. et al., 1997).

It is easy to understand that sperm centrosome has important implications in human infertility, and it has been demonstrated that sperm centrosomal dysfunctions lead to aberrant embryonic development [211,212]. This has been also proven in a

globozoospermic patient, though an attempt to restore defective sperm centrosomal function has been performed, without success[213,214].

1.4.5 Centrosome and parthenogenesis

As described above (paragraph 1.3) unfertilized oocytes can be activated by various physical or chemical stimuli and their female haploid genome can be diploidized, but the embryonic development does not proceed to term. To understand this process it is important to consider that centrosomal material of MII oocyte does not organize into unified foci and is not capable to generate astral microtubules nor a correctly oriented spindle in the absence of centrioles [215]. Accordingly previous studies carried out in *Xenopus* showed that microinjected exogenous centrosomes can function as zygotic centrosomes and induce successful parthenogenesis. It was shown that the centrosomes from various cell types are capable of inducing parthenogenesis [216-218] in case they contain intact and replication-competent centrioles [219,220]. These observations imply that the oocyte centrosome reduction has evolved as a control mechanism to suppress parthenogenetic development. Due to the loss of functional autonomy, the oocyte centrosome cannot initiate or successfully complete normal

embryonic cleavages without being supplemented by a fertilizing spermatozoon.

Yet many insect species are obligatory or facultative parthenotes [221]. Indeed in lower specie successful parthenogenesis depends upon the oocyte's ability to generate complete and functional centrosomes in the absence of male gamete. In sea urchin and insects, for instance, parthenogenetically activated eggs generate multiple cytoplasmic asters, containing centrosomal proteins and centriole, possibly due to the absence of a correct control along the process of spindle formation [222,223]. Two of the multiple astral centrosomes become associated with the female pronucleus and form the mitotic spindle, whereas the others degenerate. Furthermore in some stick insect species, spermatozoa do not contribute centrioles, so eggs replenish all of the components of the zygotic centrosome.

Currently there are only a few data in the literature about mitotic spindle organization and parthenogenesis in mammals. Mouse oocytes do not receive centrosomes from spermatozoa, yet they do not develop through parthenogenesis. Probably this block is due to another control strategy present in eutherian mammals linked to imprinting [179] (paragraph 1.3.3). All these processes suggest us to study centrosomal organization in parthenogenetic cells, comparing these with their bi-parental counterpart.

1.4.6 Centrosome alterations and chromosomal instability

Recent studies have shown that several factors like abnormal kinetochore–spindle interactions, premature chromatid separation, centrosome amplification, multipolar spindles and abnormal cytokinesis can result in chromosomal segregation defects [224].

Usually chromosomal alterations and karyotypic instability are in relation with tumors. Malignant formations were studied in depth the past century. In 1929 Theodor Boveri, in fact, while studying chromosomal segregation in Ascaris worms and Paracentrotus sea urchins, suggested that malignant tumors arise from a single cell with an abnormal genetic constitution acquired as a result of defects in the mitotic spindle apparatus [225]. He was right, the best explanation today is that numerical chromosomal instability appears to arise as a result of chromosome segregational defects [226-229], most frequently resulting from multipolar spindles. Structural chromosomal instability results from chromosome breakage and rearrangement due to defects in cell cycle checkpoints, the DNA damage response and/or loss of telomere integrity [230]. Structural chromosomal instability frequently results from breakage-fusion-bridge (BFB) cycles, first described in maize by geneticist Barbara McClintock in 1938 [231]. In this process, a chromatid break occurs, exposing an unprotected chromosomal

end which, after replication, is thought to fuse with either another broken chromatid or its sister chromatid to produce a dicentric chromosome. During the anaphase stage of mitosis, the two centromeres are pulled to opposite poles, forming a bridge which breaks, resulting in more unprotected chromosomal ends, and thus the cycle continues [232].

Since we hypothesized that parthenogenetic cells posses centrosome alterations and multipolar spindle formations (Figure 18) we carried out a comparative analysis between parthenogenetic versus bi-parental cell line chromosomal assessment.

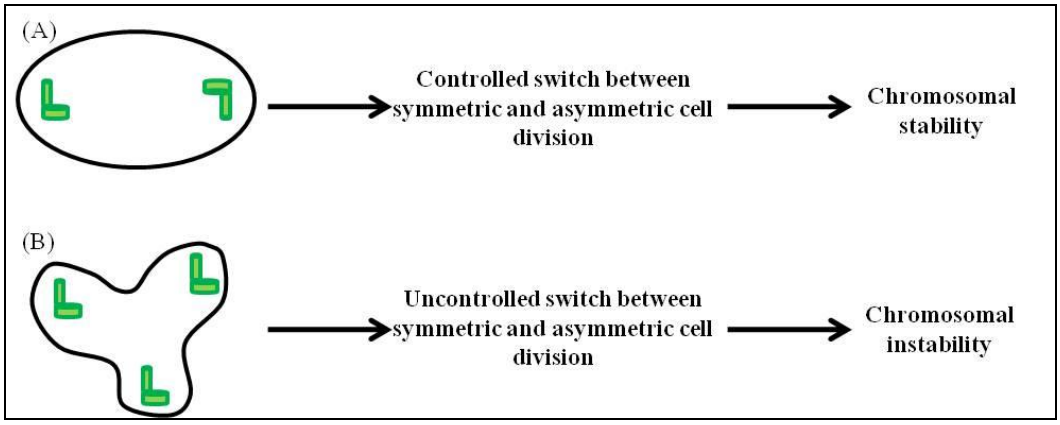


Figure 18. Normal centrosome organization ensure timely and controlled switching between symmetric and asymmetric cell division, resulting in correct chromosome segregation (A). Supernumerary centrioles are sufficient to cause chromosome malsegregation (B). (From Brevini T.A.L. et al., 2011).

Multiple chromosome malsegregations have been previously described in human oocytes after parthenogenetic activation, either spontaneous or induced by puromycin [173]. Consistent with this, a high incidence of polyploid and mixoploid chromosomal complements has been reported in parthenotes derived from bovine and porcine activated oocytes, with abnormal chromosomal complements occurring as early as completion of the first cell cycle [166,233].

Generally in all mammalian parthenotes, with the exception of mouse, the lack of paternal contribution to the centrosome is considered the most likely cause of such a high rate of aneuploidy. Furthermore since mammalian parthenote are able to achieve the 40-somite stage [126,127], it appears that centriole abnormalities does not limit cell proliferation, self-renewal and correct differentiation into a variety of tissues, indicating that the requirement of a paternal centrosome described in lower animals appears to be less stringent in mammalian cells. One explanation for this may be found in the hypothesis that genomic imprinting may represent the main mechanism that ensure bi-parental fertilization in higher mammals [179,234].

On the other hand, the link between the high percentage of parthenogenetic cells showing chromosome number alteration and mono-parental cell ability to proliferate is puzling.

1.4.7 Mitotic spindle defects and protein alterations

Several centrosomal proteins are closely related to centrosome organization and studies of their expression level alterations are often used to understand or confirm cell centrosome defects. In the studies described below in this thesis many protein related to spindle formation/organization and mitotic check point were also investigated in order to verify the alterations resulted by morphological or immunocitochemical analysis.

One family of proteins analyzed and that plays an important role in regulating centrosome function, bipolar spindle assembly, chromosome segregation and cytokinesis is the Aurora kinases [235,236] (Figure 19). The three members of the Aurora kinase family in mammals, Aurora kinases A/B/C (AURKA, AURKB and AURKC), are usually overexpressed in cancer cells and many studies were carried out on these cell type in order to characterize these kinases.

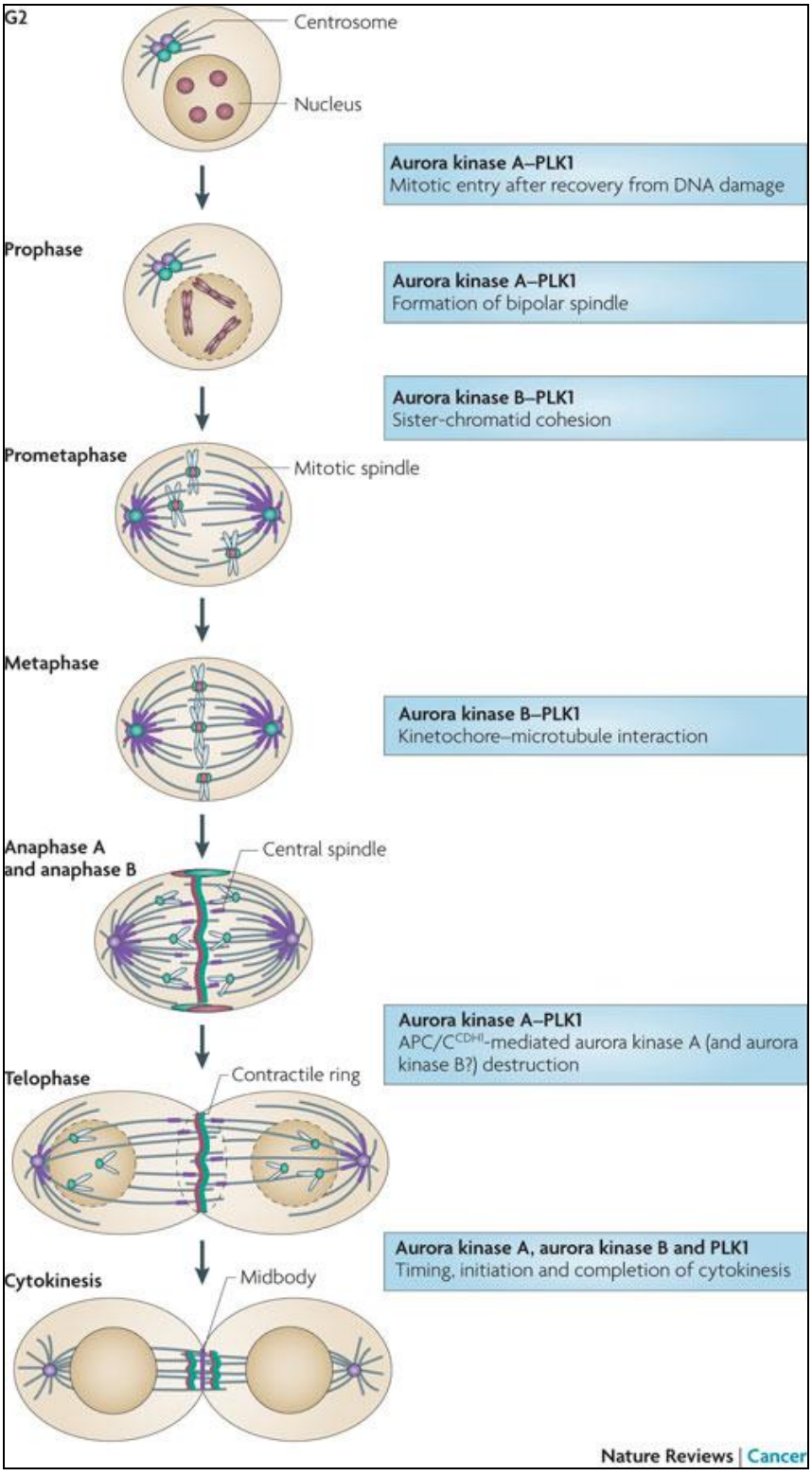

G2
Centrosome
Nucleus
Aurora kinase A–PLK1
Mitotic entry after recovery from DNA damage
Prophase
Aurora kinase A–PLK1
Formation of bipolar spindle
Aurora kinase B–PLK1
Sister-chromatid cohesion
Prometaphase
Mitotic spindle
Metaphase
Aurora kinase B–PLK1
Kinetochore–microtubule interaction
Anaphase A
and anaphase B
Central spindle
Aurora kinase A–PLK1
APC/C^{CDH1}-mediated aurora kinase A (and aurora kinase B?) destruction
Telophase
Contractile ring
Aurora kinase A, aurora kinase B and PLK1
Timing, initiation and completion of cytokinesis
Cytokinesis
Midbody
Nature Reviews | Cancer

Figure 19. From G2 phase of the cell cycle to metaphase of mitosis polo-like kinase 1 (PLK1; green) and aurora kinase A (also known as STK6; purple) co-localize at the centrosomes, but in contrast to aurora kinase A, PLK1 also localizes to kinetochores in prometaphase and metaphase. Aurora kinase B (also known as STK12; red), however, is not present on centrosomes and is first detected on chromatin in late G2. In prophase, aurora kinase B localizes to the chromosome arms and inner centromere, and in prometaphase and metaphase it localizes only to the inner centromere. In anaphase and telophase aurora kinase B co-localizes with PLK1 on the central spindle, cortex and midbody to regulate cytokinesis. Although aurora kinase A is degraded in anaphase and telophase a small proportion of the kinase is also associated with the central spindle and midbody; however, the exact function of aurora kinase A at this location is unclear. Crosstalk between aurora kinases and PLK1 is described in the blue boxes.

AURKA is localized in the centrosome from the time of duplication until the end of mitosis. Its overexpression has been associated with centrosome amplification and multipolar spindles. Indeed recent study by Anand et al. showed that AURKA overexpression, overrides the spindle assembly checkpoint, resulting in arrested mitosis with incomplete cytokinesis, leading to multinucleation [237].

AURKB is associated with the proteins, survivin and the inner centromere protein (INCENP). It is located in heterochromatin in early mitosis, in central spindle during anaphase, in cell cortex

when the contractile ring forms and then in midbody during cytokinesis. Studies by Hauf and colleagues demonstrated that this protein is involved in destabilization of improper microtubule attachments and also plays a role in maintaining the spindle assembly checkpoint [238]. Further RNA interference experiments showed that AURKB absence induces alterations of function and localization of the spindle checkpoint components, namely budding uninhibited by benzimidazoles 1 homolog beta (BUBR1/BUB1B), mitotic arrest deficient-like 2 (MAD2) and centrosome-linker motor protein (CENPE) [239] resulting in misaligned chromosomes, syntelic attachments of chromosomes to the spindle poles (in which both chromatids are attached to the same pole), cell division failure, and endoreduplication.

The last Aurora kinase, not studied in the thesis, AURKC, localizes to the centrosome from anaphase to telophase. It is essential for mitosis, but little has been published about its exact function and its substrates.

Another important protein family required for mitotic spindle assembly and function is polo -like kinase (PLK).

The first polo kinase was originally identified in a Drosphila mutants that display abnormal mitotic spindle organization [240]. Subsequently, potential homologues of Drosophila polo have been identified in yeasts and in mammals. Currently, the conserved family of PLKs consists of many members throughout various

species. Multiple PLKs are present in mammalian cells (PLK1, PLK2/SNK, PLK3/FNK/PRK, and PLK4/SAK) and the available data are consistent with the idea that these may also act earlier in the cell cycle, possibly during G1. If this hypothesis is correct, different members of the polo-like kinase family would act at several points during the cell cycle.

In this thesis particular attention was given to PLK2. It display a broad tissue distribution [241] and plays important roles in regulating cell proliferation in G1 and early S phases (Figure 20), a time when both PLK2 mRNA and protein levels peak [242,243]. Overexpression and depletion of PLK2 lead to an increase and decrease of centrosome numbers, respectively, indicating that it is fundamental for controlled centriole duplication during S phase[244].

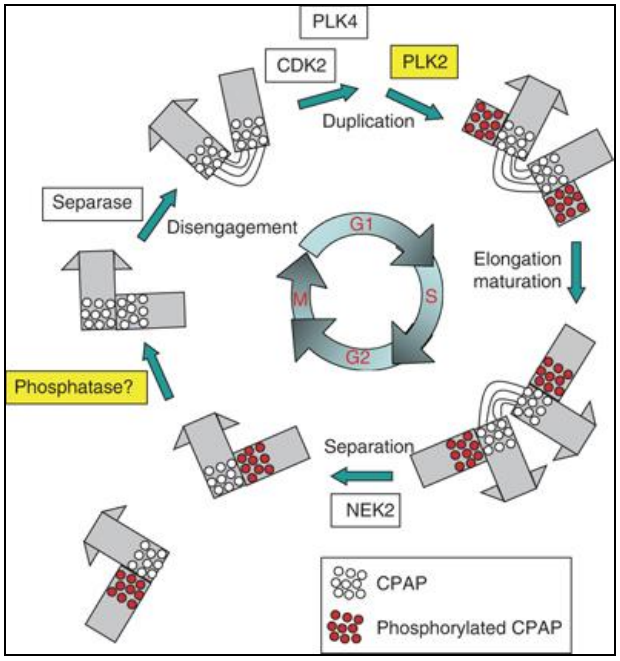


Figure 20. PLK2 control the centriole cycle in a close association with the cell cycle. PLK2 phosphorylation is important for the function of CPAP in procentriole formation at G1/S transition phase. The phosphorylated CPAP (red dots) is located at the proximal end of the procentriole. CPAP is dephosphorylated by an unknown phosphatase once the cell enters anaphase (blank dots). (From Chang J et al., 2010).

Other molecules analyzed in this thesis are TTK protein kinase (TTK), BUB1, CENPE [245] and mitotic arrest deficient-like 1 (MAD1) (Figure 21).

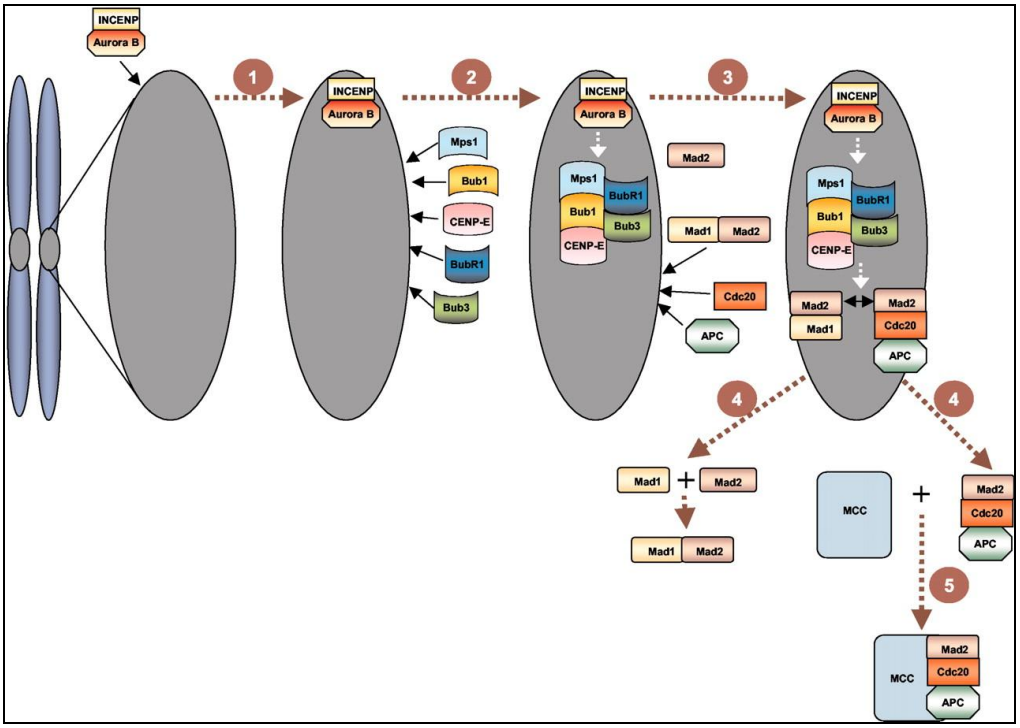


Figure 21. Proposed schematic diagram of the spindle checkpoint cascade. (From Vigneron S. et al., 2004).

All these proteins are normally involved in kinetochore-microtubule binding, correct chromosome congression and alignment as well as segregation during mitosis. In particular, studies by Abrieu showed that disruption of BUB1 and CENPE caused a reduction of tension across the centromere, an increase of incidence of spindle pole fragmentation and resulted in mono-oriented chromosomes approaching abnormally close to the spindle pole, with a mixture of aligned and unaligned chromosomes [246]. Moreover, usually the final result of CENPE inhibition is mitotic arrest, initiation of apoptosis and cell death

and this effect is more likely to affect rapidly dividing cancer cells. However redundant mechanisms have been described in mammalian cells to enable kinetochore microtubule binding and checkpoint monitoring in the absence of CENPE, although with poor positioning at the spindle poles and chronically monooriented chromosomes [247].

Another central component of the spindle assembly checkpoint and recruitment of kinetochores is MAD1 [248-250]. Deregulation of its expression has been shown to affect cell cycle progression and, in particular, the increase of its levels causes a reduction in the expression of v-myc myelocytomatosis viral oncogene homolog (MYC) in human monoblasts where a decrement of cell proliferation with a protracted G1 phase was observed [251].

A further molecule only recently identify as implicated in centrosome replication control and examined in this thesis is the Cyclin F (CCNF). Indeed, D'Angiolella et al. showed that siRNA mediated depletion of CCNF in HeLa cells induces centrosomal and mitotic abnormalities, including multipolar spindles and/or asymmetric bipolar spindles with lagging chromosomes [252] (Figure 22).

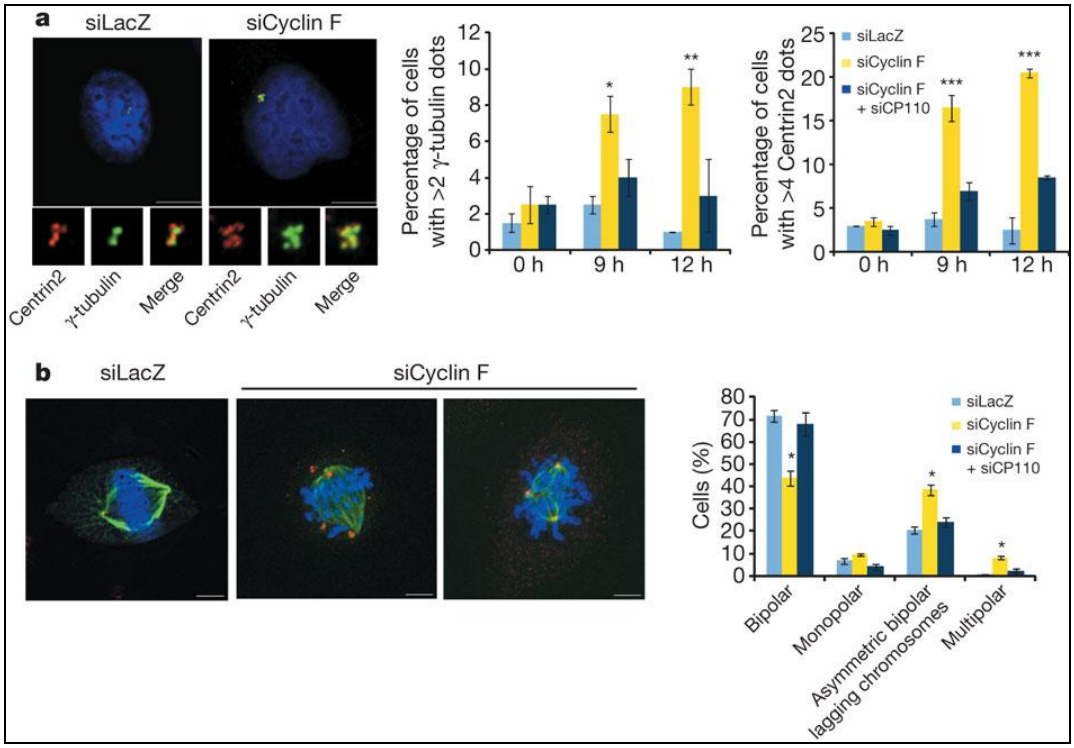


Figure 22. Cells transfected with siRNAs to Cyclin F show centrosome alterations (a) and form multipolar spindle (b). (From D'Angiolella V. et al., 2010).

1.5 Human parthenogenesis and parthenogenetic stem cells

Since it is possible to derive parthenogenetic embryos from supernumerary human oocytes, as described above (paragraph 1.3.5) recent studies also demonstrated that these embryo-like structures can be used in order to isolate pluripotent cells. First attempts at this have described low attachment of parthenogenetic embryos to feeder cells and arrest of proliferation after few cell divisions [109]. Only in the last few years several paper reported the

obtainment of parthenogenetic stem cells, displaying characteristics in common with their bi-parental counterpart [253-257]. In our laboratory we developed a protocol for isolation of ICMs from human parthenogenetic blastocyst-like structures. Parthenotes were subjected to enzymatic digestion with pronase in order to remove zonae pellucidae and ICM cells were separated from trophectoderm with microsurgical tecnique. These were plated of inactivated feeder layer and cultured as described by Brevini et al, 2009. Currently, these lines have been growing for over 4 years and possess most of the main features of bi-parental stem cells. In particular, as reported in our paper [257] (see Appendix A), these cells show the typical ESC morphology, express appropriate stem cell markers such as OCT4, NANOG, REX1, SOX2, alkaline phosphatise (AP), SSEA-4, TRA 1-81, and lack of the human ESC negative markers (e.g. SSEA-1). Furthermore our parthenogenetic cell lines also possess high levels of telomerase activity, which is turned down when cells are subjected to culture conditions that induce their differentiation, indicating that a physiologically normal control of telomerase activity is present.

In vitro differentiation potential of these cells was assessed and the results obtained demonstrated the ability of these cells to differentiate and give rise to components belonging to the three embryonic germ layers, while no expression of trophectoderm-related markers was detected, possibly reflecting parthenote

inability to generate adequate fetal membrane development and to implant correctly. Moreover, also driven differentiation, using controlled culture conditions, were tested on the cells. Data obtained in our laboratory demonstrate that parthenogenetic cells are able to differentiate and to form different cell subtypes belonging to the neural and hemopoietic lineage, not only giving rise to early cell population, but also generating more mature cell types expressing nestin, CNPase and MAP2 or showing lymphoid, erythroid and myeloid morphology, respectively. 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.

However, injection of our human parthenogenetic cells in immunodeficient SCID mice gave rise to poor differentiation or in the formation of myofibrosarcomas. This suggests the possibility of an intrinsic deregulation of the mechanisms controlling the choice between proliferation and differentiation in embryonic stem cells obtained through parthenogenesis. 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. Moreover we observed the presence of extranumerary centrioles and aberrant

ploidy. This is consistent with the high incidence of polyploid and mixoploid chromosomal complements reported in parthenotes derived from different species, including bovine, porcine and human, as described above (see paragraph 1.3.6).

2.AIM

As described above human parthenogenetic ESCs possess most of the main features of their bi-parental counterpart, including indefinite proliferation and *in vitro* differentiation abilities (see Introduction 1.5 paragraph and Appendix A). At the same time we also observed the presence of severe chromosomes abnormalities and extranumerary centrosomes.

We considered to be of biological interest to elucidate the origin of these discrepancies. To this purpose we performed a detailed characterization of parthenogenetic cell line specific properties and their differences with bi-parental ESCs.

Specific aims of this thesis were:

1. to investigate the mechanisms responsible for the severe anomalies observed in parthenogenetic cell lines;
2. to understand which adaptive mechanisms are active in parthenogenetic cells, allowing them to normally proliferate and differentiate despite the abnormalities.

To these purposes direct comparative studies were carried out on parthenogenetic and bi-parental human cell lines as well as on those obtained from animal models.

In particular we used pig and sheep because their parthenogenetic mechanisms are more similar to those of the human than mouse.

As described above (see paragraph 1.4.4), in fact, in the human and other mammalian species, the spermatozoa contribute the proximal centriole during fertilization, while in murine species functional centrioles are maternally inherited.

In the first set of the experiments we analysed if chromosome alterations are related to the use of human oocytes and the relatively old age of donors. It is well known, in fact, that advanced maternal age, as well as the interference of ovarian hyperstimulation with the natural selection of good-quality [258,259], are clear risk factors for chromosomal aneuploidy and mosaicism in the human embryo [260-264]. In this case we used the pig because oocytes can be retrieved from individual just after puberty, the age of maximal reproductive fitness in this species.

Furthermore we examined if the sever anomalies found in human parthenogenetic cells are caused by the derivation protocol and/or the culture conditions used in the experiments. To this end we transferred sheep parthenotes into synchronized recipients shortly after oocyte activation. Embryos were allowed to grow for 21 days and fibroblast cell lines were established and analysed.

In the second set of experiments reported in this thesis we investigated how such abnormal cells could survive, proliferate and correctly differentiate *in vitro*, instead of undergoing apoptosis as expected.

To this purpose we investigated the apoptotic mechanisms, the incidence of autophagy and the occurrence of special form of intercellular comunications.

3. MATERIALS AND METHODS

Unless otherwise indicated, chemicals were purchased from Sigma- Aldrich (Italy).

3.1 Human cell lines

The experiments performed in the human species were carried out on two parthenogenetic pluripotent cell lines previously obtained in our laboratory [257] (Appendix A). The bi-parental cell lines used as bi-parental counterpart were: HUES13 (generated in the Melton lab and used with permission), HES 7, HES I-3 and HES I-6 thanks to a collaboration with the group of Prof. Benvenisty N. of The Alexander Silberman Institute of Life Sciences of The Hebrew University of Jerusalem.

3.1.1 Human parthenogenetic cell line culture

Human isolated ICMs were cultured on feeder cells in human embryonic stem cell medium composed by Dulbecco's modified Eagle's medium, without pyruvate, high glucose formulation (Gibco, Italy) and supplemented with 10% Knock-out serum replacer (Gibco, Italy), 5% FBS (Gibco, Italy), 1 mM glutamine, 0.1 mM β-mercaptoethanol, 5 ng/ml human recombinant basic Fibroblast Growth Factor (bFGF; R&D System, USA) and 1%

nonessential amino acid stock (Gibco, Italy) as described by Brevini et al. [257].

3.2 Parthenogenetic activation

3.2.1 Porcine oocytes collection, *in vitro* maturation, activation and culture

Porcine ovaries were collected from gilts of approximately 120 Kg at the local abattoir and transported to the laboratory in physiological saline (9g/l NaCl) at 30-34°C. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles with an 18-gauge needle and vacuum pressure of 50 ml/min. The follicle aspirate was collected in 15 ml tubes (Terumo, VenoSafe™) and only COCs with a large, compact cumulus and homogeneous oocyte cytoplasm were selected for the *in vitro* maturation (IVM).
For parthenogenetic activation studies, IVM was performed on a total of 1221 oocytes as previously described by Grupen et al. [265] with minor modifications. COCs were cultured for 22 hours at 38.5°C in an atmosphere of 5% CO_2 in TCM-199 supplemented with 25% porcine follicular fluid, 1.1 mg/ml sodium bicarbonate, 0.1 mg/ml sodium pyruvate, 0.5 mM cysteamin, 8.2 μg/ml insulin, 10 ng/ml epidermal growth factor, 1 mM dibutryl cAMP, 0.5 IU/ml porcine FSH:LH (Pluset; Serono, Rome, Italy), 1.0 μg/ml 17 β-estradiol, 75 μg/ml penicillin and 50 μg/ml streptomycin (199-

IVM). After 22 hours COCs were washed in fresh medium and matured for an additional 24 hours in the same medium without dibutryl cAMP.

At the end of IVM (46 hours), oocytes were denuded by gently pipetting in TCM-199 HEPES buffered medium, containing 0.1% hyaluronidase at 38.5°C, washed for 10 min in the same medium supplemented with 20% (v/v) fetal calf serum (FCS, Gibco, Italy) and incubated in Tyrode's albumin lactate pyruvate medium (TALP for activation, see Table 6) for 30 min at 38.5°C. Parthenogenetic activation was performed according to the method described by Brevini et al. by sequentially exposing the oocytes to 5 mM ionomycin in TALP for 5 min at 38.5°C in the dark and to 2 mM 6-DMAP in medium NCSU-23 (see Table 7) for 3 h at 38.5°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 [266]. Presumptive parthenotes were washed thoroughly in NCSU-23 and cultured in groups of 25–35 in 50 μl NCSU-23 drops under mineral oil at 38.5°C in 5% O_2, 5% CO_2 and 90% N_2 atmosphere. On day 5 post-activation, half of the medium was replaced with fresh NCSU-23 containing 20% (v/v) FCS to reach a final FCS concentration of 10% (v/v) in the *in vitro* culture drop. On day 6/7 IVF blastocysts were obtained and ICMs were islated for culture.

TALP medium for activation	
Penicillin G	0,21 mM
Streptomycin	0,07 mM
Red Phenol	0,027 mM
D-Glucose	5,00 mM
NaCl	114,00 mM
KCl	3,16 mM
$MgCl_2.6H_2O$	0,50 mM
NaH_2PO_4 anhydrous	0,35 mM
$NaHCO_3$	25,00 mM
$CaCl_2.2H_2O$	4,72 mM
Na- lactate syrup	10 mM
Na-pyruvate	0,10 mM
Ca-lactate	3,00 mM
Caffeine Na-benzoate 50:50	2,00 mM
Bovine Serum Albumin (BSA)	4 mg/ml
pH: 7.6 OSM: 309	

Table 6. Composition of TALP medium used for parthenogenetic activation.

NCSU-23 medium	
NaCl	108,73 mM
KCl	4,78 mM
$NaHCO_3$	25,07 mM
$KH2PO_4$	1,19 mM
$MgSO_4.7H_2O$	1,19 mM
D-Glucose	5,55 mM
Sorbitol	50 mM
Red Phenol	0,027 mM
Penicillin G	0,21 mM
Streptomycin	0,07 mM
$CaCl_2.2H_2O$	1,7 mM
BSA	4 mg/ml
L-Glutamine	1mM
pH: 7.6 OSM: 280	

Table 7. NCSU-23 medium composition.

3.2.2 Sheep oocytes collection, activation and transfer in uterus

The experiments carried out in sheep were performed in collaboration with the group of Prof. Ledda S. of the Department of Veterinary Pathology and Clinic of the Università degli Studi di Sassari.

Estrous cycles of adult Sarda-breed ewes were synchronized by the standard insertion of intravaginal sponges (40 mg FGA; Intervet). Multiple ovulations were induced by the administration of Follicular Stimulating Hormone (FSH-P; 20 mg pituitary extract kindly donated by FATRO, Italy) administered intramuscular every 12 hours for 48 hours. Recently ovulated oocytes were surgically removed from the oviducts of ewes, anesthetized with pentobarbital sodium, by flushing with 10 ml PBS supplemented with 4 mg/ml BSA fraction V. Oocytes were held in TCM 199-HEPES plus 10% FBS (Boehringer Mannheim, Italy) and antibiotics (penicillin-streptomycin) at room temperature. Cumulus cells were removed with 300 UI/ml hyaluronidase in Ca^{2+}-, Mg^{2+}-free PBS and repeated pipetting.

In vivo matured oocytes were activated with 5 μM ionomycin for 5 minutes and incubated for 3 h with 2 mM 6-DMAP in TCM-199 with 10% Fetal Bovine Serum (FBS, Gibco, Italy) in standard culture conditions as previously described by Loi et al. [159]. At the end of incubation 6-DMAP was washed off and the oocytes were

cultured. A well-developed blastocysts (> 100 cells and a clear ICM) were transferred to the uteri of foster-ewes (2 blastocysts per animal) that had ovulated 6 days previously. Fetal development was monitored and foetuses were collected at 21-day of pregnancy (the maximum length of time of sheep parthenogenetic development [159]) in order to isolate their fibroblasts.

3.3 *In vitro* fertilization

3.3.1 Pig oocytes *in vitro* fertilization and culture

For *in vitro* fertilization (IVF) studies, IVM was performed on a total of 1130 oocytes as previously described (please see 3.2.1 paragraph).

Frozen-thawed spermatozoa were purified by centrifugation through two-layer Percoll gradients. Live sperm cells were washed in TALP medium for IVF (see Table 8) and then were diluted in IVF medium (see Table 9). Oocytes were divided in groups of 45-50 and co-cultured with 120000 spermatozoa/ml in IVF medium at 38.5°C in 5% CO_2 atmosphere. After 24 h, oocytes were gently washed to eliminate cumulus cells and sperm adhering to the zona pellucida. Finally, embryos were cultured in groups of 25–35 in 50 μl NCSU-23 (see Table 7) drops under mineral oil at 38.5°C in 5% O_2, 5% CO_2 and 90% N_2 atmosphere. On day 5 post-activation, half of the medium was replaced with

fresh NCSU-23 containing 20% (v/v) FCS to reach a final FCS concentration of 10% (v/v) in the *in vitro* culture drop. On day 7/8 IVF blastocysts were obtained and ICMs were isolated for culture.

TALP 10X medium for IVF	
$MgCl_2.6H_2O$	1.25 mM
NaCl	102,7 mM
KCl	3 mM
NaH_2PO_4	0,28 mM
$NaHCO_3$	5 mM
Na- lactate syrup	3,7 ml/l
Na-pyruvate	1 mM
HEPES	20 mM
Polyvinyl alcohol (PVA)	1 mg/ml
Kanamycin	1,3 mM
pH: 7.4 OSM: 266	

Table 8. Composition of TALP medium used for IVF

IVF medium	
$CaCl_2.2H_2O$	2,65 mM
$MgCl_2.6H_2O$	0,4 mM
NaCl	106,1 mM
KCl	3,08 mM
$NaHCO_3$	26 mM
NaH_2PO_4	0,33 mM
Na-pyruvate	1 mM
Na- lactate syrup	1,86 ml/l
BSA FAF	6 g/l
Kanamycin	0,13 mM
pH: 7.4 OSM: 280	

Table 9. IVF medium composition

3.3.2 Sheep oocytes *in vitro* fertilization and transfer in uterus

These experiments were performed in collaboration with the group of Prof. Ledda S. of the Department of Veterinary Pathology and Clinic of the Università degli Studi di Sassari.

In vivo matured oocytes were collected from adult Sarda-breed after ovarian hyperstimulation (see 3.2.2 paragraph). Oocytes were partially denuded of granulosa cells by gentle pipetting in

TCM-199 HEPES buffered medium supplemented with 300 IU/ml hyaluronidase. Frozen-thawed spermatozoa from a Sarda breed were used in this experiment. The IVF system was composed by synthetic oviductal fluid (SOF) medium, originally described by Tervit et al. [267], enriched with 20% heat inactivated estrous sheep serum, 2,9 mM Ca lactate and 16 nM isoproterenol [268]. Fertilization was conducted in 50 μl drops with 1 x 10^6 spermatozoa/ml swim-up derived motile spermatozoa and maximum 15 oocytes per drop. After 20/26 hours cleaved oocytes were washed three times and cultured in SOF medium supplemented with 2% BME-essential amino acids, 1% MEM-nonessential amino acids (Gibco, Italy), 1 mM glutamine and 8 mg/mL fatty acid free BSA at 39°C in 5% O_2, 5% CO_2 atmosphere. Culture was continued until 6 to 7 days post fertilization, when embryos developed at least to expanded blastocysts were transferred to the uteri of foster-ewes (2 blastocysts per animal). Fetal development was monitored and foetuses were collected at 21-day of pregnancy (the same day used for parthenogenetic foetuses) in order to obtain fibrostast primary cultures.

3.4 Porcine sperm tail microinjection in matured oocyte

These experiments were carried out in collaboration with the group of Prof. Galli C. of Avantea, Laboratory of Reproductive Technologies, Cremona.

3.4.1 Porcine oocytes maturation

Ovaries were collected from the local slaughtered. Oocytes were aspirated with the aid of a vacuum pump and transferred to a TCM199-based maturation medium supplemented with 10% of FBS (Gibco, Italy), 1 μl/ml ITS media supplement, 1 mM sodium pyruvate, 0.5 mM L-cystein and gonadotropins (0.05 IU/ml FSH and 0.05 IU/ml LH; Pergovet 75, Serono, Italy). Oocytes were cultured at 38.5°C in 5% CO_2 for 44 h. After maturation oocytes were denuded of granulosa cells and MII oocytes were selected for polar body extrusion.

3.4.2 Boar sperm preparation

Frozen-thawed pig semen was washed through a discontinous Redigrad density gradient (45%-90%) at 750 g for 30 min. The viable spermatozoa recovered from the bottom of the tube were rinsed in Ca^{2+} free TALP and centrifuged at 400 g for 10 min. The sperm pellet was suspended at a concentration of 4 million

sperm/ml in Hepes buffered SOF medium supplemented with 6 mg/mlfatty acid free BSA, MEM amino acids (Gibco, Italy), 1 μg/ml heparin, 20 μM penicillamine, 1 μM epinephrine and 100 μM hypotaurine (SOF-IVF). Just before ICSI, the sperm suspension was diluted 1:1 with a 12% solution of polyvinylpyrrolidone in SOF IVF medium.

3.4.3 Intact sperm and tail microinjection

Intact sperm and sperm tail microinjection was performed as described by Kimura and Yanagimachi [269] using a Piezo micropipette driving unit (Prime Tech, Japan). For ICSI of intact sperm, a motile sperm was aspirated into an injection pipette of approximately 7 μm, immobilized by two or three piezo-pulses to the tail-midpiece junction and injected. For sperm tail injection, just before the injection, sperm tails were detached mechanically from sperm heads with very strong piezo-pulses at the tail-sperm head junction. Then a single tail was aspirated into an injection pipette of approximately 2 μm and injected. The oocytes were held on the holding pipette by suction, with the polar body orientated to the 6 or 12 o' clock position, and the injection pipette was advanced through the zona pellucida using the piezo drill motion.

3.4.4 Activation of tail injected oocytes and embryo culture

Matured oocytes with or without injected sperm tails were activated at 48-50 h of maturation by double DC pulses of 1.2 Kv/cm for 30 sec applied in 0.3 M mannitol solution, containing 1 mM Ca [270] and 100 M Mg, followed by 3 hr culture in maturation medium with 5 g/ml cytochalasin B.

Activated and intact sperm injected oocytes (day 0) were cultured in SOF medium supplemented with FAF BSA and MEM amino acids (SOF-BSA-AA [271]) at 38.5°C in an atmosphere of 5% CO2 and 5% O2. On day 2 the cleavage rate was assessed and not cleaved oocytes were removed. Culture medium was changed at 50% at day 4 and day 6. Embryos were fixed at different time point in order to analyzed their centriole assessment (see 3.7.1. paragraph).

3.5 ICM isolation and culture

3.5.1 STO feeder cell preparation

STO fibroblasts (LGC Promochem-ATCC, Italy) were routinely cultured in high glucose DMEM (Gibco, Italy), supplemented with 2mM glutamine and 10% FBS (Gibco, Italy). For growth inactivation, sub-confluent mono-layers were exposed to the medium above containing 10 μg/ml mitomycin-C (Gibco, Italy) for 3 hours. They were re-suspended in culture medium and seeded

at a density of 25 x 10^4 cell/well in 4-well dishes, pre-coated with 0.1% gelatin. Inactivation was carried-out 24 hours before plating of ICMs or passaging of pluripotent cell lines. Two hours before use, the medium was changed and replaced with embryonic stem cell medium (see 3.1.1 and 3.5.3 paragraphs).

3.5.2 Porcine ICM isolation

Porcine parthenotes and IVF embryos were cultured up to the early blastocyst stage. Blastocysts were incubated in pronase 0.5% (w/v) in medium TCM199 (Gibco, Italy), supplemented with 6.5 mg/ml HEPES, 1.1 mg/ml sodium bicarbonate and 4 mg/ml bovine serum albumin (BSA). Incubation was carried out for 8 minutes to eliminate zona pellucida. Subsequently, blastocysts were incubated in low glucose DMEM medium (Gibco, Italy) supplemented with 10% PVA and 10% monkey anti-porcine serum (supplied by Istituto Zooprofilattico Sperimentale Lombardia ed Emilia-Romagna, Brescia) for 15 minutes. A 30 minutes incubation in DMEM medium (Gibco, Italy) containing 10 % PVA and 10% Guinea pig complement was then performed. ICMs were isolated from lysed trophoblast cells by pipetting, washed through several DMEM drops to avoid culture oil carry-overs and encourage better attachment and singly plated on feeder cells.

3.5.3 Porcine ICM culture

Porcine embryonic stem cells were culture in DMEM-low glucose : HAM'SF10 (1:1; Gibco, Italy) supplemented with 10% K.O. SERUM (Gibco, Italy), 5% FBS (Gibco, Italy), 2 mM glutamine, 0.1 mM β-mercaptoethanol, nucleoside mix, 1% non-essential aminoacid (Gibco, Italy), 1000IU/ml ES GROWTH (LIF; Chemicon, USA) and 5ng/ml bFGF (R&D System, USA) [67].

Within 3 days, circular colonies with distinct margins of small, round cells were observed and media were refreshed. 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 of an average of 500–600 cells, avoiding to obtain single cell suspension. Cells were then passaged on freshly prepared feeder-layers. Culture medium was changed every day.

3.6 Fetal fibroblast isolation and culture

This part of experiments was performed in collaboration with the group of Prof. Ledda S. of the Department of Veterinary Pathology and Clinic of the Università degli Studi di Sassari.

Primary cultures of foetal fibroblasts were obtained from 21 day-old sheep fetuses generated by the transfer of IVF or parthenogenetic blastocysts into the uterus of synchronized recipients. The isolation of fibroblasts was carried out as previous described by Denning et al. [272]. Explanted sheep fetuses were dissociated manually and then treated with 0.25% trypsin-EDTA. Primary cultures were grown on 0.1% gelatin pre-coated Petri dishes (Sarstedt, Italy) until the first passage, after which no treated Petri dishes (Sarstedt, Italy) were used. Cells were cultured in DMEM High Glucose (Gibco, Italy) supplemented with 20% FBS, 200 mM glutamine and antibiotic-antimicotic at 37°C in a humidified environment with 5% CO_2 in air.

3.7 Centrosome characterization

3.7.1 Centriole immunocytochemical localization

Number of centrioles was assessed in all cell lines as well as in pig parthenotes and IVF embryos.

Human and porcine undifferentiated cells from bi- and mono-parental origin and sheep fibroblast derived from parthenogenetic and IVF fetuses were plated directly on CultureWell Chambered Coverglass 16-well dishes (Molecular Probes, Invitrogen, Italy) and cultured for 24 hours. Sample were fixed in 100% methanol

for 10 minutes at -20°C and permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Aspecific sites were blocked with PBS containing 10% BSA. Incubation with primary antibody specific for Centrin-1 (1:100, Abcam, UK) was then carried out for 1 hour at room temperature. Microtubules were also labelled with anti-beta tubulin antibody (1:50, Abcam, UK). Incubation with suitable secondary antibodies (Alexafluor, Invitrogen, Italy) was performed for 30 minutes and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Samples were observed under an Eclipse E600 microscope (Nikon, Japan) at 100x magnification.
Porcine parthenotes and embryos at 2-cell, 4-cell, 8-cell, 16-cell and blastocyst stage were fixed and stained as previously described in small drops and mounted on slides at the end of the procedure. Samples were observed under a TCS-NT laser confocal microscope (Leica Microsystems, Germany).

3.7.2 Centriole localization with transmission electron microscopy (TEM)

This analysis was performed in collaboration with the group of Prof. deEguileor M. of the Department of Biotechnology and Molecular Sciences of the Università dell'Insubria, Varese.
Cells from all different origin were analyzed with transmission electron microscopy in order to assess their centriole number.

Samples were fixed for 2 hours 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 hours at +4°C 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).

3.7.3 Mitotic spindle check-point molecule analysis by Real Time PCR

Total RNA was extracted from bi-parental and parthenogenetic human, porcine and ovine cell lines. We used cDNA of three bi-parental embryonic stem cell lines, HES 7, HES I-3 and HES I-6 thanks to a collaboration with the group of Prof. Benvenisty N. of The Alexander Silberman Institute of Life Sciences of The Hebrew University of Jerusalem. RNA was extracted with the TaqMan®Gene Expression Cells to Ct kit (Applied Biosystem, USA). Expression of target genes was evaluated using an ABI-

Prism 7000 Sequence Detecting System (Applied Biosystem, USA). Pre-designed gene-specific primer and probe sets from TaqMan®Gene Expression Assays (Applied Biosystem, USA) were used for the following transcripts: Aurora kinase A (AURKA; RP5-1167H4.6; AIK; ARK1; AURA; AURORA2; BTAK; MGC34538; PPP1R47; STK15; STK6; STK7); Aurora kinase B (AURKB; AIK2; AIM1; ARK2; AurB; IPL1; STK5; AIM-1; STK12; aurkb-sv1; aurkb-sv2); Autophagy related 5 homolog (ATG5; ASP; APG5; APG5L; hAPG5; APG5-LIKE); Budding uninhibited by benzimidazoles 1 homolog (BUB1; BUB1A; BUB1L; hBUB1); Cyclin-dependent kinase inhibitor 1A (CDKN1A; P21; CIP1; SDI1; WAF1; CAP20; CDKN1; MDA-6; p21CIP1; CDKN1A); Centromere protein E (CENPE; CENP-E; KIF10; PPP1R61); Cyclin F (CCNF; FBX1; FBXO1); E1A binding protein p300 (EP300; p300; KAT3B); E2F transcription factor 1(E2F1; RBP3; E2F-1; RBAP1; RBBP3); Kelch-like 3 (KLHL3; FLJ40871; KIAA1129; MGC44594); Mechanistic target of rapamycin (serine/threonine kinase) (MTOR; FRAP; FRAP1; FRAP2; RAFT1; RAPT1; FLJ44809); Microtubule-associated protein 1 light chain 3 alpha (MAP1LC3A; LC3; LC3A; MAP1ALC3; MAP1BLC3); Mitotic arrest deficient 1 (MAD1; MAD1L1; PIG9; TP53I9; TXBP181); Mitotic arrest deficient 2 (MAD2; HSMAD2; MAD2L1); MYC associated factor X (MAX; MGC10775; MGC11225; MGC18164; MGC34679; MGC36767; bHLHd4; bHLHd5; bHLHd6; bHLHd7; bHLHd8, orf1); Polo-like

kinase 2 (PLK2; SNK); Sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1; SIR2L1); Translin (TSN; RCHF1; TBRBP; TRSLN; BCLF-1; REHF-1); TTK protein kinase (TTK; RP3-357D13.2; CT96; ESK; FLJ38280; MPS1; MPS1L1; PYT); Tumor protein p53 (TP53; p53; LFS1; TRP53; FLJ92943) and v-mos Moloney murine sarcoma viral oncogene homolog (MOS; MSV; MGC119962; MGC119963) (Table 10).

When not available, gene-specific primer and probe sets were custom designed. 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.

Human Genes	Catalog number
Aurora kinase A (**AURKA**; RP5-1167H4.6; AIK; ARK1; AURA; AURORA2; BTAK; MGC34538; PPP1R47; STK15; STK6; STK7)	Hs01582072_m1
Aurora kinase B (**AURKB**; AIK2; AIM1; ARK2; AurB; IPL1; STK5; AIM-1; STK12; aurkb-sv1; aurkb-sv2)	Hs00945858_g1
Autophagy related 5 homolog (**ATG5**; ASP; APG5; APG5L; hAPG5; APG5-LIKE)	Hs00355494_m1
Budding uninhibited by benzimidazoles 1 homolog (**BUB1**; BUB1A; BUB1L; hBUB1)	Hs00177821_m1
Centromere protein E (**CENPE**; CENP-E; KIF10; PPP1R61)	Hs01068241_m1
Cyclin-dependent kinase inhibitor 1A (**CDKN1A**; P21; CIP1; SDI1; WAF1; CAP20; CDKN1; MDA-6; p21CIP1; CDKN1A)	Hs99999142_m1
Cyclin F (**CCNF**; FBX1; FBXO1)	Hs00171049_m1
E1A binding protein p300 (**EP300**; p300; KAT3B)	Hs00914221_m1
E2F transcription factor 1(**E2F1**; RBP3; E2F-1; RBAP1; RBBP3)	Hs01566609_g1
Kelch-like 3 (**KLHL3**; FLJ40871; KIAA1129; MGC44594)	Hs00213589_m1
Mechanistic target of rapamycin (serine/threonine kinase) (**MTOR**; FRAP; FRAP1; FRAP2; RAFT1; RAPT1; FLJ44809)	Hs01042405_m1
Microtubule-associated protein 1 light chain 3 alpha (**MAP1LC3A**; LC3; LC3A; MAP1ALC3; MAP1BLC3)	Hs00261291_m1
Mitotic arrest deficient 1 (**MAD1**; MAD1L1; PIG9; TP53I9; TXBP181)	Hs00269119_m1
Mitotic arrest deficient 2 (**MAD2**; HSMAD2; MAD2L1)	Hs01554515_g1
MYC associated factor X (**MAX**; MGC10775; MGC11225; MGC18164; MGC34679; MGC36767; bHLHd4; bHLHd5; bHLHd6; bHLHd7; bHLHd8, orf1)	Hs00811068_m1
Polo-like kinase 2 (**PLK2**; SNK)	Hs01573408_g1
SIN3 homolog A, transcription regulator (**SIN3**; DKFZp434K2235; FLJ90319; KIAA0700)	Hs00411592_m1
Sirtuin (silent mating type information regulation 2 homolog) 1(**SIRT1**; SIR2L1)	Hs01009003_m1
Translin (**TSN**; RCHF1; TBRBP; TRSLN; BCLF-1; REHF-1)	Hs00935849_m1
TTK protein kinase (**TTK**; RP3-357D13.2; CT96; ESK; FLJ38280; MPS1; MPS1L1; PYT)	Hs01009887_m1
Tumor protein p53 (**TP53**; p53; LFS1; TRP53; FLJ92943)	Hs99999147_m1
v-mos Moloney murine sarcoma viral oncogene homolog (**MOS**; MSV; MGC119962; MGC119963)	Hs01114731_s1

Porcine Genes	Catalog number
Cyclin-dependent kinase inhibitor 1A (**CDKN1A**; P21)	Custom made
Polo-like kinase 2 (**PLK2**)	Ss03375595_u1
Tumor protein p53 (**TP53**; P53)	Custom made
Ovine Genes	**Catalog number**
Cyclin-dependent kinase inhibitor 1A (**CDKN1A**; P21)	Bt03262188_m1
Polo-like kinase 2 (**PLK2**)	Custom made
Tumor protein p53 (**TP53**; P53)	Bt03223221_g1

Table 10. List of all the genes analyzed and probe catalogue numbers (TaqMan®Gene Expression Assays, Applied Biosystem, USA).

3.8 Karyotype assesment

Chrosomome number was assessed in human, porcine and sheep parthenogenetic and bi-parental cells. Mitotically active cells in log phase were incubated in 10μg/ml colcemid (Gibco, Italy) for 40-60 minutes. Cells were dislodged with 0.25% trypsin and centrifuged at 200*g* for 8 minutes. The cell pellet was gently resuspended in 0.075 M KCl solution and incubated for 20 minutes at 37°C followed by fixation with methanol/glacial acetic acid (3:1) solution. Fixed cells were dropped on wet slides and air dried. Giemsa staining was carried out as indicated by manufacturer (Cariomax Giemsa, Gibco, Italy). Metaphases were fully karyotyped under a Leica HC microscope. Images were then

captured with digital camera Leica DC250 using a Leica CW4000 Karyo software.

3.9 TUNEL assay

Apoptotic index were tested in parthenogenetic and bi-parental human, porcine and sheep cell lines. Cells were plated directly on CultureWell Chambered Coverglass 16-well dishes (Molecular Probes, Invitrogen, Italy) and cultured for 24 hours. They were then fixed in 4% formaldehyde in PBS for 25 minutes at 4°C, washed twice in PBS and permeabilized with 0.25% Triton X-100 in PBS for 5 minutes. Samples were incubated in 100 μl of Equilibration Buffer (Roche, Italy) for 10 minutes at room temperature and then 50 μl of TdT Reaction Mix (Roche, Italy) were added for an incubation of 60 minutes at 37°C. Cells were washed in SSC (Roche, Italy) for 15 minutes and their nuclei were counterstained with DAPI. At the end of the assay, slides were washed, mounted with Vectashield® Mounting Medium (Vector Laboratories, Italy) and examined under an Eclipse E600 microscope (Nikon, Japan).

3.10 Autophagic activity detection

3.10.1 Lysotracker Red staining

To determ lysosome formation parthenogenetic and bi-parental embryonic stem cells were stained with Lysotracker Red, a red-fluorescent dye for labeling and tracking acidic organelles. It is well known that lysosomes are cellular organelles that contain acid hydrolase enzymes (4.5 pH).

In these experiments live cells were incubated with a 1 μM solution of Lysotracker Red DND-99 (Molecular Probes, Invitrogen, Italy) in complete medium for 30 minutes at 37°C. After extensive wash in PBS and distilled water, cells were mounted in Citifluor (Citifluor, UK), covered with a coverslip, and examined with an Olympus BH2 microscope. Negative control was performed by omitting Lysotracker in the medium.

3.10.2 LC3 immunostaining

Autophagic activity was evaluated using a specific autophagosome marker LC3.

Parthenogenetic and bi-parental embryonic stem cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Incubation with anti-LC3 antibody (Novus Biologicals, USA)

diluted 1:200 in 2% BSA was performed for 1 hour in a dark moist chamber. After incubation with the primary antibody, cells were washed and incubated with an appropriate Cy3-conjugated antibody (dilution 1:100; Jackson ImmunoResearch, UK) for one hour. Samples were mounted and observed with an Olympus BH2 microscope (Olympus, Italy). Negative control was performed by omitting primary antibody.

3.10.3 TEM analysis

The presence of autophagosome was also assessed though TEM in collaboration with the group of Prof. deEguileor M. of the Department of Biotechnology and Molecular Sciences of the Università dell'Insubria, Varese.
Cells were processed and observed as described in 3.7.2 paragraph.

3.11 Inter-cellular bridge detection

3.11.1 TEM analysis

The occurency of inter-cellular bridges was assessed though TEM in collaboration with the group of Prof. deEguileor M. of the Department of Biotechnology and Molecular Sciences of the Università dell'Insubria, Varese.

Cells were processed and observed as described in 3.7.2 paragraph.

3.11.2 Scanning electron microscopy (SEM)

The experiments here described were performed in collaboration with the group of Prof. deEguileor M. of the Department of Biotechnology and Molecular Sciences of the Università dell'Insubria, Varese.

Inter-cellular bridge formation was investigated in human, porcine and sheep cell lines using scanning electron microscopy (SEM). Cells were fixed and dehydrated as previously described for TEM (3.7.2 paragraph). Samples were then treated with hexamethildisilazane and mounted on polylysinated slides, air dried and subsequently covered with a 9 nm gold film by flash evaporation of carbon in an Emitech K 250 sputter coater (Emitech, USA). Specimens were examined with a SEM-FEG Philips XL-30 microscope (Philips, Netherlands).

3.11.3 Dextran single cell injection

Functional trafficking activity through inter-cellular canals were investigated in porcine and ovine bi-parental and parthenogenetic cells. Solution of 50mg/ml of Rhodamine-conjugated 10-kDa dextran (Molecular Probes, Invitrogen, Italy) was introduced into

the cytoplasm of a single cell. Eppendorf FemtoJet® Microinjector (Eppendorf, Italy) was used in order to inject a small quantity of dextran in cell cytoplasm. Movement of the molecule from the injected cell to others was observed with a Nikon Eclipse E600 microscope (Nikon, Japan).

3.12 Statistical analysis

Statistical analysis was performed using the unpaired t-test or the Mann–Whitney rank sum test, as appropriate, using the Sigma Stat statistical package (Systat Software Inc., USA). Data are presented as mean percentages (± SEM) of a minimum of three independent replicates. In all cases, differences of $P \leq 0.01$ were considered significant.

4. RESULTS

DERIVATION OF PIG AND SHEEP CELL LINES

Isolation and characterization of porcine parthenogenetic and bi-parental embryonic stem cells

ICMs were isolated from 282 parthenotes and 101 IVF blastocysts using immunosurgery technique. They were plated on inactived feeder cells and 3 out of 6 and 30 out of 41 outgrowths, derived respectively from IVF and parthenogenetic embryos, were able to give rise a stable pluripotent cell lines that could be propagated extensively *in vitro* (more than 50 passages) (Table 11).

	IVF embryos	**Parthenotes**
Oocytes	1388	1221
Blastocysts	124 (8.93%)	282 (23.1%)
Outgrowths	6 (4.84%)	41 (22.16%)*
ESC lines	3 (2.42%)	30 (16.22%)*

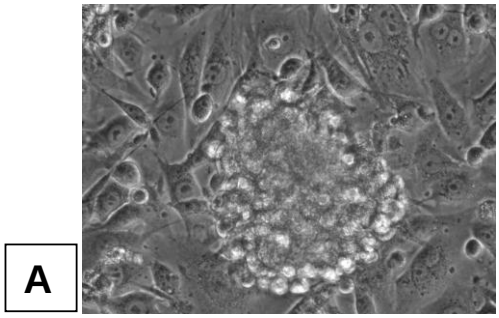
Table 11. Derivation of porcine ESC lines from IVF embryos and parthenotes. Oocyte and blastocyst number used, total number (% rate) of outgrowths and cell lines obtained. *p<0.05.

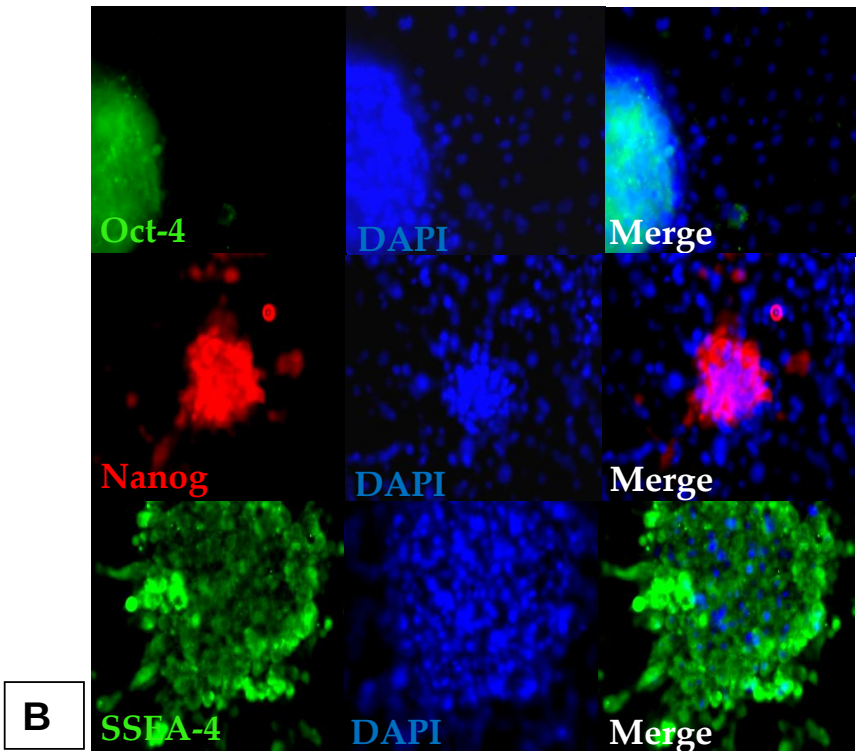
We obtained homogeneous outgrowths, consisting entirely of cells which resembled ESCs in their morphology (Figure 23 panel A and Figure 24 panel A), both using parthenogenetic and IVF ICMs.

The outgrowths grew in size and, after 7-8 days of culture, formed circular colonies of 3-5 mm in diameter, with distinct margins (Figure 23 panel A and Figure 24 panel A). At this stage they were passaged onto a fresh feeder layer to establish cell lines or were subjected to further characterization. Cells obtained from both origin showed many pluripotency aspects common to mouse and human ESCs. They expressed many of the known pluripotency related transcription factors and surface markers. More in detail molecular analysis demonstrated that both IVF and parthenogenetic derived cells transcribed for gene like OCT4, NANOG, SOX2 and REX1 (Figure 23 panel C and Figure 24 panel C). Moreover immunocytochemical studies confirmed the presence of Oct4, Nanog and SSEA4 in our porcine cells (Figure 23 panel B and Figure 24 panel B).

Three cell lines for each origin were analyzed in the experiments described below.

For more detailed information see Appendix B titled: "Culture conditions and signalling networks promoting the establishment of cell lines from parthenogenetic and bi-parental pig embryos" by Brevini et al. [67].


A


Oct-4
DAPI
Merge
Nanog
DAPI
Merge
SSEA-4
DAPI
Merge
B

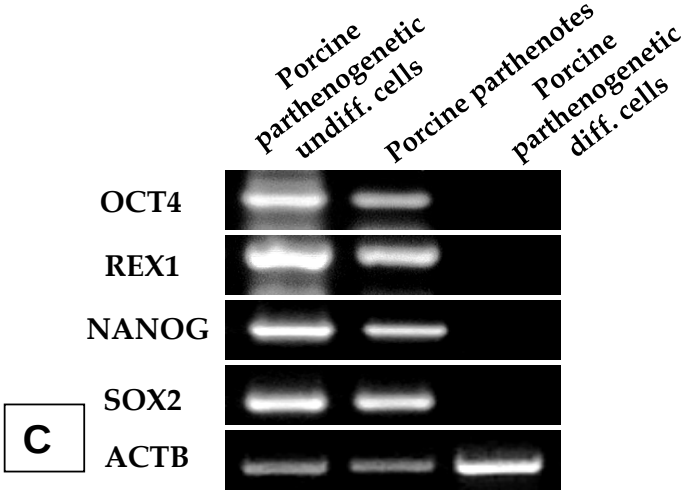
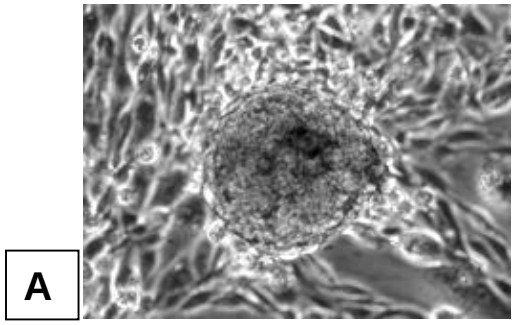
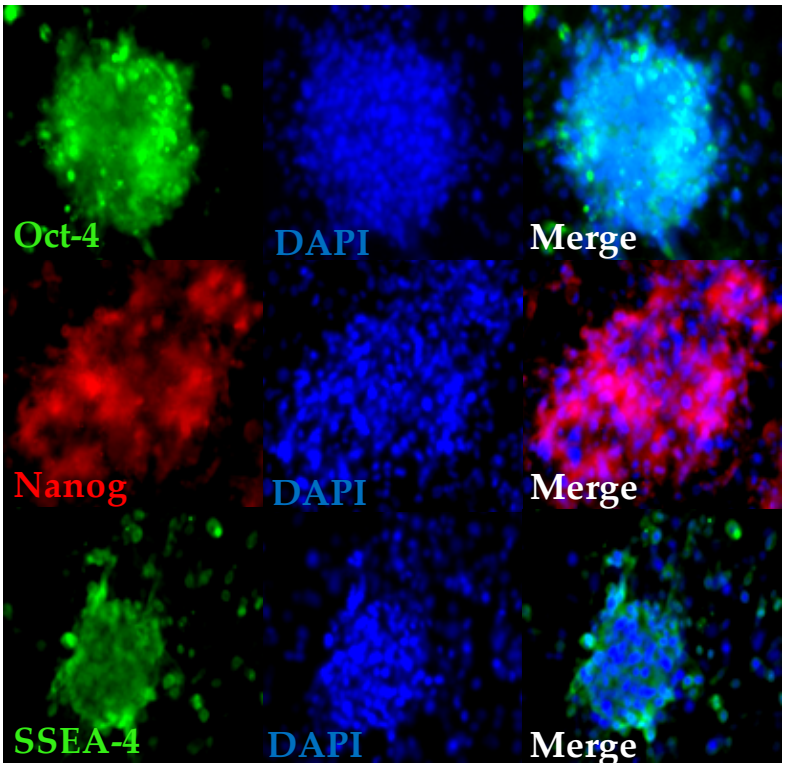

Porcine parthenogenetic undiff. cells
Porcine parthenotes
Porcine parthenogenetic diff. cells
OCT4
REX1
NANOG
SOX2
ACTB
C

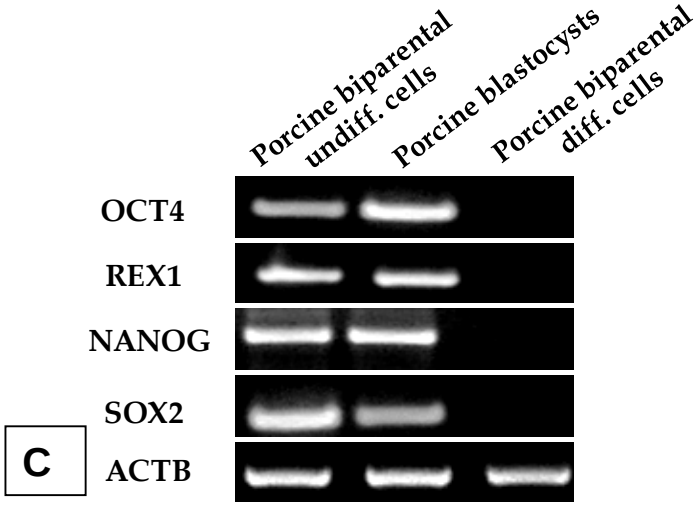
Figure 23. Porcine ESCs derived from parthenotes. Parthenogenetic ESCs formed circular colonies with distinct margins (panel A). They were positive for several known pluripotency-related markers. The figure is representative of all passages (more than 50 passages at present). Immuno-cytochemical analysis with specific antibodies demonstrated positivity of parthenogenetic cells for Oct-4, Nanog and SSEA-4. Cell nuclei, stained with DAPI, are coloured in blue (panel B). Molecular studies confirm the expression of OCT4, NANOG, REX1, SOX2 (panel C). Genomic DNA was used as a positive control; negative control was represented by differentiated parthenogenetic ESCs.



A


Oct-4
DAPI
Merge
Nanog
DAPI
Merge
SSEA-4
DAPI
Merge

B


Porcine biparental undiff. cells
Porcine blastocysts
Porcine biparental diff. cells
OCT4
REX1
NANOG
SOX2
ACTB

C

Figure 24. Porcine ESCs derived from IVF blastocysts. Bi-parental ESCs formed circular colonies with distinct margins (panel A). They were positive for several known pluripotency-related markers. The figure is representative of all passages (more than 50 passages at present). Immuno-cytochemical analysis with specific antibodies demonstrated positivity of porcine ESCs for Oct-4, Nanog and SSEA-4. Cell nuclei, stained with DAPI, are coloured in blue (panel B). Molecular studies confirm the expression of OCT4, NANOG, REX1, SOX2 (panel C). Genomic DNA was used as a positive control; negative control was represented by differentiated bi-parental ESCs.

Isolation of sheep fibroblasts from IVF and parthenognetic fetuses

In collaboration with the group of Prof. Ledda S. of the Department of Veterinary Pathology and Clinic of the Università degli Studi di Sassari, 2 IVF embryos and 2 parthenotes were transferred to the uteri of each of 4 recipients. Fetuses were constantly monitored and the sizes of the conceptuses after parthenogenic activation were compared with those of conceptuses obtained from normally fertilized oocytes. No difference in fetal sac size between parthenogenetic emrbyos and controls was detected. On day 21 fetuses were recovered from recipient ewes and fibroblasts were isolated as described above (paragraph 3.6). 6 cell lines, 3 from parthenogenetic and 3 from bi-parental fetuses, were established and analyzed in the following experiments.

PART 1: ORIGIN OF CHROMOSOME ABNORMALITIES

Katyotype assessment in parthenogenetic and bi-parental cells

We looked at the incidence of chromosomal abnormalities in parthenogenetic cell lines and compared it with their bi-parental counterparts. A total of 192, 172, 237 parthenogenetic and 200, 165, 172 bi-parental human, pig and sheep cells were analysed respectively. The diploid configuration represented the modal value in the bi-parental cell lines of all species, whereas hypo-haploid and hypo-diploid configurations where the most frequent in all parthenogenetic cell lines, irrespective of the species and of their origin: cell line or primary culture (Figure 28).

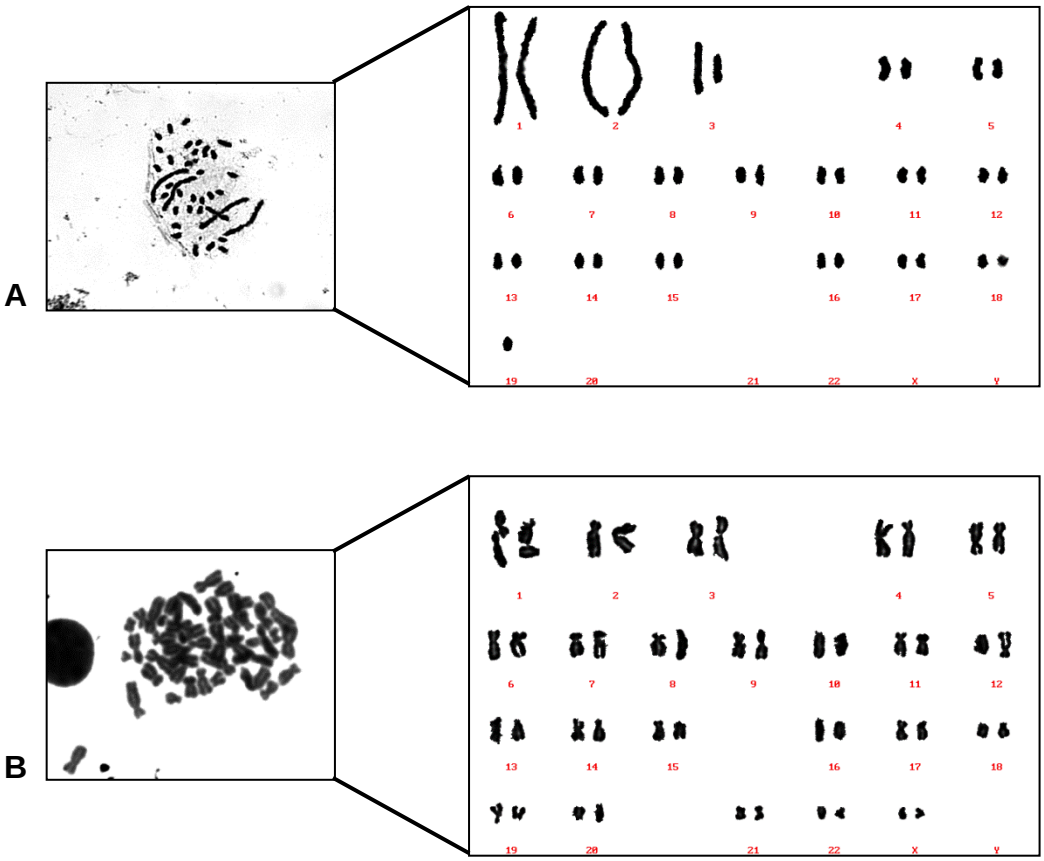


Figure 25.Representative pictures of human parthenogenetic (panel A) and bi-parental (panel B) cell karyotype.

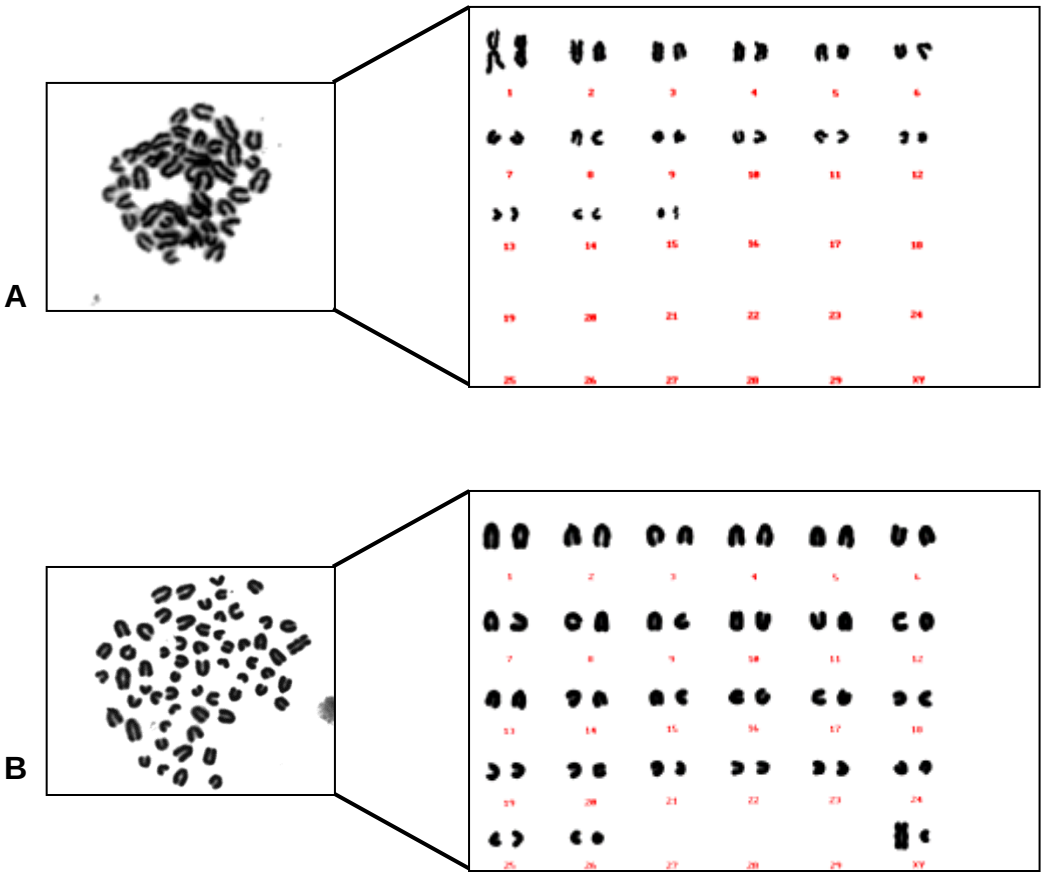


Figure 26.Representative pictures of porcine parthenogenetic (panel A) and bi-parental (panel B) cell karyotype.

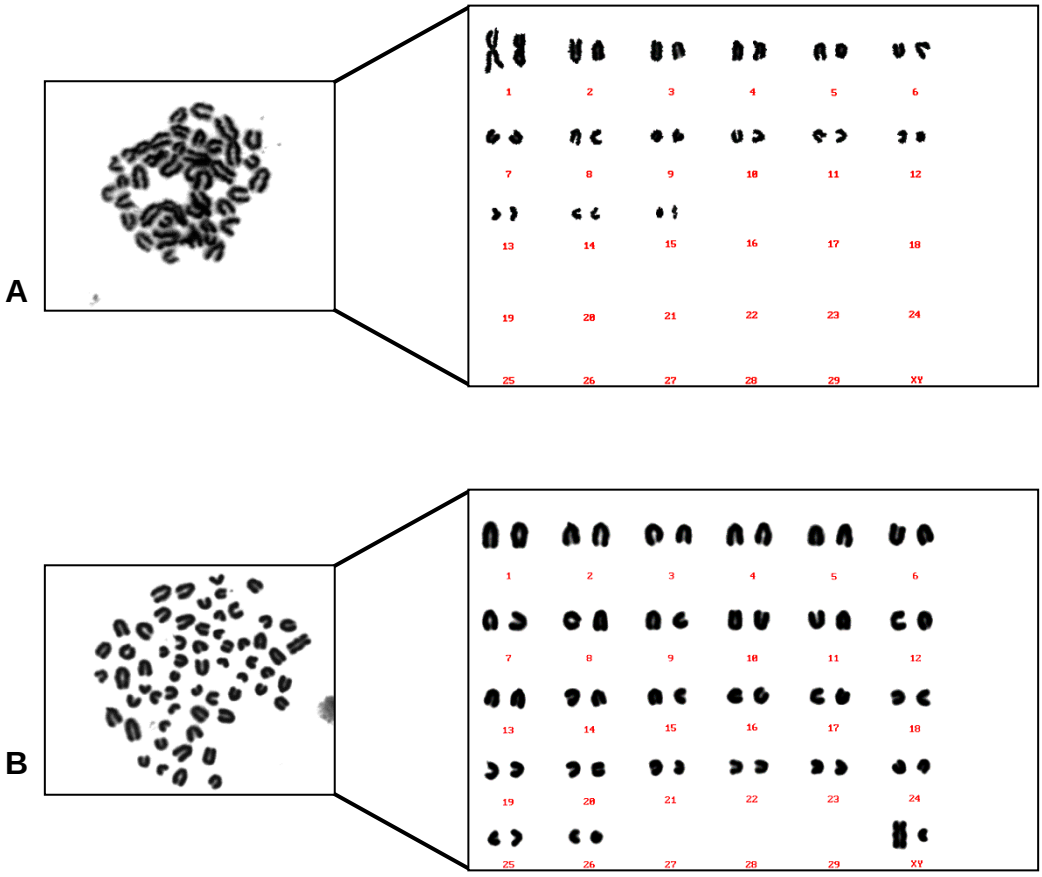


Figure 27.Representative pictures of sheep parthenogenetic (panel A) and bi-parental (panel B) cell karyotype.

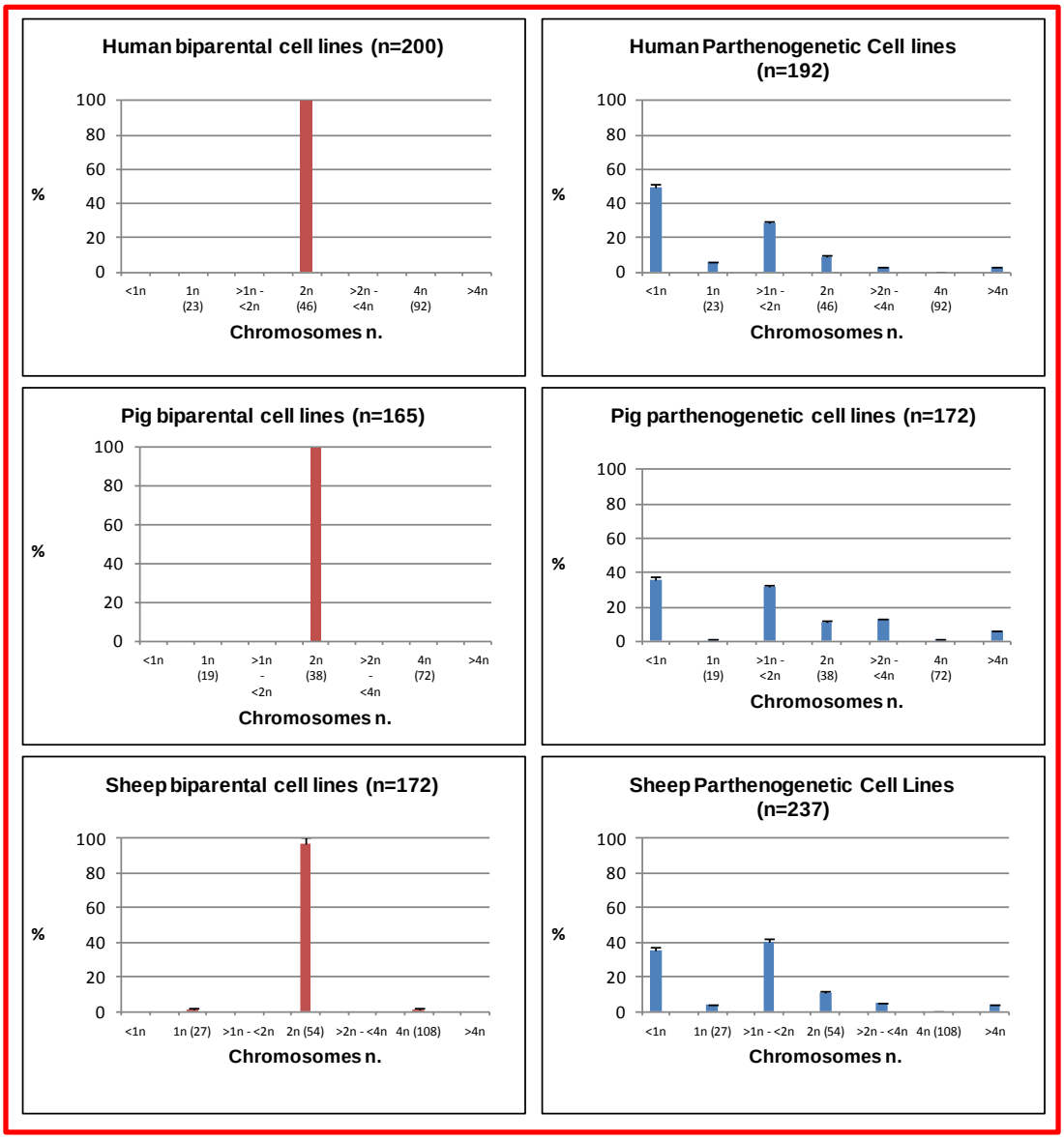


Figure 28. Chromosome counting in parthenogenetic and bi-parental cell lines highlights the higher incidence of chromosomal abnormalities in monoparental cells of all examined species. Hypodiploid and hypoaploid configurations were the most common ones and indicate the occurrence of multipolar mitosis.

Comparative studies of centrosome organization in parthenogenetic and bi-parental cell lines

A systematic comparison of the incidence of multiple centrosomes after IVF or parthenogenetic activation was performed. To this purpose we analyzed human and porcine pluripotent cell lines as well as sheep foetal fibroblasts. Centriole number was analyzed after immune-localization of centrin, a centriole-associated calcium-binding protein that plays a fundamental role in centrosome duplication. Punctuate centrin localization were counted on 343, 237, 267 parthenogenetic and 150, 224, 416 bi-parental human, pig and sheep cells respectively. A high and significantly different incidence of supernumerary pairs of centrioles was observed in parthenogenetic cell lines of all species, whereas only 20 of 847 bi-parental cells showed an abnormal number of centrioles (Figure 30).

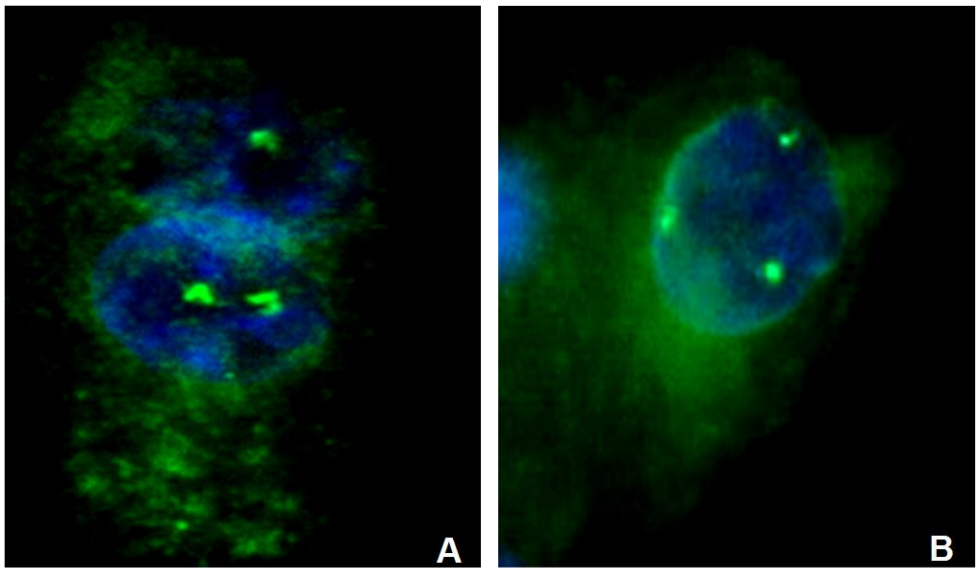


Figure 29.Representative pictures of cells showing 1, 2 (normal centriole number) and 3 centriole pairs (abnormal centriole number), localized with centrin-specific antibody.

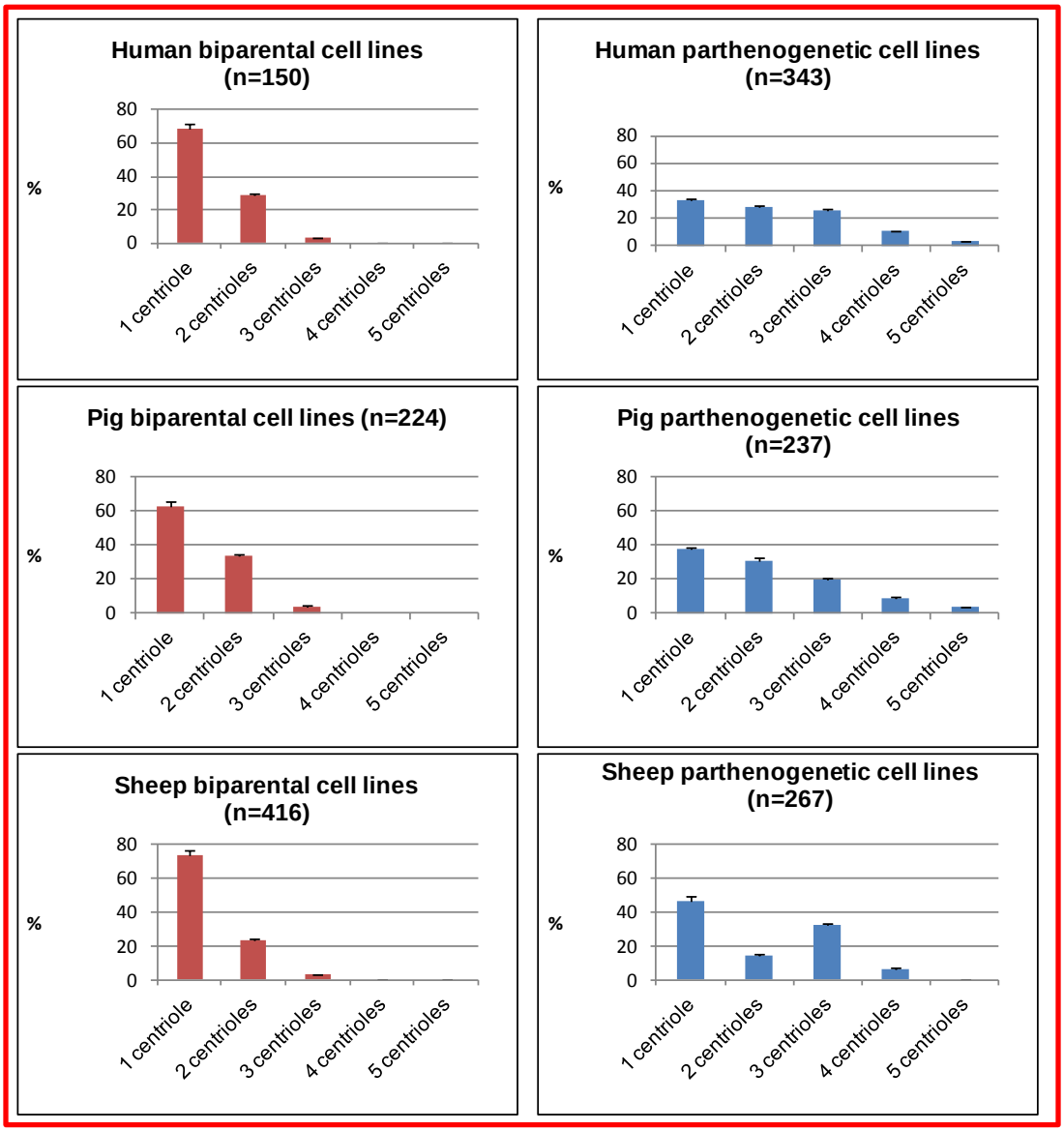


Figure 30. A significant increase of abnormal number of centriole pairs (more than 2) is observed in cell lines derived from human. pig and sheep parthenotes as established by a direct comparison with the respective bi-parental counterparts.

Furtheremore, the presence of multipolar asters (Figure 31) confirms that extranumerary centrosomes are functional and able to form multipolar spindles.

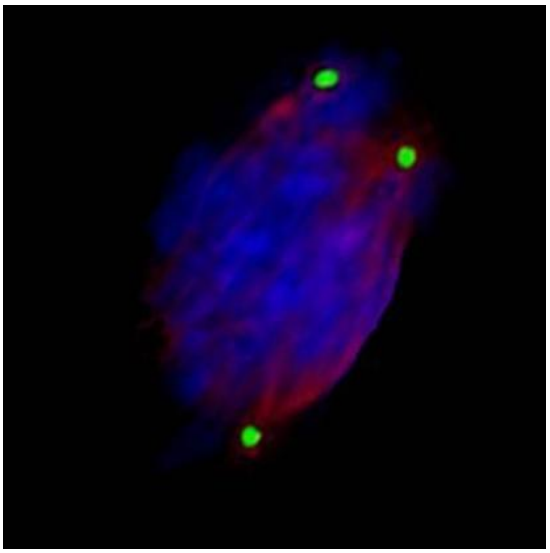


Figure 31. Multipolar asters in human parthenogenetic pluripotent cells confirms that extranumerary centrosomes are functional and able to form multipolar spindles.

To further confirm these results we also analysed the expression levels of PLK2, a molecule required for centriole duplication [244], and CCNF, depletion of which cause centrosomal and mitotic abnormalities, including multipolar spindles and asymmetric, bipolar spindles with lagging chromosomes [252]. In our studies we detected up regulation of PLK2 in human (15,9-fold) pig (15,7-fold) and sheep (13,9-fold) parthenogenetic cell lines and, in

parallel, we could observe a down-regulation (6,5 folds) of CCNF expression in human mono-parental cells (Figure 32).

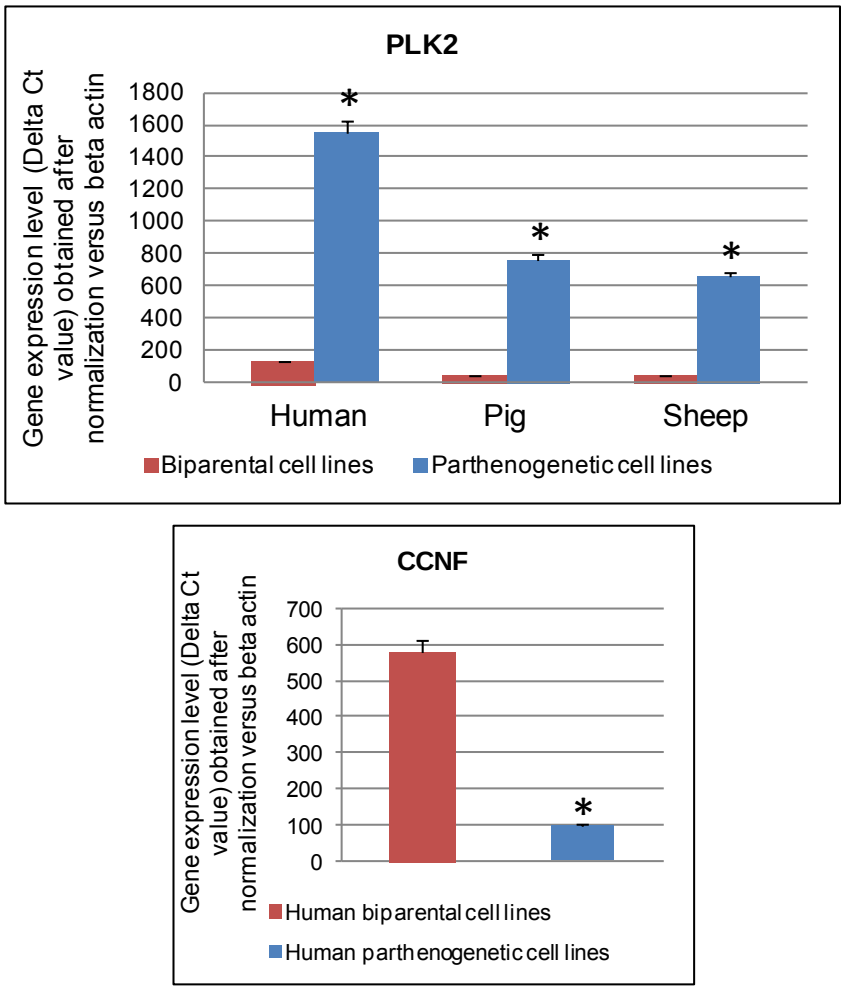


Figure 32. A significant increase of PLK2 expression in human, pig and sheep parthenogenetic cell lines that confirms at the molecular level the centrosome amplification detected by morphological analysis. In parallel, down-regulation of CCNF further support the presence of centrosomal and mitotic abnormalities. $^{*}p<0.05$.

Quantitative PCR experiments also demonstrated aberrant levels of molecules related to spindle formation in human parthenogenetic cells, when compared to those of three bi-parental embryonic stem cell lines. 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 CENPE, TTK and Aurora A kinase, indicated abnormalities at different spindle check points in parthenogenetic cell lines (Figure 33).

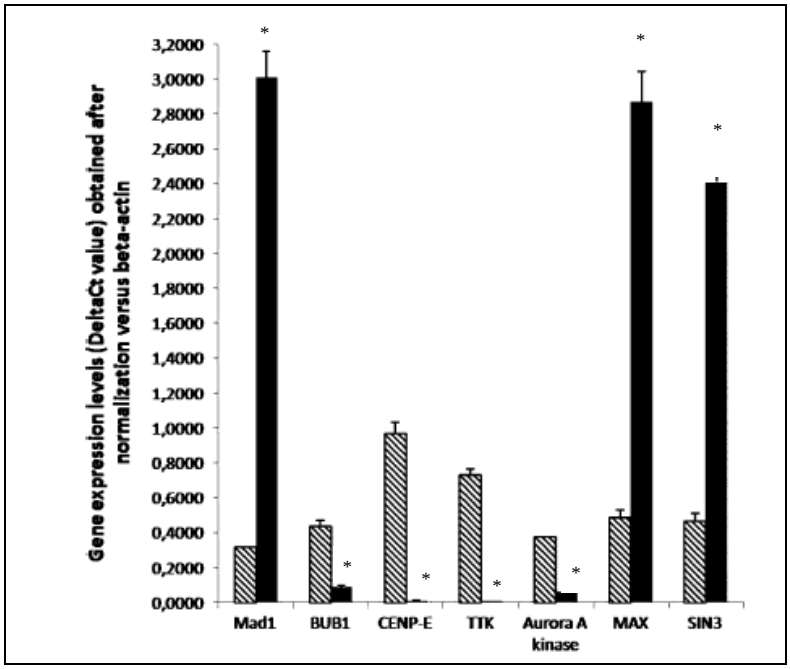


Figure 33. Expression level of molecules related to spindle formation and chromosome segregation in human parthenogenetic and bi-parental cell lines. Bars represent the average ΔCt of HP cells (solid bars) and bi-parental cells (striped bars) related to the genes examined. $^{*}p<0.05$.

Centriole number assessment in porcine IVF and parthenogenetic embryos

In order to determine when centriole number alterations take place, we analyzed the number of centrioles present in IVF and parthenogenetic pig embryos. The evaluation was carried out on 48 IVF embryos and 56 parthenotes at different developmental stages (2-, 4-, 8-, 16-cell and blastocyst) for a total of 364 and 376 blastomers respectively. An abnormal centriole number was observed in 59.6% (224/376) of parthenote blastomers, while only 2.7% (10/364) of the IVF blastomers examined, showed supernumerary centrioles (Table 12). In particular centrioles were detectable in parthenotes before 4-cell stage and a high incidence of abnormalities was immediately observed, while no centriole anomalies were visible in IVF embryos. In the latter case centrioles appeared at morula/blastocysts stage, as previous demonstrated for porcine species [178].

	IVF	Parthenotes
Embryos analyzed	48	56
Blastomer analyzed	364	376
Blastomers with >2 centrioles	10/364 (2.7%)	224/376 (59.6%)*

Table 12. Comparative analysis between IVF embryos and parthenotes. Centriole number alteration is significantly higher in parthenotes. *p<0.05.

Sperm tail microinjection in porcine matured oocytes

To confirm the hypothesis about the link between the absence of paternal centrioles contribution in parthenogenetic cells and the chromosome/centriole number alterations described above, we carried out specific experiment in collaboration with the group of Prof. Galli C. (Avantea, Laboratory of Reproductive Technologies, Cremona), injecting sperm tail in porcine mature oocytes.

In this species, in fact, as well as in human and sheep, paternal centrioles were localized in the proximal end of the sperm tail [178]. We separated head from tail spermatozoa and only tail, containing the proximal centrioles, was injected into MII oocytes.

After this, activation was performed in order to stimulate oocytes and to trigger parthenogenetic development.

In the first set of experiment we compared 16-cells and morulas obtained from ICSI embryos, parthenotes and parthenotes in which sperm tail were injected.

The results obtained showed that only partenothes display balstomers with more than 2 centrioles, while no centrioles were detected in ICSI embryos or parthenotes in which sperm tail was injected (Table 13). These results are in agreement with a previous study revealing that in the pig sperm centrioles are lost in zygote after *in vitro* fertilization and they are not detectable until the late blastocysts stage [178]. Furthermore these data demonstrated that in parthenotes centriole alterations take place at the very early stage of parthenote development.

	ICSI	Parthenogesis	Tail injection plus activation
Oocytes	56	30	53
48h	43	25	42
96h	19	25	17
Blastomer analyzed	225	376	79
Blastomers with >2 centrioles	no centrioles	224 (59.57%)*	no centrioles

Table 13. Centriole number in blastomers of 96h ICSI embryos, parthenotes without or with sperm tail. Centriole number alteration (more than 2 pair of centrioles) was observed only in parthenogenetic blastomers, while no centrioles were detected in ICSI embryos or parthenotes with injected sperm tail. *p<0.05.

In the second set of experiment we compared blastocysts obtained from ICSI embryos and parthenotes with injected sperm tail. The number of blastomers with an altered number of centrioles were not statistically different between the two groups (Table 14). This results validated our hypothesis demonstrating that the injection of centriole is sufficient to regulate and control *de novo* centriole formation.

	ICSI	**Tail injection plus activation**
Oocytes	34	30
48h	24	25
144h	4	10
Blastomer analyzed	139	79
Blastomers with >2 centrioles	10 (7.2%)	5 (6.3%)

Table 14. Centriole number in blastomers of 144h ICSI embryos and parthenotes with sperm tail. Centriole number alteration is not statistical different between ICSI embryos and parthenotes in which sperm tail was injected.

These results indicate that chromosomal instability in parthenogenetic cells is caused by the presence of functionally active extranumerary centrioles. These centriole abnormalities are present in all cell types analyzed (ESCs and foetal fibroblasts), independently from the species (human, pig and sheep). These alterations are already present in embryos since the first mitotic divisions and, consequently, before the derivation of cell lines, demonstrating that are not caused by the isolation protocol or culture conditions used for ESCs. Furthermore, data obtained after sperm tail injection demonstrated that all these alterations are cause by the absence of male proximal centrioles contribution.

PART 2: ADAPTIVE MECHANISMS ACTIVE IN PARTHENOGENETIC CELLS

The high rate of aneuploidy together with multipolar centrosome should lead to the inhibition of cell proliferation and to a rapid cell death through the activation of the mitotic checkpoint [273]. However we observed that both human and porcine parthenogenetic cells undergo unlimited cell divisions and maintain the ability to differentiate *in vitro* into normal cell types if cultured in the appropriate conditions, as described above[257,274]. Furthermore, the capacity of cell proliferation and differentiation was certainly maintained also in sheep parthenotes developed *in utero* since they showed a normal morphology (Figure 34) with a beating heart as previously described by Loi et al. [159].

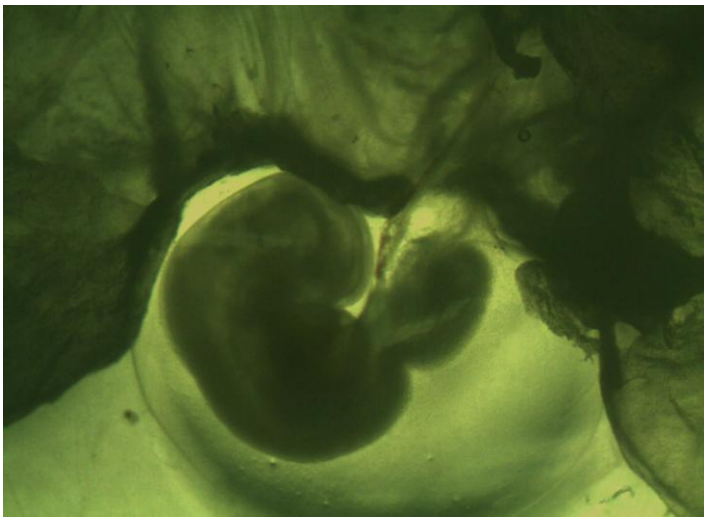


Figure 34. Sheep parthenote after 21 days of gestation in utero shows normal morphological features and a beating heart.

Apoptotic index and expression levels of apoptosis-related genes

In agreement with our observation TUNEL assay showed a low apoptotic index of parthenogenetic cells, that was not significantly different from their bi-parental counterpart (Figure 35).

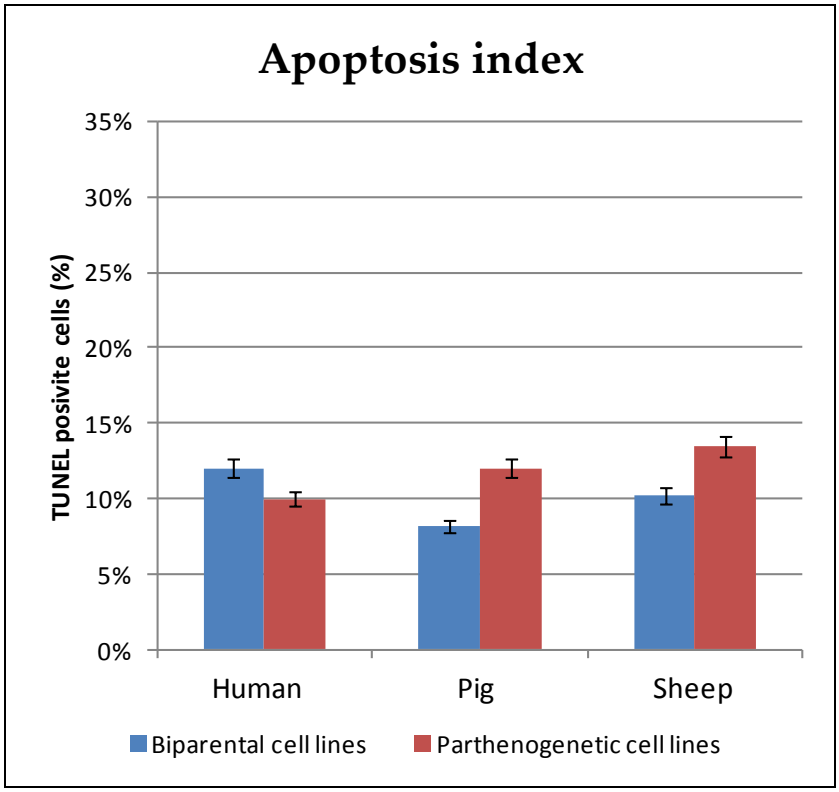


Figure 35.Quantification of apoptotic index in human, pig and sheep bi-parental and parthenogenetic cell lines. TUNEL-positive cells were scored randomly choosing five microscopic fields to reach a total of at least 900 cells and averaged. Error bars represent SD. The high rate of aneuploidy and multipolar centrosome does not increase TUNEL-positive cell number and the apoptotic index in parthenogenetic is not significantly different from that of corrisponding bi-parental counterpart.

To confirm this observation we also examined by Real Time-PCR the expression levels of TP53 and TP21, the two genes mainly involved in apoptosis mechanism activation. Results obtained clearly showed that both transcripts are significantly down regulated in parthenogenetic lines of all species, suggesting this as a possible way used by these kind of cells to continue their proliferation and to prevent apoptotic process activation even in the presence of high levels of aneuploidy (Figure 36).

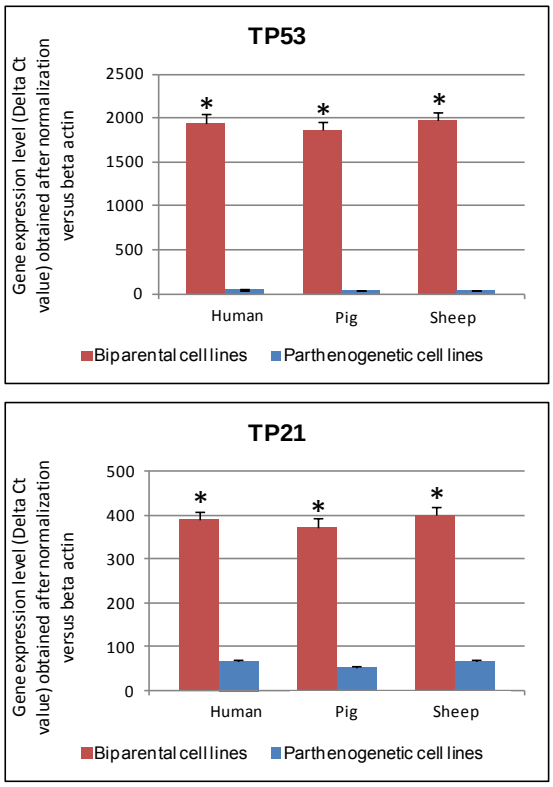


Figure 36. Aneuploidy-related stress limits cell proliferation through a p53- and p21- dependent mechanism. On the contrary, we observed a down-regulation of p53/p21 pathway in all parthenogenetic cell lines. $^{*}p<0.05$.

Characterization of autophagic activity

Aneuploidy in yeast and mammalian cells leads to a stress response caused by the unbalance in cellular protein composition [275]. For this reasons we also investigated for the presence of autophagic degradation in parthenogenetic cell lines. This mechanism may help them to eliminate extranumerary centrioles and probably may represent an alternative way to re-establish a correct centrosome complement and to preserve the diploid status.

This phenomena, in fact, is known as an evolutionary conserved and strictly regulated lysosomal pathway utilized in cells in order to eliminate aberrant and dysfunctional organelles [276]. Results obtained using TEM and immunostaining analysis showed that parthenogenetic cells contained fagosome structures and exhibited a strong punctuate signal following the incubation with a lysosome-specific probe (LysoTracker Red DND-99) as well as with an anti-LC3 antibody, both of which indicate the presence of high amounts of lysosomes and autophagosomes in the cytoplasm (Figure 37).

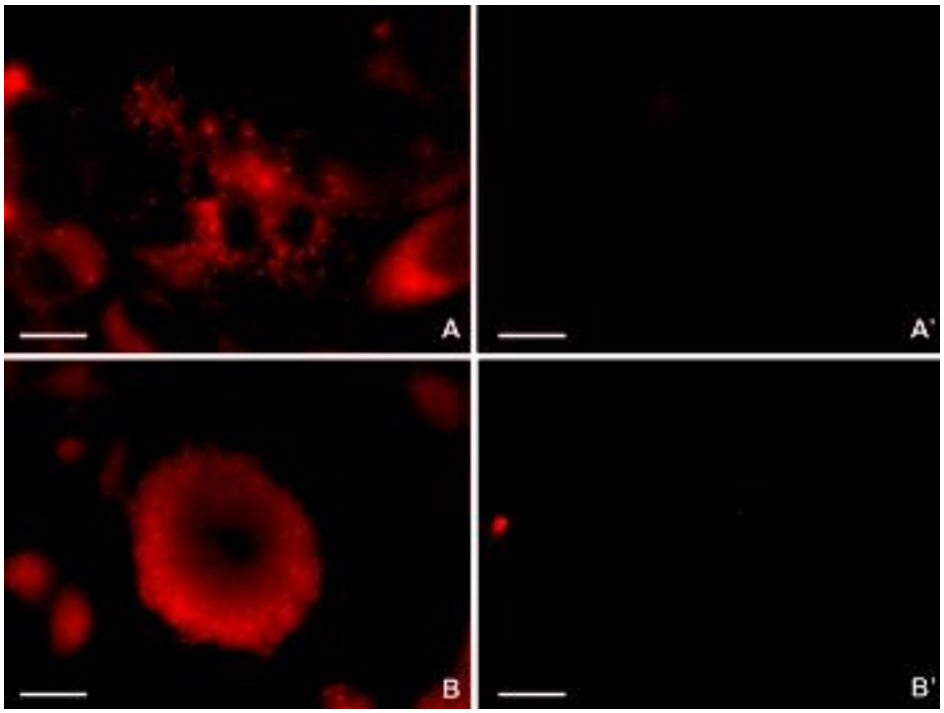


Figure 37. Autophagic activity in parthenogenetic cells. The punctuate positivity in cells after Lysotracker Red staining (panel A) and immunocytochemical localization of LC3 (panel B) indicate the presence of numerous lysosomes and autophagosomes. Panels A′,B′: negative controls. Bar: 10 μm.

Consistent with morphological observations, LC3 expression was nearly 8 times higher in mono-parental cell lines than in bi-parental (Figure 38). We also observed a significant increase in the expression levels of other specific genes that correlate with the activation of the autophagic program, such as Autophagy related 5 homolog (ATG5), Sirtuin1 (SIRT1), E1A binding protein p300 (EP300) and E2F transcription factor (E2F1) (Figure 38). At the same time the analysis of Mechanistic target of rapamycin

(MTOR), a well characterized autophagy inhibitor [277], showed specific mRNA levels 10 times lower in parthenogenetic than in bi-parental cells (Figure 38).

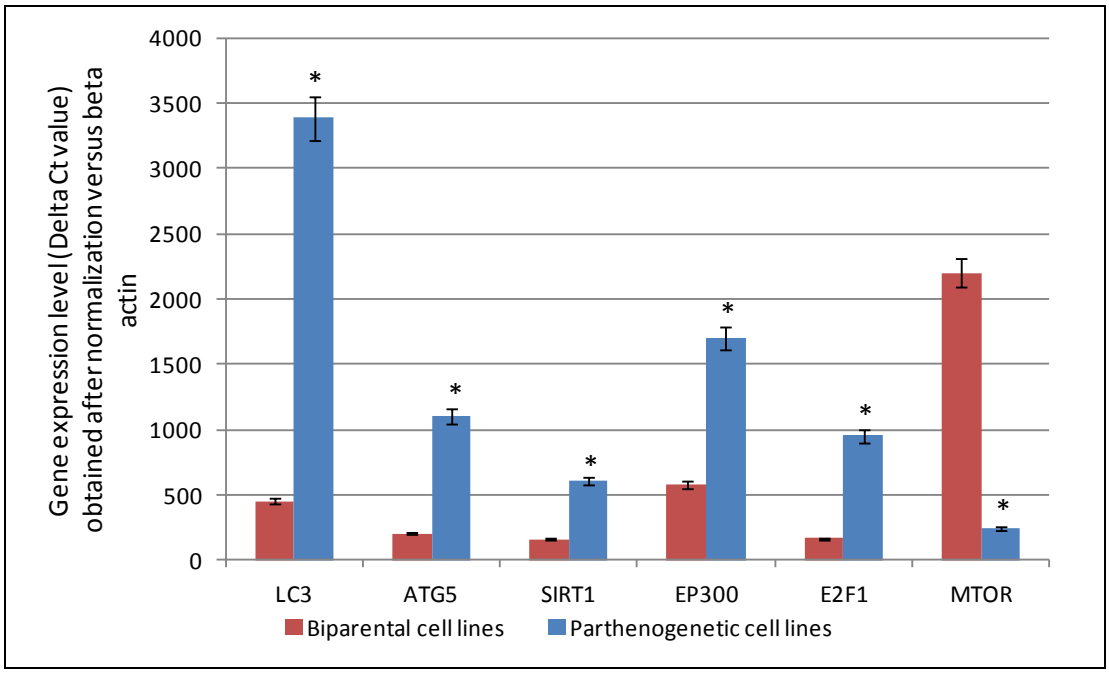


Figure 38. The significant difference of autophagy levels between human parthenogenetic and bi-parental cell lines can be appreciated through the several fold difference in the expression levels of autophagy-related genes. *p<0.05.

The presence of inter-cellular bridges

The high rate of supernumerary centrosomes and of severe aneuploidy of parthenogenetic cells, is inconsistent with the in continuous proliferation and ordinate *in vitro* differentiation as yet described. We hypothesized that this could be also possible through a wide communication between cells that provides mutual exchange of missing cell products and, at the same time, alleviates the unbalance in cellular protein composition that would hamper normal cell functions. Ultrastructural analysis clearly showed the existence of intercellular bridges variable in size that are compatible with the notion of reciprocal cell support, similarly to what occurs among germ cells (Figure 39). In addition in parthenogenetic cell lines and sheep post-implantation parthenote cells displayed the presence of communications besides the wide cytoplasmic characterized by actin bundles, unbalanced chromatid separation and chromosomal bridges (Figure 39).

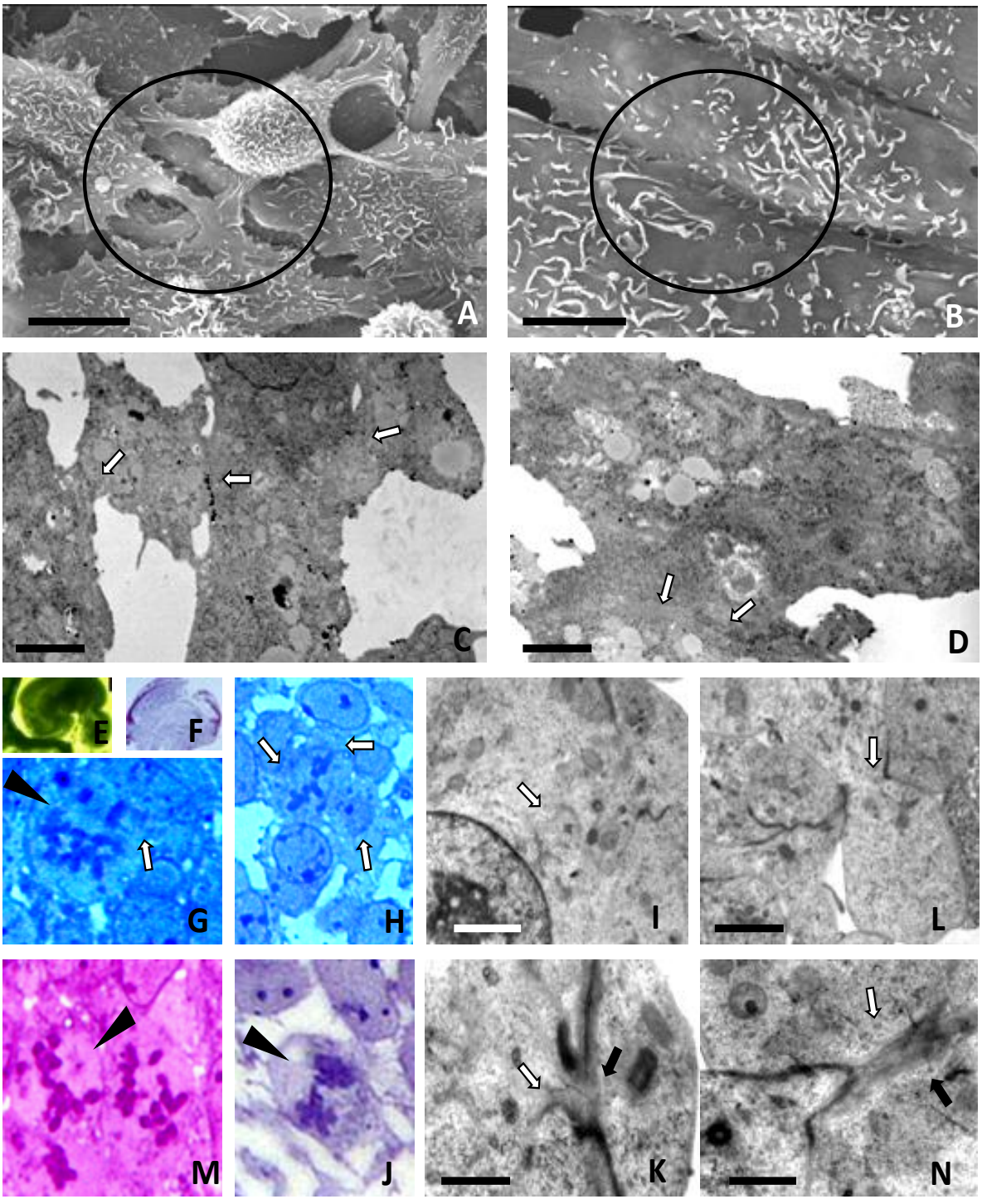

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Figure 39. Electron microscopy of human and sheep parthenogenetic cells demonstrate intercellular bridges, allowing a cytoplasmic continuity between the cells: SEM (encircled areas), TEM (white arrows). In utero sheep parthenote (21 days of gestation) (panel E) and semithin sagittal section counterpart (panel F). Light microscopy of sheep post-implantation parthenote cells shows cytoplasmic bridges (panels G-H white arrows) and unbalanced chromatid separation (panels G-Jarrowheads). Tripolar metaphase cell as a result of the presence of multiple centrioles (panel M, arrowhead). Anaphase cell with one intact chromatin bridge (panel J, arrowhead). TEM ultrastructure of sheep parthenote cells show the presence of intercellular bridges (panels I and N-white arrows), wide open cytoplasmic communication, and in detail (panels K and N) actin patches (black arrows) at the level of the intercellular canal. Bars (panel A)10 μm; (panel B) 6 μm; (panel C) 1.25 μm; (panel D) 1 μm; (panel I) 1 μm; (panel L) 1.5 μm; (panel K) 800nm; (panel N) 800nm.

Consistent with the morphological observations, human parthenogenetic cells showed up regulation of both AURKB (9-fold) and KLHL3 (5-fold), compared with their bi-parental counterpart. These two molecules are known to actively stabilize intercellular canals [278]. Furthemore, 5-fold increase of TSN expression level was detected in mono-parental cells (Figure 40). This up regulation is compatible with an active mRNA distribution between connected cells (Figure 40).

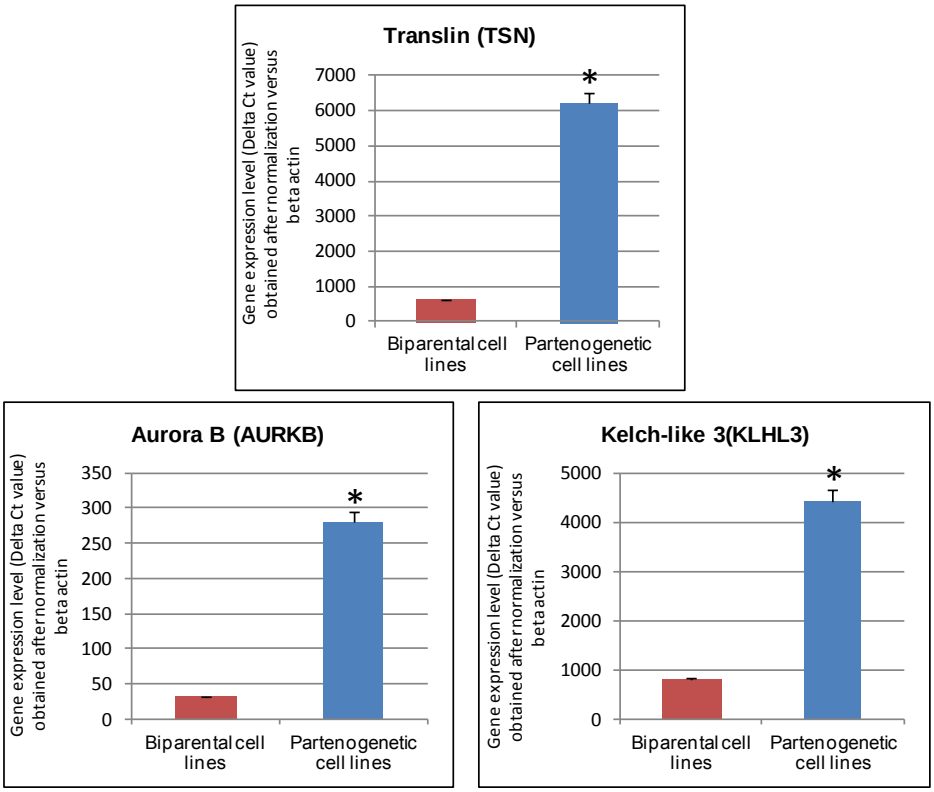

Figure 40. Up regulation of TNS (a well known RNA-binding protein that transports mRNA molecules between cells, through intercellular canals), of AURKB and of KLHL3 (recently described as an essential stabilizer of intercellular bridges) validate the morphological features. *p<0.05.

Functional intercellular trafficking activity through intercellular canals identified with ultra-structural analysis was demonstrated by an extensive migration among cells of fluroscent 10-kDa dextran that was injected into the cytoplasm of a single cell. This molecule in fact moved from the cell injected to others (Figure 41) suggesting their use for transfer of mRNAs, proteins and cell products among cells.

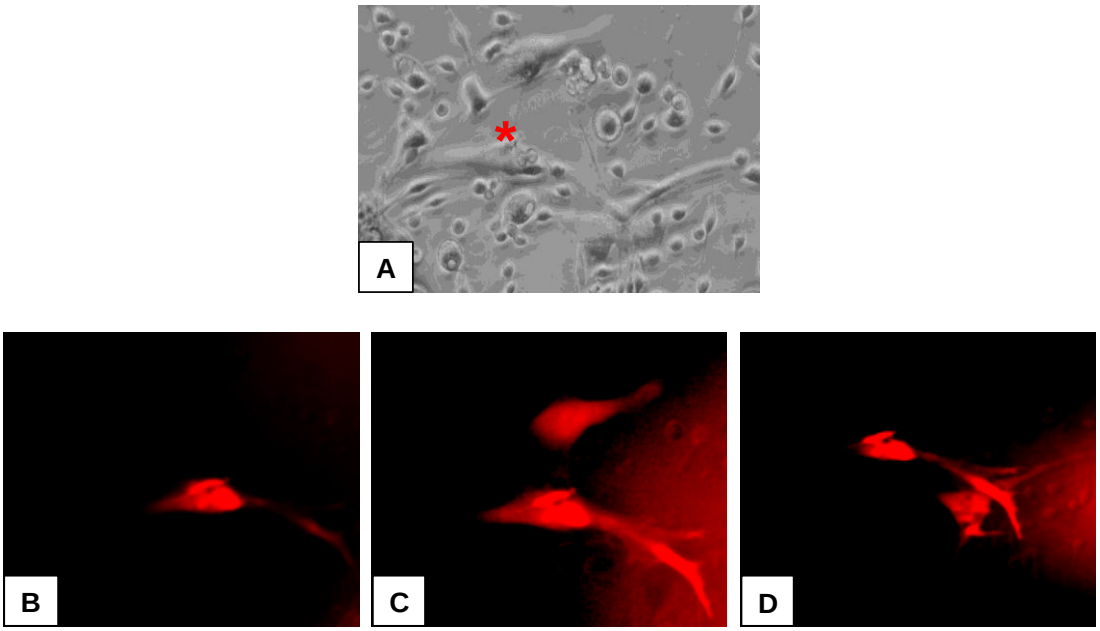

Figure 41. Cells injected with 10-kDa dextran. Functional activity of intercellular bridges in parthenogenetic cells (panel A) is demonstrated by migration of dextran molecules from the injected cell (panel B) to adjacent cells (panels C and D).

All the results obtained in this second part of thesis demonstrate that, despite ploidy alteration, parthenogenetic cell lines continue to proliferate without active apoptosis mechanism. This is possible thanks to significant down regulation of p53 and p21 genes observed in our uni-parental cells when compared with their bi-parental counterpart.

Furthermore parthenogenetic cells show a dramatic increase of autophagic activity which helps them to eliminate extranumerary centrioles and probably may represent an alternative way to re-establish a correct centrosome complement and to preserve the diploid status.

Since this mechanism was insufficient to prevent an incidence of chromosome number alterations, but cell proliferation and differentiation was not compromised, the extensive network of intercellular bridges identified among parthenogenetic cells may have a crucial role.

5. DISCUSSION

The development of parthenotes to the blastocyst stage was reported for several species [109,153,158-162,279], but limited data related to the potential plasticity of the cell lines derived from them and their biological characteristics, with specific regards to the potential abnormalities associated with their origin, are available.

Since our previous studies demonstrated the presence of abnormal karyotype in human parthenogenetic cell lines [257], in the experiments reported in this thesis we looked at the incidence of chromosomal abnormalities in human, porcine and sheep parthenogenetic cell lines and compared it with their bi-parental counterparts.

The results described in this thesis showed that hypo-haploid and hypo-diploid configurations were the most frequent in all parthenogenetic cell lines, irrespective of the species and of their origin: pluripotent cell line or primary culture (Figure 28), while the diploid configuration represented the modal value in the bi-parental cell lines of all species. In particular, we were able to identified not only chromosome number alteration in human parthenogenetic ESC lines, where the donor age is relative old, but also in parthenogenetic porcine ESCs. In the latter case oocytes were retrieved from young animals just after puberty (the age of high reproductive fitness in this species), suggesting that chromosome alterations are independent from the donor age.

Consistent with our results, a similar incidence of aneuploidy was recently described also in cell lines obtained from bovine parthenotes [280], which were derived from oocytes isolated from animals in their full reproductive fitness.

Moreover we detected hypo-haploid and hypo-diploid configurations in primary cultures of fibroblasts obtained from parthenotes transferred into the uterus of synchronized recipients and allowed to develop *in vivo* for 21 days, the maximum length of time of sheep parthenogenetic development [159]. These results give us the possibility to rule out the experimental protocols used in our laboratory for establishing and maintaining of embryonic stem cell lines as possible causes of aneuploidy.

In agreement with these data, the presence of frequent chromosomal abnormalities in pre-implantation mammalian parthenotes has been previously described both in human [281-283] and animal species [284-286].

Furthermore the results obtained in the experiment carried out in this thesis demonstrated that parthenogenetic cell lines of all species showed a high and significantly different incidence of supernumerary pairs of centrioles (Figure 30). Furthermore the presence of multipolar asters in parthenogenetic cell lines (Figure 31) confirms that extranumerary centrosomes are functional and able to form multipolar spindles similar to that of tumour cell lines [287].

We also observed a significant high rate of parthenote blastomers (59,57%) showing an abnormal centriole number. In particular our results showed that centriole formation were evident in parthenotes from the 4-cell stage and a high incidence of abnormalities was immediately visible. This is the first description of centriole dynamics during parthenote pre-implantation development in a non-rodent species and indicates that centriole abnormalities are not linked to a prolonged culture, but are already present at the blastocyst stage, when embryonic cell line derivation takes place.

Similar abnormalities have been previously described in human parthenogenetic embryos, that were obtained from oocytes spontaneously activated and/or induced with puromycin [282,283]. 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 has been reported in parthenotes derived from bovine activated oocytes, with abnormalities occurring as early as completion of the first cell cycle [166].

All these observations are in agreement with the recent observations that centriole *de novo* assembly is normally turned off when one centriole, acting as template, is present [288]. 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 detect in these cell lines.

The relationship between parthenogenesis and centrosome, in fact, is complex. It is important to point out that in mammals, with the notable exception of mice [206], centrosome of the male and female gametes undergo a reciprocal reduction. In fact, centrioles degenerate and are lost during oogenesis and while oogonia and growing oocytes display normal centrioles until pachytene stage, they are absent in the mature oocytes [199]. At the time of fertilization, the distal centriole is carried into the ooplasm by the sperm, where it mixes with a stockpile of centrosomal proteins and generates a functional zygotic centrosome [179,204,208,209]. This process has been described in rhesus monkeys [200], rabbits [201], cows [99], sea urchins [202], Xenopus [203] and several other species [204].

Indeed in mammalian parthenotes, 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. However, in many insects where parthenogenetic development is an obligatory or facultative form of reproduction, oocyte ability to generate a functional centrosome, in the absence of the sperm contribution, has evolved [221].

The increased number of centrioles determined with the localization of punctuate centrin, was also confirmed by the

alterated expression levels of PLK2 and CCNF in our parthenogenetic cell lines. In particular, we detected up regulation of PLK2, a molecule required for centriole duplication [244], in human (15,9-fold) pig (15,7-fold) and sheep (13,9-fold) derived lines and a down-regulation of CCNF (6,5 folds) in human cells (Figure 32). These results are consistent with the recent finding that siRNA mediated depletion of CCNF in HeLa cells induces centrosomal and mitotic abnormalities, including multipolar spindles and asymmetric, bipolar spindles with lagging chromosomes [252], similar to the alterations we found in parthenogenetic cell lines and described in this thesis. Altered expression levels of mitotic check point molecules were also found in human parthenogenetic cells (Figure 33). In particular, the comparison of uni-parental pluripotent cells with their bi-parental counterpart (HES 7, HES I-3 and HES I-6) indicated a much higher level of expression of MAD1, and the related molecules MAX and SIN3 in parthenogenetic cells. MAD1 is a central component of the spindle assembly checkpoint and recruitment of kinetochores [248-250] and its de-regulation has been shown to affect cell cycle progression with a decrement of cell proliferation and a protracted G1 phase [251]. The altered levels of such molecules may be related to the lack of paternal contribution in spindle assembly. A similar explanation could account for the very low transcription of BUB1, CENPE and TTK detected in these cells. These two

molecules, in fact, are normally involved in kinetochore-microtubule binding, correct chromosome congression and alignment, as well as segregation during mitosis. In particular, BUB1 and CENPE disruption usually causes a reduction of tension across the centromere and an increased incidence of spindle pole fragmentation, resulting in a mixture of aligned and unaligned chromosomes [246].

Since the lack of a paternally derived centriole, able to recruit the egg centrosomal material to form a functional centrosome, was the common feature in all the three species examined here, we suggest that parthenogenetic activation *per se,* rather than the oocyte donor age or the culture method, is responsible for the centrosome alterations.

This hypothesis was definitively validate by the results obtained after sperm tail injection in porcine matured oocytes. Immunostaining carried out on parthenogenetic activated embryos previously injected with tail containing male proximal centrioles, displayed a normal centriole number comparable to that of the *in vitro* fertilized embryos. This confirm that the presence of male centriole can restore the negative regulatory mechanism that suppress *de novo* centriole assembly.

Furthermore, since the presence of multipolar spindle is usually linked with ploidy alterations, these results may explain the high degree of aneuploidy characterizing parthenogenetic cell lines.

Generally a high rate of aneuploidy together with multipolar centrosome should lead to the inhibition of cell proliferation and to a rapid cell death through the activation of the mitotic checkpoint [273]. Aneuploidy in yeast and mammalian cells leads to a stress response caused by the unbalance in cellular protein composition [275]. Recent results indicate that aneuploidy-related stress limits cell proliferation through a p53-dependent mechanism [289].

On the contrary, we observed that human and pig parthenogenetic ESC lines undergo unlimited cell divisions and maintain the ability to differentiate into normal cell types if cultured in the appropriate conditions [67,257]. Furthermore, the capacity of cell proliferation and differentiation was certainly maintained also in the sheep parthenotes developed *in utero* since they showed a normal development and organogenesis with a beating heart [159], in the absence of any overt sign of malignant transformation. Consistently with these observations the apoptotic index of all parthenogenetic cell lines described in this thesis is low and not significantly different from their bi-parental counterparts (Figure 35). This is made possible thank to a significant down regulation of p53 and p21 genes identified in parthenogenetic lines of all species (Figure 36). Indeed these gene expression alterations may represent the possible way used by

these cells to continue their proliferation even in the presence of high levels of aneuploidy and centrosome alterations.

Similar findings were recently described in a Drosophila transgenic strain that contains extra centrosomes in around 60% of somatic cells but that is morphologically normal, except for a long delay in development compared to the wild type [234]. Drosophila cells, however, did not show an increased incidence of aneuploidy, probably because most of the extra centrosomes were able to cluster forming a bipolar spindle by metaphase. This is different from the cells examined in the current experiments which showed a high and steady rate of aneuploidy. Indeed we have been unable to detect the clusterization of supernumerary centrioles in our cells.

By contrast we identified through morphological and molecular analysis the presence of autophagic activity, an evolutionary conserved and strictly regulated lysosomal pathway for cleansing aberrant and dysfunctional organelles [276].

LC3 positivity, in fact, indicates the presence of high amounts of lysosomes and autophagosomes in the cytoplasm of parthenogenetic cells (Figure 37). This molecule (also known as MAP1LC3) assists autophagosome formation and is present in sealed autophagosomes [276]. Consistent with the morphological results, expression levels of autophagy related genes (ATG5, SIRT1, EP300 and E2F) were higher in parthenogenetic cells than

in bi-parental ones (Figure 38), while MTOR, a well characterized autophagy inhibitor [277], showed specific mRNA levels 10 times lower in uni-parental cell lines than in bi-parental. In particular, ATG5 has a pivotal role in autophagosome formation, through its recruitment together with ATG12, ATG16 and LC3 to the phagophore. The expression of this autophagic modulator can be up-regulated by E2F1 [290] and it can be deacetylated by SIRT1 [291]: both mechanisms result in enhanced autophagy, with potential positive effects on cell life span [292]. It is interesting to note that SIRT1, not only promotes autophagy [293] but also inactivates p53 by deacetylation [294] therefore reinforcing the down-regulation of p53 observed in parthenogenetic cell lines.

This very intense autophagic activity is probably an alternative way to re-establish a correct centrosome complement and to preserve the diploid status, providing a way to eliminate the extranumerary centrioles and chromosomes. This process is likely to be used as an active self-protective strategy in order to eliminate highly abnormal organelles, thus contributing to cell survival and ensuring the maintenance of a "normal" population [295]. Since we have previously shown that centrosome amplification is already taking place at the blastocyst stage, when ICM are isolated for cell line derivation, it is interesting to note that autophagic degradation has recently been described in early embryonic development and it was suggested to be used for

removal of obsolete maternal factors [296]. We hypothesize that parthenogenesis-related anomalies prolong and amplify the ongoing autophagic processes and therefore result as a preferred pathway for counteracting the deleterious effects of centrosome amplification, as opposed to other systems where centrosome clustering is commonly observed [297].

Optical and ultrastructural analysis clearly showed the existence of intercellular bridges (Figure 39). Injection of fluroscent 10-kDa dextran also confirmed the functional activities of this canals (Figure 41). Moreover our results demonstrated that parthenogenetic cell lines displayed up regulation of molecules essential for the stabilization of the intercellular canals containing chromosome filaments (AURKB and KLHL3) [278]. These connections can be considered as a reminiscent of the midbodies converted into stable canals and correlated to chromatin bridges [278]. Similar structures have been described in the form of stable intercellular bridges between germ cells [298] and provide a mean for transfer of mRNAs, proteins, and cell products [299]. In particular, mRNAs are transported through intercellular bridges by a testis-brain-RNA-binding protein called Translin (TSN) [300]. Arrested cytokinesis is a common process taking place during spermatogenesis [299]. However gene expression pattern and morphological analysis indicated that the communications observed between parthenogenetic cells are more similar to the

recently described intercellular canals that persists between two cells when chromosome segregation is altered for any reason [278], as it occurs in about 1% of dividing somatic cells and, at higher rate, in transformed cells [301].

Since the dramatic increase of autophagic activity was insufficient to prevent an incidence of aneuploidy, but cell proliferation and differentiation was not compromised, we hypothesized that this canals may provide a mutual exchange of missing cell products between adjacent cells [300,302] and, at the same time, alleviates the unbalance in cellular protein composition that would hamper normal cell functions.

6. CONCLUSIONS

Our data provides evidence that the lack of paternal centriole in the parthenogenetic zygote leads to a centrosome amplification due to the lack of appropriate inhibition mechanisms and the ensuing multipolar mitosis cause severe chromosomal instability (CIN). Despite it is becoming clear that CIN probably plays a causative part in a substantial proportion of malignancies [303] we found that it is surprisingly compatible with parthenogenetic embryonic stem cells *in vitro* proliferation and differentiation as well as with parthenotes post-implantation development *in vivo*. Our data support the view that this is possible through a series of compensatory mechanisms that suppress apoptosis and enable cells to support each other through wide and stable network of intercellular communications.

Our data lead us to propose that deriving cell lines from human parthenotes, and from animal species that share the same fertilization model, can help us to understand the mechanisms that link parthenogenesis, pluripotency and oncogenesis. We think that this has previously gone largely unnoticed because of the substantial difference in the fertilization process between the mouse and all other mammalian species, of the very limited number of human and non-rodent parthenogenetic cell lines available and of the absence of obvious phenotypic alterations. However the extensive concordance between our observations

and a wide amount of experimental data obtained in completely different experimental models, support the physiological relevance of our data. The findings also indicate that parthenogenesis could be an unexpected but very powerful model to study the onset of malignant transformation.

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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

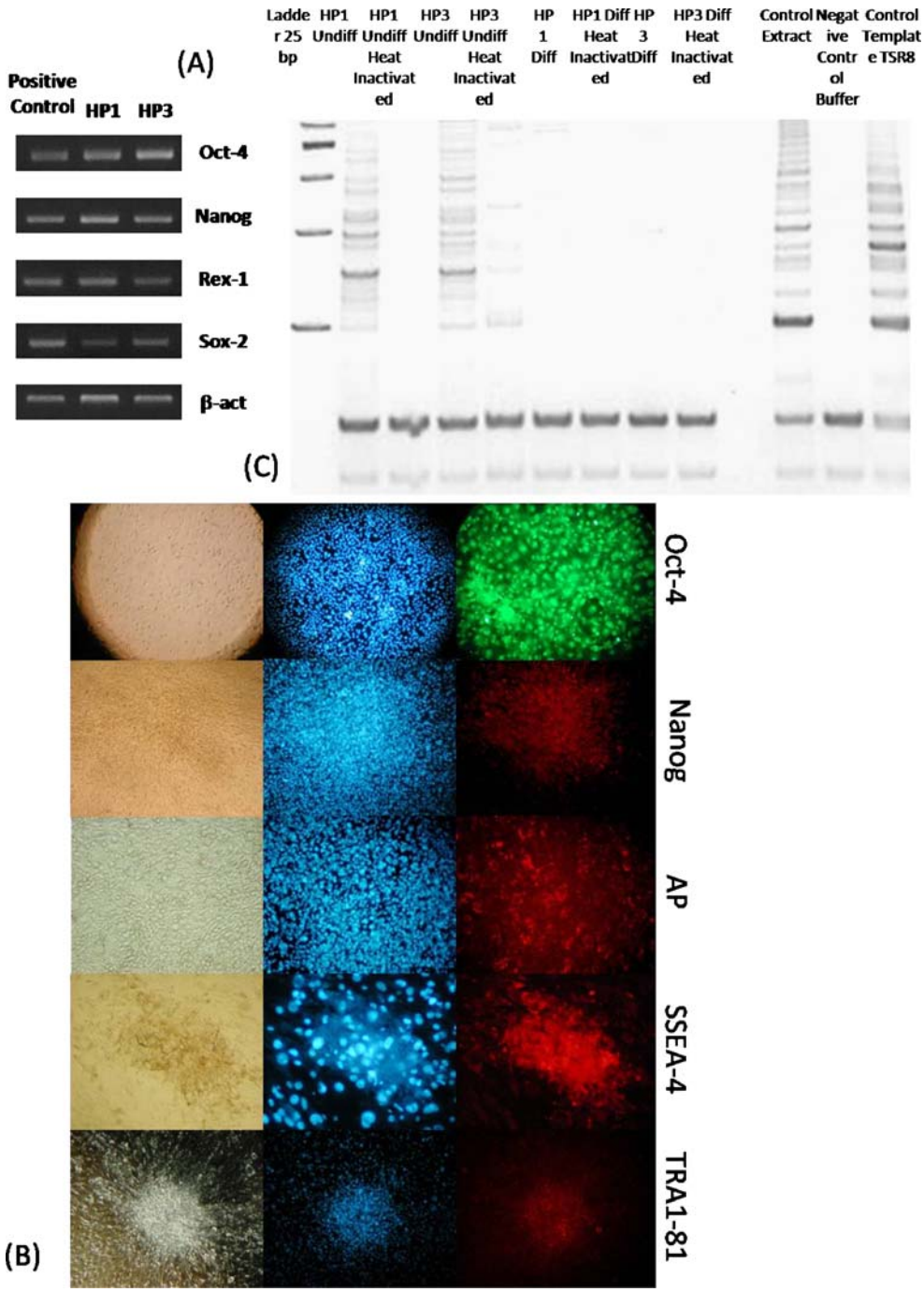

Fig. 1 Pluripotency of HP cells is demonstrated by their positivity to several known pluripotency-related markers and by their telomerase activity. The expression of Oct-4, Nanog, Rex-1, Sox-2 is shown by RT-PCR screening of RNA extracted from HP cells. Beta actin and RNA from bi-parental embryonic stem 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

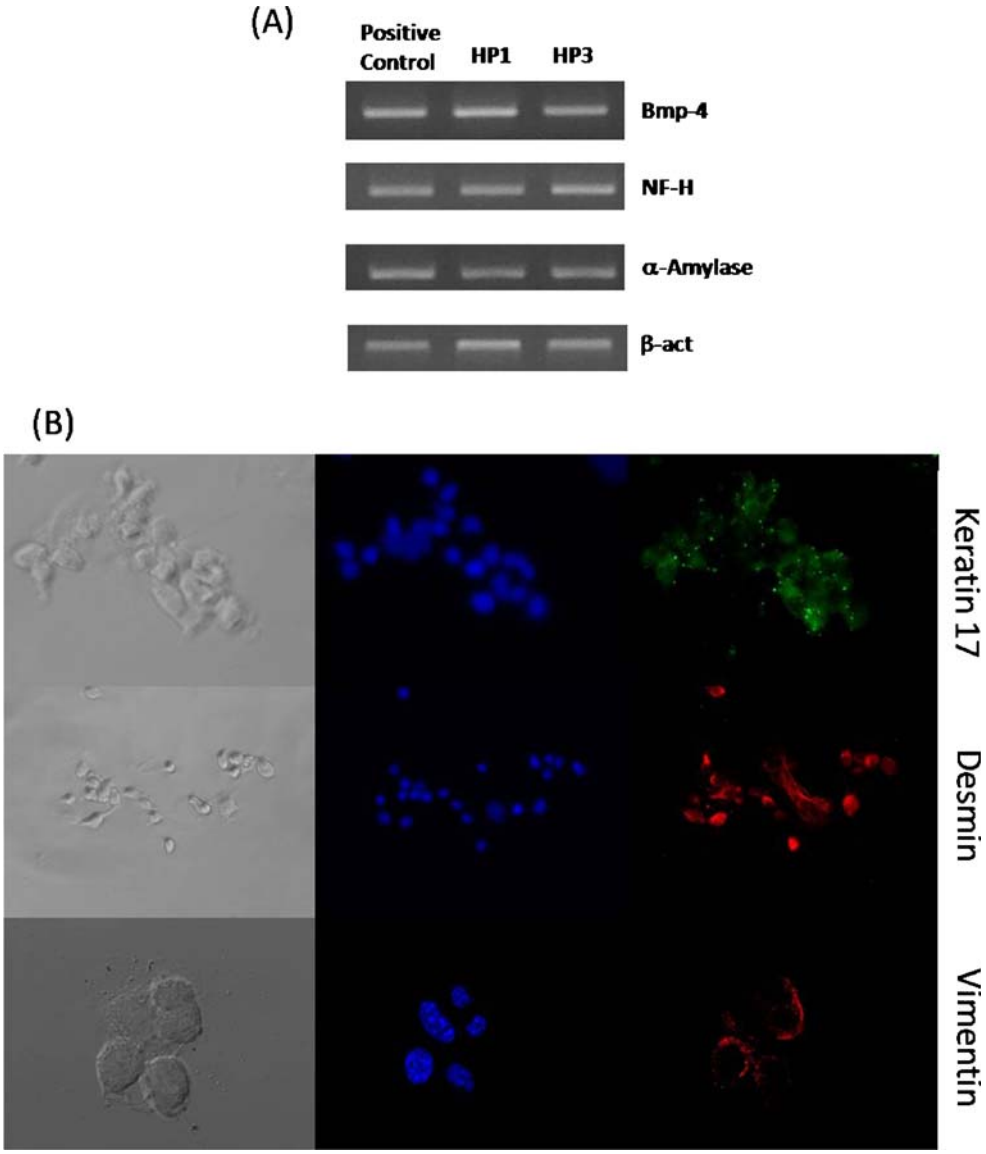

Fig. 2 Differentiation ability of human parthenogenetic cells after induction of EB formation. RT-PCR analysis of RNA extracted from EBs consistently demonstrated expression of markers specific for the three germ layers and, more in details bone morphogenetic protein-4 (Bmp-4, mesoderm), neurofilament 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

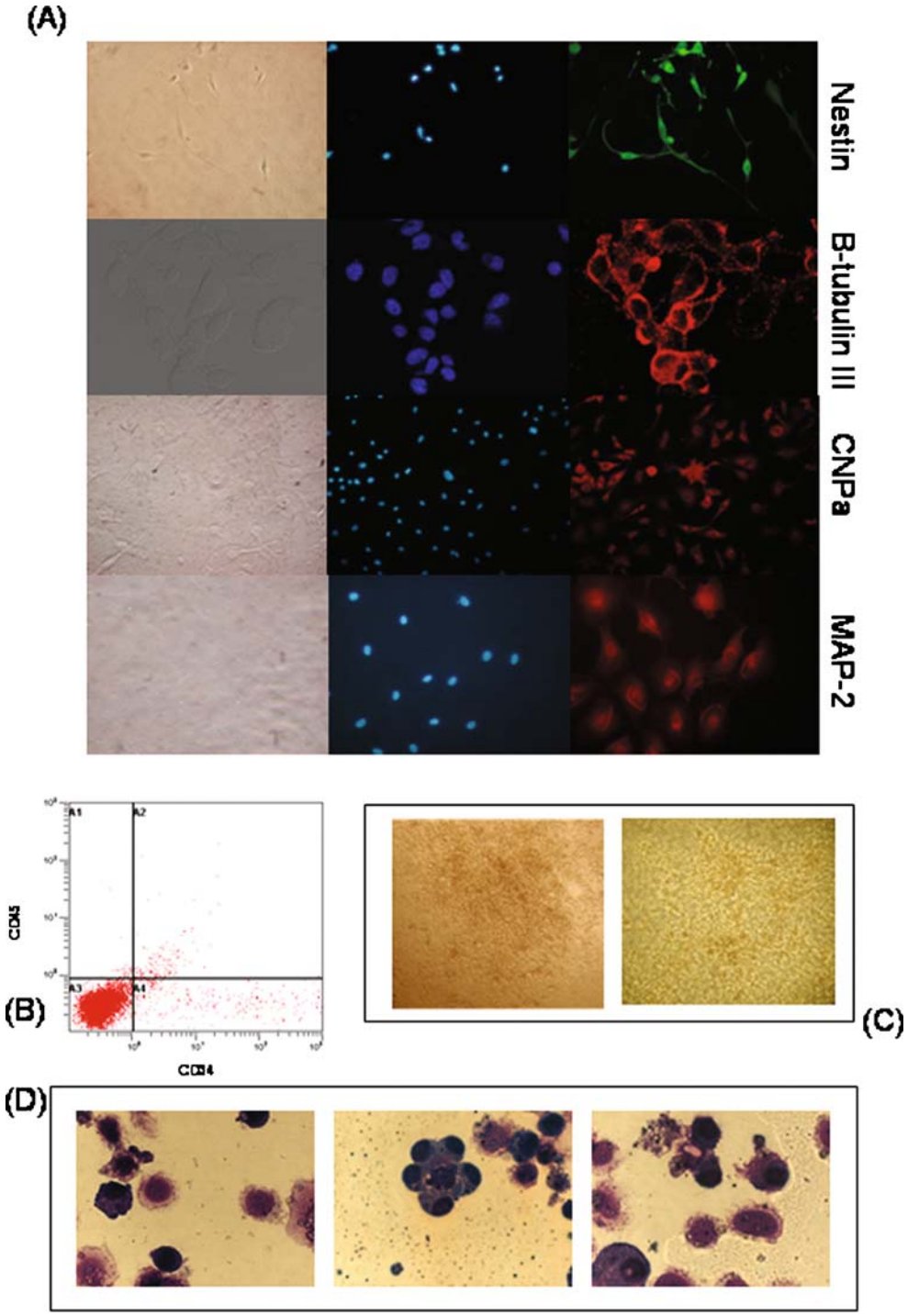

Fig. 3 Neural and hematopoietic differentiation of HP cells. Culture conditions routinely used to address bi-parental embryonic stem cells towards neural differentiation successfully drove HP cells to form different cell subtypes belonging to the neural lineage, with cells displaying immune-positivity 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

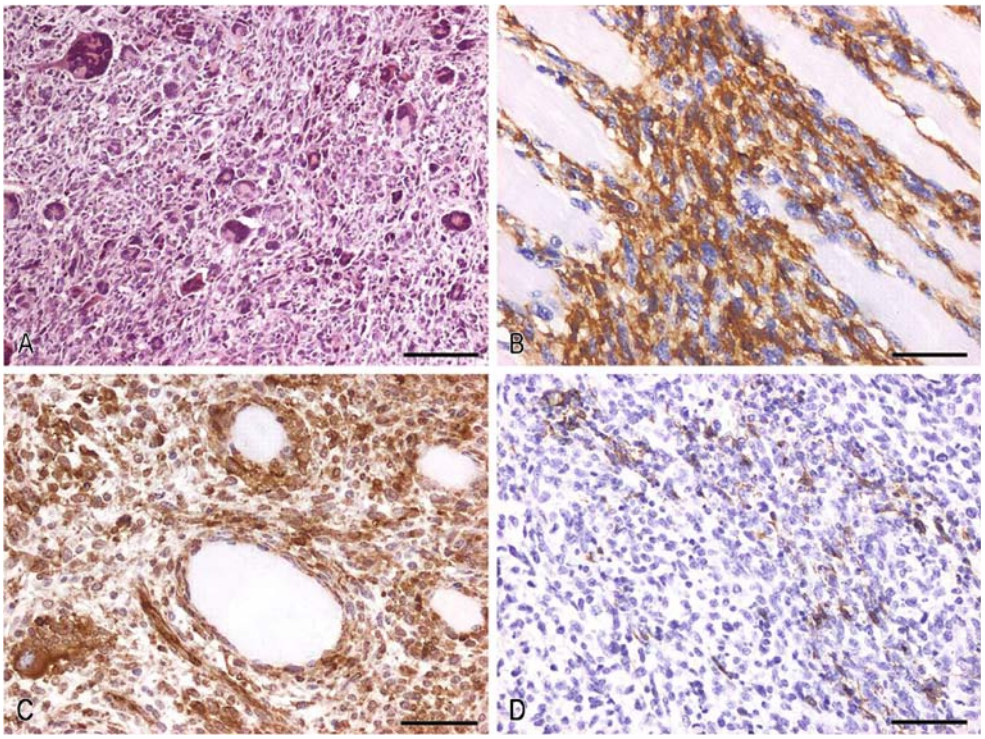

Fig. 4 Formation of myofibrosarcoma-like tumors following injection of undifferentiated HP cells in the hind leg of SCID Beige and Fox Chase SCID mice. Numerous multinucleated cells showing aberrant mitotic figures (**a**). Vimentin-positive (**b**) and Smooth Muscle Actin-positive (**c**) cells dissecting 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.

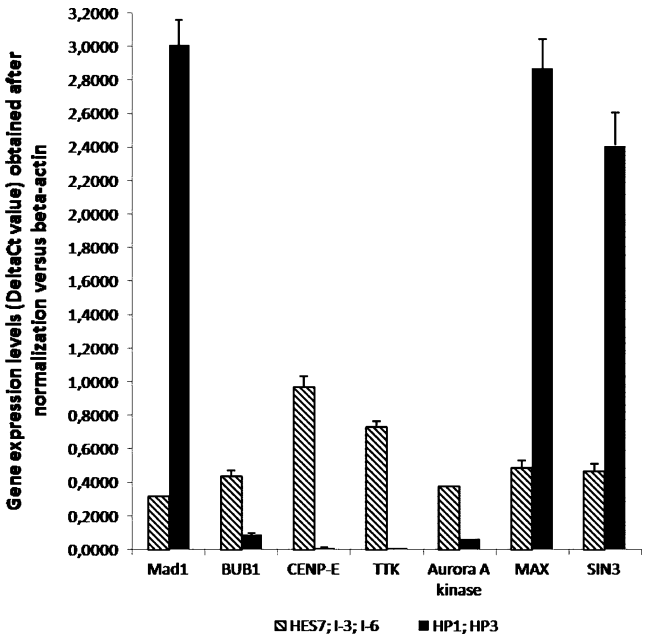

Fig. 5 Expression level of molecules related to spindle formation and chromosome segregation in HP cells and bi-parental cell lines HES 7, HES I-3 and HES I-6. Bars represent the average ΔCt of HP cells (*solid bars*) and bi-parental cells (*striped bars*) related to the genes examined. ΔCt value was 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

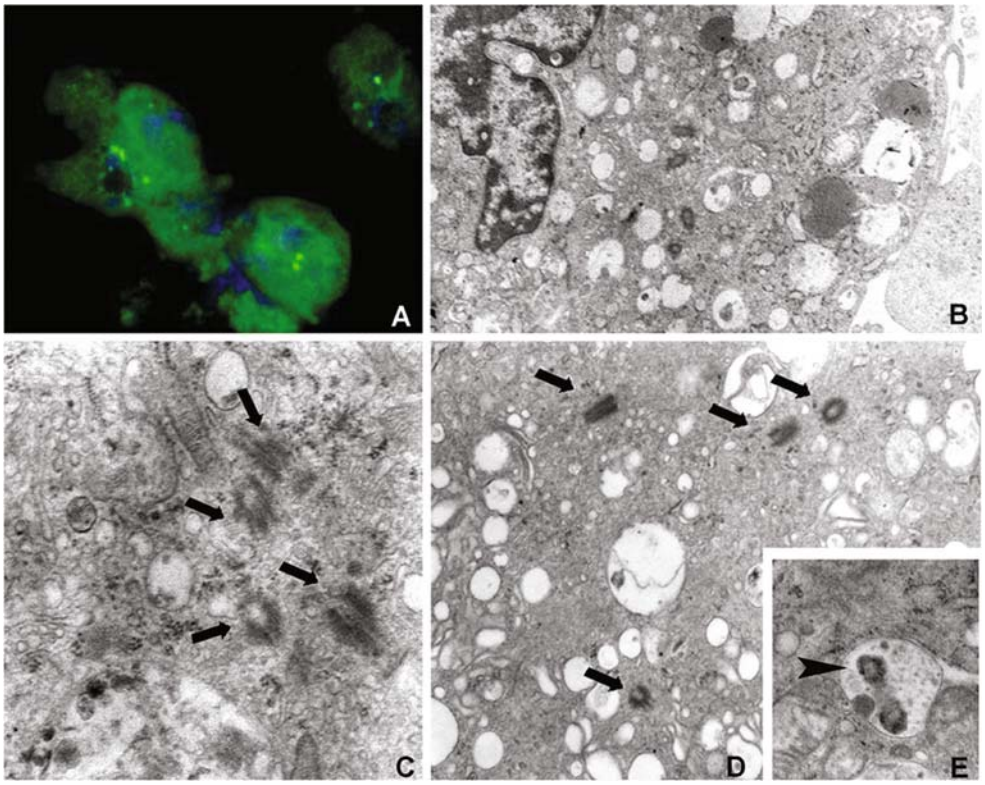

Fig. 6 Immunohistochemical (**a**) and ultrastructural (**b-c-d-e**) analysis of human parthenogenetic cells. The presence of amplified centrosomes loosely dispersed in the cytoplasm can be appreciated. Supernumerary centrioles (*arrows*) and massive autophagic processes generally coexist (**d**). Often 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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Culture Conditions and Signalling Networks Promoting the Establishment of Cell Lines from Parthenogenetic and Biparental Pig Embryos

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Abstract The generation of porcine embryonic stem cells (pESC) would potentially have great impact in the biomedical field given the long-standing history of the pig as a prime animal model for pre-clinical biomedical applications. These cells would also be beneficial for the agricultural area, allowing efficient genetic engineering of this animal, to improve health and production traits. Despite numerous reports, no conclusive results have been obtained on the isolation and propagation of pESC lines and the establishment of pluripotent cells from the pig has remained an elusive goal. In the present study we performed a systematic analysis of different culture media for their ability to support the establishment of homogenous outgrowths from in vitro-produced embryos. Furthermore, we investigated which molecular networks are responsive to the factors contained in the most efficient media, since the identification of dominant signaling pathways that regulate porcine stem-cell pluripotency is likely to facilitate the generation of genuine pESC. Finally we compared IVF blastocysts versus parthenotes as a possible source for putative pESC in terms of blastocyst rate, resilience to immunosurgery procedures, ability to attach to the feeder, to generate outgrowths and to establish stable cell lines.

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Introduction

The establishment of embryonic stem cells (ESC) in domestic animals could potentially have great impact both in agricultural perspective and biomedical fields. In particular, pig is a desirable species to create pluripotent cell lines because of its value as a biomedical model, due to its immunological, morphological, physiological and functional similarities to the human [1–4]. However, despite numerous reports, no conclusive results have been obtained on the isolation and propagation of putative porcine ESC (pESC) lines and no validated pESC lines are available as yet. Putative pESC established by Notarianni et al. [5] were poorly defined. In other studies no ESC-like cells survived passage 10, while epithelial-like lines survived up to passage 42, but failed to differentiate [6]. Talbot et al. [7] reported that pluripotency was difficult to maintain in ESC-like and epithelial-like cell cultures for more than a few passages. Further studies have been conducted by other authors [8, 9], however, the general consensus is that none of these lines were truly ESCs and pluripotent [10, 11] and a number of technical questions are still to be answered. The use of conventional protocols for culture of mouse and human ESC does not appear to sustain extended growth nor pluripotency of cultured porcine cells. Recently it has been demonstrated that medium components play a pivotal role in regulating intrinsic and extrinsic factors involved in the control of pluripotency and that specific pathways may be up-regulated, or down-regulated, in response to the addition of specific molecules [12]. It is evident that one important

aspect that needs to be addressed is the identification of appropriate in vitro culture conditions that may encourage establishment of homogeneous outgrowths, and ensure a favorable environment, as a preliminary step towards the isolation of genuine pig pluripotent cells. Here we present results of experiments designed in order to perform a systematic study of different media formulations. We identified ten different medium compositions and tested them for their ability to promote outgrowth formation. Furthermore, since the identification of dominant signaling pathways that regulate stem-cell pluripotency in the pig is likely to facilitate the generation of genuine pESC, we studied the expression pattern of molecular networks known to play a key role in the maintenance of pluripotency (LIFR, gp130, STAT3, FGFR-1, FGFR-2, PI3K, AKT and PTEN) in response to factors contained in the most efficient media. Finally, given the fact that in the literature a higher efficiency of ESC derivation is reported for parthenogenetic activated oocytes compared to biparental blastocysts [13–15], we compared embryos obtained from IVF versus parthenogenetic activation, to verify if this occurs also in pig. Furthermore we tried to identify what mechanism affects efficiency since it could help the derivation of cell lines regardless to the source of the embryo.

Material and Methods

Oocyte Collection and In Vitro Maturation (IVM)

Unless otherwise indicated, chemicals were purchased from Sigma- Aldrich (Milan, Italy).

Ovaries were collected from gilts of approximately 120 Kg at the local abattoir and transported to the laboratory in physiological saline (9g/l NaCl) at 30–34°C. Cumulus- oocyte complexes (COCs) were aspirated from antral follicles with an 18-gauge needle and vacuum pressure of 50 ml/min.

The follicle aspirate was collected in 15 ml tubes (Terumo, VenoSafe™) and only COCs with a large, compact cumulus and homogeneous oocyte cytoplasm were selected. In vitro maturation (IVM) was performed as previously described [16] on a total of 2351 oocytes.

Embryo Production

At the end of IVM (46 h), oocytes were denuded by gently pipetting in TCM-199 HEPES buffered medium, containing 0.1% hyaluronidase at 38.5°C and washed for 10 min in the same medium supplemented with 20% (*v/v*) fetal calf serum (FCS). Subsequently oocytes were fertilized with frozen-thawed spermatozoa or parthenogenetically activated as described below.

In Vitro Fertilization (IVF)

Frozen-thawed spermatozoa were purified by centrifugation through two-layer Percoll gradients. Live sperm cells were washed in Tyrode's albumin lactate pyruvate medium (TALP medium), consisting of 1.25 mM $MgCl_2.6H_2O$, 102.7 mM NaCl, 3 mM KCl, , 0.28 mMNaH_2PO_4, 5 mM $NaHCO_3$, 3.7 ml/l of Na lactate, 1 mM Na pyruvate, 20 mM HEPES, 1 mg/ml of polyvinylalcohol (PVA) and antibiotics.

Spermatozoa were then diluited in IVF medium (2.65 mM $CaCl_2$-$2H_2O$, 0.4 mM $MgCl_2.6H_2O$, 106.1 mM NaCl, 3.08 mM KCl, 26 mM $NaHCO_3$, 0.33 mMNaH_2PO_4, 1 mM Na pyruvate, 1.86 ml/l of Na lactate, 6 g/l BSA FAF and antibiotics). A total of 1130 oocytes were divided in groups of 45–50 and co-cultured with 120000 spermatozoa/ml in IVF medium at 38.5°C in 5% CO_2 atmosphere. After 24 h, oocytes were gently washed to eliminate cumulus cells and sperm adhering to the zona pellucida. Finally, embryos were cultured in groups of 25–35 in 50 µl NCSU-23 drops under mineral oil at 38.5°C in 5% O_2, 5% CO_2 and 90% N_2 atmosphere.

Parthenogenetic Activation

1221 oocytes were denuded and incubated in TALP medium for 30 min at 38.5°C. Parthenogenetic activation was performed according to the method described by Brevini et al. [16] by sequentially exposing the oocytes to 5 mM ionomycin in TALP for 5 min at 38.58C in the dark; and to 2 mM 6-DMAP in medium NCSU-23 for 3 h at 38.5°C in an atmosphere of 5% CO2, 5% O2, and 90% N2. Presumptive parthenotes were washed thoroughly in NCSU-23 and cultured as described above.

On day 5 post-activation or fertilization, half of the medium was replaced with fresh NCSU-23 containing 20% (*v/v*) FCS to reach a final FCS concentration of 10% (*v/v*) in the in vitro culture drop.

Preparation of STO Fibroblast Feeder Layers

STO fibroblasts (LGC Promochem-ATCC, Italy) were routinely cultured in high glucose DMEM (Gibco, Italy), supplemented with 2 mM glutamine and 10% Foetal Bovine Serum (FBS; Gibco, Italy). For growth inactivation, sub-confluent mono-layers were exposed to the medium above containing 10 µg/ml mitomycin-C (Gibco, Italy) for 3 h. They were re-suspended in culture medium and seeded at a density of 25×10^4 cell/well in 4-well dishes, pre-coated with 0.1% gelatin. Inactivation was carried-out 24 h before plating of ICMs or passaging of pluripotent cell lines. Two hours before use, the medium was changed and replaced with putative pESC medium (see below).

Immunosurgery and Outgrowth Derivation

Parthenotes and IVF embryos were cultured up to the early blastocysts stage (6 days for parthenotes and 7 days for IVF embryos). Blastocysts were incubated in pronase 0.5% (*w/v*) in medium TCM199 (Gibco, Italy), supplemented with 6.5 mg/ml HEPES, 1.1 mg/ml sodium bicarbonate and 4 mg/ml bovine serum albumin (BSA). Incubation was carried out for 8 min to eliminate zona pellucida. Subsequently, blastocysts were incubated in low glucose DMEM medium (Gibco, Italy) supplemented with 10% PVA and 10% monkey anti-porcine serum (supplied by Istituto Zooprofilattico Sperimentale Lombardia ed Emilia-Romagna, Brescia) for 15 min. A 30 min incubation in DMEM medium (Gibco, Italy) containing 10% PVA and 10% Guinea pig complement was then performed. ICMs were isolated from lysed trophoblast cells by pipetting and washed through several DMEM drops to avoid culture oil carry-overs and encourage better attachment.

Outgrowth Culture Conditions

Isolated ICMs, derived from IVF embryos and parthenotes, were plated on freshly inactivated STO fibroblast feeder layers. The different culture formulations tested for their ability to promote outgrowth attachment are described in Table 1.

After 3 days from primary outgrowth establishment, media were refreshed. 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 of an average of 500–600 cells, avoiding to obtain single cell suspension. Cells were then passaged on freshly prepared feeder-layers. Culture medium was changed every day.

Putative pESC Characterization

Gene Expression Analysis of Putative pESC

A small aliquot of the cell suspension was isolated at each passage and was subjected to Reverse Transcription-Polimerase Chain Reaction (RT-PCR), screening for the expression of the genes listed in Table 2. Total RNA was extracted and cDNA was obtained using Superscript-™ II Reverse Transcriptase (Invitrogen, Italy). Amplifications were carried out in an automated thermal cycler (iCycler, Biorad), using the conditions appropriate for each set of primers. Accession numbers, sequences of the primers used, annealing temperatures and fragment sizes are summarized in Table 2. Amplification products were purified in Spin-X centrifuge tube filters (Corning, Netherlands), sequenced (SEQLAB, Gottingen, Germany) and aligned using Clustal W 1.82 (EMBL-EBI service).

Real-time PCR was performed on 10 parthenotes and 10 IVF ICMs using ABI- Prism 7000 Sequence Detecting System (Applied Biosystem) and gene-specific primer (Table 3). 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 (β-actin), to give ΔCt value.

Immunocytochemical Analysis

Parthenogenetic and IVF-derived putative pESC were rinsed with PBS, fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Primary antibodies, diluted in blocking solution (10% serum in PBS), were incubated over night at 4°C.

Table 1 Formulation of the culture media tested. * Nutrient mix was composed by 2 mM glutamine, 0.1 mM β-mercaptoethanol, nucleoside mix and 1% non-essential aminoacid (Gibco, Italy)

	DMEM-low glucose (Gibco, Italy)	DMEM – K.O. (Gibco, Italy)	HAM'S-F10 (Gibco, Italy)	NUTRIENT MIX *	1000IU/ml ES-GROWTH (LIF; Chemicon, USA)	5ng/ml bFGF (R&D System, USA)	K.O. SERUM (Gibco, Italy)	FBS (Gibco, Italy)
A		+		+	+		15%	
B		+		+	+		10%	5%
C		+		+		+	10%	5%
D	+		+	+	+	+	15%	
E	+		+	+	+	+	10%	5%
F	+			+	+	+	15%	
G	+			+	+		10%	5%
H	+			+			10%	5%
I	+			+				
L	+			+		+	10%	5%

Table 2 Primers used for RT-PCR

GENE	EMBL Accession No.	Primer sequence	Annealing temperature	Fragment size
Oct-4	NM_001113060.1	For 5′- aggtgttcagccaaacgacc-3′ Rev 5′- tgatcgtttgcccttctggc-3′	64°C	335bp
Nanog	DQ447201.1	For 5′- atccagcttgtccccaaag-3′ Rev 5′- atttcattcgctggttctgg-3′	60°C	438bp
Sox -2	EU503117.1	For 5′ -gccctgcagtacaactccat-3′ Rev 5′- gctgatcatgtcccgtaggt-3′	60°C	216bp
Rex - 1	AM410991	For 5′-cttcaaggagagcgcaaaac-3′ Rev 5′-tgtccccaatcaaaaatgct-3′	56°C	299bp
BMP-4	EU549864.1	For 5′-tgagcctttccagcaagttt-3′ Rev 5′- caacgcacagatcaggaaga-3′	60°C	298bp
NF-H	NM_021076.3	For 5′-agagctggaggcactgaaaa-3′ Rev 5′-tccgacactcttcaccttcc-3′	60°C	248bp
α-amylase	AF064742.1	For 5′-cgctccatgattgctgatta-3′ Rev 5′-cctcaccacccaaatcaatc-3′	57°C	196bp
LIFR	SSU97364	For 5′- atcatcagtgtggtggcaaa-3′ Rev 5′- gcagggtccagactgagatg-3′	60°C	201bp
gp130	NM_001097432	For 5′- aaagctgcctcaacttggaa-3′ Rev 5′- accagaaacttggtgccttg-3′	60°C	211bp
FGFR-1	AJ577088.1 (Hyttel, 2009)	For 5′- actgctggagttaataccaccg-3′ Rev 5′- gcagagtgatgggagagtcc-3′	55°C	125bp
FGFR-2	NM_001099924	For 5′-cgtgtacacccaccagagtg-3′ Rev 5′- agaggctgactgaggtccaa-3′	60°C	139bp
STAT-3	DQ470570.1	For 5′- cgcagagttcaaacacctga-3′ Rev 5′- agttcacgttcttggggttg-3′	60°C	260bp
PTEN	FJ436380.1	For 5′-cgacgggaagacaagttcat-3′ Rev 5′-aggtttcctctggtcctggt-3′	60°C	163bp
PI3K	NM_001012956.1	For 5′- atgggggaagcagagaagtt-3′ Rev 5′- ggcagtttcagccattcatt-3′	60°C	281bp
AKT	AB499527.1	For 5′- atcgtgtggcaggatgtgta-3′ Rev 5′- ctggccgagtaggagaactg-3′	60°C	200bp
Actin	NM_007393.3	For 5′- tgaaccctaaggccaaccgtg-3′ Rev 5′- tgtagccacgctcggtcagga-3′	60°C	267bp

The following markers of pluripotency were analized: Oct-4 (1:50, Chemicon, USA); Nanog (1:20, R&D System, USA); SSEA-4 (1:100, Chemicon, USA). The specificity of these antibodies on pig tissues was previously validated [17–19]. 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). Samples were observed either under a TCS-NT laser confocal microscope (Leica Microsystems, Germany) or an Eclipse E600 microscope (Nikon, Japan), depending on the experiment.

Telomerase Activity

Telomerase activity measurement was performed using the TRAPeze® Telomerase Detection Kit (Chemicon, USA), following the manufacturer's instruction. Briefly, lysates were obtained from IVF and parthenogenetic putative pESC. Reactions were separated on non-denaturing TBE-based 12% polyacrylamide gel electrophoresis and visualized with SYBR Green staining. Buffer alone was used as negative control, telomerase-positive control cells (provided in the kit) was the positive control.

Table 3 Primers used for Real Time-PCR

Vitronectin	NM_214104.1	For 5′-gagctgctgcactgactacg-3′ Rev 5′-aaccgttcttgaggttggtg-3′	60°C	299bp
ITGB1	NM_213968.1	For 5′-cgggagaaaatgctccaata-3′ Rev 5′-cacactcaaacgtcccattg-3′	60°C	227bp
Actin	NM_007393.3	For 5′- tgaaccctaaggccaaccgtg-3′ Rev 5′- tgtagccacgctcggtcagga-3′	60°C	267bp

Differentation Ability of Putative pESC

Derivation of Embryoid Bodies

To induce the formation of embryoid bodies (EBs), cells were cultured in 30 μl hanging droplets of putative pESC medium without LIF and bFGF. The medium was refreshed every day and after 10–12 days, EBs were detectable. Differentiation of EBs was confirmed, through RT-PCR screening for the expression of markers related to mesoderm (Bone Morphogenetic Protein-4, BMP-4), ectoderm (Neurofilament-H, NF-H) and endoderm (α-amilase) (see Table 2).

Spontaneous In Vitro Differentiation

EBs 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. After one week, cells were processed for immunocytochemical analisys. They were subjected to the same process previously described for immunocytochemical analisys and were screened for the presence of differentiation markers: Desmin (1:200, Chemicon, USA); Keratin 17 (1:200, Chemicon, USA); Vimentin (1:200, Chemicon, USA). The ability of Keratin 17 antibody to recognize pig cells is documented by the manufacturer. Desmin and Vimentin antibody specificity was previously validated by other Authors [17, 20].

Statistical Analysis

The X^2 test ($P < 0.05$) was used to compare the results obtained from IVF and parthenogenetic embryos.

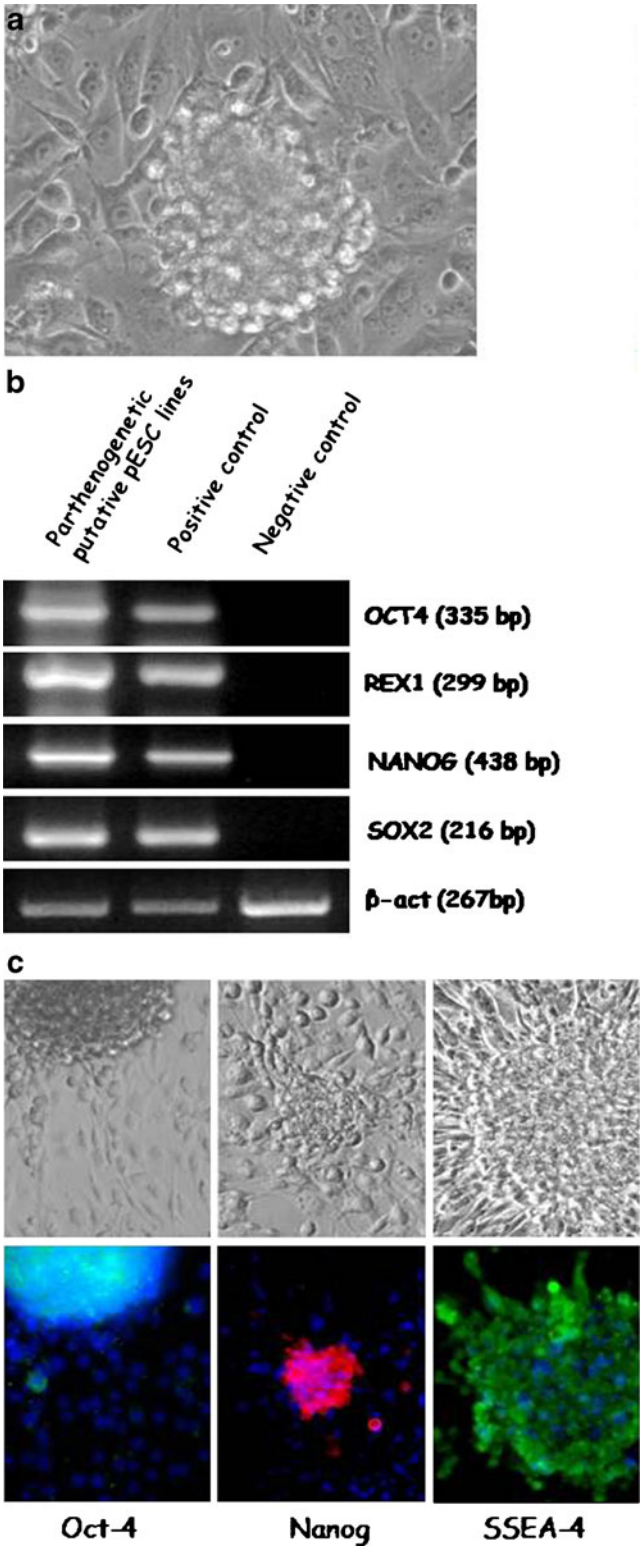
Results

Assessment of Different Culture Conditions for Outgrowth Formation and Cell Line Derivation

In this set of experiments we examined the effect of different medium formulations (Table 1) through the evaluation of primary outgrowth formation, outgrowth expansion and cell line establishment. We tested the different media using ICMs derived from both parthenogenetic and IVF embryos. The results obtained are summarized in Table 4. No primary outgrowths were obtained with media A, B, C, G, H, I and L. Using these media ICMs did not attach or adhered loosely to the feeder layer, with no increase in outgrowth cell number. Intermediate results were obtained with media D and F. In these two groups, we could observe attachment of ICMs. However all cells differentiated after 9–12 days of culture. The best results were obtained with medium E that allowed the derivation of homogeneous outgrowths, consisting entirely of cells which resembled ES cells in their morphology (Figs. 1 and 2, panel A), both using parthenogenetic and IVF ICMs. The newly produced outgrowths grew in size and, after 7–8 days of culture, formed circular colonies of 3–5 mm in diameter, with distinct margins (Figs. 1 and 2, panel A). At this stage they were passaged onto a fresh feeder layer to establish cell lines or were subjected to further characterization. Putative pESC showed many pluripotency aspects common to mESC and hESC. They were maintained for many passages (45 passages at present). They presented high telomerase activity, which disappeared when cells were induced to differentiate (Fig. 3), indicating a physiologically normal control of telomerase activity in these cells. They expressed many of the known pluripotency related transcription factors and surface markers (Figs. 1 and 2, panel B and C).

Table 4 Effect of different culture media formulation. Total number of parthenogenetic and IVF ICMs plated, outhgrowth attachment and pluripotent cell lines obtained

Medium	Parthenogenetic ICM	Parthenogenetic outgrowth	IVF ICM	IVF outgrowth	Note
A	12	0	5	0	No
B	13	0	4	0	No
C	10	0	5	0	No
D	39	7	18	1	Differentiated after 9–12 days
E	36	30	16	2	Homogeneous and ESC-like morphology
F	24	4	10	1	Differentiated after 9–12 days
G	12	0	6	0	No
H	15	0	8	0	No
I	11	0	6	0	No
L	13	0	5	0	No



◀ **Fig. 1** Putative pESC derived from parthenotes. Putative pESC were positive for several known pluripotency-related markers. The figure is representative of all passages (45 passages at present). The expression of Oct-4, Nanog, Rex-1, Sox-2 is shown in panel **b**. Genomic DNA was used as a positive control; negative control was represented by differentiated parthenogenetic putative pESC. Cytochemical analysis with specific antibodies demonstrated immunopositivity of parthenogenetic cells for Oct-4, Nanog and SSEA-4. Cell nuclei, stained with DAPI, are coloured in blue (**c**)

Altogether, this result indicates that the use of medium E, containing a combination of LIF, bFGF and FBS is the most effective to promote outgrowth attachment and support cell line expansion.

Characterization of Molecular Networks Involved in the Inhibition of Differentiation

We analyzed the expression profile of a set of molecules responsive to LIF and bFGF. The results obtained by RT-PCR analysis are shown in Fig. 4 and indicate that putative pESC express STAT-3, but mRNAs for LIF receptor subunits (LIFR and gp130) are not present. On the contrary expression of three genes (PI3K, AKT and PTEN), representative of the PI3K/AKT pathway, a signaling cascade known to be responsive to LIF, was detected consistently. Further analysis demonstrated that putative pESC express FGFR-2, while no positivity was obtained for FGFR-1.

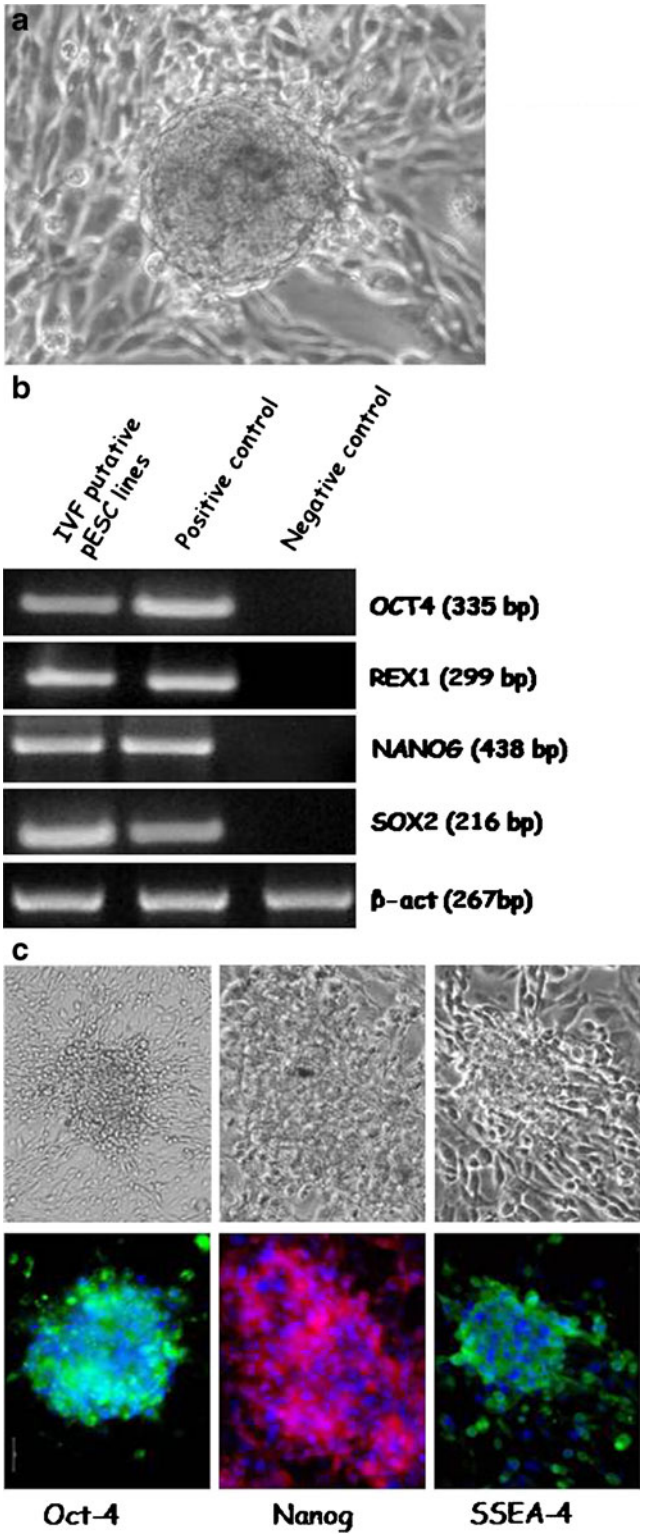
Derivation of EBs and Differentiation Plasticity of Putative pESC

Putative pESC lines obtained from IVF embryos and parthenotes were analyzed for differentiation ability. Formation of EBs was observed regularly after 10–12 days of hanging droplets culture for both types of cell lines (Figs. 5 and 6, panel A). EBs expressed tissue-specific markers like BMP-4, NF-H, and α-amilase (Figs. 5 and 6, panel B). Furthermore when EBs were disaggregated and grown in monolayer conditions, they presented the expression of keratin 17, vimentin and desmin (Figs. 5 and 6, panel C), indicating that subpopulations of cells representative of all three germ layers could be obtained.

IVF-Embryos vs. Parthenotes: Blastocyst Rate, Immunosurgery Resilience, Outgrowth and Cell Line Formation

We compared blastocyst rate, immunosurgery resilience, primary outgrowth formation and cell lines derivation using IVF blastocysts and parthenotes as a source.

The results for this comparison are shown in Table 5 and indicate that parthenogentic activation generated a significantly higher number of blastocysts than IVF (23.1% vs 8.93%). ICMs were successfully isolated from 83 IVF blastocysts (82.18%) and 185 parthenotes (65.6%). Parthenotes tended to be less resilient than IVF embryos to immunosurgery, although this difference is not statistically



◀ **Fig. 2** Putative pESC derived from IVF blastocysts. Putative pESC were positive for several known pluripotency-related markers. The figure is representative of all passages (45 passages at present). The expression of Oct-4, Nanog, Rex-1, Sox-2 is shown in panel **b**. Genomic DNA was used as a positive control; negative control was represented by differentiated IVF putative pESC. Cytochemical analysis with specific antibodies demonstrated immunopositivity of bi-parental cells for Oct-4, Nanog and SSEA-4. Cell nuclei, stained with DAPI, are coloured in blue (**c**)

significant. However, parthenogenetic ICMs had a significantly higher ability to form outgrowths (22.16% vs 4.82%) and to generate cell lines that displayed an ESC-like morphology (39 from parthenotes and only 3 from IVF embryos). All cell lines (42) were positive for pluripotency markers at the first passage. Ten of them lost their expression within two to three passages and then differentiated or degenerated. Only the remaining 32 cell lines, that maintained pluripotency markers, displayed high telomerase activity and showed differentiation capability, were considered putative pESC (Figs. 1, 2, 3, 5 and 6). They represented 2.41% of the plated IVF ICMs and 16.22% of the parthenogenetic ones.

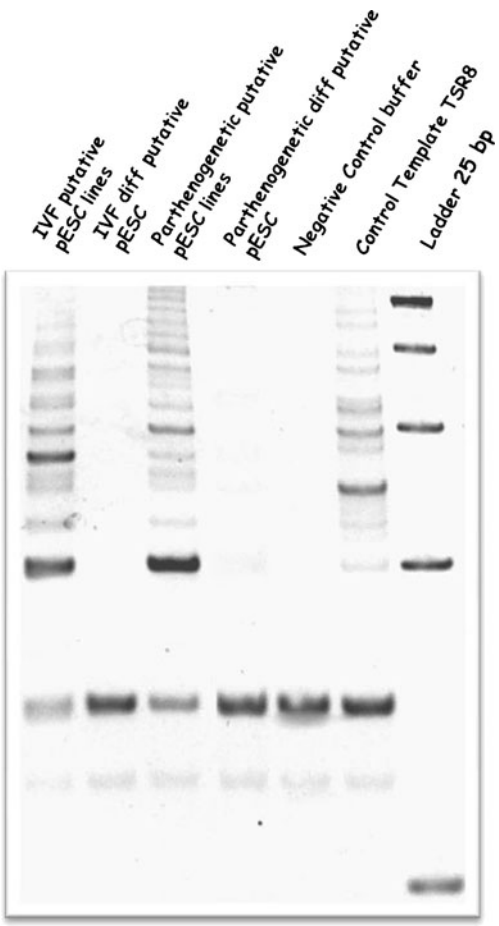


Fig. 3 IVF and parthenogenetic putative pESC telomerase activity. Undifferentiated parthenogenetic and IVF putative pESC displayed high levels of telomerase activity, while no telomerase activity could be detected in differentiated (Diff) progeny, indicating a physiologically normal control of telomerase activity in these cells

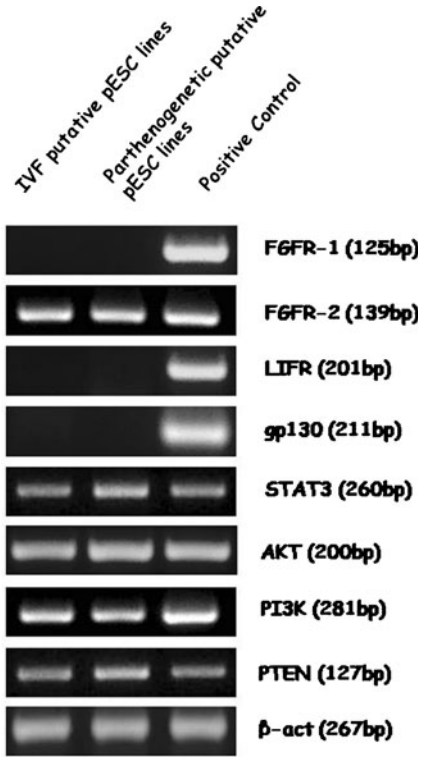


Fig. 4 Gene expression panel of pluripotency pathways related genes. Parthenogenetic and IVF putative pESC showed consistent expression of FGFR-2, STAT3, AKT, PI3K and PTEN, while no positivity was obtained for FGFR-1, LIFR and gp130

In order to understand why parthenogenetic ICM had higher ability to form outgrowths, we compared the expression level of cell adhesion molecules between parthenogenetic and bi-parental ICMs. The results indicated that the expression of beta integrin-1 (ITGB1) and vitronectin was higher in parthenogenetic than in IVF ICMs (see Fig. 7).

Discussion

We compared different media formulations using both pig parthenogenetic and IVF embryos as a source for outgrowth formation and cell line derivation. We could establish that, regardless to the different source used, 1000 IU/ml of LIF, 5 ng/ml human recombinant basic Fibroblast Growth Factor and a minimum concentration of 10% K.O. serum were required in our medium, in order to ensure ICM attachment (medium D and F). In our experimental conditions, the presence of these components allowed 16.8% (45/268) of the seeded ICMs to adhere to the inactivated feeder layer and to form outgrowths. However it is interesting to note that, only when these outgrowths were maintained also in the presence of FBS and Ham's F10 (medium E), self renewal ability was ensured and stable lines were generated

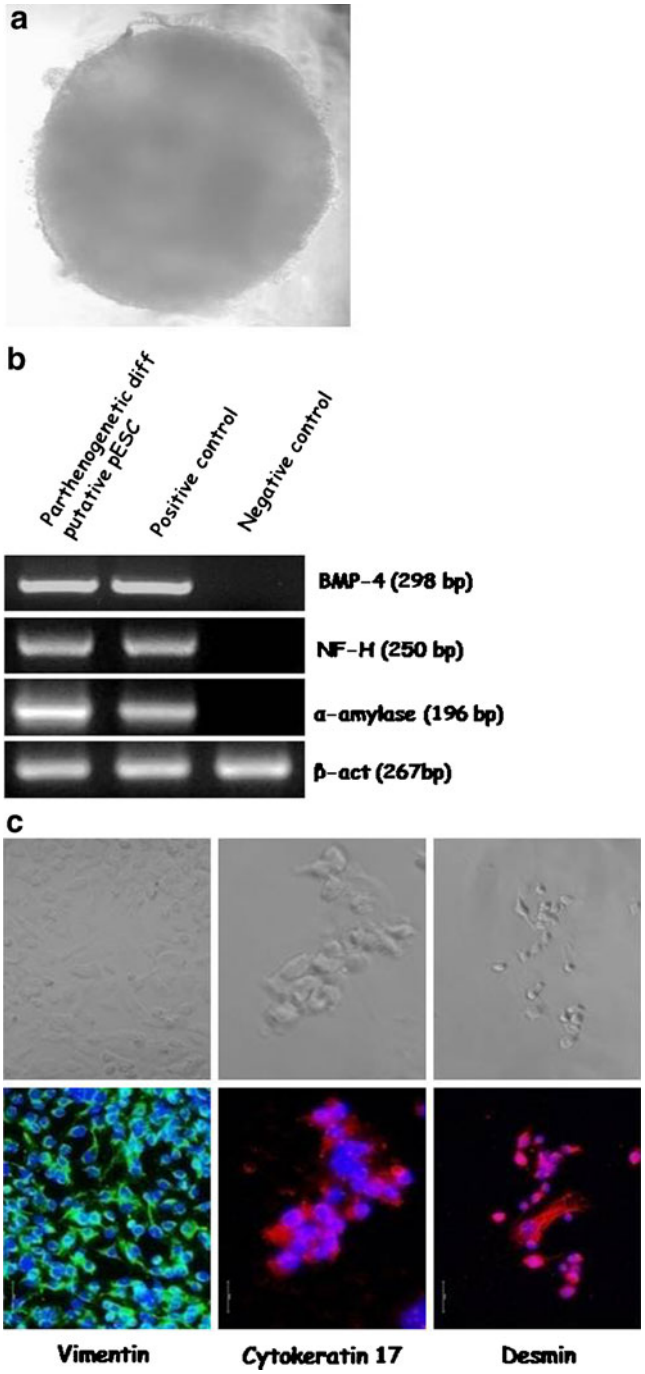


Fig. 5 Differentiation ability of parthenogenetic putative pESC after induction of EB formation. RT-PCR analysis of RNA extracted from EBs (**a**) consistently demonstrated expression of markers specific for the three germ layers and, more in details bone morphogenetic protein-4 (Bmp-4, mesoderm), neurofilament H (NF-H, ectoderm) and α-amylase (endoderm). Genomic DNA was used as a positive control; negative control was represented by undifferentiated parthenogenetic putative pESC (**b**). Disaggregation and plating of EBs confirmed parthenogenetic cell plasticity, with consistent presence in the monolayer of cells displaying immunopositivity for vimentin (ectoderm), cytokeratin 17 (endoderm) and desmin (mesoderm). Cell nuclei, stained with DAPI, are coloured in blue (**c**)

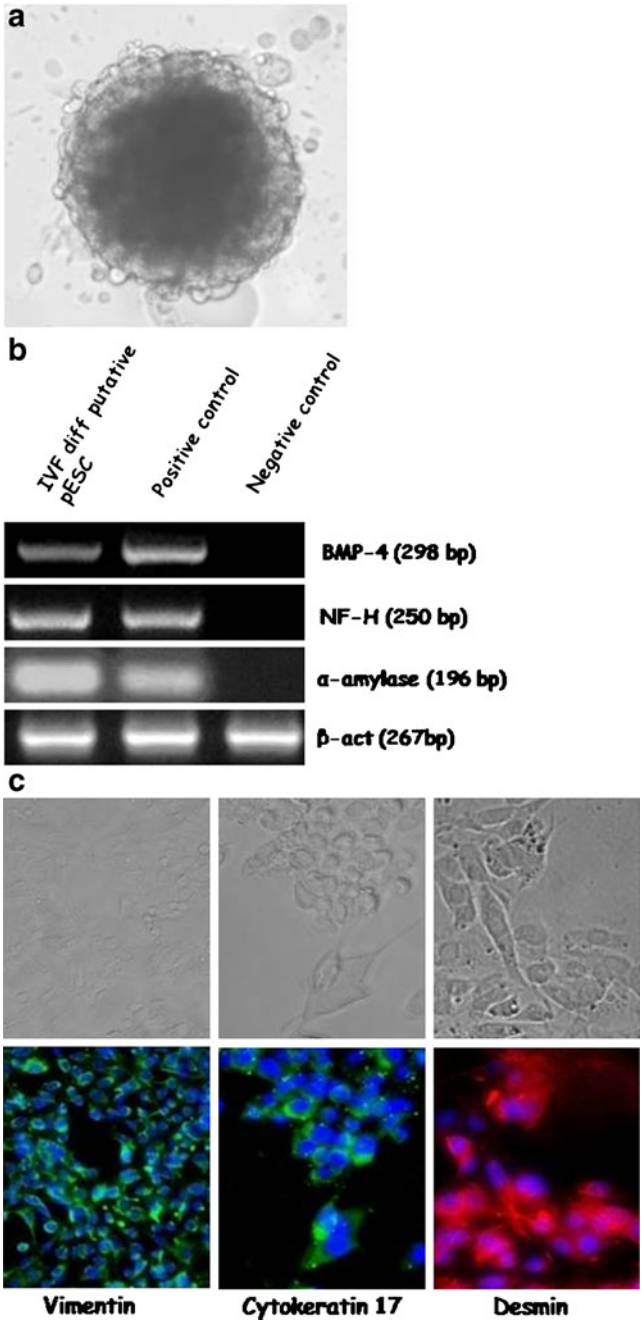


Fig. 6 Differentiation ability of IVF putative pESC after induction of EB formation. RT-PCR analysis of RNA extracted from EBs (**a**) consistently demonstrated expression of markers specific for the three germ layers and, more in details bone morphogenetic protein-4 (Bmp-4, mesoderm), neurofilament H (NF-H, ectoderm) and α-amylase (endoderm). Genomic DNA was used as a positive control; negative control was represented by undifferentiated IVF putative pESC (**b**). Disaggregation and plating of EBs confirmed IVF putative pESC plasticity, with consistent presence in the monolayer of cells displaying immunopositivity for vimentin (ectoderm), cytokeratin 17 (endoderm) and desmin (mesoderm). Cell nuclei, stained with DAPI, are coloured in blue (**c**)

(32/52). A possible role played by FBS, in combination with LIF, in the maintenance of pluripotency has recently been demonstrated in experiments carried out with mouse ESC, where the use of a medium supplemented with LIF and FBS was able to preserve the undifferentiated phenotype [21]. Consistent with this, the cell lines generated in our laboratory, displayed undifferentiated morphology, expressed pluripotency-related markers and showed high telomerase activity, indicating that the culture conditions used were adequate to maintain several aspects related to pluripotency (Figs. 1 and 2). This was further confirmed by the ability of both IVF and parthenogenetic putative pESC to give rise to EBs, that were able to differentiate into cell types belonging to the three embryonic germ layers, as shown by molecular screening and immunocytochemical staining (Figs. 4 and 5).

Cell signaling pathways that are known to govern pluripotency and that are well characterized in other species are still to be elucidated in the pig and an understanding of these aspects would indeed help to identify the conditions adequate to support self-renewal requirements in this species. Our results clearly indicated that neither LIF nor bFGF alone are sufficient to establish outgrowths and generate stable cell lines. Therefore we studied the signaling pathways responsible for the biological activities of both these molecules. Our results showed the expression of STAT3 but the absence of gp130 and LIFR transcripts, two specific subunits of LIF receptors (Fig. 3). In agreement with this, a previous report demonstrated inconsistent expression of LIFR in porcine 24 hr epiblast cells [22]. The variability among samples was explained in that study as a result of contaminating endoderm, making it difficult to ascertain a potential importance of LIF/LIFR in the porcine. This observation has been further confirmed by a recent study that could not detect LIFR in the epiblast cells of early embryos [23]. Altogether, these data would imply the dispensability of LIF in supporting pluripotency in the porcine species and would seem to disagree with our results indicating LIF as a key supplement, supporting both attachment and self renewal. However we found that putative pESC actively transcribe for PI3K, AKT (a key effector in the PI3K pathway) and PTEN (a negative regulator of the same pathway) (Fig. 3). The PI3K /AKT signaling cascade is known to be responsive to LIF and has been previously shown to trigger the expression of Nanog and to facilitate efficient proliferation and survival of murine ESC [24]. Based on these observations, we hypothesize that LIF is involved in maintenance of self renewal in porcine cells. However this cytokine is unlikely to act through the gp130/LIFR/STAT3 signaling pathway, but, rather via alternative cascades, one of which could be represented by the PI3K /AKT system.

Table 5 Effect of IVF vs. parthenogenesis on different parameter leading to the derivation of putative pESC lines. Total number (% rate) of ICMs, outgrowths and cell lines obtained from IVF and parthenogenetic blastocyst. *($P < 0.05$)

	IVF	Parthenogenetic activation
Oocytes	1130	1221
Blastocysts	101 (8.93%)	282 (23.1%)
Successful Immunosurgery	83 (82.18%)	185 (65.6%)
Outgrowths	4 (4.82%)	41 (22.16%)*
Cell lines	3 (3.61%)	39 (21.08%)*
Putative pESC	2 (2.41%)	30 (16.22%)*

A further factor that was tested in our experiments and resulted to be beneficial for pig outgrowth attachment and colony formation was bFGF. This observation is supported by previous reports showing that FGF pathway is necessary for proliferation and pluripotency [25, 26] and can fully replace LIF for maintenance of hESC self-renewal [27]. Inhibition of the bFGF signaling has been demonstrated to repress Oct3/4 expression, suppress downstream kinases and drive ESC into differentiation [28, 29]. bFGF exerts its effects, binding to one or more of its several specific receptors [30, 31]. The expression studies carried out in our experiments demonstrated the presence of FGFR-2 in pESC (Fig. 3), indicating the ability of porcine cells to bind bFGF and explaining the beneficial effect exerted by the addition of this factor to the culture medium. These experimental data suggest a possible role of the FGF signaling pathway in self renewal of porcine cells. Furthermore it is noteworthy that bFGF can also bind and activate the PI3K /AKT cascade [32, 33], which is expressed in putative pESC. It cannot be ruled out that bFGF could exert its pluripotency related effect through this pathway as well.

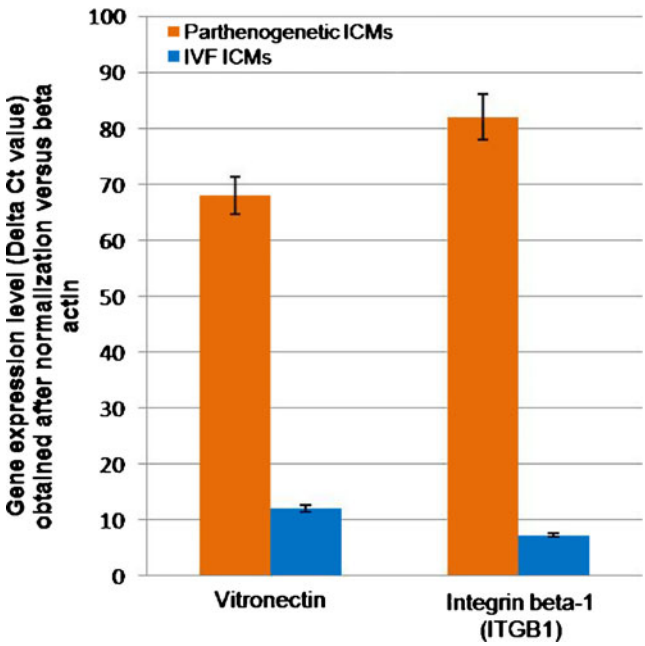


Fig. 7 Expression level of adhesion molecules involved in the implantation cascade. Bars represent the average ΔCt of parthenogenetic ICMs (orange) and IVF ICMs (blu) related to vitronectin and ITGB1 genes. ΔCt value was obtained from the Ct of the target gene normalized with the Ct value for β-actin of the same sample

Expression of the molecules involved in the signaling pathways described above was detected both in parthenogenetic and IVF derived cells, implying the existence of a number of common regulatory pathways between the two cell populations. Striking diversities were however evident in a second set of experiments where differences between parthenotes and IVF embryos as a source for the establishment of putative pESC were assessed. In these studies we used the medium formulation previously selected as the one ensuring the most adequate support for both and scored the ability to generate stable outgrowths/cell lines from embryos of different origins.

We observed that parthenogenetic ICMs appeared to be less resilient than IVF ones to the standard immunosurgery protocol used (Table 5). We have no clear explanation for this, however it may be useful to remember that a significantly higher number of apoptotic cells has been reported in pig parthenogenetic blastocysts, compared with fertilized embryos [34]. This may represent a disadvantage and leads to a decreased ability of these embryos to withstand the stress related to immunosurgery treatment. By contrast, isolated parthenogenetic ICMs generated a significantly higher number of outgrowths than IVF ones (22.16% vs 4.82%). This observation suggests a better ability of parthenogenetic embryos to adhere to the feeder cells and to form outgrowths. Although, further studies are needed in order to better elucidate this aspect, we think that one of the possible explanation of this trend may be found in the higher expression levels of beta integrin-1 and vitronectin, that we consistently detected in parthenogenetic ICMs (see Fig. 7). Both these molecules are involved in the implantation cascade and stabilize cell to cell adhesion [35]. Their increased expression in parthenogenetic ICMs may therefore be one of the possible factors accounting for the significantly higher number of outgrowths obtained from parthenotes. Consistent with these data, previous studies carried out in mouse ESC have shown that altered expression of integrins lead to a reduced ability of the cells to adhere and to maintain stable association with fibroblast feeder layers [36]. The higher ability of parthenogenetic

ICMs to form outgrowths represents an obvious advantage for the generation of cell lines. However, it may as well increase cell ability to improperly colonize a substrate and needs to be further studied. Several reports in the literature correlates increased expression of cell adhesion molecules to the process of invasion and tumor formation [37, 38]. The higher ability of parthenogenetic ICMs to form outgrowths described in the present report does not appear to be specific of the pig and is common to many other species. In the mouse, Kim K. et al. [15] describe an efficiency of ESC de novo isolation from parthenogenetic activated oocytes of 65%, compared to the much lower success (averaging 15%) reported when IVF blastocysts were used as a source [39]. Consistent with these data, also in human systems, embryos obtained with parthenogenetic activation protocols were more effective than those obtained from regular IVF, with a significantly higher number of ICMs that attached to MEF feeder layer and generated stable cell lines [14]. Similarly, in rabbit, while only 1 line was isolated from ten IVF blastocysts, 3 lines were recovered from ten parthenogenetic embryos [40].

These observations suggest that similar regulatory pathways are likely to exist among different species and modulate many of the mechanisms involved in the control of cell adhesion, outgrowth formation and cell line generation. However species related differences appear to drive the coupling of these common mechanisms with the specific timing of embryo development, the distinct expression and relative concentration of pluripotency-related molecules and the diverse needs of the cell in its micro environment. We think that the understanding of these subtle but meaningful diversities within species may provide beneficial information towards the isolation of genuine ESC.

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Embryonic Stem Cells in Domestic Animals

No shortcuts to pig embryonic stem cells

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Abstract

The establishment of embryonic stem cell (ESC) lines in domestic species could have great impact in the agricultural as well as in the biomedical field. In particular, derivation of pig ESC would find important applications aimed at improving health and production traits of this species through genetic engineering. Similarly, the immunological, morphological, physiological, and functional similarities to the human make the pig a very effective and suitable animal model for biomedical studies and pre-clinical trials. While proven blastocyst-derived mouse and human ESC lines have been established, no validated porcine ESC (pESC) lines are available. In the present manuscript we briefly discuss some of the factors that make the establishment of ESC lines in the pig, and in animal species other than mouse and human, a very slow process. The paucity of information related to morphology, pluripotency markers, differentiation capability hampers a thorough evaluation of the validity of putative lines.

These difficulties are further increased by the lack of reliable antibodies, reagents, and *in vitro* culture systems that could ensure reliable results in the pig and allow for the screening and long-term maintenance of pESC.

Data from the literature suggest that similar regulatory pathways are likely to exist among different species. Coupling of these pathways with their distinct expression patterns, the relative concentrations of pluripotency-related molecules, and timing of embryo development, along with supportive micro-environmental conditions, would appear to vary in a species-specific manner. We feel that the understanding of these subtle but meaningful diversities may provide beneficial information about the isolation of genuine porcine embryonic stem cells.

Keywords: Pig; Embryo; Stem cells

Contents

1. Introduction

During the past 15 years, many reports of porcine ESC lines, or what are often presented as “ES-like” cell lines, have been published. However, validated pig ESC (pESC) lines still do not exist and no conclusive results have been obtained, despite numerous

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reports related to the isolation and propagation of putative pESC lines (reviewed in Brevini et al, 2007) [1].

Putative pESC established by Evan et al (1990) [2] and their relative differentiated cells, were poorly defined. In other studies no ESC-like cells proliferated beyond passage 10, while epithelial-like lines survived up to passage 42, but failed to differentiate [3]. In other studies the pluripotent features of both ESC-like and epithelial-like cells were difficult to maintain for more than a few passages [4]. The general consensus is that none of these lines were truly ESCs and pluripotent [1,5] and a number of technical questions are still to be answered. None have been successfully used as a biological reagent in a manner similar to the use of human, monkey or mouse ES cells, i.e., directed pluripotent *in vitro* differentiation [6,7] or as a means of genetically engineering through embryonic chimera formation [8].

The majority of mouse and monkeys [9] ESC lines have been established from *in vivo*-derived embryos, while human ones originate from *in vitro*-fertilized (IVF) and *in vitro*-cultured (IVC) blastocysts [10–12].

In the peer-reviewed reports on establishment of porcine ES, ES-like, or ICM cell lines, all used *in vivo*-derived blastocysts as their primary culture material [2,13–18]. These *in vivo* blastocysts were acquired from the reproductive tract, at various stages, but generally at the early blastocyst stage to the later elongated or filamentous stage. Only in the last years studies reported on the isolation of putative pESC using *in vitro* produced blastocysts [1,19–21].

Given the high cost and the low efficiencies of ESC derivation from *in vivo* derived embryos [22], more researchers are working with domestic species to generate greater numbers of *in vitro* embryos (*in vitro* fertilized embryos, parthenotes, somatic cell nuclear transfer embryos) for use in ESC isolation. However, difficulties are evident in the production of pESCs *in vitro*: while IVF of bovine embryos is well established, production of porcine embryos *in vitro* is still challenging, with low efficiency and quality [23].

2. Different species, different timing

The optimal embryonic developmental stage for the initiation of ICM cultures for establishing pESC lines is not known.

At the time of implantation, the mouse blastocyst contains three cell types: epiblast, trophectoderm, and primitive endoderm [24]. The epiblast will give rise to the embryo and it has been shown to be the source for ESCs [25]. These three early embryonic lineages are present in all eutherian species. However, compared to mice and humans, where the epiblast never gets exposed to the uterine environment, blastocysts of ungulates have an extended period of preimplantation development, during which exposure takes place (Fig. 1). In man, formation of the three early lineages takes approximately 6 d post fertilization [26]. In contrast, in the pig, sheep, and cow, epiblast formation starts at hatching and is completed around day 12 [27]. This implies that no defined epiblast is likely to be present in pig blastocysts before hatching (day 6 or 7 of development *in vivo*) [28]. During the following days there is only a modest increase in the number of epiblast cells in the ICM of the blastocyst compared with the increase in trophectoderm and visceral endoderm cells.

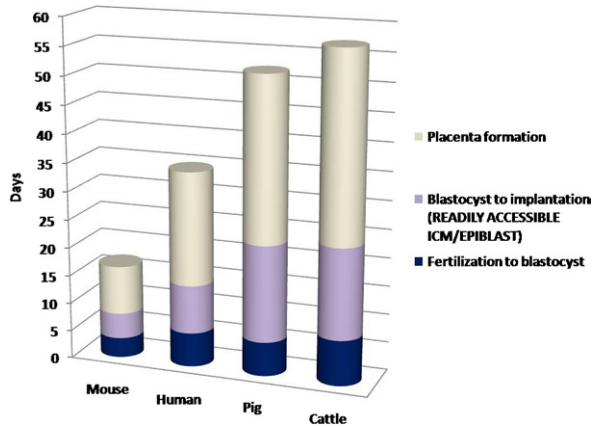

Fig. 1. Species specific periods of early embryos development.

In pig conceptuses, the thin layer of trophectoderm, the Rauber's layer, covering the epiblast and separating it from the uterine lumen and visceral endoderm, slowly starts to degenerate by day 9 post-fertilization. This structure progressively disappears, leaving the epiblast directly exposed to the uterine lumen [29]. The first signs of polarity become evident along with the formation of a crescent-shaped thickening within the posterior third. This thickening will differentiate into the primitive streak. This event accompanies the appearance of defined mesoderm and endoderm layers [30]. It follows a gradual downregulation of the pluripotency marker OCT-4 with a concurrent upregulation of β-tubulin III expression, a marker of neural differentiation. This suggests that embryos at this stage are no longer suitable for ESCs derivation.

The question that needs to be answered is: what point in the preimplantation development is best for the isolation of stable pESC lines? Examining this problem, Chen et al [17] found that early hatched *in vivo*

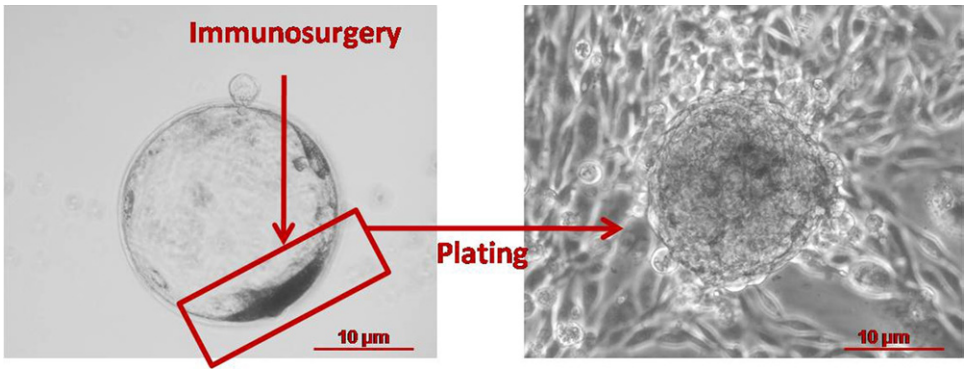

Fig. 2. Isolation of ICM from a preimplantation pig blastocyst, plating on feeder cells and derivation of a colony of putative pluripotent cells.

blastocysts had a relatively small number of trophectoderm cells and a less flattened ICM. The success rate for the establishment of pig ES-like cell cultures was decidedly greater (12 cultures (21%) vs. none) from recently hatched blastocysts than from late-hatched blastocysts [17]. Another report used *in vivo* pig blastocysts from day 5–6 to day 10–11 of gestation and found that while day 10–11 blastocysts yielded ES-like cell cultures, few or none were propagated from day 5–6 embryos [15]. Our experience confirms the possibility of establishing stable pluripotent cell lines using day 6–7 blastocysts. This was demonstrated, using both *in vivo* and *in vitro* (parthenogenetic and IVF) derived embryos, which were treated with pronase for 7 min to remove the zona pellucida and then subjected to immunosurgery, using a custom made pig antiserum. ICMs were seeded on inactivated SIM mouse embryo-derived and thioguanine and ouabain resistant (STO) fibroblast feeder layers and monitored for attachment and derivation of outgrowths (Fig. 2). Although cell lines were derived from the different sources, the rate of success varied and parthenogenetic ICMs displayed a higher ability to form outgrowths and generate stable cell lines [1,31].

3. Which culture conditions?

Compared with the large number of studies exploring the appropriate culture conditions for mouse and human ESCs, there is a minimal amount of data available for domestic species ESC. That limited information is mainly based on mouse ESC culture systems. As a result, such conditions did not appear to be effective for maintaining stable undifferentiated ESC lines in domestic animals. We are convinced that a major goal at present is to develop better culture formulations in order to obtain homogenous pluripotent outgrowths from pig embryos and identify the best *in vitro* environment that would facilitate derivation of stable pESC culture.

Several authors highlighted the need for a feeder-layer (STO cells or mouse embryonic fibroblasts) in order to ensure the survival of pig (and bovine) epiblast cells in primary culture [32,33]. Without feeder-cell support, cultures of primary pig epiblast cells failed to grow, and instead senesced and died over a 10–14 d period. Similar results were reported with feeder-free, short-term, primary cultures of pig ICMs, with or without the addition of leukemia inhibitory factor (LIF) to the medium [34]. It is plausible that ungulate ES cell line establishment will therefore require feeder cells, at least in their initial culture, as has been true for the establishment of most mouse and primate ES cell lines.

The need for a feeder layer does not seem to be related only to the release of specific factors by the feeder cell populations, since the use of conditioned medium did not exert a comparable effect. The presence of feeder cells appeared to be necessary in order to ensure good culture conditions. Therefore, at present, most laboratories use protocols similar to those described in the mouse, and ungulates ESCs are grown on a feeder layer, in medium supplemented with various other nutrients or components like basic fibroblast growth factor (bFGF) [35–37], LIF [23,35–38], epidermal growth factor (EGF), and stem cell factor (SCF) [38]. We have found that pig cell lines do not express LIF receptor, indicating that the addition of this cytokine to the culture medium is not essential for the maintenance of pluripotency. However, LIF appears to inhibit the differentiation process [21] since its presence in the standard medium used for embryoid body (EB) formation, results in preventing cell commitment to germ layer specification and inhibited cell aggregation (see Fig. 3).

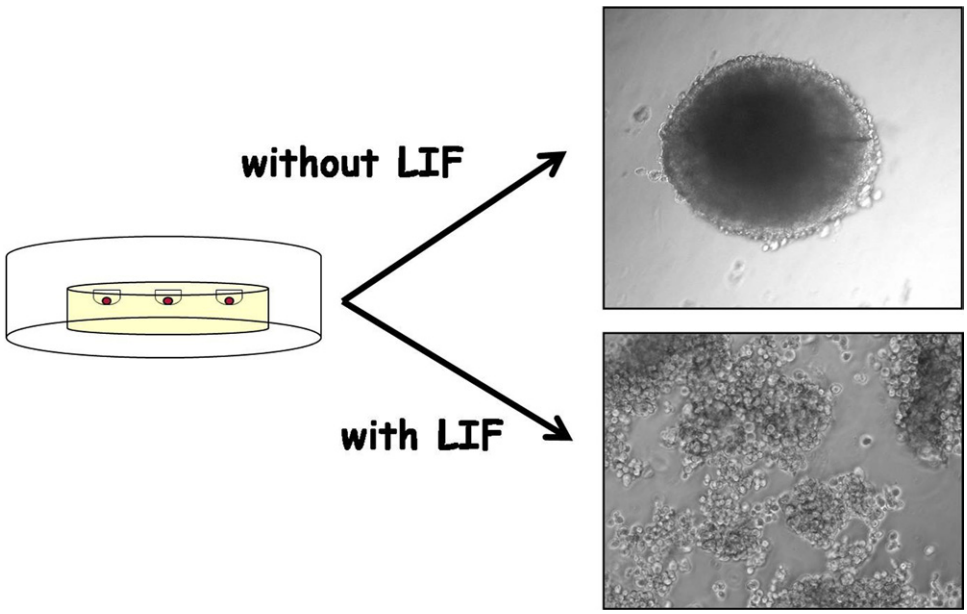

Fig. 3. Pluripotent cells, cultured in hanging droplets, generate EBs after 9–10 days of co-culture. The addition of LIF to the medium inhibited differentiation and cell aggregation.

It is likely that the role of LIF in pluripotency maintenance of pESC does not occur through a classical LRβ-gp130 and STAT3 activation pathway [39]. In agreement with this, Blomberg et al demonstrated inconsistent expression of LIFR in porcine epiblast cells cultured for 24 h [40]. The variability among samples that was observed in that study was explained as the result of contaminating hypoblast, making it difficult to ascertain a potential importance of LIF/LIFR in the porcine. This observation has been further confirmed by a recent study that could not detect LIFR in the epiblast cells of early porcine embryos [41]. Within this scenario, we suggest that LIF effect is likely to be mediated through alternative signaling pathways that have been shown to participate in maintaining pluripotency. Our data indicate a possible involvement of the PI3K/AKT signaling cascade [31], which is known to be responsive to LIF and has been shown to trigger the expression of *NANOG* and to facilitate efficient proliferation and survival of murine ESC [42].

Another key aspect in long term cell culture maintenance is sensitivity of cell to cell contacts. Indeed, the inability to withstand dissociation of primate ES cells into single-cell suspensions is a complicating factor in their culture. Mouse ESCs are in general dissociated with trypsin–EDTA and this procedure does not seem to affect their plating efficiencies [43]. In contrast, enzymatic and chemical dissociation of human or monkey ES cells typically gives re-plating efficiencies of less than 1% [44] and seems to render the cells more exposed to chromosome abnormalities [45,46].

This is apparently even more pronounced in ungulate epiblast cells. Primary cultures of AP positive, undifferentiated, ungulate epiblast cells, prepared by the successive immunodissection, culture, and physical dissection method, are extremely sensitive to lysis, by either physical manipulation, withdrawal of calcium, or exposure to trypsin-EDTA [32,33,47]. Primary cultures of pig epiblast cells, in particular, will rupture and lyse after only 5 min exposure to Ca^{2+}/Mg^{2+}-free PBS, with cells completely disintegrating in 30–60 min [47]. This inability to withstand dissociation is a critical point for passaging protocols of porcine epiblast cells. In line with this requirement, we disaggregate pig ICMs using mechanical dissociation and passage pig cell line colonies using micro-loops and mechanical pipetting, avoiding completely the exposure to enzymatic digestion. Furthermore, while performing these procedures, we always make sure to keep clumps of cells and never reach single cell suspension that would result in immediate differentiation.

4. How do we assess pluripotency?

For many years, characterization of ESC in domestic species was mainly carried out on the basis of morphological criteria, given the fact that, until recently, no

specific molecular marker has been identified. Although evidence based on the comparison between the transcriptomes of mouse and human ESC leads to the conclusion that mouse ESCs are substantially different from human cell lines, they express the same set of factors, known to be required for pluripotency in mouse ESCs, including, for example, the two homeodomain proteins OCT-4 and *NANOG* [48].

Furthermore, classic human and mouse ESC markers such as OCT-4, SSEA-1, SSEA-4 and alkaline phosphatase are indeed expressed by ungulates ICM and embryo-derived cell lines; however, the same genes are also expressed in the trophectoderm and endoderm [49–52]. The paucity of specific information available to authenticate appropriate ESC markers hampers thorough evaluation of the validity of putative pESC lines.

OCT-4 protein expression mode in porcine is controversial. Many authors report data demonstrating that both ICM and trophectoderm express this marker [1,40,50,53–58]. Furthermore, recent studies show OCT-4 gene expression in porcine trophectoderm and endoderm cell lines [59]. On the other hand, according to Vejlsted et al, in embryos at the expanding hatched blastocyst stage, OCT4 is confined to the inner cell mass [60]. In our studies we find that OCT-4 mRNA is detectable at the time of porcine ICM plating and during the first passages, while, by passage seven to ten, OCT-4 mRNA expression is completely turned off or, when expression persists, immune-positivity is common to the cytoplasmic compartment and not only restricted to the nucleus [1]. Of course a consistent limitation in these findings is the lack of reliable antibodies for ungulates that could ensure consistent results, which makes the interpretation of the data even more confused. It is interesting to note that, unexpectedly, downregulation of OCT-4 does not seem to affect these cells, which have been cultured for several further months without showing changes in their morphology and with no expression of specific differentiation markers. These observations suggest that, even though OCT-4 is likely to be a marker of stemness also in the pig, it does not seem to be the only or the key factor playing a role in the maintenance of pluripotency in this species [39]. It may be hypothesized that OCT-4 presence plays an indispensable role in plating and early culture of pig epiblasts, but may then be replaced by other pluripotency factors like *NANOG*, which is a well-characterized marker in human and mouse ESC lines.

NANOG is constantly expressed in the cell lines that were generated in our laboratory and, in contrast to OCT-4, it is detectable at every passage [1]. Interestingly, it is also strongly downregulated in caprine trophectoderm, while being strongly expressed in the ICM [52] and does appear to be a specific marker of pluripotency for ruminants because both its mRNA and protein are found in the ICM and strongly downregulated in the trophectoderm of caprine blastocysts [52].

Altogether, these observations lead us to hypothesize that *NANOG* may be able to maintain pig pluripotent cells in an undifferentiated state, also in the absence of the simultaneous expression of OCT-4. This is further supported by recent studies indicating that *NANOG* over expression is sufficient to support mouse ESC self-renewal [61].

On the other hand, it may be hypothesized that *NANOG* expression, in the absence of OCT-4, indicates a "stand-by" mode, where a cell is prevented from committing to differentiation but, at the same time, is not fully pluripotent and only the simultaneous expression of both factors (and, possibly, many others) has to be present in order to maintain cells in a genuine pluripotent state.

Conflicting data regarding the expression of other pluripotency markers (SSEA1, SSEA4, Alkaline Phosphatase, Sox2, Rex1) further complicates our understanding of pESC. Although these factors are considered characteristic of ESC in other species, they cannot be regarded as definitive markers in the pig [1,50,59].

5. Perspectives and strategies

Many factors, some of which are briefly discussed in the present manuscript, make the establishment of ESC lines in the pig, and in animal species other than mouse and human, a very slow process. The paucity of conclusive information related to morphology, pluripotency markers, differentiation capability that should be distinctive of pESC, hampers thorough evaluation of putative lines at present. These difficulties are further increased by the lack of antibodies, reagents and tools that ensure reliable screening in the pig and, as a result of this, misleading assessments are a real hazard.

Further investigation is required to identify the optimal time for the initiation of pig ICM cultures and to set up better in-vitro culture systems for the establishment and long-term maintenance of pESC. The interactions with the feeder layer still need to be fully understood. It is reasonable to assume that similar regulatory pathways are likely to exist among different species and modulate many of the mechanisms involved in the control of cell adhesion, outgrowth for-

mation and cell line generation. However species related differences may drive the coupling of these common mechanisms with the specific timing of embryo development, the distinct expression and relative concentration of pluripotency-related molecules and the cell needs in its micro environment. We feel that the understanding of these subtle but meaningful diversities in the pig species may provide beneficial information towards the isolation of genuine embryonic stem cells. Possibly, these aspects can be better evaluated if we concentrate our research on the identification of porcine specific pathways involved in the control of self-renewal, focusing on a close re-exam of the mechanisms driving pig early embryo development and differentiation. This approach should comprise the evaluation of both intrinsic and extrinsic factors that may not be the same molecules that have been shown to be effective in other species. There is still a lot of work to be done and there are no shortcuts.

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Parthenogenetic Cell Lines: An Unstable Equilibrium Between Pluripotency and Malignant Transformation

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Abstract: Human parthenogenetic embryos have been recently proposed as an alternative, less controversial source of embryonic stem cells. However many aspects related to the biology of parthenogenetic cell lines are not fully understood and still need to be elucidated. These cells have great potentials; they possess most of the main features of bi-parental stem cells, show the typical morphology and express most of the pluripotency markers distinctive of ESC. They also have high telomerase activity, that disappears upon differentiation, and display great plasticity. When cultured in appropriate conditions, they are able to give rise to high specification tissues and to differentiate into mature cell types of the neural and hematopoietic lineages. However, their injection in immunodeficient mice has been reported to result in tumour formations. Aberrant levels of molecules related to spindle formation, cell cycle check points and chromosome segregation have also been detected in these cells, that are characterized by the presence of an abnormal number of centrioles and massive autophagy. All these observations indicate the presence of an intrinsic deregulation of the mechanisms controlling proliferation versus differentiation in parthenogenetic stem cells. In this manuscript we summarize data related to these aberrant controls and describe experimental evidence indicating their uniparental origin as one of the possible cause. Finally we propose their use as an intriguing experimental tool where the pathways controlling potency, self renewal and cell plasticity are deeply interconnected with cell transformation, in an unstable, although highly controlled, equilibrium between pluripotency and malignacy.

Keywords: Centriole, mitotic spindle, parthenogenesis, pluripotency, tumorigenic.

INTRODUCTION

Parthenogenesis is the process by which an oocyte can develop without the intervention of the male counterpart. It 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.

Mammals are not spontaneously capable of this form of reproduction, but, mammalian oocytes can be successfully activated *in vitro,* mimicking the intracellular calcium wave induced by the sperm at fertilization, which triggers cleavage divisions and embryonic development [1]. Mammalian parthenotes, however, are unable to develop to term and arrest their development at different stages after activation, depending on the species [2]. The reason for this arrest is believed to be due to genomic imprinting which causes the repression of certain genes [3], that are normaly expresse by the paternal allele.

Because mammalian parthenotes are inherently unable to form a new individual, following the success reported in mouse [4, 5] and non-human primate models [6, 7], human parthenogenetic embryos have recently been proposed as an alternative, less controversial source of embryonic stem cell lines [1, 8-11]. 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). However many aspects related to the biology of parthenogenetic embryos and parthenogenetic derived cell lines still need to be elucidated. The assembly of the new embryonic centrosome in the absence of the sperm centriol can lead to aberrant progression of cell cycle and spindle formation [12]. The decreased extent of heterozygosis may amplify any negative genetic component potentially present in the genotype [13, 14]. The very high incidence of chromosome instability and aberrant chromatid separation in oocytes retrieved from IVF patients, especially when over 34 year old [15-18] also represents a concern, given the fact that these represent a large part of the population accessing assisted reproductive therapy and, hence, are a major potential source of oocytes for parthenotes derivation.

In an attempt to better elucidate some of these aspects, human parthenogenetic (HP) cell lines, recently derived in our laboratory [2, 19], were characterized for their pluripotency and differentiation plasticity, both *in vitro* and *in vivo*.

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Here we discuss the characteristics of these cell lines that are common to bi-parental embryonic stem cells. However we also describe the presence of an intrinsic deregulation of the mechanisms controlling proliferation versus differentiation and suggest their uniparental origin the possible cause.

PLURIPOTENCY AND *IN VITRO* DIFFERENTIATION ABILITY OF HP CELL LINES

The development of human parthenotes to the blastocyst stage was reported only recently [20-22] and not many data are available because of the limited accessibility of 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. The lines derived in our laboratory have been growing for over two years and possess the main features of bi-parental stem cells. They are capable of renewing themselves; they can be continuously cultured in an undifferentiated state and can be propagated extensively *in vitro*. For over 70 passages they constantly expressed markers that characterize and are distinctive of human embryonic stem cells, such as Oct-4, Nanog, Rex-1, Sox-2, alkaline phosphatase, SSEA-4, TRA 1-81, indicating a pluripotency signature that is not influenced by the exclusive presence of the maternal genome. In common with biparental cell lines they also display high telomerase activity, which is often correlated with self-renewal, regeneration and immortality and is typically expressed in germ cells and embryonic stem cells [23, 24] (Table **1**). In line with this, undifferentiated HP cells displayed high levels of telomerase activity, while no telomerase activity could be detected when cells were subjected to specific stimuli in order to induce tissue differentiation *in vitro* . These data indicate that a physiologically normal control of telomerase activity is present in HP cells and that, if subjected to culture conditions that induce differentiation, they turn down their telomerase activity. Consistent with this, HP cells were able to form embryoid bodies (EBs) that actively transcribed RNAs involved in specification of the three embryonic germ layers, demonstrating the potential of these cells to differentiate in the main tissue types of the body. Interestingly, very low or no expression of trofectoderm-related markers was detected, suggesting that human parthenogenetic cells have a poor capability to differentiate towards trofectoderm and this may reflect parthenote inability to give rise to adequate fetal membrane development and to implant correctly.

In our opinion a crucial point was to assess the possibility to drive differentiation of human parthenogenetic cells towards a specific lineage, in controlled culture conditions. Data from our lab demonstrate that 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 experiments showed that HP cells were able, not only to give rise to early neural lineages, as previously described [8-11], but also to more mature cell types expressing nestin, CNPase and MAP2 [19]. This is consistent with the observation that murine androgenetic embryonic stem cells are able to differentiate into neuronal and glial cells [25]. 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 lymphoid, erythroid and myeloid sub-populations. Our results further expanded current knowledge on HP *in vitro* differentiation plasticity showing the formation of mature hemopoietic cell lineages and confirms what has been recently reported 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 [25]. 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.

Table 1. Comparison of Pluripotency Signature in Biparental and Parthenogenetic (HP) Cell Lines

	Human Biparental ESC	Human Parthenogenetic Cell Lines (HP)
Oct-4	+	+
Nanog	+	+
Sox-2	+	+
Rex-1	+	+
TRA 1-81	+	+
SSEA-4	+	+
Telomerase activity	high	high
EB derivation	yes	yes

PARTHENOGENETIC CELLS SHOW A DEREGULATION OF THE MECHANISMS CONTROLLING PROLIFERATION VERSUS DIFFERENTIATION

Injection of HP cells in immunodeficient mice gave rise to the formation of malignant sarcomas with myofibroblastic differentiation [26]. This is consistent with the observation that the subcutaneous injection of androgenetic mouse embryonic stem cells in immunodeficient mice generates sarcomas with muscular differentiation [27]. These findings suggest the presence of an intrinsic deregulation of the mechanisms controlling the choice between proliferation and differentiation in embryonic stem cells obtained through parthenogenesis and androgenesis. However, it is interesting to note that this appeared to be modulated by the microenvironment and, while it was undetectable when cells were differentiated *in vitro*, it became evident once cells were exposed to the *in vivo* milieu.

These results are in contrast to what previously described in other human parthenogenetic cell lines [8-11] and the possibility exists that these anomalies derive from differences in the procedures used for the derivation of the cell lines. We think that an important factor to be considered is the age of the oocyte donors. This aspect could be important, given the

high incidence of chromosome instability and aberrant chromatid separation in oocytes retrieved from IVF patients, especially when over 34 year old [15-17]. Unfortunately no details on donors age were given in the other reports, therefore the relevance of this parameter cannot be evaluated.

However, based on a series of experiments carried out in our lab [19], 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 of the altered behaviour of parthenogenetic cell lines upon *in vivo* transplantation, with the use of relatively old donors as a potential aggravating factor.

PARTHENOGENETIC CELLS AND DE-NOVO CENTRIOLE ASSEMBLY

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 [28]. With the notable exception of mice [29] this degenerative process has been described in rhesus monkeys [30], rabbits [31], cows [32], sea urchins [33], *Xenopus* [34], and several other species [35]. 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. In Sciara parthenogenetic embryos, the spindles assembled without centrosomes are anastral, and the distance between the daughter nuclei is greatly reduced [36]. This limitation is common to many other species that are obligatory or facultative parthenotes, 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 [37-39]. 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 [40], 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, also HP cells display multiple centrioles Fig. (**1**) suggesting that the ability to rearrange functional centrosomes is altered in these cells. Marshall [41], suggested that centriole de novo assembly is normally turned off when centrioles are present in a cell. Therefore the absence of sperm centriole in parthenotes may lead to the lack of the negative regulatory mechanism that normally 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 the obligatority of ensure biparental fertilization [42]. However these abnormalities in spindle rearrangement may explain the high percentage of cells showing misshaped, mal-segregated chromosomes in our cell lines. Multiple chromosome malsegregations have been previously described in human oocytes after parthenogenetic activation, either spontaneous or induced by puromycin [43]. Consistent with this, a high incidence of polyploid and mixoploid chromosomal complements has been reported in parthenotes derived from bovine and porcine activated oocytes, with abnormal chromosomal complements occurring as early as completion of the first cell cycle and, again, it was linked to the absence of a paternally supplied centrosome [44, 45].

The patterns of abnormal centrosome reformations observed in HP cells closely resemble those seen in cancer cells which argues that structural defects of centrosomes can account for the formation of abnormal mitosis and multipolar cells frequently observed in cancer [46]. Interestingly enough these abnormalities do not seem to affect the overall proliferation rate and *in vitro* differentiation plasticity of HP cells. Although this needs to be further elucidated, it could be partially explained by the massive autophagocitic activity present in these cells. This process is likely to be used as an active self-protective strategy in order to eliminate highly abnormal organelles thus contributing to cell survival and ensuring "normal" population [47].

PARTHENOGENETIC CELLS AND MITOTIC SPINDLE CHECKPOINT MOLECULES

We previously showed that HP cells display altered expression levels of mitotic check point molecules Fig. (**2**). 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 [48-50]. Increased level of Mad1 has been previously shown to cause a reduction in the expression of Myc in human monoblasts where a decrement of cell proliferation with a protracted G1 phase of the cell cycle was observed but Mad1 was neither demonstrated to cause spontaneous differentiation nor to enhance induced differentiation but rather inhibited differentiation induced by retinoic acid [51]. On the other hand Mad1 dimerizes with MAX and, through a specific association with the SIN3 co-repressor, decreases transcription and, indirectly inhibits cell proliferation [52]. Taken together these observations support the hypothesis that the altered levels of such molecules present in HP cells cause anomalies in their control of proliferation and differentiation.

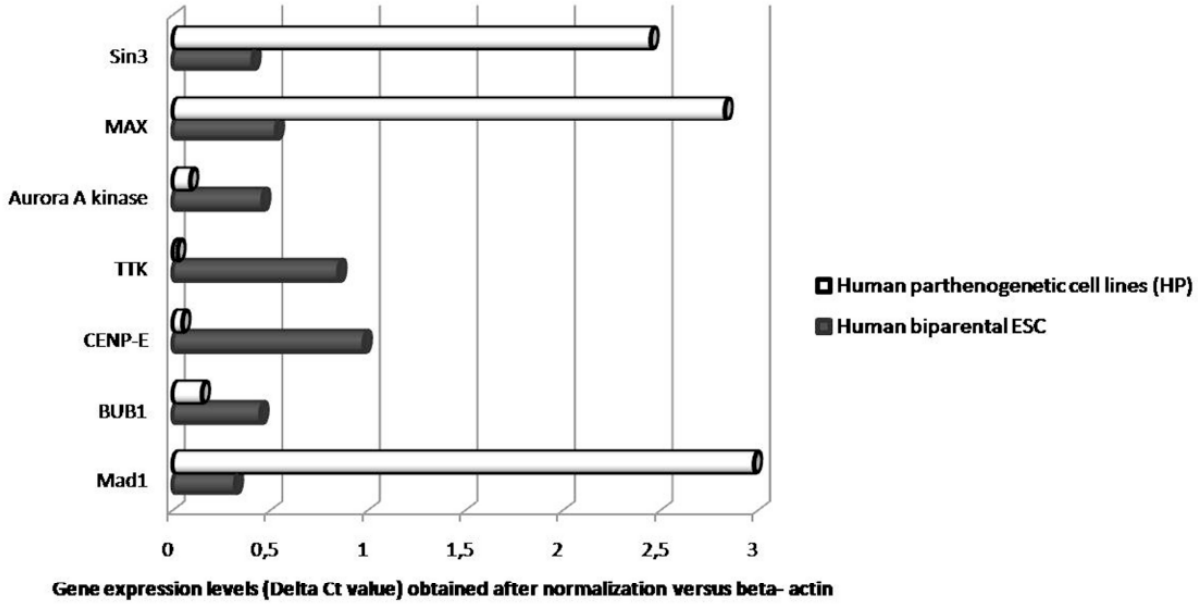

Fig. (1). Expression level of molecules related to spindle formation and chromosome segregation in HP cells and biparental cell lines. Bars represent the average ΔCt of HP cells (white)and biparental cells (grey) related to the genes examined. ΔCt value was obtained from the Ct of the target gene normalized with the Ct value for β-actin of the same sample.

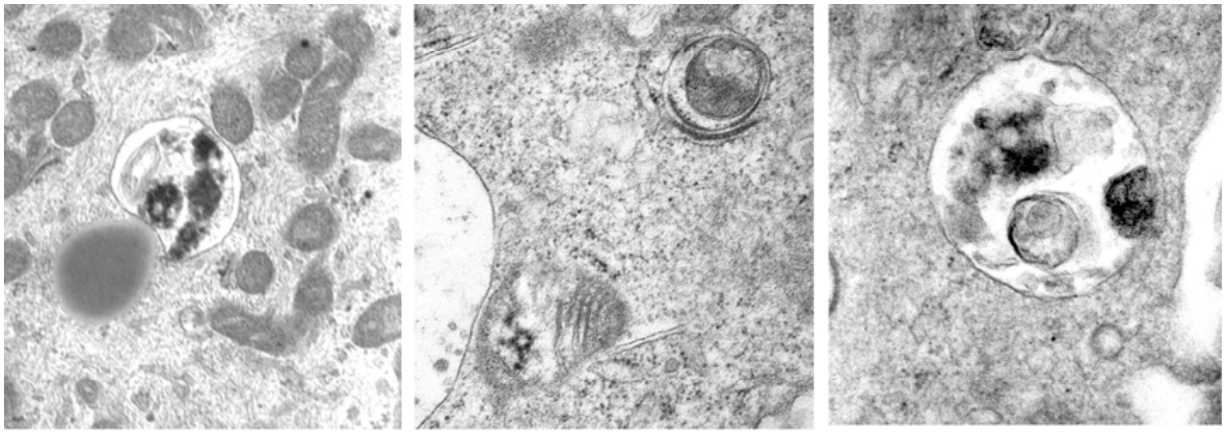

Fig. (2). Ultrastructural analysis of human parthenogenetic cells. The presence of amplified centrosomes loosely dispersed in the cytoplasm can be appreciated. Supernumerary centrioles and massive autophagic processes generally coexist.

Disturbances in the control of spindle formation and cell division are also consistent with the observation that HP cells showed very low transcription for TTK and CENP-E, molecules normally involved in kinetochore-microtubule binding, correct chromosome congression and alignment as well as segregation, during mitosis. Disruption of CENP-E, in fact, has been shown to reduce tension across the centromere, increase the incidence of spindle pole fragmentation, and result in mono-oriented chromosomes approaching abnormally close to the spindle pole, with a mixture of aligned and unaligned chromosomes [53]. Normally the final result of CENP-E inhibition is mitotic arrest, initiation of apoptosis and cell death and this effect is more likely to affect rapidly dividing cancer cells. However redundant mechanisms have been described in mammalian cells to enable kinetochore microtubule binding and checkpoint monitoring in the absence of CENP-E, although with poor positioning at the spindle poles and chronically monooriented chromosomes [54].

CELLS IN AN UNSTABLE EQUILIBRIUM BETWEEN PLURIPOTENCY AND MALIGNANT TRANSFORMATION

The data presented in this review, and many evidence available from the literature, demonstrate that cell lines obtained from parthenogenetic embryos display intriguing, although somehow contraddictory features. The main pathways controlling potency, self renewal and cell plasticity are active and functional in these cells and, in line with this, HP cells are able to self renew and maintain unaltered stable pluripotent cell colonies *in vitro*. In this highly controlled self renewal equilibrium, where symmetric cell division is predominant, abnormal centrioles and chromosome mal-segregations do not result in aberrant growth and/or dedifferentiation. Our data suggest that authophagy is one of the resources that these cells utilize in order to eliminate supernumerary centrioles in these conditions. When cultured *in vitro*, they are able to respond to differentiation media and

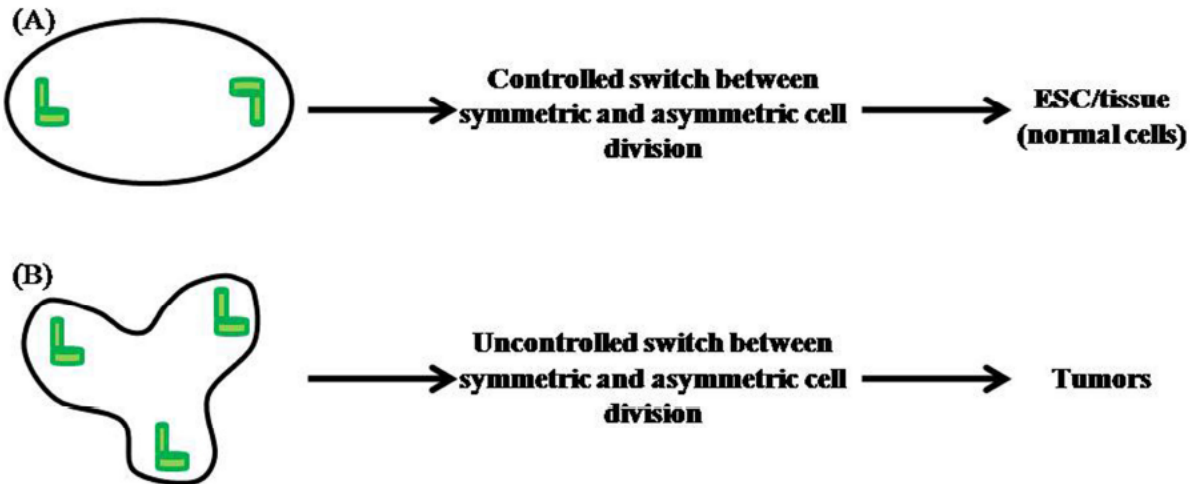

Fig. (3). Normal centrosome ensure timely and controlled switching between symmetric and asymmetric cell division, resulting in correct tissue homeostasis (**A**). Supernumerary centrioles are sufficient to cause cell transformation and tumors (**B**).

give rise to populations representative of the three germ layers, switching between symmetric and asymmetric cell divisions in a timely and controlled manner. HP cells can be driven though standard protocols to differentiate into hematopoietic and neural cells. However this pluripotency/high plasticity status seems to be related to a strictly controlled environment, such as the one which is recreated in *in vitro* culture conditions, and is lost when cells are transplanted in SCID mice. The *in vivo* milieu appears to interfere with this mode and releases cells to aberrant growth, with unrestrained expansion of the stem cell compartment that leads to the formation of malignant sarcomas.

Work from Boveri at the turn of the 20th century postulated that centrosomes play an essential role in the maintenance of genome stability and that defects in centrosome biogenesis could lead to aneuploidy and potentially favor tumor formation. For over 100 years, however this hypothesis has not been tested. Basto and colleagues [55] recently demonstrated that the induction of supernumerary centrosomes is sufficient to cause transformation in flies, with large and invasive tumors as a result. Castellanos and collegues [56] used an unbiased approach to study the tumorigenic potential of panel of Drosophila mutants, defective in various aspects of centrosome biogenesis. These authors found that most of mutants generated a high frequency of tumors but Polo-like kinase and Aurora A mutants gave rise to especially large and invasive tumors that could be serially re-transpalnted and were able to generate an almost infinite tumor mass. Parallel studies from Weaver *et al.* [57] demonstrated that reduced levels of centrosome-linker motor protein CENP-E result in elevated levels of aneuploidy. Although it will be important to determine how specie with high numbers of chromosomes that need to be segregate to progeny during cell divition respond to centrosome dysfunction, we think it may be of interest to note that HP cells were found to express dramatically decreased levels of Aurora A and CENP-E [19], indicating that similar molecular pathway are likely to be affected by the presence of supernumerary centriols in human cells.

Interpreting the results we generated with HP cells, in the light of the data mentioned above, we can hypothesize that parthenogenetic cells may represent an interesting model where the control of asymmetric cell division is lost, due to the presence of abnormal centrosome. Since switching between symmetric and asymmetric cell divisions in a timely and controlled manner is crucial for normal tissue homeostasis, deregulation of this process can cause continued expansion of the stem cell compartment, with the activation of the tumorigenic process [58], as we detect when HP cells are transplanted to SCID mice.

In this line, the tumorigenic process detected in HP cells may be related to the presence of supernumerary centrioles that would create misalignment between the spindle and the asymmetric cell determinants, resulting in enforcement of the tumorigenic process. We think that further studies on the link between supernumerary centriols, multipolar spindles and genetic instability leading to cancer in mammalian specie could provide important information, especially if we consider that most of the data available at the present, are limited to lower species. In our opinion the use of HP cell lines could represent an intriguing and unique experimental tool where the pathways controlling potency and self renewal vs cell transformation can be investigated in a human model, and where the use of different milieaux can activate mechanisms that control the switch between pluripotency and malignancy.

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Parthenogenesis in mammals: pros and cons in pluripotent cell derivation

Mini-Review

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Abstract: Embryonic stem cells (ESCs) represent a useful tool for cell therapy studies, however the use of embryos for their derivation give rise to ethical, religious and legal problems when applied to the human. During the last years parthenogenesis has been proposed as an alternative source to obtain ESCs. Based on the fact that parthenotes avoid many concerns surrounding the *"ad hoc" in vitro* production and following destruction of viable human embryos. Unfortunately many aspects related to parthenogenetic cell biology are not fully understood and still need to be elucidated. In this review we describe advantages and limits of these cells. We discuss their typical ESC morphology and high telomerase activity, which disappears after differentiation. We examine the pluripotency signature that they share with bi-parental ESCs. We review their high differentiation plasticity that allow for the derivation of several mature cell type populations when we expose these cells to adequate conditions. On the other hand, in-depth analysis demonstrated chromosome mal-segregation and altered mechanisms controlling centriole arrangement and mitotic spindle formation in these cells. We hypothesize their mono-parental origin as one of the possible cause of these anomalies and suggest a great caution if a therapeutic use is considered.

Keywords: *Centriole • Embryonic Stem Cell • Parthenogenesis • Pluripotency*

1. Introduction

Stem cells are characterized by their ability to proliferate indefinitely and, in adequate conditions, to give rise to specialized cell types. Their isolation can be obtained using different sources, with different advantages and limitations [1]. Embryonic stem cells (ESCs) are pluripotent cells derived from the inner cell mass (ICM) of pre-implantation blastocysts, as previously demonstrated in the mouse [2,3] and humans [4]. These cells present unlimited self-renewal and are able to differentiate into all cell types of the body [5,6]. These properties are not shared by adult stem cells isolated from differentiated adult tissues. Adult stem cells, in fact, show a limited capacity for self-renewal *ex vivo* [7] and are very difficult to expand *in vitro*. Consequently ESCs are considered to be an unlimited source of transplantation materials for the replacement cell therapy. These characteristics have resulted in efforts aimed at establishing ESCs in species other than the human and the mouse, including rabbit, hamster, cattle, sheep, goat and non-human primates. However, in order to generate ESCs lines, the creation and destruction of an embryo are needed. Moving from animals to man, the embryo's potency to develop into a complete human being, represents a concern and raises serious ethical, religious, legal and political issues. This imposes the necessity to identify alternative sources of pluripotent cells for research and therapeutic purposes [1]. During recent years different methods have been suggested in order to obtain pluripotent cells without the generation and destruction of a viable human embryo. To this purpose, ESCs have been successfully derived, removing a single cell (blastomere) from an eight-cell-stage embryo, using a

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biopsy procedure routinely utilized in *in vitro* fertilization clinics, in order to carry out pre-implantation genetic screenings [8,9]. Similar results were obtained deriving stem cells from growth-arrested embryos. Zhang *et al*. recently used such 'blocked' embryos (considered non-viable and discarded) to derive human ESCs that met all the criteria for pluripotency and had normal karyotype [10]. Another approach proposes the use of non-embryonic entities obtained through altered nuclear transfer (ANT). This technique is conceptually based on somatic cell nuclear transfer (SCNT), but the somatic cell used is genetically altered before being transferred to an enucleated oocyte, obtaining an abnormal blastocyst, unable to implant into the uterus but capable of generating customized embryonic stem cells [11,12]. Most recently, studies suggest the possibility to induce the pluripotent status (iPS) in somatic cells by direct reprogramming [13], using retroviruses or lentiviruses that "force" the expression of factors considered essential for pluripotent status maintenance, such as Oct3/4 [14,15], Sox2 [16] and Nanog [17,18]. Several pioneering studies suggest cellular reprogramming also through fusion of somatic cells with embryonic cell lysates [19] or loading permeabilized cells with lysates of donor cells (somatic or pluripotent) [20] as an alternative to the derivation of ESCs from human embryos, and a solution to both the ethical and legal problems.

The use of artificial parthenogenesis has been recently proposed as a possible alternative source of ESCs. While many features common to ESCs have been identified in these lines, several aspects related to parthenogenetic cell biology still need to be elucidated [21]. In this review we summarize data present in the literature related to parthenogenetic cell line derivation in human species. In an attempt to better clarify some important biological aspects, we discuss characteristics, pluripotency, differentiation plasticity, but also evaluate limits of these cell lines, recently derived in our laboratory [22].

2. Parthenogenesis in mammals

Parthenogenesis is a form of asexual reproduction, by which an oocyte can develop without the intervention of the male counterpart. This process may routinely occur naturally in some animal species such as fish, ants, flies, honeybees, amphibians, lizards and snakes. By contrast, in mammals this form of reproduction is not spontaneous, but oocytes can be successfully activated *in vitro,* using various electric, mechanical or chemical stimulations, which mimic the intracellular calcium wave induced by sperm at fertilization [23]. This phenomenon causes cleavage divisions and embryonic development [1]. However, mammalian parthenotes are unable to develop to term due to genomic imprinting alteration [24] that result in the repression of paternally expressed genes [25]. The consequent developmental abnormalities cause an arrest of parthenote development at different stages after activation - depending on the species [26] - giving embryo-like structure unable to form a new individual. In this scenario, after the successful isolation of embryonic parthenogenetic cell lines in the mouse [27,28] and non-human primate models [29,30], human parthenotes have recently been proposed as an alternative, less controversial source of stem cell lines [1,31-34]. Furthermore it must be noted that they may also be used as a new experimental tool for studies on the biological mechanisms involved in human early embryo development and assisted reproduction [21].

3. Human parthenogenesis as a source of pluripotent cell lines

In recent years the possibility to obtain ESCs after parthenogenetic activation in humans has been demonstrated [1,31-34], although the success of this approaches has been strongly limited by the restricted accessibility to unfertilized oocytes. We firstly reported the generation of a human parthenogenetic (HP) cell line in 2006 [35] and were then able to characterize these cells for their pluripotency and differentiation plasticity, both *in vitro* and *in vivo* [22]. In particular, we were able to show their typical ESC morphology and the main features of bi-parental stem cells, expressing appropriate stem cell markers such as Oct-4, Nanog, Rex-1, Sox-2, alkaline phosphatase, SSEA-4, TRA 1-81, and lack of the human ESC negative markers (e.g. SSEA-1) [36,37]. Altogether these results indicate a pluripotency signature that does not appear to be influenced by the exclusive presence of the maternal genome. HP cells also possess high levels of telomerase activity, which is turned down when cells are subjected to culture conditions that induce their differentiation, indicating that a physiologically normal control of telomerase activity is present.

In vitro differentiation potential of these cells was assessed throughout their culture in hanging droplets, which induces formation of embryoid bodies (EBs). When EBs were plated and grown as a monolayer, both gene expression and immunocytochemical analysis demonstrated the ability of these cells to differentiate and give rise to components belonging to the three embryonic germ layers (Figure 1). Interestingly no expression of trophectoderm-related markers was detected, possibly reflecting parthenote inability to generate adequate fetal membrane development and to implant correctly.

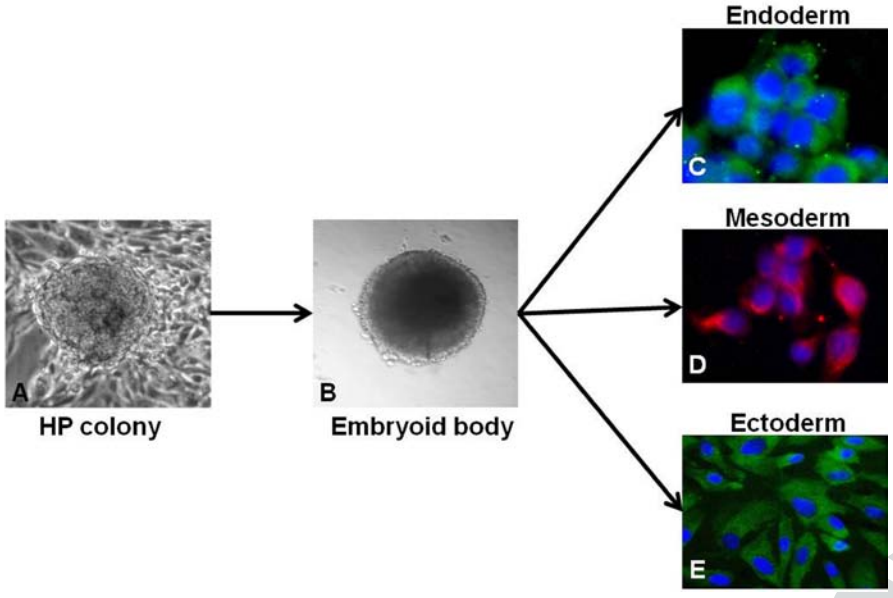

Figure 1. Pluripotency and differentiation plasticity of HP cell lines. HP cells grow in colonies and show a typical ESC morphology (A). They are able to form EBs (B) that differentiate into cells belonging to the three embryonic germ layers: endoderm (C); mesoderm (D) and ectoderm (E).

A crucial point in ESC studies is, in our opinion, to assess the possibility to drive cell differentiation towards a specific lineage, using controlled culture conditions. Data obtained in our laboratory demonstrate that HP cells were able to differentiate and to form different cell subtypes belonging to the neural lineage [22]. We are able to show that, when exposed to the appropriate *in vitro* microenvironment, these cells, not only gave rise to early neural population, but also generated more mature cell types expressing nestin, CNPase and MAP2 [22]. Moreover our experiments demonstrated HP *in vitro* differentiation capability to form mature hemopoietic cell lineages - lymphoid, erythroid and myeloid sub-populations [22]. All these data are in agreement with the observation that murine uni-parental ESCs are able to differentiate into neuronal and glial cells as well as to generate adult-transplantable hematopoietic stem cells that can re-populate the hematopoietic system of adult transplant recipients [38]. Moreover these findings indicate that uni-parental cells may be differentiated and may represent a powerful tool in order to study the mechanisms controlling the human lineage determination *in vitro*.

4. Parthenogenetic pluripotent cell and mitotic spindle alteration

It has been demonstrated that centrioles degenerate and are lost during mammalian oogenesis. These organelles are present in oogonia and growing oocytes until pachytene stage, while they disappear in the mature oocytes, where they persist as a peri-centriolar disaggregated material [39]. They are then replaced at fertilization, when the sperm contributes the centriole that joins and re-organize the peri-centriolar material present in the oocyte to re-form a fully functional centrosome [40,41].This event causes an aberrant progression of cell cycle and spindle formation in the newly synthesized embryonic centrosome in the absence of the sperm centriol [42]. With the notable exception of mice [43], the lack of centrioles on parthenogenetic development have been described in rhesus monkeys [44], rabbits [45], cows [46], sea urchins [47], *Xenopus* [48], and several other species [49]. In these species oocytes are unable to form astral microtubules and a correctly oriented spindle (Figure 2). These observations are in agreement with our findings in HP cells that lack correct control of the spindle formation process. These cells displayed multiple centrioles as well as an altered expression level of mitotic check point related-molecules, suggesting that the ability to rearrange functional centrosomes is altered in these cells. In particular HP cells showed a higher level of Mad-1 and the related molecules MAX and SIN3, when compared with bi-parental cells. These molecules represent a central component of the spindle assembly checkpoint and recruitment of kinetochores [50-52], suggesting the possibility of severe disturbances in the control of spindle formation and mitosis in these cells. Furthermore HP cells showed very low

transcription for TTK and CENP-E, which are involved in correct chromosome aggregation and alignment as well as segregation, during mitosis. Altogether these observations support the hypothesis that the altered levels of such molecules in HP cells cause anomalies in their control of proliferation and differentiation.

5. Parthenogenetic pluripotent cell and karyotype alteration

Abnormalities in spindle rearrangement is usually linked to a high percentage of cells showing mal-segregated chromosomes. Previous studies demonstrated the presence of multiple chromosome malsegregations in human oocytes after parthenogenetic activation, either spontaneous or induced by puromycin [53]. A high incidence of polyploid and mixoploid blastomers has also been reported in parthenotes derived from bovine and porcine, suggesting the absence of a paternally supplied centrosome as a possible cause [54,55]. Consistent with this, chromosome instability was observed in HP cell lines. However, these abnormalities do not seem to affect the overall proliferation rate and *in vitro* differentiation plasticity of these cells that, when cultured *in vitro*, are able to respond to specific media, routinely used for bi-paretntal ESCs differentiation, and to give rise to populations representative of the three germ layers in controlled manner. Furthermore it is interesting to note that the use of an alternative protocol of activation has been shown to better ensure chromosome stability. In this line, Fulka et al. recently reported the derivation of karyotypically normal cell lines from mouse MI maturing oocytes exposed to Butyrolactone I, which converts meiotic metaphase I chromosomes into chromosomes with mitotic-like morphology [56].

The data available from the literature as well as that described in this review indicate that cell lines isolated from parthenotes display interesting, even though contradictory aspects. Despite the exhibition of abnormal centrioles and chromosome mal-segregations, these cells are indeed able to proliferate and maintain their pluripotent status and respond in a controlled manner to specific stimuli, generating a vast array of differentiated cell populations. We think they may represent a precious model where the pathways controlling self-renewal and differentiation plasticity deeply interrelate with cell instability and abnormality.

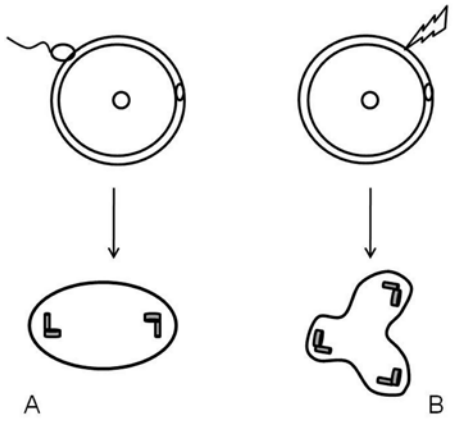

Figure 2. Centriole organization in fertilized embryos and parthenotes. At fertilization the sperm contributes the centriole that joins and re-organizes the peri-centriolar material present in the oocyte to re-form a fully functional centrosome (A). Parthenogenetic activation causes the formation of mulpiple centrioles that result in aberrant spindles, due to the absence of the centriole contributed by the sperm (B).

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