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Chapter 3

De-Differentiation of Somatic Cells to a Pluripotent State

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Additional information is available at the end of the chapter

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1. Introduction

The gametes are highly specialized haploid cells that harbor genetic background of each individual. During fertilization, the fusion of female and male gametes occurs in order to produce zygote. These zygotes are diploid cells and have genetic material of both individuals. Zygotes start to divide and undergo further pre-implantation development through the formation of morula, blastocysts and finally fetus (Fig. 1). When intrauterine fetal development is finished, the organism is already formed and ready to birth. The zygotes can be considered primordial stem cells, which originate the whole organism through unequal divisions to produce blastomeres, the cells resulting by cleavage of a zygote. Sixteen blastomeres constitute a morula, the spherical embryonic mass surrounded by the zona pellucid, which further became a blastocyst. Blastocyst is a thin-walled hollow structure surrounded by trophoblasts layer that contains a cluster of cells called the inner cell mass (ICM) from which the embryo arises and the scientists isolate embryonic stem (ES) cells for *in vitro* cultivation and for study the process of differentiation. However, ES cells are pluripotent cells able to produce any cell type raise ethical concerns about the destruction of human embryo to produce stem cell lines. To get the better concept of pluripotent cells for stem cell based therapies the reprogramming of patient specific adult cells to embryonic stage was suggested (Takashi & Yamanaka,2006). Cell reprogramming is a process of de-differentiation of somatic cells into pluripotent state whereby they adopt features of ES cells. De-differentiation of adult cells can be achieved through i. somatic cell nuclear transfer; ii. cell fusion - somatic cell hybrids and; iii. production of induced pluripotent stem (iPS) cells through the activation of essential stemness genes (reprogramming factor), over-expression in fibroblasts and/or other adult cells. Small molecules and other technologies are also exploring to repro-

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gram cells without the use of viral vectors primarily by Yamanaka. The methods used for cell de-differentiation induce the expression of genes that are not normally expressed in adult cell but are expressed in pluripotent stem cells, leading to the activation of pluripotent cell transcriptional networks. A cascade of transcriptional activity switch on the changes in gene expression profile in the adult cells, which begins to express a repertoire of genes that are commonly, identified in pluripotent ES cells. Following reprograming the adult cells undergo morphological changes and begin to grow as a tightly packed cluster of cells known as a colony, which mirrors how undifferentiated ES cells grow in culture. Both processes of reprograming and resulting pluripotency of reprogrammed cells vary significantly and elucidation of different approaches can clarify the reprograming process. In this chapter we will describe different methods of reprogramming of differentiated cells to pluripotent cells and the knowledge gain from each. Additionally, we try to provide a functional vision on reprogramming process and to analyze different types of stem cell niches produced by natural and reprogrammed cells. The better comprehension of stem cell niches will allow us to improve the reprogramming technology and to put more close in production of natural pluripotent stem cells using molecular biology approaches.

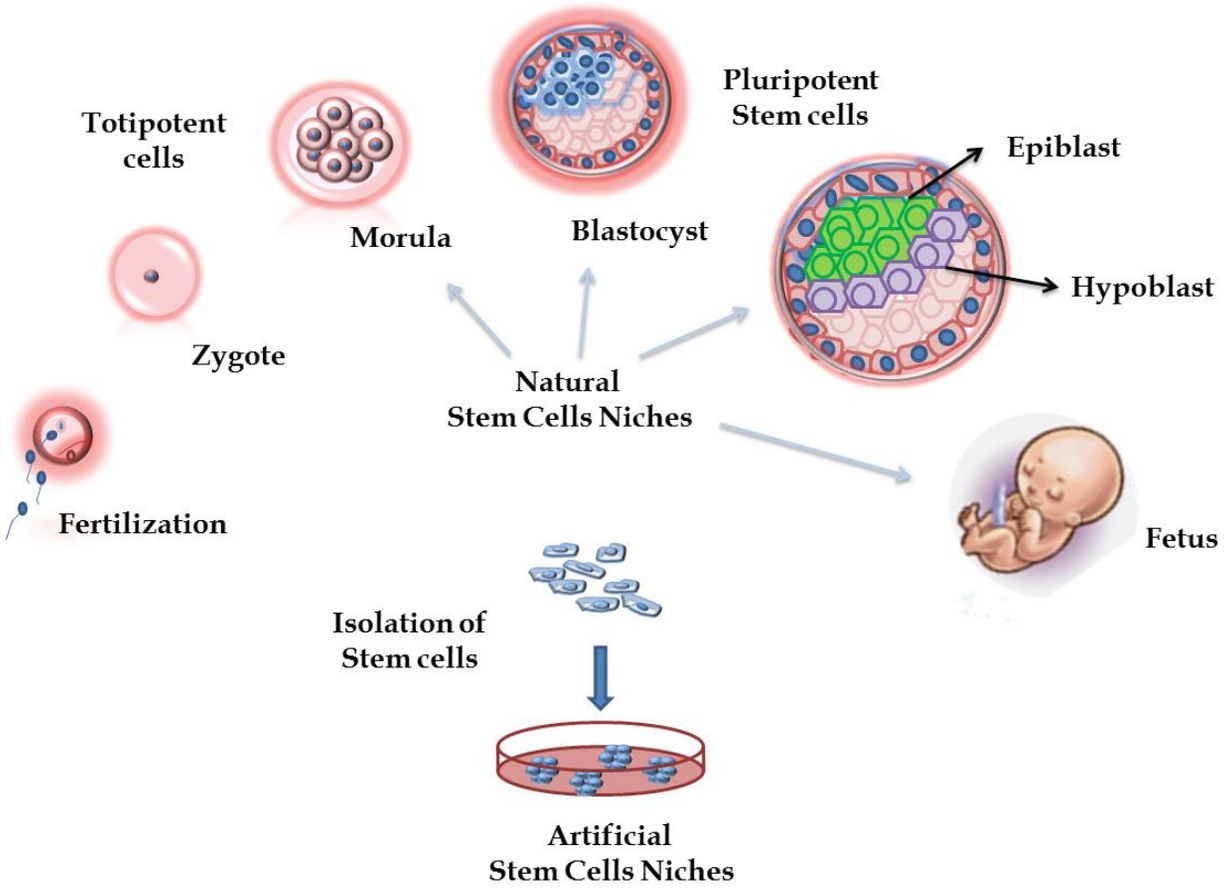

Figure 1. Early development of stem cells niches. According to current knowledge there are natural stem cells niches during development: morula, blastocyst, epiblast and fetus, and artificial stem cells niches: stem cell culture *in vitro*.

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2. Natural *in vivo* pluripotent stem cell niches

2.1. Morula stem cell niche

Here we proposed that starting from morula, when zona pellucid arises, a first specific compartment called stem cell niche is formed. This niche can be defined as a microenvironment in which stem cells are found. Stem cell niche provides the both interaction between the cells and their interaction with local microenvironment, by which their fate regulates and occurs. In morula, stem cell niche consists of pluripotent stem cells that provide expression of specific transcription factor, such as POU domain transcription factor (Oct3/4) responsible for self-renewal capacity and pluripotency of these cells. In mammals morula first cell fate decisions is governed by key transcriptional factor: Oct3/4 (Palmieri et al., 1994). Oct3/4 is unique because it requires maintaining the pluripotency in both conditions *in vivo* and *in vitro* (Nichols et al., 1998) and it is essential for epigenetic reprogramming (Niwa et al., 2000). The depletion of transcription factors leads to increased expression of genes that are involved in the processes of development and cell differentiation (Niwa et al., 2000).

2.2. Blastocyst stem cell niche

The first lineage segregation is resulted in the formation of trophectoderm and ICM (Wobus, et al., 2005). Upon silencing of Oct3/4, a part of morula cells spontaneously inactivates the self-renewal process and start to differentiate into trophoblast cells, thus forming pluripotent stem cell niche in blastocyst. This blastocyst niche is a dynamic structure which follows developmental program of an organism in parallel with Oct3/4, expression of other transcriptional factor such as Nanog occurs in ICM. in early blastocyst (Nichols et al., 1998; Avilon et al., 2003).

2.3. Epiblast stem cell niche: Naive and primed pluripotent stem cells

In mice in late blastocyst transcription factor Sox2 starts to express in the cells of ICM in addition to GATA6 and Nanog, which lead to formation of two distinct populations: epiblast and hypoblast (Mitsui et al., 2003). These cell populations are considered the precursors of the primitive endoderm and the pluripotent epiblast (Morrissey et al., 1998). Recent studies suggest that stem cells in rodent epiblast have two distinct stable states of pluripotency: naïve and primed, thus establishing epiblast stem cell niche (Tesar et al., 2007; Nichols, 2009). According to these classification both of states exhibit features of bona fide pluripotent stem cells, such as have indefinite self-renewal, tri-germ layer potential and depend on expression of all three transcription factors, such as Oct3/4, Sox2 and Nanog (Tesar et al., 2009; Nichols, 2009; de Los Angeles et al., 2012). Naïve (more immature) pluripotent stem cells can be obtained from pre-implanted stage of embryo in rodents (Okamoto et al., 2003). These cells have both sex X chromosomes activated and are able to produce high-grade chimeras after their reintroduction into the host blastocyst. In contrast, in humans primed pluripotent ES cells are isolated from human pre-implantation blastocysts stage of development. In these cells one of female X chromosome is inactivated, albeit human ES

cells are self-renewing and express key transcription factors and are able to form teratoma (Okamoto et al., 2003; Brons et al., 2007; Tesar et al., 2005). Studies of X chromosome inactivation in pre-implantation human embryos reported that *XIST* transcript accumulation on this chromosome occurs in the eight-cell stage embryo, however the identity of the cells, which show *XIST* accumulation is not clear. *In vitro* studies of *XIST* accumulation in human ES cells lines revealed three different patterns of X chromosome inactivation. The naïve state - with both active X chromosomes, intermediate state - with both *XIST* accumulation and last - state, when the cells never undergo X chromosome inactivation even under differentiation and *XIST* accumulation does not occur (Dvash and Fan, 2009). Therefore, using current technologies "true" pluripotent stem cells can be exclusively isolated from mouse and may be from some other rodents, which present similar pattern of early embryonic development with mice. It is not obvious if it is possible to obtain "true" human ES cells, once we cannot test their contribution into developing human embryo (due to ethic consideration). However, the lack of X chromosome reactivation indicates that probably these cells will never be able to reintegrate into early development events *in vivo* similar to rodent.

3. *In vitro* pluripotent stem cell niches

After isolation, pluripotent stem cells start to organize *in vitro* stem cell niche, which up to a certain degree simulates experience of these cells *in vivo*. *In vitro* these cells showed similar morphology with ICM, forming islands of juxtaposed cells and expressing pluripotent stem cell markers such as Oct3/4, Nanog, Sox2 (Tesar et al., 2005; Tesar et al., 2007). However, to distinguish *in vitro* naïve and primed pluripotent cells are difficult or even impossible task due to high heterogenety of pluripotent cell lines established *in vitro* (Brons et al., 2007; Tesar et al., 2007). Different factors may contribute to this heterogeneity, such as natural polymorphism of the cells, selection of colonies in vitro, which can be pluripotent at different degrees, cell culture conditions adopted in each work as well as pluripotent cells, itself, may produce an "imperfect" *in vitro* microenvironment again due to their natural heterogeneity.

4. Generation of artificial pluripotent stem cell – Reprogramming strategies

4.1. Reprogramming by means of differentiated cells nuclear transfer

Several strategies can be provided in order to reprogramming differentiated or committed somatic cell genome. One of these strategies is a nuclear transfer (NT) of differentiated cell nucleus to oocyte whose maternal DNA was removed (Campbell et al., 1996). This type of reprogramming uses the natural components without any previous genetic or molecular modification of nucleus–donor and oocyte-recipient. NT is relatively efficient and frequently depends on technical experience of researcher (Galli et al., 2012). There are two kinds of nuclear transfer trial: egg-NT involves the transfer of a single somatic nucleus to an unfertil-

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ized enucleated oocyte and oocyte-NT involves the transplantation of multiple somatic cell nuclei into immature oocyte of amphibian. Nevertheless are important differences between the two types of nuclear transfer experiment. In oocyte-NT experiments extensive cell division take places and new functional cell types appear as soon as the nuclear transplant embryo start to develop. In this experiment somatic cell chromatin is directly reprogrammed to express pluripotency genes within a day. In contrast to oocyte-NT experiments, in egg-NT no new cell types are formed, and neither oocyte nor nuclei divide, however direct transition of reprogrammed nuclei that transcribe genes of pluripotency into differentiated cells occurs. Analysis of the mechanism of reprogramming in egg-NT experiments, which involves transcription pluripotency genes and others, is complicated owing to rapid DNA replication and numerous cell divisions (Halley–Scott et al., 2010; Julien et al., 2010).

The NT process leads to direct reprogramming of pluripotent stem cell and expression of such markers as Oct3/4, Nanog, and Sox2 that are silent in differentiated somatic cell nucleus. In general, the reactivation of silent pluripotency genes starts around 24 and 48 hours after NT (Halley–Scott et al., 2010; Julien et al., 2010; Byrne et al., 2003). Upon NT occurs the series of events when oocyte cytoplasm induces changes in the structure of donor chromatin toward pluripotent state, which became more appropriate for embryonic development. However, synchronization process which should happen between genomic DNA of donor cell and cytoplasm of recipient cell is complex and may affect significantly pluripotency of reprogrammed cells. Attempts to facilitate this reprogramming process have been made using chemicals that alter the methylation status of the chromatin, such as TSA (trichostatin A), azacytidine, scriptaid, either before or after NT. In the mouse, the use of TSA (a histone deacetylase inhibitor, HDACi) significantly increased the success rate of mouse cloning (Kishigami et al., 2007).

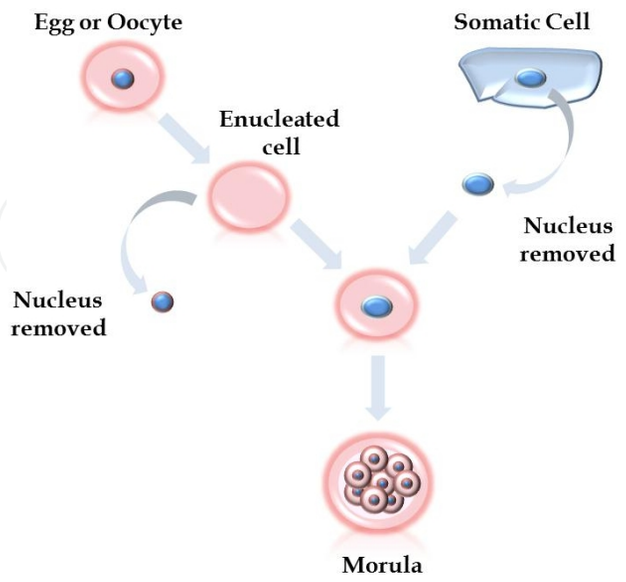

Figure 2. Stages of nuclear transfer. The nucleus is removed from an egg (or oocyte) and replaced by a nucleus from a donor cell (somatic cell).

In mammals, embryo obtained by NT and transferred into foster mother can result (or not) in full term development. The clones, obtained by NT method, are genetically identical to donor organism, which provide a nucleus. The sheep Dolly was the first successfully cloned farm animal. Dolly was obtained from NT of terminally differentiated mammary epithelial cell (Campbell et al., 1996). However the generation of animals by NT is not very efficient, once many clones are dying soon after implantation, and only few clones survive and born (Galli et al. 1999; Ritchie 2006). These clones frequently affected with severe abnormalities, they die prematurely and often obese. The survival rate of clones depends on species, on donor cell type, method of NT and varied significantly between different laboratories (Oback & Wells, 2002; Wilmut et al., 2002). However, pre-implantation development does not seem to be a problem (Ono et al., 2001; Ono et al., 2001a) the majority of the term losses occurs during the post implantation period and/or after birth. It has been reported in some experimental studies, that only 2-3% of the transferred embryos develop to term in mice (Ono et al., 2001a; Sakai et al., 2005). Over time the methods were improved and other species have been cloned with success from differentiated donor cells, such as cattle (Galli et al., 1999); mouse (Wakayama & Yanagimachi, 1999); pig (Polejaeva et al., 2000a); cat (Shin et al., 2002); goat (Keefer et al., 2002); mule (Woods et al., 2003); horse (Galli et al., 1999); rabbit (Challah-Jacques et al., 2003); rat (Zhou et al., 2003) and dog (Lee et al., 2005). In humans, the attempt to NT has been achieved using animal oocytes as recipients for human genetic material. The reprogramming of human somatic cell nuclei did not occur after NT into bovine and rabbit oocytes. These oocytes with human genome were not able to follow early embryonic development. The up-regulation of human pluripotency-associated genes did not occur. These data raised a question about the potential use of animal embryonic environment to generate patient-specific stem cells using NT technology. Ethical implications also should be taken in consideration (Chung et al., 2009).

4.2. Reprogramming by means of stem cells nuclear transfer

In 1998, Cibelli performed stem cells nuclear transfer (SCNT) using nucleus of bovine fibroblasts and enucleated bovine oocytes. They obtained 330 reconstructed oocytes, generated 37 cloned blastocysts, which served for isolation of 22 ES-like cell lines. These ES-like cells were injected into bovine oocytes, cultured cultured to produce embryos that further which were transferred into recipient females. In six out of seven calves at least one tissue originated from ES cell has been found. Other authors demonstrated the ability of karyoplast of ES cells induce Oct4 expression in the somatic genome (Tada, 2001).

In humans (Hall et al., 2007) and non-human primate (Mitalipov et al., 2002) the SCNT efficiency of blastocyst formation has typically been very low, thus suggesting a lack in or complete nuclear reprogramming. In order to overcome these difficulties modified SCNT approach was used to produce rhesus macaque blastocysts from adult skin fibroblasts and to isolate from this blastocyst two ES cell lines. This was achieved thought non-invasive approaches for meiotic spindle detection in oocytes and their removal using high-performance imaging. Spindle imaging system supports rapid and highly efficient real-time enucleation of primate oocytes. In this experiment spindle removal efficiency was 100%. The investiga-

tion of karyotype, microsatellite and single nucleotide polymorphisms (SNP) analyses confirmed that both ES cell lines were originated from SCNT embryos and were not from parthenotes. These ES cell lines demonstrated typical pluripotent cells morphology, self-renewal capacity and expression of stem cell markers. They were also transcriptionally similar to ES cells derived from fertilized blastocysts, and pluripotent, as demonstrated by the generation of several tissues from three germ layers after *in vivo* teratoma formation (Byrne 2007). Additionally, the experiments using mouse pluripotent primordial germ (PG) and ES cells as nuclei donors have also been performed using single-cell NT method. The results showed that embryos obtained from PG or ES cells NT method cannot develop and complete pre-implantation stage (Kato and Tsunoda, 1995). Possibly that long term *in vitro* culture can affect the karyotype of these cells accumulating chromosomal abnormalities, thus resulting in formation of abnormal embryos (Balbach et al., 2007).

The main goal of NT technology was to multiply the genotypes of high genetic value in farm animals and species, which are under the risk of extinction. Further, this technology was used as a tool for genome reprogramming of somatic differentiated cells into pluripotent state. The principles of cloning, which were developed by Willadsen (1986), are also important today. All the cloning studies provided the first experimental evidence for reprogramming (Kono et al., 1997; Gurdon, 2008). Currently, NT technologies can be applied in two different ways, to produce animal clones and to reprogram the nuclei of differentiated somatic cell, which can be used for basic research to analyze X chromosome inactivation or to study the dynamics of imprinting process during reprogramming and in some cases for preclinical evaluation of these cells in animal models (Hochedlinger and Jaenisch 2006). This technology yet holds medical interest to produce patient-specific stem cells, which can be used in cell therapy and regenerative medicine.

4.3. Reprogramming by means of early embryonic environment

The pluripotency, characteristic feature of ES cells, can be evaluated by their capacity to differentiate into cells of the three germ layers. More precisely, ES cells pluripotency can be evaluated by generation of chimaeras, organisms composed of cells from two or more individuals from the same or different species (Kaufman, 1981; Keller, 1995; Wobus, 2005). Production of human/animal chimaeras is a method currently in use to analyze developmental potency of mammalian ES in biomedical research (Behringer, 2007; Lensch et al., 2007). James et al (2006) showed for the first time that a nonhuman embryo surrogate environment could be used to study developmental potential of human ES cells as well as biological compatibility between human ES cells and the mouse ICM. Adult stem cells (ASC) are now seen as an alternative to ES cells, which can raise a number of ethical objections due requires destruction of human embryo. Populations of multipotent ASC that express ES cell markers, such as Oct3/4, Nanog and Sox2, presenting a differentiation capacity similar to that of ES cells *in vitro*, can be isolated from different fetal and adult animal and human tissues (Wenceslau et al., 2011). For example, we have reported the isolation of human immature dental pulp stem cells (hIDPSC) from deciduous (baby) teeth, which express the aforementioned pluripotent markers and can differentiate into several cell types *in vitro*, such as bone, carti-

lage, skeletal, smooth muscles and neurons (Kerkis et al., 2006; Lizier et al., 2012). We found that after their transplantation into adult mice, they engrafted within different mouse organs, such as the liver, heart, spleen, kidney and the brain. Although hIDPSC express pluripotent cell markers they present fibroblast-like morphology and were isolated from adult tissues (Kerkis et al., 2006; Lizier et al., 2012). We demonstrated that hIDPSC are truly multipotent cells, which were able to undergo further development similar to mouse ES cells in nonhuman embryo surrogate environment. These cells were able to contribute *in vitro* into ICM of mouse blastocyst, thus undergoing cell divisions, and *in vivo* into fetus development thus generating pretermed human/mouse chimaera, which a prerequisite to characterizing pluripotency similar for ES cells. In this study in order to analyze the ability of hIDPSC (46, XY) to contribute to ICM and trophectoderm of mouse early embryos, 6–8 cells stained with vital Vibrant fluorescent dye (Fig. 3A) were injected into the perivitelline space and/or the blastocell of 8 compacted morulae and 20 early blastocysts (Fig. 3B). After injection these cells have adopted similar size to those of the recipient mouse embryo. They proliferated in the recipient mouse embryonic environment and showed a contribution to the ICM and also to the trophoblast cell layer (Fig. 4A). To determine the developmental and pluripotent capacity of hIDPSC, six to eight stained cells were injected into the blastocele of 57 early blastocysts (Fig. 4B) and were immediately transferred to the uterus of five foster mothers. Three mice achieved pregnancy and, according to ethical recommendations, human/mouse chimaeras were collected before birth. The 18 d.p.c. mouse foetuses seemed to be well formed based on their morphological appearance (Fig. 4C).

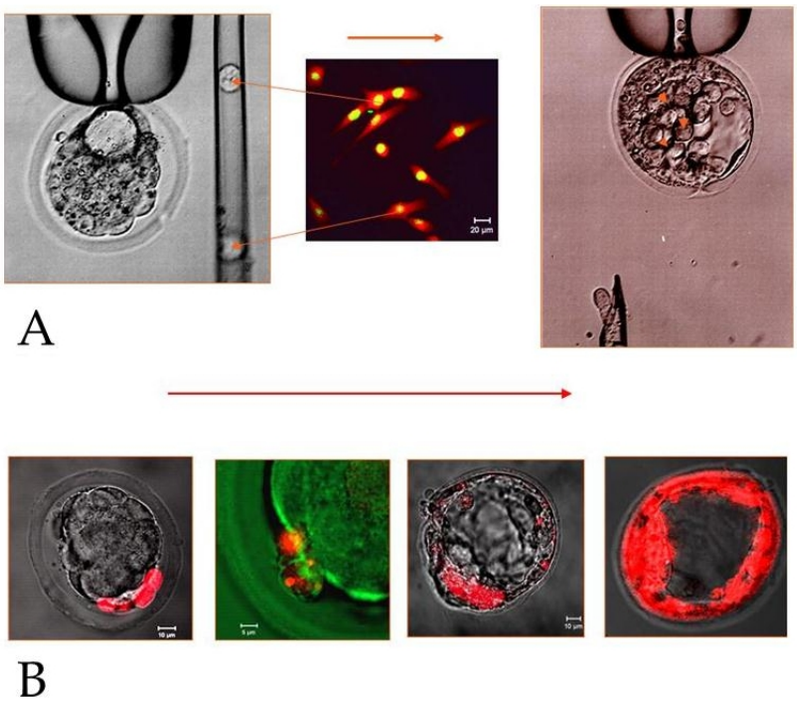

Figure 3. The hIDPSC injection in early embryonic environment. (A) hIDPSC stained with vital Vibrant fluorescent dye were injected into the perivitelline space and/or compacted morulae (B) hIDPSC showed a contribution to the ICM and also to the trophoblast cell layer.

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Additionally to Vibrant fluorescent dye the anti-hIDPSC antibody, (this identifies exclusively hIDPSC), was used to detect the presence of these cells in 18 d.p.c. mouse foetuses). Strong fluorescent signals were observed in different organs of the chimaeras, such as the brain, liver, intestine and muscles (Fig. 4D and Fig. 5B). Using a variety of methods we demonstrated hIDPSC contribution to mouse embryos, which did not present any type of morphological deficiency (Fig. 5A). We were able to produce evidence, that these cells accomplished differentiation within local tissues, by the presence of human-specific tissue proteins, such as myosin and cytokeratin. Moreover, we used a specific antibody against human nuclei to confirm, again, that the cells were indeed of human origin (Siqueira da Fonseca et al., 2009). Little is known about the initial reprogramming events that occur after transference of ASC into mouse blastocysts (Yokoo et al., 2005). In our experiment, hIDPSC were capable of engrafting and proliferating inside mouse morulae and blastocysts and forming pretermed chimaeras. These cells contributed not only to ICM, as do human ES cells, but also to the trophoblast cell layer – without any embryo damage.

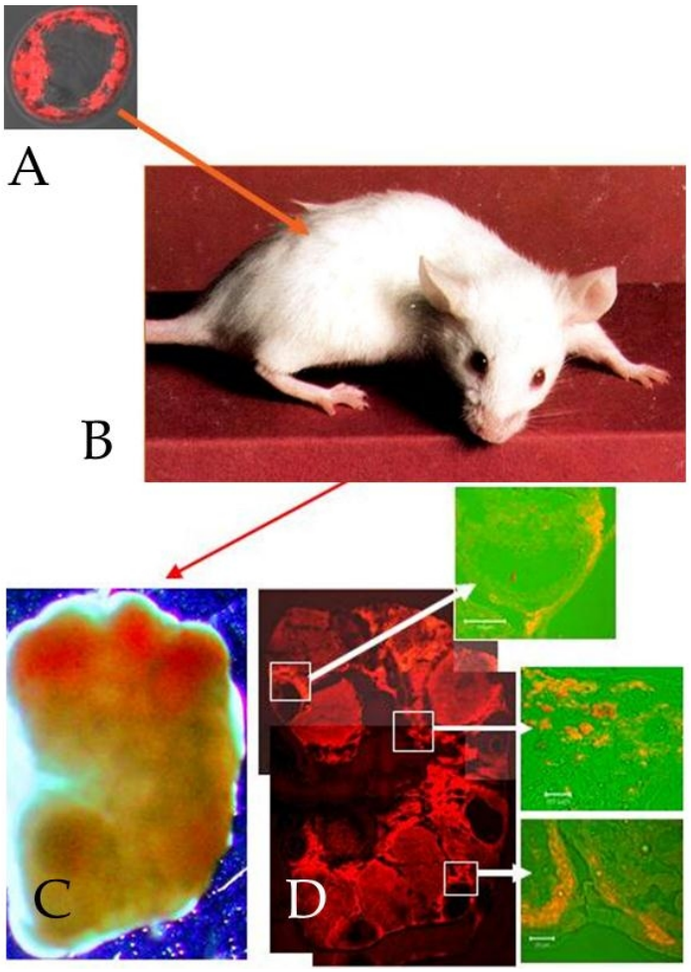

Figure 4. Developmental and pluripotent capacity of hIDPSC to generating pretermed human/mouse chimaera. Early chimera blastocyst (A) were transferred to the uterus of foster mother (B). Human/mouse chimaeras (C) were collected before birth and fluorescent signals were observed in different organs of the chimaeras (D).

Furthermore, hIDPSC integrated into host embryos and developed foetuses, undergoing the process of differentiation. Obviously that due to the difference in cell cycle dynamics between mouse and human cells, the number of human cells during mouse pre-natal development is decreased in comparison with hIDPSC contribution in ICM of blastocyst. However, it is not clear if hIDPSC can really undergo reprogramming into ES-like cells within nonhuman embryo surrogate environment.

Our finding suggests that expression of such pluripotent markers, as nanog and oct4 by hIDPSC is enough condition for these cells to contribute into different mouse tissues in early embryo-fetal development, to differentiate properly and to express human proteins within mouse fetal an immune privileged environment (Siqueira da Fonseca et al., 2009).

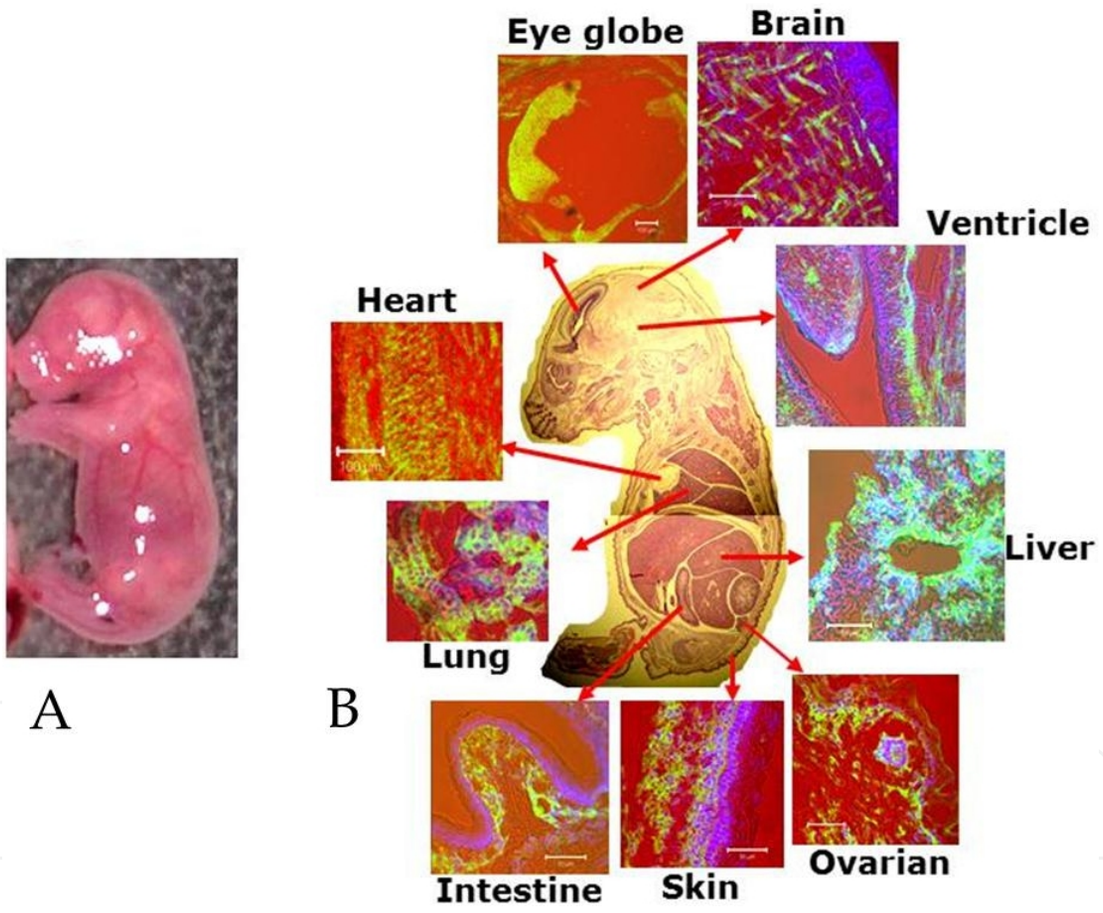

Figure 5. The hIDPSC contribution in pretermed human/mouse chimaera. (A) 18 d.p.c. mouse fetus. (B) Strong fluorescent signals were observed in different organs of the chimaeras, such as the brain, liver, intestine, muscles and others.

4.4. Reprogramming by means of cell fusion

First pluripotent hybrid cells have been isolated by fusion of pluripotent teratocarcinoma (TC) cells with differentiated somatic cells, which served as a tool for investigating the interaction between different genomes. These TC cells are similar to ES cells in morphol-

ogy and gene expression pattern, thus maintaining variable levels of pluripotency, however not all TC cells able to generate chimaeras and to contribute to germ line (Papaioannou and Rossant 1983). These cells frequently have abnormal karyotype, such as loss of the Y chromosome, trisomy, deletions or translocations (Takagi et al. 1983, Rousset et al. 1983, Modlinski et al. 1990). The hybrid cells obtained from pluripotent TC cells and somatic cell partner, which express embryonic antigens, were able to produce teratomas containing derivatives of all three embryonic germ layers (Andrews and Goodfellow 1980, Atsumi et al., 1982; Rousset et al. 1983; Forejt et al., 1984; Takagi, 1983) and to form embryoid bodies (EBs) in suspension culture (Takagi, 1983). These hybrid cells showed also reactivation of particular genes after reprogramming (Miller and Ruddle, 1976, 1977; Andrews and Goodfellow, 1980; Rousset et al., 1983) and reactivation of inactive X chromosome originated from the somatic partner (McBurney and Adamson, 1976; McBurney and Strutt, 1980; Takagi et al., 1983, Takagi, 1988; Mise et al., 1996). However, pluripotent hybrids were obtained when lymphocytes or thymocytes, not fibroblasts, were used as the somatic parents in fusion (Rousset et al., 1979). These studies indicate that hybrid cells generated by ES cells and differentiated cells, which have less cytoplasm, seem to be more adequate systems to undergo reprogramming.

Matveeva et al. (1996) has obtained cultures of intraspecific embryonic hybrid cells by fusion of mouse ES cells, denominated HM-1 cells, which were derived from HPRT-deficient strain 129 mice (Magin et al., 1992) and characterized as highly pluripotent (Magin et al., 1992; Selfridge et al.1992) with splenocytes derived from an adult DD/c female. These hybrids were denominated as hybrid embryonic stem and somatic (HESS) cells and characterized as pluripotent and HPRT positive (Matveeva et al., 1996; 1998). Our group used three mouse hybrid clones HESS-1, HESS-2 and HESS-3 in order to study their karyotypes and investigate the influence of the karyotypes on the differentiation of these cells through the formation of embryonic bodies (Mittmann et al., 2002). The hybrid cells used in our study were near diploid (HESS-2 and HESS-3) and near tetraploid (HESS-1) and chromosome analysis showed different trisomies. The trisomies of chromosomes 1 and 11 were found in near diploid hybrids. These trisomies are probably typical of these pluripotent cells, and have previously been described in the mouse ES cells line (Crolla et al., 1990) and in TC cells (McBurney and Rogers, 1982). We found that the sex chromosome constitution in the HESS-2 line was predominantly XY, while in the HESS-3 line it was XO. Interesting that in HESS-2 and HESS-3 lines the segregated X chromosome was of embryonic origin. Indeed, it has been demonstrated by Ringertz and Savage (1976) that hybrids lose the chromosomes originating from differentiated, more slowly dividing cells. In our experiments, hybrids showed the capacity to form EBs *in vitro*, even at late passages (Fig. 6). The EBs formed by the hybrid cells could be considered as complex as those derived from the HM-1 line and the cystic-type EBs formed by pluripotent cells (Martin and Evans, 1975; Van der Kamp et al., 1984; Doetschman et al., 1985; Pease et al., 1990).

In the EBs derived from hybrids we observed haematopoietic-like cells, cells resembling skeletal and smooth muscle and others (Fig. 7). Cells of ectodermal origin (e.g. nerve cells) were not identified in EBs derived from hybrids. Our data shows that the 'embry-

onic' X chromosome may be lost in pluripotent hybrids, but reprogramming of the 'somatic' X chromosome may still occur, thus allowing restricted pluripotency. The normal karyotype may be a prerequisite for the efficient contribution of these cells to the germ line in transgenic and chimeric animals and for their ability to differentiate *in vitro* into a wide spectrum of cell types (Papaioannou et al., 1978; McBurney Rogers, 1982; Pease et al., 1990; Bronson et al., 1995; Liu et al., 1997; Suzuki et al., 1997). Therefore, we further tested the capacity of near diploid HESS-2 to differentiate *in vitro* in putative germ cells (GC) (Fig. 8). We demonstrated that two days after induction of differentiation by retinoic acid, the HESS-2 derived GC-like cells presented expression patterns of a gene set, involved in the progression of early stages of gametogenesis (Vasa, Stella, Dazl, Piwil 2, Tex14, Bmp8b, Tdrd1 and Rnf17). This finding is similar to previous descriptions of GC obtained *in vitro* from mouse ES cells (Hübner et al., 2003; Geijsen et al., 2004; Kerkis et al., 2007). HESS-2 generates GC *in vitro*, which were able to differentiation into sperm- and oocyte-like cells. These structures resembling the formation of presumptive oocytes appeared floating in the culture medium. FISH analyses indicate that several GC derived from HESS-2 hybrid cells were able to undergo sex chromosome reduction. The expression of ZP2 and ZP3, oocyte-specific markers, was also detected supporting our morphological observation. Hence our observations indicate that HESS-2 cells can progress into both female- and male- GC differentiation, however, the female developmental program could be achieved only in early stages (Lavaginolli et al., 2009).

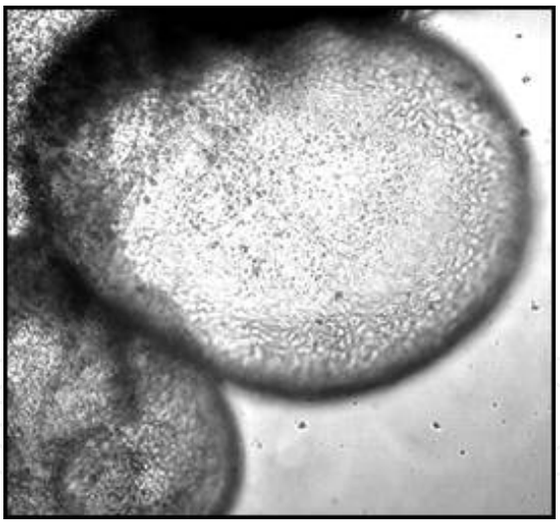

Figure 6. Cystic embryoid body - a globular cell cluster cultured from mouse ES cells.

Therefore, we demonstrated that near diploid somatic cell hybrids obtained by the fusion of ES cells with differentiated cell can be fully reprogrammed and able to produce *in vitro* even GCs. It is not likely that these cells will be able to generate live offspring after fertilization of normal oocyte due to abnormal karyotype. However, they represent an interesting model to study the influence of karyotype on the process of GC *in vitro* formation. More recently the reprograming of somatic cell nucleus after the fusion with induced pluripotent stem (iPS)

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cells has been reported (Takahashi and Yamanaka, 2006). These iPS-somatic cell hybrids demonstrated the expression of markers of pluripotent cells, such as Oct4, SSEA-1, and alkaline phosphatase and were able to differentiate into multiple cell types similar to ES cells, thus confirming the reprogramming ability of iPS cells (Takahashi and Yamanaka, 2006).

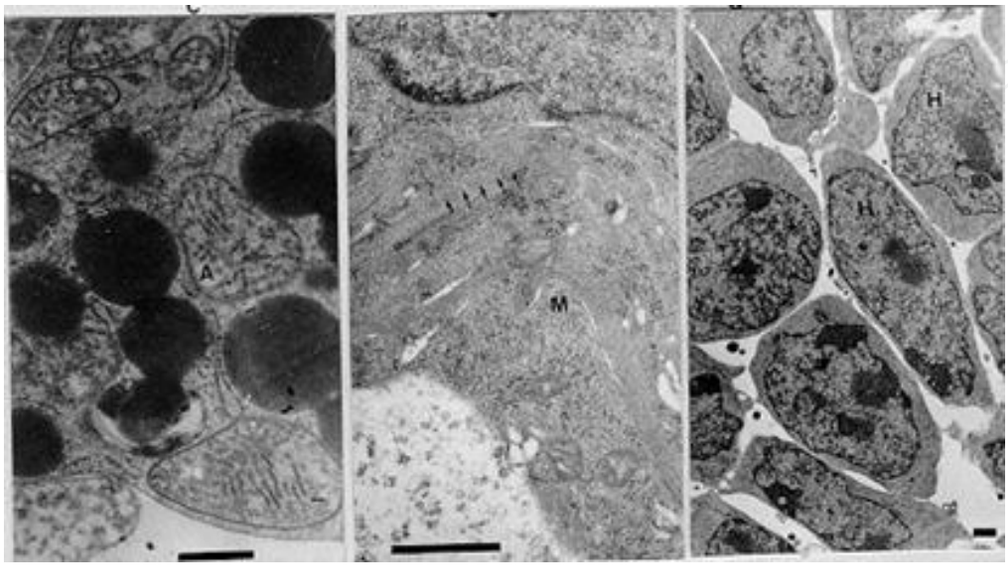

Figure 7. Electron microscopy demonstrates differentiation within cystic embryoid bodies derived from somatic cell hybrids (HESS-2).

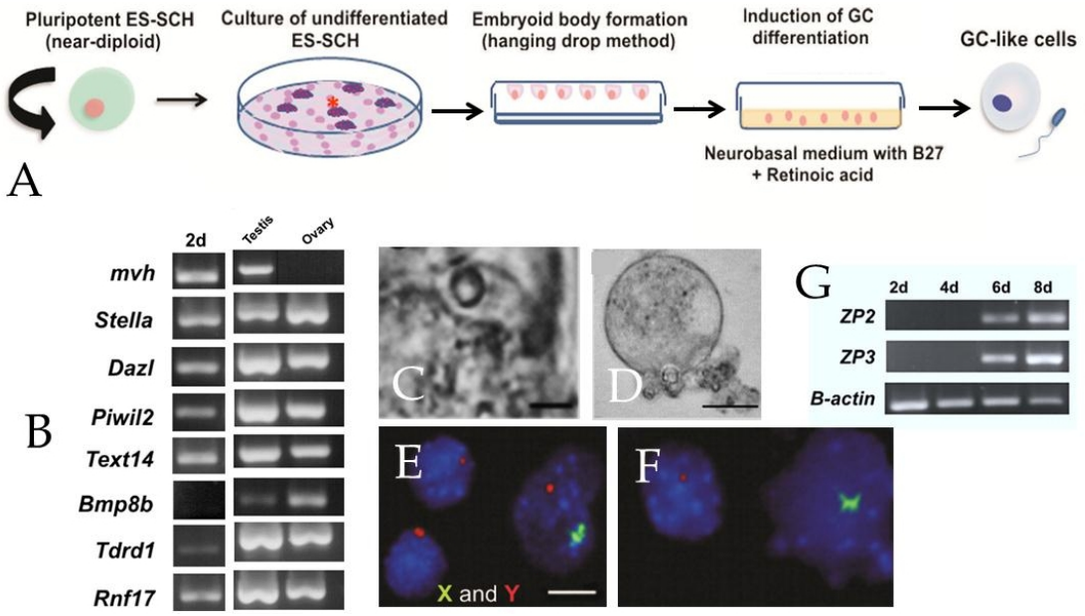

Figure 8. Germ cell derived in vitro from HESS-2. (A) Protocol of differentiation. (B) Expression of genes involved in the progression of early stages of gametogenesis during HESS-2 differentiation. (C) Sperm-like structure. (D) Oocyte-like structure. (E and F) Haploid cells with X or Y chromosomes. (G) Expression of oocyte-specific markers in oocyte-like structures obtained from HESS-2.

4.5. Reprogramming by means of Yamanaka's factors

The pluripotency manifests during short time of early mammalian development (Choen et al., 2011; Dejosez et al., 2012). Such powerful, pluripotent cells can be obtained *in vitro* from early embryo and they are very promising for the future of regenerative medicine and even for organ generation. However, in humans isolation of these cells implicates with ethical problem of embryo destruction. Thus the idea to obtain such pluripotent cells artificially took the minds of the researchers. The technologies to obtain such alternative pluripotent cells are growing continuously. Yamanaka 's group using the combination of different factors performed the first reprograming of mouse embryonic fibroblasts. The resulted iPS cells showed gene-protein expression of ES-cell markers, teratoma formation, differentiation into the tissues of three germ layers, beside chimaeras generation. This reprogramming strategy, using defined factors (i.e. Klf4, Oct4, Sox2, and c-Myc, termed "KOSM"), is conceptually and technically simple (Takahashi & Yamanaka, 2006). However, it is a low efficient and reproducibility process, which is influenced by several variables and also could affect the quality, such as completely or non-completely reprogrammed iPS cells. These variables are the age donor, cell type, different delivery systems and reprogramming cocktail choice, factors used for reprogramming (Daley et al., 2009). Currently, several strategies, based on genes, proteins, iRNA, as well as on different chemicals, are available for the reprogramming of somatic cells (Nakagawa et al., 2008; Yu et al., 2007). In the original method of iPS generation developed by Yamanaka's group used the moloney murine leukemia virus (MMLV) retrovirus for transgene expression (Takahashi and Yamanaka 2006). This vector has cloning capacity of around 8 kb allows delivery of genes into the genome of cells and expected to be silenced after reprogramming and induction of endogenous genes activation. The efficiency of iPS cells generated using MMLV retroviruses Expressing the KOSM set genes is around 0.1% in mouse embryonic fibroblasts and approximately 0.01% in human fibroblasts (Jahner et al., 1982; Stewart et al., 1982; Hotta et al., 2008). Lentiviral vector is also used in reprograming experiments thus exhibiting slightly higher (8–10 kb) cloning capacity and usually have higher infection efficiency than MMLV retroviruses (Blelloch et al., 2007). However, carcinogenesis may be caused by genomic integration of retro- or lentiviral fragments into host DNA (Varas et al., 2009) and use of c-Myc oncogene, which after reactivation might cause malignant tumor formation (Okita et al., 2007; Brambrink et al., 2008). Thus viral systems are still unsafe for therapeutic application. Therefore, a number of reports demonstrates that iPS cells can be generated by reducing the use of viral constructs and/or minimize viral integration through substitution of key reprogramming factors by chemical compounds or employing less differentiated cells, which already express endogenously one or more of the key pluripotency factors (Hota et al., 2008). Our group reprogrammed hIDPSC-fibroblast-like cells isolated from deciduous (baby) teeth, which express endogenous Oct3/4 and Nanog, using retroviral vector and four Yamanaka' s factors (Fig. 9).

Reprogrammed hIDPSC presented all key characteristics of pluripotent cells: formed juxtaposed colonies of ES-like morphology and produce teratoma with derivates of all three germ layers. These cells did not integrate retroviral vector in their genome and express lower levels of Oct4, Nanog and Sox 2. In contrast to iPS cells derived to fibroblast cell, the hIDPSC

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derived iPS cells were generated to in shorter time and presented higher efficiency of colonies formation And were able to form under iPS colonies feeder –free conditions conditions. For example, the time of fibroblasts reprogramming using retrovirus vectors takes 20–25 days (Aesen et al., 2008), while reprogramming of hIDPSC occurs only in eleven days after infection (Beltrão-Braga, 2011). These results suggest that age of donor and differentiation status of cell type used for reprograming may also affect reprogramming efficiency. Accordingly, Maherali and Hochedlinger et al., (2007) compared skin fibroblasts reprograming efficiency from two-month-old and two years-old mice. Older cells produced half as many iPS cell colonies as young skin fibroblasts. It has been shown that iPS cells have so-called epigenetic memory, which means that after reprograming their differentiation potential can reflect on their lineage commitment before reprograming. Therefore, hIDPSC showed strong neural commitment, which is due to their ectomesodermal origin. After reprograming strong neural commitment was evidenced within teratomas as well as spontaneous *in vitro* differentiation into neurons hIDPS-iPSC was also detected. It was expected that ordinary human adult cells reprogrammed as iPSC may revolutionize medicine by creating new therapies unique to individual patients. However, important questions have persisted about the safety of these cells, such as it is not clear the degree to which these cells are homologous to ES cells in respect of the genes expression pattern, differentiation capacities, epigenetics and in particular interest is the question whether iPSCs genetic material is altered during the reprogramming process. The researchers, which examined 22 different human iPSC lines obtained from seven research groups showed that these cells present 10 times more mutations than they expected to find. While some of the mutations appeared to be silent, the majority did change specific protein functions, including those in genes associated with causative effects in cancers. Anyway, the studies of iPSC provide an important new tool in the fight against human disease, but to use these cells directly in the clinic, we must ensure that they are safe.

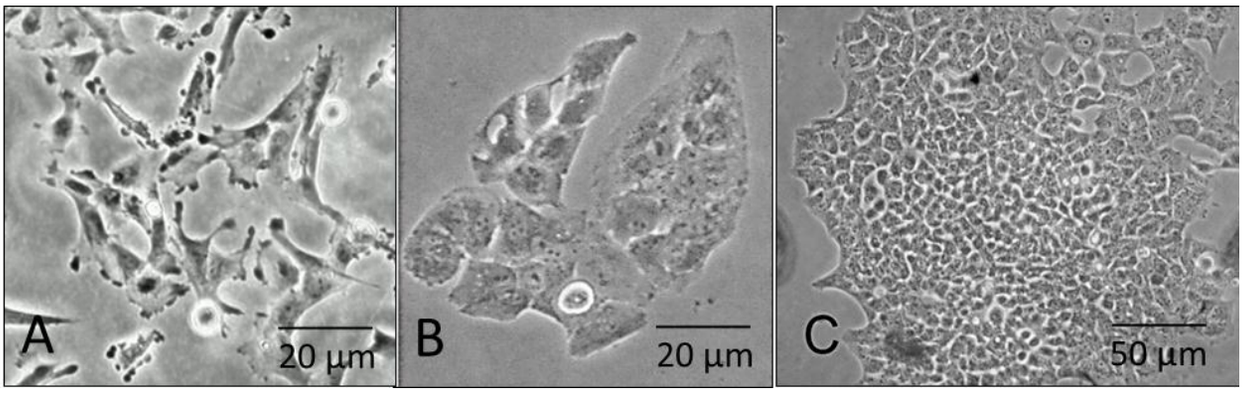

Figure 9. hIDPSC-derived iPS cell. (A) Representative figure of morphological characteristics of hIDPSC *in vitro* culturing. (B) iPS cell derivation were shown to be obtained under feeder-free condition on matrigel-coated dishes. (C) A typical hIDPSC-derived iPS cell colony. Light microscopy.

5. Final considerations

All reprogramming strategies are aimed at genomic reprogramming, which is a key biological process. It is still unknown, how many and what reprogramming factors, which initiate a cascade of reprogramming events, are involved in NT, SCNT, in cell fusion and even in iPS cell production. Yamanaka's study suggests that these factors may be mainly proteins of the nucleus; however the cytoplasm factors also should be taken in consideration. NT technique, which was used for Dolly the Sheep and many other species, has been abandon by many researches due to the low efficiency. Some researches try to use SCNT in stem cells research in order to obtain stem cells that are genetically matched to the donor organism. However, up to data no human ES cells were obtained using SCNT. Another limitation of this method is that resulting cells retain mitochondrial structures, which originally belonged to the egg. The great limitation of cell fusion technology is chromosome set composed by different genomes. Currently many scientists, which used all these methods moved to iPS cell production.

We started this chapter with simplified description of the concept of stem cell niches formation during early development. This conception lead to comprehension that such niches are very complex and composed by heterogeneous population of different somatic and stem cells. We know, that at least two different populations of pluripotent stem cells naïve and prime can be identified *in vivo* and isolated *in vitro* in rodents. In humans these two populations are difficult to identified and isolate. Additionally, the data on the pattern of X chromosome activation of *in vitro* cultured human ES cells suggest the existence of may be three such populations. In adult organism the number of stem cell niches increased dramatically, the examples are neuronal, hematopoietic, hair follicle, skeletal muscle, dental pulp and many other stem cells niches. In order to obtain stem cells of the most excellent quality the scientist try to re-create stem cell niche *in vitro*, which enables ad of control of culture conditions, including oxygen tension and hydrostatic pressure and various factors believed to be involved in self-renewing, division, migration, recruitment and lineage commitment of stem cells. Any strategies of reprogramming are closely related with the conception of stem cell niche, because in all strategies of reprogramming the nucleus or the cell with different developmental histories and from different cells niches are used. In order to translate the potential of reprogrammed cells into to the clinical reality our knowledge about reprogrammed stem cells microenvironment should be significantly improved.

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