13

Reprogramming Somatic Cells by Fusion with Embryonic Stem Cells: Present Status and Prospects in Regenerative Medicine

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1. Introduction

Both embryonic and somatic stem cells have attracted many scientists' interest as they appear to be ideal candidates for cell therapy to treat various degenerative diseases and tissue injury for which there are no cures available. However, during the last few years, extensive amount of publications have focused on somatic cell reprogramming, a reversal of cell fate from a mature differentiated state to an undifferentiated state or directly to that of progenitors or mature cells of a different cell type. The advantage of that strategy is to obtain an autologous source of cells, which when differentiated and/or transplanted will not be rejected by the recipients. Several strategies have been applied for reprogramming purpose, including somatic cell nuclear transfer (SCNT), somatic-stem cell fusion, cell-free extract treatment and induced pluripotent stem cell (iPSC) generation. As a model for studying nuclear reprogramming, cell-cell fusion has been studied since the 1970's. Recent observations demonstrate that transplanted stem cells can differentiate into multiple cell types *in vivo* and have brought investigators to a new stage for applying cell fusion to regenerative therapies, cherishing the idea that fusion-based reprogramming may possess more potential than the original belief. In this chapter, we have briefly discussed the current progress of somatic cell reprogramming induced by cell fusion. In our lab, using human embryonic stem cells (hESCs) and human fetal fibroblasts (HFFs) as fusion candidates, we have compared the fusion efficiency *in vitro* using different methods and successfully generated hybrid cells. Research publications to date suggest that cell fusion between stem cells and somatic cells may restore regenerative capacity of terminally differentiated cells and can be applied for transplantation and cell therapy. However, certain aspects of disadvantages cannot be neglected. The limitations of cell fusion based reprogramming includes the presence of stem cell nuclei in the hybrid cells, the possibility of causing immune-rejection and the genetic instability of hybrid cells. Insights of both pros and cons have been described in this chapter and we conclude that reprogramming induced by cell fusion can be achieved at different efficiencies when using various fusion methods *in vitro*. The hybrid cells show ESC-like properties with double nuclear content. More detailed studies will be needed to remove ESC nuclei before these cells can be used for clinical purposes.

2. Stem cells and regenerative medicine

Stem cells have been identified as clonal cells that have the capacity to self-renew as well as the ability to generate more than one type of specialized cells (Weissman, 2000; Cowan and Melton, 2006; Sidhu and Ryan *et al.*, 2010). These properties make them important in wound healing and in the processes of regeneration throughout life. Regenerative medicine is an emerging field with the aim of repairing tissue/organs caused by injury, disease or aging by restoring the function of cells, tissues and organs faster and better. The techniques being investigated and applied include manipulation of stem cell behaviour by genetic (gene therapy) or non-genetic means, followed by transplantation of stem cells (cell therapy) and *in vitro*-grown cells/tissues/organs taking the dynamics and microenvironment into account (tissue engineering).

The traditional approach to reduce disability and improve health of patients has been organ transplantation, which started with the successful transplantation of the kidney between identical twins (Guild and Harrison *et al.*, 1955). With the development and usage of immunosuppressant drugs, improved survival rate of patients have been reported (Chkhotua and Klein *et al.*, 2003; Kim and Kwon *et al.*, 2004) and solid organ types have expanded to the heart, liver, pancreas and lung (Hariharan and Johnson *et al.*, 2000; Sayegh and Remuzzi, 2007). However, despite these positive aspects, there is shortage of transplantable organs. According to the official U.S. Government information on organ and tissue donation and transplantation (http://www.organdonor.gov/), by June 2010 more than 108,000 people are waiting for transplantation surgeries and this number continues to rise by approximately 300 people per month due to scarcity of available donors.

Regenerative medicine is a broad phrase used to describe the field of medicine that covers a diverse range of research including cell therapy, tissue engineering and transplantation. The use of stem cells in regenerative medicine provides unlimited cell sources for clinical application that overcome the shortage of tissue/organs. There are three classes of stem cells according to their origin: embryonic, germinal and adult stem cells (also known as somatic stem cells). Depending on their capacity to differentiate into other cell types, stem cells may be classified into totipotential, pluripotential and multipotential stem cells (Rao and Mattson, 2001). Despite the fact that more and more research are showing evidence for the potential use of stem cells in regenerative medicine (Bajada and Mazakova *et al.*, 2008), it is not clear which type of stem cell provides the best approach for cell therapy.

2.1 Adult stem cells in regenerative medicine

There are different types of adult stem cells, some of these include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), adipose tissue-derived stem cells (ADSCs), cardiac stem cells (CSCs), neural stem cells (NSCs), pancreatic stem cells (PSCs), hepatic oval cells (HOCs) and bronchioalveolar stem cells (BASCs). Adult stem cells are located within a stem cell niche and upon injury, proliferate to maintain the stable stem cell number and generate differentiated cells to replace damaged tissue cells. Adult stem cells have had applications in the treatment of many diseases including nervous system disorders, heart disorders, diabetes, muscular disorders, vascular disorders and interstitial lung diseases (Mimeault and Batra, 2008). An easy and straightforward approach for applying stem cells in regenerative medicine is to transplant/inject adult stem cells or differentiated cells into the targeted diseased or injured site, whereas the approach of tissue engineering is designed to

generate solid tissue or organ by using bioreactors, scaffolds and other biomaterials *in vitro* before transplantation.

Bone marrow (BM) has long been known to contain at least three types of stem cells: HSCs, MSCs and endothelial progenitor cells (EPCs) (Alison and Islam, 2009). HSCs are able to reconstitute the hematopoietic system by differentiation into all types of blood cells and MSCs are able to differentiate into skeletal tissues (Bonnet, 2003). Currently, BM or BM-derived stem cell transplantation is the best-known and well-established stem cell-based therapy in regenerative medicine. When HSCs are transplanted for either allogenic or autologous therapy, function of bone marrow that was damaged can be re-established through engraftment and continuous generation of a new hematopoietic system (Armitage, 1994; Shizuru and Negrin *et al.*, 2005). HSC transplantation method has been established and has become a standard therapy for the treatment of many hematopoietic diseases including Hodgkin's and non-Hodgkin's lymphoma, acute and chronic myelogenousleukemia and myelodysplastic syndromes (Armitage, 1994; Copelan, 2006).

A concept that has brought much attention recently in the field of adult stem cell research is plasticity, which describes the phenomenon that restrictions in cell fates are flexible and the capacity of stem cells to differentiate into various cell types, including those not of their lineage of origin (Raff, 2003). Being the most extensively studied adult stem cells, HSCs have received much more attention as a result of recent transplantation studies, showing their plasticity to give rise to many non-hematopoietic cells both *in vivo* and *in vitro* (Quesenberry and Dooner *et al.*, 2010). This will be discussed in further details in Section 3 of this chapter.

2.1.1 Skeletal muscle

Being the largest tissue in the body, skeletal muscle contains a population of stem cells known as satellite cells (SCs). SCs are normally quiescent but can be activated to proliferation upon injury or disruption of the basal lamina resulting in the generation of multinucleated myofibers for muscle regeneration (Shi and Garry, 2006). Many regulatory factors, including SC niche, stimulatory and inhibitory growth factors are involved in muscle regeneration. Allogenic SCs have been transplanted for therapeutic purposes. However, limited migration ability and poor survival of the injected cells have hampered their application in regenerative medicine (Broek and Grefte *et al.*, 2010). Transgenic strategy to promote proliferation of SCs and derivation of other precursor cells involved in skeletal muscle regeneration have been investigated, showing great potential for the treatment of skeletal muscle injury and diseases.

2.1.2 Heart

The heart of human adults has been shown to contain resident adult stem cells with differentiation and regeneration capacity (Leri and Kajstura *et al.*, 2005; Lyngbæk and Schneider *et al.*, 2007). As precursors of cardiac muscle, transplantation of autologous myoblast cells and other cell types, for example, cardiomyocytes and BM-derived cells have also been suggested to be potential therapy for the treatment of myocardial dysfunction (Rubart and Field, 2006).

Apart from cell therapy, scientists have been attempted to create three dimensional scaffolds and engineering heart tissue. A recent improvement in this aspect was the establishment of a bio-artificial heart with a perfusion-decellularized matrix obtained from real heart (Ott and Matthiesen *et al.*, 2008).

2.1.3 Liver, kidney and pancreas

Both whole liver and hepatocytes have been transplanted to treat various liver diseases. However, due to limited number of available liver and hepatocytes, stem cells (especially BM-derived stem cells) have been considered a valuable source for obtaining sufficient number of cells for transplantation. Yet, the existence or absence of a stem cell population in adult liver is still not clear. Studies on liver development suggested the presence of progenitors in fetal liver (Schmelzer and Zhang *et al.*, 2007) and another side population (SP) cells have also been found to contribute to hematopoietic and epithelial lineages (Terrace and Hay *et al.*, 2009), representing the existence of a second potential hepatic progenitor cells.

The adult kidney consists at least 26 types of different cells with low proliferative potential. Thus, kidney is considered a highly terminally differentiated organ. However, the kidney has regenerative and repair potential upon injury. Much effort has been made in looking for renal stem/progenitor cells residing in the adult kidney. Renal epithelial stem cells have been reported to exist and SP cells have been isolated from adult kidneys (Iwatani and Ito *et al.*, 2004; Benigni and Morigi *et al.*, 2010). In addition, there has been controversy as to whether the adult pancreas contains stem cells. In normal adult pancreas, β-cells are known to renew at a low rate, but the mechanism of this cell renewal is not clear and has been suggested to be either replication of differentiated β-cells or neogenesis of pancreatic progenitor cells. The presence of pancreatic stem/progenitor cells has been proposed in the adult pancreatic exocrine, pancreatic duct and islets (Efrat, 2008). However, further characterization of these cells is essential and to date, whether there is a true renal and pancreatic stem cell population still remains elusive.

In addition, BM-derived stem cells have been reported to transdifferentiate to repair kidney and to generate insulin producing cells (Efrat, 2008; Iwatani and Imai, 2010).

2.1.4 Cartilage and Bone

When damaged, bone repair occurs within a compressed time frame and in a precise location, whereas damaged cartilage has shown apparent lack of self-renewal. Current research on cell-based skeletal tissue regeneration focuses on identification of an ideal cell type for tissue repair. Bone has been found to contain stem cells as well as osteoprogenitor cells that produce bone and cartilage (Nuttall and Patton *et al.*, 1998; Gronthos and Zannettino *et al.*, 1999). At the same time, BM-derived MSCs have been widely investigated for their potential to differentiate into bone and cartilage tissue (Arinzeh, 2005).

2.1.5 Nervous system

It has been suggested that diseases of the nervous system including spinal cord injury and degenerative diseases can be treated or at least improved by replacing dysfunctional cells with new ones derived from NSCs. The existence of NSCs in the central nervous system (CNS) was supported by the fact that the CNS can regenerate neuronal axons, replenish lost neural cells as well as recovery of neuronal functions. More specifically, NSCs reside in the subventricular zone of the forebrain and the dentate gyrus of the hippocampus, where they consistently generate new neural cells (McKay, 1997; Temple, 2001). To repair the nervous system, scientists have applied two main methods：transplantation of stem cells and allow them to differentiate into neurons/glial cells *in vivo* or induce differentiation *in vitro* before transplantation; alternatively, growth factors that are involved in brain development have been used to stimulate stem cells within the brain to repair damage (Okano, 2006). NSCs

have been considered an important source of neurons for transplantation in treating Parkinson's disease. Other adult stem cells, including BM-derived stem cells have also been shown to differentiate towards neuronal lineage and possess the potential to be used for the relief from Parkinson's symptoms (Morizane and Li *et al.*, 2008).
The major advantage of using adult stem cells is that these cells can be autologous (isolated from the patients themselves), lessening the possibility of rejection by the immune system. However, it is difficult to isolate pure population of adult stem cells due to the extremely low numbers existing in the adult tissue of interest (approximately 1 in 10,000 cells) (Marshak and Gardner *et al.*, 2001). Another problem that hinders research on adult stem cells has been maintaining and expanding long-term cultures of these cells *in vitro*.

2.2 Pluripotent stem cells in regenerative medicine

Embryonic stem cells (ESCs) derived from spare embryos can be cultured in large numbers on feeder layers or extracellular matrices in defined medium (Yu and Thomson, 2008). These cells are pluripotent in that they are able to form all three germ layers when directly and spontaneously differentiated. A number of cell types, including neural progenitors, dopaminergic (DA) neurons, insulin-producing cells, cardiomyocytes, endothelial cells, hematopoietic cells and many others, have been generated from ESCs (Choumerianou and Dimitriou *et al.*, 2008; Sidhu, 2008). Yet, destruction of embryos to isolate human embryonic stem cells (hESCs) is considered a major ethical concern regarding the use of these cells. Another limitation is the allogenic resource of these cells, which may result in immune rejection when transplantation is carried out. New technologies such as somatic cell nuclear transfer (SCNT) and somatic cell reprogramming (cell fusion and induced pluripotent stem cell, iPCS generation) offer opportunities to overcome transplantation issues (Figure 1). However, SCNT technique raises other ethical issues regarding the use of human eggs while the latter is being investigated extensively. The past couple of years have witnessed the progress of reprogramming and re-differentiation of these reprogrammed cells, although research on reprogramming is still in its early stages and much more work needs to be done before somatic cell reprogramming can be used in clinical therapy.
One of the most exciting report in reprogramming was the generation of iPSCs from terminally differentiated somatic cells by transduction of four transcription factors (*OCT4*, *SOX2*, *KLF4* and *c-MYC*) into fibroblasts (Takahashi and Yamanaka, 2006; Takahashi and Tanabe *et al.*, 2007). Later, it was shown that the four factors can be substituted with different combinations or with small molecules and a variety of somatic cells were successfully reprogrammed (Amabile and Meissner, 2009; Cox and Rizzino, 2010). The iPSCs closely resemble ESCs and are pluripotent (Robbins and Prasain *et al.*, 2010). The approach of generating patient-specific and disease-specific iPSCs lines has already been achieved (Dimos and Rodolfa *et al.*, 2008; Park and Arora *et al.*, 2008; Ebert and Yu *et al.*, 2009; Lee and Papapetrou *et al.*, 2009). The use of these cells could pave the way for regenerative medicine without immune response that limits allogenic cell therapy or the ethics with human embryos destruction when isolating hESCs.
However, one of the most important questions in iPSC generation is to identify the starting cells amongst somatic tissue that give rise to iPSCs, and this remains elusive. In addition, although new techniques using non-integrating viruses, transient plasmid transfection or small molecules have been developed to avoid changes to the somatic cell genome, the results need to be further confirmed. Most recently, research revealed a gene expression signature in

iPSCs that is different from ESCs, suggesting that iPSCs are not identical to ESCs; rather, they could be a unique subtype of pluripotent cell (Chin and Mason *et al.*, 2009).

Another strategy being explored to induce reprogramming of differentiated cells is through fusion with pluripotent cells (Figure 1). Fusion of human fibroblasts with hESCs generates tetraploid hybrid cells with gene expression patterns, epigenetic status and differentiation ability characteristic of hESCs (Cowan and Atienza *et al.*, 2005). A reprogramming study based on hESC claimed re-expression of enhanced green fluorescent protein (EGFP) in OCT4-EGFP knock-in ESC-derived myeloid precursor cells after cell fusion (Yu and Vodyanik *et al.*, 2006). Most recently, it was reported that iPSCs generated from fibroblasts also possess the ability to reprogram other somatic cells after fusion (Sumer and Jones *et al.*, 2010). In the following sections of this chapter, we will discuss fusion between different cell types and prospect future challenges and application of fusion-based reprogramming in regenerative medicine.

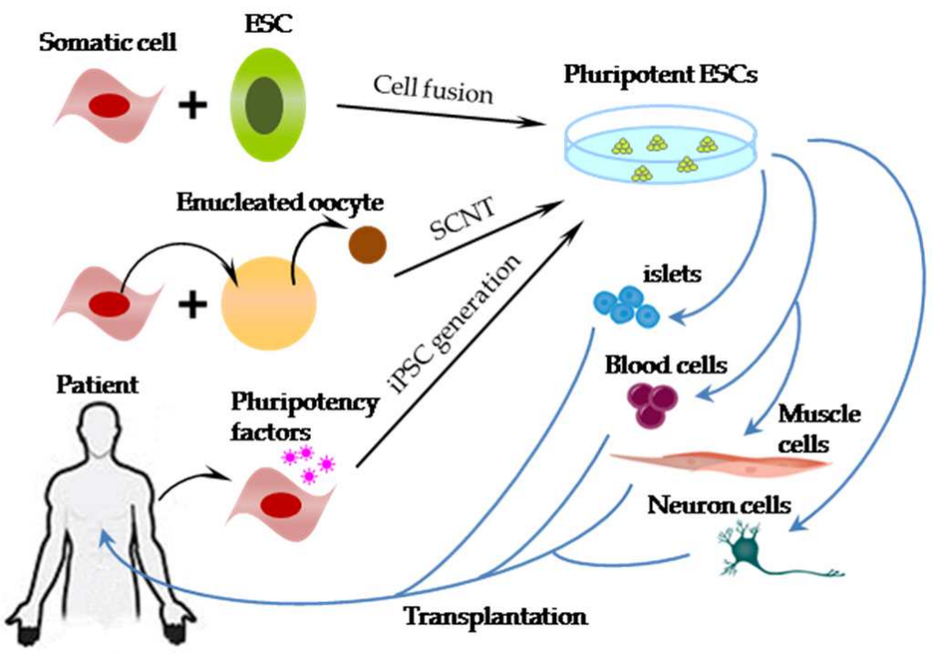

Fig. 1. Approaches to reprogram somatic cells and differentiation of the resulting pluripotent cells for regenerative medicine.

3. Cell-cell fusion

3.1 Cell fusion during development *in vivo*

Cell-cell fusion is a process that occurs in a range of normal development and infection conditions *in vivo*. The beginning of a new life and the first step of development is the event of fusion between the sperm and the egg, known as fertilization. While sperm-egg fusion has been a subject of intense investigation, the mechanism and factors that are essential for cell fusion are still not clear. Knockout studies on mouse models have shown that CD9 on the egg plasma membrane is essential for fusion (Boucheix, 2000; Kaji and Oda *et al.*, 2000; Miyado and Yamada *et al.*, 2000). Later, a sperm-specific glycoprotein Izumo, which is only detectable after the acrosome reaction, was found to be required for sperm-egg fusion

(Inoue and Ikawa *et al.*, 2005). Later, during normal development, the formation of syncytia, where multiple distinct nuclei exist, was observed in placenta, skeletal muscle and bone formation.

Trophoblast cells of mammals are known to fuse with neighbouring cells to form the syncytiotrophoblast. These syncytiotrophoblasts are maintained by further fusion with cytotrophoblasts and they serve as a good candidate for transportation of nutrients and hormones across the maternal and fetal blood vessels (Huppertz and Frank *et al.*, 1998; Pötgens and Schmitz *et al.*, 2002).

Skeletal muscle is composed of multinucleate muscle fibres, which are the products of multistep fusion process during development. Fusion starts with mononucleatemyoblasts fusing with each other to form nascent myotubes containing a small number of nuclei. This is then followed by additional fusion between the multinucleate myotubes and myoblasts, leading to the formation of large and mature myotubes (Horsley and Pavlath, 2004). At a later stage of development, SCs can fuse with muscle fibres for regeneration and maintenance of the skeletal muscle. The mechanism and regulation of myoblast fusion is reviewed in detail recently (Pavlath 2010).

Another example of cell fusion that occurs *in vivo* in mammals is macrophage fusion, which results in the formation of multinucleate osteoclasts and giant cells (Vignery, 2000). During osteoclastogenesis, macrophages fuse with each other to form multinucleate osteoclasts in the bone, or giant cells in chronic inflammatory sites. As a result, macrophages increase in size and consequently endow them enhanced capacity of resorption, which is illustrated by the difference in gene expression patterns of mono-/multi-nucleated macrophages (Teitelbaum and Ross, 2003). This resoption capacity has been shown to be important for bone remodelling and failure in fusion induces thick and brittle bone formation in osteopetrosis.

3.2 Cell-cell fusion in tissue regeneration

It has been reported that different types of cells can fuse spontaneously when co-cultured *in vitro* (Weiss and Green, 1967; Ying and Nichols *et al.*, 2002). More recently, cell fusion has also been suggested to be involved in transdifferentiation and tissue regeneration. Transdifferentiation is a concept describing the process of conversion of cells from a specific lineage to that not of their lineage of origin and has been demonstrated by the plasticity of adult stem cells. BM-derived HSCs and MSCs are the most extensively studied adult stem cells, which have also been considered to possess the most plasticity and can give rise to non-hematopoietic cells. After BM transplantation, cells carrying markers indicating their origin can be found in different tissues, including the liver, lung, pancreas and muscle (Quesenberry and Dooner *et al.*, 2010). Some scientists proposed that this is the consequence of fusion between transplanted cells and local somatic/precursor cells, rather than real transdifferentiation.

An early transplantation study aimed to treat a muscle defective disease, Duchenne muscular dystrophy (DMD), suggested that fusion occurred between implanted cells and host myoblasts/muscle fibres. Later, transplantation of BM-derived progenitors suggested possible fusion between the progenitors and myofibers and their contribution in muscle regeneration (Gibson and Karasinski *et al.*, 1995; Ferrari and Cusella-De Angelis *et al.*, 1998). In humans, it was shown that exogenous BM cells fused with myofibres *in vitro* after BM transplantation contributed to tissue repair (Gussoni and Bennett *et al.*, 2002). Additional

data also showed that repair of muscle injury was not the result of HSC transdifferentiation, but was the consequence of fusion between transplanted HSCs and muscle fibres, further confirming the importance of cell fusion during tissue repair at the site of damage (Camargo and Green *et al.*, 2003; Corbel and Lee *et al.*, 2003).

Results from two groups using a hepatic lethal murine models showed regain of normal liver function after BM transplantation. The newly generated hepatocytes express genes of both donor and host, indicating fusion of donor and host cells. Southern blot and karyotyping analysis further confirmed that the hepatocytes derived, arise from BM and somatic cell fusion and not by transdifferentiation of HSCs (Vassilopoulos and Wang *et al.*, 2003; Wang and Willenbring *et al.*, 2003). Together with the studies of muscle repair, it is accepted that in a damaged tissue model, BM can fuse with somatic cells at the site of injury and regenerate organ function.

It was then indicated that this transdifferentiation induced by cell fusion is injury-independent and could occur under normal conditions. Analysis of female brains that had male BM transplantation, revealed the existence of tetraploid Purkinje neurons (XXXY), indicating fusion between HSCs and existing Purkinje neurons. Similarly, after transplantation of green fluorescent protein (GFP)-positive BM into lethally irradiated wild-type mice, GFP-positive Purkinje-BM cell heterokaryons containing two nuclei were observed, suggesting fusion had occurred (Weimann and Charlton *et al.*, 2003; Weimann and Johansson *et al.*, 2003). BM-derived MSCs have also been transplanted into Niemann-Pick mice. The transplanted MSCs were shown to fuse with Purkinje neurons and develop into functional neurons to promote brain function (Bae and Furuya *et al.*, 2005).

BM cell fusion was also investigated using transgenic mouse models carrying either Cre-recombinase or LacZ reporter gene, which is under the control of LoxP-flanked stop cassette mediated by Cre recombination. Spontaneous fusion was observed in the brain, liver and heart of irradiated healthy mice (Alvarez-Dolado and Pardal *et al.*, 2003). More recently, it was suggested that fusion between BM cells and Purkinje cells and the formation of heterokaryon occurs at a much higher efficiency upon tissue injury than under normal conditions (Johansson and Youssef *et al.*, 2008). Then another group sought to address fusion under strictly physiological conditions using the Cre-LoxP system. They claimed absence of binucleated cell generation after BM transplantation and favour the idea that fusion between HSCs and Purkinje neurons is only a transient event and under non-invasive conditions, no stable heterokaryons are formed (Nern and Wolff *et al.*, 2009).

Other studies on BM transplantation suggested that cell fusion could also be the mechanism of tissue repair for heart (Nygren and Jovinge *et al.*, 2004), kidney (Fang and Alison *et al.*, 2005) and intestinal epithelium (Rizvi and Swain *et al.*, 2006). However, there are also studies reporting HSC plasticity with no cell fusion observations (Newsome and Johannessen *et al.*, 2003; Bailey and Jiang *et al.*, 2004; Harris and Herzog *et al.*, 2004).

While some may ask whether stem cells regenerate tissue by fusing with other cells or by transdifferentiation, it will be fair to say that cell fusion and transdifferentiation do not exclude each other. In transplantation and transdifferentiation studies mentioned above, each group used a different cell population, including whole BM cells, HSCs and MSCs isolated based on expression of different cell markers. The diversity thus makes it hard to compare the results and conclude. Transdifferentiation of BM cells have been shown *in vitro*; however, cell fusion does occur during tissue repair as well as in normal tissues although at a low frequency. It is most possible that the mechanism of plasticity involves both fusion and transdifferentiation. For one thing, upon fusion of BM cells with another cell type, the

latter may induce differentiation of BM cells; for another, fusion may result in transient hybrid cells with plasticity, which can differentiate into different cell types. Further research will be needed to provide more information on stem cell plasticity and tissue regeneration.

3.3 Fusion-induced reprogramming

3.3.1 Fusion between pluripotent and somatic cells

It has long been known that under co-culturing system, different cell types can fuse together, although at rather low rates. Somatic cells were shown to fuse spontaneously with ESCs *in vitro* and were reprogrammed (Pells and Di Domenico *et al.*, 2002).

The pioneer study was designed to fuse pluripotent embryonic carcinoma cells (ECCs) with primary thymocytes with the resultant hybrid cells showing similarities to the parent ECCs in their multipotent differentiation abilities (Miller and Ruddle, 1976). This indicated that pluripotency was not lost by the introduction of a somatic cell; on the contrary, pluripotency prevailed the cell fate and overwhelmed the differentiation pathways.

More recent research has shown that pluripotency can be re-established in somatic cells by fusion with ECCs, embryonic germ cells (EGCs) and ESCs, suggesting that these pluripotent cells possess the same or similar components that mediates reprogramming (Han and Sidhu, 2008). Compared to ECCs and EGCs, ESCs may represent a better source for this fusion-based reprogramming and have been paid much attention. The programming abilities of mouse ESCs (mESCs) were first illustrated by showing that mouse spleen cells obtained pluripotency after fusion with ESCs (Matveeva and Shilov *et al.*, 1998). After fusion with mESCs, the Oct4 gene was reactivated 48 hours later in mouse lymphocytes, which suggest pluripotent properties of the hybrid cells. This was also testified by the hybrids' contribution to all three germ layers as well as the epigenetic status as assessed by DNA methylation pattern of imprinted and non-imprinted genes (Tada and Takahama *et al.*, 2001). Similar to mESCs, hESCs can also reprogram differentiated somatic cells to an embryonic state by fusion (Figure 2). This was first achieved by Eggan's group in which hESCs were fused with human fibroblasts, resulting in tetraploid hybrid cells with gene expression patterns, epigenetic status and differentiation ability characteristic of hESCs (Cowan and Atienza *et al.*, 2005). Another fusion-induced reprogramming based on hESCs claimed re-expression of EGFP in OCT4-EGFP knock-in ESC-derived myeloid precursor cells after fusion and ESC-specific marker gene expression in the hybrid cells. Furthermore, pluripotency of the hybrids were demonstrated by the formation of three germ layers and extra-embryonic tissues (Yu and Vodyanik *et al.*, 2006). These results provide an alternative pathway to SCNT and iPSCs generation for reprogramming somatic cells and especially for studying the mechanisms of nuclear reprogramming.

Fusion between same or similar cells results in synkaryons or homokaryons (Figure 2). The former are single nucleated cells in which chromosome loss or re-sorting has occurred to homokaryons, an intermediate cell type in the process of synkaryon formation. Similarly, the cells formed by the fusion of different types of cells are either synkaryons or heterokaryons. Heterkaryons contain multiple nuclei and each nucleus remains separate and stable over time. Heterokaryon formation has been used as a tool to overcome chromosome loss and rearrangement after fusion and as a model to study the role of cytoplasmic factors on gene expressions. When nuclear fusion occurs, the fused nucleus initially contains chromosomal content of both fusion partners, but then chromosome loss or re-sorting leads to the formation of snykaryons.

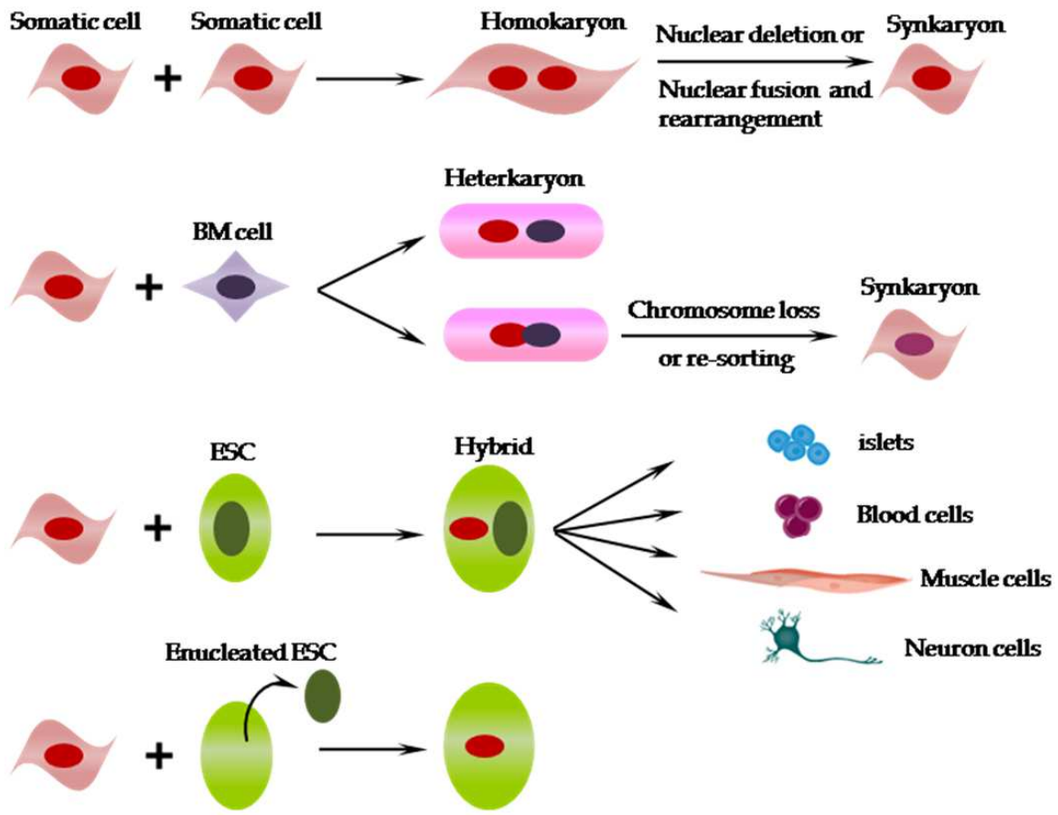

Fig. 2. Fusion between same or different types of cells and the resulting cell types.

3.3.2 Comparison of different fusion methods

Reactivation of previously silenced genes in the somatic cells after fusion with another cell type was first reported in heterokaryons of muscle and amniotic cells, indicating plasticity of differentiated cells can be changed by fusion (Blau and Chiu *et al.*, 1983). Subsequent studies fusing different cell types demonstrated that silenced genes in differentiated cell types can be reactivated after fusion and the differentiated state is not fixed. Instead, the differentiated state of cells can be reset and regulated in the heterokaryons (Yamanaka and Blau, 2010). In the case of somatic-stem cell fusion, reprogramming of somatic cells have been reported as mentioned in this chapter and shown in Figure 2.

Three approaches have been developed and applied for fusion-induced reprogramming: electro-fusion, polyethylene glycol (PEG)-induced fusion and Sendai virus-induced fusion. Electro-fusion, which involves delivery of an electric pulse to the fusing cells, was developed in the 1980s. It allows the two types of cells in a low conductivity medium suspension, be brought into close contact by dielectrophoresis, which uses high frequency alternating current and aligns the cells in a chain. Then, the high voltage electrical field pulses applied brings about an abrupt change in the ionic conductivity and membrane permeability, which results in temporary reversible membrane breakdown, pore formation on the membranes and finally combining of the membranes. The cells are held in place transiently by an alternating voltage to allow fused membranes to mature and components of the cell cytoplasm to mix. A number of studies reported high fusion rate and reproducibility when using electro-fusion (Radomska and Eckhardt, 1995). Electro-fusion

has been applied to induce fusion between mouse EGCs/ESCs and somatic cells for reprogramming (Tada and Tada *et al.*, 1997; Tada and Takahama *et al.*, 2001).

PEG is a chemical that has long been used to induce cell-cell fusion (Ahkong and Howell *et al.*, 1975). Several mechanisms were suggested on how PEG mediates cell fusion. Being a linear polymer of ethylene oxides and hydroxyl terminals, PEG has the ability of binding to water molecules and form compounds. It is now widely accepted that PEG aggregates cell membranes as well as dehydrating in the areas of contact (MacDonald, 1985). At the same time, PEG also promotes fusion via positive osmotic pressure that likely helps stabilize fusion intermediates (Lentz, 2007). Fusion experiments mediated by PEG between both mouse and human ESCs have demonstrated that 50% PEG can be applied to reprogram somatic cells although at a rather low efficiency (Cowan and Atienza *et al.*, 2005; Do and Schöler, 2005).

Sendai virus (Hemagglutinating Virus of Japan, HVJ) is a type of enveloped virus that can bind to cell surface receptors on the target membrane and fuse with cell plasma membrane at neutral pH with the mediation of viral envelope glycoproteins (Robert Blumenthal, 1991). Cell fusion induced by HVJ was first reported to induce fusion among tumor cells, resulting in the formation of giant polynuclear structures (Okada, 1962). Then, cell fusion between mouse and human was induced by virus in 1965 (Harris and Watkins *et al.*, 1965). The HVJ-Envelope (HVJ-E) is inactivated and purified HVJ, with only the cell membrane-fusing capability retained. When HVJ-E are added to the cells at a concentration of several hundred per cell, they are absorbed on the cell surface immediately by the receptor acetyl type sialic acid (at the terminal of sugar chains), which is recognized by haemagglutinin–neuraminidase (HN) protein at 4°C. This leads to distortion of the cell membranes due to the penetration of an F protein of the envelope into the lipid layer of the cell membrane, which allows an inflow of ions. At this stage, the cell/HVJ-E complex is brought to a 37°C environment to induce temporary alteration of the cell membrane structure and cell fusion (Hoekstra and Klappe *et al.*, 1985; Henis and Gutman, 1987; Loyter and Chejanovsky *et al.*, 1989; Düzgüneş and Shavnin, 1992). Because the genomic RNA of the Sendai virus has been inactivated, there is no potential infective or proliferative harm to the cells.

However, the fusion efficiency of these methods in reprogramming has not been compared. It is in fact hard to compare the results from the above mentioned studies directly. First of all, in the reports of different groups, different somatic cell types and different ESC lines were used, which may affect fusion efficiency. Secondly, even for the same fusion approach, conditions applied by each group differ slightly.

We modified protocols of electro-fusion, virus induced fusion and PEG-induced fusion to suit two hESC lines and a human fetal fibroblast (HFF) cell line that have not been used for fusion-based reprogramming with the aim of determining their effectiveness of reprogramming human somatic cells. The results indicated that by optimizing fusion methods, hybrid cells between hESCs and HFFs could be generated at different efficiencies. These hybrid cells formed stem cell-like colonies and expressed stem cell specific markers.

To identify the two types of cells, an auto-fluorescent hESC line Envy and CMFDA- or SNARF-labelled cells (MEL1 and HFF10T, respectively) were used. An additional drug selection process was applied for hybrid selection, which enabled screening of stem cells that fused with puromycin-resistant HFFs (HFF10T). Envy cells contain a GFP sequence and the constitutive expression of GFP makes them easily visible and distinguishable from feeder HFFs (Costa and Dottori *et al.*, 2005). MEL1 hESCs were labelled with CMFDA to express a similar level of fluorescence as Envy under the microscope and flow cytometry

conditions, whereas HFF10T cells were labelled fluorescent red with SNARF. As shown in Figure 3A, cells that were dual fluorescent represent the fused hybrid cells. After induced fusion of hESCs and puromycin-resistant HFFs, cells were cultured in normal hESC growth medium with puromycin supplemented from day 2 to 14 days post-fusion to screen for hybrid cells. Morphological changes during this period were assessed at different time points and shown in Figure 3B (day 5 and passage 2 day 7). Puromycin is toxic to wild type hESCs and the cell death was observed within 24 h. Only hESCs that had fused with

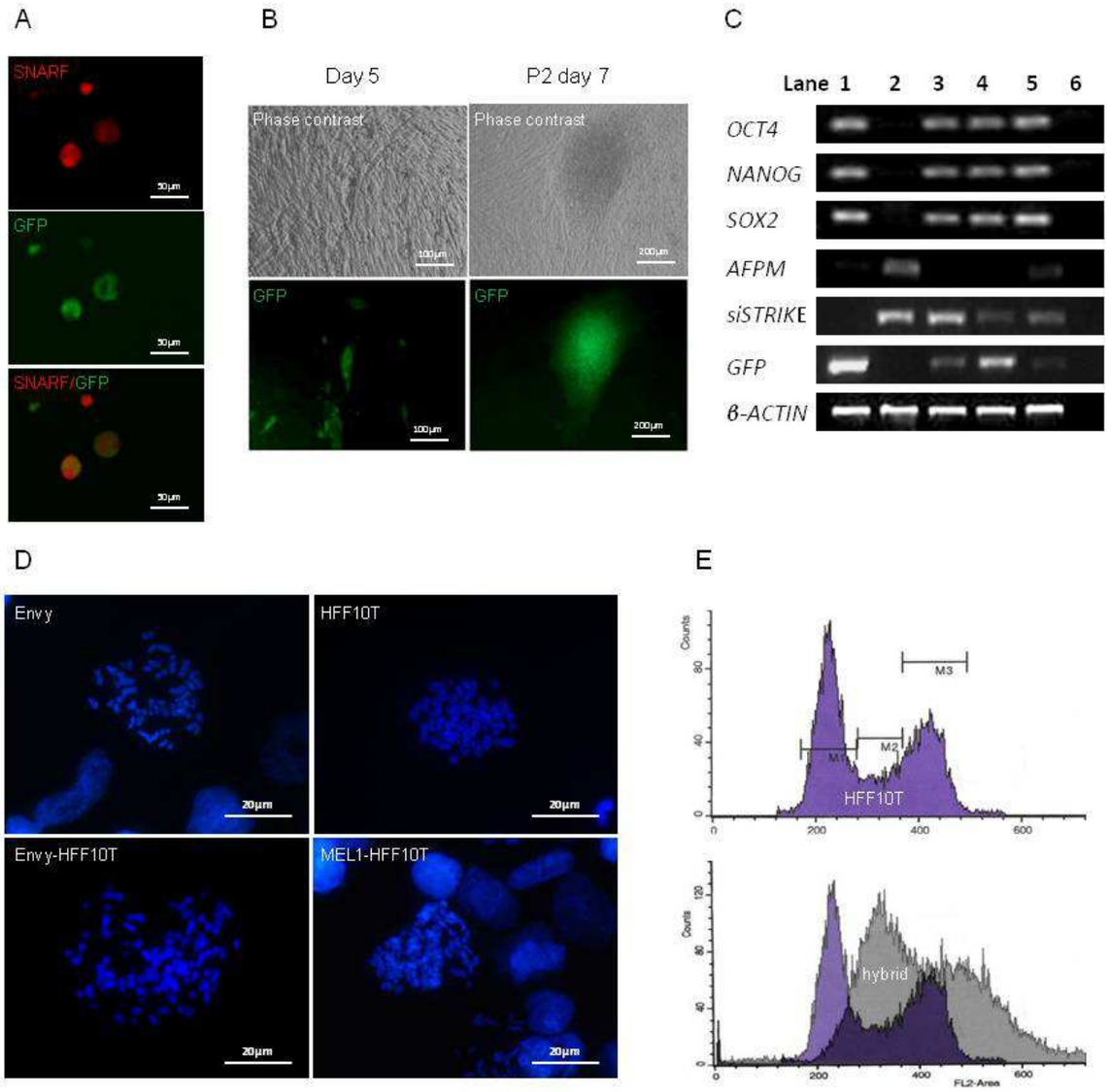

Fig. 3. hESC-HFF fusion. (A) A typical example of hybrid generation induced by cell fusion. (B) Formation of hybrid colony after fusion between Envy and HFF10T induced by PEG. (C) RT-PCR analysis of gene expression. Lanes 1 to 6 were loaded with samples (1) Envy, (2) HFF10T, (3) hybrid P1, (4) hybrid P2, (5) hybrid P3 and (6) water. (D) Chromosome numbers in hESCs, HFFs and hybrid cells. (D) Analysis of DNA content in HFF10T cells and hybrids (Han, unpublished data).

HFF10T cells that bear the puromycin-resistant plasmid sequence can survive the antibiotic selection system. Stem cell-like colonies started to form on day 4 and after 2 weeks, the colonies can be manually dissected for sub-culture and they illustrated the compact, phase-bright cell cluster feature of hESCs (Figure 3B).

RT-PCR (Figure 3C) shows that through the culturing process, pluripotency markers such as *OCT4*, *NANOG* and *SOX2* were expressed in the putative hybrids at a comparable level to that in the hESC control, indicating their ESC properties. In contrast, AFPM, a somatic cell marker, was strongly expressed in HFF10T cells but not in hESCs. In the hybrid cells at P1 and P2, this AFPM expression was removed, most probably due to reprogramming. However, a weak expression of AFPM was observed from P3 hybrid cells, suggesting re-differentiation. Primers that specifically amplify GFP sequence that was inserted into Envy cells' DNA and the puromycin-resistant sequence of the plasmid (siSTRIK) that was transferred into HFF10T cells were used to examine the origin of the hybrid cells.

DNA content analysis and chromosome number counting reflects the ploidity of the cells. As shown in Figure 3D and 3E, a normal human diploid cell has 46 chromosomes (44 autosomes and 2 sex chromosomes), whereas in a typical hybrid cell generated from PEG induced fusion between Envy/MEL1 and HFF10T cell, 92 chromosomes were contained within a single nucleus. The DNA content of hybrids showed an obvious loss of S phase cells and a trend of increased DNA content.

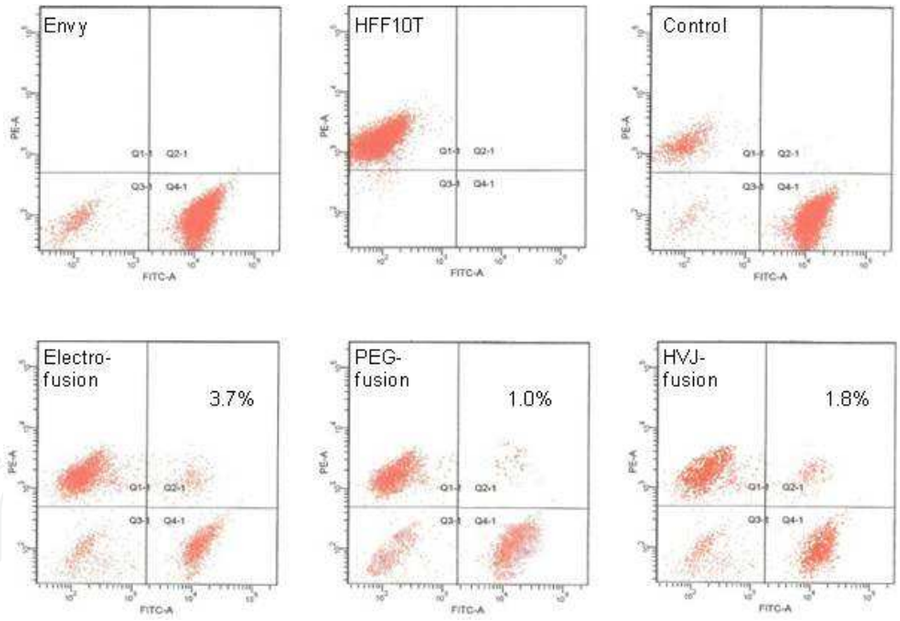

Fig. 4. Quantification of fusion efficiency by flow cytometry between Envy and HFF10T cells induced by different methods (Han, unpublished data).

When flow cytometer was applied, as shown in Figure 4, the GFP positive Envy and the SNARF-labelled HFF populations are separate and distinct. The fusion products appeared as a dual fluorescing population with magnitudes that are approximately equal to those of the stained fusion partners. The fusion efficiencies of the three methods (electro-fusion, virus-induced fusion and PEG-induced fusion) were compared using two different hESC lines, Envy and MEL1. There were no considerable differences in fusion results between the

two cell lines (data not shown). However, when fusion strategies are considered, it was found that in electro-fusion, voltage applied for generating pores on the membranes of cells caused cell lysis and resulted in less survival of hybrid cells or generation of stem cell-like colonies. Furthermore, PEG 1500 was able to generate the highest number of colonies with typical stem cell characteristics despite its cytotoxicity and low percentage of double-stained hybrid cells (Figure 4). Sendai virus fusion was shown to have a moderate fusion ability as determined by hybrid generation efficiency and number of colony formation. In the study, it was concluded that fusion using PEG was still superior compared to other fusion methods used. Our results showed a fusion rate of 1.0 ± 1.2% and 1.2 ± 1.0 % for two different hESC lines (Envy and MEL1 respectively). When colony number was counted, only 0.0003 ± 0.0001% of the cell population was observed to form stem cell-like colonies. This was not improved by optimization attempts which involved changing the PEG concentration or modifying the incubation time. Nor did the density of the cells in the PEG solution have any effect on the fusion rate. This could be because PEG mediated fusion is affected by many factors that are hard to normalize, such as the size and shape of the cell pellet and the speed and vigor with which PEG is stirred into the resuspended cell pellet (Radomska and Eckhardt, 1995).

4. Prospects of cell fusion in future regenerative medicine

After injury or under pathological conditions, stem cells can proliferate and differentiate in order to regenerate and repair damaged tissues. During regeneration process, adult stem cells normally differentiate to cells that reside in the damaged site. As discussed earlier, in the organs of muscle, brain and liver, cell fusion has been demonstrated during tissue regeneration, despite of controversial reports. Stem cells from the BM origin have been of particular interest because they have shown to migrate to alternative locations and contribute to functional cells. Transdifferentiation was postulated as the mechanism of generation of the differentiated cell types after HSC and MSC transplantation. However, the finding that HSCs and MSCs can fuse with other cell types *in vivo* indicated that cell fusion could be the mechanism to explain the low number of newly generated functional cells in tissue repair after injury. Currently, it is still not clear whether the improvement of organ function after damage was a result of fusion-mediated recovery or production of growth factors and cytokines of the residue stem cells. Furthermore, it is possible that fusion occurs in more organs than we know and at a higher frequency than we expected. In addition, the function of cell fusion may be more important than what we have known in the process of organ or tissue repair.

In vitro cell fusion studies have shown that fusion between different cell types can change cell fate and gene expression profiles. Fusion reverses the differentiated state of mature cells towards a more immature or even embryonic-like state (Tada and Takahama *et al.*, 2001; Cowan and Atienza *et al.*, 2005).

Despite successful reprogramming, the strategy of inducing reprogramming by cell fusion has been problematic in the following aspects. Firstly, although the somatic cell genome is reprogrammed, the existence of ESC or other pluripotent cell genome in the tetraploid hybrids is a technical hurdle for therapeutic application. When transplanted or injected into somatic donors, immune rejection caused by the ESC genome could happen at a high frequency. Secondly, after fusion of two cells, both heterokaryons and synkaryons can be formed. Chromosome loss or spitting a nucleus of a heterokaryon results in the formation of

synkaryons and chromosomal instability and genetic instability have been reported (Vasilkova and Kizilova *et al.*, 2007; Nowak-Imialek and Kues *et al.*, 2010). This needs to be taken into consideration when clinical transplantation is the aim of generating these hybrids. Thirdly, the efficiency of fusion induced reprogramming has been low. Since the first study reporting the success of reprogramming human somatic cells after fusion with hESCs, efforts have been made in order to increase reprogramming efficiency in the system. Over expression of certain transcription factors, such as Nanog and Sall4, as well as manipulation of histonemethylase Jhdm2a and methyltransferease G9a, have been claimed to significantly enhance the ability to generate reprogrammed hybrids from fusion (Silva and Chambers *et al.*, 2006; Ma and Chiang *et al.*, 2008; Wong and Gaspar-Maia *et al.*, 2008). However, these approaches need to be further tested in different cell lines and cell types. Lastly, fusion induced reprogramming was evidenced by morphological changes, gene expressions and epigenetic states, yet only limited functional reprogramming has been demonstrated (Yu and Vodyanik *et al.*, 2006).

To fully explore the mechanisms of fusion induced reprogramming for cell therapy and regenerative medical application, there are important questions yet to be answered. For example, the subcellular location of reprogramming factors has always been a subject of interest. When the nucleus and the cytoplasm of mESCs were fused with neurosphere cells separately, only karyoplasts could reactivate Oct4 in the somatic genome (Do and Scholer, 2004). A conflicting observation was made later by Strelchenko et al., showing that hybrid cells generated by fusion of human somatic cells with enucleated ESCs express pluripotency markers OCT4 and TRA-2-39 (Strelchenko and Kukharenko *et al.*, 2006). Most recently, it was reported that hESC cytoplast fusion can only initiate but unable to complete reprogramming, indicating the importance of the ESC nucleus in fusion-induced reprogramming (Hasegawa and Zhang *et al.*, 2010) (Figure 2). Another issue is the possibility of immune rejection caused by the existence of the allogenic genome in the hybrid cells when they are transplanted into the somatic donor. In this case, removal of the pluripotent genome would be necessary. However, this would be hindered if the major elements responsible for reprogramming within ESCs are in the nucleus. Although a remedial approach has been developed in the mouse by allowing enough time for ESC nucleus to reprogram the somatic genome before removing the ESC nucleus (Pralong and Mrozik *et al.*, 2005), the reprogramming ability of those nuclei-removed ESCs needs to be confirmed and it is not known if this approach could also be applied to hESCs. Targeted elimination of ESC chromosomes from mouse somatic cell-ESC hybrids is introduced as an alternative method to expel the effect of ESC genome (Matsumura and Tada *et al.*, 2007). It was also reported that human B-lymphocytes can be reprogrammed towards a multipotent state in transient heterokaryons before nuclear fusion (Pereira and Terranova *et al.*, 2008). In that case, it may be possible to remove ESC genome after reprogramming has occurred although this needs further investigation. Finally, the lacking of effective approaches to monitor early reprogramming events and being unaware of the molecules that mediate reprogramming in the ESCs, makes it challenging to achieve efficient fusion and reprogramming. However, considering that cell fusion is technically simple and reprogramming occurs fast after fusion, it is widely accepted that it is an ideal way to investigate the regulatory mechanisms of reprogramming. For example, it was demonstrated by heterokaryoyic cell fusion between mESCs and human fibroblasts that activation-induced cytidinedeaminase (AID), a DNA demethylation enzyme, is required for reprogramming initiation (Bhutani and Brady *et al.*, 2010).

5. Conclusion

In vivo cell fusion is a tightly regulated process that occurs in normal development or under pathological conditions. There are indications that induced *in vitro* cell fusion could be potentially applied for regeneration when stem cells are fused with somatic cells. A better understanding of the process will enable us to use cell fusion as an approach for cell therapy. Fusion between ESCs and somatic cells is considered a way of reprogramming somatic cells, with the generation of pluripotent cells that can be further differentiated into desired cell types. Cell-cell fusion may be faster and more efficient than iPSC technique as hESCs provide all the necessary factors for reprogramming. However, enucleation of ESC nucleus must be achieved before the hybrid cells can be used clinically. In addition, cell fusion is a useful tool for studying the molecular mechanism that controls somatic cell reprogramming.

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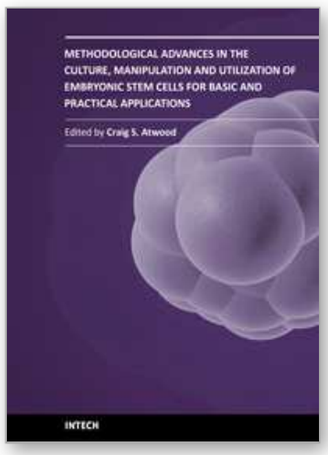

Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes methodological advances in the culture and manipulation of embryonic stem cells that will serve to bring this promise to practice.

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