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Cryopreservation of Human Pluripotent Stem Cells: Are We Going in the Right Direction?

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1. Introduction

The first derivation of human embryonic stem cells (hESCs) (Thomson et al., 1998) and the more recently development of human induced pluripotent stem cells (iPSCs) (Park et al., 2008; Takahashi et al., 2007; Takahashi & Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007) have marked the beginning of a new era in biomedical research. These two types of human pluripotent stem cells (hPSCs) are characterized by an unlimited capacity to self-renew while retaining their potential to differentiate into almost all cell types of the body (Odorico et al., 2001; Reubinoff et al., 2000; Silva & Smith, 2008). These remarkable properties turn hPSCs into one of the most interesting cell types for toxicology and drug discovery, tissue engineering and regenerative medicine (Battey, 2007; Mountford, 2008). In fact, work with hPSCs has already provided new and exciting developments that may eventually lead to the creation of novel cell-based therapies for the treatment of a wide range of human diseases including Parkinson's and other neurodegenerative diseases, diabetes, cardiac and vascular diseases (Kiskinis & Eggan, 2010; Ronaghi et al., 2010). However, a major challenge for the widespread application of hPSCs is the development of efficient protocols for cryopreservation.

To date, two techniques are mainly applied for the cryopreservation of hPSCs: conventional slow freezing and vitrification. The conventional slow-freezing and rapid-thawing procedure using dimethylsulfoxide (DMSO) as a cryoprotectant is the most commonly used method (Grout et al., 1990; Meryman, 2007). While this established technique is effective for somatic cell lines and even murine embryonic stem cells (mESCs), hematopoietic and mesenchymal human stem cells, this is not the case for hPSCs, due to low recovery rates and high levels of differentiation (Berz et al., 2007; Reubinoff et al., 2001; Richards et al., 2004; Thirumala et al., 2010). In contrast, vitrification of hPSCs by the "open pulled straw" method

using high cryoprotectant concentrations together with flash-freezing in liquid nitrogen has reported higher cell survival rates (Li et al., 2010b; Reubinoff et al., 2001; Richards et al., 2004). However, there are several disadvantages preventing the widespread use of this technique. First, high concentrations of cryoprotectors, which are cytotoxic above 4ºC, are needed. Second, these procedures are tedious to perform manually. Additionally, as vitrification is mostly performed in open pulled straws, contact between the liquid nitrogen and the cells is unavoidable, which carries the risk of contamination. Finally, and one of the most limiting disadvantages of this technique is that it is clearly unsuited for freezing bulk quantities of hPSCs.

During the last decade, several groups have been studying different approaches to improve the above described cryopreservation protocols. In the present work we will review the recent advances in the cryopreservation field trying to point out how a better understanding of the sensitivity of hPSCs to the cryopreservation process will help to develop more efficient protocols.

1.1 Human pluripotent stem cells

1.1.1 Human embryonic stem cells

The pioneering work on mESCs, and later advances in culturing techniques that were developed to culture nonhuman primate embryonic stem cell lines eventually led to the first successful generation of hESC lines by Thompson and coworkers and two years later by Reubinoff and coworkers (Evans & Kaufman, 1981; Martin, 1981; Reubinoff et al., 2000; Thomson et al., 1995; Thomson et al., 1996; Thomson et al., 1998). These hESCs were derived from human embryos that were produced by *in vitro* fertilization for clinical purposes. HESC lines were karyotypically normal and maintained the developmental potential to contribute to derivatives of all three germ layers, even after clonal derivation and prolonged undifferentiated proliferation (Amit et al., 2000). Since then, hundreds of stem cell lines have been derived world-wide from morula, later blastocyst stage embryos, fresh and cryopreserved supernumerary embryos, single blastomeres and parthenogenetic embryos (Klimanskaya et al., 2006; Lin et al., 2007; Mai et al., 2007; Revazova et al., 2007; Stojkovic et al., 2004; Strelchenko et al., 2004).

HESCs grow in tightly packed colonies and maintain defined borders at the periphery of colonies. High nucleus to cytoplasm ratio and prominent nucleoli are typical features of individual hESCs within colonies. HESCs are also characterized by high telomerase activity and expression of a number of cell surface markers and transcription factors including stage-specific embryonic antigen (SSEA)-4, SSEA-3, TRA antigens, Oct3/4, Nanog and absence of hESCs negative markers such as SSEA-1 (Carpenter et al., 2003; Chambers et al., 2003; Draper et al., 2004; Heins et al., 2004; Nichols et al., 1998). Functional confirmation of the multipotent nature of hESCs is generally achieved by examining their potential to differentiate into all three germ layers (ectoderm, mesoderm and endoderm) both *in vitro* and *in vivo. In vitro*, hESCs are allowed to randomly differentiate as embryoid bodies (EBs), which are aggregates of cells grown in suspension culture, followed by immunocytochemical analysis, or measurement of expression of genes associated with the three germ layers by RT-PCR (Reubinoff et al., 2000). The *in vivo* test for pluripotency of hESCs is normally teratoma formation in immunocompromised mice (Bosma et al., 1983).

1.1.2 Human induced pluripotent stem cells

An important revolution in the stem cell research field was accomplished when several groups in different studies demonstrated that using a cocktail of four factors, somatic cells could be reprogrammed into iPSCs (Maherali et al., 2007; Okita et al., 2007; Park et al., 2008; Takahashi et al., 2007; Takahashi & Yamanaka, 2006; Wernig et al., 2007; Yu et al., 2007). Consequently, it was shown that such cells could be generated from patient-specific cells for a wide variety of diseases (Kiskinis & Eggan, 2010; Raya et al., 2009; Raya et al., 2010) and from a wide variety of somatic cell types (Sun et al., 2010). These cells are morphologically similar to hESCs, express typical hESC-specific cell surface antigens and genes, differentiate into multiple lineages *in vitro*, and form teratomas containing differentiated derivatives of all three primary germ layers when injected into immunocompromised mice. Indeed, these new pluripotent cell lines satisfy all the original criteria proposed for hESCs (Thomson et al., 1998). Nevertheless, some differences have been observed between hESCs and iPSCs (Chin et al., 2009); but it remains unclear whether the small percentage of genes that are differentially expressed between these two types of hPSCs are shared among different lines and whether these differences are biologically significant.

Developing iPSCs into therapeutic reagents faces a number of practical hurdles, including risks associated with cell processing, the difficulty of ensuring the purity and characteristics of the reprogrammed population and the safety and efficacy of reprogrammed cells *in vivo* (Condic & Rao, 2008; Rao & Condic, 2008; Rao & Condic, 2009). Moreover, a case of rejection has been recently described after iPSCs autologous transplantation (Apostolou & Hochedlinger, 2011). Nonetheless, there is cause for considerable optimism that patient-specific iPSC lines will both enhance the study of human diseases and advance these studies toward clinical applications.

1.2 Cryopreservation of hPSCs

Cryopreservation is the process of cooling and storing cells, tissues or organs at sub-freezing temperatures, below −80°C and typically below −140°C, to maintain viability (Baust et al., 2009). The freezing process involves complex phenomena of water crystallization and changes in solute concentration both outside and inside the cell that can be detrimental to cell survival. In addition, exposure to low temperatures has been reported to induce a stress response resulting in biomolecular-based cell death for different cell types (Baust et al., 2001; Fu et al., 2001; Paasch et al., 2004; Xiao & Dooley, 2003).

In general, the major steps used in cryopreservation of most cell types can be summarized as follows (figure 1): i) harvesting the cells, ii) addition of cryoprotective agents within a carrier media to the cell suspension, iii) ice crystal induction in cell suspension following a determined cooling rate (ranging from -1 to -10^{o}C/min), iv) long-term storage at cryogenic temperatures (normally in liquid nitrogen), v) rapid thawing by warming the cell suspension in a 37-40^{o}C water bath, vi) removal of cryoprotective agent by centrifugation and vii) seeding down the cells to allow culture growth (Gao et al., 1998; Hubel, 1997).

Cryoinjury can be due to one or a combination of the following processes: 1) cytotoxicity of cryoprotective agents (Muldrew & McGann, 1994; Schneider & Maurer, 1983); 2) osmotic injury due to excursion of cryoprotective agents upon freeze-thawing (Gao et al., 1995; Mazur & Schneider, 1986); 3) intracellular ice formation in the cooling process (Fujikawa,

1980; Mazur et al., 1972) and 4) recrystallization of the intracellular ice during the warming process (Mazur & Cole, 1989; Trump et al., 1965). In addition, recent studies have linked numerous stress factors associated with cryopreservation to known initiators of molecular-based apoptotic cell death processes (Baust et al., 2009).

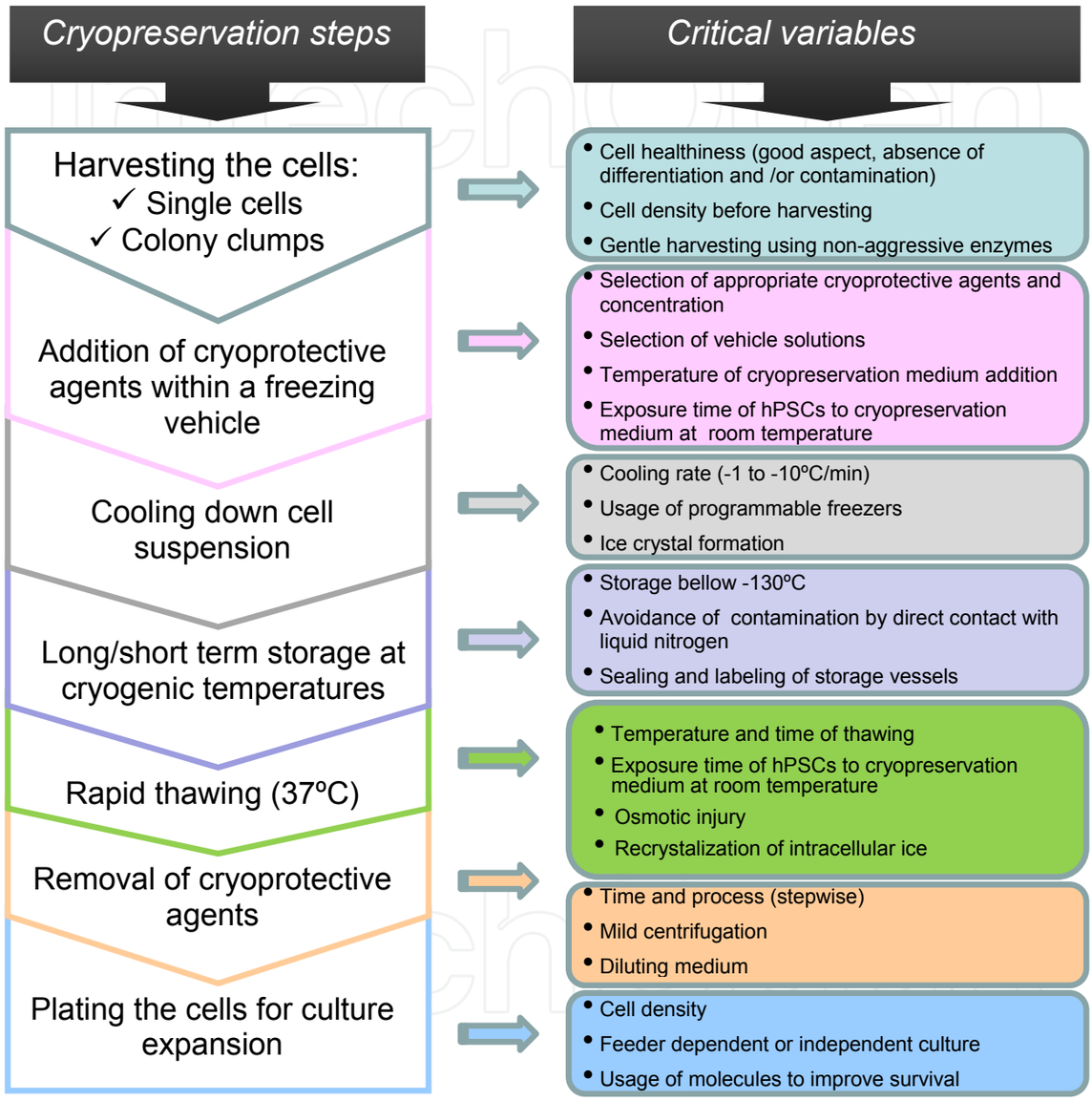

Fig. 1. Representative diagram of the main steps involved in a general cryopreservation process and the critical variables that should be considered in order to preserve cells with good recovery rates.

2. Sensitivity of hPSCs to cryopreservation

The techniques employed for the cryopreservation of hPSCs which include slow freezing-rapid thawing and vitrification, have been shown to be refractory for these cells that present

very low survival rates (5-20% and 25-75% respectively) and many of the cells that do survive differentiate upon thawing and expansion (Reubinoff et al., 2001; Richards et al., 2004). The low efficiency of hPSCs cryopreservation has been attributed, in part, to the highly "cooperative" nature of these cells (as comparable with mESCs), which appear to require intimate physical contact between them within the colony (to permit cell-cell signaling) and an optimum clump size of about 100-500 cells during cryopreservation and serial passage (Amit et al., 2000; Reubinoff et al., 2000). All these statements mean that we are dealing with a cell type that presents extremely high sensitivity to cryopreservation. Therefore, the arising questions are: why are hPSCs so vulnerable to the cryopreservation process? And which are the processes involved in the low survival rates of hPSCs after cryopreservation?

Heng et al postulated for the first time that apoptosis instead of cellular necrosis, was the major mechanism inducing the loss of viability of cryopreserved hESCs during freeze-thawing with conventional slow-cooling protocols (Heng et al., 2006). They showed that most of the cells were viable (~98%) immediately after thawing (determined by the Trypan blue dye exclusion method) and that cell viability was gradually decreasing with time in culture at 37ºC. Moreover, the kinetics of cell death could be reversibly slowed by a reduction in the temperature at which the cells were held post-thaw, indicating an apoptotic mechanism for cell death rather than an unregulated necrotic process. Based on these previous results, Xu et al investigated the apoptotic pathways activated during cryopreservation (Xu et al., 2010a). They described that the largest effect observed, mainly due to the freezing step, was an increase in the level of reactive oxygen species in hESCs. This presumably leads to the activation and translocation of p53 as strong expression of this protein was seen in the nucleus of thawed cells. Consequently, Caspase 9 was activated and a significant increase was also observed after thawing. In addition, Caspase 8 activity showed a similar increase post-thaw, indicating the possible activation of the extrinsic apoptotic pathway. They also stated that the elevated levels of F-actin observed during freezing could result in changes in apoptotic signals. These results led the authors to conclude that apoptosis in cryopreserved hESCs was induced through both, the intrinsic and extrinsic pathways (Xu et al., 2010a).

However, a remaining question is unanswered: why hPSCs are so sensitive to apoptosis compared to mESCs or other cell lines? In order to answer this issue, Wagh et al performed detailed microarray studies on hESCs at different time points after thawing and compared their transcriptomes with control cells that did not go through the cryopreservation process (Wagh et al., 2011). Viability, stemness, colony morphology and proliferation were also monitored at different times post-thawing. They observed a full recovery of the phenotypes of cryopreserved hESCs after 5 days of cultivation. However, the number of colonies was significantly smaller in the frozen hESCs compared to control groups. Furthermore, the colony growth rate was also reduced. Gene expression analysis showed very similar transcriptomes for the surviving fraction of 30 minutes frozen-thawed hESCs and the control unfrozen cells. Therefore, they concluded that the transcriptome of the surviving hESCs is preserved during cryopreservation. On the other hand, increases in the number of the up- and down-regulated genes occur continuously within 24 h after thawing and culturing, and those genes are declined or maintained within 48 h. This observation favored the hypothesis that physical cellular damage induced by freezing and/or thawing inhibits proper attachment during cultivation resulting in an induction of anoikis apoptotic cell

death despite an almost stable transcriptome. Supporting this theory the analysis of the microarray showed differences in the expression of genes involved in cell communication, cell growth and maintenance, cell death, cell differentiation and cell proliferation (Wagh et al., 2011). In agreement, Li et al showed that increased cellular adhesion induced by the Rho associated kinase (ROCK) inhibitor Y-27632 enhances the survival of single hESCs after thawing (Li et al., 2009). To demonstrate this, they treated cryopreserved hESCs simultaneously with Y-27632 and EGTA, a calcium chelant that disrupts cadherin activity and therefore cell adhesion. This double treatment significantly reduced the capacity of hESCs to form colonies and cell viability after thawing (Li et al., 2009). These results point to a high sensitivity of cryopreserved hPSCs to the loss of adherence between cells and/or to the substrate, resulting in a detachment induced apoptosis or anoikis.

According to the high differentiation rates experienced by hPSCs after cryopreservation, Wagh et al observed a down-regulation of pluripotency markers such as nanog, sox2 or klf4 (Wagh et al., 2011). In agreement with these results, it has been shown that the pluripotency marker Oct-4 was significantly decreased after culturing cryopreserved hESCs for several days (Katkov et al., 2006). In addition, the freeze-thaw stress increases the expression of several genes involved in the differentiation processes such as embryonic morphogenesis, neurogenesis, ossification, tissue morphogenesis, regeneration and vasculature development (Wagh et al., 2011).

3. Improvement of existing cryopreservation protocols

Many laboratories have been working over the last 10 years in the development of new cryoprerservation protocols for hPSCs. The main aim of the vast majority of these protocols has been the improvement of cell recovery including: an enhancement of cell survival and a reduction of cell differentiation. To this end different approaches have been adopted: development of new cryopreservation protocols such as vitrification, usage of different cryoprotective agents or molecules to improve survival, xeno-free cryopreservation media, cryopreservation of adherent hPSC colonies or single cells and/or utilization of devices to control changes in temperature. Although each of these works (summed up bellow), provide some improvements in hPSCs recovery after cryopreservation, not many of them have addressed the key question: how the changes introduced in the cryopreservation protocols contribute to the enhancement in cell recovery?

3.1 Vitrification and optimizations of the technique

One of the first attempts to overcome the low survival rates experienced by hPSCs after cryopreservation using the standard slow freezing-rapid thawing method was the adaptation of the vitrification protocol. This technique was developed for the cryopreservation of bovine ova and embryos (Vajta et al., 1998) and it was successfully adapted for hESCs freezing by Reubinoff and colleagues some years ago (Reubinoff et al., 2001). The protocol requires stepwise exposure of colony fragments to two vitrification solutions of increasing cryoprotectant concentrations. This exposure is sequential and brief (60 and 26 seconds respectively either at room temperature or at 37ºC). The common components of the vitrification medium are DMSO and ethylene glycol. The composition of the vehicle solution varies, with differences in sucrose concentration, the presence or absence of serum and the buffer used. Using mixtures of cryoprotectants helps to reduce the

intrinsic toxicity of each, and the method published by Reubinoff et al utilized 20% DMSO, 20% ethylene glycol and 0.5 mol/l sucrose (Reubinoff et al., 2001). However, no studies have been reported so far to determine the permeability of the cells (or colony fragments) to either cryoprotectant, or the intrinsic toxicity of these components to hESCs (Hunt, 2011).

Extremely rapid cooling rates are required to achieve vitrification using this two-component system. This is accomplished by direct immersion into liquid nitrogen of open-pulled straws containing small droplets (typically 1-20 µl) of vitrification solution within which the colony fragments (< 10) are held. Straws are then generally transferred to liquid nitrogen for long term storage (Vajta et al., 1997; Vajta et al., 1998).

The thawing process has to be as well, as rapid as possible to avoid ice crystallization. This is accomplished by direct immersion of the vitrified samples into pre-warmed culture medium containing sucrose, followed by stepwise elution of the cryopreotectants using sucrose as an osmotic buffer. An alternative method with direct exposure to growth medium without stepwise elution of the cryoprotectants has also been used with no noticeable deleterious effects (Hunt & Timmons, 2007; Reubinoff et al., 2001).

Vitrification has been adopted by many groups as the method of choice for hPSCs cryopreservation based on several comparative studies reporting recovery rates of undifferentiated colonies of more than 75% after vitrification compared to the 5-10% obtained after slow-cooling and rapid thawing (Li et al., 2010b; Reubinoff et al., 2001; Richards et al., 2004; Zhou et al., 2004). However, this technique presents some limitations: it is labor intensive and technically challenging, it is not suitable for large amounts of cells and the contact between the liquid nitrogen and the cells carries the risk of contaminations (Vajta & Nagy, 2006). Some attempts to improve these limitations have been done so far. One approximation was proposed by Heng et al for the cryopreservation of adherent hESCs colonies (Heng et al., 2005). They designed a culture plate made of detachable screw-cap culture wells resistant to storage at low temperatures in liquid nitrogen envisioned to develop automated systems for handling bulk quantities of cells (Heng et al., 2005). Alternatively, a method combining the large holding volume of slow-cooling rapid-thawing in cryotubs with the high efficiency of vitrification was described by Li et al (Li et al., 2008a). In this protocol hESCs clumps (>70um) were harvested after passage and transferred to a nylon cell strainer, exposed to vitrification solutions and vitrified by direct immersion in liquid nitrogen. Using this bulk vitrification method, 30 times more hESCs clumps (100-150) can be vitrified in a cell strainer compared to the open pulled straws. In addition, comparable results to those obtained for the classical vitrification method were reported for the recovery rate, the degree of differentiation and the maintenance of the pluripotency of the surviving cells (Li et al., 2008a). A refinement of this technique, using a cryovial fitted with stainless steel mesh, produced similar results (Li et al., 2010a). Although this method is easy and efficient to perform it still presents the limitation of direct contact of the cells with liquid nitrogen increasing the possibility of contamination and cell infection.

In order to avoid direct contact of the vitrification solution with the liquid nitrogen several methods have been developed. Usage of embryo straws sealed in both ends with a commercial plastic bag heat sealer was reported by Richards et al (Richards et al., 2004). This

improvement of the cryopreservation technique presents a similar yield of hESCs recovery after thawing with low differentiation rates comparable with the results of Reubinoff et al (Reubinoff et al., 2001). The usage of cryovials for vitrification has also been explored showing interesting results. Nishigaki et al used a DMSO-based and serum-free vitrification medium to cryopreserve iPSCs in cryovials (Nishigaki et al., 2011). They compared various vitrification solutions containing different concentrations of DMSO, ethylene glycol and polyethylene glycol with knockout serum replacement (KSR) in both DMEM and Euro-collins vehicle solutions. Analysis of the thermal properties of the cryopreservation solutions during the cooling process by differential scanning calorimetry (DSC) indicated that they would vitrify at an optimal cooling rate of ~ -125 ^{o}C/min. Recovery rates between 20-30% are described one day after thawing using 40% ethylene glycol and 10% polyethylene glycol in Euro-Collins solution. Furthermore, cryopreserved cells express undifferentiation markers and keep pluripotency (Nishigaki et al., 2011). Therefore, using this protocol the vitrification of large amounts of cells is feasible and avoids the risk of contamination.

3.2 Usage of different cryoprotective agents and vehicles

During the cryopreservation course, as the cell suspension is cooled below the freezing point, ice crystals form and the concentration of the solutes in the suspension increases, being both processes damaging for the cells. Cryoprotective agents (CPAs) are necessary to minimize or prevent the damage associated with the freezing process. The mechanisms providing this protection in slow cooling-rapid thawing protocols, although not completely understood, appear to work primarily by altering the physical conditions of both the ice and the solutions immediately surrounding (external to) the cells. In contrast, vitrification overcomes the problems associated with ice crystallization in a different manner. Here cryoprotectants are used in high concentrations preventing ice formation entirely (Baust et al., 2009; Hunt, 2011).

Different CPAs have been identified so far to be used for the cryopreservation of mammalian cells (Klebe & Mancuso, 1983; Matsumura et al., 2010); however, the two most commonly used substances are glycerol and DMSO. Other substances used include sugars, polymers, alcohols and proteins. CPAs can be divided roughly into two different categories: (1) permeating CPAs: substances that permeate the cell membrane (e.g. DMSO and glycerol); and (2) nonpermeating CPAs: impermeable substances (e.g. polyethylenen glycol and trehalose); both types present a different impact on the freezing process. Permeating CPAs have a low molecular weight and thus can penetrate the cell membrane and gradually substitute the water present in the cells. The osmolality of the cells is thereby increased, and subsequently, the percentage of extracellular water that can form ice crystals before reaching the osmotic equilibrium is reduced and total dehydration of the cells is prevented. Nonpermeating CPAs cannot penetrate the cell membrane and stabilize the cell by forming a viscous glassy shell around its surface (Hubel, 1997; Karlsson, 2002; Karlsson & Toner, 1996; Meryman, 2007). Therefore, the selection of an appropriate CPA or a combination of them used in optimal concentrations within an effective vehicle solution will determine the efficiency of the cryopreservation process for a given cell type. In this sense, alternatives to DMSO as the cryoprotectant of choice for hPSCs have been tested due to the known effect of this solvent on inducing differentiation and cytotoxicity (Adler et al., 2006). See Table 1 for an overview of CPAs and freezing vehicles used in different protocols describe here.

Trehalose is a natural disaccharide that has been selected as an attractive CPA for several reasons. First of all, it has been shown to be effective in mammalian cell stabilization at low temperatures and water contents. Secondly, trehalose preserves cell viability by different mechanisms than DMSO (Crowe et al., 2001; Sum et al., 2003; Sum & de Pablo, 2003). Finally, trehalose addition to the cryopreservation medium containing DMSO and fetal bovine serum (FBS) has been proven to increase the viability of hematopoietic precursor cells from 7% to 20% and improved membrane integrity in cryopreserved fetal skin cells (Erdag et al., 2002; Zhang et al., 2003). Ji et al showed that trehalose loading into adherent colonies of hESCs prior to cryopreservation results in small, but significant improvements in cell viability when combined with DMSO treatment and high FBS concentrations (Ji et al., 2004). In the same line of results, it has been demonstrated that trehalose addition to the freezing and post-thawing medium of hESC colonies cryopreserved in suspension in freezing medium containing 10% DMSO, increased the recovery rate by ~3 folds (from 15 to 48%) (Wu et al., 2005). These results suggested that the protective mechanism of trehalose addition might be the reduction of osmotic changes during the freezing and thawing process, although this hypothesis has not been demonstrated. The addition of trehalose did not affect the normal karyotype of the cells neither their pluripotency capacity tested by teratoma formation (Wu et al., 2005).

A comparison between four different types of CPAs for iPSCs cryopreservation has recently been described: DMSO, ethylene glycol, propylene glycol and glycerol (Katkov et al., 2011). Interestingly, the toxicity of these four CPAs was analyzed after 30 minutes exposure of a 10% CPA solution at 37ºC. The results showed that DMSO was the most toxic CPA for iPSCs while glycerol was the least harmful being the other two CPAs in between. Surprisingly, the protective effect exerted by the same CPAs after cryopreservation of small iPSC clumps by the slow cooling-rapid thawing protocol was the opposite, being DMSO the most protective CPA together with ethylene glycol while glycerol was the least protective one. The same result was obtained when iPSCs previously dissociated with Accutase™ were cryopreserved in the presence of a ROCK inhibitor in combination with the previous mentioned CPAs. Therefore, ethylene glycol was selected as the cryoprotectant of choice since it presents less toxicity than DMSO and exerts similar levels of protection (Katkov et al., 2011). In addition, these results give clear evidence that the low hPSCs recovery rate obtained after cryopreservation is mainly caused by the freezing-thawing procedure, rather than by the process of CPA addition/removal.

The combination of different CPAs has also been tested in comparison to the conventional freezing solution containing 10% DMSO in slow cooling-rapid freezing protocols. Ha et al performed a detailed study about the composition of the cryopreservation medium, initially analyzing the impact of both DMSO and FBS concentration in hESCs recovery (Ha et al., 2005). They reached the conclusion that a combination of 5% DMSO plus 50% FBS was the most effective one sustaining survival rates of 10%. Afterwards, they used this freezing medium composition as a starting point to test different concentrations of other CPAs such as ethylene glycol or glycerol. An increase of 3 fold in the survival rate (around 30%) was obtained when using a combination of 5% DMSO + 50% FBS +10% ethylene glycol that was selected as the most effective cryopreservation medium. Three passages after thawing cryopreserved hESCs retained the key properties and characteristics of hPSCs (Ha et al., 2005).

CPA composition	Freezing vehicle	Addition of other molecules	Cell type	Cell processing	Type of culture	Recovery rate	Reference
10 % EG	Not described	ROCK inhibitor	iPSCs	Colony clumps and single cells	MEF feeder layer	~20-50% recovery(1)	(Katkov et al., 2011)
		No		Adherent colonies		~60% recovery (1)	
10% DMSO	Growth medium	No	hESCs	Colony clumps	MEF feeder layer	60% (30 min) <10% (24 h) (2)	(Wagh et al., 2011)
						0-55% recovery (16)	(Li et al., 2010b)
		ROCK inhibitor	hESCs	Single cells	Human foreskin feeder layer	50-60% survival(4)	(Martin-Ibanez et al., 2008)
			hESCs and iPSCs		MEF feeder layer and feeder-free culture	7-8 fold increase in the number of recovered cells or colonies (8)	(Claassen et al., 2009)
		ROCK inhibitor + P53 inhibitor	hESCs	Single cells	MEF feeder layer and feeder-free culture	~80% survival(5)	(Xu et al., 2010a)
	90% FCS	ROCK inhibitor	hESCs and iPSCs	Single cells	Feeder-free culture	90% viable cells(4)	(Mollamohammadi et al., 2009)
	90% KSR	No	hESCs	Colony clumps	MEF feeder layer	8-53% survival(17)	(Lee et al., 2010)
		ROCK inhibitor	hESCs	Colony clumps	MEF feeder layer	85-95% survival(6)	(Li et al., 2008b)
				Single cells	Feeder-free culture	53-65% (7)	(Li et al., 2009)
	90% (DMEM/F12 + 20% FBS)	Z-VAD-FMK	hESCs	Adherent colonies	MEF feeder layer	18.7% (11)	(Heng et al., 2007)
		No				~98% (5 min) 20-30% (90 min) (12)	(Heng et al., 2005)
	60 % growth medium + 30%FBS	No	hESCs	Adherent colonies (microca-rriers)	MEF feeder layer and feeder-free culture	1.5-1.9 fold increase in recovery rate(13)	(Nie et al., 2009)
5% DMSO 5% HES	80% (DMEM/ F12 + 20% KSR)	No	hESCs	Colony clumps	MEF feeder layer	~80% recovery (9)	(T'joen et al., 2011)
10% DMSO + 0.2 mol/l Trehalose	90% KSR	No	hESCs	Colony clumps	MEF feeder layer	37-48% recovery(10)	(Zhang et al., 2003)

10% DMSO+ 35 mM Trehalose	40% Growth medium + 50% FBS	No	hESCs	Adherent colonies	MEF feeder layer and feeder-free culture	25% viability increase in respect to DMSO alone(14)	(Ji et al., 2004)
7.5% DMSO 2.5% PEG	Growth medium	ROCK inhibitor + P53 inhibitor	hESCs	Single Cells	MEF feeder layer and feeder-free culture	80-90% survival(5)	(Xu et al., 2010b)
10% DMSO + another undisclosed CPA	Phosphate buffer	No	hESCs and iPSCs	Colony clumps	Human foreskin feeder layer	90-96% viability(15)	(Holm et al., 2010)
5% DMSO 10% EG	50%FBS and DMEM/ F12	No	hESCs	Colony clumps	MEF feeder layer	30% colony recovery (3)	(Ha et al., 2005)

Table 1. Cryoprotectant agents and freezing vehicles used for the cryopreservation of hPSCs using the slow-freezing rapid-thawing protocol. The conditions and recovery rates showed in the table correspond to the best condition tested in the referenced works. (DMSO: dimethyl sulfoxide; EG: ethylene glycol; PEG: polyethylene glycol; HES: Hydrosyethylstarch; FBS: fetal bovine serum; FCS: fetal calf serum; KSR: Knockout serum replacement; MEF: mouse embryonic fibroblasts). The recovery rates were determined using different tests: (1) % cell recovery determined by QUANTA Coulter Counter measurement of Calcein-PM+/7AAD-. (2) % viability determined with FDA/EB staining at different time points after thawing. (3) Number of colonies 10 days after plating. (4) Cell viability was determined counting the number of cells by the Trypan blue exclusion method immediately after thawing. (5) Cell viability was determined by FACS using propidium iodide staining immediately after thawing. (6) Number of colonies at day 5/total colonies replated. (7) Flow cytometry analysis of apoptotis using Anexin V and propidium iodide immediately after thawing. (8) Fold increase in the number of recovered cells determined using a Z2 Coulter Counter and Size analyzer 4 days after thawing. Fold increase in the number of colonies determined by microscopy. (9) Recovery rate was calculated as follows: the amount of Grade A+B colonies at day 7 post-thawing versus the amount of frozen Grade A+B colonies. (10) Number of colonies 7 days after thawing. (11) MTT assay to measure % survival rate 24 h post-thawing. (12) Viability determined by Trypan blue exclusion method of adherent colonies at different time points after thawing. (13) Recovery was calculated as the number of cells one week after thawing divided by the number of cells at the time of freezing. The recoveries of hPSCs frozen using microcarriers are normalized to the recoveries of hPSCs frozen as free colonies. (14) Cell viability was measured by MTT assay or Alamar Blue assay several days after thawing. (15) Viability or percentage of surviving cells was calculated as a ratio between live hPSCs after thawing and total number of initially frozen cells. Cells were counted using the Trypan blue exclusion method. (16) Recovery rates were estimated as the % of attached and undifferentiated clumps counted 7-8 days after-thawing respect to the initially frozen. (17) Viability was assessed counting the number of colonies stained for alkaline phosphatase.

A new cryopreservation formula containing 7.5% DMSO plus 2.5% polyethylene glycol was analyzed in another work (Xu et al., 2010b). This study resulted in slight but significant increase in the hESCs recovery determined by counting the number of cells or colonies in

feeder-independent or feeder-dependent culture respectively (Xu et al., 2010b). Recently, an alternative cryopreservation medium combining intracellular (5% DMSO) and extracellular (5% Hydrosyethylstarch) CPAs has been proven to be highly effective for the cryopreservation of small hESC clumps by the classical slow-freezing rapid-thawing method. These clumps are obtained by a combination of hESC colony detachment with Collagenase IV followed by 5 minutes dissociation using an undisclosed solution. This protocol is suitable for handling bulk amounts of hPSCs (T'joen et al., 2011).

Comparison of different freezing vehicles using DMSO as a cryoprotectant has also been studied for the cryopreservation of dissociated hESCs (Mollamohammadi et al., 2009). Three preservation media containing 10% DMSO plus: 90% fetal calf serum (FCS), 90% KSR or 90% hESCs medium containing 20% KSR and ROCK inhibitor were analyzed. The percentage of viable cells obtained by the Trypan blue exclusion method after thawing showed that cells were better preserved in the presence of 90% FCS as a vehicle (~90%). The other two freezing solutions caused lower survival rates (60-80%) (Mollamohammadi et al., 2009). Following a similar approach, Ha et al studied the impact of different FBS concentrations (5, 50 and 95%) in the vehicle freezing solution using a 5% DMSO as a CPA (Ha et al., 2005). A decrease in the survival rate is observed as the FBS concentration is reduced although no differences were found between 50 and 95%. Therefore, the authors established 50% of FBS as the optimal concentration to support hPSCs survival during the cryopreservation process (Ha et al., 2005).

3.3 Addition of molecules to enhance survival

One of the first molecules studied in the cryopreservation process to enhance cell survival was the caspase inhibitor Z-VAD-FMK (Heng et al., 2007). Results obtained in a previous work from the same group showing that apoptosis rather than necrosis was the responsible mechanism involved in the loss of viability during hESCs cryopreservation encouraged them to test a broad-spectrum irreversible inhibitor of caspase enzymes (Heng et al., 2006). Exposure to 100 mM Z-VAD-FMK in the freezing solution alone did not significantly enhace the post-thaw survival rate. However, when Z-VAD-FMK was added to the freezing solution as well as to the post-thawing solution a significant enhancement in the cell survival rate (~two fold) was observed. Nevertheless, the differentiation rates of cryopreserved hESCs were not reduced and therefore the culture recovery was not improved (Heng et al., 2007). Similarly, the addition of a specific Caspase-9 inhibitor to the post-thawing recovering medium failed to increase hESCs colony formation 4-5 days after thawing, although it did reduce Caspase 8 and 9 activity 2 h after cryopreservation (Xu et al., 2010a). These results suggested that Caspase activity was not the triggering mechanism contributing to the low hPSCs recovery after cryopreservation, but it could be a downstream effector.

A significant improvement in the cryopreservation field came up with the addition of a ROCK inhibitor. ROCK have been found to play a role in the regulation of multiple biological pathways such as apoptosis, cell cycle, differentiation, cell adhesion as well as gene expression (Amano et al., 1997; Hall, 1994; Ishizaki et al., 1997; Krawetz et al., 2009; Maekawa et al., 1999). Watanabe et al reported for the first time, that addition of the ROCK inhibitor Y-27632 improved the cloning efficiency of dissociated hESCs more than 25-fold when the cells were plated at low density (Watanabe et al., 2007). One year later, the same inhibitor was tested for the cryopreservation of hESCs. Li et al demonstrated that 10 μM Y-27632 added to the post-thaw medium during 1 day increased hESCs survival when

cryopreserved as small clumps (Li et al., 2008b). In parallel our group reported that dissociated hESCs could be cryopreserved in the presence of ROCK inhibitor (Martin-Ibanez et al., 2008; Martin-Ibanez et al., 2009). The addition of Y-27632 to the freezing medium did not increase the formation of hESCs colonies compared to the control non treated cells although it increased cell survival. In contrast, the presence of ROCK inhibitor in the post-thawing recovery medium did increase the formation of hESCs colonies significantly (50-100 times). The addition of Y-27632 to both, the cryopreservation and the post-thawing medium was the condition tested contributing to the highest cell recovery after freezing.

Rock inhibitor addition	**Type of cell and culture**	**Cell processing**	**Recovery rate (method of analysis)**	**Time of analysis**	**Reference**
Freezing and post-thawing recovery media (1 day)	hESCs on human foresking feeders	Single cells	50-60% survival % survival after thawing analyzed by Trypan Blue exclusion method	Immediately after thawing	(Martin-Ibanez et al., 2008)
	hESCs and iPSCs in feeder-free culture	Single cells	60-80% viable cells % survival after thawing analyzed by Trypan blue exclusion method	Immediately after thawing	(Mollamohammadi et al., 2009)
	hESCs on MEF feeders and feeder-free culture	Single cells	80-90% survival % survival after thawing analyzed by propidium iodide staining	Immediately after thawing	(Xu et al., 2010a) (Xu et al., 2010b)
	iPSCs on MEF feeders	Single cells, clumps and adherent colonies	20-60% recovery depending on the type of culture % recovery after thawing by QUANTA Coulter Counter measurement of Calcein-PM+/7AAD-	Not stated	(Katkov et al., 2011)
Post-thawing recovery medium (1 day)	hESCs on MEF feeders	Colony clumps	85-95% survival Number of colonies at day 5/total colonies replated	5 days after thawing	(Li et al., 2008b)
	hESCs in feeder-free culture	Single cells	53-65% Flow citometry analysis of apoptosis using Anexin V and propidium iodide	Immediately after thawing and 24 h after thawing	(Li et al., 2009)
Post-thawing recovery medium (4 days)	hESCs on MEF feeders and feeder-free culture	Single cells	7-8 fold increase in the number of recovered cells 4 days after thawing (Z2 Coulter Counter and Size analyzer)	4 days after thawing	(Claassen et al., 2009)
Post-thawing recovery medium (2 days)	iPSCs on MEF feeders	Single cells	7 fold increase in the number of colonies 48 h post-thawing	2 days after thawing	(Claassen et al., 2009)

Table 2. Overview of the ROCK inhibitor treatments tested to improve the recovery rates after cryopreservation of hPSCs. Survival rates showed in the table are obtained using the best condition tested in the work referenced.

Moreover, we described a complete avoidance of hESCs differentiation just after cryopreservation showing that most of the colonies expressed the undifferentiation markers: Oct-4, nanog, SSEA-4, TRA-1-81 and TRA-1-60. The addition of Y-27632 increased the growth rates to control levels, did not affect hESCs normal karyotype and kept their pluripotency (Martin-Ibanez et al., 2008). Similar results have been shown not only for hESCs but also for iPSCs in both feeder-associated and feeder-free conditions (Claassen et al., 2009; Katkov et al., 2011; Mollamohammadi et al., 2009). See table 2 for a sum up of all the ROCK inhibitor treatments used for cryopreservation of hPSCs.

ROCK inhibitors have also been used in combination with other molecules such as Caspase inhibitors, p53 inhibitors or Bax inhibitors added always to the post-thawing culture medium. Xu et al showed that none of the three combinations pan-Caspase inhibitors + Y-27632, Caspase 9 inhibitor + Y-27632 and Bax inhibitor + Y-27632 enhanced the protective effect of ROCK inhibitor alone for cryopreserved hESCs (Xu et al., 2010a). Only the treatment with a p53 inhibitor + Y27632 induced a cell recovery similar to that of ROCK inhibitor. However, treatment with p53 alone did not account for an increase in cell survival (Xu et al., 2010a). Similar results were obtained by the same group in another report where they observed an enhancement of hESCs recovery when cryopreserved in 10% DMSO or 7.5% DMSO + 2.5% polyethylene glycol and treated with p53 inhibitor + Y-27632 in the post-thawing medium (Xu et al., 2010b).

Although most of the works studying the effect of ROCK inhibitor during cryopreservation did not address the mechanism of action of this molecule, at least two of them showed some interesting results (Li et al., 2009; Xu et al., 2010a). Both of them reported a reduction in hESCs apoptosis and/or Caspase activity one day after cryopreservation driven by Y-27632. This is in agreement with the previous report of Watanabe et al who pointed to an antiapoptotic role of this ROCK inhibitor (Watanabe et al., 2007). In addition, Li et al demonstrated that Y-27632 treatment increased the adherent properties of cryopreserved hESCs favoring cell aggregate formation and adhesion to the substrate. This effect, in turn, prevented anoikis and enhanced hESCs survival (Li et al., 2009; Mollamohammadi et al., 2009).

3.4 Cryopreservation of adherent versus suspension hPSC colonies

In view of the poor survival rates obtained after cryopreservation of hPSCs in suspension using the slow-cooling rapid thawing method, some authors decided to test cryopreservation of adherent cells. This decision was based on previous studies done with certain cell types difficult to preserve. For example, hepatocytes cryopreserved in alginate gels display a higher viability and lower apoptotic activity than hepatocytes cryopreserved in suspension (Mahler et al., 2003). Similarly, hepatocytes sandwiched between two layers of collagen provide enhanced viability and protein secretion compared with cells preserved in solution (Birraux et al., 2002; Koebe et al., 1990; Koebe et al., 1999). Taking these results as a proof of principle, hESCs were successfully cryopreserved as adherent colonies in 24 well plates in medium containing 10% DMSO + 30% FBS by Ji et al (Ji et al., 2004). This approach demonstrated that hESCs frozen as adherent colonies were five times more viable than clumps of colonies frozen in suspension. In addition, encapsulation of hESCs colonies inside Matrigel™ for 1 or 2 days increased viability significantly respect to unencapsulated adherent frozen colonies or colonies encapsulated for just 1 h. The percentage of adherent hESC colonies recovered 1 to 2 weeks after cryopreservation was about 80-90% and almost no differentiation was detected. In

contrast, less than 2% of hESC colonies attached when frozen in suspension. A recent work by Katkov et al reported a refinement of the technique cryopreserving adherent iPSC colonies in the presence of ethylene glycol as a cryoprotectant and using a six-step programmed protocol (Katkov et al., 2011). Preservation of iPSCs under these conditions induced a six-fold increase in cell recovery after thawing respect the standard cryopreservation of cell clumps by the slow-freezing rapid thawing method (Katkov et al., 2011). Two mechanisms are postulated to explain the increased viability obtained preserving hESCs as adherent colonies. The first is that hESC colonies do not have to settle to the surface and attach. This is a decisive process for the survival of hPSC colonies frozen in suspension that is rarely achieved due to the massive cell death or cell damage experienced within the colony during cryopreservation. Second, the maintenance of a continuous extracellular matrix signaling may also play a role in the enhanced viability and reduced differentiation of hESCs cryopreserved in an adherent state (Ji et al., 2004). The disadvantage of this technique is that large scale storage is not feasible because hPSCs attached to plates cannot be stored at high density. In addition, culture plates are unable to be sealed like cryovials, increasing the risk of sample cross-contamination during storage in liquid nitrogen. However, methodologies such as preservation on microcarriers might provide the advantages of freezing adherent cells at higher densities that are not possible on flat surfaces. This is what has been described by Nie et al, who used Cytodex 3 microcarriers to cryopreserve adherent hESCs (Nie et al., 2009). These microcarriers consisted of a thin layer of denatured collagen covalently coupled to a matrix of cross-linked dextran. They were modified with Matrigel™ or irradiated MEF to enhance the adhesion of hESC colonies. In this work it was first demonstrated that hESCs colonies were effectively expanded in a pluripotent, undifferentiated state on both types of microcarriers (Matrigel™ and MEF coated). Then cryopreservation utilizing this system was compared to standard freezing of hESC colonies in suspension. hESCs-microcarriers were suspended in freezing medium consisting in 10%DMSO and 30%FBS at a cell density of $1x10^6$ cells/ml on 10 cm^2 microcarriers. The suspension was transferred to cryovials, frozen inside a freezing container at a cooling rate of -1ºC/min and moved into liquid nitrogen. Seven days after thawing viability was assessed by counting the number of cells. This number was compared to that of the conventional hESCs slow freezing method. Cryopreservation on microcarriers resulted in 1.7 times the recovery of hESCs frozen in free suspension (Nie et al., 2009). Although the enhancement of cell recovery is not very promising, further optimization of this methodology holds a great potential for future larger-scale cryopreservation.

3.5 Cryopreservation of dissociated single hPSCs versus clumps of colonies

hPSCs are colony-forming social cells that present a high vulnerability to apoptosis upon cellular detachment and dissociation (Amit et al., 2000; Watanabe et al., 2007). These characteristics could explain why most of the cryopreservation protocols rely on hPSCs small clumps to improve survival rates (Heng et al., 2006; Reubinoff et al., 2001; Richards et al., 2004; Zhou et al., 2004). However, the cryopreservation of clumps presents some associated problems such as limitations on cryoprotectant exposure inside the clump. In this sense, T'Joen et al demonstrated that the application of a cell dissociation solution before freezing, thereby creating a mixed population of very small hESC clumps and single cells, increased the recovery rate after cryopreservation (T'joen et al., 2011). In addition, the use of hPSC colony clumps also prevents a good estimation of freezing-thawing efficiency, as precise cell numbers cannot be estimated. Therefore, development of cryopreservation protocols for dissociated hPSCs is a pre-requisite for the widespread use of these cells in basic or clinical research.

Most of the studies carried out so far for the cryopreservation of dissociated hPSCs involved the usage of ROCK inhibitors (usually 10μM of Y-27632), since in its absence very few or none colonies are obtained. This inhibitor has been reported to significantly increase the survival rate of frozen/thawed single hESC as well as iPSCs (Claassen et al., 2009; Li et al., 2009; Martin-Ibanez et al., 2008; Mollamohammadi et al., 2009; Xu et al., 2010a; Xu et al., 2010b). Recent studies have demonstrated that Y-27632 increased not only the survival rate but also the adhesion of frozen–thawed dissociated single hPSCs in the presence and absence of feeder cells (Claassen et al., 2009; Katkov et al., 2011; Li et al., 2009; Martin-Ibanez et al., 2008; Mollamohammadi et al., 2009; Xu et al., 2010a; Xu et al., 2010b). In fact, Li et al. proposed that Y-27632 does not block apoptotic pathways, but rather prevents hPSCs from sensing their external environment, giving them time to make important cell-cell interactions and thus allowing them to escape anoikis (Krawetz et al., 2009; Li et al., 2009). Moreover, Mollamohammadi et al showed by RT-PCR analysis that the expression of integrin chains aV, a6 and b1 increased significantly in the presence of ROCK inhibitor (Mollamohammadi et al., 2009). They proposed that this increase in integrins expression may account for the maintenance of an undifferentiated state and an increase in cell adhesion of hESCs and iPSCs to the substrate allowing better cloning efficiency (Mollamohammadi et al., 2009).

The usage of ROCK inhibitors for continuous treatments has not induced any adverse effects on hPSCs pluripotency or chromosomal stability, even after substantial number of passages (Mollamohammadi et al., 2009; Watanabe et al., 2007). ROCK inhibitors such as Y-27632 or Fasudil are already used clinically in cardiovascular therapies (Hu & Lee, 2005), suggesting that they are safe for the treatment of hPSCs. Although the exact mechanism of action of ROCK inhibitors is, at the moment, unknown and a lot of cross talks between signaling pathways occur, the usage of this compound opens a new field of study to improve hPSCs cryopreservation and culture protocols.

3.6 Controlled-rate cryopreservation

The cooling rate is one of the cryobiological variables associated with damage during slow-cooling (Figure 2). When the cooling process is rapid, intracellular ice crystals form before complete cellular dehydration has occurred. These ice crystals disrupt cellular organelles and membranes and lead to cell death during the recovery (thawing) process. On the other hand, when the cooling process is slow, free intracellular water is osmotically pulled from the cells resulting in complete cellular dehydration and shrinkage. This can also cause cellular death but there is little agreement on the mechanisms involved. However, when the cooling rate is slow enough to prevent intracellular ice formation, but fast enough to avoid serious dehydration effects, cells may be able to survive the freezing and thawing process. This survival zone or window is readily observed in many bacteria and other prokaryotes, but for most eukaryotic cells it is nonexistent or very difficult to find without using cryoprotectants. These agents have little effect on the damage caused by fast freezing (intracellular ice crystal formation), but rather prevent or lessen the damage caused by slow freezing (dehydration and shrinkage) (Figure 2) (Mazur, 1984). Thus, a tight control of the cooling rate is crucial to reduce cellular damage during cryopreservation, even in the presence of CPAs. This is achieved by the usage of programmable freezers. These devices, although expensive and not always available, are technically more reliable and reproducible. Several works have studied the relevance of programmable freezers for the cryopreservation of hPSCs. Ware et al reported survival rates of 60-70% with no apparent increase in differentiation using DMSO as a cryoprotectant, a control rate freezing device and straws as containers (Ware et al., 2005). The

results of this study identified three critical factors for successful hESCs freezing: ice crystal seed at some point above the temperature of spontaneous intracellular ice formation (between -7ºC and -12ºC), an appropriate freezing rate (between -0,3ºC and -1,8ºC/min) and rapid thawing (at 25-37ºC) (Ware et al., 2005). Another study optimizing the same critical factors described an improved protocol consisting in: cooling the sample from 0 ºC to -35 ºC at a cooling rate of -0.5ºC/min, seeding at -10 ºC before being plunged immediately into the liquid nitrogen and rapid thawing. Under these conditions a survival rate of 80% was obtained (Yang et al., 2006). A successful usage of programmable freezing for the cryopreservation of adherent iPSCs has also been recently described (Katkov et al., 2011). The authors developed a six step programmed protocol including : 1) -1ºC/min from 0ºC (addition of CPA on ice) to -10ºC; 2) hold for 30 min at -10ºC; 3) -3ºC/min to -40ºC; 4) -1ºC/min to -60ºC; 5) -0.33ºC/min to -80ºC and 6) hold at -80ºC for 5 min and then transfer to liquid nitrogen. Adherent iPSC colonies cryopreserved using ethylene glycol as a CPA under these conditions showed a 63% recovery, which represents a 6 fold increase respect the preservation without a programmable freezer using DMSO (Katkov et al., 2011).

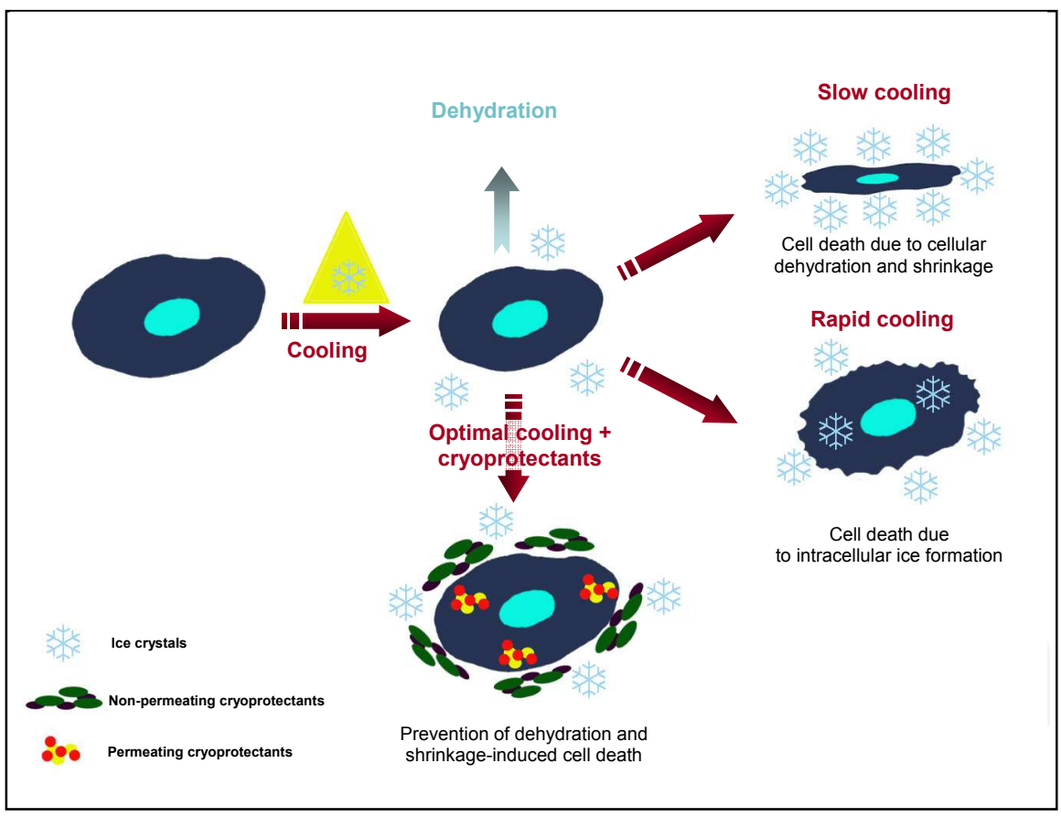

Fig. 2. Effects occurring during the cryopreservation of cells at different cooling rates. When the cooling process starts, ice crystals formation is induced and free intracellular water is osmotically pulled from the cells. If the cooling process is slow this effect lead to cellular cell death by dehydration and shrinkage. In contrast, if the cooling process is rapid, intracellular ice crystals form before complete cellular dehydration has occurred. These crystals induce cell death by cellular organelles and membrane disruption during the thawing process. An optimal cooling rate together with the usage of cryoprotectants in the freezing media avoids dehydration effects and intracellular ice formation allowing cell survival after thawing.

A recent report made an interesting comparison of three methods of cryopreservation of hESC clumps including: conventional slow freezing-rapid thawing using cryovials, vitrification and programmable cryopreservation in plastic straws (Li et al., 2010b). Assessing the efficiency of cryopreservation by counting the number of attached undifferentiated colonies 1-2 days and 7-8 days after thawing they reached the conclusion that conventional cryopreservation may not be appropriated for hESCs preservation since few colonies attached and most of them were differentiated. The usage of a programmable freezer increased significantly the cryopreservation efficiency (~50% colony recovery respect to ~5% of conventional freezing), although it was not better than the high efficiency obtained by vitrification (80-90% colony recovery). Both methodologies maintain unaffected the pluripotency and normal karyotype of the cells (Li et al., 2010b). Another comparative study published at the same time reported lower survival rates after programmable cryopreservation of hESC clumps (10-20% survival colonies), although they were significantly higher than the ones obtained after conventional slow-freezing (4-8%) (Lee et al., 2010). In this study the best cryopreservation condition was obtained using a stepwise transfer method for hESC clumps, which consisted in using a series of solutions with increasing serum replacement and DMSO concentrations to achieve a stepwise equilibration before freezing. The same inverse process was performed after thawing in order to gradually rehydrate the cells. The combination of stepwise methods with programmable freezers yielded survival rates of 30-50% with low numbers of differentiated cells (Lee et al., 2010).

3.7 Cryopreservation in xeno-free conditions

Clinical application of hPSCs would need hESC and iPSC lines derived, cultured, differentiated and cryopreserved in xeno-free conditions following good manufacturing practice (GMP) regulations. Several attempts to improve hPSCs culture conditions have been reported. These advances include: the derivation of clinical grade hESC and iPSC lines, the use of conditioned media together with Matrigel™ as an attachment substrate for hPSCs culture and the derivation and propagation of hESC lines on human feeder layers in xeno-free culture media (Amit et al., 2004; Hovatta et al., 2003; Rajala et al., 2007; Rajala et al., 2010; Richards et al., 2002; Richards et al., 2003; Skottman et al., 2006; Unger et al., 2008). Some approaches have also been done in the cryopreservation field towards the development of xeno-free effective cryopreservation protocols. The first one was an optimization of the established vitrification method previously described by Reubinoff et al (Reubinoff et al., 2001; Richards et al., 2004). In this new method they reported the successful vitrification of hESCs in sealed closed straws, their storage in the vapor phase of liquid nitrogen and the substitution of FCS with human serum albumin as the major protein source in the cryoprotectant solution. This refinement of the technique allowed the removal of animal components from the cryopreservation medium, therefore lowering the risk of cross-transfer of viruses and other pathogens to the hESCs. Moreover, sealing the straws the authors also prevented contact with potentially contaminated liquid nitrogen during cooling and storage. The efficiency of hESCs preservation was similar to the original vitrification protocol (Richards et al., 2004).

An effective serum and xeno-free chemically defined freezing procedure for hESCs and iPSCs has been recently developed (Holm et al., 2010). This protocol describes the usage of a commercially available freezing and post-thaw washing solution that presents the

advantage of being chemically defined, sterile and batch tested. The cryopreservation solution named STEM-CELLBANKER™ contains 10% DMSO, glucose and a high molecular weight polymer (undisclosed) used as a second cryoprotectant, all dissolved in phosphate-buffered saline. hPSCs are preserved using this solution in cryovials and the slow-cooling rapid-thawing method, without any programmed freezer. After thawing, cells are recovered in the washing solution named CELLOTION™ containing NaCl, centrifuged to eliminate cryoprotectants and plated down on a feeder layer of human mitotically inactivated fibroblasts. Post-thaw recovery was substantially increased without any detrimental impact on proliferation or differentiation (Holm et al., 2010). Similar cryopreservation yields were obtained for both hESCs preserved as clumps and iPSCs preserved as single cells without ROCK inhibitor treatment. Therefore, this is a simple and efficient system that enables the cryopreservation of large quantities of hPSCs in a chemically defined medium that is clinical grade compatible (Holm et al., 2010). Employing a similar protocol but using a home-made cryopreservation solution containing 10% DMSO and 90% KSR, Li et al reported the preservation of single hESCs in serum and feeder-free conditions in the presence of ROCK inhibitor during the first day after thawing (Li et al., 2009).

4. Conclusion

Understanding the mechanisms involved in the high vulnerability of hPSCs to the cryopreservation process is essential to develop efficient protocols for cryopreservation. Most of the research being undertaken over the last years is still empirical and few advances have been achieved in the identification of the pathways involved in the enhancement of cell survival induced by different factors, cryoprotectants or preservation systems. However, from the results obtained in these studies it is becoming increasingly clear that cell-cell adhesion and/or paracrine signaling between hPSCs are essential for survival and control of their undifferentiated state (Amit et al., 2000; Reubinoff et al., 2000; Thomson et al., 1998). Gap junctions and cell adhesion molecules are highly expressed in hESCs and have been implicated in these processes (De et al., 2002; Richards et al., 2004; Sathananthan et al., 2002; Wong et al., 2004; Wong et al., 2008). Therefore, disruption of these structures during cryopreservation due to ice crystal formation outside the cells may induce anoikis contributing to the poor recovery of hPSCs after slow cooling. However, a better understanding of this process together with a systematic study of the critical cryobiological variables is still needed to improve the already existing cryopreservation protocols. Further advances in the field would also require the development of reliable and standardized assays to measure not only immediate post-thaw recovery but also the ability of single cells or clumps to re-attach, proliferate and maintain pluripotency. Moreover, it is necessary to establish the n-points at which these assays should be applied, in order to allow direct quantitative comparisons between different cryopreservation methods that are not feasible at the moment. Thus, all present and future investigations would likely provide a reproducible effective and efficient cryopreservation protocol for hPSCs large-scale storage that will fulfill GMP requirements, permitting the widespread use of hPSCs in basic and/or clinical research.

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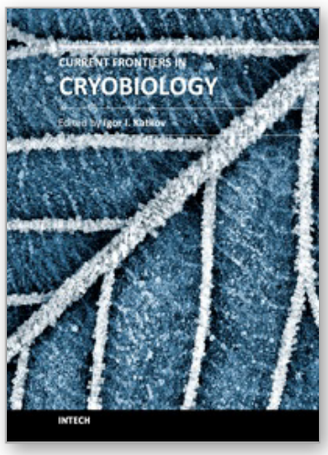

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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