Chilling without regrets

Deciphering the effects of cryopreservation on the epigenetic properties of frozen cells will benefit the applications of cryo-technology

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In 2014, Facebook and Apple announced that they would pay for female employees to have their oocytes frozen to allow them to delay having children and instead focus on their careers. Whatever motivated the companies to make their offers, the fact that they did so highlights a prevalent problem faced by many young women: Their most fertile years are also a crucial period for building a career, when time off work may disadvantage them.

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To fulfill their offers, Facebook and Apple will need to offer their employees access to cryopreservation technologies that profoundly change the dynamics of family planning. Such technologies are not new, but work over the past decades has been aimed at increasing safety and efficacy and has reduced costs to the point that companies can now offer cryopreservation as a way to attract and retain female workers.

Of course, the potential of cryopreservation goes far beyond freezing the eggs or sperm of ambitious young technology workers—it is a ubiquitous technology used in research and medicine for a wide variety of applications (Fig 1). For example, cryopreservation is used to store and transport biological material, including adult stem cells or stem cells from umbilical cord blood or bone marrow—both of which can later be

used to treat disease or extend lifespan in the same patient—blood donations, especially of rare blood types, tissues, and organs. It is also offered as a crucial service for cancer patients to preserve their gametes before they undergo therapy that may render them infertile and, generally used in assisted reproduction to store oocytes, fertilized eggs, or embryos. Cryopreservation can contribute to environmental preservation efforts, where it is used to conserve the tissues or gametes of important or endangered plant and animal species, such as the work of the Svalbard Global Seed Vault in Norway, which stores frozen seeds from important food crops. It is also an important technology for the food industry, which offers frozen vegetables and frozen whole meals.

However, despite the widespread use of cryopreservation for storing cells and tissues, there are concerns about the safety of the procedure [1]. In particular, cryopreservation is known to affect cell survival after thawing, which can have an impact on the subsequent clinical applications of frozen cells. For example, cryopreserved embryos or oocytes are less likely to successfully develop after *in vitro* fertilization compared to “freshly” prepared ones [2,3]. It is therefore important to understand the details of the cryopreservation process and its effect on cellular structures and processes, including genetic and epigenetic effects, in order to increase the safety and effectiveness of many research and clinical applications of cryo-technology.

The cryopreservation of cells and tissues generally uses temperatures below -140°C and a cryoprotective agent (CPA), although some groups have claimed to store cells for long periods at temperatures of around -80°C .

In addition to the deep cold storage, a major damaging aspect of cryopreservation is the cooling process itself, which can lead to cellular injury owing to ice crystals that begin to form both inside and outside cells. Biological materials are therefore often cryopreserved using slow-cooling protocols—during which cells are typically cooled at a rate of $1\text{--}2^{\circ}\text{C}/\text{min}$ in the presence of CPAs. An alternative method is vitrification by quickly increasing the concentration of solutes in the medium—the rapidly increasing viscosity in turn prevents the growth of ice crystals.

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The outcome of both cooling methods in terms of the survival and downstream functionality of frozen cells and tissues depends on various variables including the cell type and shape, the type and dosage of CPA used, the cooling and thawing rates, nucleation temperature, and sample geometry. The commercial use of clinically applicable cells—such as stem cells and reproductive cells—uses cryopreservation protocols in order to meet regulatory standards. However, even optimized, well-established freezing protocols still affect cell survival and functionality after thawing owing to CPA-induced cytotoxicity, intra- and extracellular ice crystals, dehydration, and damage from solution effects.

Figure 1. Various applications of cryopreservation of cells and tissues.

The efficiency and safety of cryopreservation methods is usually assessed by measuring cell survival rates immediately after thawing, but this parameter does not measure the impact of more subtle effects on cellular processes, and in particular on epigenetic mechanisms. Such epigenetic marks control the expression of genes and reflect the influence of developmental and environmental factors. Moreover, epigenetic marks can be passed on to daughter cells through cell division. There is also increasing evidence that epigenetic markers can be passed on through sexual reproduction via gametes and can influence disease risk or even cause disease in the next generation. Epigenetics has become a focus of intense research during the past decade to understand how environmental factors trigger stable changes in gene expression and to find therapeutic interventions against cancer and other diseases by activating or shutting down specific genes. While there is already considerable data about and insight into how nutrition, exposure to toxic compounds, or lifestyle can alter epigenetic markers, there is

very little data and research on the impact of cryopreservation on epigenetic mechanisms in eukaryotic chromosomes.

In terms of inheritance, the most well-known epigenetic mechanisms are DNA methylation and histone post-translational modifications. The first process is implemented by DNA methyltransferases that alter DNA directly by methylating nucleotides, mostly in the promoter regions of gene sequences, to silence the target genes. Chemical modification of histone proteins is done by a suite of enzymes that alter the chromatin structure by condensing or relaxing the tight packaging of DNA around histones. Both DNA methylation and histone modifications regulate gene expression and are important components of cell development and differentiation, switching on or shutting down gene activity when needed.

Various studies suggest that cryopreservation might affect epigenetic mechanisms that alter both DNA methylation status and histone modification. For example, exposure to the widely used cryoprotectant dimethylsulfoxide (DMSO) was shown to alter global

DNA methylation patterns in mouse embryoid bodies [4]. Both slow freezing and vitrification protocols decrease DNA methylation levels in bovine oocytes [5]. Cryopreserved boar spermatozoa show reduced mRNA expression of DNA methyltransferases, lysine acetyltransferase, and protamines [2]. Bovine embryos cryopreserved by either conventional slow cooling or vitrification show a clear decrease in specific DNA methyltransferases [5]. Similar studies also show effects of cryopreservation on histone modification. After somatic cell nuclear transfer in fresh and cryopreserved oocytes, histone 3 lysine 9 (H3K9) acetylation levels appeared to be associated with survival rates of implanted embryos [6]. Elevated levels of acetylated H3K9 were associated with enhanced viability of cloned sand cat embryos. Hyperacetylation of H4 (histone 4) and demethylation of H3K9, two hours after devitrification, has been reported in pig oocytes [7]. These changes might be associated with abnormal fertilization and impaired development. Yet, these are only patchy results and do not provide a clear picture of the extent and impact of epigenetic changes resulting from cryopreservation, or whether the same changes are relevant for human cells or tissues. Some studies even showed that routine cryopreservation of sperm from healthy men had no impact on the DNA methylation status of maternally or paternally imprinted genes, repetitive elements, genes associated with spermatogenesis or male infertility [8].

Of particular interest are the possible effects of cryopreservation on imprinted genes. These genes, the expression of which is determined by their parental origin and tightly controlled by DNA methylation, play a crucial role in embryo development and genomic integrity; imprinting errors have been linked to disorders such as Angelman, Beckwith–Wiedemann, or Prader–Willi syndromes. As genomic imprinting takes place during gametogenesis, it is crucial to understand whether the cryopreservation of gametes and embryos affects imprinting and how. Indeed, there is some evidence that it does: Vitrification of mouse embryos resulted in the loss of methylation at the control regions of the imprinted H19 gene along with subsequent altered H19 expression and studies with bovine 2-cell embryos revealed increased methylation at these imprinting control regions [9].

However, these are just a few studies from different species and different cryopreservation protocols with differing parameters. Such sparse data make it nearly impossible to find, let alone analyse, reliable relationships between the various variables of cryopreservation protocols and epigenetic changes, particularly in human cells and tissues. Moreover, while there is some data on cryopreservation-induced alterations in global DNA methylation and specific histone modifications in reproductive tissue, the biological implications of such effects are largely elusive. Similarly, while IVF seems to increase the risk for some imprinting diseases, it is not yet clear which factors or processes might cause the epigenetic effects and if and how parental genes contribute [10].

“Understanding the process of cryopreservation and identifying the variables that are linked with abnormal epigenetic marks should help to make the procedure safer for patients...”

In addition to its use in reproductive technologies, cryopreservation is also used to store and transport stem cells for clinical use. There are already concerns that genetic and epigenetic changes in stem cells used in medicine could cause those cells to fail to differentiate correctly or lead to cancer, but whether these changes come from the biological processing or cryogenic storage of the cells is not fully clear. In fact, stem cells appear to be particularly sensitive to biophysical cues in their environments in a manner that affects their epigenome and related mechanisms. Studies show that even the surface microtopology of scaffolds used to grow induced pluripotent stem cells can significantly influence their differentiation potential by altered histone H3 acetylation and methylation. And yet, there is a severe dearth of information on how cryopreservation affects stem cells.

We therefore need systematic studies that analyse the effects of cryopreservation on DNA methylation and histone modifications in stem cells, gametes, embryos, and any other clinically relevant cell types. As mentioned above, cryopreservation is a

Box 1: Further reading

General information on cryopreservation

Baust JC, Gao D, Baust JM (2009) Cryopreservation: An emerging paradigm change. *Organogenesis* 5: 90–96
 Schattman GL (2015) CLINICAL PRACTICE. Cryopreservation of oocytes. *N Engl J Med* 2373: 1755–1760
 Petropoulos A, Cattapan A, Baylis F, Leader A (2015) Social egg freezing: risk, benefits and other considerations. *CMAJ* 187: 666–669
 Sparks AE (2015) Human embryo cryopreservation—methods, timing, and other considerations for optimizing an embryo cryopreservation program. *Semin Reprod Med* 33: 128–144

Effects of cryopreservation on stem cells and germ line cells

Hofmann N, Sun H, Chatterjee A, Saha D, Glasmacher B (2015) Thermal pretreatment improves viability of cryopreserved human endothelial cells. *Biopreserv Biobank* 13: 348–355
 Downing TL, Soto J, Morez C, Houssin T, Fritz A, Yuan F et al (2013) Biophysical regulation of epigenetic state and cell reprogramming. *Nat Mater* 12: 1154–1162
 Roberts SA, Hann M, Brison DR (2012) Factors affecting embryo viability and uterine receptivity: insights from an analysis of the UK registry data. *Reprod Biomed Online* 32: 197–206
 Hu W, Marchesi D, Qiao J, Feng HL (2012) Effect of slow freeze versus vitrification on the oocyte: an animal model. *Fertil Steril* 98: 752–760.e3

Potential impact of assisted reproductive technologies on the risk of imprinting-related diseases

Choufani S, Shuman C, Weksberg R (2010) Beckwith–Wiedemann syndrome. *Am J Med Genet C Semin Med Genet* 154C: 343–354
 Buiting K (2010) Prader–Willi syndrome and Angelman syndrome. *Am J Med Genet C Semin Med Genet* 154C: 365–376
 Odom LN, Segars J (2010) Imprinting disorders and assisted reproductive technology. *Curr Opin Endocrinol Diabetes Obes* 17: 517–522
 Amor DJ, Halliday J (2008) A review of known imprinting syndromes and their association with assisted reproduction technologies. *Hum Reprod* 23: 2826–2834

multi-parametric procedure and systematic studies of how individual variables and synergies between multiple factors affect epigenetic marks will help to further improve cryopreservation protocols for individual cell types and their application, making procedures safer for patients.

There is also a more general problem: Even detailed and verifiable information about alterations in global DNA methylation and histone modification levels do not provide knowledge on how this affects the activity of specific genes and how this ultimately influences the biological fate of thawed cells. Despite some knowledge of the effects of cryopreservation on promoter DNA methylation of specific genes—especially imprinted genes—the effects on protein–DNA interactions in the context of histone acetylation and methylation marks have not yet been explored. Next-generation sequencing in combination with methylation-sensitive restriction enzyme treatment, immunoprecipitation of methylated DNA, or bisulfite conversion might help to decipher the impact of specific variables of cryopreservation protocols on DNA methylation. Similarly, chromatin immunoprecipitation

(ChIP)-based methods such as ChIP-chip and ChIP-seq or ChIP-qPCR should be used to analyze genome-wide and promoter-specific histone modifications.

Understanding the process of cryopreservation and identifying the variables that are linked with abnormal epigenetic marks should help to make the procedure safer for patients who are in urgent need of cryopreserved cells or tissues. Such knowledge would also benefit many other socioeconomic and ecological applications of cryopreservation from safer egg banking for working women, to safer food production and better preservation of the genetic diversity of ecosystems.

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Conflict of interest

The authors declare that they have no conflict of interest.

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