CIRM - MRC

Human Somatic Cell Nuclear Transfer

Workshop Report

June 13-14, 2010

CHAPTER 1. EXECUTIVE SUMMARY

Somatic cell nuclear transfer (SCNT), or nuclear reprogramming achieved through transplantation of a somatic cell nucleus into the cytoplasm of an egg, has led to breakthroughs in our understanding of cellular reprogramming and other normal developmental processes. While SCNT was first developed in the 1950s and 60s using frogs (Gurdon, 1962a, 1962b, 1962c) and then successfully applied to mammals in 1996 (Campbell, et al., 1996), the therapeutic possibilities of this technology were not appreciated until after the derivation of the first embryonic stem cells from mouse SCNT embryos (Munsie, et al., 2000), and the availability of human embryonic stem cells (hESC) (Thomson, et al., 1998). These developments led to a new paradigm in regenerative medicine, the idea of creating self-renewing pluripotent stem cell lines which contained an exact copy of a patient's own nuclear genetic material. In spite of this promising beginning, human SCNT (hSCNT) remains challenging and it has not yet led to the production of any pluripotent stem cell lines. Meanwhile, increased understanding of the molecular mechanisms that govern cell fate has led to recent breakthroughs in our ability to reprogram human somatic cells by other methods, such as generation of induced pluripotent stem cells (iPSCs) by forced expression of transcription factors (Takahashi, et al., 2007; Takahashi & Yamanaka, 2006; J. Yu, et al., 2007). Thus, the role of hSCNT in a new world of factor-mediated reprogramming needs to be reexamined in light of the significant technical and ethical challenges facing the field.

In June 2010, the California Institute for Regenerative Medicine and the Medical Research Council of the United Kingdom called a joint meeting to examine the topic of hSCNT and its role in stem cell research. This workshop brought together many of the preeminent researchers in the field of SCNT to discuss whether SCNT is achievable in humans, what challenges exist, and whether hSCNT offers any scientific or medical advantages over other methods of cellular reprogramming. In the following report, we document the topics discussed at this workshop and summarize the conclusions that emerged from these discussions.

This meeting focused on scientific aspects of SCNT research. Non-scientific considerations, including policies governing the use of human oocytes in research, were discussed in terms of their impact on SCNT studies, but the meeting was not intended to analyze or propose changes in those areas.

OUTCOMES

Status of the Field

SCNT has been achieved in many different animals, from sheep to non-human primates, and has led both to cloned organisms and to the production of ESC. In humans, the technique has proved technically challenging and a unique set of hurdles has precluded the derivation of viable stem cell lines from hSCNT embryos. Speakers presented the most recent advances, and suggested that the technical challenges to achieving hSCNT are being addressed through blossoming collaborations within the field. They highlighted knowledge gained from working with non-human primates and other animals, and stressed the importance of sharing information across the few laboratories that are still pursuing hSCNT. In addition new laboratory methods that might increase the availability of large numbers of human eggs (oocytes) were presented. Finally, participants pointed to several non-scientific issues that have impacted progress in the field, including funding constraints, the limited availability of occytes for research use, and geographical constraints that limit opportunities for collaboration. Overall, researchers were

confident that hSCNT could be achieved and consideration should be given to addressing both scientific and non-scientific challenges.

Potential Mechanistic Insights and Therapeutic Benefits from SCNT Research

Participants highlighted several potential benefits that could emerge if SCNT were to be achieved in humans. First, hSCNT research could lead to important breakthroughs in our mechanistic understanding of pluripotency and cell fate regulation. The reprogramming events involved in hSCNT could provide important insights into the molecular mechanisms regulating nuclear reprogramming, and could help optimize methods of inducing pluripotency by other means.

Workshop participants noted that SCNT, the derivation of iPSCs, and other forms of reprogramming represent relatively new technologies that have not been thoroughly explored, and it is not yet clear which method would be most appropriate for understanding or treating a specific human disorder. It is possible that iPSCs or cells reprogrammed directly from one differentiated cell type to another differentiated cell type may be more restricted than SCNT-derived lines in their ability to produce certain cell lineages, thereby limiting their therapeutic potential. Participants acknowledged that substantial improvements in the efficiency of SCNT are required before it could be considered a viable therapeutic option. However, given that our knowledge of reprogramming is in its infancy, it is still possible that hSCNT will emerge as the technology of choice for specific human stem cell therapies or for developing particular disease models.

SCNT might be therapeutically important for certain disorders, such as mitochondrial diseases, with no alternative therapeutic options. The potential application of SCNT technology for mitochondrial disorders was thoroughly discussed at the workshop. During reproduction, mitochondria are transmitted cytoplasmically from the mother; and during cell division, multiple organelles are randomly segregated to daughter cells. Human mitochondria carry some of their own DNA, which encodes 13 proteins of the respiratory chain. Mutations in these genes lead to several mitochondrially transmitted diseases (see chapter 4.3) such as Leber's hereditary optic neuropathy (LHON) and Myoclonic Epilepsy with Ragged Red Fibers (MERRF). The heterogeneity and random transmission of mitochondria during cell division means that mitochondrial disorders are very difficult to prevent or eliminate. In theory, the transfer of the nuclear genetic material from an egg carrying mitochondrial defects into an enucleated egg with normal mitochondria can be used to prevent the transmission of mitochondrial defects from a mother to her child. Mitochondrial replacement was recently achieved in non-human primates, resulting in reconstructed oocytes capable of fertilization and generation of healthy offspring (Tachibana, et al., 2009). Optimization of hSCNT techniques may advance this approach, termed spindle transfer, as a therapy for replacing diseased mitochondria in an affected human egg with healthy ones (Craven, et al., 2010; Gardner, et al., 2007).

A further reason to continue hSCNT research rests in its potential to shed light on the earliest stages of human development. Even the first few events after fertilization differ among species (Haaf, 2006), and increasing evidence suggests that subtle defects in these early stages of development can have serious repercussions on health and viability (Reefhuis, et al., 2009). Although some of these events can be studied using other *in vitro* models, hSCNT could be particularly useful for understanding the influence of genetic background on early development and help us understand environmental and genetic contributions to developmental disorders.

This research could lead to improved treatments for reproductive failure and insights into the major causes of birth defects in humans.

Alternative techniques for deriving patient-specific or immune-matched cells

The discovery, half a century ago, that egg cytoplasm can convert a nucleus from a differentiated cell type into that of a totipotent cell opened up new therapeutic possibilities. Autologous cell therapy could now be envisioned for a wide range of disorders. For example, hESCs derived from embryos generated by SCNT could in principle be immunogenically matched to the nucleus donor, thus allowing cell therapy without immune suppression. In addition, previously inaccessible cells such as neurons or glia could be derived by hSCNT from patients with relevant diseases, which would allow the development of new human *in vitro* disease models. The success of SCNT in animals thus led to a new therapeutic paradigm based on the concept of patient-derived pluripotent stem cells.

As hSCNT remained elusive, however, several alternative methods were considered for generating these pluripotent stem cells. Currently, transcription factor-induced reprogramming (iPSC technology) is the major alternative to SCNT, but a number of other techniques have also been explored over the years and could potentially be considered for specific research and therapeutic applications. At the workshop, speakers presented data on SCNT achieved using a nuclear donor from one species and an egg donor from another (interspecies SCNT), and discussed the potential of this approach for human applications. Other speakers debated the properties of stem cells derived from an unfertilized egg, or human parthenogenetic stem cells. Finally, one group presented a thorough evaluation of the possibility of using one-cell human zygotes as recipients for SCNT. Although each of the techniques presented some unique advantages, participants agreed that SCNT and iPSC technologies remain the most viable options for deriving medically relevant, immune-compatible stem cells.

RECOMMENDATIONS AND FUTURE PROSPECTS

Much of the SCNT Workshop centered on the technical and ethical obstacles to achieving hSCNT, and on a discussion of approaches to overcome or address these obstacles.

Technically, hSCNT has proved challenging. Nevertheless, workshop participants were very positive about the robust experience with animal SCNT. In addition, there was hope that current limitations in the availability of human oocytes might be overcome through the potentially revolutionary approach of deriving mature oocytes in the laboratory from more available, immature human gametes, which could speed up technical optimization of hSCNT. Participants stressed the importance of collaboration and sharing of technical knowledge between researchers working with different animal models and with different sources of human eggs. They expressed a strong belief that SCNT can be achieved in humans.

In addition to technical issues, a lack of funding and the legal restrictions to accessing suitable oocytes in many areas of the world were highlighted as roadblocks to success for hSCNT. Significantly, it was perceived that in some agencies reviewers do not tend to consider hSCNT a priority on review panels. On the other hand, hSCNT is complicated by ethical and political restrictions on work with human eggs. Given the constraints, researchers proposed that funding a concerted collaborative effort to achieve hSCNT would be the most effective means of

supporting the field. This funding should occur relatively rapidly, as researchers with experience working with hSCNT are being driven away from the field due to logistical issues.

CONCLUSIONS

In conclusion, this workshop highlighted the important roles for hSCNT research in understanding nuclear reprogramming and in therapeutic development. hSCNT could lead to the identification of pathways or molecules important for effective reprogramming. In addition, it may offer unforeseen advantages over human (h)iPSCs for certain applications, and a related technology, spindle transfer, remains the only putative therapeutic option for specific disorders such as mitochondrial disease. Finally, hSCNT could provide insights into some of the molecular, epigenetic and functional characteristics of the earliest stages of human development under normal and disease conditions. The technical and logistical challenges to achieving hSCNT would be best addressed through a collaborative consortium of researchers from around the world that could bring different comparative advantages to bear on the technique. Overcoming the barriers to hSCNT is probably achievable and continues to be an important gap in our quest for improved knowledge of human development and regenerative medicine.

CHAPTER 2. INTRODUCTION AND BACKGROUND TO THE SCNT WORKSHOP

During normal development, the epigenetic modifications associated with germ cell identity are reset to those associated with toti- and then pluripotency following fertilization; this process of genomic reprogramming is essential for generating the diversity of cells required to produce an organism. In the 1950s, Briggs and King showed that transplanting the nucleus of an early embryonic cell (blastomere) into the cytoplasm of an enucleated egg could support the development of normal tadpoles (Briggs & King, 1952). Soon after, John Gurdon was able to generate cloned frogs from tadpole cells using somatic cell nuclear transfer (SCNT) (Gurdon, 1962a, 1962c), illustrating that reversion of a differentiated somatic cell nucleus to an undifferentiated totipotent state is possible. Extensive research over the next 40 years led to the development of techniques to clone mammals from blastomeres, and eventually to the cloning of a sheep named Dolly from an adult cell using SCNT (Campbell, et al., 1996; Wilmut, et al., 1997). Since Dolly, researchers have cloned several small and large animals including mice, cows, pigs, goats, rabbits, and gaurs, indicating that SCNT is a viable reprogramming technique for many organisms (Lewis, et al., 2000; Niemann & Kues, 2007).

SCNT was proposed as a possible means of deriving hESC genetically identical to patients, which could be used to develop *in vitro* disease models and for cell therapy. Dr. Renee Reijo Pera commented that hSCNT also offers an exciting opportunity to understand the earliest stages of human development and may provide a unique tool for studying disorders relevant to the first few cell divisions in the embryo. However, very few reports document SCNT in humans (French, et al., 2008; Lavoir, et al., 2005; McElroy, et al., 2008; Stojkovic, et al., 2005; Y. Yu, et al., 2009), and there are no published reports of hESC lines derived following SCNT. In this Human SCNT Workshop, CIRM and the MRC sought to re-evaluate the importance and priorities of hSCNT and related technologies in research and human therapeutic development. Participants discussed both the technical hurdles to achieving robust SCNT in humans, and the potential benefits that hSCNT might bring.

2.1. Totipotency, pluripotency and tetraploid complementation

Throughout the workshop, participants commented on the fact that SCNT can lead to reprogramming of somatic nuclei to totipotency, whereas other methods of reprogramming yield pluripotent stem cells instead. Totipotency refers to the ability of a cell to produce all the differentiated cells in an organism, plus extraembryonic tissues. For example, the one cell zygote is a totipotent cell, which, upon transfer to a receptive uterus, can produce a viable offspring. Pluripotency refers to the ability of a cell to generate cells from all three germ layers (endoderm, mesoderm and ectoderm). Pluripotent stem cells can give rise to any cell type in the fetal or adult body, but they cannot develop into a fetus on their own because they lack the potential to create some of the extraembryonic tissues such as the trophoblast cells of the placenta.

The most stringent test of pluripotency in mouse is a tetraploid complementation assay (Kubiak & Tarkowski, 1985; Nagy, et al., 1990; Nagy, et al., 1993; Tam & Rossant, 2003). In this assay, an embryo at the two-cell stage is fused to produce one tetraploid cell, which contains four copies of each chromosome, rather than two. Such tetraploid embryos, when transplanted to a uterus, can form all of the extra-embryonic tissue required for supporting fetal growth and can form blastocysts, but do not survive beyond mid-gestation. However, if diploid pluripotent stem

cells are mixed with tetraploid embryos at the morula or blastula stage, the resulting embryos then develop normally; the fetus is exclusively derived from the diploid pluripotent stem cells, supported by extra-embryonic tissues derived from the tetraploid embryo.

2.2. Reproductive and therapeutic cloning by SCNT

SCNT-derived blastocysts have been used to generate embryonic stem cells (SCNT-ESC) in mice and non-human primates (Byrne, et al., 2007; Cram, et al., 2007; French, et al., 2006; Markoulaki, et al., 2008; Rideout, et al., 2002). These SCNT-ESCs are very similar to ESCs derived from normal embryos (Brambrink, et al., 2006; French, et al., 2006; Kim, et al., 2010) and can be used to generate cells for cell therapy in mice (Rideout, et al., 2002). Using SCNT to generate ESCs for the purpose of replacing damaged tissues or cells is referred to as cell nuclear replacement (CNR) therapy or therapeutic cloning, and, together with developing models of disease, is the focus of most of the research on human SCNT. However, to date there are no published reports of successful derivation of hESCs by SCNT.

Although the goal of this workshop was to consider the use of human SCNT for therapeutic cloning purposes, much of the research that was discussed involved the *reproductive* cloning of animals. Reproductive cloning refers to the duplication of an organism using SCNT. Reconstructed eggs that successfully develop into a blastocyst after SCNT of a somatic nucleus can be transplanted into a receptive uterus, where they generate viable progeny that are genetically identical to the nucleus donor. Reproductive cloning in animals was essential to proving that the genetic material from an adult cell can be fully reprogrammed to a totipotent state. It also proves that the egg contains all of the factors necessary to reprogram a nucleus, which is yielding important information about the molecular mechanisms involved in reprogramming. Although animals produced by SCNT often have health issues, their progeny appear to be normal indicating that SCNT is capable of almost complete developmental reprogramming (Watanabe & Nagai, 2009). Workshop sponsors and participants concurred that research aimed at reproductive cloning to produce a human genetically identical to a donor is ethically unacceptable and should not be conducted. Human reproductive cloning is illegal in California and the UK.

2.3. Progress and remaining questions in nuclear reprogramming of somatic cells

SCNT was championed as a promising means for deriving patient-specific hESCs, which could be used both to develop cellular models of intractable human diseases as well as for potential cell therapy applications. The potential of SCNT to develop patient-derived cells has been stalled, however, as hSCNT is technically challenging and access to a critical resource, human eggs, is very limited. In the mean time, factor-mediated reprogramming of human somatic cells toward pluripotency by making hiPSCs has become relatively straightforward and is easily performed in many labs. A similar method of direct reprogramming was recently used to convert fibroblasts directly into functional neurons and cardiac cells in mouse (Ieda, et al., 2010; Vierbuchen, et al., 2010), and it is likely this will soon be achieved with human cells. hiPSCs reprogrammed by transduction with transcription factors are unspecialized, they regenerate themselves (self–renew), and they can be differentiated into derivatives of the three primary germ layers – endoderm, mesoderm and ectoderm - both *in vitro* and *in vivo*, suggesting that they are functionally pluripotent. hiPSCs have been generated from patients carrying a host of genetic disorders, and many laboratories are using them to develop *in vitro* models of human diseases. In addition, iPSC-derived cells have been used successfully for cell therapy in animal models (Swistowski, et al., 2010). In light of these stunning advances in nuclear

reprogramming, it is necessary to re-examine the role of human SCNT in basic mechanistic research and in therapeutic development.

As various methods are being developed to derive pluripotent stem cells, an important question revolves around their equivalency, which can be most rigorously analyzed using animal models. ESC derived from fertilized mouse embryos (fESC) serve as the gold standard for pluripotent stem cells, and their ability to give rise to mice entirely comprised of ESC derivatives (all-ESC mice) in the tetraploid complementation assay proves their pluripotency. Although SCNT-derived (cloned) animals exhibit developmental abnormalities in many species, ESC derived from SCNT embryos (SCNT-ESC) in the mouse are functionally indistinguishable from fESC, and are capable of producing normal all-SCNT-ESC mice (Brambrink, et al., 2006). Initial analysis of iPSCs suggested that they were functionally and molecularly distinct from fESC, as they were not successful in the tetraploid complementation assay and, especially at low passage number, they retain epigenetic memory of the original mature donor cell (Polo, et al., 2010). However, recent progress in factor-mediated reprogramming has led to the generation of some iPSC lines that are more similar to fESC and that are capable of producing all-iPSC mice by tetraploid complementation (Kang, et al., 2009; Stadtfeld, et al., 2010). Comparing stem cells derived in these different ways has allowed researchers to derive more fully pluripotent stem cells. Workshop participants commented that comparisons of fESCs, SCNT-ESCs and iPSCs will continue to help scientists optimize methods in the dynamic field of cellular reprogramming.

Another important question in stem cell biology is whether nuclear reprogramming *in vitro* can yield stem cells with appropriate epigenetic phenotypes. Epigenetic differences in stem cells produced by different methods can affect the cellular products derived from these cells, as well as influence their utility for developing disease models. For example, Nissim Benvenisty's group has shown that hiPSCs may not be useful for modeling the acquisition of the Fragile X disease phenotype (Urbach, et al., 2010). Although neurons derived from hiPSCs from Fragile X patients fail to express FMR1, a defect that recapitulates a neuronal phenotype in Fragile X patients, undifferentiated hiPSCs from these patients do not reactivate the FMR1 gene. Therefore, hiPSCs cannot model the differentiation-dependent epigenetic silencing of the FMR1 locus. This is in contrast to hESCs derived from embryos identified by preimplantation genetic diagnosis to carry Fragile X, which do express the gene and silence it upon differentiation *in vitro*. These observations suggest that hiPSCs might not be completely appropriate models for specific diseases with epigenetic contributions. As our knowledge of cellular dedifferentiation and nuclear reprogramming increases, it may be possible to ensure that epigenetic information relevant to a particular disease model is preserved during reprogramming.

In conclusion, human SCNT has the potential to contribute important insights for cellular reprogramming and the treatment of human disease. Importantly, further mechanistic research is needed to understand nuclear reprogramming, and to understand the relative merits of SCNT-ESCs, iPSCs and cells derived through direct reprogramming from one differentiated cell type to another.

WORKSHOP ON HUMAN SCNT

The objectives of this workshop were: **a**) to re-evaluate the role of human SCNT in research and therapeutic development; and **b**) to identify roadblocks to achieving human SCNT. To meet these goals, CIRM and the MRC invited leading experts in human and animal SCNT to discuss progress and remaining hurdles to human SCNT.

HUMAN SCNT WORKSHOP REPORT

The Human SCNT Workshop took place over two days, and was organized into a series of panels that addressed specific areas of relevance (see Workshop Agenda, page 34). Panelists gave brief presentations and participated in moderated discussions on the relevant topic. This report summarizes the results of these discussions.

CHAPTER 3: NUCLEAR REPROGRAMMING AND SCNT

The concept of duplicating an organism from a somatic nucleus was first proposed as a "fantastical experiment" by Hans Spemann in 1938. Twenty-four years later, John Gurdon achieved this breakthrough in frogs and reported the first successful cloning by transferring a nucleus into an egg, thus opening the door to the field of somatic cell nuclear transfer. Appropriately, Sir John Gurdon opened the CIRM - MRC Human SCNT Workshop with a lecture on the past and present of nuclear transfer, and thus began the discussion on the future of SCNT.

3.1.1 Key questions in nuclear reprogramming

Sir John Gurdon presented an overview of how the development of a fertilized egg into a multicellular organism occurs through a series of cell divisions, and progressive commitment of daughter cells to different cell lineages and eventually to a stable cell fate. During the process of cellular differentiation, changes in chromatin modifications and transcription factor expression alter the pattern of gene expression from that of a naïve pluripotent cell to that of a differentiated cell that only produces proteins relevant to a specific cell type. In animals, cell differentiation is generally thought to be a one-way process, as differentiated cells cannot normally revert to a progenitor cell fate. However, cell differentiation must be reversed for reproduction to occur. DNA that supports germline-specific gene expression in the egg and sperm is, upon fertilization, converted to naïve DNA with the potential to express genes from every cell type in the body. Converting a nucleus with a restricted pattern of gene expression and a specified cell fate into a nucleus with a different capacity for gene expression and more general cell fate potential is called nuclear reprogramming.

We are beginning to replicate nuclear reprogramming in the laboratory. Developmental biologists have shown that the cytoplasm of an egg is capable of reprogramming adult somatic cells into totipotent cells, and transfer of a nucleus from an adult animal into an egg cytoplasm has led to successful reprogramming in many different species including mouse, sheep, cow, gaur, pig, rabbit, and dog (Lewis, et al., 2000; McEvoy, et al., 2003; Niemann & Kues, 2007). In addition, expressing a handful of select factors, mostly transcription factors, in differentiated cells can also lead to nuclear reprogramming, either directly into another differentiated cell type (Davis, et al., 1987; Ieda, et al., 2010; Wernig, et al., 2007; Zhou, et al., 2008) or into pluripotent stem cells (Muller, et al., 2009; Takahashi, et al., 2007; Takahashi & Yamanaka, 2006; J. Yu, et al., 2007). Such induced pluripotent stem cells (iPSCs) are substantially similar to pluripotent stem cells derived from the inner cell mass of blastocyst stage embryos (ESC) (Chin, et al., 2010; Guenther, et al., 2010; Newman & Cooper, 2010). Importantly, some iPSC lines are capable of reconstituting a mouse in the most rigorous assay of pluripotency, the tetraploid complementation assay (Stadtfeld, et al., 2010). The advent of human iPSCs has allowed for the derivation of previously inaccessible human cell types from adult patients with a host of disorders. These advances have led to an explosion of research on stem cells, and to an interest in using reprogrammed cells to improve the study and the treatment of human diseases.

In spite of this progress, our knowledge of the mechanisms that control nuclear reprogramming remains limited. Factor-mediated reprogramming techniques suffer from very low efficiency and require several weeks of culturing. Workshop participants cautioned that accumulation or even selection of detrimental mutations may occur during prolonged cell culturing. Furthermore, iPSC generation is most efficient when the factors, which include oncogenes, are introduced using genome-integrating vectors. Such iPSC are not considered safe for use in humans and

alternate techniques for factor delivery or activation are under investigation, with a recent breakthrough reported using synthetic modified mRNA (Warren, et al., 2010). Eggs contain all of the cellular factors needed to reprogram somatic nuclei to totipotency in a rapid and relatively physiological fashion, without the use of oncogenes or integrating viruses. ESCs derived from SCNT mouse embryos are competent for tetraploid complementation and give rise to normal mice using that approach, suggesting that mouse ESC derivation following SCNT in this species selects for faithfully reprogrammed cells (Brambrink, et al., 2006; Kim, et al., 2010). Dr. Hans Schöler mentioned that in his hands, SCNT-ESC exhibit a far better developmental potential than iPSC. For these reasons, workshop participants stressed that understanding the mechanisms regulating SCNT might lead to important advances in our understanding of nuclear reprogramming and improve the procedures for generating iPSCs.

It is important to point out that, in spite of certain advantages apparent through comparing SCNT-derived ESCs with iPSCs, SCNT does not faithfully recapitulate normal developmental reprogramming. Animals produced by SCNT suffer from abnormally high levels of developmental defects and unexplained deaths, which can be reversed after a round of normal reproduction (French, et al., 2006; Kubota, et al., 2004; Panarace, et al., 2007). However, workshop participants agreed that the benefits originally envisioned of hSCNT were real, and it was too early to determine whether hiPSC- or hSCNT-derived cells will ultimately be more useful for studying or treating human disorders.

3.1.2 Mitochondria

Studies of developmental biology and cellular reprogramming have led to significant insights as to how the actions of specific gene networks contribute to cell fate and identity. Much less is known, however, about the role mitochondria play in these processes and how they are, in turn, affected by them. To explore this topic, Dr. Eric Shoubridge provided an overview of mitochondrial biogenesis and behavior as it relates to mammalian development and cellular function. These details provided the necessary context to discuss an interesting set of observations made by Drs. Mitalipov, Campbell, Cibelli and others in the course of their studies on animal cloning.

As the energy producers of the cell, mitochondria hold a special place in eukaryotic cell biology through a long, shared history with the nuclear genome. While possessing their own DNA (mtDNA), mitochondria have evolved species-specific mutual dependencies with their host cell nuclei whereby nuclear and mitochondrial genomes must work together to coordinate energy production for the cell. Equally important is the fact that in many cell types, mitochondrial replication has become uncoupled from nuclear division. Instead, mitochondria replicate their DNA and divide in response to the energetic needs of a cell. Mitochondria can be quite dynamic and often undergo fission and fusion with one another, which leads to sharing of DNA contents as well as variations in the total number of individual mitochondria within a given cell. These properties lend a stochastic nature to mitochondrial inheritance whereby daughter cells receive their mitochondrial complement from the cytoplasm of the mother cell at the time of cytokinesis. If the mother cell contains mitochondria with differing genetic contents, a phenomenon known as heteroplasmy, cells may inherit differing pools of mitochondria.

The mutation rate of the mitochondrial genome is much higher than that of the nucleus, possibly due to its proximity to damaging free radicals produced by the electron transport chain. While certain DNA repair mechanisms are known to be active, mtDNA will inevitably accumulate mutations with age, many of which will have deleterious consequences on cell health and

viability. Mitochondrial heteroplasmy can develop when individual pools of mitochondria harbor differences in their genetic content due to spontaneous accumulation of mutations within a subset of the organelles. Depending on the energetic state of the cell and other mitigating factors, certain mtDNA mutations may have selective advantages over others, leading to preferential accumulation or loss of specific sequences over time. In situations where intragenic complementation occurs or when the cells' energy needs are low, the effects of mtDNA mutations may go unnoticed and may be propagated without immediate consequence.

SCNT necessitates the introduction of a nuclear genome to a potentially foreign mitochondrial environment. Oocytes, which are the typical vesicle for nuclear transfer, contain a very high but variable number of mitochondria. These numbers reflect an extensive amplification that occurs during female germline development, which is thought to ensure that daughter cells inherit sufficient number of organelles through multiple rounds of subsequent division (Shoubridge, 2000; Shoubridge & Wai, 2007). Prior to the primordial germ stage, however, germ cells and gametes do not replicate their mtDNA and thus become progressively depleted of their mitochondrial content. At the same time, the energetic requirements of these cells are minimal, providing little basis for negative selection against mitochondria with compromised function. While evidence from animal studies suggests that there is a "filter" of unknown mechanism that eliminates the most devastating classes of mitochondrial mutation from the germline (Fan, et al., 2008; Stewart, et al., 2008), it is not uncommon for detrimental mitochondrial mutations to segregate invisibly to daughter cells only to be revealed later in development. The implications of heteroplasmy and stochastic transmission on mitochondrial disease will be discussed further in a subsequent section.

After reviewing the state of the field, panelists discussed a number of uncertainties that could impact the overall potential and utility of SCNT with respect to research and practice. There is no doubt that under some circumstances, mitochondrial mutations and heteroplasmy could lead to deleterious effects in cells derived from SCNT. However, some panelists asserted that there are additional concerns relating to the origin of the organelles themselves. They argued that mitochondria from somatic cells may not be compatible with early development due to poorly understood changes in mitochondrial character that may occur as cells differentiate. They posited that such changes act as a "biological clock" that could interfere with accurate resetting of the developmental program. They further believed that mitochondria of oocytes are maintained in a perpetually youthful (developmentally potent) state that is key to a cell's ability to support normal development. Other panelists expressed skepticism however, citing a lack of evidence to support this hypothesis over alternative explanations.

In addition to developmental competence, panelists questioned the extent to which differences between nuclear and mitochondrial background would affect the fitness and fate of reprogrammed cells. Most evidence to date suggests that mitochondria from one species are unable to be maintained in the cellular context of another, largely due to bioenergetic incompatibilities or an inability to functionally integrate with the host cell genetic program (Beyhan, et al., 2007; Kenyon & Moraes, 1997; Yoon, et al., 2007). While species-specific compatibility is generally accepted, there is growing evidence that variations in mitochondrial haplotypes are correlated with differences in developmental potential of SCNT-derived embryos (Ferreira, et al., 2009; Ferreira, et al., 2007; Yan, et al., 2010). On a related note, Dr. Keith Campbell shared his observation that genetically distinct, cloned sheep showed differences in mitochondrial enzyme levels, despite possessing identical organelle haplotypes. It was clear from these discussions that without a better understanding of the intricacies of such relationships, it will be difficult to factor in the additional consequences of donor mitochondrial

carryover when evaluating merits and risks of the SCNT procedure. There is also uncertainty as to whether parental imprinting of nuclear genes might play any role in compatibility or fitness of a specific nuclear-mitochondrial combination.

In conclusion, the panelists agreed that mitochondrial biology represents a significant hurdle to our understanding of SCNT and other forms of cellular reprogramming, both from a mechanistic standpoint as well as a practical one. Unfortunately, the lack of molecular tools and genetic models has greatly slowed our ability to understand many of the fundamental questions that must be addressed before we can fully realize the potential that these techniques have to offer.

3.2. SCNT in animals: what we have learned

One key advantage of SCNT is that it allows us to study nuclear reprogramming in a physiological context. Sir John Gurdon highlighted experiments using frog eggs that are resulting in important advances in our understanding of the mechanisms regulating nuclear reprogramming. Immediately following, Dr. Campbell led a session to explore the lessons learned from achieving SCNT in different animal species. Overall, participants presented exciting breakthroughs in our understanding of the molecular aspects of nuclear reprogramming that are being made through SCNT research, and explained the potential impact that these discoveries could have on the field of human somatic cell reprogramming. In addition, they highlighted some of the technical factors that contribute to effective SCNT and that could affect factor-mediated nuclear reprogramming.

3.2.1. Molecular mechanisms of nuclear reprogramming

SCNT is being used to answer questions about the molecular regulation of nuclear reprogramming. An essential aspect of reprogramming is converting a highly repressed chromatin with a restricted potential for gene expression into a decondensed chromatin with more unrestricted gene expression potential. Sir John Gurdon presented data supporting the importance of the oocyte-specific linker histone B4 for chromatin decondensation during reprogramming of mouse nuclei in a frog oocyte model. The somatic linker histone H1 is replaced by B4 within 2-3 hours of nuclear transfer, and B4 inhibition abrogates activation of pluripotency genes (Jullien, et al., 2010). This rapid histone exchange is consistent with the rapid reprogramming kinetics observed during SCNT. Dr. Gurdon also discussed experiments using frog eggs that suggest that the activity of histone H3.3, which is abundant in eggs and responsible for maintaining active genes in an open chromatin state, may contribute to the maintenance of differentiation-specific gene expression from transferred somatic nuclei, thus enabling epigenetic memory (Ng & Gurdon, 2008). Such gene activity is detrimental to proper early embryonic development, and is thought to contribute to developmental failures observed in many SCNT embryos. By contrast to SCNT embryos, mouse ESCs derived from SCNT embryos (SCNT-ESC), although they maintain some epigenetic memory, generally support normal development in the tetraploid complementation assay (Brambrink, et al., 2006; Kim, et al., 2010).

A second essential mechanism that occurs during reprogramming involves the upregulation of pluripotency markers, such as the transcription factors Nanog, *Oct4* and *Sox2*. This induction occurs with faster kinetics following SCNT than during factor-mediated reprogramming, e.g. in mouse as early as the one cell stage for SCNT, as compared with 14-21 days for iPSCs (Hanna, et al., 2009). However, Dr. Schöler pointed out that *Oct4* expression in mouse SCNT-derived blastocysts is often abnormal, which correlates with the developmental failures of SCNT

embryos, while normal *Oct4* signal in SCNT blastocysts correlates with their ability to form SCNT-ESC (Boiani, et al., 2002). Experiments such as these are yielding important mechanistic information about the processes controlling nuclear reprogramming during normal development as well as those that drive reprogramming during SCNT. Sir John Gurdon and other researchers in the SCNT field are exploring how egg-specific factors, including transcription factors, are involved in the reprogramming process.

Research into the molecular mechanisms regulating nuclear reprogramming and SCNT will impact factor-mediated methods of generating stem cells. The factors used currently to convert differentiated cells into pluripotent stem cells were originally identified by comparing the proteins and RNAs in differentiated cells with those of ESCs (Takahashi & Yamanaka, 2006; J. Yu, et al., 2007). Current factor-mediated iPSC techniques developed from these findings are of relatively low efficiency and often lead to incomplete reprogramming, and several of the transcription factors used to reprogram are oncogenic. An important area of scientific exploration in the SCNT field is the identification of factors in the egg cytoplasm that permit nuclear reprogramming, and conversely the search for factors in the cytoplasm of differentiated cells that repress reprogramming and stabilize differentiation. For instance, Dr. Jose Cibelli described a research strategy to identify reprogramming factors using SCNT in zebrafish, which is an excellent genetic model that until recently had not been used for SCNT. Dr. Shoukhrat Mitalipov indicated that OCT4, a transcription factor commonly used for the generation of iPSC, does not seem to be expressed at high levels in his non-human primate model of SCNT. His laboratory is trying to identify other molecular factors that are important for SCNT reprogramming, which could yield more efficient and safer reprogramming techniques for human cells.

3.2.2. Technical optimization of SCNT

SCNT is a challenging technique, and important information about reprogramming is learned in the process of overcoming technical hurdles. Dr. Mitalipov pointed out that in primates, including humans, donor cell preparation techniques and developing less invasive protocols are as important to achieving SCNT as egg quality. Dr. Cibelli stressed that cellular conditions such as epigenetic state of the donor cell have also been reported to affect reprogramming. Both groups are in the process of identifying experimental variables that contribute to high efficiency SCNT, and they suggested that some of the resulting outcomes might be transferable to iPSC reprogramming. As researchers are trying to identify cellular factors that facilitate reprogramming in model systems, utilizing in particular the large eggs of frogs and the genetically tractable zebrafish, we need to keep in mind that some factors might be of different importance in different species. Many participants were of the opinion that efforts to understand human reprogramming would benefit from working out the conditions for human SCNT.

Comparing cells produced through SCNT and iPSC technologies could lead to important cell biological insights and a better understanding of the value of each technique. For instance, it appears that iPSCs are more likely to retain epigenetic memory of the donor cells than ESCs derived following SCNT (Kim, et al., 2010; Polo, et al., 2010). If this is the case, SCNT-ESCs might be better for studying the developmental progression of a disorder that involves the reproducible acquisition of epigenetic errors. On the other hand, iPSC might be better at preserving clinically relevant epigenetic changes that have occurred in patients, but whose acquisition may not be recapitulated in development achieved *in vitro*. Much of the research comparing SCNT and iPSC is being conducted in mouse, but as discussed below there may be considerable value to exploring these differences in human cells as well.

CONCLUSIONS

Both SCNT and iPSC technologies are useful for producing pluripotent stem cells from somatic cells. It is unclear which technology will be more useful for therapeutic development, and the answer may differ for different disorders. Comparing SCNT and iPSCs will deepen our understanding of the mechanisms regulating nuclear reprogramming, and will expand the options available for understanding and treating human disease.

CHAPTER 4. HUMAN SCNT: PROGRESS, PROMISE AND ROADBLOCKS

Dr. Reijo Pera emphasized that human development differs in important aspects from that in animals, and Dr. Campbell pointed out that one of the outcomes of SCNT research has been our recognition of species-specific differences in the earliest stages of development. SCNT in each species requires unique technological breakthroughs, suggesting that reprogramming is slightly different among animals. Furthermore, human and mouse ESCs express some different protein markers and require different growth factors for their maintenance, and, similarly, there have been some published differences between human and mouse cells reprogrammed by iPSC methods (see (Loh & Lim, 2010). For these reasons, most participants in the workshop agreed that human SCNT would have a significant impact on our ability to control reprogramming in human cells.

Workshop participants were of the strong opinion that further research is required to establish the potential of hSCNT. There may be therapeutic advantages to cells produced through hSCNT as compared to hiPSC or direct reprogramming from one differentiated cell type to another. For instance, a technique related to SCNT, spindle transfer, is unique in its potential to interrupt the mother-to-child transmission of mitochondrial disorders. In addition, stem cells produced using different methods or from different cell populations might be functionally distinct, and Dr. Irv Weissman cautioned that such differences may include detrimental cellular behaviors that may not be apparent until the cells are differentiated into certain cell types. Considerations such as these indicate that further comparisons of hSCNT and hiPSC are warranted, given that the relative therapeutic value of these cells is still uncertain.

In this section, we summarize the workshop participants' discussion on the state of hSCNT research, roadblocks to achieving SCNT in humans, and the potential therapeutic value of hSCNT technology for treating mitochondrial disorders.

4.1. Status of human SCNT (hSCNT)

Research into hSCNT has been challenging technically, politically, and ethically. Current methods of hSCNT are unreliable, and there are no published reports of human cell lines produced by SCNT. In the session on human SCNT, Dr. Jeannie Fontana and Dr. Sam Wood presented results of the only hSCNT experiment published to date that has resulted in successful reprogramming of adult human somatic cell nuclei by human oocytes, as assessed by DNA fingerprinting (French, et al., 2008). Progress by this group since the paper's publication in 2008 has been severely limited due to lack of funding. Dr. Mary Herbert presented preliminary data on the optimization of hSCNT methods, based on collaboration with Dr. Mitalipov who developed conditions for SCNT in non-human primates. Dr. Qi Zhou presented a study that established morphological criteria for classifying human oocytes in terms of their developmental potential for hSCNT embryo development (Y. Yu, et al., 2009). His group was in the process of deriving and characterizing hESC lines from hSCNT embryos, but due to lack of funding he was unable to finish these studies.

Contrary to predictions that hSCNT would require hundreds of oocytes, speakers commented that the efficiency of reprogramming by SCNT in humans appears to be high. As of the workshop date, Stemagen had used only 57 donated oocytes to produce 12 blastocysts following SCNT, translating to ~ 20% efficiency. Five blastocysts were used for DNA

fingerprinting and mitochondrial DNA sequencing, which confirmed that at least one of these blastocysts contained nuclear DNA from the donor cell and mitochondrial DNA from the host oocyte (French, et al., 2008).

Workshop participants with experience in human work agreed that the quality of the egg is key in determining the proportion of *in vitro* fertilized or nuclear transfer embryos that grow to the blastocyst stage. Dr. Wood indicated that factors affecting an oocyte's intrinsic developmental potential are similar to those that affect *in vitro* fertilization (IVF), and include the age, reproductive history, and physical characteristics of the donor. However, even among women of optimal profiles, there are marked and consistent differences in egg quality and in the percentage of oocytes displaying aneuploidy. Dr. Wood and Dr. Herbert stressed the importance of oocyte handling and other technical issues. Dr. Zhou developed a classification system that appears to predict the quality of an oocyte based on its morphology (Y. Yu, et al., 2009). Workshop participants agreed that further research is required to identify factors that contribute to egg quality for both hSCNT and IVF.

Other technical issues also affect the rate of successful development of an embryo following SCNT. Dr. Herbert described some of the solutions that her team, in collaboration with Dr. Mitalipov, has developed to optimize some of the steps in hSCNT:

- Enucleation: Adapting techniques developed for monkey SCNT, Dr. Herbert used polarized light birefringence to visualize the oocyte spindle and laser ablation to facilitate insertion of the enucleation pipette. The Stemagen group has also successfully optimized enucleation rates using extrusion or direct aspiration methods (French, et al., 2008).
- Nuclear transfer and fusion: Electrofusion techniques developed for SCNT in Rhesus monkeys resulted in high rates of oocyte lysis in human. Dr. Herbert showed that the addition of the membrane-fusing agent Hemagglutinating Virus of Japan Envelope (HVJ-E) increased the rates of reconstruction. Dr. Wood commented that both subzonal injection (SUZI) and direct cytoplasmic injection were effective for nuclear transfer, and that different fusion and activation procedures had been tested.
- Blastocyst development: Dr. Herbert and Dr. Wood reported that their groups were testing biological and technical variables to determine the optimal conditions for blastocyst development. Variables include donor cell type and cell cycle synchronization of the donor cells, as well as more physiological methods of reconstructed oocyte activation.
- Evaluation: Dr. Herbert noted that blastocyst formation is an unsatisfactory method of measuring the efficiency of reprogramming following SCNT. She commented on the need to develop readouts such as semi-quantitative analysis of Histone-3 Lysine-4 methylation status.

Speakers were very positive about current progress in the methods to achieve hSCNT. They also pointed to the importance of developing methods to generate hESC from hSCNT embryos. Dr. Wood indicated that there could be technical issues in this process, as the Stemagen group has been unsuccessful in generating hESCs from seven hSCNT embryos developed after their 2008 publication. Dr. Zhou commented that hESCs had been generated from his hSCNT embryos, but did not present characterization of these lines.

4.2. Challenges and Innovative Approaches

The most direct hurdles to achieving SCNT in humans are technical, as described above. However, workshop participants also cited the procurement of human eggs and logistical issues as significant roadblocks. Recent efforts to derive human oocytes from immature gametes were considered particularly promising for the field.

4.2.1. Procurement of oocytes

Workshop participants emphasized that mature human oocytes are a limited resource, and that good quality oocytes are essential for improving the rate of blastocyst formation from hSCNT embryos. Donor characteristics such as age and fertility profile contribute to oocyte quality. Recruiting sufficient donors with an optimal profile and optimizing oocyte collection procedures were cited as the main factors that could improve the availability of eggs suitable for research.

Legal and social considerations have also limited the availability of viable oocytes for research. Importantly, in some jurisdictions, law prohibits the use of human oocytes for SCNT experiments. In California, human SCNT is allowed but there are restrictions on compensation to donors. Donors may be compensated for expenses but cannot be provided with additional compensations for research participation (see permissible expenses http://www.cirm.ca.gov/reg/pdf/Reg100020_SM_Acct_Standards.pdf). Participants raised questions about the extent that these regulations might limit hSCNT research in California.

Drs. Herbert, Wood and Scott Noggle showed the results of independent surveys indicating that lack of compensation limits the number of individuals willing to donate oocytes for research, with Dr. Wood emphasizing the relatively high levels of discomfort, inconvenience and risk that women must undertake to donate eggs. Surveys presented at the workshop suggested that the low rates of egg donation for research reflected women's reluctance to participate in the time-consuming and uncomfortable procedures required to procure eggs, rather than an ethical objection to the use of their eggs for research.

Workshop participants described a handful of current programs designed to simultaneously support fertility treatment and research. Referred to as "egg sharing," these programs offer donors access to IVF for infertility treatment at reduced cost in return for authorizing some of the retrieved oocytes to be used in research. Dr. Herbert reported that most oocytes for her studies were donated by women in the United Kingdom undergoing IVF treatment who agree to share half of their oocytes in return for a 50% contribution towards the cost of their treatment. Women undergoing IVF treatment in this program were more likely to donate their excess eggs to research than to other infertility patients, although these results were sensitive to the financial incentives offered for each option. This egg sharing agreement includes provisions to prioritize reproductive success. For example, egg donation only occurs after six or more oocytes have been collected. Egg sharing programs have resulted in successful donation of eggs for research, but funding issues limit their impact (see: http://www.hfea.gov.uk/egg-donation-and-egg-sharing.html). This unique study has been funded by MRC in conjunction with a social science study to evaluate donor attitudes and provide a firmer evidence base for future work.

Another approach, implemented by the state of New York, involves direct payments to donors for providing oocytes for research. Dr. Noggle from the New York Stem Cell Foundation (NYSCF) presented the results of a survey involving 230 donors from the Columbia University program for assisted reproduction.

The following survey results were highlighted:

93% believe women should be able to donate eggs for medical research,
74% believe women should be compensated equally for donations to reproduction or research,
43% would still consider donating to research if compensation was limited,
9% prefer to donate their eggs to research rather than for reproductive purposes, and
40% expressed no preference.

The Columbia University program offers interested donors the choice between donation for research or reproduction. Regardless of choice, compensation equal to typical compensation for reproductive donation ($8000) is offered. The Columbia University program has received 15 research donations to date. Overall, the Columbia program remains very positive about the results of their oocyte policy and the possible impact it could have on procuring eggs for research.

4.2.2. Research breakthroughs: alternative sources of oocytes

Given the medical and ethical barriers to procuring eggs from human donors through ovarian stimulation, described above, researchers have explored other methods of obtaining mature human eggs. Dr. Aaron Hsueh and Dr. Bernie Tuch described successful maturation of human oocytes using a combination of *in vitro* and xenotransplantation techniques. Other research has addressed problems relating to maturation of oocytes obtained as a clinical byproduct, that require additional maturation (McElroy, et al., 2010). These alternative methods represent a critical advance that could impact the pace of development of hSCNT.

Dr. Tuch presented several approaches for procuring oocytes that are being developed at the Stem Cell Therapy Foundation in Australia. A small number of oocytes deemed unfit for IVF because of their immaturity were successfully matured *in vitro*. In addition, the group is developing conditions to mature oocytes from fetal ovarian tissue, which contains oocyte precursors. Dr. Tuch commented that this research has been delayed due to regulatory and funding issues. He suggested that there is a critical need to educate both the regulators and the public about the need for oocytes for research.

Dr. Hsueh presented recently published results from a CIRM-funded study that described the maturation of dormant oocytes present in human ovarian fragments removed from patients with benign ovarian tumors (Li, et al., 2010). This tissue is normally discarded following surgery. Based on data from mouse studies, Hsueh and colleagues treated the ovarian fragments with an inhibitor of the Phosphatase with TENsin homology (PTEN) protein. These activated fragments were then xenotransplanted into ovariectomized, immune-deficient mice and induced to mature by treating mice with Follicle Stimulating Hormone (FSH). His group found a dramatic increase in the number of mature oocytes produced from ovarian fragments stimulated with the PTEN inhibitor. Although the functionality of these oocytes was not tested in human, mouse oocytes produced in a similar way were fertilized, and the resulting embryos developed into normal mouse pups. These exciting results suggest that it may be possible to activate dormant human oocytes, and produce mature human oocytes, by a combination of PTEN inhibition and xenotransplantation.

Although this research is still preliminary, alternative methods of deriving mature human oocytes could have a profound impact on the pace of hSCNT research by making human oocytes more readily available.

4.2.3. Logistical roadblocks

Throughout the workshop, participants cited a handful of logistical issues that are faced by researchers engaged in hSCNT research. Most significantly, funding is scarce, unreliable, and often insufficient to support an adequate research group. hSCNT projects are perceived to suffer in the competition for funding in comparison to hiPSC or hESC projects under the existing review criteria for grants, as the research is expensive, the materials are limited, and the collaborators are few and sparsely distributed. In addition, funded grants can sometimes be withdrawn due to changing political or ethical concerns. Research led by multiple groups, including Stemagen and Professor Zhou in the US and China, respectively, was halted due to lack of funding.

These interruptions in funding are destabilizing to the field not only because of their impact on the individual research project, but also because they affect the community engaged in the research. Graduate students cannot complete their training, and young scientists are wary of dedicating their labs to research in an area of unreliable funding. Lack of personnel and a dwindling research community were cited as other hurdles impacting the progress of hSCNT research. Participants agreed that collaboration was essential to progress under these conditions of scarcity.

4.3. Therapeutic advantages to hSCNT technology: Mitochondrial disorders

SCNT technology remains unique in its potential to serve as a tool for addressing or preventing certain pathologies of the mitochondria. As reported by Dr. Shoubridge and Dr. Robin Lovell-Badge, mitochondrial diseases represent an important class of disorders that affect approximately 1 in 5000 individuals. Unlike typical inherited diseases, mitochondrial disorders can be caused by mutations in the mitochondrial genome as opposed to nuclear DNA, a fact that has important implications on the transmission, manifestation and severity of disease. Importantly, mitochondria are dynamic organelles that undergo frequent fusion and fission in response to energetic needs and can vary in copy number from cell to cell. These properties lead to the phenomenon of heteroplasmy, in which mitochondria within an individual cell or tissue may possess a mixture rather than a homogenous pool of DNA. Because mitochondria are transmitted cytoplasmically, the degree of heteroplasmy affects the extent to which mutations will be transmitted from the mother to her child as well as from cell to daughter cells, and ultimately determines the level to which organelle function is compromised. In general, mitochondrial mutations do not lead to disease symptoms unless a critical threshold is reached, typically representing around 80% of a cellular complement. This poses a problem when a small founding population of maternal mitochondria, potentially harboring mutations, becomes expanded during germline development, thereby surpassing the threshold and ultimately leading to disease in the next generation (Poulton, et al., 2010).

Because nuclear transfer can lead to the elimination of donor mitochondria if care is taken to avoid their transmission during the procedure, scientists have long recognized the possibility of using this technique to “cure” mitochondrial disorders by replacing the diseased mitochondrial environment of an oocyte with that of a healthy one. Evidence supporting the feasibility of this approach was obtained when Dr. Mitalipov’s group (Tachibana, et al., 2009) achieved

mitochondrial genome replacement in Rhesus macaques using spindle transfer from one oocyte to another. While similar approaches could be envisioned for humans, and the related technique of pronuclear transfer has been reported in humans (Craven, et al., 2010), there are a number of important factors that must be considered before they could be put into practice. Dr. Lovell-Badge emphasized that there must be a clear and quantitative means for identifying those oocytes with the potential to develop disease, such as those with known homoplasmic mutations or those where the level of heteroplasmy is sufficiently high. For cases where the mitochondrial mutation is undetermined, not quantifiable, or the degree of heteroplasmy is low, it would not be possible to predict with any accuracy whether or how an offspring would be affected. Given the technical and ethical challenges of oocyte spindle transfer, it is not clear how such uncertainties would impact the complex risk-benefit ratio considerations from the diverse perspectives of the mother, the provider of oocytes with healthy mitochondria, and potential offspring. While such challenges are significant, it is clear that a better understanding of mitochondrial biogenesis combined with technical improvements in diagnostics and technological capabilities could pave the way for oocyte spindle transfer to be used as a treatment for cytoplasmic disorders for which there are no realistic alternatives.

4.4 Species-specific differences in pluripotent stem cells

Interestingly, recent evidence suggests that the inherent species-specific differences between mouse and human ESC may affect reprogramming. Under standard culture conditions, both the growth factor requirements for maintaining pluripotency and the gene expression patterns displayed by ESCs and iPSCs differ substantially between mouse and human (Ohtsuka & Dalton, 2008). Although both mouse and human ESCs are derived from blastocysts, human ESC more closely resemble mouse stem cells extracted from a later developmental stage, the epiblast stage (EpiSC) (Brons, et al., 2007; Ohtsuka & Dalton, 2008; Rossant, 2008; Tesar, et al., 2007). Although mouse fESC and SCNT-ESC display differential methylation in a few genomic regions (Kim, et al., 2010), they have been reported to be transcriptionally and functionally indistinguishable (Brambrink, et al., 2006). However, Dr. Roger Pedersen reported on recent results suggesting that the transcriptional and epigenetic profiles of mouse EpiSC derived from fertilized embryos (fEpiSC) and SCNT-EpiSC differ more drastically (Maruotti, et al., 2010). Namely, SCNT-EpiSC lines had abnormal expression levels of some imprinted genes as compared to fEpiSC. The functional consequences of such differences are consistent with the developmental arrest and abnormalities typically seen in postimplantation development after SCNT. Based on the fact that human ESC resemble mouse EpiSC, Dr. Pedersen postulated that unlike mouse SCNT-ESC, which resemble fESCs, human SCNT-ESCs may harbor epigenetic abnormalities that distinguish them from hESCs derived from IVF embryos. He suggested that further studies are also needed on hiPSCs to determine the extent of epigenetic perturbations induced by reprogramming, which could affect their therapeutic potential.

Recent data in mouse illustrate that reprogramming cells by different methods results in different outcomes. Low passage iPSCs retain some epigenetic memory of the donor cell type and preferentially differentiate into that same lineage, whereas ESC derived from embryos reprogrammed through SCNT are more purely pluripotent (Kim, et al., 2010; Polo, et al., 2010). Dr. Hans Schöler and others further emphasized that SCNT reverts the reprogrammed genome to totipotency, whereas iPSC technology results in pluripotency. Given these observations in mouse, and the species-specificity of some cellular behaviors, most participants in the workshop agreed that human SCNT research would have a significant impact on our ability to understand and consequently control reprogramming in human cells.

CONCLUSION

Workshop participants provided strong arguments regarding the scientific merit of pursuing research to achieve SCNT in human cells. In spite of important work on reprogramming in animals, there are species differences in the earliest stages of development that might make these studies not fully applicable to human reprogramming. A rigorous comparison of SCNT-hESCs, IVF-hESCs and hiPSCs would further our understanding of the mechanisms regulating somatic cell reprogramming in human cells and could increase the therapeutic potential of stem cells. hSCNT is also the only technology that has been proposed for treating mitochondrial disorders. Panelists indicated that technical, logistical, and legal/ethical issues present serious roadblocks to SCNT research in humans at the moment. However, some of these obstacles could be overcome if reliable funding were made available for research to optimize human SCNT and characterize hSCNT-ESCs.

CHAPTER 5. ADDITIONAL STRATEGIES FOR DERIVING PATIENT-SPECIFIC OR OTHERWISE IMMUNE-MATCHED STEM CELLS

One of the great advantages of achieving cellular reprogramming in human cells is the possibility of deriving patient-matched stem cells for use in regenerative medicine. In theory, stem cell products derived from individual patients could be used for cell therapy or organ replacement without the need for life-long immune suppression. Since derivation of hESC lines following human oocyte-mediated hSCNT has not yet been achieved, and iPSC technologies face their own limitations, the workshop considered alternative strategies for the derivation of patient-specific or otherwise immune-matched stem cell lines. Workshop participants addressed the potential merits of interspecies SCNT (iSCNT), nuclear transfer into zygotes, and parthenogenesis.

5.1. Interspecies SCNT (iSCNT)

To avoid the need for human oocytes, several groups have attempted to use readily available animal eggs as recipients for human somatic nuclei to achieve human/animal iSCNT. Workshop participants agreed that iSCNT-ESC-derived products would not be suitable as direct therapies, given the likelihood of immune rejection due to the presence of animal mitochondria as well as the potential for transmission of animal pathogens. However, many felt that iSCNT approaches could lead to useful insights about mechanisms underlying human cell reprogramming and are therefore worthy of further consideration.

In the animal kingdom, it has been possible to generate viable iSCNT offspring using very closely related species. Although early embryos have been generated from more distant pairings, including some human/animal combinations, they rarely progress through development to the blastocyst stage. For instance, a few horse nuclei in enucleated cow oocytes develop to blastocysts (and occasional cell lines form but cannot be maintained), but no blastocysts are formed when horse nuclei are incorporated into enucleated mouse oocytes (Tecirlioglu, et al., 2006). These observations, combined with the paucity of reports on ESC derivation following iSCNT, fuel skepticism as to whether ESC can be reliably derived following iSCNT using human somatic nuclei and easily available animal oocytes such as those from rabbit, cow or mouse. While success may be more likely with oocytes from non-human primates, the ethical and practical considerations of such approaches arguably preclude their merits.

Efforts to investigate the high failure rate of iSCNT-ESC derivation have been hampered by experimental variability and conflicting data. While some studies suggest that a failure to activate the pluripotency program may be to blame (Chung, et al., 2009), others have shown that pluripotency genes do, in fact, become activated in the early iSCNT embryo (Koziol, et al., 2007), and thus other mechanisms are likely to be involved. A second potential cause of developmental arrest in iSCNT embryos may lie in failures of mitochondrial function, presumably due to heteroplasmy or a lack of compatibility between mitochondrial and nuclear genomes. Interestingly, Dr. Hui Sheng, who successfully derived human/rabbit iSCNT-ESC-like cells in 2003 (Chen, et al., 2003), briefly described recent findings suggesting that the human mitochondria become dominant in those cells, presumably because mitochondrial DNA replication is under species-specific nuclear control. These new observations raise the possibility that incompatibilities between mitochondrial and nuclear genomes may not ultimately pose a critical roadblock for iSCNT.

A third potential hurdle to the derivation of iSCNT constructs was discussed by Dr. Fontana, who posited that differences in timing of embryonic genome activation (EGA) may lie at the heart of their developmental arrest. She argued that porcine oocytes might prove more suitable for use with human nuclei than those from other species, as both undergo EGA at the 4-cell stage. To support this contention, Dr. Fontana presented morphological evidence for human/porcine iSCNT blastocyst development.

5.2. Nuclear transfer into zygotes

Fertilized human eggs (one cell zygotes) represent a potentially useful source of recipient cells for hSCNT, as they are more readily available for research purposes than are oocytes. Early attempts to achieve nuclear transfer into enucleated zygotes were not successful in mice. Dr. Dieter Egli and colleagues hypothesized that important cellular factors required for developmental reprogramming might have become sequestered in the zygotic nuclei and would therefore have been lost when they were removed prior to SCNT. They addressed this potential issue by utilizing zygotes that are arrested in mitosis and have had their chromosomes removed. At this stage, the nuclear envelope breaks down and presumably releases such factors back into the cytoplasm where they become available for reprogramming the donor nucleus. Using this approach, Egli et al. were able to successfully derive germline-competent mouse SCNT-ESC lines (Egli, et al., 2007). However, attempts to transfer this protocol to human cells using normal cryopreserved and dispermic fresh human zygotes have not been productive. Dr. Egli presented extensive data showing that, despite pursuit of various experimental strategies, resulting hSCNT embryos failed to develop beyond cleavage stages, displaying karyotypic and transcriptional abnormalities.

5.3. Parthenogenesis

Although patient-specific cell lines are the ideal choice for achieving graft acceptance in cell transplant settings, large scale banking of therapeutic hESC lines for allogeneic transplants would be economically more viable. hESC cell banks would also be useful for applications that require rapid access to cells for transplant. To decrease the likelihood of allogeneic transplant rejection, researchers have proposed developing a cell bank of multiple cell lines carrying specific human leukocyte antigen (HLA) alleles that could provide large-scale access to cell products with at least partial histocompatibility for many patients (matching HLA alleles increases the success of bone marrow and solid organ transplants, although it does not obviate the need for immune suppression due to the effects of minor histocompatibility loci). Complexity of tissue matching could be further reduced if those alleles were present in a homozygous state (Nakajima, et al., 2007; Taylor, et al., 2005).

One promising approach to achieve HLA-matched cells involves the derivation of hESCs from parthenogenetic embryos, i.e. embryos developed from oocytes without fertilization (Revazova, et al., 2007) under conditions that lead to some loci or the entire genome becoming homozygous (Revazova, et al., 2008). Additionally, the techniques used to activate human oocytes for SCNT can cause parthenogenetic activation, leading to the derivation of parthenogenetic hESCs in which many loci are homozygous (Kim, et al., 2007). The goal would be to derive human parthenogenetic ESCs (hpESCs) homozygous for common HLA alleles. To illustrate the potential utility of hpESCs for regenerative medicine, Dr. Nikolai Turovets presented data demonstrating that several of the hpESC lines that he and his colleagues have generated display a normal diploid karyotype and behave similarly to authentic (biparental) hESCs in pluripotency assays, and that like other ESCs, they can be induced to differentiate

into hepatocyte and retinal pigmented epithelial (RPE) lineages. Importantly, he noted that it was necessary to optimize these protocols for use with hpESCs as opposed to their conventional hESC counterparts.

Although Dr. Tiziana Brevini concurred with Dr. Turovets on the differentiation plasticity of hpESCs, she provided evidence for the presence of multiple centrioles in these cells (Brevini, et al., 2009). She also showed data that suggested that parthenogenetic embryos, and thus potentially the lines derived from them, form incorrect mitotic spindles and contain chromosomal instabilities. She pointed out that in most mammals, including humans, the centriole in the oocyte degenerates. Under normal circumstances, the functional centriole, which is required for proper establishment of the first mitotic spindle and suppression of supernumerary centriole formation, is provided by the sperm upon fertilization. These facts suggest that defects observed in hpESCs may stem from the absence of paternal contribution to the parthenogenetic process. Dr. Turovets noted that his hpESC lines have been cultured up to 100 passages without acquiring aneuploidies, but commented that his group would be very interested in having them tested for mitotic spindle abnormalities.

In addition to addressing its scientific merits, workshop participants discussed the practicality of covering a significant segment of the human population with a collection of HLA homozygous hpESC lines. Despite the existence of a large number of HLA alleles at 6 loci, Dr. Jeffrey Janus suggested that 10 cell lines would be sufficient to match the 3 most common HLA alleles, which cover about 30% of the Caucasian population (Taylor, et al., 2005). Participants also acknowledged the need to test the actual immune response to HLA homozygous hpESC-derived cells, and to address the potential for aberrant cell behavior that may be caused by the lack of paternal imprints or induced homozygosity at loci carrying detrimental recessive mutations. Some expressed the opinion that regulatory hurdles for the clinical use of hpESC would be high, and felt that their utility rather lies in serving as tools for studying imprinting in humans.

Overall, participants felt that without direct comparisons, it remains unknown which method of stem cell line derivation will be most suitable for various applications. They proposed that until these comparisons are made, it is too early to abandon any one approach.

CONCLUSIONS

The success of interspecies SCNT has been limited and remains controversial, and it appears to require closely related species. At the moment, the technique is probably not viable for producing hESCs because human-primate fusions are ethically difficult sources. Despite rigorous attempts by one group, the use of one-cell human zygotes for SCNT has not been successful to date. hpESCs produced by parthenogenesis compare favorably to conventional hESCs in pluripotency and ability to differentiate. While these cells may face additional regulatory challenges due to spindle and/chromosomal abnormalities, it may be possible to ameliorate or overcome these concerns through improved methodologies or judicious choice of application. Beyond their potential for regenerative medicine, hpESCs could also be valuable for studying human imprinting and other developmental phenomena.

CHAPTER 6: SUMMARY OF CONCLUSIONS

Participants in the Human SCNT Workshop concluded that hSCNT is a technically challenging but feasible research goal. However, significant effort must be made to support the research or the technical expertise required to achieve it could be lost. This chapter presents the major conclusions and recommendations discussed at the workshop.

Although the bulk of research on cellular reprogramming using SCNT will continue to occur in animal models, there are fundamental questions about human processes that would benefit from the use of human cells. Workshop participants identified the following areas of impact:

1. hSCNT is a good tool to deepen our understanding of the processes regulating nuclear reprogramming in human cells. Recent research indicates that there are differences between stem cells generated through iPSC and SCNT technologies in mouse, which points to their use as complementary approaches for studying reprogramming of somatic cells. In addition, there are known differences between animal (particularly mouse) and human stem cells. Participants stressed the great value of hSCNT for understanding reprogramming in human cells.
2. hSCNT is a useful tool to study the early stages of human development and disease processes, and a unique tool for understanding the role of mitochondria in human disease. Species differences are observed as early as fertilization, so studying normal and disease processes in human cells is of tremendous value. Furthermore, there may be some diseases, particularly diseases of epigenetic etiology, which are more accurately modeled using methods other than hiPSC.
3. hSCNT technology could have therapeutic potential for ameliorating specific disorders, such as mitochondrial diseases. In addition, further research is required on the biology of mitochondria, both in animal and human models, to better understand how these essential organelles affect, and are in turn affected, by cellular reprogramming. hSCNT would provide an important tool to study mitochondrial biology in humans.

hSCNT faces significant barriers. Given the small number of laboratories that actively pursue hSCNT research, the stem cell field is in danger of losing the few technical advances that have been made to date. Workshop participants cited the most significant roadblocks to achieving hSCNT as:

1. Technical: Some of the technical hurdles to achieving SCNT are species-specific. Experience in achieving animal, and particularly primate, SCNT has helped researchers overcome some of the technical hurdles faced by hSCNT, but significant barriers remain. Issues surrounding egg quality were consistently brought up when discussing lack of progress in hSCNT. Workshop participants suggested that these technical hurdles are surmountable, especially given new methods of procuring higher numbers of quality oocytes.
2. Legal/ethical: Human egg procurement is difficult in most areas in the world and seriously hampers research on hSCNT. Lack of financial compensation to donors was viewed as the major cause of these supply limitations. In areas where eggs are more readily available, there is a general lack of sufficient funding to support hSCNT research.
3. Logistical: Lack of access to funding was cited as the most significant of the logistical barriers in the US, China and Australia. Participants indicated that both the lay public as well as members of the research community are relatively uninformed about the status and potential merits of hSCNT and are not convinced that hSCNT can be achieved. The

lack of funding and lesser status granted to hSCNT research has seriously hampered recruitment of new graduate students and young scientists into the field, a second key barrier to success.

The three technologies discussed in addition to conventional hSCNT did not fully recapitulate the benefits that hSCNT could offer. Nuclear transfer into zygotes has not been achieved with human cells. Similarly, interspecies SCNT has not yet reproducibly yielded human/animal ESC lines. Even if successfully derived, such lines were not considered suitable for direct therapeutic application but may have potential to serve as useful disease models in cases where iPSC models are not effective. While parthenogenetic stem cells might in principle be of value for cell therapy, it will be necessary to first address concerns regarding their molecular and genetic integrity. Overall, aside from hiPSC, hSCNT appeared to be the most compelling of the reprogramming methods under consideration.

In discussing the future of hSCNT research, workshop participants were positive about the prospects of achieving the technique in humans in a very short timeframe, if the logistical and ethical barriers could be overcome. Solutions to the specific roadblocks identified above were:

1. Collaboration will be essential to transfer hands-on knowledge about technical issues surrounding egg quality and nuclear transfer in human cells.
2. New methods of deriving appropriate eggs are being developed, but egg donation is still a roadblock. Surveys of potential egg donors suggest that financial compensation is significantly increasing the availability of eggs in areas such as New York. Participants suggested that collaboration between researchers in these different areas could help advance the field by making eggs available to a wider research community. They also suggested that further research on oocyte quality could enable the best use of this limited resource.
3. Targeted funding for achieving hSCNT might be required, as hSCNT research proposals are not competitive with hiPSCs under the current review procedures of many funding agencies. Research proposals that focus on the activity of a single laboratory are generally not competitive, as the resources and expertise required for achieving hSCNT is distributed worldwide.

Importantly, collaboration was viewed as essential to achieving hSCNT. Researchers indicated that technical issues and legal barriers might be more easily overcome collectively. Egg procurement issues could also be addressed more effectively, as collaboration could diversify the use of oocytes in areas where they are more readily available. Finally, collaboration would bring energy to the field in a time when young scientists are exploring their career options. The workshop participants envisioned that a well funded, targeted collaboration of researchers with access to high quality eggs would lead to the final technical breakthroughs necessary to achieve hSCNT, thus opening the door to further understanding of early human development and disease etiology.

REFERENCES

Beyhan, Z., Iager, A. E., & Cibelli, J. B. (2007). Interspecies nuclear transfer: implications for embryonic stem cell biology. *Cell Stem Cell, 1*(5), 502-512.

Boiani, M., Eckardt, S., Scholer, H. R., & McLaughlin, K. J. (2002). Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev, 16*(10), 1209-1219.

Brambrink, T., Hochedlinger, K., Bell, G., & Jaenisch, R. (2006). ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. *Proc Natl Acad Sci U S A, 103*(4), 933-938.

Brevini, T. A., Pennarossa, G., Antonini, S., Paffoni, A., Tettamanti, G., Montemurro, T., Radaelli, E., Lazzari, L., Rebulla, P., Scanziani, E., de Eguileor, M., Benvenisty, N., Ragni, G., & Gandolfi, F. (2009). Cell lines derived from human parthenogenetic embryos can display aberrant centriole distribution and altered expression levels of mitotic spindle check-point transcripts. *Stem Cell Rev, 5*(4), 340-352.

Briggs, R., & King, T. J. (1952). Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. *Proc Natl Acad Sci U S A, 38*(5), 455-463.

Brons, I. G., Smithers, L. E., Trotter, M. W., Rugg-Gunn, P., Sun, B., Chuva de Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A., & Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature, 448*(7150), 191-195.

Byrne, J. A., Pedersen, D. A., Clepper, L. L., Nelson, M., Sanger, W. G., Gokhale, S., Wolf, D. P., & Mitalipov, S. M. (2007). Producing primate embryonic stem cells by somatic cell nuclear transfer. *Nature, 450*(7169), 497-502.

Campbell, K. H., McWhir, J., Ritchie, W. A., & Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature, 380*(6569), 64-66.

Chen, Y., He, Z. X., Liu, A., Wang, K., Mao, W. W., Chu, J. X., Lu, Y., Fang, Z. F., Shi, Y. T., Yang, Q. Z., Chen da, Y., Wang, M. K., Li, J. S., Huang, S. L., Kong, X. Y., Shi, Y. Z., Wang, Z. Q., Xia, J. H., Long, Z. G., Xue, Z. G., Ding, W. X., & Sheng, H. Z. (2003). Embryonic stem cells generated by nuclear transfer of human somatic nuclei into rabbit oocytes. *Cell Res, 13*(4), 251-263.

Chin, M. H., Pellegrini, M., Plath, K., & Lowry, W. E. (2010). Molecular analyses of human induced pluripotent stem cells and embryonic stem cells. *Cell Stem Cell, 7*(2), 263-269.

Chung, Y., Bishop, C. E., Treff, N. R., Walker, S. J., Sandler, V. M., Becker, S., Klimanskaya, I., Wun, W. S., Dunn, R., Hall, R. M., Su, J., Lu, S. J., Maserati, M., Choi, Y. H., Scott, R., Atala, A., Dittman, R., & Lanza, R. (2009). Reprogramming of human somatic cells using human and animal oocytes. *Cloning Stem Cells, 11*(2), 213-223.

Cram, D. S., Song, B., & Trounson, A. O. (2007). Genotyping of Rhesus SCNT pluripotent stem cell lines. *Nature, 450*(7169), E12-14.

Craven, L., Tuppen, H. A., Greggains, G. D., Harbottle, S. J., Murphy, J. L., Cree, L. M., Murdoch, A. P., Chinnery, P. F., Taylor, R. W., Lightowlers, R. N., Herbert, M., & Turnbull, D. M. (2010). Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. *Nature, 465*(7294), 82-85.

Davis, R. L., Weintraub, H., & Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell, 51*(6), 987-1000.

Egli, D., Rosains, J., Birkhoff, G., & Eggan, K. (2007). Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. *Nature, 447*(7145), 679-685.

Fan, W., Waymire, K. G., Narula, N., Li, P., Rocher, C., Coskun, P. E., Vannan, M. A., Narula, J., Macgregor, G. R., & Wallace, D. C. (2008). A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations. *Science, 319*(5865), 958-962.

Ferreira, C. R., Burgstaller, J. P., Perecin, F., Garcia, J. M., Chiaratti, M. R., Meo, S. C., Muller, M., Smith, L. C., Meirelles, F. V., & Steinborn, R. (2009). Pronounced segregation of donor mitochondria introduced by bovine ooplasmic transfer to the female germ-line. *Biol Reprod, 82*(3), 563-571.

Ferreira, C. R., Meirelles, F. V., Yamazaki, W., Chiaratti, M. R., Meo, S. C., Perecin, F., Smith, L. C., & Garcia, J. M. (2007). The kinetics of donor cell mtDNA in embryonic and somatic donor cell-derived bovine embryos. *Cloning Stem Cells, 9*(4), 618-629.

French, A. J., Adams, C. A., Anderson, L. S., Kitchen, J. R., Hughes, M. R., & Wood, S. H. (2008). Development of human cloned blastocysts following somatic cell nuclear transfer with adult fibroblasts. *Stem Cells, 26*(2), 485-493.

French, A. J., Wood, S. H., & Trounson, A. O. (2006). Human therapeutic cloning (NTSC): applying research from mammalian reproductive cloning. *Stem Cell Rev, 2*(4), 265-276.

Gardner, J. L., Craven, L., Turnbull, D. M., & Taylor, R. W. (2007). Experimental strategies towards treating mitochondrial DNA disorders. *Biosci Rep, 27*(1-3), 139-150.

Guenther, M. G., Frampton, G. M., Soldner, F., Hockemeyer, D., Mitalipova, M., Jaenisch, R., & Young, R. A. (2010). Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. *Cell Stem Cell, 7*(2), 249-257.

Gurdon, J. B. (1962a). Adult frogs derived from the nuclei of single somatic cells. *Dev Biol, 4*, 256-273.

Gurdon, J. B. (1962b). Multiple genetically identical frogs. *J Hered, 53*, 5-9.

Gurdon, J. B. (1962c). The transplantation of nuclei between two species of Xenopus. *Dev Biol, 5*, 68-83.

Haaf, T. (2006). Methylation dynamics in the early mammalian embryo: implications of genome reprogramming defects for development. *Curr Top Microbiol Immunol, 310*, 13-22.

Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C. J., Creyghton, M. P., van Oudenaarden, A., & Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. *Nature, 462*(7273), 595-601.

Ieda, M., Fu, J. D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., & Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell, 142*(3), 375-386.

Jullien, J., Astrand, C., Halley-Stott, R. P., Garrett, N., & Gurdon, J. B. (2010). Characterization of somatic cell nuclear reprogramming by oocytes in which a linker histone is required for pluripotency gene reactivation. *Proc Natl Acad Sci U S A, 107*(12), 5483-5488.

Kang, L., Wang, J., Zhang, Y., Kou, Z., & Gao, S. (2009). iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. *Cell Stem Cell, 5*(2), 135-138.

Kenyon, L., & Moraes, C. T. (1997). Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids. *Proc Natl Acad Sci U S A, 94*(17), 9131-9135.

Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M. J., Ji, H., Ehrlich, L. I., Yabuuchi, A., Takeuchi, A., Cunniff, K. C., Hongguang, H., McKinney-Freeman, S., Naveiras, O., Yoon, T. J., Irizarry, R. A., Jung, N., Seita, J., Hanna, J., Murakami, P., Jaenisch, R., Weissleder, R., Orkin, S. H., Weissman, I. L., Feinberg, A. P., & Daley, G. Q. (2010). Epigenetic memory in induced pluripotent stem cells. *Nature, 467*(7313), 285-290.

Kim, K., Ng, K., Rugg-Gunn, P. J., Shieh, J. H., Kirak, O., Jaenisch, R., Wakayama, T., Moore, M. A., Pedersen, R. A., & Daley, G. Q. (2007). Recombination signatures distinguish embryonic stem cells derived by parthenogenesis and somatic cell nuclear transfer. *Cell Stem Cell, 1*(3), 346-352.

Koziol, M. J., Garrett, N., & Gurdon, J. B. (2007). Tpt1 activates transcription of oct4 and nanog in transplanted somatic nuclei. *Curr Biol, 17*(9), 801-807.

Kubiak, J. Z., & Tarkowski, A. K. (1985). Electrofusion of mouse blastomeres. *Exp Cell Res, 157*(2), 561-566.

Kubota, C., Tian, X. C., & Yang, X. (2004). Serial bull cloning by somatic cell nuclear transfer. *Nat Biotechnol, 22*(6), 693-694.

Lavoir, M. C., Weier, J., Conaghan, J., & Pedersen, R. A. (2005). Poor development of human nuclear transfer embryos using failed fertilized oocytes. *Reprod Biomed Online, 11*(6), 740-744.

Lewis, I. M., McClintock, A. E., French, A. J., Zuelke, K. A., Harford, B. A., & Trounson, A. O. (2000). Cloning and transgenesis in farm animals--an Australian perspective. *Aust Vet J, 78*(10), 694-697.

Li, J., Kawamura, K., Cheng, Y., Liu, S., Klein, C., Liu, S., Duan, E. K., & Hsueh, A. J. (2010). Activation of dormant ovarian follicles to generate mature eggs. *Proc Natl Acad Sci U S A, 107*(22), 10280-10284.

Loh, K. M., & Lim, B. (2010). Recreating pluripotency? *Cell Stem Cell, 7*(2), 137-139.

Markoulaki, S., Meissner, A., & Jaenisch, R. (2008). Somatic cell nuclear transfer and derivation of embryonic stem cells in the mouse. *Methods, 45*(2), 101-114.

Maruotti, J., Dai, X. P., Brochard, V., Jouneau, L., Liu, J., Bonnet-Garnier, A., Jammes, H., Vallier, L., Brons, I. G., Pedersen, R., Renard, J. P., Zhou, Q., & Jouneau, A. (2010). Nuclear transfer-derived epiblast stem cells are transcriptionally and epigenetically distinguishable from their fertilized-derived counterparts. *Stem Cells, 28*(4), 743-752.

McElroy, S. L., Byrne, J. A., Chavez, S. L., Behr, B., Hsueh, A. J., Westphal, L. M., & Pera, R. A. (2010). Parthenogenic blastocysts derived from cumulus-free in vitro matured human oocytes. *PLoS One, 5*(6), e10979.

McElroy, S. L., Kee, K., Tran, N., Menses, J., Giudice, L. C., & Reijo Pera, R. A. (2008). Developmental competence of immature and failed/abnormally fertilized human oocytes in nuclear transfer. *Reprod Biomed Online, 16*(5), 684-693.

McEvoy, T. G., Ashworth, C. J., Rooke, J. A., & Sinclair, K. D. (2003). Consequences of manipulating gametes and embryos of ruminant species. *Reprod Suppl, 61*, 167-182.

Muller, L. U., Daley, G. Q., & Williams, D. A. (2009). Upping the ante: recent advances in direct reprogramming. *Mol Ther, 17*(6), 947-953.

Munsie, M. J., Michalska, A. E., O'Brien, C. M., Trounson, A. O., Pera, M. F., & Mountford, P. S. (2000). Isolation of pluripotent embryonic stem cells from reprogrammed adult mouse somatic cell nuclei. *Curr Biol, 10*(16), 989-992.

Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M., & Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development, 110*(3), 815-821.

Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., & Roder, J. C. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci U S A, 90*(18), 8424-8428.

Nakajima, F., Tokunaga, K., & Nakatsuji, N. (2007). Human leukocyte antigen matching estimations in a hypothetical bank of human embryonic stem cell lines in the Japanese population for use in cell transplantation therapy. *Stem Cells, 25*(4), 983-985.

Newman, A. M., & Cooper, J. B. (2010). Lab-specific gene expression signatures in pluripotent stem cells. *Cell Stem Cell, 7*(2), 258-262.

Ng, R. K., & Gurdon, J. B. (2008). Epigenetic memory of an active gene state depends on histone H3.3 incorporation into chromatin in the absence of transcription. *Nat Cell Biol, 10*(1), 102-109.

Niemann, H., & Kues, W. A. (2007). Transgenic farm animals: an update. *Reprod Fertil Dev, 19*(6), 762-770.

Ohtsuka, S., & Dalton, S. (2008). Molecular and biological properties of pluripotent embryonic stem cells. *Gene Ther, 15*(2), 74-81.

Panarace, M., Aguero, J. I., Garrote, M., Jauregui, G., Segovia, A., Cane, L., Gutierrez, J., Marfil, M., Rigali, F., Pugliese, M., Young, S., Lagioia, J., Garnil, C., Forte Pontes, J. E., Ereno Junio, J. C., Mower, S., & Medina, M. (2007). How healthy are clones and their progeny: 5 years of field experience. *Theriogenology, 67*(1), 142-151.

Polo, J. M., Liu, S., Figueroa, M. E., Kulalert, W., Eminli, S., Tan, K. Y., Apostolou, E., Stadtfeld, M., Li, Y., Shioda, T., Natesan, S., Wagers, A. J., Melnick, A., Evans, T., & Hochedlinger, K. (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotechnol, 28*(8), 848-855.

Poulton, J., Chiaratti, M. R., Meirelles, F. V., Kennedy, S., Wells, D., & Holt, I. J. (2010). Transmission of mitochondrial DNA diseases and ways to prevent them. *PLoS Genet, 6*(8).

Reefhuis, J., Honein, M. A., Schieve, L. A., Correa, A., Hobbs, C. A., & Rasmussen, S. A. (2009). Assisted reproductive technology and major structural birth defects in the United States. *Hum Reprod, 24*(2), 360-366.

Revazova, E. S., Turovets, N. A., Kochetkova, O. D., Agapova, L. S., Sebastian, J. L., Pryzhkova, M. V., Smolnikova, V. I., Kuzmichev, L. N., & Janus, J. D. (2008). HLA homozygous stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells, 10*(1), 11-24.

Revazova, E. S., Turovets, N. A., Kochetkova, O. D., Kindarova, L. B., Kuzmichev, L. N., Janus, J. D., & Pryzhkova, M. V. (2007). Patient-specific stem cell lines derived from human parthenogenetic blastocysts. *Cloning Stem Cells, 9*(3), 432-449.

Rideout, W. M., 3rd, Hochedlinger, K., Kyba, M., Daley, G. Q., & Jaenisch, R. (2002). Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell, 109*(1), 17-27.

Rossant, J. (2008). Stem cells and early lineage development. *Cell, 132*(4), 527-531.

Shoubridge, E. A. (2000). Mitochondrial DNA segregation in the developing embryo. *Hum Reprod, 15 Suppl 2*, 229-234.

Shoubridge, E. A., & Wai, T. (2007). Mitochondrial DNA and the mammalian oocyte. *Curr Top Dev Biol, 77*, 87-111.

Stadtfeld, M., Apostolou, E., Akutsu, H., Fukuda, A., Follett, P., Natesan, S., Kono, T., Shioda, T., & Hochedlinger, K. (2010). Aberrant silencing of imprinted genes on chromosome 12qF1 in mouse induced pluripotent stem cells. *Nature, 465*(7295), 175-181.

Stewart, J. B., Freyer, C., Elson, J. L., Wredenberg, A., Cansu, Z., Trifunovic, A., & Larsson, N. G. (2008). Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLoS Biol, 6*(1), e10.

Stojkovic, M., Stojkovic, P., Leary, C., Hall, V. J., Armstrong, L., Herbert, M., Nesbitt, M., Lako, M., & Murdoch, A. (2005). Derivation of a human blastocyst after heterologous nuclear transfer to donated oocytes. *Reprod Biomed Online, 11*(2), 226-231.

Swistowski, A., Peng, J., Liu, Q., Mali, P., Rao, M. S., Cheng, L., & Zeng, X. (2010). Efficient Generation of Functional Dopaminergic Neurons from Human Induced pluripotent Stem Cells under Defined Conditions. *Stem Cells*.

Tachibana, M., Sparman, M., Sritanaudomchai, H., Ma, H., Clepper, L., Woodward, J., Li, Y., Ramsey, C., Kolotushkina, O., & Mitalipov, S. (2009). Mitochondrial gene replacement in primate offspring and embryonic stem cells. *Nature, 461*(7262), 367-372.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell, 131*(5), 861-872.

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*(4), 663-676.

Tam, P. P., & Rossant, J. (2003). Mouse embryonic chimeras: tools for studying mammalian development. *Development, 130*(25), 6155-6163.

Taylor, C. J., Bolton, E. M., Pocock, S., Sharples, L. D., Pedersen, R. A., & Bradley, J. A. (2005). Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. *Lancet, 366*(9502), 2019-2025.

Tecirlioglu, R. T., Guo, J., & Trounson, A. O. (2006). Interspecies somatic cell nuclear transfer and preliminary data for horse-cow/mouse iSCNT. *Stem Cell Rev, 2*(4), 277-287.

Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner, R. L., & McKay, R. D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature, 448*(7150), 196-199.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science, 282*(5391), 1145-1147.

Urbach, A., Bar-Nur, O., Daley, G. Q., & Benvenisty, N. (2010). Differential modeling of fragile X syndrome by human embryonic stem cells and induced pluripotent stem cells. *Cell Stem Cell, 6*(5), 407-411.

Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Sudhof, T. C., & Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. *Nature, 463*(7284), 1035-1041.

Warren, L., Manos, P. D., Ahfeldt, T., Loh, Y. H., Li, H., Lau, F., Ebina, W., Mandal, P. K., Smith, Z. D., Meissner, A., Daley, G. Q., Brack, A. S., Collins, J. J., Cowan, C., Schlaeger, T. M., & Rossi, D. J. (2010). Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified mRNA. *Cell Stem Cell*.

Watanabe, S., & Nagai, T. (2009). Death losses due to stillbirth, neonatal death and diseases in cloned cattle derived from somatic cell nuclear transfer and their progeny: a result of nationwide survey in Japan. *Anim Sci J, 80*(3), 233-238.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E., & Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature, 448*(7151), 318-324.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., & Campbell, K. H. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature, 385*(6619), 810-813.

Yan, Z. H., Zhou, Y. Y., Fu, J., Jiao, F., Zhao, L. W., Guan, P. F., Huang, S. Z., Zeng, Y. T., & Zeng, F. (2010). Donor-host mitochondrial compatibility improves efficiency of bovine somatic cell nuclear transfer. *BMC Dev Biol, 10*, 31.

Yoon, Y. G., Haug, C. L., & Koob, M. D. (2007). Interspecies mitochondrial fusion between mouse and human mitochondria is rapid and efficient. *Mitochondrion, 7*(3), 223-229.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II, & Thomson, J. A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science, 318*(5858), 1917-1920.

Yu, Y., Mai, Q., Chen, X., Wang, L., Gao, L., Zhou, C., & Zhou, Q. (2009). Assessment of the developmental competence of human somatic cell nuclear transfer embryos by oocyte morphology classification. *Hum Reprod, 24*(3), 649-657.

Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J., & Melton, D. A. (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature, 455*(7213), 627-632.

CIRM - MRC Human SCNT Workshop
Agenda

Purpose

- Solicit perspectives from leaders in the field of stem cell research and regenerative medicine on the current role of SCNT technology
- Provide an opportunity for leaders in the field to exchange ideas
- Inform CIRM and the MRC about the status of the human SCNT field, so that they can make informed decisions about future funding priorities for cellular reprogramming

June 13, 2010	
11:30 am	**Lunch**
12:30 pm	**Welcome** Lord Naren Patel, UK – **Chair** Hosts: Rob Buckle (MRC) and Alan Trounson (CIRM)
CONFERENCE OPENING **40 minute talk / 10 minutes for questions**	
1:00 pm	Sir John Gurdon, University of Cambridge, UK *The past and present of nuclear reprogramming by eggs and oocytes*
Session 1. Mammalian and non-human primate SCNT: What are the lessons for the field? **20 minute talks / 30 minute discussion**	
Chair	Keith Campbell, University of Nottingham, UK
1:50 pm	Jose Cibelli, Michigan State University
2:10 pm	Shoukhrat Mitalipov, Oregon Health & Science University *SCNT in the rhesus macaque model*
2:30 pm	**Discussion**
Questions to consider for presenters and discussion • What evidence do we have from non-human SCNT studies to suggest there is a scientific imperative to do human studies? • What do we observe in non-human SCNT studies that appears important or useful to replicate in human studies? • How do observations in non-human SCNT studies contribute or relate to the development of cell based therapies?	
3:00 pm	**Coffee Break**

Session 2. Xenotransfer human DNA to animal oocyte: Is this a viable alternative to human oocytes? **15 minute talks / 30 minute discussion**	
Chair	Roger Pedersen, University of Cambridge, UK
3:15 pm	Hui Sheng, Shanghai Jiao Tong University, Xinhua Hospital, China
3:30 pm	Jeannie Fontana, Burnham Institute for Medical Research, CA
3:45 pm	**Discussion**
Questions to consider for presenters and for discussion • What is the viability of xenotransfer for human DNA; has it been successful? • What is the potential of xenotransfer technology for disease modeling? • For potential clinical applications: How can we address animal mitochondrial DNA and other animal products? • What types of clinical products (cellular, sub-cellular) are envisioned from this process?	
Session 3. Mitochondrial diseases: Does SCNT / Spindle Transfer (ST) represent a unique therapy? **15 minute talks / 30 minute discussion**	
Chair	Shoukhrat Mitalipov, Oregon Health & Science University
4:30 pm	Eric Shoubridge, McGill University, Canada
4:45 pm	Robin Lovell-Badge, National Institute for Medical Research, UK
5:00 pm	**Discussion**
Questions to consider for presenters and for discussion • What are alternative options for treating mitochondrial diseases? • Are there other clinical settings that may specifically benefit from SCNT / ST? • What is the prevalence of mitochondrial diseases? What effect does that have on the decision whether to pursue human SCNT / ST? • Is SCNT / ST technology for treatment of mitochondrial diseases technically comparable to SCNT technology for therapeutic cloning? • Is this a model for how funding agencies might promote SCNT research (specific disease with specific intervention) • Does SCNT / ST technology lend itself to modeling mitochondrial diseases?	
6:30 pm	**Dinner**

June 14, 2010	
7:30 am	**Breakfast**
Session 4. Procurement of human oocytes: What has been the experience to date? 15 minute talks / 30 minute discussion	
Chair	Renee Reijo-Pera, Stanford University, CA
8:00 am	Jeffrey Janus, International Stem Cell Corporation, CA *Procurement of human oocytes in California and Russia: five years experience of International Stem Cell Corporation.*
8:15 am	Scott Noggle, New York Stem Cell Foundation
8:30 am	Bernie Tuch, Research Consortium at Fertility East, Australia
8:45 am	Aaron Hsueh, Stanford University, CA
9:00 pm	**Discussion**
Questions to consider for presenters and for discussion • What levels of donor reimbursement are necessary to support research? (Is it feasible for CIRM to support SCNT research without allowing reimbursement) • Are there reasons for donors to prefer research donation over reproductive use of oocytes? • What is the experience of research donors with regard to OHSS or other adverse outcomes? • Are research donation programs being evaluated for donor satisfaction?	
Session 5. Parthenogenesis: A viable road to immune compatible cell lines? 15 minute talks / 30 minute discussion	
Chair	Fulvio Gandolfi, University of Milan
9:30 am	Nikolay Turovets, International Stem Cell Corporation *Derivation of highly enriched cultures of differentiated cells from human parthenogenetic stem cells.*
9:45 am	Tiziana Brevini, University of Milan *Parthenotes as a source of embryonic stem cells: pros and cons*
10:00 am	**Discussion**
Questions to consider for presenters and for discussion • What is the efficiency of producing parthenogenetic lines? • What are the similarities / differences between embryo-derived and parthenogenetically derived hESC lines? What impact do the differences have on therapeutic suitability? • What is the perceived value of parthenogenetic lines for basic and clinical research and therapies?	
10:30 am	**Coffee Break**

Session 6. Human SCNT: What is the status of the science? 20 minute talks / 30 minute discussion	
Chair	Miodrag Stojkovic, University of Kragujevac, Serbia
10:50 am	Mary Herbert, Newcastle University, UK
11:10 am	Sam Wood, Stemagen, CA
11:30 am	Qi Zhou, Chinese Academy of Sciences, Beijing
11:50 am	Dieter Egli, New York Stem Cell Foundation *Nuclear transfer into human zygotes and oocytes*
12:10 am	**Discussion**
Questions to consider for presenters and for discussion • What are the technical hurdles to human SCNT? • What advances / technologies are needed to overcome the hurdles?	
12:40 pm	**Lunch**
Session 7. SCNT – iPSC comparisons in non-human mammals Are there critical differences? 20 minute talks (discussion in following panel)	
Chair	Robin Lovell-Badge, National Institute for Medical Research, UK
2:00 pm	Hans Schöler, Max Planck Institute, Germany
2:20 pm	Roger Pedersen, University of Cambridge, UK
2:40 pm	**Coffee Break**

PANEL DISCUSSION

Non-embryonic alternatives for patient matching: Can iPSC replace SCNT?

Chair	Lord Naren Patel, UK
3:00 pm	Hans Schöler, Max Planck Institute, Germany Roger Pedersen, University of Cambridge, UK Sir John Gurdon, University of Cambridge, UK Irv Weissman, Stanford University, CA Robin Lovell-Badge, National Institute for Medical Research, UK

Questions to consider for discussion

- Are there unique basic/ translational research questions that may only be addressed through SCNT?
- If so, can mouse / non-human primate SCNT answer those questions?
- Is there a need for SCNT-derived disease in the dish models?
- What advantages does SCNT have over iPSC for therapeutic applications?

4:45 pm	**Wrap Up**