Cloned embryonic stem cells: a long journey from mice to humans (2000–2013)

The prospect of cloning human embryos by somatic cell nuclear transfer (SCNT) into oocytes has engendered a mix of fear, excitement, controversy and extremely polarized opinions in spite of the fact that it never worked robustly let alone efficiently, until now. In the May issue of Cell, Tachibana et al. (2013) reported on the unprecedented derivation of karyotypically normal embryonic stem cells (ESCs) from cloned human embryos. This was not the first report of ESCs generated after SCNT into human oocytes (Noggle et al., 2011), but it is the first case in which these cell lines are diploid and their nuclear genome is perfectly matched to the donors of the somatic nuclei. Not only that, but when one looks at the cases of SCNT in mammals reported to date, it becomes obvious that human SCNT in the hands of Tachibana et al. was also quite efficient in comparison with other species. For example, in their first round of human SCNT Tachibana et al. produced 10 cloned blastocysts from 42 oocytes (24%); of these blastocysts, 8 were used for derivation, yielding 4 ESC lines (50%); in 2 subsequent rounds of SCNT with oocytes from different women, the rates of blastocyst formation were 27 and 60%, and the rates of ESC derivation were 25 and 33%.

Cloned ESCs are the tools used for therapeutic cloning, which is designed to remedy disease, not as a means for reproduction. Therapeutic cloning was first accomplished in mice in 2002 (Rideout et al., 2002) after the demonstration 2 years earlier that mouse ESCs could be derived after SCNT (Munsie et al., 2000). Similar attempts in humans resulted in two reports of cloned ESCs, which were later revealed as being fabricated and were retracted (Kennedy, 2006). Except for a very few reports, rather a long period of silence ensued in human SCNT. This vacuum occurred even though the experimental protocols were advanced enough to allow the derivation of ESC lines from single human blastomeres (Chung et al., 2008). It seemed that, in humans, making immunocompatible cells to regenerate tissues or to treat disease would require something other than SCNT into oocytes. If 242 human oocytes did not suffice to produce a genuine cloned ESC line (Hwang et al., 2004), then one should consider that persevering in SCNT might never pay off, suggesting that there may be some biological roadblock that first needs to be removed. Research on the putative roadblocks to human SCNT came to a standstill when, in 2006, the direct induction of pluripotency in somatic cells (iPSCs) provided an easier alternative to cloned ESCs (Takahashi and Yamanaka, 2006) without the technical and ethical limitations that are associated with the use of oocytes and the generation of totipotent embryos. iPSCs and ESCs are functionally interchangeable, although they are distinguishable on the molecular level (Kim et al., 2010; Polo et al., 2010; Ohi et al., 2011). In 2007 the report that non-human primate ESCs could be derived by SCNT (Byrne et al., 2007) revived the SCNT method, but it was not sufficient to overcome the mistrust which had followed the fabricated reports of cloned human ESCs. Paraphrasing Cibelli (2007) therapeutic cloning was on the death bed, although death has not been pronounced. Tachibana et al. have now raised human therapeutic cloning from the death bed, reporting that cloned human blastocysts support the derivation of ESCs. Cloned human blastocysts had been obtained before (French et al., 2008; Fan et al., 2011), but the derivation of ESCs was either not attempted, not successful or not reported. To enable ESC derivation, cloned human embryos have to be produced that not only develop to the morphological blastocyst stage, but also harbor a functional inner cell mass (ICM) capable of forming an epiblast. At face value this is the prime achievement of Tachibana et al. and it prompts the first question: What did the authors do differently to previous investigators? In fact, there is more to it, as I am going to illustrate below. It is difficult to predict the future based on one paper, but provided that the experiments are reproducible in the hands of others, the results of Tachibana et al. also prompt a second question: what does the achievement of cloned human ESCs mean for basic science and for biomedicine? I reflect on the pros and cons of the new achievement and try to identify open issues that the revived field of human SCNT will likely have to confront.

What did the Tachibana group do differently to previous investigators?

Without robust protocols for production of cloned human blastocysts, Tachibana et al. had to work out several critical steps on their own, albeit not from scratch. The authors built on their expertise on non-
human primate oocytes, transposed it to human and applied a combination of chemical treatment and skillful oocyte manipulation. The authors recognized the great sensitivity of human oocytes to micromanipulation. In particular, the removal of the chromosome-spindle complex (enucleation) triggers a partial activation of the human oocyte, which is then less apt to reprogram the somatic nucleus, drastically reducing the quality of cloned blastocysts.

It may be noted that Noggle et al. (2011) also recognized the enucleation of human oocytes as critical step in the cloning procedure, although they reached a different conclusion than Tachibana et al. Upon replacement of the oocyte chromosomes with the nucleus of a somatic cell, Noggle et al. observed developmental arrest and mRNA transcription abnormalities, in contrast to when the oocyte chromosomes remained in place. The authors concluded that the oocyte genome is necessary for the reprogramming of the somatic genome in humans. Embryo culture conditions did not seem to play a significant role to the different outcomes of Noggle et al. and Tachibana et al., as both studies used Global medium under low oxygen atmosphere. However, most of the oocytes in Noggle et al.’s study had been stained with Hoechst dye and irradiated with UV, raising the possibility that cellular damage had occurred in the enucleated oocytes in the form of damaged cytoplasmic organelles, e.g. mitochondria. Tachibana et al. instead enucleated the oocytes using an Oosight Imaging System, which is based on polarized light and does not require DNA staining and UV irradiation. It is also possible that the oocytes of Noggle et al. had undergone partial activation described by Tachibana et al.

Tachibana et al. counteracted the oocyte activation with caffeine, a phosphodiesterase inhibitor which preserves high levels of maturation-promoting factors in the ooplasm (Choi and Campbell, 2010). Nuclear transfer was made as gentle as possible by virus-mediated membrane fusion instead of stabbing the oolemma with an injection needle. Activation of the nucleus-transplanted human oocytes was performed by a modified protocol in which dimethyl amino purine, a protein kinase inhibitor, is combined with an electric pulse that can replace the calcium ionophore, ionomycin. It appears that a conventional protocol lacking the pulse, while able to activate intact human oocytes and to yield parthenogenetic embryos, would not work on SCNT oocytes. During the activation of the reconstructed oocytes, a carefully titered concentration of trichostatin A (TSA, an inhibitor of histone deacetylases) led to increased blastocyst rates. Previous work in the mouse model had shown that TSA relieves at least some of the chromatin roadblocks to reprogramming (e.g. by improving histone acetylation) and thereby facilitates mRNA transcription in cloned embryos of mice and other mammalian species (Kishigami et al., 2006; Ding et al., 2008; Li et al., 2008; Bui et al., 2010). Following treatment of cloned mouse embryos with histone deacetylase inhibitors, the rate of ESC derivation rose from ≈20% up to 60% (Ono et al., 2010).

The results described above demonstrate that there is no irreversible biologic barrier that prevents an enucleated human oocyte from reprogramming a somatic nucleus. Instead, important technical refinements are key to realize this reprogramming potential. Yet it is unlikely that these important technical refinements alone explain the whole outcome. The oocyte donors in Tachibana et al.’s study were of the very young age of 23–31 years and had no infertility history, similar to the donors in Noggle et al.’s study (22–33 years old). I return to this aspect when reflecting on the principles that should, in my opinion, guide the allocation of human oocytes for reproductive versus non-reproductive purposes. The oocytes were also carefully screened, and the oocytes of donors who produced fewer oocytes supported better development than those of donors who produced more oocytes. Perhaps it is not surprising that quality and quantity are inversely related. In the mouse, the few oocytes obtained after natural ovulation performed better after SCNT than the larger number of oocytes ovulated after hormone stimulation (Hiragi and Solter, 2005).

What does this achievement mean for basic science and biomedicine?

For basic science the derivation of cloned human ESCs means that reprogramming is compliant with normal morphology of the cloned embryo (formation of a blastocyst with trophectoderm and ICM requires the expression of specific genes from the silent somatic state), but also that the ICM is functionally pluripotent in at least some of its cells. Thus, from now on, the human oocyte can be mined for molecular factors that enact reprogramming, knowing that these factors are present. Cloned human blastocysts had been produced before, but evidence of functional reprogramming was limited to expression of pluripotency markers. Since the molecular analysis of embryos almost invariably destroys the embryo itself, the analysis is subjected to obvious restrictions in humans. The ethical aspects of working with human embryos deserve to be mentioned. Human embryos may not be created on purpose for scientific research in most Western jurisdictions (as in the ‘Oviedo’ Convention on Human Rights and Biomedicine of the Council of Europe, 4 April 1997), but they can be used for research if they are spare embryos from, e.g. IVF, pending consent of the parents. There are couples who do not feel comfortable with the idea of their biological child growing up in another family, or with the idea to that this child may want to meet them one day, so they prefer to donate their surplus embryos for research after a successful pregnancy. Despite these donations, gene expression studies in human embryos remain far less common than in mice.

For biomedicine and particularly for regenerative medicine the ability to clone human ESCs means that iPSC’s quality can be tested against a reference within the realm of reprogramming. Without cloned human ESCs, the quality of human iPSCs can only be tested by epigenetic and gene expression markers, by cell differentiation in vitro, and by teratoma formation in mice. The teratoma assay is paradoxical because the feature that is sought in the teratoma assay as proof of cellular pluripotency is the same feature that poses a safety concern therapeutically. Although there is a risk that residual pluripotent cells might escape the differentiation protocol, get reintroduced in the organism and form a tumor, this risk does not invalidate the importance of the teratoma assay in characterizing the cloned cells. There is always a risk, however small, associated with therapy, and protocols do improve. Additionally, differentiated cells would ideally be transplanted that have lost their ability to form teratoma. Another interesting feature of the cloned ESCs is that the somatic mitochondria are lost and almost all of the embryo’s mitochondria are oocyte derived. The strong purifying selection that operates against mtDNA mutations in the maternal germline means that the few remaining germ cells have ‘high-quality’ mtDNA compared with somatic tissues (Stewart et al., 2008). This difference may play an important role in distinguishing iPSCs from ESCs, since the mitochondria of the former are somatic. The almost complete loss of somatic mitochondria
after SCNT, reported by Tachibana et al., is at variance with the report of
cloned human embryos produced by cross-species SCNT from human
to rabbit, in which the human somatic mitochondria coexisted with the
resident mitochondria of the rabbit oocyte (Chen et al., 2003).

**A great leap forward, and still needed, but at what price?**

Even if we cannot say yet whether it will eventually be possible to safely
use cloned human ESCs for therapeutic purposes, do we need them at all?
Since we already have human iPSCs, human ESCs produced by
SCNT into an oocyte may seem redundant for applications, although
the oocyte remains the only system that can reprogram a somatic
nucleus to a full extent. Therefore, we still need the system of
oocyte-mediated reprogramming as reference. The oocyte is likely to point to
us to oocyte-specific molecules that enhance reprogramming in iPSCs, as
exemplified by the maternal factor Gli1 in mouse oocytes (Maekawa
et al., 2011), although it is questionable if iPSCs need to be perfect in
order to serve clinical applications. Having said so, I think that we
should not put science on a leash and stop research just because we
do not (yet) see obvious benefits at the present time, as long as this
science is done under conditions protecting the autonomy and wellbeing
of oocyte donors. This brings me to the price, metaphorically speaking,
paid for the great leap forward.

The oocyte donors in Tachibana et al.’s study were of the very young
age of 23–31 years, an age when women typically have children. In 2009,
the mean age of women at the birth of their first child was 27–28 years in
OECD countries and 25 years in the USA (OECD family database; www.
oecd.org/social/family/database). Donor oocytes are typically recov-
ered after gonadotropin stimulation, which increases the yield but also
entails health risks for the donors, even though modern stimulation pro-
tocols are much safer than in the past (Devroey et al., 2011; Mertes and
Pennings, 2011). If we deem that the main value of the oocyte lies in
serving sexual reproduction, then the donation of oocytes for SCNT
may be more acceptable when women donate oocytes in excess of
their own reproductive needs rather than when they donate in the
sole interest of science. In the case of egg-sharing programmes, in
which oocyte donation may deprive the woman of her own chance of
having a child, donation still serves sexual reproduction. In Tachibana
et al.’s study, donors were recruited via print and web-based advertising
apparently unrelated to clinical programs of assisted reproduction. In the
case of surplus oocytes from ART programs, allocation of oocytes for
SCNT was subordinate to their use in assisted reproduction, whereby
the health risks—however small—associated with oocyte donation
were balanced with the benefit of establishing a family; donation for
SCNT of surplus oocytes harvested in the context of assisted reproduc-
tion is documented (French et al., 2008). In the case of oocytes used to
generate cloned ESCs via SCNT, the risks are not balanced for the oocyte
donor since the ESC lines are matched to the donor of the somatic nuclei
not the donor of the oocytes.

Personally I would consider understanding the mechanisms of human
oocyte-mediated nuclear reprogramming an important goal, and I would
not welcome a worldwide ban on creating human embryos for research
provided this is done under conditions protecting the autonomy and
wellbeing of oocyte donors; but at the same time I think the science of
SCNT (e.g. therapeutic cloning) and the clinical practice of assisted
human reproduction should not compete for the same resource, i.e.
for the good oocytes. Otherwise, it becomes difficult to choose the
former in the face of the demographic crisis of western countries. One
possible way to reconcile the two applications may be to allocate the
good oocytes for reproduction, and the not-so-good oocytes for
SCNT. Since oocytes are stripped of their chromosomes prior to
SCNT, defective oocytes that are at high risk of meiotic aneuploidy
and have poor reproductive prospects could still be suitable for re-
programming. Although Tachibana et al. used the oocytes of young
donors, it has been shown in mice that the oocytes of old donors
support better reprogramming than the oocytes of young donors
(Esteves et al., 2011).

**Open issues**

The current breakthrough raises exciting questions. Some are mechan-
istic, others are more application oriented, and still others are philosoph-
ical. Can a human oocyte alone (without help from chemistry, e.g.
caffeine and TSA) reprogram a somatic nucleus? For Tachibana et al.,
none of the transplanted oocytes could make it without caffeine or
TSA. In my view, the scientific value of SCNT into oocytes is to identify
oocyte-borne factors that may help refine iPSC reprogramming. In this
respect, even if such human oocytes are missing some components
and need some molecular additions, it will still be exciting to see
their proteome analysis compared with that of other species (Pfeiffer
et al., 2011).

Can the experimental protocol of Tachibana et al. be successful on any
human oocyte? In a recent study, three human oocytes were compared
with each other for transcriptome, and only 30% of the total expressed
genes were detected in all three oocytes, suggesting that each human
oocyte may have its own molecular signature (Shaw et al., 2013).
Maybe the oocyte variability between individuals and between ethnic
groups is as large as the oocyte variability within individuals, a challenging
puzzle for scientists.

Do cloned human ESCs set a new quality standard with which to
compare the products of other reprogramming systems? If ESCs
derived from normal IVF fertilized embryos are assigned the highest
level of quality, it is questionable if cloned ESCs have a real chance to
compete at that level: SCNT lines are probably going to be of better
quality than iPSCs, but worse than IVF-based human ESCs. The starting
material in IVF and SCNT—somatic versus sperm chromatin—is simply
different. The oocyte tries to process the somatic nucleus as if it were
sperm, engaging in a ‘battle for supremacy’ (Gurdon, 2013). However,
if the starting points are different when the means of transportation
and the duration of the journey are the same, it logically follows that
the end-points will also be different. For this reason I doubt that
cloned embryos and derivative ESCs will ever attain the same quality
as their IVF counterparts, but this remains to be assessed experimentally.

Do Tachibana et al. envision the treatment of inherited mitochondrial
diseases via germline gene therapy, e.g. via the transplantation of a
nucleus from a mitochondrially diseased fertilized embryo into a rescuing
oocyte? I should stress the fact that this procedure is not SCNT since no
somatic material whatsoever is introduced into the oocyte. While this
mitochondrial rescue is considered beneficial for reproduction, the pres-
ence of mitochondrial antigens from the oocyte donor may reduce the
immunocompatibility of the cloned ESC-derived tissues with the somatic
nucleus donor/potential patient.
Does the derivation of pluripotent cloned human ESCs mean that the precursor embryo, in addition to a normal ICM, also has a normal trophectoderm and qualifies therefore as totipotent? The ESCs in the study of Tachibana et al. had many features common in ESCs derived from fertilized human blastocysts (e.g. immunocytochemistry for OCT4, NANOG, SOX2, SSEA4, TRA-1-60, TRA-1-81; microarray expression profile; embryoid body formation; cardiac differentiation). Formation of teratoma tumors after transplantation of cloned human ESCs into SCID mice is another strong hint that the cells are functional, although inferring normalcy from a tumor is an oxymoron. It is worthwhile to recall that human ESCs, in contrast to mouse ESCs, are very well capable of generating trophectoderm and tropoblast spontaneously in vitro (Golos et al., 2013); however this avenue of differentiation was not explored by Tachibana et al. All in all, these data do not show that the precursor embryos in the study of Tachibana et al. were totipotent. We can discuss forever the potency of the cloned blastocysts of Tachibana et al. but the litmus test, i.e. the only conclusive assay remains the transplantation of the cloned embryo into a uterus followed by full development. There is no automatism and no need for such undertaking, while the prospect of therapeutic applications is far more appealing than creating a genetic copy of an individual (Cibelli et al., 2001). Can cloned human ESCs really be used for therapeutic purposes? It is likely not possible in the short term as long as the risk of teratoma formation is significant, but differentiation protocols will improve and the risk will become more acceptable.

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References


