The ups and downs of somatic cell nucleus transfer (SCNT) in humans

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Abstract Achieving successful somatic cell nuclear transfer (SCNT) in the human and subhuman primate relative to other mammals has been questioned for a variety of technical and logistical issues. Here we summarize the gradual evolution of SCNT technology from the perspective of oocyte quality and cell cycle status that has recently led to the demonstration of feasibility in the human for deriving chromosomally normal stem cells lines. With these advances in hand, prospects for therapeutic cloning must be entertained in a conscientious, rigorous, and timely fashion before broad spectrum clinical applications are undertaken.

Keywords Nucleus · Oocyte · Nucleus transfer

Simply stated, SCNT in mammals should in practice be a very straightforward technique. First, cytoplasts are prepared by enucleating the metaphase II stage oocytes, i.e. metaphase II chromosomes are removed from the oocyte. Second, the selected nucleus is introduced into the cytoplast either by direct injection or by induced cell fusion. The reconstructed SCNT products are then activated in ways mimicking fertilization and if everything goes well cloned embryos develop [6].

Capsule This paper is dedicated to the memory of Keith Campbell (1954–2012), a pioneer in the field of somatic cell nuclear transfer.

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Logically, since following the birth of Dolly [7], and the production of additional clones in other mammalian species, there is ample confirmation that Dolly was not an exceptional case, prompting widespread discussion regarding the pros and cons of human SCNT. Given this prospect, it has been commonly accepted and broadly emphasized that the production of cloned human individuals must be banned (reproductive cloning). On the other hand, the production of embryos from which patient compatible embryonic stem cells could be obtained seemed to be acceptable, at least in some countries (therapeutic cloning). However, once the discovery of induced pluripotent stem cell production (iPSC) was realized by the work of Takahashi and Yamanaka [see 8 for review], illustrating that differentiated or even terminally differentiated cells can be converted (dedifferentiated) into a pluripotent state by induced overexpression of four factors (Oct 4, Sox 2, Klf 4, c-Myc; OSKM), greater resistance against the use of SCNT in humans has emerged, including the prospect of therapeutic cloning.

Although many papers on modifications of the original iPSC technology seeking improved efficiency and safety have been published, it should be emphasized that our understanding of the mechanisms by which iPSC elicits dedifferentiation remain woefully incomplete. Instead, it is commonly accepted that the oocyte cytoplasmic milieu is the most direct means to obtaining reprogramming of a differentiated somatic cell nucleus [9, 10]. This fact—the quality of the oocyte cytoplasmic milieu—may well underscore the reason that while research on human SCNT continues, reports are rare in this species relative to the many papers published in other mammals.

Birth of Dolly

The work that led to the birth of Dolly was antedated by many years of experiments using laboratory and domestic animals to test many methodological combinations and schemes. Initial

experiments used almost exclusively differentiating nuclei of early developing embryos and cytoplasts that were produced by enucleating pronuclear stage embryos. The use of one-cell (pronucleus) stage embryo cytoplasts resulted in completely negative and discouraging results prompting Nobel laureate John Gurdon [11] to state that: “For reasons I have never fully understood, the next major attempts to transplant nuclei in mammals used fertilized eggs as recipients, rather than unfertilized eggs as had proved successful in frogs.”

The first scientist who successfully used cytoplasts produced by enucleation of metaphase II oocytes was Steen Willadsen [12]. In his experiments, blastomeres isolated from 8-cell stage embryos were fused to mature oocyte cytoplasts and several lambs were born. This fundamental scheme, or modifications of it, has been adopted in all subsequent experiments. The trend from this point on was to combine mature oocyte cytoplasts with more and more developmentally advanced cells as donor karyoplasts. In tandem, many technical improvements were realized, such as the piezo injector for nuclear transfer, which collectively accelerated progress in this field [6]. But even after birth of several offspring from cultured inner cell mass cells (ICM) or embryonic fibroblasts, used as a source of somatic cell nuclei for SCNT [13, 14], it was widely assumed that the production of offspring from nuclei of already newborn or adult individuals would be impossible in the near future or not possible at all. This fatalistic opinion amongst the scientific community was not altered even after the birth of Dolly with many prominent scientists within and without the field claiming that Dolly was a rare and unrepeatable case. This attitude was soon to change upon the birth of clones from SCNT in a number of different mammalian species. Concomitantly, and quite logically, discussion and discourse entered the arena of SCNT technology with respect to the prospect of human cloning. For better or worse, the stage was set for the potential of SCNT to become blurred with the manipulation of human reproduction.

Big problems in humans

As mentioned above, the birth of Dolly was becoming perceived as hardly a chance event having been preceded by many years of empirical testing of different experimental schemes and combinations. The nearly unlimited supply of oocytes and somatic cells enabled scientists to study animal SCNT in more detail, always with the aim in mind to improve its efficiency. In sharp contradistinction, it was recognized and appreciated to be a nearly impossible task to achieve SCNT in humans given the formidable obstacle of supply—where would a sufficient number of oocytes be obtained for cytoplast production?

In laboratory and domestic animals these oocytes can be relatively easily obtained either after induced superovulation

(rodents) or after aspiration of immature oocytes with their subsequent *in vitro* culture (ungulates). Patients in human assisted reproduction clinics are similarly hormonally stimulated to yield many oocytes upon egg retrieval but the mature oocytes are primarily and almost exclusively destined for embryo production and infertility treatment. Moreover, it has become clear that, generally speaking, the quality of human oocytes is lower when compared to oocytes of the other mammalian species studied so far. One of several manifestations for this is the frequency of aneuploidies causing embryonic failure which tends to be very high in human oocytes and rather low in other mammalian species [15]. The reasons for this are not yet fully explained. It is, however evident that the quality of oocytes decreases with the maternal age, and this is for example manifested by the loss of cohesins. Environmental factors (endocrine disruptors, bisphenol A—BPA) also play an important role. It has been also shown that the mitochondrial DNA (mtDNA) content in oocytes is highly variable and ranges from 11.000 to 903.000 mtDNA molecules. Mitochondria are the powerhouse in the cell (production of ATP) but they are also engaged in many other cellular processes [16]. Moreover, analyses of epigenetic characteristics of chromatin (histone methylation and acetylation) clearly show that the population of human oocytes retrieved after controlled ovarian hyperstimulation (COH) is rather inconsistent and variable even between oocytes from the same patients [17] and certainly some other oocyte characteristics will influence the success of SCNT in humans [18]. It should be emphasized, however, that studies of this kind involve predominantly oocytes deemed unsuitable for IVF or ICSI, so it remains unclear whether these oocytes are abnormal *per se*. Human oocytes seem to be also very sensitive to external changes—for example the fluctuation of temperature. Taken together, these confounding factors have limited progress in the human SCNT field and have most recently prompted redirecting the sourcing of human oocytes to those donated by reimbursed and generally younger donors.

Inconsistent results

In spite of the obstacles mentioned above, several papers reporting SCNT in humans have been published. Not all will be mentioned here, for example the papers by Hwang et al. [19, 20] because these results have been questioned in the literature (for example, see commentaries by David Cyranoski in *Nature*). It is difficult to draw firm conclusions from these papers. In general, embryos produced by NT underwent few cleavage divisions and only exceptionally reached the blastocyst stage [21–24]. It must be noted, however, that cytoplasts were sourced from oocytes that were either immature at the time of egg retrieval and were subsequently *in vitro* matured to metaphase II stage in culture, or

from oocytes deemed unsuitable for IVF. Eventually, enucleated pronuclear stage embryos were used. The first paper with promising results was published by French et al. [25]. These studies reported production of several SCNT blastocysts (23 %) when male fibroblast cell nuclei were introduced into 29 enucleated oocytes obtained from three young donors. When compared to previous reports, a likely reason for success in this instance can be traced to the source of oocytes used for cytoplasm preparation. Oocyte donors were young and demonstrated to have been successful in previous COH stimulation cycles. In light of this, the results published later by Noggle et al. [3] were rather surprising. These authors studied several aspects of human SCNT and concluded that the ordinary NT scheme cannot lead to the production of blastocysts because the reconstructed embryos ceased their development after several cleavages. The reason for arrested development was suggested to be aberrant activation of embryonic genome in reconstructed embryos. Interestingly, when the oocyte genetic material (chromosomes, oocytes were not enucleated) was not removed and nuclei were introduced into intact oocytes, the embryos developed quite well and several embryonic stem cell lines, albeit triploid, were produced. Somewhat surprisingly then, another paper published soon after this report [4] reported successful production of human SCNT blastocysts using the conventional SCNT scheme. No ESC lines were derived from these blastocysts. One telling difference in these latter experiments was the use of in vitro matured oocytes for cytoplasm production. When these three papers are compared, it is very difficult to find a satisfactory explanation for these inconsistent results. Moreover, we can no longer presume that humans are so exceptional or unique such that SCNT would be impossible to achieve in our species.

Or are human oocytes so different when compared to oocytes of other species? Some results indicate that they are, since the frequency of aneuploidies is much higher than in oocytes of other species (mouse 5 %, humans about 50 %) [15]. Again, it must be noted here that these results reflect studies based on analyses of patients treated for different forms of infertility. So, the conclusion is again very difficult. On the other hand, the production of parthenogenetic human ESC lines is a routine procedure and here the oocytes used are not that much different from those that are used for nuclear transfer. The supporting role of the oocyte nuclear material is also not surprising. Essentially, in early days of nuclear transfer experiments the reconstructed embryos only developed when nuclei were injected into non-enucleated zygotes [26, 27]. Thus, one may conclude that more experiments may shed some light on human NT and explain the inconsistent results.

The very recent paper published by Mitalipov's groups confirmed that this is true [5]. In these experiments the oocytes from paid donors were used and some minor, but

probably very essential modifications, were introduced into their NT scheme. First, the oocytes were enucleated in the presence of caffeine and second, after NT the reconstructed embryos were cultured in a medium containing TSA (trichostatin A). In fact, both these treatments were already used in animal NT experiments and improved their efficiency [9]. In general, the NT scheme has been perfected in previous experiments performed by this group and some other technical details can be found in a given paper [5]. Taken together, the efficiency of a given NT scheme was quite high and this was confirmed by a very good embryonic development and high efficiency when ESC lines were established from them. Interestingly, the NT blastocysts exhibited poorly developed trophoblast but this does not prevent the efficient establishment of ESC lines. In our opinion, there are some additional interesting points mentioned in this paper that may have the implication in a conventional human assisted reproduction. The authors conclude that only those oocytes having a premium quality have a chance to develop well after NT. Second, the best oocytes were obtained from donors from which 10 or less oocytes were collected. Does the same apply to conventional IVF (ICSI)?

Summary

It has not been our intention to review the scant literature on SCNT in humans with respect to detailing experimental procedures so as to provide a modicum of speculation that might help to explain a dichotomy— that is why there are some studies reporting production of blastocysts whilst others suggested that SCNT is impossible. For non-human primates, failure to produce cloned offspring by SCNT has been reported. However, blastocysts produced by SCNT have been used to derive embryonic stem cell lines [28]. This now applies for humans too. It is also clear, that the human and nonhuman primates present subtle but important distinctions in the physiology of the cell cycle and egg activation but this would not come as such a surprise. It is, however, our opinion that the production of blastocysts in humans by SCNT is and will be very difficult. A key factor seems to be the insufficient number of oocytes available for cytoplasm production, even with the use of donor eggs. Moreover, as noted above, the incidence of genetic and epigenetic instability in human oocytes is disconcerting, further contributing to the overall problem of poor oocyte quality especially as a function of advancing age or environmental factors [15]. Nevertheless, we also do believe that experiments aiming at producing blastocysts by SCNT in humans and primates [28] will certainly accelerate the introduction of some technical improvements, that might be very useful in solving certain problems of assisted human

reproduction, into clinical practice. These approaches are now almost exclusively oriented on the elimination of mutated mitochondrial DNA (mtDNA), that can cause some devastating diseases [29]. It has been demonstrated quite recently in humans that the transfer of pronuclei or spindles, isolated from the cytoplasm containing mutated mtDNA, into healthy cytoplasts seems to be very promising and healthy looking blastocysts were obtained [30–33].

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