nature portfolio

Peer Review File

Induction of Experimental Cell Division to Generate Cells with Reduced Chromosome Ploidy

Corresponding Author: Professor Shoukhrat Mitalipov

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The present manuscript, authored by Marti Gutierrez et al., investigates the potential for experimental reduction of somatic chromosome ploidy in human oocytes through the process of mitomeiosis.
This study proposes a novel method for reducing chromosome number in human somatic cells through somatic cell nuclear transfer (SCNT), with the objective of facilitating in vitro gametogenesis (IVG) for individuals who are deficient in functional oocytes.
The researchers transferred non-replicated (2n2c) somatic nuclei into enucleated, metaphase II (MII) human oocytes, inducing a premature metaphase-like state without DNA replication—a process termed mitomeiosis. As the authors hypothesise, under typical conditions, such SCNT oocytes are incapable of proceeding to fertilisation due to spindle checkpoint arrest. However, by applying an artificial activation process (involving electroporation and roscovitine), the research team was able to successfully trigger chromosome segregation.
Chromosome tracing revealed random segregation of somatic chromosomes, with an average of 23 retained, mimicking haploidization. These SCNT oocytes were then fertilised with sperm, developing into early embryos, with some reaching the blastocyst stage. Despite the absence of recombination and the incomplete chromosome distribution, this study signifies a pioneering endeavour in the field of reproductive biology, demonstrating the potential for the reconstitution of functional gametes from somatic cells in humans. This advancement presents novel therapeutic prospects for the treatment of infertility.

The findings of the research employing human oocytes and embryos are of considerable value and significance to researchers in the field of reproductive biology and its application to infertility treatment.
A crucial point that merits correction is the assertion made by the authors, which lacks a solid foundation.

The authors concluded that, in contrast to the results observed in mice, human embryos subjected to SCNT have been shown to undergo chromosome segregation only 25% of the time upon signal from sperm fertilisation. The argument was posited that this phenomenon was attributable to the spindle checkpoint for non-replicated chromosomes in M-phase. However, the M-phase checkpoint is a spindle assembly checkpoint (SAC), and there is no spindle checkpoint for non-replicated chromosomes. It is requested that further elucidation be provided on the rationale behind the exclusion of the mitotic phase from consideration in human subjects, in contrast to its inclusion in murine models.
In the event of a spindle assembly checkpoint, the SAC protein of the kinetochore can be examined by means of immunostaining. In the event of calcium oscillation being rendered impossible due to damage sustained from enucleation or double injection, the utilisation of a calcium indicator or the examination of cyclin activity is recommended.

Specific points:

L271-L273: “These results suggest that partition of somatic chromosomes, consisting of single chromatids after activation of human SCNT oocytes is more or less equal. Similar to conventional meiosis, mitomeiosis allows the reduction to half the diploid chromosome number in SCNT zygotes.”
It is an oversimplification to say that mitomeiosis is similar to conventional meiosis, since conventional mitosis divides evenly and causes few chromosome segregation error. Please, tone it down.

Fig.2e:
Please also indicate the percentage of abnormal SCNT spindle formation. Does the percentage in the graph match the

percentage of SCNT oocytes in Fig. 1f that can't extrude PB?
Also discuss why these spindles are formed.

Fig.4g:
This shows that chromosome length was not biased, but what about shape?
It has recently been shown that acrocentric chromosomes (a type of chromosome) are more likely to have problems (https://doi.org/10.1038/s41467-024-54659-3).

Fig.4h:
Why is there a bias for chromosome 8?
Could you please explain a little more?

All graphs:
All graphs should be in a format where individual values are visible.
It is also advisable to include specific P values as well as asterisks.

Reviewer #2

(Remarks to the Author)
Review of MS "Induction of Experimental Cell Division to Generate 1 Cells with Reduced
2 Chromosome Ploidy", by Gutierrez et al.

The manuscript explores, in a deeply, unprecedented way, the direct haploidization of somatic cells, leveraging on the meiotic machinery of the MII oocytes, with the long-term goal to generate artificial gametes for the treatment of infertile couples. Segregation of the chromatids and their parental origin in Polar Bodies (PB) and ProNuclei (PN) has been tracked (previous PB and PN individual sampling by micromanipulation!) by chromosome sequencing of genetically characterized gamete and cell donors to precisely examine ploidy reduction mechanisms. The analysis at individual blastomere finally revealed total number/balance in somatic and sperm chromosomes in preimplantation embryos derived from fertilized human SCNT oocytes.
The manuscript addresses a long term sought in assisted reproduction, that is for the direct production of haploid gamete through the transfer of somatic cell into enucleated oocytes. The experimental work is logically conceived, and brings useful information for specialist working on artificial gamete production.

There are several concepts expressed in the text that I do not fully share, for the following reasons.

At Page 3, lines 62-64, is it stated: “including rapid and efficient reprogramming of transplanted somatic cell genomes by natural oocyte cytoplasmic factors, leading to robust erasure of the somatic epigenetic program and the establishment of an oocyte-like epigenome within hours”. I disagree with the wording. The transplanted somatic genome is NOT efficiently reprogrammed by the oocyte’s cytoplasmic factors, NOR is its somatic epigenetic program erased to an oocyte-like epigenome, as stated. Rather, the efficiency is very low in all the published data available, including the present one, as demonstrated by the 8.8% blastocyst development of fertilized human SCNT oocytes.

Page 3, lines 70-73, it is stated “It is a common misconception that SCNT immediately generates a totipotent embryo-like entity; in reality, embryonic development is initiated only upon activation of the SCNT oocyte”. This is pleonastic, everyone knows it. Please, amend of remove.

Page 4, line 90: “In this study, we adapted mitomeiosis for human SCNT oocytes”. The term mitomeiosis is a synthesis coined by the Authors, that might sound exotic, but in reality, the asymmetric EXTRUSION and/or RETENTION of a set of chromosomes – as accomplished in this work - belongs ONLY to the meiotic process. I found the wording unappropriated.

Page 5, lines 147-149: “that, following fusion with enucleated MII oocyte cytoplasm, somatic 2n2c chromatin directly enters metaphase, bypassing S-phase.” Again, this is what physiologically takes place; S-phase occurs later, after PN organization; please, remove or amend.

Page 14, lines 387-390. “However, human SCNT oocytes did not respond to the conventional activation stimulus induced by fertilization with sperm possibly caused by the spindle checkpoint arrest that can sense presence of nonduplicated chromosomes”. The reason presented in here to justify the absence of activation induced by the spermatozoa does no stand at all. In ALL meiotic MII oocytes fertilizing chromosomes are unduplicated; DNA synthesis starts ONLY after PN organization.
The fact that sperm-induced activation is insufficient to release SCNT oocytes from the MII arrest is very interesting, but the reasons must be searched somewhere else. The fact that a second spindle is assembled following removal of the original one, and the transfer of a somatic nucleus might be a track.

The use of roscovitine and electric activation in SCNT are not original, but well established and dated activation protocols.

Page 15, lines 429-31. ”It is likely that many of these critical components of meiosis I are absent in the MII oocyte cytoplasm

thus precluding somatic homolog pairing and accurate segregation in human SCNT oocytes". I do not agree. The structure of somatic chromosomes is by far more structurally complex than an oocytes' chromatids; it might be well that homolog paring is precluded by this structural conformation.

What is missing in the manuscript are comments on the fact that, besides the difficulty to achieve a balanced haploidization, the major problem is that the haploid set of somatic chromosomes must be also fully reprogrammed. The quality of the blastocyst displayed in figure E is very poor, overlapping typical SCNT blastocysts; further, only 8.8% of fertilized SCNT embryos developed to blastocysts, clearly indicated a defective nuclear reprogramming of the somatic chromosomes. What has been achieved in this work is essentially what was defined time ago a "semi cloning". While an unbalanced chromosome composition might result in early developmental failures, the problems of having a set of not fully reprogrammed chromosomes might instead appear later in live, with much more serious problems for clinical applications.

Reviewer #3

(Remarks to the Author)
I have been asked to conduct a bioethics review of this manuscript, "Induction of Experimental Cell Division to Generate Cells with Reduced Chromosome Ploidy." The authors offer a compelling and novel alternative to standard IVG approaches to human oocyte derivation. Given the potential benefits of this alternative approach, the following questions should be addressed, first around the generation of research embryos in this protocol, and second around the procurement of human biomaterials used in this study.

Could the authors explain how many research embryos (SCNT zygotes) in total were created during the course of this study? Also, what was the furthest developmental timepoint that any one of these research embryos was studied and why that particular timepoint?

The research team used donated oocytes (procured after hormonal induction of healthy female volunteers), donated sperm, and donated somatic cells.

The authors describe in Methods that the oocyte donors were compensated $5,000-$8,000 per donation cycle. The fact that financial compensation was provided is not an issue for this reviewer, as compensation for time, effort and inconvenience is permitted by the ISSCR guidelines for healthy oocyte donors for basic research. But what accounts for this $3,000 difference in the range of the compensation amount? Did they not all undergo basically the same burdens and inconveniences? Furthermore, why not use frozen eggs (with informed consent) rather than ask healthy volunteers to undergo hormonal induction and egg retrieval specifically for this study? Are there specific scientific reasons not to use frozen oocytes for this study?

The authors also explain that somatic cells were procured through skin biopsy and blood. Did the somatic cell donors each provide both skin and blood or were there separate skin and blood donors? Why was there a need to have skin donation at all - why not just rely on blood donors as a source of somatic cells?

Could the authors provide me with the informed consent forms used for each of these procurement practices? It may also be a good idea to provide these consent forms as part of the authors' supplemental information files.

In summary, this is a fascinating manuscript and the questions above are only meant to offer this reviewer a deeper understanding of the ethical care taken around this important study.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I appreciate your thorough responses to my previous inquiries. I appreciate your efforts to revise the manuscript and clarify certain aspects of the process. However, I believe that some fundamental issues remain unresolved.
Firstly, it appears that there is some confusion regarding the spindle assembly checkpoint (SAC). The SAC does not act as a checkpoint for unreplicated chromosomes. Instead, it functions as a surveillance mechanism that monitors microtubule attachment to kinetochores and the establishment of proper tension across sister chromatids. This helps regulate the timing of anaphase onset. The process is controlled by proteins such as Mad1, Mad2, BubR1, and Mps1. These proteins are found in structures called kinetochores when attachments are wrong or missing. However, in your response, you mention "the presence of kinetochore, SAC protein, and spindle microtubules," but you do not mention whether you checked for SAC-specific components (like Mad2). The use of general markers for centromeres or tubulin alone is insufficient for demonstrating SAC activation.
I would like to express my concern that there appears to be no clear connection between the observed arrest and SAC activity or failure. However, merely examining spindle structures or chromosome shapes does not provide sufficient evidence to determine whether SAC is being utilized. Interpreting the arrest as due to a tension-based mechanism is

speculative at this point, and as it is written now, it lacks proper referencing or experimental validation.
If the primary assertion is that the MII arrest in SCNT oocytes is attributable to a tension-sensitive checkpoint that differs from the standard SAC, then this claim must be explicitly delineated and substantiated by the appropriate citations. These citations should include previous research that shows such mechanisms in mammalian oocytes.
In summary, the manuscript still does not adequately resolve the core mechanistic questions raised in the initial review. These inquiries focus on the nature of the cell cycle arrest and the role (or absence) of the SAC in this context. In the revised discussion, I recommend addressing these concerns with either direct experimental evidence or more cautious, well-referenced interpretations.

Reviewer #2

(Remarks to the Author)
I noticed that the authors answered more o less convincingly all my criticisms.
However, I deeply disagree with the sentence in the reply letter "However, due to protocol optimizations in cattle and many other farm animals, blastocyst and full-term development of SCNT embryos now days is similar or even higher thanfor IVF embryos.". I am one of the bunch of guys doing nuclear tranfer in the 90ties, and still doing it, and the efficiency in terms of offspring is unfortunately more o less the same the 26 years ago.
I still maintain my perplexities over the term "mitomeiosis".
Having state that, I am fine with its publication, however, a clear warning should be added on the likely risks of using haploidization as a "gamete" making tool, caused by the lack of reprogramming of the somatic cell chromosomes.

We would like to thank all reviewers for their time and efforts to review our manuscript and for helpful suggestions to improve the conclusions and presentation of the paper. We conducted additional analyses, revised the figures and text to clarify some points as suggested by the reviewers. Please find the uploaded revised manuscript files along with a point-by-point response to the critiques detailing how we have addressed the reviewers' concerns.

Reviewer #1 (Remarks to the Author):

The present manuscript, authored by Marti Gutierrez et al., investigates the potential for experimental reduction of somatic chromosome ploidy in human oocytes through the process of mitomeiosis.
This study proposes a novel method for reducing chromosome number in human somatic cells through somatic cell nuclear transfer (SCNT), with the objective of facilitating in vitro gametogenesis (IVG) for individuals who are deficient in functional oocytes.
The researchers transferred non-replicated (2n2c) somatic nuclei into enucleated, metaphase II (MII) human oocytes, inducing a premature metaphase-like state without DNA replication—a process termed mitomeiosis. As the authors hypothesise, under typical conditions, such SCNT oocytes are incapable of proceeding to fertilisation due to spindle checkpoint arrest. However, by applying an artificial activation process (involving electroporation and roscovitine), the research team was able to successfully trigger chromosome segregation.
Chromosome tracing revealed random segregation of somatic chromosomes, with an average of 23 retained, mimicking haploidization. These SCNT oocytes were then fertilised with sperm, developing into early embryos, with some reaching the blastocyst stage. Despite the absence of recombination and the incomplete chromosome distribution, this study signifies a pioneering endeavour in the field of reproductive biology, demonstrating the potential for the reconstitution of functional gametes from somatic cells in humans. This advancement presents novel therapeutic prospects for the treatment of infertility.

The findings of the research employing human oocytes and embryos are of considerable value and significance to researchers in the field of reproductive biology and its application to infertility treatment.
A crucial point that merits correction is the assertion made by the authors, which lacks a solid foundation.

The authors concluded that, in contrast to the results observed in mice, human embryos subjected to SCNT have been shown to undergo chromosome segregation only 25% of the time upon signal from sperm fertilisation. The argument was posited that this phenomenon was

attributable to the spindle checkpoint for non-replicated chromosomes in M-phase. However, the M-phase checkpoint is a spindle assembly checkpoint (SAC), and there is no spindle checkpoint for non-replicated chromosomes. It is requested that further elucidation be provided on the rationale behind the exclusion of the mitotic phase from consideration in human subjects, in contrast to its inclusion in murine models.

In the event of a spindle assembly checkpoint, the SAC protein of the kinetochore can be examined by means of immunostaining. In the event of calcium oscillation being rendered impossible due to damage sustained from enucleation or double injection, the utilisation of a calcium indicator or the examination of cyclin activity is recommended.

Response: We thank the reviewer for his/her time and efforts put to evaluate our manuscript and very helpful suggestions to further elucidate the rationale behind the explanation of the MII-phase arrest observed in our study in human SCNT oocytes. Our results indicate that most interphase somatic cell nuclei introduced into the cytoplasm of enucleated MII-arrested oocytes do form M-phase-like spindles suggesting that they bypass the M-phase checkpoint or spindle assembly checkpoint (SAC). These results are illustrated in the Figure 2 of our manuscript including presence of kinetochore, the SAC protein, and spindle microtubules detected by immunostaining and time-lapse video (Supplemental Video 1, 2). However, low fertilization and cleavage rates of human SCNT oocytes (ICSI alone) suggests problems in exiting from the oocyte-specific MII-phase arrest and entrance into interphase of next cell cycle. We agree that the problem seems to be not in the spindle assembly checkpoint (SAC) per se but rather in MII cell cycle arrest. During natural meiosis II, sister chromatids generate bi-polar tension when attached to opposite spindle poles. In contrast, single-chromatid chromosomes in SCNT oocytes cannot generate this bi-polar tension, potentially preventing exit from MII arrest and progression to anaphase. Observed abnormal structural spindle morphologies in approximately 60% of human SCNT oocytes could be caused by multiple factors including tension-based checkpoint arrest. Therefore, we revised the manuscript to clarify that we are proposing a tension-based checkpoint mechanism rather than SAC **(Pages 14-15, Lines 409-414)**.

Regarding the exact mechanisms of why SCNT oocytes fail to respond to sperm induced activation and exit from the MII arrest, we conducted series of experiments targeting the known pathway of oocyte activation (Figure 3). We ruled out potential issues with calcium oscillations since in most species (except mice!) including bovines, nonhuman primates and humans, exposure of SCNT oocytes to calcium treatments alone cannot induce exit from the MII arrest (Mitalipov et al., Biol Reprod, 2001; Byrne et al., Nature, 2007; Tachibana et al., Cell, 2013). For example, electroporation, calcium ionophore or ionomycin treatments all induce efficient calcium oscillations in intact MII or SCNT oocytes and briefly release from MII arrest. However, MPF

activity is quickly restored with recondensation of chromosomes and reentry of oocytes into a new M-phase arrest, known as metaphase III. This persistent arrest can be circumvented by additional treatments that inhibit protein synthesis or phosphorylation resulting in high activation and development rates. In our current study, we demonstrate that fertilization with sperm aided with supplemental activation with cyclin-dependent kinase inhibitor roscovitine is required to circumvent the MII arrest and resume cell cycle in human SCNT oocytes.

Specific points:

L271-L273: “These results suggest that partition of somatic chromosomes, consisting of single chromatids after activation of human SCNT oocytes is more or less equal. Similar to conventional meiosis, mitomeiosis allows the reduction to half the diploid chromosome number in SCNT zygotes.”
It is an oversimplification to say that mitomeiosis is similar to conventional meiosis, since conventional mitosis divides evenly and causes few chromosome segregation error. Please, tone it down.

Response: We revised this sentence: "While similar to meiosis, mitomeiosis achieves numerical chromosome reduction, the random segregation pattern and absence of recombination represent fundamental differences from conventional meiotic mechanisms." **(Page 10, Lines 279-283).**

Fig.2e:
Please also indicate the percentage of abnormal SCNT spindle formation. Does the percentage in the graph match the percentage of SCNT oocytes in Fig. 1f that can't extrude PB?
Also discuss why these spindles are formed.

Response: The percentage of human SCNT oocytes exhibiting abnormal spindle morphology is up to 60%. This percentage does not match the 77% of SCNT oocytes that did not extrude the PB in Fig. 1f **(Page 6, Lines 146-147; Page 6, 170-173; Page 21, 523-524)**.

Fig.4g:
This shows that chromosome length was not biased, but what about shape?
It has recently been shown that acrocentric chromosomes (a type of chromosome) are more likely to have problems (https://doi.org/10.1038/s41467-024-54659-3).

Response: As suggested, we conducted an additional analysis for possible segregation bias by chromosome morphology (metacentric vs. acrocentric) but we did not find a significant correlation likely due to insufficient number of samples. We added a sentence in results section to indicate this observation **(Page 11, Lines 297-301)**.

Fig.4h:
Why is there a bias for chromosome 8?

Could you please explain a little more?

Response:

At this point, we don't have any reasonable explanation regarding the bias observed for chromosome 8 segregation rates as this chromosome is relatively moderate in size and aneuploidy rate in human IVF embryos is relatively low compared to some other chromosomes. We added a sentence in the text to reflect this phenomenon **(Page 11, Lines 304-309)**.

All graphs:
All graphs should be in a format where individual values are visible.
It is also advisable to include specific P values as well as asterisks.

Response: We modified all graphs to show individual data points and included specific p-values alongside significance indicators as requested.

Reviewer #2 (Remarks to the Author):

Review of MS "Induction of Experimental Cell Division to Generate 1 Cells with Reduced
2 Chromosome Ploidy", by Gutierrez et al.

The manuscript explores, in a deeply, unprecedented way, the direct haploidization of somatic cells, leveraging on the meiotic machinery of the MII oocytes, with the long-term goal to generate artificial gametes for the treatment of infertile couples. Segregation of the chromatids and their parental origin in Polar Bodies (PB) and ProNuclei (PN) has been tracked (previous PB and PN individual sampling by micromanipulation!) by chromosome sequencing of genetically characterized gamete and cell donors to precisely examine ploidy reduction mechanisms. The

analysis at individual blastomere finally revealed total number/balance in somatic and sperm chromosomes in preimplantation embryos derived from fertilized human SCNT oocytes.
The manuscript addresses a long term sought in assisted reproduction, that is for the direct production of haploid gamete through the transfer of somatic cell into enucleated oocytes. The experimental work is logically conceived, and brings useful information for specialist working on artificial gamete production.

There are several concepts expressed in the text that I do not fully share, for the following reasons.

At Page 3, lines 62-64, is it stated: "including rapid and efficient reprogramming of transplanted somatic cell genomes by natural oocyte cytoplasmic factors, leading to robust erasure of the somatic epigenetic program and the establishment of an oocyte-like epigenome within hours". I disagree with the wording. The transplanted somatic genome is NOT efficiently reprogrammed by the oocyte's cytoplasmic factors, NOR is its somatic epigenetic program erased to an oocyte-like epigenome, as stated. Rather, the efficiency is very low in all the published data available, including the present one, as demonstrated by the 8.8% blastocyst development of fertilized human SCNT oocytes.

Response: We agree that SCNT based reprogramming, while the only known platform inducing the totipotency that operates by natural oocyte factors, is low and often erroneous in some species. However, due to protocol optimizations in cattle and many other farm animals, blastocyst and full-term development of SCNT embryos now days is similar or even higher than for IVF embryos. Low human SCNT blastocyst rates in our study is likely affected by high aneuploidy rates resulted by random segregation. Our focus was on demonstrating proof-of-concept for chromosome reduction rather than optimizing SCNT reprogramming efficiency. Nevertheless, we revised this sentence to reflect this point **(Page 3, Lines 62-66)**.

Page 3, lines 70-73, it is stated "It is a common misconception that SCNT immediately generates a totipotent embryo-like entity; in reality, embryonic development is initiated only upon activation of the SCNT oocyte". This is pleonastic, everyone knows it. Please, amend of remove.

Response: We removed this sentence as the reviewer correctly notes that this is well-established knowledge for experts **(Page 3, Lines 72-74)**.

Page 4, line 90: “In this study, we adapted mitomeiosis for human SCNT oocytes”. The term mitomeiosis is a synthesis coined by the Authors, that might sound exotic, but in reality, the asymmetric EXTRUSION and/or RETENTION of a set of chromosomes – as accomplished in this work - belongs ONLY to the meiotic process. I found the wording unappropriated.

Response: We chose "mitomeiosis" to distinguish this artificial cell division from natural meiosis and mitosis and to emphasize that while it achieves asymmetric chromosome number reduction (meiotic-like), it occurs through direct entry of somatic chromosomes into metaphase without S-phase. We acknowledge this is a synthesis term and ensured clear definition throughout.

Page 5, lines 147-149: “that, following fusion with enucleated MII oocyte cytoplasm, somatic 2n2c chromatin directly enters metaphase, bypassing S-phase.” Again, this is what physiologically takes place; S-phase occurs later, after PN organization; please, remove or amend.

Response: Here, we referred to bypassing the S-phase for the somatic cell nucleus that would from the G0/G1 normally progress to the S-phase. But during the SCNT, the somatic cell nucleus from the G0/G1 enters directly the M-phase, thus bypassing the S-phase. We clarified this sentence: "By transferring G0/G1-arrested nuclei directly into MII cytoplasm, we bypass the S-phase that would normally precede somatic cell division, forcing premature metaphase entry **(Page 6, Line 151)**.

Page 14, lines 387-390. “However, human SCNT oocytes did not respond to the conventional activation stimulus induced by fertilization with sperm possibly caused by the spindle checkpoint arrest that can sense presence of nonduplicated chromosomes”. The reason presented in here to justify the absence of activation induced by the spermatozoa does not stand at all. In ALL meiotic MII oocytes fertilizing chromosomes are unduplicated; DNA synthesis starts ONLY after PN organization.

Response: By duplicated and nonduplicated chromosomes, we meant here that mammalian MII oocytes carry haploid chromosomes but still contain two sister chromatids (duplicated). The second meiotic division in MII oocytes, triggered by sperm entry, induces segregation of sister chromatids. In contrast, the SCNT oocytes carry diploid chromosome set, but each chromosome is presented by a single chromatid since we bypassed the previous the S-phase and did not allow the chromosome duplication to occur **(Page 14, Lines 407-409)**.

The fact that sperm-induced activation is insufficient to release SCNT oocytes from the MII arrest is very interesting, but the reasons must be searched somewhere else. The fact that a second spindle is assembled following removal of the original one, and the transfer of a somatic nucleus might be a track.

Response: We agree that this phenomenon is specific to the SCNT and thus proposed that the reason could be the unusual somatic spindle consisting of chromosomes each carrying a single chromatid. See also our reasoning for the reviewer 1 above.

The use of roscovitine and electric activation in SCNT are not original, but well established and dated activation protocols.

Response: We agree that combination of electroporation followed by roscovitine has been used before for SCNT including by us for nonhuman primates and we provided a couple of references (references #10 and #19) in the manuscript.

Page 15, lines 429-31. "It is likely that many of these critical components of meiosis I are absent in the MII oocyte cytoplasm thus precluding somatic homolog pairing and accurate segregation in human SCNT oocytes". I do not agree. The structure of somatic chromosomes is by far more structurally complex than an oocytes' chromatids; it might be well that homolog paring is precluded by this structural conformation.

Response: Thanks for your suggestion, this is an interesting alternative hypothesis that could contribute to the random segregation pattern. The condensed, differentiated state of somatic chromatin may indeed prevent the homolog recognition mechanisms that operate during natural meiosis. We incorporated this hypothesis into our Discussion **(Page 16, Lines 454-459)**.

What is missing in the manuscript are comments on the fact that, besides the difficulty to achieve a balanced haploidization, the major problem is that the haploid set of somatic chromosomes must be also fully reprogrammed. The quality of the blastocyst displayed in figure E is very poor, overlapping typical SCNT blastocysts; further, only 8.8% of fertilized SCNT embryos developed to blastocysts, clearly indicated a defective nuclear reprogramming of the somatic chromosomes.

Response: This is an important distinction that we acknowledge we did not specifically address. Given that all analyzed embryos showed chromosomal abnormalities, it is impossible in our current study to separate developmental arrest due to aneuploidy from the developmental arrest due to failed reprogramming. Future studies will require embryos with balanced chromosome complements to assess reprogramming quality independently. We acknowledged this limitation and suggested in the Discussion that it represents a critical area for future investigation **(Page 17, Lines 471-479)**.

What has been achieved in this work is essentially what was defined time ago a “semi cloning”. While an unbalanced chromosome composition might result in early developmental failures, the problems of having a set of not fully reprogrammed chromosomes might instead appear later in live, with much more serious problems for clinical applications.

Response: As we indicated above, we revised the discussion to include this point **(Page 17, Lines 471-479)**.

Reviewer #3 (Remarks to the Author):

I have been asked to conduct a bioethics review of this manuscript, "Induction of Experimental Cell Division to Generate Cells with Reduced Chromosome Ploidy." The authors offer a compelling and novel alternative to standard IVG approaches to human oocyte derivation. Given the potential benefits of this alternative approach, the following questions should be addressed, first around the generation of research embryos in this protocol, and second around the procurement of human biomaterials used in this study.

Could the authors explain how many research embryos (SCNT zygotes) in total were created during the course of this study? Also, what was the furthest developmental timepoint that any one of these research embryos was studied and why that particular timepoint?

Response: 328 oocytes were utilized in this study to generate 155 research embryos for purposes of studying developmental potential and genetic analyses. Embryos were not cultured beyond day 5 or 6 after fertilization (blastocyst stage). The blastocyst stage is the stage at which trophoblastic biopsy and preimplantation genetic testing is typically conducted in a clinical context and also the stage at which the embryo is typically transferred to the uterus **(Page 31, Lines 630-634)**.

The research team used donated oocytes (procured after hormonal induction of healthy female volunteers), donated sperm, and donated somatic cells.

The authors describe in Methods that the oocyte donors were compensated $5,000-$8,000 per donation cycle. The fact that financial compensation was provided is not an issue for this reviewer, as compensation for time, effort and inconvenience is permitted by the ISSCR guidelines for healthy oocyte donors for basic research. But what accounts for this $3,000 difference in the range of the compensation amount? Did they not all undergo basically the same burdens and inconveniences? Furthermore, why not use frozen eggs (with informed consent) rather than ask healthy volunteers to undergo hormonal induction and egg retrieval specifically for this study? Are there specific scientific reasons not to use frozen oocytes for this study?

Response: The range in compensation reflects the change in compensation over the last 15 or so years that we have been conducting human oocyte studies. Our goal has always been to compensate research oocyte donors the same amount that we compensate fertility oocyte donors since they are undergoing the same procedures and risks. Clinic competition for fertility oocyte donors and market forces have resulted in an increase in compensation over the last several years. We have adjusted our compensation to match these increases over time. For this particular study, which was conducted during the last 3-4 years, the compensation has increased roughly from $7,000 to $8,000. We have edited this section of the manuscript to clarify this **(Page 32, Lines 658-660)**.

Since oocyte freezing for fertility preservation is a relatively recent phenomenon, there is no large pool of frozen eggs available for research. Most patients freeze eggs for their own personal use and most egg banks freeze eggs for fertility purposes. One might anticipate that at some point in the future there may be some patients who have completed their childbearing and may choose to donate their eggs to research.

Furthermore, in our experience, the development (blastulation rate) of SCNT embryos made using frozen eggs is a lot lower that the blastulation rate using fresh oocytes. Using frozen eggs would therefore necessitate using a much larger number of oocytes.

The authors also explain that somatic cells were procured through skin biopsy and blood. Did the somatic cell donors each provide both skin and blood or were there separate skin and blood

donors? Why was there a need to have skin donation at all - why not just rely on blood donors as a source of somatic cells?

Response: Both a peripheral blood sample and skin biopsy were obtained from all somatic donors. Skin biopsy was necessary because fibroblasts offer several advantages as donor cells for SCNT such as ease of isolation, growth, and maintenance.

Could the authors provide me with the informed consent forms used for each of these procurement practices? It may also be a good idea to provide these consent forms as part of the authors' supplemental information files.

Response: Yes, we would be happy to provide a copy of the consent form used for this study. (see attached).

In summary, this is a fascinating manuscript and the questions above are only meant to offer this reviewer a deeper understanding of the ethical care taken around this important study.