Characterization of Spermatogonial Stem Cells Lacking Intercellular Bridges and Genetic Replacement of a Mutation in Spermatogonial Stem Cells

Naoki Iwamori1,5, Tokuko Iwamori1, Martin M. Matzuk1,2,3,4,5,6*

Introduction

Stem cells are defined as cells that possess both self-renewal activity and differentiation potential. Because of these characteristics, stem cells are expected to be therapeutic targets for regenerative medicine and can be used in the future for the correction of genetic defects [1,2]. Once the mutation is repaired, either the stem cells or differentiated cells could be transferred to the individual to reconstitute or correct the damaged cells or organs. At present, there have been several reported trials utilizing stem cells as a means of regenerative therapy [3–5]. One problem for the utilization of stem cells for regenerative medicine is the difficulty of culture and expansion of stem cells in vitro. Indeed, stem cells used in the regenerative therapies described above are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), for which efficient culture systems have been established [3–5]. Among the various stem cells, spermatogonial stem cells (SSCs) are the only stem cells derived from an adult animal and for which both long-term culture systems and transplantation techniques have been developed [6–9].

SSCs arise from gonocytes during postnatal development of the testis, possess self-renewal potential, and allow spermatogenesis to continue throughout adult life [10,11]. SSCs can be isolated from both postnatal and adult testes and be maintained long-term in vitro in an undifferentiated state in the presence of glial cell line derived neurotrophic factor (GDNF) [7–9]. SSCs can reconstitute spermatogenesis when they are transplanted into germ cell-depleted testes [6]. A number of genes, including cell surface markers, transcription factors, and other proteins, have been identified as markers of undifferentiated spermatogonia, and some of these proteins are essential for self-renewal of SSC, although the molecular mechanisms regulating SSCs still remain elusive [12–27].

During spermatogenesis, a unique structure called the intercellular bridge is observed [28,29]. As germ cells begin the process of differentiation, they are connected with neighboring sister germ cells through the intercellular bridge. We previously identified testis-expressed gene 14 (TEX14) as an essential component in the intercellular bridges during spermatogenesis [30–32]. When we produced a knockout of TEX14, spermatogenesis was arrested and only spermatogonia remained even in 1-year-old testes [30]. Moreover, most PLZF-positive spermatogonia existed as single cells in TEX14 null testes, suggesting that TEX14 null SSCs were at least maintained even in aged testes. However,
whether SSCs without intercellular bridge can self-renew, differentiate, and proliferate remains unclear.

Herein, we analyzed the characteristics of testes without intercellular bridges by examining the expression of SSC marker genes, established long-term cultures of SSC with a mutation in *Tex14*, and showed that we could correct a genetic defect in *Tex1f*” spermatogonia by homologous recombination to reveal the therapeutic potential of SSCs for regenerative medicine.

**Materials and Methods**

**Animals and Cell culture**

TEX14 heterozygous mice [30], which were C57BL/6J:129S5/SvEvBrd hybrid background, were crossed to actin-GFP transgenic mice. *Tex1f”* and GFP double-mutant mice were backcrossed to DBA strain for at least two generations before they were intercrossed. All mouse experiments were performed in accordance with protocols approved by the Institutional Care and Use Committee of Baylor College of Medicine.

Establishment of spermatogonial stem cell lines was performed as previously described [7]. Briefly, testes and tails were collected from GFP-positive pups at postnatal 7 days and processed for establishment of spermatogonial stem cell lines and genotyping, respectively. After removal of the tunica, isolated testes were treated with a two-step enzymatic digestion with collagenase and trypsin [33]. Testicular cells were suspended in SSC media supplemented with 10 ng/ml rat glial cell derived neurotrophic factor (GDNF; R&D systems), 20 ng/ml mouse epidermal growth factor (EGF; eBiosciences), 10 ng/ml human basic fibroblast growth factor (bFGF; eBiosciences), and 10^3 U/ml mouse leukemia inhibitory factor (LIF; Millipore) and seeded onto gelatinized dish. After seven days of culture, SSCs were collected by gentle pipetting and seeded onto inactivated mouse embryonic fibroblast (MEF) feeder layers. SSCs were cultured for at least one month before use in additional experiments.

**Magnetic Activated Cell Sorting**

Testicular cells prepared as described above were incubated with rat anti-CD9 antibodies (BD Biosciences) on ice for 30 min, followed with anti rat IgG microbeads (Miltenyi Biotec) on ice for 20 min. CD9-positive cells were separated by MACS cell separation system (Miltenyi Biotec) according to the manufacturer’s instructions.

**Quantitative RT-PCR**

Total RNA from testes and SSCs were extracted using the RNA mini and micro kit (Qiagen), respectively, according to the manufacturer’s instructions and reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and an oligo-dT primer Green PCR Master Mix (ABI) and analyzed by the ABI 7500 sequence detection system. The primers used in the experiments were shown in Table 1.

**Immunofluorescence**

For visualization of intercellular bridges, cells were fixed with 2% paraformaldehyde for 10 min and permeabilized with 0.5% NP-40 for 15 min. After incubation with goat anti-TEX14 antibodies [30] at 4°C overnight, intercellular bridges were visualized by Alexa 488 and Alexa 564 conjugated secondary antibodies (Invitrogen). Following primary antibodies were used; rabbit anti-ZBTB16 (PLZF; Santa Cruz Biotechnology), mouse anti-INK4A (p16; Santa Cruz Biotechnology), mouse anti-CDKN1A (p21; Santa Cruz Biotech.), and mouse anti-CCNE (cyclin E; Santa Cruz Biotech.) antibodies. For immunofluorescence of tissue sections, paraformaldehyde fixed sections were retrieved by microwave, and then incubated with goat anti-TEX14 [30] and guinea pig anti-CEP55 [32] antibodies overnight at 4°C, followed by Alexa 488 and Alexa 594 conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature. Fluorescent sections were mounted with VECTASHIELD containing DAPI (VECTOR Laboratories).

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**Table 1. Primer sequences used in the study.**

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| Used in qPCR |                |                |
| Cdh1      | ACCATGATGAGAAGCTGCCG | ACCATCCTAACACAGAGCTCC |
| Epcam     | TGCTCTAATTGCGGTGTTA | TCCGAGCTTGGCTGTAGTC |
| Gfra1     | TACACACAGATGCTCAATGAA | GTAGCTGTGGTGGCTGACTC |
| Ret       | GACCAAGGAGTAGTGGTATAGT | GACCTATGGCACTTCACTGA |
| Zbtb16    | CACACTCAAGGCCAACAG | ATCATGGCCGATGTCCTCG |
| Bcl6d     | CCGCAGGAAGATGTTTCT | GCTGAGCCGCTAGG |
| Id4       | GAGACTCACTCCTGGTCTG | ATGCTGCTACCTGGTGT |
| Nanso2    | AAATCTCTGCAACCAATG | CGGAGAATGCTACACAG |
| Tafl4b    | AGCCCTAACGCCCAAACCC | TGAATTTCTACAGGCATG |
| Neurog3   | GCCCTATTGGAAGATTC | AGATGGTGGAGACCTCAC |
| Stra8     | ACAAGAGGTAGGCCACGCT | CTCGTGATTTTGGTGGC |
| Kit       | GCCACGCTACAGGTCATC | GTGCGGAATCATGACGTC |
| Ddx4      | AGAGGAGATAGTGGTGGTCTG | GGGCAAAGAAGATGCAGT |
| Tex14     | AAATAGTAGGAGTGGGGTC | CTATCAGCAGACTGTCCTC |
| Ink4a     | GTGGTCGATCGTGCGG | GCAGTGCCACTGACGGTAG |
| Arf       | GCTCTGATCACCTGGAAATG | TGAATCCTGACCTGAGT |
| Cdkn1a    | GCAGATCCACAGGATATCC | CAATCGCTACTGACGG |
| Cdkn1b    | AGGGCGCAACGAAAGAAG | GGTGCTCACTTGGAGGT |
| Cldn1     | GCTTTATCCCAACCCCCAC | CTACAGTGATCACCTGCC |
| Cldn2     | GATCACACACAGGCTCTC | ATGAAGCAAGCGTCTC |
| Cldn3     | ATGTCAGAGCCATCTTGG | CTGTTGAGTGGAAGAAG |
| Cine      | CGTTGATGAGGGAAGAAG | TAGAGCAACACATCAGG |
| Used in genotyping |                |                |
| Tex14 F1  | GAGCCTACTTCTGCTCTGG |                |
| Tex14 F2  | GTACCTCTGACGGGCCAAG |                |
| Tex14 F3  | CGAGGTTGATCTACGGTGG |                |
| Tex14 R1  | CAGACCCAGGGTTCAGAAG |                |
| Tex14 R2  | GGTTGTTTACAGCGGATCTA |                |
| Tex14 R3  | GGGGAATCCTCCAGCTAGG |                |
| Tex14 RN  | ATGCGGAGGAGCATCCCTATCC |                |

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Figure 1. Expression profiles of SSC markers in Tex14<sup>−/−</sup> testis and spermatogonia. Relative expression patterns of the indicated genes in Tex14<sup>−/−</sup> (blue) and Tex14<sup>−/−</sup> (red) testis at 8 weeks of age (A) and in CD9-positive Tex14<sup>−/−</sup> (blue) and Tex14<sup>−/−</sup> (red) spermatogonia (B) were quantitatively analyzed. All of the expression levels were normalized to Gapdh expression. * indicates that there is significant difference (P<0.05). doi:10.1371/journal.pone.0038914.g001
Figure 2. Detection of intercellular bridge formation and establishment of spermatogonial stem cells in culture. A. Detection of intercellular bridge proteins in Tex14\textsuperscript{+/+} and Tex14\textsuperscript{−/−} testis. Immunofluorescence images of CEP55 (red) and TEX14 (green) and nucleus (DAPI) in 8-week old Tex14\textsuperscript{+/+} (+/−) and Tex14\textsuperscript{−/−} (KO) testis are shown. Insets indicate higher magnification images. B. Detection of intercellular bridges in short
Generation of a Genetic Rescue Construct and Homologous Recombination in SSCs

A rescue construct was generated as follows: 8.3 kb of the 12987/SvEvBrd-Hprt-m2 genomic region containing exons 9 and 10 of the mouse Tex14 gene [34] was inserted into pBluescript SK containing diphtheria toxin A for negative selection (pDTA.3 kindly provided by Dr. Pumin Zhang, Baylor College of Medicine) and a loxp-flanked Pgk1-Neo cassette was inserted 2 kb downstream of exon 10 of Tex14. The linearized rescue construct was electroporated into Tex14−/− SSCs. SSC clones were selected in SSC media supplemented with G418 and 6-thioguanine. Targeted clones were screened by genomic PCR using primers shown in Table 1.

Results

Molecular Analysis of SSC Markers in Tex14 Null Testes

Expression patterns of genes, known to be transcriptionally active in spermatogonial stem cells, were analyzed in TEX14 null testes, because characteristics of Tex14−/− spermatogonial stem cells, such as self-renewal activity, differentiation potential, and proliferative activity, remained elusive. For the expression of cell surface markers of spermatogonial stem cells, Cd9, Gfra1, and Ret were significantly increased in Tex14−/− testes, although Epcam was decreased (Fig. 1A). Up-regulation of Gfra1 and Ret suggests enrichment of undifferentiated spermatogonias in Tex14−/− testes, since Gfra1 and Ret are receptors for GDNF, which is an essential growth factor for SSCs. For the expression of intercellular markers of spermatogonial stem cells, Zbb16 (also called Ptfla), Stra8, and Tgfβb were increased in Tex14−/− testes, whereas B6hb, Id4, and Nanos2 were decreased in Tex14−/− testes (Fig. 1A). Enhanced expression of the three former genes suggests that undifferentiated spermatogonia might be enriched in Tex14−/− testes. Suppressed expression of the latter three genes, however, suggests that the ratio of undifferentiated spermatogonia to differentiated spermatogonia remaining in Tex14−/− testes might not be very high, or that spermatogonia remaining Tex14−/− testes might have both differentiated and undifferentiated characteristics even if they were existed as single spermatogonia. Expression of Ddx4 (also known as Mvh) was extensively lower in Tex14−/− testes, probably because Ddx4 is expressed not only in spermatogonia but also in later stage germ cells, and there were only spermatogonia enriched in Tex14−/− testes (Fig. 1A).

Next, spermatogonia were enriched from whole testes using CD9 as a marker of undifferentiated spermatogonia. When expression of cell surface markers was analyzed, Epcam was significantly lower in Tex14−/− spermatogonia, and Gfra1 and Ret were significantly higher (Fig. 1B). However, differences of expression of those genes between Tex14−/− and Tex14+/− spermatogonia were decreased. For the expression of intracellular markers, expression of Zbb16, B6hb, and Nanos2 were not significantly changed between Tex14−/− and Tex14+/− spermatogonia (Fig. 1B). Although Id4 expression was significantly lower in Tex14−/− spermatogonia, the difference between Tex14−/− and Tex14+/− spermatogonia was decreased (Fig. 1B). When differentiation related markers were analyzed, Neurog3 expression was higher in Tex14−/− spermatogonia, but Kit expression in Tex14−/− spermatogonia was as high as Tex14+/− spermatogonia (Fig. 1B).

These results for the expression profiles in enriched spermatogonia suggest that spermatogonia lacking intercellular bridge might not be very different from normal spermatogonia and that CD9-positive Tex14−/− spermatogonia might have both undifferentiated- and differentiated characteristics.

Morphological Analysis of Tex14 Heterozygous and Homozygous Mutant SSCs in Culture

The disappearance of intercellular bridges in Tex14−/− testes was previously confirmed by immunostaining of TEX14 and CEP55, both of which are essential components for intercellular bridges to be stabilized [32]. There is no clear localization of both TEX14 and CEP55 in Tex14−/− testes, indicating that no stable intercellular bridges form in Tex14−/− testes (Fig. 2A).

Before establishment of long-term cultures of spermatogonial stem cells, characteristics of spermatogonia without intercellular bridges were analyzed in short-term cultures of Tex14−/− spermatogonia. Surprisingly, Tex14−/− spermatogonia could form chain-like structure, even though no intercellular bridges formed (Fig. 2B). Tex14−/− spermatogonia could also form cluster-type colonies, although the size of these clusters was small and the number of cells in these clusters was less than ten (data not shown). When intercellular bridges were visualized in isolated Tex14−/− and Tex14+/− spermatogonia using TEX14 staining, clear TEX14-positive intercellular bridges were detected in both chains and clusters of Tex14−/− colonies but not in Tex14+/− colonies (Fig. 2B). To confirm lack of intercellular bridges in Tex14−/− spermatogonia, alpha-tubulin (TUBA) in cultured spermatogonia was observed to be stretched and showed that cells were connected with neighboring cells, whereas TUBA expression in Tex14−/− spermatogonia was round shaped and showed that each cell was not connected with neighboring cells (Fig. 2C). These results indicate that Tex14−/− spermatogonia definitely lack intercellular bridges although they could form small clusters and chain-like structures without interconnections to neighboring cells.

Molecular Analysis of Tex14 Heterozygous and Homozygous Mutant SSCs in Culture

Gene expression of several genes, which are essential for spermatogonial stem cells, was examined in short-term cultures of spermatogonia. A number of stem cell related genes (Epcam, Ret, Zbb16, B6hb, Tgfβb, Neurog3, and Stra8) were significantly upregulated in Tex14−/− spermatogonia, while differentiation related genes (Kit and Ddx4) were simultaneously upregulated (Fig. 3A). The expression of Id4 and Nanos2, whose expression are restricted to single cells and clusters with less than four aligned spermatogonia, respectively, were not significantly changed in Tex14−/− spermatogonia, even though single spermatogonia were enriched in Tex14−/− intercellular bridge less spermatogonia (Fig. 3A). Upregulation of Neurog3 and Stra8 was also intriguing, because these genes are known to be expressed at a higher level in differentiating spermatogonia rather than undifferentiated spermatogonia [17,25,33]. These results suggest that Tex14−/− spermatogonia are already committed to differentiation, even
Figure 3. Expression profiles of SSC markers and cell cycle regulators in Tex14+/− and Tex14−/− SSCs. Relative expressions of SSC markers (A) and cell cycle regulators (B) in Tex14+/− (blue) and Tex14−/− (red) SSCs were quantitatively analyzed. All of the expression levels were normalized to Gapdh expression. * indicates that there is significant difference (P<0.05).

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Figure 4. Alterations of cell cycle regulators in spermatogonial stem cells by lack of intercellular bridges. Protein expression of cell cycle regulators in Tex14<sup>+/−</sup> and Tex14<sup>−/−</sup> SSCs were analyzed by immunoblot (A) and immunostaining (B). Immunofluorescence images (B) of indicated cell cycle regulators (green) and ZBTB16 (red) in Tex14<sup>−/−</sup> (+/−) and Tex14<sup>−/−</sup> (KO) SSCs are shown.

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though they possessed characteristics of undifferentiated spermatogonia.

We also analyzed the expression of cell cycle related genes in Tex14<sup>−/−</sup> spermatogonia. While expression of CDK4 inhibitors, p16<sup>ink4a</sup> and Cdkn1a, were increased, p19<sup>Arf</sup> and Cdkn1b were decreased in Tex14<sup>−/−</sup> spermatogonia (Fig. 3B). When expression levels of cyclins were analyzed, cyclin D1 and cyclin D2 were significantly upregulated, and cyclin D3 and cyclin E were not detected in Tex14<sup>−/−</sup> spermatogonia (Fig. 3B). Down-regulation of p19<sup>Arf</sup>, cyclin D3, and cyclin E and up-regulation of p16<sup>INK4A</sup> and CDKN1A were confirmed at protein level (Fig. 4). Notably, CDKN1A in Tex14<sup>−/−</sup> spermatogonia was undetectable, whereas CDKN1A in Tex14<sup>+/−</sup> spermatogonia was high, suggesting that lack of intercellular bridges inhibit cell proliferation by induction of CDKN1A. Down-regulation of cyclin E could be induced by inhibition of CDK4 activity. These results suggest that cell cycle progression of Tex14<sup>−/−</sup> spermatogonia was inhibited by two different CDK4 inhibitors even though CDK4 activity in Tex14<sup>−/−</sup> spermatogonia could be activated by expression of its related cyclins.

Establishment of Tex14 Heterozygous and Homozygous Mutant SSCs in Culture

We next attempted to establish long-term cultures of Tex14<sup>+/−</sup> and Tex14<sup>−/−</sup> spermatogonial stem cells. We successfully established long-term cultures of spermatogonial stem cell from wild-type and GFP-positive Tex14<sup>+/−</sup> mice (Fig. 2D). Tex14<sup>+/−</sup> spermatogonial stem cells showed similar colony morphology as wild-type cells (Fig. 2D). However, Tex14<sup>−/−</sup> spermatogonia could not be maintained for long-term, although they could form colonies and grow for short-term as shown above.

Replacement of the Genetic Defect in Tex14 Heterozygous Mutant SSCs in Culture

To replace the genetic defect in Tex14<sup>−/−</sup> spermatogonial stem cells in which exon 10 was replaced with a Pgkl-Hprt cassette in one allele of genome, a replacement construct to “knockin” exon 10 into the deleted genome was generated (Fig. 3A). In this replacement construct, the Pgkl-Neo cassette was flanked with loxP sequences so that it could be removed after insertion into the genome. After transfection of this replacement construct into Tex14<sup>−/−</sup> spermatogonial stem cells, the stem cells were expanded and genotyped using genomic PCR. Seventeen colonies could be expanded and one colony was confirmed to have correct replacement of the deleted exon 10 of Tex14 in the genome (Fig. 5). Although these genetically replaced spermatogonial stem cells were transplanted into germ cell-depleted testes, unfortunately no pups were obtained.

Discussion

In the present study, characteristics of spermatogonia without intercellular bridges were analyzed. By gene expression profiling, Tex14<sup>−/−</sup> spermatogonia possessed not only characteristics of undifferentiated spermatogonia but also characteristics of differentiated spermatogonia. To conclude if spermatogonial stem cells without intercellular bridges could self-renew or not, further experiments using transplantation technique could be required. However, it is very difficult to evaluate self-renewal activity of Tex14<sup>−/−</sup> SSCs by detection of reconstituted colonies after transplantation because our results suggest that proliferative activity of Tex14<sup>−/−</sup> SSCs is low and Tex14<sup>−/−</sup> SSCs may only form small colonies. At least in vivo, Tex14<sup>−/−</sup> SSC are able to self renew, since there are a number of spermatogonia even in one-year-old Tex14<sup>−/−</sup> testes [30].

In our study, one surprising finding is the formation of chain-like colonies by Tex14<sup>−/−</sup> SSCs, although our previous reports showed that most spermatogonia in Tex14<sup>−/−</sup> testes lack chain formation and exist as a single state but not in paired and aligned forms as observed in wild-type adult testes [30]. In addition, we confirmed Tex14<sup>−/−</sup> spermatogonia were not connected with neighboring cells, even if they form chain-like structure. This suggests that there may be adhesion proteins expressed on the surface of the dividing SSCs that allow them to adhere to one another in a chain-like structure. Alternatively, the chain-like structure may be beneficial for survival and/or cell division of the SSCs because of paracrine growth factors secreted from neighboring SSCs. There may also be some differences in the characteristics between spermatogonial stem cells in vivo and in vitro. All postnatal male germ cells are surrounded by Sertoli cells in vivo, whereas cultured SSCs are free to move and to divide and their proliferation could be accelerated in vitro.
Figure 5. Genotyping of the rescued Tex14 allele. A. Strategy of gene replacement of the Tex14 mutation. The PgkHprt, which was inserted into exon 10 of the Tex14 gene, was replaced with exon 10 of Tex14 and a loxP flanked Pgk-Neo cassette. Locations of primers used for genotyping and predicted size of the PCR products are shown. B and C. Genotyping of the Tex14 mutation. Mutation of the Tex14 gene was confirmed by genomic
previously showed, Tex14 functions as a stabilizer of intercellular bridge to avoid cell abscission by interaction with and inhibition of CEP55 function [32]. Midbody formation occurs during cellular division even in Tex14−/− spermatogonia. As shown in Figure 2A, there was no localization of CEP55 to midbodies in 8-day old testes where spermatogonia actively proliferate. These results suggest that proteins required for cell abscission might not be expressed at very high levels in the Tex14−/− midbody, and this midbody might act as a transient intercellular bridge. Alternatively, intercellular bridge might regulate the movement of spermatogonia, because previous reports showed that chains of spermatogonia extensively move around in seminiferous tubules [36,37]. Tex14−/− spermatogonia might not be able to move actively after cell division because of a lack of intercellular bridges.

Tex14−/− spermatogonia display both activation and repression of proliferative activity according to our expression profiling. Tex14−/− spermatogonia can proliferate even though expression of CDK inhibitors was repressed, because some chains of Tex14−/− spermatogonia were detected in the culture in vitro. However, there must be a reduced rate of proliferation since the Tex14−/− SSCs could not be expanded in long-term culture. Otherwise, Tex14−/− SSCs could proliferate in their first few divisions and might subsequently enter cellular senescence. Since down-regulation of cyclin E expression could be affected by up-regulation of CDK inhibitors, especially CDKN1A, up-regulation of CDKN1A may be critical for inhibition of cellular proliferation of Tex14−/− spermatogonia. As shown in Figure 2C, cytoskelton of Tex14−/− spermatogonia were dramatically changed by lack of intercellular bridges. Checkpoint mechanisms monitoring cellular cytoskelton might induce up-regulation of CDKN1A. Formation of intercellular bridges, at least, could be important for continuous proliferation of spermatogonia.

An attempted replacement of a genetic defect in spermatogonial stem cells was also presented in this study. This is the first report showing a genetic correction in vitro using spermatogonial stem cells, although there are reports of homologous recombination in spermatogonial stem cells [38,39]. Unexpectedly, Tex14−/− spermatogonial stem cells could not be expanded, although there are many spermatogonia in Tex14−/− tests. Therefore, we used Tex14−/− spermatogonial stem cells as a model of genetic correction. If spermatogonial stem cells could be easily expanded from infertile animals or patients, this methodology could be applicable. Of course, there are some ethical issues that need to be considered if this technique is used in the therapy of infertile patients since this method could produce genetically engineered offspring. Therefore, at this time, this technique is appropriate for therapy of infertile animals except for human. If rare animals are infertile or subfertile because of genetic defects, this methodology would be very powerful to solve this problem.

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Author Contributions

Conceived and designed the experiments: NI MMM. Performed the experiments: NI TI. Analyzed the data: NI MMM. Wrote the paper: NI MMM.

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