—Mini Review—

Transgenesis Via Sperm

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Abstract: *The idea of using a sperm cell to introduce exogenous DNA into an oocyte at the time of fertilization is of interest for simple production of transgenic mice. Since 1989, contradictory reports have appeared in the literature, but now this technology, so-called sperm-mediated gene transfer (SMGT), is considered reproducible. Concomitantly, in vivo transfection of sperm cells has also proceeded, including direct gene delivery into a testis, so-called testis-mediated gene transfer (TMGT), and gene transfer into seminiferous tubules. This review summarizes what has been achieved in the field of gene transfer via sperm cells.*

Key words: *Gene transfer, Seminiferous tubules, Sperm, Testis, Transgenics*

Introduction

Transgenic animals have become valuable tools for both research and applied purposes. The most widely used method to create transgenic animals is to microinject foreign DNA into a pronucleus of the fertilized eggs. However, this pronuclear microinjection method has only had limited success in producing transgenic animals of larger species. It also requires high technical skills and is labor intensive. The idea of using a sperm cell as a vehicle to introduce exogenous DNA into an oocyte is of interest for simple production of transgenic mice. This method is very simple and convenient. The possibilities and limitations of this technology are discussed.

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Sperm-mediated Transgenesis and the Mechanism of Binding of Sperm to DNA

Historical background of development of sperm-mediated transgenesis

In 1971, Brachett *et al.* [1] demonstrated that when DNA was added directly to sperm prior to their use for fertilization, it could be transferred to the egg by the sperm. In 1989, Lavitrano *et al.* [2] further reported that exogenous pSV2CAT plasmid DNA could be transmitted to offspring by simply incubating sperm cells with transgene molecules prior to *in vitro* fertilization (IVF). In the same year, Arezzo *et al.* [3] reported similar results with sea urchins. Using Southern blot hybridization, Lavitrano *et al.* [2] found that of 250 progeny arising from IVF of eggs with pSV2CAT-treated sperm cells, approximately 30% exhibited the presence of the transgene. Additionally, restriction mapping and sequence analysis confirmed the presence of transgenes, as did the detection of CAT gene expression. Further, the transgene was detected in the F1 progeny. Gene delivery to offspring by this method is now called sperm-mediated gene transfer (SMGT). However, despite the above successes, SMGT has not yet become established as a reliable method for the production of transgenic animals. Attempts to reproduce SMGT by other laboratories have failed to achieve the same results [4, 5]. Nevertheless, there have been several reports on attempts at transfer of DNA to sperm cells in a broad range of animals including mice, pigs, buffalo, rams, goats, roosters, chicken, cattle, fishes, and insects (honeybees) [reviewed by Lauria and Gandolfi [6], Smith [7] and Gandolfi [8]]. Remarkably, Maione *et al.* [9] repeated the experiments performed by Lavitrano and colleagues in 1989 and published the results of work carried out in their laboratory and in Kiessling's laboratory in Boston. They were able to show that SMGT is repeatable, and observed that, depending on the experiment, between 0 to 100% of progeny were transgenic as determined by

genomic Southern blot analysis, with a mean of 7.4%. Recently, Lavitrano and colleagues [10] reported that 53 of 93 animals born after artificial insemination (AI) of freshly ejaculated semen which had been incubated with plasmid DNA [encoding human decay accelerating factor (hDAF)] were transgenic (57%). Remarkably, 34 of 53 animals transcribed the transgene appropriately. Furthermore, more than 80 of the founders in which mRNA was detected expressed the protein at a level similar to that found in the normal human heart. Despite the above successes, it is still far from clear how useful such SMGT is for the efficient production of transgenic animals. This is simply because only a few groups, including Lavitrano's group [11–14] and Wang's group [15], have reported successful SMGT using several animals such as pigs, gilts, and rabbits.

The mechanism of gene delivery to spermatozoa

That spermatozoa are capable of DNA uptake is now well documented [2, 16], but both the mechanism involved in this process and its regulation are still poorly understood. A portion of the DNA remains bound to the sperm head plasma membrane, but part is integrated and in mammals is consistently found in the postacrosomal region of the sperm nucleus [17, 18]. This high degree of affinity of sperm for DNA appears to be mediated by the complex structure of the MHC class II molecules and the antigen CD4, located in the posterior region of the mouse sperm head [19, 20]. Once in the nucleus, the DNA becomes tightly associated to nuclear proteins [21, 22] and can then be cleaved by sperm endonucleases and finally integrated into the genome [23]. Lavitrano and colleagues identified a set of 30- to 35-kDa sperm proteins, conserved in many vertebrates, which specifically bind DNA [24] and might participate in the uptake of DNA by spermatozoa. There are also molecules that act as negative regulators in interaction between sperm and DNA. Zani *et al.* [25] also showed that a protein in seminal plasma they called IF-1 blocks the uptake of exogenous DNA by sperm. This accounts for the observation that seminal plasma must be removed as part of the SMGT protocol. In addition, they presented evidence that the incoming DNA binds the sperm nuclear matrix prior to becoming integrated into the sperm genome.

Use of electroporation and liposomes for enhancement of gene delivery

Although in most cases spermatozoa are simply incubated in the presence of foreign DNA, electroporation or cationic liposomes are sometimes used to enhance uptake [26–32]. These methods have been used on several species including mice [26], salmon [27], finfish and shellfish [28], cows [29, 32], zebrafish [30] and chicken [31]. For example, Bachiller *et al.* [26] first demonstrated that cationic liposomes can enhance uptake of exogenous DNA by spermatozoa. Analysis using laser confocal microscopy revealed that about 80% of liposome/DNA-treated spermatozoa exhibit a signal specific for the exogenous DNA inside the sperm head. After IVF with DNA-introduced sperm cells, the resulting 2-cell embryos were transferred to recipients to obtain offspring. Southern blot analysis of the offspring born failed to detect any signal for the exogenous DNA. Although there is no clear explanation for this failure, the majority of sperm with low motility may have been more frequently labeled with the exogenous DNA than sperm which exhibit active motility and potentially are capable of fertilizing oocytes but are not susceptible to transfection by DNA/liposome complex. Rottman *et al.* [31] performed liposome-mediated transfection of rabbit, cow and chicken sperm cells with the alkaline phosphatase gene followed by insemination. The transgene was expressed at least several months after the birth of the only transgenic calf. Shemaesh *et al.* [32] bound transgenes to liposomes before exposing DNA to bovine sperm. That treatment did not yield transgenic animals, but when restriction enzymes were added to the mixture, transgenic bovines were successfully produced. It appears that their work is in progress since no direct molecular evidence (e.g., Southern or northern blot data) was presented.

Use of viral vector in the SMGT system

The possibility of infection of sperm by viral vectors to create transgenic animals is of interest. Retroviral vectors (RVVs) are one of the most frequently employed forms of gene delivery in animal transgenesis [33, 34]. As in SMGT, zygotes may be incubated in media containing high concentrations of the resultant retroviral vector. Alternatively, RV-producing cell monolayers may be used, upon which zygotes are co-cultivated. In either case, up to ca. 90% of embryos will be infected. Farre *et al.* [35] used adenoviral vector to introduce foreign DNA into pig sperm. When spermatozoa were exposed to replication-defective adenoviral vector bearing lacZ gene, gene transfer was observed in the heads of the spermatozoa. Of the 2- to 8-cell embryos obtained after *in vitro* fertilization with adenovirus-exposed sperm, 21.7% expressed the lacZ gene [encoding β-galactosidase (β-gal)]. Four of 56 piglets

(approximately 7%) after AI with adenovirus-exposed spermatozoa were positive on PCR analysis, but none of the PCR-positive piglets exhibited the lacZ gene after genomic Southern blot analysis. Furthermore, the offspring obtained after mating the PCR-positive animals did not carry the exogenous DNA. These studies show that adenovirus is able to deliver exogenous DNA into pig spermatozoa, but the use of pig spermatozoa carrying replication-defective adenovirus does not appear to be practically useful as a system for obtaining transgenic pigs. Hall *et al.* and Gordon [36, 37] have demonstrated that germ-cell transduction fails when spermatogenic cells and mature sperm are exposed to a high titer of adenovirus and subsequently subjected to *in vitro* fertilization.

Use of lentiviral or adeno-associated viral (AAV) vectors, both of which are recognized as promising vectors for human somatic gene therapy [38], appears to be of interest. Single-cell mouse embryos were successfully infected *in vitro* with recombinant lentiviral vectors to generate transgenic mice carrying the green fluorescent protein (GFP) gene [39]. Eighty percent of founder mice carried at least one copy of the transgene, and 90% of these expressed GFP at high levels. Furthermore, lentiviral vectors could be used to express genes in a tissue-specific manner [39]. However, this vector could not infect isolated mouse epididymal spermatozoa (personal communication from Dr. Masato Ikawa at Osaka University). Couto *et al.* [40] demonstrated that direct exposure of murine spermatozoa to very high concentrations of AAV failed to lead to germ cell transduction. If these vectors were able to transduce sperm, exposure of sperm to such large amounts of vectors prior to fertilization would be expected to lead to early integration. It remains unclear why mature sperm cannot be infected by these viral vectors.

Gene transfer into oocytes after intracytoplasmic sperm injection (ICSI) of spermatozoa treated with DNA in vitro

Perry *et al.* [41] first reported use of the combination of DNA-bound sperm and ICSI (called "TransgenICSI") as an alternative to the traditional gene transfer method, pronuclear microinjection [42]. They microinjected sperm exposed to naked DNA molecules into oocytes, and demonstrated that approximately 20% of founder mice had integrated and expressed the transgenes. The transgene behaved as expected (transgene inheritance was approximately followed by Mendelian rule). Perry *et al.* [41] proposed that with their methodology transgenes were not necessarily integrated into the sperm genome prior to transport by sperm into the oocyte. Szczygiel *et al.* [43] demonstrated that the expression of foreign DNA is associated with paternal chromosome degradation in TransgenICSI. Chan *et al.* [44, 45] obtained a similar finding using primates; ICSI of rhodamine-labeled DNA-treated sperm resulted in production of transgenic rhesus embryos. Transgene uptake and expression using TransgenICSI has been reported in other animals such as porcine embryos [46, 47]. Nevertheless, the technique still requires manual injection of the DNA-coated sperm into oocytes.

Testis-mediated Transgenesis

Gene transfer to male germ cells by direct introduction of DNA into mammalian seminiferous tubules

A promising approach to production of transgenic animals appears to be gene transfer into male stem cells such as spermatogonia by direct introduction of foreign DNA into seminiferous tubules. Yamazaki *et al.* [48, 49] first demonstrated using direct introduction of plasmid DNA and subsequent *in vivo* electroporation (EP), that almost all spermatogenic cells within seminiferous tubules could be transfected by DNA, but that males undergoing DNA introduction failed to produce transgenic mice despite mating with normal females. Kim *et al.* [50] demonstrated that a certain percentage of sperm cells including spermatogonia could be transfected by lacZ expression plasmid encapsulated by liposomes when injected into seminiferous tubules of mice and pigs that had been treated with busulfan, an alkylating reagent, to destroy developing male germ cells. In mice, 8–14.8% of seminiferous tubules expressed the foreign gene, and 7–13% of epididymal spermatozoa were found to possess the DNA by PCR. In pigs, foreign DNA was also incorporated into male germ cells, since 15.3–25.1% of seminiferous tubules expressed lacZ gene. However, no evidence was obtained to suggest that the foreign DNA is transmitted to offspring after mating. Celebi *et al.* [51] demonstrated that circular plasmid DNA/liposome complex introduced into mouse seminiferous tubules transfected spermatogenic cells efficiently, and that these transfected sperm led to transmission of a transgene in mouse offspring. However, this transmission was transient and the transferred plasmid DNA disappeared between the young to adult stages, due probably to its episomal state in mice. They suggested that circular plasmid confers survival in mouse tissues and is diluted out

along with cell proliferation.

Huang *et al.* [52] employed gene delivery to seminiferous tubules and ICSI of DNA-transfected sperm cells. They first infused seminiferous tubules of young mice aged 2 weeks old with transgene and then used electroporation to encourage transfection. Two weeks later, testes were harvested and "transgenic sperm" expressing yellow fluorescent protein (YFP) were selected and used for ICSI. Transgenic pups that were YFP-positive were produced. This suggests that exogenous DNA can be incorporated into the genome of maturing sperm, probably spermatocytes and spermatids. Transfection of spermatogonia by such a procedure is thought to be difficult (personal communication from Dr. Kentaro Yomogida at Osaka University).

As mentioned previously, attempts to infect germ-line stem cells with viral vectors or transfect them by other techniques such as introduction of plasmid DNA and subsequent whole-testis electroporation [48, 49] have met with little success. Blanchard and Boekelheide [53] reported that introduction of adenoviral vector carrying a lacZ gene into seminiferous tubules by rete testis injection resulted in lacZ gene expression in Sertoli cells, but not in germ cells. A possible explanation for the inability of virus or plasmid to transduce spermatogonial stem cells and generate colonies expressing the transgene is that supporting somatic Sertoli cells cover and shield stem cells from the virus present in the tubule lumen. Nagano *et al.* [54] injected a mixture of Gen-PGKβgal retrovirus-producing cells and freshly collected testis cells into the seminiferous tubules of recipient mice, and first demonstrated the expression of a retrovirally delivered reporter lacZ transgene in male germ line stem cells and that differentiated germ cells persisted in the testis for more than 6 months. They further demonstrated that introduction of retroviral vector into seminiferous tubules of mice resulted in germ cells in 35% of seminiferous tubules and in production of transgenic mice with an efficiency of 35% [55]. The introduced DNA was integrated into the host chromosome when Southern analysis was performed in offspring, and the gene transmission pattern from one generation to the next was Mendelian. This alternative route for transgenesis is now applied to species including rats [56, 57], pigs [58] and goats [59].

Testis-mediated gene transfer (TMGT) based upon direct introduction of DNA into the interstitial space of mammalian testis

Sato *et al.* [60] first attempted to transfect testicular spermatozoa by direct introduction of Calcium phosphate-precipitated circular plasmid DNA (pSV2-CAT) into the testes of adult mice, but this attempt failed to produce transgenic mice. PCR analysis revealed that the introduced DNA could be detected in the freshly isolated epididymal spermatozoa from caput and cauda epididymides as early as 6 h after injection. The DNA was also detectable even in the ejaculated spermatozoa recovered from the uteri of females. However, we failed to detect foreign DNA in the fertilized 1-cell eggs obtained by mating with superovulated females by PCR. These findings suggest that the DNA solution injected into the testis is rapidly transferred to its epididymal portion, where it is taken up by epididymal spermatozoa.

Ogawa *et al.* [61] demonstrated that injection of linearized lacZ expression plasmid mixed with cationic liposome Lipofectin™ into testes of adult mouse *via* the scrotum resulted in transmission of the foreign DNA sequences to F0 progeny (blastocysts) through fertilization. They found that 80.0% (16/20) of blastocysts derived from mating with males receiving the DNA were stained for X-Gal. These findings clearly demonstrate that DNA exogenously introduced into the mouse testis is transferred to eggs via spermatozoa with relatively high efficiency. At the time, we termed this technology "testis-mediated gene transfer (TMGT)" as a novel method of *in vivo* gene transfer [61].

Sato *et al.* [62] showed that a single injection of circular plasmid DNA complex with Lipofectin™ into mature mouse testes is sufficient for transfection of spermatozoa (epididymal spermatozoa), and for relatively high efficiency (50–100%) of gene delivery to mid-gestational fetuses (F0) obtained by mating of injected males with normal females. However, the DNA introduced appeared to be present mosaically in the TMGT-derived fetal tissues, since it was estimated to be present at less than 1 copy per diploid cell [62]. Sato *et al.* [63] also showed that the exogenous DNA introduced to a testis was transmitted at least to the second generation.

Expression of introduced DNA was first evident only in F0 early blastocysts, but almost absent in F0 mid-gestational fetuses and organs of adult F0 mice [61, 63]. We failed to detect any gene expression in these F0 fetuses at northern blot level, and succeeded in detecting it only in a limited number of samples when

nested RT-PCR was performed [63]. Furthermore, we tested several commercially available reagents used for *in vitro* gene transfer to examine which is best for introducing high numbers of copies of exogenous DNA into the fetal mouse genome *via* TMGT [64]. Unfortunately, we were unable to find any candidate reagents for this purpose.

Another group [65] demonstrated with use of a TMGT method essentially the same as ours that plasmid DNA singly injected into testes was transmitted *via* fertilization to F0 offspring with efficiencies of 5.6–17.6% for rats and 9.7% for mice. Notably, Chang *et al.* [65] demonstrated using genomic Southern blot hybridization that these positive samples had more than 1 copy of the DNA per diploid cell, in contrast to our findings described above. Furthermore, they successfully detected gene expression at northern blot level in F0 and F1 offspring [65]. This is in marked contrast to our findings, for we have failed to detect expression of exogenous DNA in F0 offspring (including fetuses and adult organs), as noted above. Recently, the same group tested the efficiency of each of 8 commercially available liposomes in associating exogenous DNA with sperm, and found that only 2 liposomes, DMRIE-C and SuperFect™, led to detection of exogenous DNA on rat epididymal spermatozoa [66]. When TMGT was performed on rat testes using enhanced green fluorescent protein (EGFP) expression plasmid/DMRIE-C (or SuperFect™) complex, the resulting F0 neonates carried the foreign DNA at more than 1 copy per diploid cell [66]. In addition, expression of EGFP was present in more than 80% of morula-stage embryos examined when DMRIE-C or SuperFect™ was used. Kojima *et al.* [67] injected adenoviral DNA into the mouse testis using the TMGT method and found that Leydig cells, but not Sertoli or spermatogonic cells, were efficiently infected. They claimed that gene transfer of adenovirus into a testis via TMGT may be effective for *in vivo* gene therapy for male infertility caused by Leydig cell dysfunction.

Chang *et al.* [68] first examined the mechanism of TMGT using confocal microscopy of frozen sections of epididymis prepared 4 days after testis injection with fluorescence-labeled DNA, and found that the exogenous DNA was bound to the surface of spermatozoa in the cauda epididymis. This finding suggests that exogenous DNA introduced into a testis is transferred to the epididymis within 4 days and then binds to epididymal spermatozoa. We recently assessed the mechanisms of TMGT in greater detail by injection of trypan blue (TB), a dye generally used for staining dead cells in cell culture systems, and Hoechst 33342, a fluorescent dye generally used in staining cell nuclei, into adult murine testes. It was observed that the solution introduced into the testis was transported to the ducts of the caput epididymis *via* the rete testis and efferent ducts immediately after testis injection and reached the corpus and cauda epididymis within 3–4 days after injection [69]. These findings suggest that exogenous DNA introduced directly into the testis is mainly taken up by epididymal spermatozoa, which subsequently transfer the DNA to oocytes through fertilization.

It is of interest to determine the state of the exogenous plasmid DNA in the mid-gestational F0 fetuses derived from superovulated females mated to DNA-injected males. PCR analyses of yolk sac DNA revealed that most (78–100%) of these fetuses possessed the exogenous DNA. However, genomic Southern blot analysis of these PCR-positive samples failed to detect any hybridizable band despite repeated trials [63]. In a preliminary test, we found that foreign DNA (Cre expression vector termed “pCAG/NCre”) was present intact in mouse offspring when PCR using several primer sets which could cover an entire region of pCAG/NCre DNA was performed [70]. However, some other samples had a deleted form of pCAG/NCre in which both end regions of the CAG/NCre inserted together with a pBluescript SK(–) vector backbone had been lost [70]. Based on these findings, we speculate that exogenous DNA introduced into the adult mouse testis may be present in the fetal tissues mosaically. If this DNA is present in each cell, it may be located in close association with host chromosomes and/or in part integrated into host chromosomes. Our previous findings [63] demonstrated that the pattern of transmission of exogenous DNA to the next generation is not Mendelian. Yonezawa *et al.* [66] obtained a finding similar to ours; more than 80% of morula-stage rat embryos exhibited EGFP fluorescence, but the ratio of animals carrying the foreign DNA decreased as animals developed. They also observed that only some postpartum progeny were foreign-DNA-positive with a high incidence of mosaicism. These findings suggest that there is extensive loss of the foreign DNA introduced *via* TMGT during transition from the preimplantation to postimplantation stages. In this sense, the gene delivery pattern in the TMGT system appears to differ from that in the previous system, which depended on pronuclear microinjection of DNA [42].

Further improvement of TMGT itself is still required for use of it as an alternative method for transgenic animal

production. For example, introduction of DNA into the testis of a very young male, probably around 5 to 10 days of age, appears to be interesting, since young testes are smaller than adult ones and have actively proliferating spermatogonial cells. Therefore, it appears that sperm cells in a testis would be easily accessible for exogenous DNA contact. Repeated injections of DNA are also interesting. This strategy is aimed at increasing the frequency of DNA contact with testicular spermatozoa. However, in our preliminary trial of repeated injections (up to 6 times) of plasmid/liposome (FuGENE6™) complex failed to improve the TMGT system [71]. Choice of reagents used for gene transfer may be important for improvement of TMGT: in particular, use of DMRIE-C and TransFect™ appears to be promising, as suggested by others [66]. If efficient methods for TMGT are developed in mice, we believe that this technology will provide a powerful tool for production of domestic transgenic animals such as cows, pigs and horses, which is considered difficult with presently available microinjection techniques [72].

TMGT in fishes

The most successful form of TMGT appears to be gene delivery to fishes. For example, Lu *et al.* [73] presented interesting data on production of transgenic silver sea bream (*Sparus sarba*) by the TMGT method. They injected the liposome-transgene mixture into the gonads of male sea bream at least 48 h before spawning. The males were mated to reproductively active females, and fertilized eggs were collected for further incubation. PCR analysis revealed that 59 to 76% of the hatched fry possessed the transgene. Southern blot analysis also confirmed that the transgene had been integrated into the host genome. Gene expression was also confirmed. Lu *et al.* [73] concluded that TMGT would be of great value for modifying the phenotype of marine fishes.

Gene transfer to vas deferens

Another way to transfect sperm cells with exogenous DNA *in vivo* is direct introduction of DNA into spermatozoa in the vas deferens. Huguet and Esponda [74, 75] injected a plasmid DNA encoding the GFP into mouse vas deferens. The night after injection, males were mated with normal estrus females. When the resulting offspring were analyzed, four of 53 newborn were found to be positive for presence of the GFP gene by PCR. In the positive animals, some tissues exhibited GFP expression. They suggested the possibility of use of this method as a simple alternative to the creation of transgenic animals. These studies also demonstrate that epididymal and vas deferens secretions do not block binding of DNA to spermatozoa. This appears to be in agreement with our previous finding that DNA introduced into the interstitial space of a testis can bind to epididymal spermatozoa [60, 62, 69].

Conclusion

Several studies have demonstrated the occurrence of a putative transgenic event in preimplantation embryos with the subsequent disappearance of any trace of exogenous DNA in the resulting offspring. In fish species, a similar observation was made. The rate of chimerism is very high [up to 75%; [76, 77]], and the occurrence of persistence of plasmid DNA extrachromosomally, even through 2 generations, is very common [76]. This resulted in the lack of expression of the transferred gene [76, 78] or even complete absence of DNA in the embryo [79]. We observed a similar phenomenon in mice when DNA was directly introduced into testis and the TMGT-derived offspring were examined [70, 71]. However, animals such as sea urchin [3], *Xenopus* [80], and chicken [31] appear more susceptible to transfection with exogenous DNA than mice and rats, since offspring derived from oocytes fertilized by DNA-exposed spermatozoa express the DNA and possess it at a level detectable by genomic Southern blot analysis.

It is now clear that DNA molecules can associate with and even penetrate sperm cells. In most cases, however, exogenous genes are completely or partially degraded and/or rearranged. The most common fate of the DNA appears to be persistence outside the chromosomes. Under certain conditions, exogenous DNA introduced into oocytes by transformed fertilizing sperm may be integrated, leading to production of a transgenic animal in at least some instances. Interestingly, Perry *et al.* [41] suggested that this takes place when exogenous DNA reaches the pronucleus, and that this DNA is sometimes protected from immediate degradation.

The SMGT and TMGT technologies described in this review open important new perspectives in the field of animal transgenesis. The conventional approach using pronuclear microinjection is still effective as a means of producing transgenic animals. However, it is both costly and labor-intensive and requires long periods of time to perform it. The use of either SMGT or TMGT would be cheaper and more rapid, with quick and effective delivery of genes to target tissues. If a method

to introduce exogenous DNA into the genome of immature sperm cells or mature spermatozoa can be developed, SMGT will together with TMGT undoubtedly contribute to the development of new methods of transgenesis for research and biotechnology, as well as to the field of gene therapy for treating human male infertility.

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