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Gene Therapy: The Potential Applicability of Gene Transfer Technology to the Human Germline

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Abstract

The theoretical possibility of applying gene transfer methodologies to the human germline is explored. Transgenic methods for genetically manipulating embryos may in principle be applied to humans. In particular, microinjection of retroviral vector appears to hold the greatest promise, with transgenic primates already obtained from this approach. Sperm-mediated gene transfer offers potentially the easiest route to the human germline, however the requisite methodology is presently underdeveloped. Nuclear transfer (cloning) offers an alternative approach to germline genetic modification, however there are major health concerns associated with current nuclear transfer methods. It is concluded that human germline gene therapy remains for all practical purposes a future possibility that must await significant and important advances in gene transfer technology.

Key words gene targeting; gene therapy; genetic modification; pronuclear microinjection; sperm-mediated gene transfer

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1. Introduction

Human germline genetic modification is theoretically possible: the technologies of animal transgenesis (pronuclear microinjection, sperm-mediated gene transfer, nuclear transfer, etc) could in principle be applied to humans. The purpose of this paper is to consider the potential for applying the available genetic modification (GM) technologies to the goal of achieving human germline gene therapy.

If germline gene therapy does become a technically viable proposition, one crucial question must be asked: Why do it? There is in effect a 'golden rule' applying to disorders potentially amenable to germline gene therapy: in any disorder with enough molecular knowledge available to allow the prospect of germline gene therapy, that same knowledge should also be sufficient to allow detection of the disease-causing sequences via embryo pre-screening. Given the low transfer efficiencies and safety risks available at present (i.e. extrapolating from animal transgenesis), candidate disorders would have to be severe and unavoidable by pre-screening. However, it is conceptually possible that gene transfer technologies improve to the point at which it becomes easier and safer to perform germline gene therapy than to carry out embryo pre-screening. In this futuristic scenario of expanded genetic knowledge coupled with effective gene transfer technology, germline GM might become the preferred therapeutic route.

The possibility of human germline genetic modification raises several important and vexing bioethical issues, including questions of responsibility towards future generations, difficulties of distinction between gene therapy and genetic enhancement, and the spectre of eugenics [1, 2, 3]. Thus, human germline genetic modification is far more ethically contentious than somatic gene therapy. However, such bioethical matters are beyond the scope of the present discussion. Instead, this paper focuses solely upon *scientific* issues related to human germline gene therapy.

2. Criteria for Assessing Applicability to Human Germline Gene Therapy

An ideal gene transfer system in the context of human germline gene therapy would have the following features: (a) the ability to deliver transgenes in a highly efficient manner; (b) non-prohibitive cost and expertise requirements; (c) minimal risk of causing insertional DNA damage; (d) low rate of mosaicism; (e) high DNA carrying capacity; (f) the ability to permit adequate and controlled transgene expression; and (g) the ability to target transgenes to precise genomic loci.

Unfortunately, no single system amongst the presently available systems is able to provide all of features (a-g) above. Indeed, some gene transfer systems are so thoroughly unsuited to human germline gene therapy that they are not considered here. Of the systems that offer some positive features, in every case major drawbacks exist. In each case, particular scientific advances are required before the methods would be suitable for use in human germline gene therapy. In this respect, methods that require a relatively small degree of scientific research should be seen as more plausible than methods requiring many years of progress towards distant (possibly unobtainable) goals.

3. Gene Transfer to Human Embryos

Most transgenic animals have been produced via the introduction of transgenes into embryos, and the associated technology and underpinning science is accordingly well developed. Thus, the human embryo is a potential candidate for human germline gene therapy.

Unless dramatic improvements in the technologies are forthcoming, certain transfection methods are not at present a realistic proposition for gene transfer into human zygotes. Such methods include liposome-mediated gene transfer, electroporation, naked DNA uptake and many viral vectors. The problem is one of low transfection frequencies, coupled with fact that zygotes must be harvested (as opposed to grown in vitro). This leaves pronuclear microinjection and retroviral transfer as the only contenders presently available that might be adapted for use with embryos in human germline gene therapy.

Pronuclear Microinjection

Jon Gordon in 1980 demonstrated that exogenous DNA could be introduced into the germline simply by the physical injection of a solution of cloned DNA into zygote pronulei [4]. Subsequently,

pronuclear microinjection has become the most widely used method of germline gene transfer, despite the fact that it remains an intrinsically costly and laborious approach. The technique is most established with mice, however gene transfer via pronuclear microinjection has also been carried out with a wide range of other mammals including rats, rabbits, and farmyard animals. Accordingly, it is to be expected that the human zygote should in principle be similarly amenable to gene tranfer via pronuclear microinjection. The microinjection technique is intrinsically simple, although it requires expensive equipment and high levels of skill [5]. A fine glass needle is loaded with DNA solution. Under the microscope, the needle is guided through the cytoplasm towards one of the zygote's pronuclei. A nanolitre quantity of DNA solution is injected, bringing typically two hundred DNA molecules into the pronucleus.

Pronuclear microinjection would be an obvious choice of transgene delivery method for human embryos. The technique is well established in animals, and is likely to be directly applicable to the human zygote [6, 7, 8]. Zygotes from various mammalian species have particular characteristics that necessitate amendments to the basic (murine) technique. For example, bovine and porcine zygotes are optically opaque, due to the presence of lipid granules in the cytoplasm; this necessitates centrifugation to displace the obscuring cytoplasmic material such that the pronuclei become visible. Similarly, the pronuclei in ovine zygotes are very difficult to visualise, due to sharing a very similar refractive index with the cytoplasm; this necessitates the use of top-quality optics, such as differentiation interference contrast (DIC) microscopy, instead of standard phase contrast microscopy. Thus, empirical adjustments enable pronuclear microinjection to be employed with zygotes from essentially any mammal. It would be surprising and unfortunate if the human zygote proved to be an exception to this rule. Indeed, visualisation of the pronuclei in human fertilised eggs is not problematic.

Although pronuclear microinjection would probably be usable with human zygotes, the major inherent problems of the method render it less than ideal for human germline gene therapy. A major problem is the relatively low rate of transgene integration: in mice, the overall efficiency of transgenesis (taking into account embryo loss in vitro and in vivo) is typically ca. 2% [9, 10, 11]. This level of efficiency is perfectly practicable for animal transgenesis, but it would be problematic for humans. Moreover, murine pronuclear microinjection transgene uptake values are several times higher than those achieved with other (non-rodent) species. Accordingly, even with hormonal induction of superovulation, the numbers of zygotes available per woman would be a strongly limiting factor in the potential use of pronuclear microinjection for human germline gene therapy.

Embryo pre-screening (preimplantation genetic screening) might be one possible way around the problem of low transgene uptake efficiency. Using established techniques, one or two blastomeres could be taken from 8 cell stage embryos and analysed by PCR for the presence of transgene DNA. However, such pre-screening would not be 100% reliable, due to mosaicism within the early embryo. Following microinjection and successful integration of the transgene sequences, the transgene would be expected to be present in only 50% of the resulting blastomeres. Assuming that in humans, as with mice, 3 blastomeres are recruited to form the entire inner cell mass (ICM) [12, 13], then 1 in 8 of the resulting individuals would contain no transgene sequences, another 1 in 8 would contain transgene sequences in 100% of their cells, and the remaining 6 from 8 individuals would be mosaics, consisting of $1/3^{rd}$ or $2/3^{rd}$ transgene-containing cells. Accordingly, pre-screening would have a failure rate of more than 50%. The only feasible way that pre-screening might work at an acceptable level of efficiency would be to screen blastocyst-stage embryos. However, blastocyst biopsy techniques are in their infancy, and it remains to be seen whether such techniques could be applied to ICM cells (as opposed to trophoblast cells).

Extrapolating from murine data, it would typically require ca. 50 zygotes to produce one genetically modified individual. Assuming 8 eggs per superovulation cycle, it would take approximately 6 months per woman to obtain 50 eggs. Pronuclear microinjection involving such a period of time, if coupled with effective blastocyst pre-screening to select for the small number of transgene–containing embryos, might be a feasible means of performing human germline gene therapy. However, reported pronuclear microinjection efficiency values are significantly lower for most mammals other than mice. If human pronuclear microinjection turned out to have a similar efficiency as that obtained with sheep or pigs, then the time taken per genetically modified individual would be ca. 5-fold longer – i.e. more than 2.5 years. And if the rate of transgenesis turned out to be similar to that obtained with cattle, the time would extend beyond 8 years. The efficiency of transgene uptake through

pronuclear microinjection is simply not known for humans, nor can it be known a priori. Thus, a circular problem exists: only if the efficiency turned out to be fortuitously high (i.e. similar to murine rates) would there be any point in attempting the technique with humans – but the necessary data on efficiency could only come from actual attempts with humans.

Another problem associated with pronuclear microinjection concerns transgene expression. Only around 60% of pronuclear microinjection-derived mice show transgene expression. Furthermore, in the animals showing expression, there are frequently problems of low-level expression or inappropriate expression (e.g. non-tissue-specific, non-temporal). Accordingly, pronuclear microinjection as a means to human germline gene therapy requires improvements in transgene expression. It is the non-targeted nature of transgene integration associated with pronuclear microinjection that is the root cause of expression problems. Some improvements may come from advances in transgene design, such as the use of matrix attachment regions (MARs) or locus control regions (LCRs): placed on either side of a gene within a transgene construct, these 'insulator' sequences appear to allow the gene to occupy a separate chromosomal domain and thus avoid position-related expression problems [14]. However, the best solution would be to target transgenes to precise genomic loci, and at present this is not possible with pronuclear microinjection. Given the fact that even the best designs of targeting transgene undergo random integration more frequently than targeted integration, the only foreseeable way to achieve high efficiency gene targeting with pronuclear microinjection would be to stimulate homologous recombination (HR) by co-injecting appropriate recombinase enzymes with the transgene. However, elucidation of such enzymes is at an early stage, and it remains to be seem whether this approach could ever provide the quantum leap improvements in targeting efficiency that would be required in the case of human germline gene therapy.

Random integration also raises the concern that an endogenous gene will be damaged by transgene insertion. The degree of risk for any one insertion event must approximate to the proportion of coding sequences (plus controlling elements) within the human genome, a figure of no more than 2%. Thus, endogenous gene damage may be expected to occur in around 1 in every 50 human zygotes integrating transgene DNA. In embryos sustaining such damage, there are several possible outcomes: (a) where a developmentally crucial gene is damaged, the result is likely to be embryo death, and the subsequent non-appearance of a genetically modified individual; (b) where one allele of an important gene is affected, haplosufficiency may permit the development of a normal or near-normal genetically modified individual; (c) where a non-essential gene is affected (such as an allele for hair colour, or a repeated gene), the resulting genetically modified individual may contain a phenotypic change that has no health implications; or (d) where an important gene is affected, debility is likely to occur in the resulting genetically modified individual. Outcomes (a-c), while not desirable, would not necessarily be highly problematic, and the occurrence of these outcomes means that the undesirable outcome (d) would occur at a frequency significantly lower than 1 in 50 genetically modified individuals. Nevertheless, such magnitude of risk implies that pronuclear microinjection in its present stage of development is not acceptable as a means to human germline gene therapy.

Retroviral Transfer

The genome of retroviruses can be manipulated to carry exogenous DNA. Retroviral vectors (RVVs) are one of the most frequently employed forms of gene delivery in somatic gene therapy [15, 16, 17]. Additionally, RVVs are able to deliver genes to the germline, as established in animal transgenesis [18, 19, 20]. Zygotes may be incubated in media containing high concentrations of the resultant retroviral vector. Alternatively, retroviral vector-producing cell monolayers may be used, upon which zygotes are co-cultivated. In either case, up to ca. 90% of (surviving) embryos will be infected. Following zygote transfer into pseudopregnant females, the infected embryos should give rise to transgenic offspring. Molecular genetic analysis of transgenics produced in this way usually show integration of a single proviral copy into a given chromosomal site. Rearrangements of the host genome are normally restricted to short direct repeats at the site of integration. In many embryos the germline cells contain viral integrants: thus, transmission of the transgene to the next generation will often occur. Methods have also been developed to allow infection into postimplantation embryos. In this context, virus uptake is effective for many somatic cell lines, however germline cells are infected at low frequency, due to a high level of mosaicism [15, 16].

Retroviral transfer remains an alternative to pronuclear microinjection in the context of human germline gene therapy. Traditional RVVs would be of minimal potential use, due to the high levels of mosaicism associated with these vectors. However, the new generation of lentiviral vectors would avoid such problems [21, 22, 23]. These vectors have the additional advantage of high gene transfer rates (70-80% of animals born are transgenic). Accordingly, lentiviral vectors represent plausible candidates for human germline gene therapy. However, the small insert capacity (9-10 kb) would preclude the transfer of many human genes. Additionally, control possibilities are less with RVV delivered transgenes compared with transgenes delivered by microinjection.

The safety problems associated with RVVs (insertional oncogenesis, viral reactivation) would also be a major concern [24, 25, 26]. In principle, judicious genetic alteration of the lentivirus genome would ensure that the resultant vector would have a very high level of safety. However, given the critical context of human germline gene therapy, one would have to question whether our basic scientific understanding of retroviruses is sufficiently advanced to empower rational vector design. Somatic gene therapy provides a salutary lesson here. Human trials involving several hundred patients have been carried out for over a decade using RVVs. Despite the theoretical risks referred to above, a lack of reports of serious adverse affects has resulted in a growing acceptance of the practical safety of RVVs. However, it has been recently reported that two patients (both young children) being treated for X-linked severe combined immunodeficiency disease (SCID-X) using RVV-based vectors have developed leukaemia. In both patients, RVV had integrated into a gene (LMO2) known to cause leukaemia if activated inappropriately. It is not known why the same endogenous gene had been targeted by the RVV concerned. The full cause of leukaemia in these patients is still under investigation, however the fact that both patients share the same integration site, coupled with the fact that the patients were both from the same (10-patient) trial, strongly implicates the particular RVVs employed in this trial [27, 28, 29]. Indeed, clinical trials involving this particular RVV-based therapy have been halted pending further investigations and pre-clinical trials [30]. It is to be hoped that enhanced RVV design will prevent any recurrence of iatrogenic leukaemia or similar serious adverse affects in somatic gene therapy. However, the occurrence of such adverse RVV effects lends weight to the argument that more basic virology is needed before any potential human germline gene therapy RVV could be deemed sufficiently safe. At the very least, extensive in vitro (cell culture) and in vivo (mammalian transgenesis) experimentation would be required in order to establish the safety of any proposed RVV (lentiviral-based or otherwise) for human germline gene therapy.

Microinjection of Retroviral Vector

A combination of microinjection with retroviral vectors has proved successful with bovines [31] and primates [32]. In the primate case, microinjection was used to deliver a retroviral vector into the perivitelline space of 224 mature rhesus oocytes. (The oocytes were subsequently fertilized by intracytoplasmic sperm injection.) The retroviral vector particles had an envelope type known to recognise and bind to the membrane of all cell types. The retroviral vector was microinjected at a developmental stage at which the oocyte nuclear membrane was absent, thus permitting nuclear entry. From 20 embryo transfers, three animals were born, one of which was transgenic. Additionally, a miscarried pair of twins was transgenic. Although this ‘combined’ method of gene transfer is laborious, it is the only approach that has permitted the generation of transgenic primates thus far.

Given the success with primates of microinjection of RVV into oocytes, this approach is likely to be effective for human germline gene therapy. The drawbacks would be similar to those associated with (a) microinjection (i.e. embryo loss) and (b) RVVs (i.e. transgene size limitations, problems with control of expression, safety risks). The process would also be laborious, but it would be expected to avoid the problems of mosaicism associated with most RVVs. Additionally, this form of gene transfer might require fewer eggs than required for pronuclear microinjection. The reported overall rate of transgenesis with rhesus monkeys was 1.3%; although this compares unfavourably with murine efficiencies (up to ca. 6% transgenic), it is significantly better than the rates achieved for animals such as sheep, cows and pigs. Moreover, this ‘combined’ technique is in its infancy, and its efficiency may well improve with use.

4. Sperm-mediated Gene Transfer

The scientific literature contains over forty reports of the successful in vitro uptake of exogene constructs (transgenes) by animal sperm cells [33, 34]. A majority of these reports provide evidence of post-fertilisation transfer and maintenance of transgenes. Several of the studies report the subsequent generation of viable progeny animals, the cells of which contain transgene DNA sequences. While a minority of studies have used 'augmentation' techniques (electroporation or liposomes) to 'force' sperm to capture exogenes, the standard methodology is very straightforward: prior to in vitro fertilisation (IVF) or artificial insemination (AI), 'washed' sperm cells are simply incubated in a DNA-containing solution. As a potential tool for genetically manipulating animals, sperm-mediated gene transfer (SMGT) has the advantages of simplicity and cost-effectiveness, in contrast with more established methods of transgenesis such as pronuclear micrinjection.

However, despite the above successes and regardless of its potential utility, SMGT has not yet become established as a reliable form of genetic modification. Concerted attempts to utilise SMGT have often produced negative results. The most notable example of such a failure is to be found in the collated results of several independent research groups: of 890 mice analysed, not a single animal contained transgene DNA [35].

Indeed, some biologists have expressed scepticism of the fundamental basis for SMGT [36, 37]. Such scepticism is posited on the assumption that major evolutionary chaos would result if sperm cells were able to act as exogene vectors. Given that the reproductive tracts contain 'free' DNA molecules (originating from natural cell death and breakage), it seems reasonable to expect sperm cells to be highly resistant to the risk of picking up such molecules [38].

Nevertheless, there exists a fairly well established body of empirical data showing that sperm cells are able, at least under particular experimental circumstances, to interact with and carry exogenes [39, 40]. Furthermore, isolated reports of the successful use of SMGT for genetic modification continue to be published. A notable recent example is the generation of several transgenic pigs following the artificial insemination of sows with sperm cells preincubated with transgene DNA [41, 42].

There are two possible ways to make sense of the above experimental and theoretical considerations. The first possible explanation is that SMGT is fundamentally unattainable. If so, the empirical evidence in support of SMGT must be faulty. For example, perhaps sperm can associate with exogenous DNA but cannot convey the DNA into the oocyte; and transgene sequences may have been erroneously identified in tissue samples, perhaps due to DNA contamination affecting sensitive detection methods such as PCR. This scenario is certainly not impossible: scientific research contains several examples of theory being misled by mistaken data. Indeed, early reports of SMGT were compared with the (then contemporary) claims of "cold fusion" in physics [36]. By contrast, the second possible explanation is that SMGT is viable, and that the claims of experimental success were not made in error. In this case, the explanation for the successful results must be that certain favourable factors applied in the fortuitous cases in which transgenes were taken up and transferred by sperm. Accordingly, several researchers have made efforts to elucidate such hidden parameters.

Underpinning such research into hidden factors has been the notion of the existence of 'inhibitory' factors (IFs) associated with sperm cells. These IFs are envisaged to prevent exogenous DNA uptake so as to protect the genetic integrity of the conceptus. The corollary of this notion is that successful instances of sperm cells taking up exogenous DNA may be attributed to the fortuitous removal or inhibition of IF(s) [43].

Seminal fluid reportedly contains an inhibitory factor (IF-1) that appears to actively block the binding of exogenous DNA to sperm and to the above-mentioned proteins [44]. Additionally, three classes of proteins identified in sperm cells have been claimed to exhibit DNA-binding properties [44, 45]. There is also some evidence that the binding of transgene DNA can trigger the activation of endogenous nucleases in sperm cells, which cleave both transgene and sperm chromosomal DNA [46, 47, 48]. The possible existence of IF(s) or other mechanisms against foreign DNA may explain the varied and often negative results obtained from attempts to use sperm to act as transgene vectors.

A superficial binding of exogenous DNA to sperm cells would be very unlikely to result in successful transgenesis, given the rigours of fertilisation. Conceptually, therefore, it is necessary to envisage the exogenous DNA being actively taken up by the sperm cell. Ultrastructural autoradiographic studies have indicated that exogenous DNA becomes concentrated within the posterior part of the nuclear area of the head, the inference being that binding of DNA by the sperm is followed by internalisation [49, 50].

One very interesting possibility is the combination of naked DNA autouptake with microinjection, a process that has been termed 'transgenICSI'. In this recent approach, sperm exposed to naked transgene molecules are microinjected into oocytes. Success has been reported with mice, with approximately 20% of founder animals integrating and expressing the transgene [51, 52]. Transgene uptake and expression following transgenICSI has also been reported in rhesus monkey embryos [53, 54] and porcine embryos [55, 56], although transgenic offspring did not result.

The success of transgenICSI provides support for the notion that sperm are indeed able to act as transgene vectors. However, some caution is required in making such a conclusion. Firstly, the experiments conducted need to be repeated and built upon before it can be said with certainty that the effect is a real one. Secondly, it could be the case that the trangene molecules bound only weakly to the sperm cells, such that only direct delivery (by ICSI) permitted the DNA to remain in place. If so, then this would not support the notion that SMGT can work when used with IVF or AI, because weakly bound or superficially located DNA might be stripped away and lost from the incoming pronucleus during fertilisation. If it were correct that ICSI is an indispensable part of the process, then SMGT would appear to have little advantage over pronuclear microinjection in terms of inherent technical difficulties and expense. However, the efficiency of the process does appear to be somewhat better than that of pronuclear microinjection. The available experimental data on standard human ICSI (i.e. not involving genetic modification) indicate that: (a) the majority (ca. 75%) of eggs are successfully fertilised; and (b) lysis following ICSI occurs at a relatively low rate (ca. 10%) [57, 58, 59]. For transgenICSI, the reported rates of success (i.e. transgenics per transfer) vary, but a figure of around ca. 35% is fairly typical [51, 52, 53, 54, 55, 56].

Whereas it would be somewhat surprising if sperm cells have the inherent ability to easily capture and transfer naked transgene molecules such that the DNA remains in place during fertilisation, it remains conceptually possible to use transfection techniques to 'force' sperm cells to capture (and thus transfer) exogenous DNA. Success has been claimed in this regard using electroporation and liposome-mediated gene transfer. Since 1990, several reports claiming successful transgene uptake and/or transfer following electroporation of sperm cells have been published, and there have been a number of reports of sperm cells taking up liposome-encapsulated DNA [34]. More research is clearly needed to determine whether and to what extent transfection techniques such as liposome-mediated gene transfer or electroporation may be able to augment SMGT. Nevertheless, given that these gene transfer techniques have been shown to work with a wide range of somatic cell types, in vitro and in vivo, there is no reason to presume that sperm cells are inherently unable to be transfected using such methods.

An alternative possibility could be to introduce the transgene into testicular (sperm) stem cells in vivo. This would in principle remove the need to collect, manipulate or transfer eggs, thus providing a major streamlining of germline GM. Preliminary results have been reported in mice, where transgene constructs were directly injected into the testis. For example, 60-70% of sperm were reported to carry the transgene following injection of naked DNA into the vas deferens [60], with a follow-up report claiming detection of the transgene in the cells of 7.5% of offspring animals produced following fertilisation with the transgene-bearing sperm [61]. Similar results were reported by Sato et al, using liposome-encapsulated transgene molecules injected close to the epididymis [62, 63, 64].

In vitro gene delivery into ex vivo spermatogonial stem cells of both adult and immature animals has recently been reported [65]. Nagano et al obtained stable transgene integration and expression in up to 20% of murine spermatogonial stem cells following retroviral transgene delivery [66]. Genetically modified stem cells were transferred into the testes of infertile recipient mice, leading to transgeneity in ca. 4.5% of the resultant progeny, plus successful transmission to subsequent generations. Similar results were obtained by Orwig et al in rats [67]. Although this form of transgenesis is at an early stage of development, preliminary work with spermatogonial stem cells in other mammals such as pigs and goats suggests that the approach is likely to be widely applicable [68, 69]. If human ex vivo spermatogonial stem cells are similarly able to pick up and transmit transgenes, an exciting potential route to germline gene therapy might emerge.

If SMGT does indeed work as reported, or if it can be made to work, then this would have very profound implications for human germline gene therapy. Gene transfer into embryos (using pronuclear microinjection or RVVs) is inherently very costly and technically demanding, due in large part to the need to remove embryos from the female, to manipulate the embryos and finally to return the embryos to the reproductive tract. By contrast, SMGT coupled with AI would permit germline GM with

minimum levels of expense and expertise, as would SMGT coupled with testicular injections. Thus, SMGT would in principle permit the widespread use of human germline gene therapy: relatively poor countries would be able to use the technique, and highly centralised facilities would not be required. Of course, such easily available human germline gene therapy would raise serious ethical concerns.

However, even if SMGT were to prove effective as a means to gene transfer, it would be fundamentally limited in the context of human germline gene therapy due to its unsuitability as a means of gene targeting. This limitation is of course shared with the embryo-based gene transfer methods considered above. However, there are at least some glimmers of hope for future gene targeting possibilities in the case of embryo-based approaches: some (albeit very limited) success has been achieved with targeting RVVs [70], and the low natural rate of HR in zygotes might conceivably be increased if appropriate recombinase enzymes were to be discovered and co-injected [71]. By contrast, there have been no reports of gene targeting using SMGT, and it is difficult to envisage even in outline how this might ever be achieved.

5. Episomal Vectors

Various extrachromosomal plasmid vectors (episomes) have been used as transgenes [72, 73]. Such vectors have been employed to produce transgenic animals, via a variety of routes including pronuclear microinjection and SMGT [74, 75, 76]. In the context of human germline gene therapy, such vectors offer the potential advantage of eliminating the threat to genome integrity associated with uncontrolled genomic integration. However, in transgenic animals, episomal plasmid vectors tend to behave in an unstable fashion [76, 77]. During development, plasmid copy numbers fluctuate and plasmids are lost from some cells. Plasmid inheritance to subsequent generations of animals is similarly problematic. Moreover, worrying health problems (such as tumour formation) have been associated with some episomal vectors [78]. Of course, the behaviour of an episome must relate in large part to its genetic constitution, and therefore stability problems and safety limitations may in principle be surmounted by improved plasmid design. However, until such improvements are realised, episomal plasmid vectors could not be considered for human germline gene therapy.

Autonomous artificial ‘mini-chromosomes’, (mammalian artificial chromosomes, MACs) have been constructed and successfully introduced into mammalian cells [79, 80]. MACs comprise centromeres, telomeres and replication origins, and are maintained autonomously within the host cell. Structural genes, promoters and enhancers (etc) can be included in MACs. Preliminary research indicates that MACs can be used, via pronuclear microinjection, to create transgenic animals in which the MACs are maintained autonomously [81]. In the context of human germline gene therapy, these specialised constructs would be expected to give a number of benefits compared with integrated transgenes, including higher and more controllable expression. More speculatively, MACs may be able to function as genetic ‘platforms’ for the safe subsequent receipt of incoming transgenes. Although this technology is in its infancy, MACs would appear to hold significant future potential for human germline gene therapy [81, 82, 83, 84, 85].

6. Totipotent Cells

At present, gene targeting requires the use of in vitro selection in order to enrich for rare targeted cells amongst a majority of random integration cells. In vitro selection cannot be conducted on embryos or sperm cells. Consequently, gene targeting in the context of human germline gene therapy would require gene transfer to be carried out with some form of dividing cells in vitro.

ESCs

Inner cell mass (ICM) cells from the mouse blastocyst can be propagated in vitro as embryonic stem (ES) cells [86, 87]. In contrast to other cultured cell lines, ESCs retain their normal karyotype even after many months in culture, during which time they remain totipotent. Furthermore, ESCs are capable of colonising the embryo. These unique properties allow ESCs to form chimeras when injected into blastocysts or aggregated with morulae. The resultant embryos can be transferred to the uterus of a pseudopregnant female mouse for gestation. In cases where an ESC has successfully contributed to the embryo, the resultant offspring will be chimeric (up to ca. 50% of animals). The ESC contribution to a mouse can high (up to ca. 80% of the cells), and will often include the germline cells. However, it should be noted that, with some transgenes, the production of chimeras can be problematic or even

unattainable, especially when germline transmission is required to breed pure lines of heterozygous or homozygous animals [86, 87, 88, 89].

It is during the in vitro culture stage that ESCs may be transgenically manipulated [90, 91]. Many gene delivery systems are effective with ESCs, including viral vectors, liposomes, and electroporation. The great advantage of ESCs is that they can be subjected to a range of selective agents in vitro, which allows the selection of particular transgenic modifications. This ability makes ESCs extremely useful for gene targeting experiments and applications [92], [93].

However, the use of ESCs is limited due to the fact that, to date, the mouse is the only animal from which ESC lines have been unequivocally established. It would be surprising if this limitation represents a fundamental biological barrier. However, further empirical work is needed before totipotent ESC lines become available for other species. Indeed, efforts to isolate non-murine ESCs have been ongoing for nearly two decades but to date no germline-competent ESCs have been isolated in other vertebrates [94, 95].

In 1998, Thomson et al isolated ESCs from human blastocysts [96].

Subsequently many other researchers have also isolated human ESCs [97, 98, 99, 100], and a new field in biology has resulted. Furthermore, gene targeting has been achieved in human ESCs [101]. However, totipotency has not been demonstrated in any human ESC line. Unfortunately, this may prove to be a rather intractable situation: proof of totipotency could only (given current technology) come from the establishment of a chimeric human being. It is manifest that the necessary experiments required to pursue this goal would be ethically unacceptable. Thus, the ESC route presently remains firmly closed against human germline gene therapy.

Nuclear Transfer

The successful transfer of ‘reprogrammed’ sheep donor nuclei has recently been achieved [102, 103, 104]. Unfertilised, metaphase-stage enucleated (‘universal recipient’) eggs received the transferred nuclei. Donor nuclei originated from somatic cells that had been forced into a form of cell cycle stasis (by incubating the cells in a minimal nutrient medium), such that DNA replication and gene expression were halted (or virtually so). Nuclear transfer was conducted by depositing a donor cell under the zona pellucida of a universal recipient egg, and fusing the two cells by electrical stimulation. This process resulted (in some cases) in successful embryo development, the donor nuclei having been ‘reprogrammed’ into totipotency. Offspring were produced following the transfer of such ‘reconstructed’ embryos to recipient ewes. Subsequent molecular genetic testing showed that the lambs’ DNA had originated from the donor cells. In some of the experiments, the donor nuclei were obtained from embryo-derived cultured cell lines. Following these groundbreaking experiments, successful cloning from cultured cells of various mammals including cattle, goats and pigs has been reported [105, 106]. Interestingly, a human ESC line has recently been derived from cloned human blastocysts produced by NT [107], pointing to a possible new field of application for NT technology.

The prospects for germline GM via NT are very significant: transgenes can be introduced to somatic donor cells in vitro, permitting germline genetic modifications. This has been achieved in animals [103]. Several gene delivery systems are applicable to NT, including liposomes and electroporation. Moreover, because selection can be applied to cultured donor cells, NT can be used to achieve germline gene-targeting. Gene targeted transgenic animals have been created in this way [108, 109]. Thus, NT is potentially able to provide the same range of transgenic manipulations presently available in mice (via the ESC route) to all mammal species. Accordingly, it seems probable that the technique could in principle be readily applied to humans as a means to achieving germline modifications.

However, in comparison with ESC transgenesis, NT has thus far proved to be relatively inefficient: only a small proportion of reconstructed embryos survive to become live animals. For example, McCreath et al produced live targeted sheep at an efficiency of less than 4% [109], and Lia et al produced live targeted pigs at an efficiency of less than 2% [110]. Such low efficiencies, presuming reconstructed human embryos to behave similarly, represent a potential problem for human NT-based germline gene therapy. Although embryo pre-selection could be used to ensure that only transgene-containing embryos were allowed to gestate, the problem would remain that a large number of valuable donor eggs would be required for each GM attempt.

The health status of NT-derived animals is also proving to be problematic [111, 112]. Developmental abnormalities are very common, and frequently result in death (foetal or postnatal) or debility. For example, of fourteen live-born lambs, seven died within 30 hours of birth, and four died within twelve weeks [109]. Similarly, out of seven piglets, two piglets died shortly after birth, and one died at 17 days; only one appeared to be entirely free of developmental abnormalities [110]. Transgenesis and gene targeting are not of themselves implicated: the health problems are associated with NT *per se*. During the in vitro (cell culture) stage, the pattern of chromosomal imprinting may change; there are indications that inappropriate expression of imprinted genes following such epigenetic alteration may be mainly responsible for the poor health of NT-derived animals [113, 114, 115]. Research into epigenetic reprogramming in NT embryos is in progress, and it is to be hoped that developmental abnormalities arising from NT will eventually be eliminated or reduced in frequency. Meanwhile, it is anticipated that NT-related health problems, to the extent that the basis for such is epigenetic, are unlikely to affect the offspring of surviving first-generation animals. However, until such time as the first-generation health problems are solved, NT appears to be too dangerous to consider for human germline gene therapy. This is unfortunate, because without NT (or human ESCs) the in vitro selection required for germline gene targeting cannot be conducted. Thus, germline GM in humans would be restricted to 'add-in' alterations; gene knockout and gene repair germline alterations in humans are not a practical proposition with the technology available at present.

Non-selective gene targeting

In embryonic stem cells and in certain somatic cells in vitro, unusually high levels of gene targeting have been reported. Isogenic transgenes, derived from the same (syngenic) laboratory animal strain as the target animal, contain homology blocks that are genetically identical (or virtually identical) to the target homology regions. Riele et al [116] reported a 20-fold improvement in targeting efficiency when an isogenic transgene was used to target the retinoblastoma susceptibly gene (*Rb*) in murine ESCs, yielding a remarkably favourable ratio of random to targeted integration (approximately 1:4). Similar results were obtained from a systematic study by Vandeursen and Wieringa [117], in which the creatine kinase M gene (*CKM*) in ESCs was targeted.

More recently, adeno-associated virus (AAV) vectors have been used to gene target somatic cells at high frequencies. Hirata et al used AAV vectors to introduce transgenes into the hypoxanthine phosphoribosyl transferase (*HPRT*) and Type I collagen (*COL1A1*) loci in normal human fibroblasts [118]. The transgenes were targeted at high frequencies, such that the majority of transgene-containing cells had undergone gene targeting with an appropriately designed vector. AAV targeting frequencies have been further improved by selective creation of double-strand DNA breaks in the target site [119, 120]. Most recently, adult human mesenchymal stem cells (MSCs) have also been targeted with high efficiency using AVV vectors [121].

The foregoing reports suggest that in ESCs and in certain somatic cells the efficiency of gene targeting can be sufficient to bypass the need for selection. Selection-based gene targeting places limits on transgene design, due to the need to engineer the requisite selective elements into the transgene. Therefore, the ability to conduct gene targeting in somatic cells without the need for selection would be a welcome addition to the armamentarium of gene transfer technologies that may in future permit human germline gene therapy.

7. Conclusions

It is probable that the human germline could be readily manipulated using current transgenic techniques. To achieve this, pronuclear microinjection would probably be effective, as would retroviral transfer, particularly using lentivirus-based vectors. A combination of microinjection and RVVs would probably be most effective – indeed such a combination has recently given rise to the first transgenic primates. SMGT may be effective also, at least in some forms of the approach, such as transgenICSI. However, AI-based SMGT is not yet an established method of transgenesis, therefore the prospects for this potentially very important form of gene transfer are less certain. Totipotent human ESCs have not been established for humans, thus ESC-based gene transfer remains – despite its effectiveness in mice – unavailable for human germline gene therapy. The lack of human ESCs leaves NT-based gene transfer as the only method that might be able to permit gene targeting in human germline gene therapy. NT could probably be readily applied to humans; however, the high level of health problems observed in

first generation NT-derived animals render the approach in its present form unfeasible for human germline gene therapy. Table 1 summarises the key features of the major candidate methods that might serve to achieve human germline gene therapy.

If human gene transfer technology is limited to adding-in gene functions via non-targeted transgene integration, and if the process needs be performed on individual embryos isolated from the reproductive tract, it is likely that human germline gene therapy will remain insufficiently safe, excessively inefficient and of inadequate clinical value to permit its use. The widespread availability and applicability of safe and effective human germline gene therapy would require the development of gene transfer methods that would (a) permit gene targeting while (b) avoiding the need for ex vivo embryo isolation and manipulation. Unfortunately, at present these two requirements are mutually exclusive. Laborious manipulations involving large numbers of embryos would in principle best be avoided by the use of SMGT, either in vivo or ex vivo. However, high-efficiency gene targeting is not available at present without the use of in vitro selection. Thus, the widespread use of human germline gene therapy does not appear likely to flow from incremental improvements in current GM methods. Rather, widespread human germline gene therapy would appear to be a future possibility that must await substantial scientific advancement. Naturally, it is impossible to predict when such improvements might be forthcoming.

Conflict of interest:

The author has declared that no conflict of interest exists.

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Tables

TABLE 1. Gene Transfer Methods

	Pronuclear micro-injection	**Retroviral transfer**	**Micro-injection of Retroviral Vector**	**Sperm-mediated Gene transfer**	**Embryonic Stem Cells**	**Nuclear Transfer**
Gene targeting possible?	No (rate is too low)	No (not yet established)	No (not yet established)	No (not possible)	Yes	Yes
Overall efficiency	Up to ca. 6%	Up to ca. 80%	Presently 1.3%	Up to ca. 80%	Up to ca. 25%	Up to ca. 4%
Cost / Expertise requirements	Very high	High	Very high	Low (except for transgenICSI and SMGT+IVF)	Very high	Very high
Genetic damage risk	< 2%	High: varies depending upon vector	High: expected to vary depending upon vector	< 2%	Low if gene targeting involved; some epigenetic problems	Low if gene targeting involved, but serious epigenetic problems
Mosaicism (F_0)?	Yes: ca. 65%	Not necessarily (lentiviral vectors)	No	Yes (likely to be similar to pronuclear micro-injection)	Chimeric	No
Transgene capacity	Unlimited: could even be used to deliver MACs	9-10 kb	9-10 kb	Not known	Depends on transfection method	Depends on transfection method

Expression	Often low or aberrant, due to random integration	Control possibilities limited by viral sequences	Control possibilities limited by viral sequences	Likely to be low or aberrant, due to random integration	No problems in gene targeted outcomes	No problems in gene targeted outcomes
Scientific status	Fully established in non-primate animals	Well established in non-primate animals	Early success reported in primates	Not well established despite several reports of success – theoretical difficulties	Fully established – but only in mice	Becoming well established
Use in human germline GM conditional upon scientific break-throughs?	No	No	No	No	Yes: human ESCs required	No
Scientific advances required before method could become a practical proposition for human germline gene therapy	Incremental improvements in efficiency ICM pre-screening technology Use of 'insulator' sequences in transgenes	RVV design improved and tested to ensure safety Engineering of RVV genome for improved transgene expression	RVV design improved and tested to ensure safety Engineering of RVV genome for improved transgene expression	Establish-ment of SMGT (must be reliable and repeatable) Development of augmented uptake methods	n/a	Improve-ments in reprog-ramming to avoid epigenetic problems Incremental improve-ments in efficiency
Ideal improve-ments	Gene targeting via recombinase use	Gene targeting by engineering RVV genome	Gene targeting by engineering RVV genome	Establishment of AI-based SMGT as a reliable form of GM	n/a	n/a