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RNA interference in embryonic stem cells and the prospects for future therapies

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In 1998, two distinct and exciting scientific fields emerged which have profoundly shaped the current direction of biomedical research. The discovery of RNA interference (RNAi) and the derivation of human embryonic stem (ES) cells have yielded exciting new possibilities for researchers and clinicians alike. While fundamentally different, aspects from these two fields may be combined to yield extraordinary scientific and medical benefits. Here, we review the prospects of combining RNAi and ES cell manipulation for both basic research and future therapies, as well as current limitations and obstacles that need to be overcome.
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Introduction

The path from discovery to therapy is a long and difficult one. The development of any potential therapy requires: (1) a good model and exhaustive *in vivo* characterization of the disease to be treated, (2) disease target identification and drug discovery and (3) therapeutic development and clinical testing. The combinatorial use of RNA interference (RNAi) and embryonic stem (ES) cells may provide new tools for all of the stages of therapeutic development and yield extraordinary benefits. However, the use of biological tools in potential therapies requires an extensive knowledge of their biological functions. While much work remains to be done in both of these fields before the biology of these systems is clearly defined, significant insights have been gained in the last few years.

Biological function

RNAi

RNAi is a mechanism of post-transcriptional silencing which acts through degradation of mRNA transcripts by the action of homologous short RNA species. Since its characterization in 1998 by Fire *et al*.,[1] RNAi has been the subject of intense investigation, the driving force behind which is twofold. First, RNAi is an ancient evolutionarily conserved mechanism of gene regulation, which is thought to be present in many, if not all, eukaryotic model systems. It has been shown to play an essential role in processes ranging from developmental regulation of gene expression to viral immunity. The second reason RNAi has intrigued the scientific and biomedical communities relates to its practical applications, both in the lab as well as in potential therapies.

Early studies investigated the ability of long dsRNA (generally ranging from 500 to 1000 nucleotides) to initiate an RNAi response in *Caenorhabditis elegans* and plants. These studies showed that dsRNA was able to silence homologous mRNA transcripts,[1,2] resulting in a measurable decrease in gene-specific expression. Additional studies, however, have shown that, in many types of mammalian cells, exposure to long dsRNA generates a non-specific immune response directed by dsRNA-dependent protein kinase (PKR) (for a review, see Kumar and Carmichael[3]). As a result, instead of the sequence-specific mRNA degradation seen in *C. elegans*, the PKR-directed interferon pathway can trigger a global shutdown of translation and apoptosis. Gene-specific silencing by RNAi was successfully achieved in mammalian tissue culture cells in 2001 through the introduction of shorter dsRNA species (less than 21 bp) into cells.[4] These short interfering RNAs (siRNAs) can specifically inactivate genes, minimizing the interferon response. This discovery enabled the use of RNAi-based tools for the large-scale manipulation of gene expression in mammalian systems. It is, however, worth noting that dsRNA does not induce the interferon pathway in all mammalian cell types. Notably, specific silencing has been reported in mouse oocytes/zygotes, ES cells and embryonal carcinoma (EC) cells using long dsRNA.[5–7]

While the general RNAi mechanism is conserved throughout the plant and animal kingdom, some variations in the pathway do exist. In addition to its function as a post-transcriptional gene silencer, there have been several studies which suggest that RNAi may play an important role in the nucleus as a transcriptional gene regulator (for recent reviews, see Matzke and Birchler,[8] Verdel and Moazed,[9] and Bernstein and Allus[10]). In

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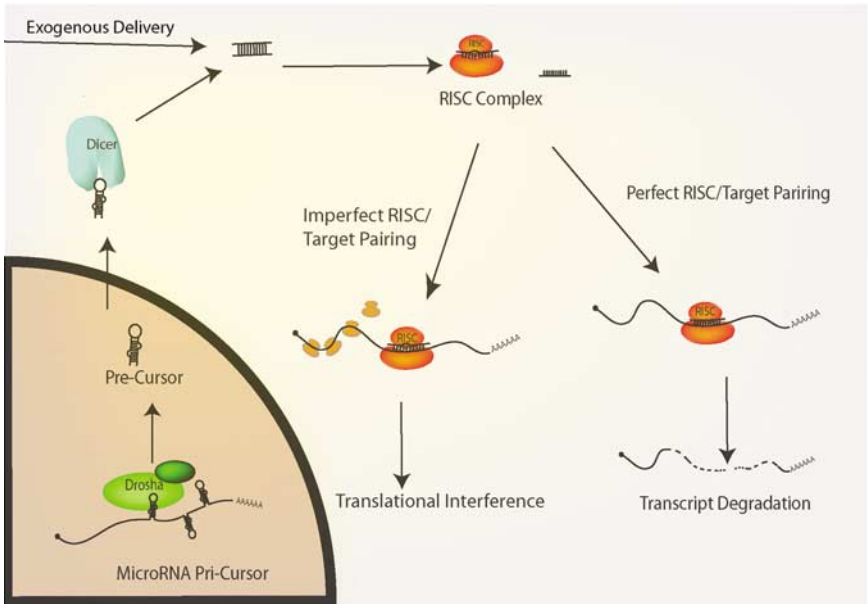

Figure 1 miRNA processing and RNAi in mammals. miRNA processing begins in the nucleus where long primary miRNAs are recognized by the associated proteins Drosha and Pasha. These enzymes cleave the long pre-cursor into short ~75 nucleotide (pre-cursor) hairpins which are then transported out of the nucleus. In the cytoplasm, the enzyme Dicer recognizes the pre-cursor and cleaves it into a 19–21 nucleotides RNA duplex with characteristic 2 nucleotides 3′ overhangs. A single strand of the duplex originating from a Dicer-processed pre-cursor or an exogenous siRNA is incorporated into the RNAi silencing complex (RISC). Depending on the degree of complementarity between the siRNA and its target mRNA, RISC may either block the translation machinery or cleave the target.

particular, the mechanism by which long dsRNA is tolerated and processed in various organisms differs. In plants and invertebrates, where mRNA transcript degradation is far more common than in mammalian systems, siRNAs are processed from either long dsRNA or very long hairpin species. In these systems, siRNAs can be generated from both strands of the RNA duplex and multiple siRNAs can be generated from a single long RNA. In fact, an important function of the plant 'immune' system is dependent upon its ability to take exogenous dsRNA, like that introduced by an invading virus, and process the dsRNA into siRNA. After an amplification step catalyzed by the RNA-dependent RNA polymerase (RdRP), these 'preprogrammed' siRNA species seek out and target other homologous viral particles (for recent reviews, see McManus,[11] Herr[12] and Wang and Metzlaff[13]). Instead of inducing viral immunity as seen in plants, the introduction of long dsRNA in a mammalian system induces the interferon response generating universal gene silencing and apoptosis. Such differences profoundly affect the interspecies application of RNAi. An effective long dsRNA-based gene-silencing technique in *C. elegans*, for example, could likely be ineffective or dangerous in a mammalian system. This is why a thorough understanding of such differences is essential before any potential gene therapy can make the transition from the lab to clinical trials.

Several years after the initial description of RNAi, data emerged that suggested a wide variety of organisms might use small RNAs to regulate gene expression.[14–16] This discovery shed light on the earlier finding that the important developmental gene in *C. elegans lin-4* does not encode a protein but instead a non-coding RNA species.[17] Further research has revealed that the *lin-4* gene is not a unique anomaly but only the first of thousands of microRNAs (miRNAs) to be identified.

miRNAs are transcribed as long primary transcripts (can be more than 2000 nucleotides). These primary transcripts are processed by the Drosha enzyme to yield a short hairpin miRNA precursor of approximately 75 nucleotides. This precursor is exported from the nucleus, where it is processed by the Dicer enzyme to yield a transiently existing ~21 nucleotide RNA duplex. Depending on the thermodynamic asymmetry of the duplex, a single strand is preferentially loaded into the silencing complex. This short RNA strand is considered to be the mature miRNA. The overall pathway is displayed in Figure 1, although there are many additional factors and details that are omitted (for recent reviews, see Hammond,[18] Tomari and Zamore[19] and Hutvagner[20]).

The RNAi pathway, induced through the introduction of synthetic siRNA or short hairpin RNA (shRNA), is very similar to the pathway by which the endogenous miRNAs are processed. In general, a high degree of complementarity between the mRNA transcript and the loaded silencing complex usually leads to RNAi-like degradation of the target transcript. If, however, there is a lower degree of sequence complementarity, the loaded complex may merely interfere with translational machinery, inhibiting protein production, through a largely unknown mechanism and usually leaving the mRNA intact. In mammals, miRNAs tend to mediate their effects through translational repression, although exceptions to this general rule can be found. The mouse miRNA miR-196, for example, pairs exactly with the Hoxb8 mRNA transcript and directs an RNAi-induced mRNA cleavage.[21,22]

Although the mechanism of RNAi has only very recently been elucidated, RNAi has quickly become one of the most popular methods of gene silencing in the lab. There are several benefits to utilizing RNAi over other gene silencing methods. siRNA silencing strategies, for

example, have been shown to be 100 times more effective than other antisense oligonucleotides (ODNs), at silencing the same target.[23] Although chemical modification of synthetic ODNs have led to more efficient delivery, they also tend to result in a decreased sequence specificity as well as an increase in toxic side effects. Additionally, RNAi-induced silencing tends to be more stable and have fewer toxic side effects when compared with silencing induced by other synthetic nucleotides such as DNA oligos or ribozymes, perhaps because RNAi harnesses an endogenous cellular pathway.

Studies in mouse ES cells have shown that embryos derived from RNAi-treated ES cells can recapitulate the phenotype of the conventionally derived null animal.[24] Use of RNAi to induce gene-silencing offers several advantages when compared with the practice of generating targeted genetic deletions in mouse 'knockouts'. In addition to the substantial time and cost required to produce a 'knockout', complex models involving the altered function of multiple genes may be very difficult to produce with current approaches. Techniques such as blastocyst injection of RNAi-encoding viruses or the implantation of RNAi-modified ES cells can produce transgenic animals in months rather than years. These techniques can be used to study normal tissue function and disease by varying the level of gene expression instead of completely abolishing it. In effect, this may provide researchers with a molecular 'tuning dial' instead of simply an on/off switch. It should be noted that RNAi-based knockdown strategies are not likely to replace conventional gene knockout techniques, but instead provide a complementary tool that may have particular advantages in gene therapies.[25]

Depending on the model system being studied, or the disease to be treated, a wide variety of methods may be employed to induce RNAi-mediated gene silencing, each of which has its own distinct advantages and disadvantages. In *C. elegans*, inducing stable RNAi is as simple as soaking the animals in a solution of dsRNA or feeding them transformed bacteria which produce long dsRNA.[26,27] Unfortunately, for those researchers not studying nematodes, these delivery methods cannot be applied to most model systems. Probably, the simplest and most versatile method used in the lab to silence gene expression *in vitro* is to design siRNA duplexes which target a gene of interest and insert them into cells using a variety of transfection techniques. Although this strategy is both rapid and inexpensive, there are limitations. Unlike *C. elegans*, mammalian cells do not contain RdRP for the amplification of siRNA. As a result, the effects of transfected or injected siRNA in a mammalian cell decrease as the moiety is diluted with cell division. Thus, a simple injection delivery method of siRNA does not provide stable long-lasting RNAi silencing in mammals. Long-term stability, however, may not be necessary in some gene therapies, such as two recently approved by the FDA for clinical trials, for the treatment of AIDS-induced age-related macular degeneration. These therapies involve a local injection of the 'naked' unpackaged siRNA directly into the eye.[28,29] Local delivery may reduce the likelihood of potential off-target effects elsewhere in the body and the transient nature of the treatment may actually be beneficial because it limits unknown, potentially negative side effects that may occur from long-term expression.

Local delivery of naked RNAs can be effective when targeting accessible organs such as the eye, the skin or the lungs. For less accessible organs, however, viral vectors may be a useful alternative. These systems often encode shRNAs, which are processed much like miRNAs into siRNAs. Unlike naked RNA, some of these viral vectors, such as adeno-associated viruses (AAV) and lentiviruses, can integrate into the host genome leading to a more permanent expression of a shRNA or siRNA. These viruses can infect non-dividing cells, such as primary neurons, making them important tools in therapies for diseases that target the CNS. shRNA-encoding AAVs have, for example, been used to effectively silence a deleterious gene in the brains of mice with spinocerebellar ataxia, which is similar to the human neurodegenerative disorder Huntington's disease.[30]

Lentiviral-based systems have recently become a very popular way to deliver small RNAs. A variety of lentiviral plasmids are available, containing various selectable markers driven by many different promoters (including inducible systems).[31–36] When pseudotyped with VSV-G, lentiviruses are highly tropic for stem cells and can easily be produced in high-titer if concentrated by high-speed ultra-centrifugation. These characteristics have made lentiviruses very useful in the lab both in ES cell culture as well as in the creation of transgenic animals from modified ES cells (for a review, see Pfeifer[37]). Although still a relatively new strategy, shRNA-encoding lentiviruses hold promise for therapies. Such vectors, ironically derived from the human lentivirus HIV, have shown promise in silencing various components necessary for HIV infection and replication (for a review, see Cullen[38]).

Large-scale screens of RNAi libraries are very useful tools for identifying novel gene function and dissecting the biology of cellular pathways. Various academic and commercial groups have created multiple types of RNAi libraries. These libraries range from collections of shRNAs or siRNAs designed to target a specific gene or group of genes, to constructs derived from the enzymatic digestion of cDNAs.[39–42] RNAi screens are very versatile because they can be applied to most systems. Thus far, RNAi screens have helped to identify novel genes involved in everything from cell division to apoptosis to fat metabolism. These tools can be especially useful in dissecting complex regulatory pathways. RNAi screens are usually carried out by one of two methods. One method involves the transfection (or infection) of a pooled RNAi library into cells followed by selection and analysis of cells expressing a phenotype of interest. Alternately, large-scale RNAi libraries can be arrayed and analyzed in a high-throughput manner. RNAi screens are simple and cost-effective tools for elucidating gene function and dissecting biological pathways, and they are also rapidly becoming essential in the process of identification and validation of potential gene therapy targets. The use of RNAi libraries could allow the rapid identification of effective targets minimizing investment on the development of drugs against ineffective targets.

ES cells

Stem cells are characterized by their ability to proliferate in an undifferentiated state and to give rise to differentiated progeny. There are two major kinds of stem

cells: ES cells and adult stem cells. ES cells can be expanded extensively in culture because of their self-renewing capacity. They are also pluripotent, that is, they have the capacity to generate differentiated progeny from all three embryonic germ layers (endoderm, mesoderm and ectoderm)[43,44] as well as the germ line.[45] In contrast to ES cells, adult stem cells such as neural stem cells or hematopoietic stem cells have a more restricted differentiation capacity and usually generate cells of the tissue from which they are derived. Adult stem cells are maintained throughout the life of the organism by their ability to self-renew.

ES cells were first derived in 1981 from the inner cell mass of the mouse blastocyst.[43,44] Before the derivation of mouse ES cells (mES cells), it had been shown that some tumors called teratocarcinomas behaved as a pluripotent and self-renewing population *in vitro*.[46,47] Cell lines derived from these tumors are called EC cells.[48] ES cell lines have most of the molecular, morphological and growth characteristics of EC cell lines. Unlike EC cells, mES cells can contribute to all tissues when injected into blastocysts, including to the germ line.[45] In 1992, another pluripotent cell type was isolated, this time from mouse primordial germ cells (PGCs). These cells are called embryonic germ (EG) cells and resemble both mES and EC cells.[49,50]

In 1998, the same year of the discovery of the RNAi pathway, a major event for ES cell research took place: the derivation of human embryonic stem (hES) cell lines[51] (Figure 2). hES cells are derived from the inner cell mass of blastocysts at about 1 week post-fertilization. Like mES cells, hES cells are a self-renewing and pluripotent population. Injection of hES cells into immunocompromised mice results in the formation of teratomas, containing cells from the three embryonic layers. Owing to obvious ethical reasons, it is unclear whether these cells can contribute to a human embryo when introduced into the blastocyst. Also in 1998, human embryonic germ (hEG) cells were derived from gonadal ridges containing PGCs (5–9 weeks post-fertilization).[52]

Both mouse and human ES cells can be propagated in the presence of serum and co-cultured with a layer of fibroblasts. However, they require different signals to self-renew. mES cells require leukemia inhibitor factor (LIF) and bone morphogenic proteins (BMPs), whereas hES require fibroblast growth factor (FGF) and suppression of BMP signaling.[53–57] Despite differences in the signals for self-renewal, the regulation by transcription factors appears to be conserved between mES and hES cells. In particular, the transcription factors Oct4[58] and Nanog[59,60] are required to maintain both mouse and human ES cells in an undifferentiated state. This requirement was recapitulated with RNAi: downregulation of Oct4 or Nanog in mES cells and hES cells led to the loss of pluripotency and self-renewal capacities.[61–64] This suggests that both transcription factors have similar roles in mouse and human ES cells. Little is known about the mechanisms by which these transcription factors maintain pluripotency, particularly because their targets have for the most part not been identified. In addition, the regulation of the cell cycle and of lineage commitment in ES cells remains poorly understood. Clearly, much work remains to be carried out to dissect the regulation of ES cell self-renewal and pluripotency and RNAi is likely to be a very powerful tool for this purpose.

The derivation of hES cells opened the possibility of using nuclear transfer techniques to produce cell lines that carry the genetic information of a human donor. By taking any somatic cell from an individual, and introducing its nucleus into an enucleated oocyte, it is possible to generate a new diploid oocyte with a nuclear genome identical to the donor. This oocyte can form a blastocyst, from which it is possible to derive nuclear transfer (NT)-ES cells. Proof-of-principle for this approach has been provided in the mouse.[65] These cells can then be used for therapeutic purposes which will be further discussed. For ethical reasons, it is important to distinguish between therapeutic cloning and reproductive cloning. Therapeutic cloning involves

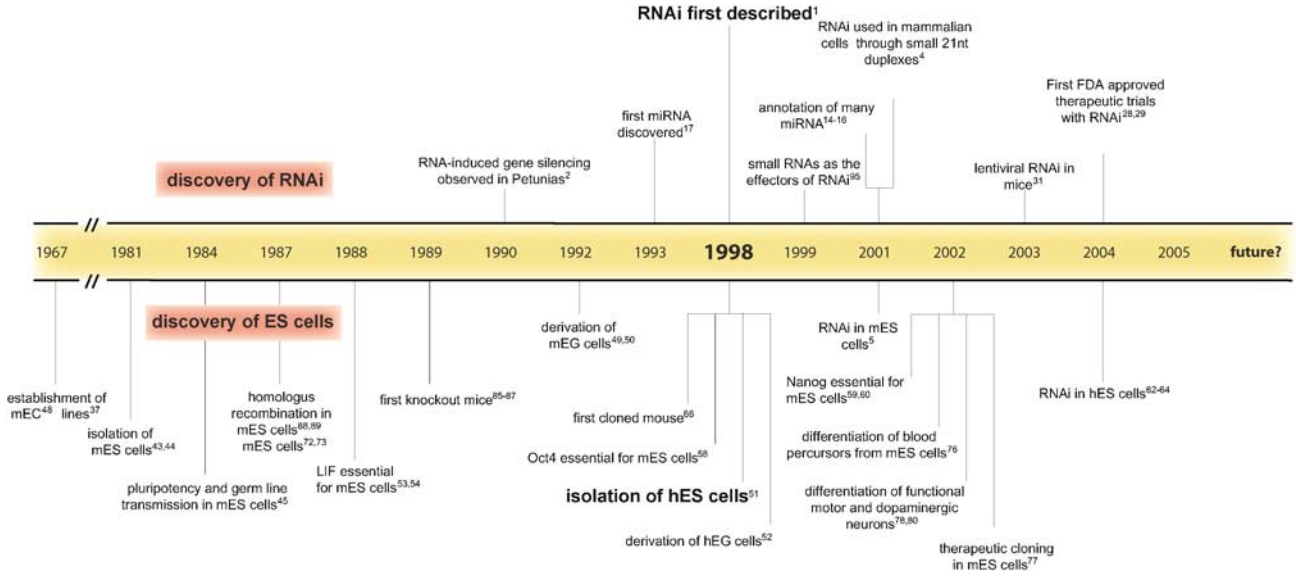

Figure 2 Timeline of RNAi and ES cell discovery. This timeline highlights some of the most important steps in the discovery of both RNAi and ES cells where the year 1998 is an important milestone. Numbers in superscript refer to references in the text.

the use of NT-hES cells for cell-based therapies. Reproductive cloning involves the implantation of a cloned embryo in the uterus to create a entire organism, which has only been reported in some animal models, like the mouse.[66]

The use of RNAi in dissecting ES cell biology and differentiation

So far, the undifferentiated state of ES cells has been studied through gain- and loss-of-function studies that have described the importance of a few genes like Oct4,[58,67] Nanog,[59,60] Foxd3[68] and Sox2.[69] This approach has been limited to the study of mice that are mutant for each of these genes. RNAi allows researchers to test the role of many genes in ES cells without the need to generate mutant mice. The specificity of RNAi can be confirmed by targeting sequences in untranslated regions of the mRNA and then rescuing the phenotype by overexpressing their coding sequence. In addition, using RNAi against various genes simultaneously can help to clarify the pathways that maintain pluripotency. Most of the genes so far shown to regulate the undifferentiated state of ES cells were chosen because of their expression patterns in the early embryo or from functional cDNA overexpression screens. With new technologies, it is possible to identify other candidate regulators of ES cells and study them with loss-of-function screens using RNAi. The availability of new ES cell lines expressing reporter genes under the control of promoters of ES cell-specific genes will allow researchers to monitor the undifferentiated state of ES cells. For example, hES cell lines have been generated that express green fluorescent protein under the control of the Oct4 promoter.[70] Microarray analysis of ES cells suggests that some genes may have an important role in determining the stem cell state, because they are upregulated in these cells when compared to somatic cells.[71] Since conditional RNAi systems are also available, analyzing candidate genes selected from various approaches or performing genome-wide screens by conditional loss-of-function analysis in both mouse and human ES cells can bring essential regulatory pathways to light (Figure 3).

Apart from understanding the undifferentiated state of ES cells, it is of great interest to understand the mechanisms that underlie lineage commitment of ES cells. Owing to the fact that they are pluripotent, ES cells can be differentiated into many if not all cell types. A popular method used to trigger differentiation *in vitro* in ES cells is through the formation of embryoid bodies (EBs), a heterogeneous aggregate of cells that is formed spontaneously in suspension after the removal of LIF. Formation of EBs from mES cells is reported to recapitulate initial steps of cell differentiation in early embryos (for a review, see Keller[72]). Therefore, through detailed study of EB formation it is possible to recapitulate the developmental context and promote differentiation of particular cell types. RNAi can help to dissect these pathways through loss-of-function genetic screens to identify critical genes involved in cell fate decision (Figure 3).

miRNAs are likely to play an important role in ES cell differentiation. It has been shown that several miRNAs are expressed in mouse ES cells. Some miRNAs are immediately suppressed upon ES cell differentiation, whereas others are expressed only after the formation of EBs.[73] Recent studies showed that ES cells lacking the critical RNase for the generation of miRNAs, Dicer, are defective in their proliferation and differentiation. It is still unclear, however, if miRNAs directly regulate the cell cycle or differentiation pathways, or have a more global effect on cell stability.[74,75]

The most successful attempts to differentiate cells from mES cells *in vitro* have shown that ES-derived cells acquire at least some of the molecular, morphological

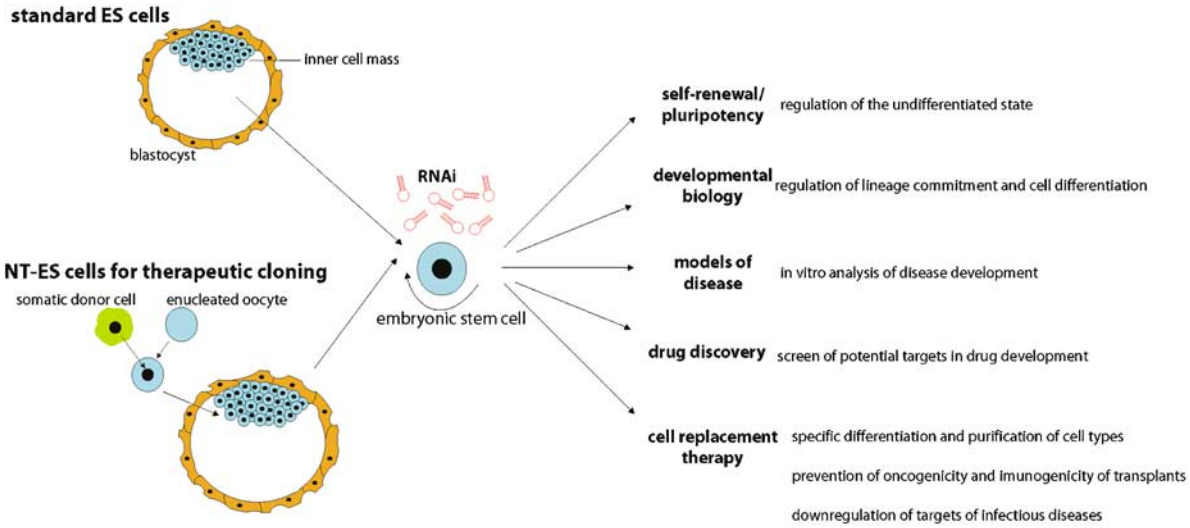

Figure 3 The potential uses of RNAi in ES cells. ES cells can be generated from normal blastocysts (standard) or through blastocysts derived from somatic cell nuclear transfer (NT-ES cells). ES cells can be expanded essentially indefinitely in culture and can give rise to all cell types of the body. RNAi may be useful in a wide variety of studies involving ES cells. Some of these areas include: understanding the basic biology of ES cells and cellular differentiation; modeling disease states *in vitro*; validating new drugs and assessing their toxicity; directing differentiation of cell types of interest from ES cells; controlling the cell cycle and immune repertoire of ES-derived cells to be transplanted and targeting infectious agents.

and functional features of differentiated cells. For example, overexpression of the transcription factor HoxB4 was shown to promote differentiation of mES cells into hematopoietic progenitors.[76] These progenitors were successfully engrafted into irradiated mice resulting in long-term multi-lineage hematopoietic progeny that persisted in secondary recipients. A parallel study showed that the Rag2 (−/−) deficiency could be repaired using homologous recombination on NT-ES cells of these mice.[77] This is an example of therapeutic cloning that combines nuclear transfer with gene correction of ES cells. Neural lineages have also been obtained from mouse ES cells. For example, exposure of differentiating cells to retinoic acid and sonic hedgehog led to differentiation into motor neurons.[78] These neurons formed functional synapses with muscle when transplanted into chick embryo spinal cords.[79] Dopaminergic neurons were also derived from ES cells and used to reverse symptoms of Parkinson's disease in rats.[80] It has been more challenging to differentiate endodermal derivatives, such as liver or pancreatic cells, from ES cells. Nevertheless, the derivation of insulin-producing cells capable of reversing diabetes in mice has been reported.[81,82] Genetic manipulation including gene silencing by RNAi may help to overcome the roadblocks to endoderm differentiation**.** Notwithstanding all the promising advances in differentiating ES cells, one should not expect that it will be possible to obtain all cell types desired or even to engineer whole organs from ES cells, at least not in the near future. For example, in the case of neurons, where there can be distinguished up to 200 different subtypes, it is unlikely that it will be possible to differentiate all of them, and to recapitulate all the neuronal networks. Nevertheless, RNAi has already been used to manipulate ES differentiation. For example, knockdown of the tumor suppressor p53 facilitates differentiation of mouse ES cells into muscle cells,[83] and knockdown of the transcription factor PU.1 favors differentiation of pro-B cells.[84]

The case studies described above lead us to believe that many of the obstacles to the generation of cell types of interest from ES cells can be overcome, and RNAi will be an important tool. Downregulation of critical genes during differentiation may induce either the growth of a specific sub-population of cells or the apoptosis of an undesired one, resulting in both cases in the enrichment for a cell type of interest.

Potential therapeutic applications and limitations of RNAi and ES cells

One may expect that, in a near future, the development of some diseases will be studied *in vitro* using ES cells. ES cells of an animal model for a specific disease, or human ES cells that were derived from a pool of patients, may enable the identification of specific genes involved in the pathophysiology of the disease, as well as the characterization of the impact of mutations or allelic diversity among different groups of patients. It should be possible to obtain hES cells that can be clonally propagated which contain exactly the same genetic information as a human donor, as it has been done in the mouse.[65] Once differentiation is well established for a particular cell type, the etiology of a disease can be studied at the molecular and cellular level using these *in vitro* models, allowing manipulations that would otherwise be impossible. RNAi-based screens will allow the identification of molecular modules essential for disease progression. RNAi screens will permit the use of ES-derived cells to validate therapeutic targets for new drugs that are cell-specific.

Historically, mouse ES cells have been very useful for generating genetically engineered animal[85–87] for research purposes using homologous recombination.[88,89] Recently, however, the focus of ES cell research has been directed towards more clinical applications, such as development of cell replacement and gene therapies. The big challenge is to be able to apply all the knowledge of ES cell biology and to obtain well-defined protocols for differentiation for cell-based therapies, where some damaged tissues may be replaced by ES-derived cells. Standardized hES cells or patient-specific NT-hES cells may be used to enrich for specific cell types using adequate genetic manipulation and culture conditions. The possibility of doing therapeutic cloning is a clear advantage of ES-derived cells therapies over those using adult stem cells. ES cells can also be propagated indefinitely and seem to be more amenable to gene manipulation, providing an inexhaustible cell source for therapy. ES cell pluripotency also enables a broader use of these cells in such therapies. Diseases that involve the loss or damage of a single or very few types of cells are the most attractive candidates for ES cell therapies. Parkinson's disease,[80] lower motor neuron loss and spinal cord injuries and[78,79] type I diabetes mellitus[81,82] are all potentially treatable by these therapies.

In addition to the conventional cell-replacement approaches aimed at repairing damaged tissues, the combination of ES cell and RNAi technologies may result in novel therapies for infectious diseases such as HIV, tuberculosis or malaria. One such strategy for combating the HIV virus has already been reported. It involves isolation of hematopoietic stem cells from an infected individual and treating them with a lentivirus that leads to expression of a shRNA targeted against either viral RNA or against the cellular receptor targeted by HIV (for reviews, see Lee and Rossi[90]). These stem cell populations are then expanded *ex vivo* and reintroduced into the patient. As hematopoietic stem cells give rise to the cells comprising the immune system, it is hoped that such a procedure will confer HIV resistance to the immune system (the main target of HIV). Alternatively, hematopoietic progenitors derived from ES cells carrying RNAi vectors that target HIV infection may be used. This approach would circumvent the need to extract hematopoietic stem cells from the patient, taking advantage of the fact that ES cells can be grown in very large numbers.

Although the potential of RNAi and stem cell-based gene therapies is extremely promising, there are issues of safety and efficiency that must be addressed before any potential therapy can be applied in humans. The combinatorial therapeutic use of RNAi and ES cells, while it may yield great benefit, also compounds the limitations and potential negative side effects which both tools may illicit. Currently, one of the most pertinent limitations involving the use of ES cells is the lack of knowledge regarding the details of ES cell developmental biology. The range of cell types that can currently be derived from ES cells is fairly limited. As a result,

the disease targets of potential stem cell-based therapies are restricted to those affecting the small subset of cell types that can be derived. It is likely that in the near future the number of cell types that can be derived from ES cells will greatly increase, but the goal of complete ES cell-based organ replacement may be far off. Most of the differentiation protocols to obtain a cell type of interest yield a heterogeneous population that contains other cell types as well. It will also be important to achieve cell purity before ES-derived cells can be transplanted into patients. Another major caveat of ES cell-based therapies is the possibility of tumor formation. If a transplant happens to contain contaminating undifferentiated ES cells, these could lead to the formation of teratocarcinomas. Downregulation through RNAi of specific genes involved in regulation of the cell cycle may be a way to avoid these tumors.

Another limitation is the immune response following engraftment of an ES-derived transplant not immunologically matched to the patient. The use of immunosuppressive drugs can prolong the survival of allogenic ES cell progeny. This is not an ideal method due to the fact that the ability of the body to heal is compromised when the immune system is suppressed. Another strategy to enhance the compatibility of the graft is to decrease ES cell expression of cell surface proteins that activate host immune responses (i.e. major histocompatibility complex and costimulatory molecules) or to increase ES cell expression of immune-inhibitory antigens (i.e. Killer cell immunoglobulin-like receptors). These approaches in isolation will not likely permit long-term engraftment of ES cell-derived cells. Another way to prevent immune rejection is through the production of patient-specific ES cells by somatic cell nuclear transfer and thus has become a very popular topic of study (Figure 3).

The use of RNAi itself also presents hurdles that must be overcome. The two main hurdles are effective RNA delivery and specificity of gene silencing. shRNA delivery for some gene therapies, such as those used in two clinical trials recently approved by the FDA (for age-related macular degeneration), could be as simple as the injection of naked RNA.[28,29] Many other techniques for RNAi delivery have been formulated including liposomal carriers, aerosolized vapors and viral vectors, but like any other potential therapeutic treatment these methods must be carefully evaluated for both efficiency as well as any possible off-target effects.

The *in vivo* delivery efficiency of the interfering RNA species to the cell of interest is an issue that deserves great attention. The second issue that needs to be addressed is how effective a construct is at silencing its target sequence and only its target sequence. The fact that miRNAs can effectively silence mRNA transcripts with which they share only partial sequence homology suggests that the problem of off-target silencing is a very real issue which needs to be addressed in any effective gene therapy design.[91] In addition, in some cases, even small 21 nt duplexes appear to be capable of inducing non-specific global silencing directed by the interferon response in mammalian cells.[92,93] On the other hand, especially with respect to viral infection, it has been reported that siRNAs can specifically silence their target RNA and not other transcripts, even when the target and those other transcripts vary in sequence by as little as a single base pair.[94] Only further studies of the relationship between miRNAs and their target sequences can help to answer questions about specificity and off-targeting, as well as define rules by which to design potential therapeutic constructs.

Conclusion

Although the fields of RNAi and ES cell research are in their infancy, it is already possible to envision cell and gene therapies combining both of these strategies. Studies of ES cells differentiation may overcome concerns about the limiting number and purity of cells available for cell-replacement therapies. For this reason it is important to understand the mechanisms that regulate ES cell differentiation. RNAi may allow the discovery of unknown genes involved in pluripotency and lineage commitment, and may be used to direct cell differentiation. In addition, genes that are implicated in the development of specific lineages can be downregulated to enrich cultures of purified cells, eliminating unwanted derivatives. By knocking down genes involved in cell proliferation, the tumorigenic potential of these ES-derived cells may be eliminated. RNAi may also be useful to manipulate the immune repertoire and reduce the probability of rejection of an ES cell-derived transplant. Finally, using RNAi in ES cells may help to model diseases *in vitro* and identify effective drug targets. Given these promising potential applications, we expect that many fascinating discoveries will be made in the years ahead through the combinatorial use of RNAi and ES cells.

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