Mini Review

Gene silencing in human embryonic stem cells by RNA interference

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ABSTRACT

RNA interference (RNAi) is a post-transcriptional conserved mechanism, which is present in a wide range of organisms and leads to specific gene silencing. The effector molecules in this process are double-stranded RNAs (dsRNAs) that are homologous in sequence to the silenced gene and are processed into small interfering RNAs (siRNAs) by an enzyme called Dicer. Consequently, siRNAs are incorporated into an RNA-induced silencing complex, which finds and cleaves the target mRNA. Because of its exquisite specificity and efficiency, RNAi is being considered as an important tool for gene silencing in living organisms. Human embryonic stem cells (HESCs) have the ability to both self-renew and differentiate into cell types of all three germ layers. HESCs open new avenues for understanding some of the very early lineage determination events that occur during embryogenesis and are also considered as an important source of cells for cell replacement therapies. Understanding how the signaling pathways orchestrate and direct HESC differentiation toward certain cell types is critical for basic research. In this way RNAi appears to be a valuable tool in stem cell biology for dissecting the pathways involved in differentiation, lineage segregation and production of cells for cell therapy. Here, we review the prospects of combining RNAi and HESC manipulation for both basic research and future therapies.

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Introduction

RNA silencing is a gene regulatory mechanism that limits the transcript level by either suppressing transcription (transcriptional gene silencing) or activating a sequence-specific RNA degradation process (post-transcriptional gene silencing) [1]. RNA interference (RNAi) is an evolutionary conserved post-transcriptional gene silencing mechanism in many eukaryotes, which plays an essential role in developmental regulation of gene expression. In this phenomenon, short double-stranded RNAs (dsRNAs), most commonly referred as short-interfering RNAs (siRNAs), elicit degradation of a homologous target mRNA [2]. In the RNAi reaction, the cellular RNase III enzyme, Dicer, cleaves the dsRNA into 21–25 bps siRNAs in an ATP dependent manner [3]. siRNAs, known as the key molecules for the RNAi effect [4], then bind to an RNAi-specific protein complex to form the RNA-induced silencing complex (RISC). This complex might undergo activation in the presence of ATP so that the antisense component of the unwound siRNA becomes exposed and paired with cognate mRNA. Subsequently, RISC cuts mRNA approximately in the middle of the duplex region and hence decreases production of the corresponding protein [5,6].

In spite of the fact that long dsRNAs could be potent silencing triggers in a diverse groups of organisms, due to their effects on inducing interferon response followed by generating universal gene silencing and apoptosis, long dsRNAs (>30 bps) are not effective in a specific manner in mammalian systems [7,8]. In contrast, siRNAs produced from DNA templates expressing short hairpin RNAs (shRNAs), can efficiently silence the corresponding gene in mammalian cells, without any interferon response [9]. On the other hand, small temporal RNA molecules (stRNAs), which represent a large group of small transcripts called microRNAs (miRNAs), mediate gene suppression by inhibiting translation of target mRNAs. Typically, stRNAs, which are derived from endogenously expressed precursor RNAs, recognize the target mRNA by a partial-complementary interaction to regions at the 3′ untranslated region (UTR) of the target mRNA [10]. However, siRNAs, which are usually processed from foreign genomes, contain a fully complementary dsRNA targeting sequence and require a perfectly matched target mRNA sequence for functionality [11].

Although the mechanism of RNAi has been elucidated recently, RNAi has quickly become one of the most popular methods of gene silencing in the laboratories. Due to its reliability and ease of use, RNAi has been used in large-scale screening projects to identify genes implicated in a variety of biological processes in several species [12–14]. In comparison with other antisense oligonucleotides, siRNA silencing strategies tend to be more effective and have fewer toxic side effects [15]. The effects of RNAi can be achieved by tran-

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sient transfection of small RNA molecules or stable transfection of a plasmid that would lead to the production of interfering molecules [16]. Rather than generating targeted genetic deletions in animal knockouts, which require substantial time and cost, blastocyst injection of siRNA-encoding viral vectors or the implantation of RNAi-modified embryonic stem cells (ESCs) could be rapid and cost effective methods to produce transgenic animals [17]. The discovery that gene-specific silencing can be successfully achieved through the introduction of siRNAs in mammalian cultured cells [18], enabled the use of RNAi-based tools to study the basic biology and manipulation of gene expression in stem cells.

HESCs, derived from inner cell mass of human blastocysts, can be expanded extensively in culture because of their self-renewing capacity [19,20]. They are also able to differentiate along all three embryonic germ layers, making them valuable tools for studying the underlying molecular mechanisms that control early development and a promising source of cells for the treatment of degenerative disorders [21,22]. A central challenge is to develop methods to direct differentiation of HESCs in a controlled manner to produce individual populations of specific cell types. Self-renewal and differentiation capacities of ESCs are regulated by the coordinated interactions between several intrinsic and extrinsic factors [23]. For instance, transcription factors Oct4, Sox2, Foxd3 and Nanog [24–28], ERK, Wnt and Notch signaling pathways [29–31], leukemia inhibitory factor (LIF) and bone morphogenetic proteins (BMPs) [32–34] form a complicated regulatory network controlling ESCs self-renewal and pluripotency. Furthermore, it has been shown that miRNAs, noncoding small RNAs that function in the post-transcriptional regulation of gene expression, also play important roles in HESCs self-renewal and pluripotency [35]. Despite these findings, little is known about the mechanisms by which these intrinsic/extrinsic factors maintain pluripotency. Moreover, the regulation of lineage commitment in HESCs remains poorly understood.

Impairment of gene expression followed by phenotypic analyses is an important way to test gene function. Homologous recombination, ribozymes and antisense technologies can be employed to generate cell lines void of a certain gene product [36,37]. However, the generation of targeting constructs and the selection and validation of clones are complicated, expensive and time consuming [38]. The discovery of RNAi has provided an alternative strategy for loss-of-gene function assays. In this way, the combinatorial use of RNAi and ESCs provides a new tool for elucidating the molecular pathways that define ES cell exclusive properties.

Salient aspects of RNAi technology

The application of siRNAs for specific gene silencing in mammalian cells involves a careful consideration of selecting the siRNA sequences in the target gene to avoid non specific complementarity to unrelated mRNAs. Depending on the method or algorithm used, it may be necessary to screen several sequences in order to identify one or more with higher efficacy [39,40]. Furthermore, for achieving an effective RNAi, it is essential to optimize transfection of the siRNAs in the target cells and monitor the efficiency of gene silencing. Due to the lack of siRNA amplification in mammalian cells, several strategies have been devised to synthesize short RNAs *in vitro* [41] or to introduce plasmids with the ability to make *de novo* siRNAs inside the cells [42,43]. Despite providing advantages over chemically synthesized siRNAs, the use of plasmid vectors remains limited because of the transient nature of siRNA expression and low and variable transfection efficiencies. To circumvent these problems, virus-based high-efficiency siRNA delivery systems have been developed [44]. Vectors containing RNA polymerase type III promoters such as the U6 small nuclear RNA promoter [45] or H1 promoter [46] have

been designed to express a single RNA transcript that folds into a hairpin RNA in mammalian cells. The efficiency of transfection itself, depends on the cell type, passage number and the confluency of cells [6]. The most commonly used transfection method for human cells is lipofection, in which nucleic acids are encapsulated by cationic lipids including Lipofectamine (Invitrogen) Oligofectamine (Invitrogen) and HiPerFect (Qiagen), added to the culture medium and taken up by the cells. Non-liposomal delivery reagents such as ExGen 500 (Fermentas) interact with nucleic acids and form particles that can enter the cells by endocytosis, are also used in RNAi studies frequently (Table 1). The advantages of these reagents are their simplicity, low toxicity and ability to transfect at low cell confluency. Microinjection and viral transduction, including the use of adenoviruses, retroviruses, lentiviruses and adeno-associated viruses (AAV) are other methods, which can be used to deliver either chemically synthesized siRNAs or shRNA constructs into HESCs (Table 1). To detect specific gene knockdown by RNAi, studying the depletion of the target protein by immunofluorescence and Western blot analyses are preferred, while the knockdown phenotype and Northern blotting are also recommended [60].

RNAi-mediated gene specific knockdown in HESCs

HESCs, derived from the inner cell mass of blastocysts at about 5 days post-fertilization, were first established in 1998 [18]. There are striking differences between mouse and human ESCs with regards to cell surface antigens [61], signaling pathways promoting self-renewal [62,63], and overall growth properties [64–66]. Therefore, it cannot be concluded that a regulatory factor active in mouse ESCs (MESCs) necessarily exhibits the same function in their human counterparts. However, similar to MESCs, RNAi-mediated downregulation of *OCT4* or *NANOG* in HESCs led to the loss of pluripotency and self-renewal capacities, and also differentiation toward trophectoderm and extraembryonic endoderm lineages [48,49,52,57]. Theses findings confirm the conserved role of OCT4 and NANOG in mouse and human ESCs. Furthermore, it was shown that reduced expression of *OCT4* in HESCs by RNAi resulted in upregulation of markers indicative of mesoderm and endoderm differentiation [59]. The observation that *OCT4*-RNAi reduced *NANOG* expression and visa versa, suggests that there is a coordinated gene regulation of *OCT4* and *NANOG* in HESCs similar to MESCs [57]. RNAi-mediated suppression of *OCT4* in HESCs was also used to identify OCT4-dependent genes in these cells. Transcriptional changes induced by *OCT4* knockdown include direct targets of this transcription factor, indirectly associated genes linked with pluripotency, and genes activated upon differentiation along the trophoblast lineage, which confirm that a network of transcription factors cooperatively maintain pluripotency in HESCs [50].

ZIC3, a zinc finger transcription factor, demonstrates differential gene expression between the pluripotent and early differentiation phases [67] and may be regulated by OCT4, NANOG, and SOX2 [68]. Targeted repression of ZIC3 in HESCs by RNA interference induced expression of several markers of the endodermal lineage and, surprisingly, reduced the expression of *NANOG*. This suggests that ZIC3 may prevent endodermal marker expression through NANOG-regulated pathways and further establishes the ZIC3 role in the maintenance of ES cell pluripotency [69]. Signal-induced proliferation associated gene-1 (spa-1), which is a principal Rap1 (Ras-proximate 1) GTPase-activating protein that regulates Rap1-related signal transduction [70], is overexpressed in HESCs [53]. HESC differentiation was induced when *spa-1* expression was knocked down by siRNA treatment, which leads to the conclusion that spa-1 may influence the maintenance and differentiation of HESCs via the Rap1/Raf/mitogen-activated protein kinase (MEK)/ERK pathway [53].

Table 1
List of publications describing different transfection methods, used to deliver either siRNA or shRNA constructs into HESCs.

Silenced genes	RNAi triggers	Transfection methods	References
hrGFP, DsRed2	Chemically synthesized siRNA	Lipofectamine 2000	[47]
OCT4, Oct4	Chemically synthesized siRNA	Lipofectamine 2000	[48]
NANOG	Chemically synthesized siRNA	Lipofectamine 2000	[49]
OCT4	Chemically synthesized siRNA	Lipofectamine 2000	[50]
OCT4, EGFP	Chemically synthesized siRNA	Lipofectamine 2000, Lipofectamine RNAiMAX, Oligofectamine	[51]
OCT4	Chemically synthesized siRNA	Oligofectamine	[52]
hrGFP, DsRed2	Chemically synthesized siRNA	ExGen 500	[47]
spa-1	Chemically synthesized siRNA	ExGen 500	[53]
OCT4	Chemically synthesized siRNA	Lipofectamine 2000	[54]
OCT4, SOX2, NANOG	Chemically synthesized siRNA	HiPerFect	[55]
GFP	ShRNA: lentiviral vector	Viral transduction	[56]
OCT4, NANOG	ShRNA: plasmid	Viral transduction	[57]
RPS19	ShRNA: plasmid	Viral transduction	[58]
OCT4	ShRNA: lentiviral vector	Viral transduction	[59]

Identification of clones with relatively different levels of gene silencing is a laborious task in stem cell studies. To make it easier, Gropp and Reubinoff used dual-promoter lentiviral vectors, co-expressing an RNAi cassette and a reporter gene for efficient and stable induction of heterogeneous levels of gene silencing in HESCs. This step was further combined with the isolation of transduced clones with different homogenous levels of gene silencing. Thus, the level of silencing in each of the clones is correlated and could be monitored by the level of expression of the reporter transgene [71]. These studies demonstrate that many of the obstacles in generation of specific cell types from HESCs using RNAi technique have overcome, but there are still some problems such as using viral vectors for delivery of RNAi cassette or producing cells that do not elicit immune response, which need to be solved, before these cells can be used in clinic.

Potential therapeutic applications and limitations of RNAi and HESCs

The discovery of HESCs had a great impact on the scientific community, mostly because they are considered to hold a great promise in transplantation medicine. However, in order to enable clinical use of these cells, their differentiation into specific cell types should be controlled through genetic manipulation methods.
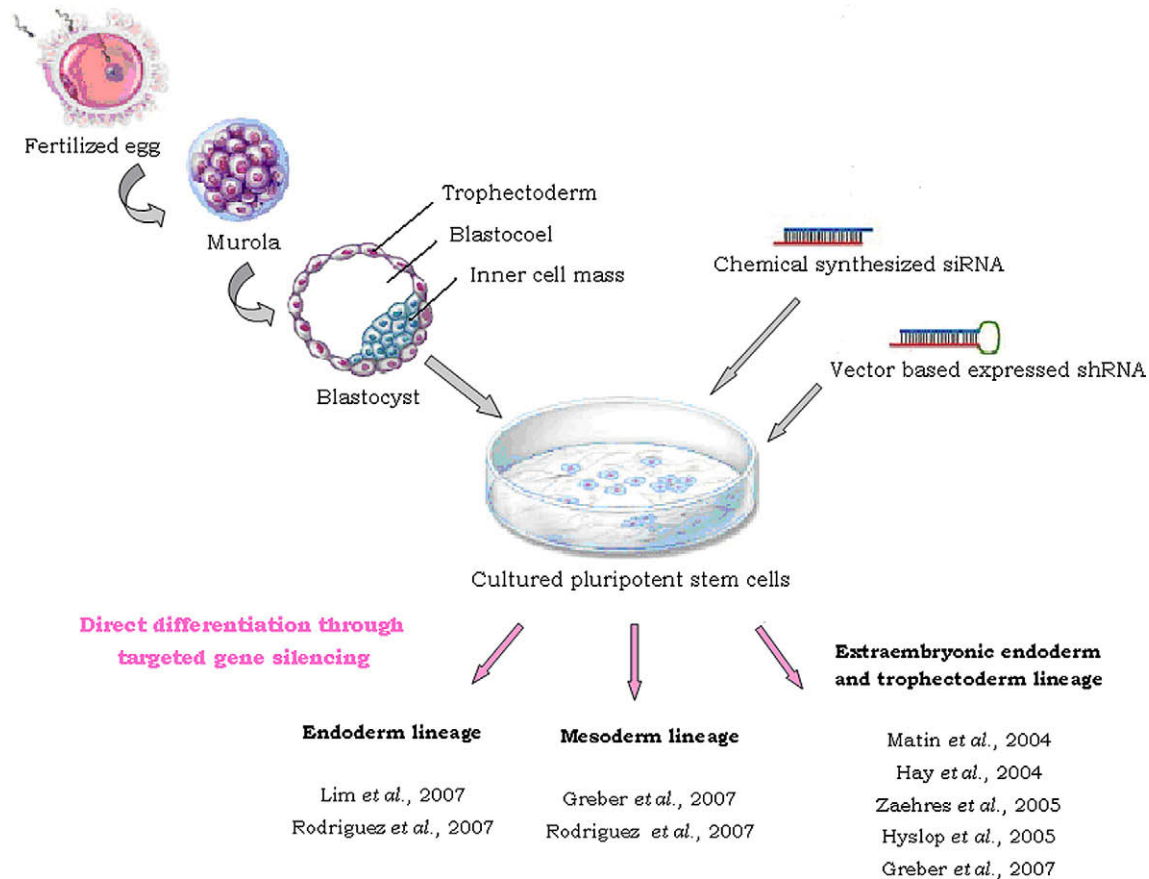

Fig. 1. The combinatorial use of RNAi and HESCs to induce pluripotent stem cells differentiation toward embryonic and extraembryonic lineages.

The use of RNAi to knockdown the expression of genes suspected to be functionally important for stem cell maintenance has facilitated efforts aimed at identifying pathways important in the maintenance of pluripotency, as illustrated for example by the results of manipulating expression of OCT4 and NANOG in HESCs [50,52,57,59]. Understanding HESCs differentiation provides the ability to direct the differentiation of HESCs to specific cell types advancing the clinical utility of HESC-derived cells and tissues. As reviewed here, RNAi mediated knockdown of several genes in HESCs induced their differentiation to cells derived from embryonic germ layers and extraembryonic lineages (Fig. 1). These results exemplify the potential of RNAi as a surrogate genetic tool to force enrichment of cell types of interest from HESCs. Another therapeutically relevant use of RNAi is genetic manipulation of HESCs for prevention or treatment of human diseases. One possible strategy will be to isolate and clonally propagate stem cell pools from patients, followed by the identification, characterization and correction of the underlying genetic defects, for example via RNAi-mediated suppression of oncogenes [46,72]. Further looming RNAi applications in HESCs are reduction of their tumorigenic potential (by knocking down proliferation genes), or manipulation of their immune repertoire to minimize rejection in patients who undergo transplantation [73].

The combinatorial therapeutic use of RNAi and HESCs also compounds the hurdles and potential negative side effects, which both tools may confront. In summary, the major limitations of ESC-based therapies are the lack of knowledge regarding the details of HESC developmental biology, heterogeneity, limited range of cell types that can be derived from HESCs, the immune response following engraftment and the possibility of tumor formation. Reducing the expression of cell surface proteins that activate host immune response or increasing the expression of immune-inhibitory antigens in HESCs are the best approaches to overcome immune response [74]. RNAi-mediated downregulation of specific genes involved in regulation of cell cycle may be a way to avoid tumor formation following engraftment of ES cell-derived transplants [73]. In the case of RNAi method, specificity of the interfering RNA species and the efficiency of in vivo delivery are issues that deserve great attention. Since it has been shown that ectopic expression of SID-1 in MESCs enabled passive cellular uptake of dsRNA/siRNA [75], SID-1 could be considered as a potential simple and rapid delivery of dsRNAs/siRNAs. Therefore, it is foreseeable that non-viral delivery vectors that encode siRNAs or cellular uptake of soaking siRNAs via SID-1 expression will pave the way for a new wave of therapeutic molecules in the years to come.

Conclusion

The phylogenetically conserved cellular phenomenon of RNAi holds substantial promise for basic research. RNAi methodology suppresses gene expression, thus mimicking loss-of-function mutations and enabling in vitro gene function analyses. Downregulation of critical genes by RNAi in HESCs 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 of the cell type of interest. The results of studies reviewed in here shed insights into the development of high-throughput experimental strategies for studying ES cell self-renewal, pluripotency, differentiation and generation of specific cell types for therapeutic purposes.

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