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Gene targeting in primary human trophoblasts

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Abstract

Studies in primary human trophoblasts provide critical insights into placental function in normal and complicated pregnancies. Mechanistic studies in these cells require experimental tools to modulate gene expression. Lipid-based methods to transfect primary trophoblasts are fairly simple to use and allow for the efficient delivery of nucleic acids, but potential toxic effects limit these methods. Viral vectors are versatile transfection tools of native trophoblastic or foreign cDNAs, providing high transfection efficiency, low toxicity and stable DNA integration into the trophoblast genome. RNA interference (RNAi), using small interfering RNA (siRNA) or microRNA, constitutes a powerful approach to silence trophoblast genes. However, off-target effects, such as regulation of unintended complementary transcripts, inflammatory responses and saturation of the endogenous RNAi machinery, are significant concerns. Strategies to minimize off-target effects include using multiple individual siRNAs, elimination of pro-inflammatory sequences in the siRNA construct and chemical modification of a nucleotide in the guide strand or of the ribose moiety. Tools for efficient gene targeting in primary human trophoblasts are currently available, albeit not yet extensively validated. These methods are critical for exploring the function of human trophoblast genes and may provide a foundation for the future application of gene therapy that targets placental trophoblasts.

Keywords

Placenta; RNA interference; transfection; lentivirus; microRNA

1. Introduction

Pharmacological agents are widely used to explore cell function, yet they have several potential disadvantages, including a lack of specificity with respect to pathways or specific proteins, partial effectiveness and limited availability of agonists or antagonists. The development of techniques designed to modify gene expression by introducing nucleic acids has revolutionized our approach to the mechanistic analysis of gene function in mammalian cells. An ideal gene transfer method is simple, cost-effective, efficient, non-toxic and results

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in long-lasting expression/silencing. Whereas cell lines are fairly easy to transfect, gene transfer into primary cells remains challenging. Here, we review gene-targeting approaches in primary cells with an emphasis on their application to primary human trophoblast (PHT) cells and placental villous tissue explants. After reviewing the basic concepts in transfection technologies, we discuss approaches used to overexpress trophoblast proteins as well as techniques used to silence genes in PHT by RNA interference (RNAi), including required control experiments and problems with off-target effects. Because of the relative ease of transfection using trophoblast cell lines [1–11], this topic is not discussed here.

2. Using PHT cells in gene targeting experiments

PHT cells represent a unique cell culture system, with special characteristics that impact gene targeting approaches. *In vivo*, villous cytotrophoblasts contain a population of progenitor cells that continue to produce daughter cells, which differentiate and fuse with the overlying syncytium (a fully differentiated, non-dividing epithelium) or develop into invasive extravillous trophoblast [12, 13]. Villous cytotrophoblast cells can be isolated from any stage of pregnancy and although term PHT cells are the most commonly studied in gene targeting experiments [11, 14–34], first [10, 35, 36] and second trimester cells [37–41] have also been used successfully. Cultured cytotrophoblasts rapidly differentiate into syncytial islands that degenerate within approximately 3–5 days. Isolated cytotrophoblasts typically do not attain an invasive proliferative phenotype, which likely requires preservation of the normal cell architecture with intact mesenchyme, basement membrane and syncytium [42]. The syncytium-like cells that develop from cultured PHT cells are believed to represent a relevant model for the syncytiotrophoblast *in vivo* (reviewed in [43]). The time dependent, rapid differentiation and transient nature of PHT cells in culture have several important implications for gene targeting. For example, it is primarily the fully differentiated syncytium that can be studied using these approaches, because the full effect of any gene targeting approach may not occur until 24–48 hours following transfection (see further below), which largely precludes the study of the cytotrophoblast. In addition, it is essential to monitor the influence of the transfection procedure on the differentiation process itself, which could confound the interpretation of the data.

3. An overview of transfection approaches in primary cells

The term transfection implies the deliberate introduction of nucleic acids into cells. In general, this procedure is commonly used to achieve (1) decreased expression of endogenous proteins by using RNAi, either involving short interfering RNAs (siRNAs), microRNAs (miRNAs) or short hairpin RNAs (shRNAs), (2) overexpression of endogenous genes, or (3) expression of foreign genes. Depending on the transfection method used and the type of nucleic acid delivered, transfections can be stable or transient. A stable transfection typically requires DNA incorporation (“transgene”) into the host cell genome. In contrast, DNA that fails to integrate into the genome and all forms of RNA are subject to degradation, resulting in transient transfection. For example, following the transfection of human cells with double-stranded siRNA, silencing is maximal by 42–54 hours and declines thereafter (reviewed in [44]). Stability of the transfected DNA is cell type dependent, with DNA being stable >72 hours in HEK293T cells [45]. In all transfection experiments, it is important to determine the transfection efficiency, which is the proportion of cells that express the transgene among all cells in the culture dish. The level of mRNA or protein overexpression, or the relative decrease in mRNA or protein expression when silencing is performed, represents an indirect indicator of the transfection efficiency. The primary approach for assessing transfection efficiency utilizes tagged proteins or RNAs, resulting in the expression of a detectable marker. Commonly used reporters include green fluorescent protein (GFP), luciferase and β -galactosidase, which can be inserted into the vector carrying

the transgene or delivered as a separate vector by co-transfection. One particularly attractive example of this approach is the use of two different luciferase genes, firefly and *Renilla* luciferase, which allow for the simultaneous quantification of the luminescent signals, one serving to inform the expression vector and one the control (normalization) reporter.

A wide array of approaches has been used to transfect primary cells (reviewed in [46–48]), some of which have been applied to PHT cells (Table 1). These techniques can broadly be categorized into chemical, physical and viral methods. The **calcium phosphate co-precipitation approach** is a chemical technique, which is based on the principle that DNA molecules are adsorbed to insoluble calcium phosphate particles [49, 68]. The calcium/DNA complexes attach to the cell surface, and are believed to be taken up by the cells through endocytosis. The calcium phosphate co-precipitation method is simple and inexpensive, and has been used for siRNA delivery in PHT cells [50]. However, this approach may be toxic to some primary cells (reviewed in [47]). In addition, the calcium phosphate co-precipitation method is frequently associated with low transfection efficiency (reviewed in [47]), as illustrated by studies of reporter gene regulation in PHT cells [24, 25]. In these experiments typically less than 2% of transfected cells expressed both the expression plasmid and reporter gene [24, 25]. **Lipid-based approaches** (lipofection) are widely used and have been associated with more efficient transfer of nucleic acids into primary cells [51, 52]. This method is based on the use of lipid formulations (typically positively charged) that associate with the negatively charged nucleic acids and form liposomes around them. Nucleic acids are efficiently delivered into the cell interior following liposome fusion with the plasma membrane. In addition to higher transfer efficiency, a major advantage of this approach is its simplicity and the availability of commercial reagents. However, toxicity of lipid formulations in some cells may constitute a disadvantage. Because lipid-based approaches have been extensively utilized to silence genes in PHT cells, this transfection method will be discussed in more detail in the RNAi section below.

Magnetofection, a physical method to transfect cells, involves the adsorption of nucleic acids into magnetic nanoparticles, which typically contain iron oxide [57, 58]. Nanoparticles are mixed with the cell culture medium, and cells are subjected to a magnetic field resulting in the intracellular delivery of nanoparticles. Although this method is associated with a high construct delivery rate, the preparation of the nanoparticles can be time-consuming and efficiency is variable. In **electroporation**, an electric impulse is delivered to cultured cells causing transient openings in the cell membrane, allowing nucleic acids to enter the cytoplasm. However, electroporation frequently diminishes cell viability, and is therefore less reliable in primary cells. **Nucleofection** is based on the electroporation technique, yet unlike conventional electroporation, nucleofection utilizes a combination of reagents and electrical parameters that are cell specific, designed to decrease cell toxicity [60, 61]. In addition, this approach allow for the delivery of DNA constructs directly to the nucleus, which may be a significant advantage for transfection of post-mitotic primary cells. Nucleic acids can also be adsorbed to the surface of nanoparticles made of noble metals such as gold. The **biolistic gene gun** utilizes an adjustable low-pressure helium pulse to “shoot” these metal nanoparticles directly into single cells or tissue slices [62], resulting in high transfer efficiencies. However, this approach can cause mechanical injury to cells. **Viral transfection methods** are associated with high transfection efficiency and stable integration of transfected DNA into the genome. Retroviral and, in particular, lentiviral vectors have been extensively used to achieve stable gene delivery in primary mammalian cells (reviewed in [64, 65]).

4. Viral and plasmid vectors

Viral vectors based on adeno-associated viruses (AAV), conventional retroviruses, adenoviruses and lentiviruses are frequently used to deliver transgenes into mammalian cells. Conventional retroviral vectors are not suitable for the transfection of PHT cells because of their reduced ability to infect non-dividing cells. This is due to the inability of the preintegration complex to cross the intact nuclear membrane, which is only disassembled during mitosis (reviewed in [69]). Furthermore, AAV vectors are the smallest known viral vectors exhibiting limited packaging capacity and are inappropriate for use in the delivery of larger constructs (reviewed in [69]). Gene delivery to PHT cells using adenoviruses has been used quite extensively [32, 38–40, 66, 67], as reviewed by Jiang and Mendelson [67]. Adenoviruses attach to the cell surface by binding to the coxsackie and adenovirus receptor (CAR) [70] with integrins, such as $\alpha V\beta 3$ [71] and $\alpha V\beta 5$ [72], acting as co-receptors that facilitate internalization. Although trophoblasts express $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins [73–75], PHT cells express CAR only in first trimester [75], which may negatively influence the ability to transfect term PHT cells using adenoviruses. Adenoviruses typically do not integrate their DNA into the host cell genome, and the expression of the transgene is characteristically transient. Moreover, a significant disadvantage of adenoviral vectors is the stimulation of immune responses, which may cause toxicity and decreased transfection efficiency [76] and as reviewed in [77]. In contrast, lentiviruses lack these potential shortcomings [63, 64] and vectors based on lentiviruses have been used to transfect PHT cells [29, 33]. We therefore focus our discussion on the use of lentiviral vectors.

The most commonly used lentiviral vectors are based on the human immunodeficiency virus (HIV). It is therefore critically important to ensure that the lentiviruses produced are replication-defective. As reviewed by Delenda, lentiviral vectors are designed with several built-in safety features that minimize the risk of generating replication-competent HIV recombinants [78]. These strategies include minimizing the number of viral genes present and physically separating transacting factors on different plasmids. Mature lentiviruses are generated in “packaging” cells (typically HEK293T cell line) by co-transfection with a plasmid containing the vector genome and two or three separate helper constructs (such as *gag-pol*, *rev* and *env*) that encode proteins that are essential for the assembly and function of the mature lentivirus. This multivector approach minimizes the risk of recombination between the vector and helper genes. To initiate transfection, target cells are incubated with medium containing mature lentiviruses. Entry of lentiviruses in CD4⁺ T cells is mediated by glycoproteins in the coat of the virus (encoded by *env*), which interact with the primary cellular receptor CD4 and its co-receptors (e.g., CXCR4 or CCR5) (reviewed in [79]). However, the expression of CD4 in PHT cells is very low or absent and the expression of the co-receptors is variable [41, 80, 81]. Indeed, HIV-1 enters human trophoblast cell lines by endocytosis [82, 83] and entry appears to be independent of both envelope glycoproteins and CD4 but requires the interaction with heparan sulfate proteoglycans [84]. However, the commonly used lentiviral vectors have been modified to allow broad tropism with the ability to infect a wide range of cell types. For example, lentiviruses expressing the vesicular stomatitis virus-G protein, which binds phosphatidyl serine in the lipid bilayer of the cell membrane of most eukaryotic cells, have been used to transfect a large number of primary cells and tissues [85–88], including PHT cells [33]. Inside the cell, degradation of the viral coat results in the release of viral single-stranded RNA, which is subjected to reverse transcription, producing double-stranded DNA. Subsequently, the DNA is integrated in the host cell genome, a process mediated by the viral integrase encoded by the *pol* gene (reviewed in [89]). A detailed account of the evolution of transfection systems based on lentiviruses is beyond the scope of this overview, and the reader is referred to available excellent reviews [78, 89–91].

Plasmid vectors can be delivered to cells without a carrier using electroporation or complex-bound to cationic lipids, polymers or peptides (reviewed in [92, 93]). Plasmid vectors have a high capacity, allowing for the insertion of large segments of genomic DNA. In general, an expression plasmid consists of two major regions: (1) an expression cassette with the transgene and any regulatory sequences needed for expression in mammalian cells and (2) the bacterial backbone that includes the origin of replication and often contains a selectable marker, such as an antibiotic resistance gene, for plasmid amplification in bacteria. Because of transgene silencing, random integration sometimes leads to poor or no transgene expression or disruption of an endogenous gene. However, site-specific, homologous and transposon-mediated integration strategies, which require the inclusion of integration enzymes or additional sequences on the plasmid, have been developed to mitigate these problems [94, 95] and as reviewed in [92, 93].

Genes can be overexpressed in PHT cells using expression plasmids transfected by non-viral methods [11, 26–28, 30, 31] or using viral vectors [29, 32, 33, 38–40, 66, 67], in particular lentiviral vectors [29, 33]. Protein trafficking and cellular localization can be investigated using plasmids encoding for green fluorescent proteins (GFP) or other proteins tagged with fluorescent or luminescent peptides [26, 29, 31, 33]. Using viral vectors, PHT cells may also be transfected with constructs that allow for studies of 5' promoters or 3'UTRs fused to a reporter gene, such as luciferase [11, 26–28, 30, 31]. Recommended control experiments for overexpression strategies include studies of untransfected cells and cells transfected with a control plasmid lacking the transgene or with a transgene coding for a mutant form of the protein [27–29, 31, 33]. It is suggested that overexpression of the transgene is confirmed indirectly using reporter genes, for example by the approach described above involving two luciferase genes [28], or directly by determining the level of target mRNAs and proteins [33].

5. An overview of RNAi

The majority of transcribed genomic DNA does not encode for proteins, and is referred to as non-coding RNA (ncRNA). A subset of these non-coding transcripts (regulatory ncRNA) regulate gene expression by affecting RNA processing, stability and translation (reviewed in [96]). RNAi, which is an endogenous process by which specific genes are silenced in response to the presence of short double-stranded RNAs, is an important mechanism for the regulation of gene expression [97]. Whereas different types of regulatory ncRNAs are capable of mediating RNAi, microRNAs (miRNAs) and small interfering RNAs (siRNAs) are of particular significance. In mammalian cells, siRNAs can be introduced into the cytoplasm as double-stranded RNA by viruses, or they can arise from endogenous loci including repeat-associated transcripts, such as transposons and hairpin RNAs. As reviewed by Bartel, miRNAs are genomically expressed as long double-stranded primary miRNA (pri-miRNA) [98]. Subsequently, pri-miRNAs are processed in the nucleus by the endonucleases Drosha and Pasha to approximately 70-nucleotide long precursor (pre-)miRNAs, which are exported to the cytoplasm by the action of exportin 5 (Fig. 1). After this initial processing, the mechanisms by which miRNAs and siRNAs result in RNAi are similar. Both pre-miRNA and double-stranded RNA are processed by the cytoplasmic endonuclease Dicer, which is bound by cofactors, such as the human immunodeficiency virus transactivating response RNA-binding protein 2 (TARBP2 or TRBP) [99], into 20–24 nucleotide long mature miRNAs or dsRNAs (Fig. 1). Subsequently, one strand of the miRNA/siRNA is incorporated into the RNA-induced silencing complex (RISC), guiding the silencing complex to target mRNAs and activating the Argonaute complex, the catalytic component of RISC. Target mRNA recognition by the miRNA/siRNA containing RISC involves several mechanisms. It has long been recognized that the Watson-Crick base pairing of the mRNA with the 5' region of the miRNA/siRNA, centered on nucleotides 2–7

(the “seed” region), is involved in target recognition. However, the 7–8 nucleotide matches to the seed region do not fully account for targeting specificity, suggesting that additional features determine functional target interactions in vivo (reviewed in [100, 101]). More recently, it has been demonstrated that factors such as the AU-rich nucleotide composition near the seed region, the proximity to sites for co-expressed miRNAs, the positioning away from the center of long untranslated regions (UTRs) and the positioning at least 15 nucleotides from the stop codon within the 3′UTR also modulate target recognition (reviewed in [100, 101]). siRNAs, which are designed to be completely complementary to the target, are believed to silence the target genes primarily by degrading the mRNA [102]. In contrast, the silencing mechanisms involved in miRNA action have been controversial, with evidence supporting both mRNA degradation and translational repression as relevant mechanisms (reviewed in [96]). However, recent evidence demonstrates that miRNA mediated silencing primarily occurs by mRNA destabilization with smaller contribution from translational repression [103].

Similar to techniques discussed earlier for plasmids, siRNAs and miRNAs can be introduced experimentally into cells using two general approaches (Fig. 2): (1) transient introduction of RNA directly into the cytoplasm of the target cells or (2) integration of silencing RNA encoding DNA into the genome of the target cell. Both siRNAs and miRNAs can be introduced into the cytoplasm of cells as double stranded 20–24 nucleotide-long RNAs (Fig. 2) using non-viral transfection methods (Table 1). When miRNAs are delivered using these approaches, they are often termed miRNA “mimics” because they have the same function as, but are not identical to, the mature endogenous miRNA. Furthermore, RNA engineered to silence endogenous miRNAs (miRNA “inhibitors” or antagomirs), can be transfected into cells. The development of vector-mediated RNAi, either by plasmids [104] or viral vectors [105, 106], particularly lentiviruses, has enabled stable and efficient gene silencing (Fig. 2). For siRNAs, a construct encoding a short hairpin RNA (shRNA) is inserted into the virus. shRNA constructs typically consist of 19–29 base pairs including a short loop and a 3′ overhang, thereby mimicking pre-miRNA, with a RNA polymerase III promoter such as H1 and U6 [33]. After integration into the target cell genome, the shRNA construct is transcribed by RNA polymerase III and exported to the cytoplasm and processed by Dicer to siRNA, as described earlier (Fig. 2). miRNA constructs that mimic pri-miRNAs consist of approximately 70 base pairs and are typically transcribed by RNA polymerase II. Like plasmid vectors, lentiviral vectors may include tetracycline-dependent tet-on/tet-off switches, Cre-LoxP nucleases that control the excision of the provirus [107], and other modifications.

There are several potential hurdles associated with the production of lentiviral vectors encoding miRNA or shRNA sequences [69]. Both miRNA and shRNA lentiviral vectors could potentially target homologous sequences that are a part of the lentiviral vector (“self-targeting”), attenuating the efficiency of vector production [69]. Because nuclear recombinant miRNAs, but not shRNAs, require processing by Drosha, a risk specific to miRNAs is degradation of the vector RNA genome by Drosha, greatly reducing virus titers [108, 109]. Furthermore, as the lentiviral vector genome and miRNA are both transcribed from RNA polymerase II, it is possible that promoter interference could decrease virus titers [69].

6. RNAi in primary human trophoblasts

Introducing synthetic siRNAs (or miRNA mimics) directly into the cytoplasm represents a powerful method to silence specific genes. Although disadvantages might include the relatively short half-lives of most siRNAs, due to their susceptibility to degradation by RNAases, this is not a major problem for cultured PHT cells because of their limited culture

time and terminal differentiation state. While transfection efficiencies may remain problematic, recent developments in lipid-based delivery of siRNAs into trophoblast cells have reduced some of these obstacles [10, 11, 14, 15, 17–23, 27, 28, 35–37, 53–56]. To identify siRNA transfection protocols that are suitable for cultured PHT and first trimester villous explants, Forbes and coworkers tested diverse lipid-based and nucleofection reagents [55]. Based on high silencing efficiency and low cell toxicity, the lipid-based reagent Dharmafect (Thermo Scientific Dharmacon, Lafayette, CO) was reported to be most efficient [55] and has been subsequently used in trophoblast research [11, 17, 19, 20, 22, 23, 28, 36, 37]. Interestingly, siRNAs have been delivered with high efficiency to the syncytium of first trimester [53, 110–113] and term villous explants [114] without applying any lipid-based reagents or nucleofection. In contrast, nucleofection was required to deliver siRNAs into cytotrophoblasts and the villous stroma of first trimester villous explants [55].

When using cationic lipid-based transfection agents, serum-free medium is recommended for siRNA preparation because sulfated proteoglycans and other proteins compete with the cationic lipids for binding nucleotides (reviewed in [115]). Due to potential toxicity, it is commonly recommended that antibiotic-free cell culture medium is used during transfection (reviewed in [115]). However, we have used Dharmafect agent with antibiotics containing cell culture medium to transfect PHT cells and did not observe cell toxicity [19, 20, 22]. Notably, transfection efficiency depends on cell plating density and the concentration of the transfection agent, as well as the siRNA. Non-specific influences on cultured cells, such as effects on cell viability, differentiation and apoptosis, should be evaluated regularly using standard assays, such as human chorionic gonadotropin (hCG), lactate dehydrogenase (LDH) exclusion and protein expression of cytokeratin-7, caspase-3 and poly ADP ribose polymerase (PARP) [20]. Cationic lipids used for transfection can activate insulin [116], signal transducer and activator of transcription 3 (STAT3) [117] and mammalian target of rapamycin (mTOR) signaling pathways [118]. Furthermore, Desforges and Westwood reported that the Dharmafect 2 transfection agent activates insulin/IGF-I signaling in PHT cells, resulting in the resistance to further stimulation by growth factors [56].

Lentiviruses have been used successfully to deliver shRNA constructs into PHT cells (Fig. 2) [33, 66]. Chen and co-workers reported that efficient transfection of PHT cells required 50-fold higher virus titers than HEK293T cells [33]. This may be related to the observation that expression of CD4, the classical receptor mediating lentivirus entry into cells, is very low or absent and the expression of the co-receptors is variable in PHT, as discussed above. In addition, infectivity was highest when PHT cells exhibited the undifferentiated, cytotrophoblast phenotype providing the rationale for the recommendation to start transfection 4 hours after plating [33].

7. RNAi: off-target effects, controls and evaluation of silencing efficiency

Although originally believed to be highly specific [119, 120], it is now recognized that siRNAs and miRNAs may have “off-target” effects, defined as effects that cannot be attributed to silencing of the specific target mRNA. Off-target effects can alter cellular function, thus complicating the interpretation of gene-silencing experiments. Strategies to minimize off-target effects need to be considered in all RNAi experiments. Off-target effects fall into three broad categories: (1) regulation of unintended complementary transcripts, (2) inflammatory response and (3) saturation of the endogenous RNAi machinery.

Using different siRNA sequences that target the same gene and microarray profiling, Jackson et al. [121] showed that “off-target” transcripts were characterized by 3′-UTRs that were complementary to the 5′ end of the transfected siRNA guide strand. This is often referred to as “miRNA-like” off-target effects because the 5′-seed sequences of the miRNAs

may regulate a diverse set of complementary 3'UTR sequences in mRNA transcripts. Although miRNA-like off-target effects are typically weaker than the on-target effects, they may become functionally relevant. There are several strategies to mitigate off-target effects. First, the use of minimally effective doses of siRNA is recommended [121–124]. Second, the use of multiple individual siRNAs with distinct seed sequences that target the same gene allows for differentiation between the shared on-target effects and siRNA-specific, variable off-target effects. Observing the same phenotype with multiple siRNAs will increase confidence that the results reflect silencing of the desired target gene. Third, multiple siRNAs can be pooled in a single experiment, thereby decreasing the concentrations of individual siRNAs. Because all siRNAs in the pool silence the same target but have disparate off-target effects, this approach will selectively reduce off-target effects. Fourth, phenotype rescue by overexpressing a functional version of the silenced gene can be instructive in identifying miRNA-like off-target effects. Fifth, chemical modification of the siRNA seed sequence can reduce miRNA-like off-target effects. In particular, 2'-O-methylation of a single nucleotide in the guide strand reduces most siRNA off-target silencing [125]. Additionally, the introduction of the bulky methyl group at position 2 of the guide strand produces a conformational change within the RISC complex, which reduces the silencing of partially complementary transcripts (i.e., off-target) without affecting the silencing of the desired target [125]. Lastly, chemically modified RNAs engineered to silence endogenous miRNAs (antagomirs) not only increases resistance to degradation but may also diminish off-target effects [126].

The innate immune system protects the host from pathogens, infectious agents and double-stranded RNA, which are recognized by Toll-like receptors (TLRs). It is therefore not surprising that siRNAs have been shown to activate immune responses in whole animals and in immune cells [127, 128]. In particular, TLR3, 7 and 8 recognize RNA molecules and are involved in the induction of interferons, TNF α and IL-6 in response to siRNAs (reviewed in [129]). TLR3, 7 and 8 are expressed at the mRNA level in the human placenta [130, 131], and first trimester PHT and trophoblast cell lines respond to TLR3-specific ligands by secreting proinflammatory cytokines [132, 133]. However, it is unknown if siRNAs elicit an immune response in these cells. Strategies designed to mitigate immune responses to siRNAs include elimination of pro-inflammatory sequences in the siRNA construct, such as avoiding GU rich motifs, and the chemical modification of the 2' position of ribose, which attenuates recognition by the TLRs [129, 134].

Components of the RNAi machinery in mammalian cells may become rate limiting for silencing. In particular, the transport of shRNAs and pre-miRNAs out of the nucleus by Exportin-5 and mRNA cleavage by AGO2 can be saturated [135, 136]. Saturation of the endogenous RNAi machinery by exogenously delivered miRNA, shRNA, or siRNA may compete with endogenous miRNA activity and even cause cellular toxicity. Such hurdles may be attenuated by the use of the lowest effective siRNA dose.

It is highly recommended to include a negative “scramble” control, in which the siRNA or shRNA have the same nucleotide composition as the targeting construct but do not match any mRNA sequence in the studied species. However, as discussed above, a scramble control is not free from off-target effects. To evaluate silencing efficiency, it is suggested that silencing is confirmed by determining the level of target mRNAs and proteins [20].

8. Conclusion and future perspectives

Efficient tools for the manipulation of gene expression in PHT cells are now available. Gene silencing by the delivery of siRNAs using lipid-based transfections or the incorporation of shRNAs and miRNAs into the genome using lentiviral vectors, as well as overexpression of

trophoblast genes mediated by viral vectors, are becoming widely used. Future studies validating these techniques in PHT cells, including studies exploring miRNA-like off-target effects, and experiments designed to identify possible inflammatory responses to RNAi, are needed. It is anticipated that these gene targeting approaches will not only become increasingly useful for exploring gene function in human trophoblasts but also provide the necessary foundation for future application of gene therapy targeting the human placenta.

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Figure 1. The mechanisms of RNA interference (RNAi) in mammalian cells

Endogenous miRNAs are expressed as primary miRNA transcripts (pri-miRNAs), which consist of imperfect hairpin structures. Pri-miRNAs are processed by the nuclear RNase II-like endonuclease Drosha into precursor miRNA (pre-miRNA) composed of approximately 70 nucleotides. Subsequently, pre-miRNAs are transported by Exportin-5 into the cytoplasm. After this initial processing, the mechanisms by which miRNAs and siRNAs results in RNAi are similar. Both pre-miRNAs and viral double-stranded RNA are processed by the endonuclease Dicer, which along with TRBP generate 20–24 nucleotide mature miRNAs and dsRNAs, respectively. One strand of the miRNA/siRNA is incorporated into the RISC, guiding the silencing complex to target RNAs, and activate Argonaute, the catalytic component of RISC. **Abbreviations:** POL II; RNA Polymerase II, AGO; Argonaute, TRBP; the human immunodeficiency virus transactivating response RNA-binding protein 2, RISC; RNA-induced silencing complex.

Figure 2. Approaches to deliver siRNA and miRNA to trophoblast cells

The modes of delivery of siRNAs and miRNAs into cells fall into two broad categories: (1) integration of DNA into the cytoplasm or genome of the target cell using vector-mediated delivery, and (2) introduction of RNA directly into the cytoplasm of the target cells. **Top: Integration of DNA into the genome of the target cell.** For delivery of siRNA using viral vectors, such as lentiviruses, a construct encoding a short (19–29 base pairs) hairpin RNA (shRNA) is inserted into the virus. After integration into the target cell genome, the shRNA construct is transcribed by RNA polymerase III to shRNA and further processed by Dicer to siRNA. miRNA constructs mimic pri-miRNAs and are typically transcribed by RNA polymerase II. The pri-miRNA is subsequently processed by Drosha and Dicer to mature miRNA. DNA constructs encoding shRNAs and miRNAs can also be integrated into the target cell genome using plasmid vectors and non-viral transfection approaches. **Bottom: Introduction of RNA directly into the cytoplasm.** Both siRNAs and miRNAs can be introduced into the cytoplasm of cells as double-stranded 20–24 nucleotide long RNA sequences using non-viral transfection methods (Table 1). When miRNA is introduced using these approaches, they are termed miRNA “mimics”. Furthermore, RNA engineered to silence endogenous miRNAs (miRNA “inhibitors” or antagonists) can be similarly transfected into the cytoplasm. **Abbreviations:** POL II/III; RNA Polymerase II/III.

Table 1

Common methods to transfect primary cells

Transfection technique	Advantages	Disadvantages	General references	PHT references
Chemical techniques				
Calcium phosphate co-precipitation	<ul style="list-style-type: none"> • Simple • Low cost • Low toxicity, at least in some primary cells 	<ul style="list-style-type: none"> • Low transfection efficiency • Cell toxicity, at least in some primary cells • Poor reproducibility 	[49]	[24, 25, 34, 50]
Lipid-based approaches	<ul style="list-style-type: none"> • Simple, easy-to-use kits commercially available • Typically high transfer efficiency 	<ul style="list-style-type: none"> • Toxic to certain cells 	[51, 52]	[10, 11, 14, 15, 17–23, 26–28, 30, 31, 35–37, 53–56]
Physical techniques				
Magnetofection	<ul style="list-style-type: none"> • High transfer efficiency • Low toxicity in most cases 	<ul style="list-style-type: none"> • Time consuming preparation of nanoparticles • Toxic to certain cells 	[57, 58]	
Electroporation	<ul style="list-style-type: none"> • High transfer efficiency in single cells 	<ul style="list-style-type: none"> • Relatively expensive equipment • Affects the viability of many cells 	[59]	[16]
Nucleofection	<ul style="list-style-type: none"> • High transfer efficiency • Low toxicity in most cases • Often delivery of DNA directly to nucleus • Reproducible 	<ul style="list-style-type: none"> • Relatively expensive reagents and equipment • Toxic to certain cells 	[60, 61]	[55]
Biolistic gene gun	<ul style="list-style-type: none"> • High transfer efficiency in single cells 	<ul style="list-style-type: none"> • Can cause mechanical damage to cells • Expensive equipment 	[62]	
Viral techniques				
Lenti- and adenoviruses	<ul style="list-style-type: none"> • High transfer efficiency 	<ul style="list-style-type: none"> • Preparation of viral particles for transfection is time consuming 	[63–65]	[29, 32, 33, 38–40, 66, 67]

Transfection technique	Advantages	Disadvantages	General references	PHT references
	<ul style="list-style-type: none">• Often low toxicity	<ul style="list-style-type: none">• Special safety precautions		