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Transposase-mediated gene modulation in the placenta

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Abstract

The placenta is an organ vital to fetal development as well as the maintenance of a healthy pregnancy and plays a crucial role in developmental programming of the fetus. The mechanisms that link intrauterine milieu, fetal health and disease development later in life are poorly understood. Placenta-specific gene modulation, both by generating transgenic animals as well as by developing methods for *in vivo* genetic modifications is a growing area of interest as this approach provides the opportunity to investigate the role of particular genes or gene networks in regulating placental function and fetal growth. Furthermore, *in vivo* placental gene transfer may be adapted to treat humans in the future and could be used as an early intervention strategy for a wide range of pregnancy complications. This review is an overview of transposase-based methods available for both transgenic animal generation and *in vivo* placental gene modifications with an emphasis on piggyBac-based systems.

Keywords

placenta; gene; transposon; transposase; transgene; transgenesis; *piggyBac*; gene therapy; gene modulation; virus; CRISPR/Cas9

Introduction

The placenta serves as an interface between fetal and maternal circulation and is essential in promoting fetal growth and the maintenance of pregnancy. It is responsible for mediating nutrient, gas and waste product transfer between the mother and developing fetus as well as a myriad of endocrine signaling and immunological functions [1]. Serious pregnancy complications as well as the long term well-being of the child have been linked to the health and physiology of the placenta [2]. Multiple studies have observed that a sub-optimal uterine

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environment can lead to increased disease risk across a child's lifespan [3]. The placenta is implicated in pregnancy complications such as intra-uterine growth restriction (IUGR), fetal growth restriction (FGR) and fetal overgrowth [4]. Despite the widespread prevalence of these conditions, only some of the genetic components, molecular mediators and signaling pathways associated with changes in placental structure and function, fetal health and disease development later in life have been identified [5] and there are no specific interventions available to mitigate pregnancy-related complications.

To investigate the role of individual genes in health and disease, studies using whole-body gene manipulation such as transgenic knock-out mice have delivered invaluable insights. However, in mice, about 30–40% of the germ line gene knock-outs are embryonic or perinatal lethal [6]. The development of improved animal models will allow the scientific community to address both longstanding mechanistic and physiological questions about the placenta as well as to further understand the placenta's contribution to human health and disease. It is now understood, that the development of a deeper understanding of the placenta and the exploration of the function of specific trophoblast genes *in vivo* is an important component for improving human health. Until recently, delivery of DNA via viral vector methods were the most common strategies for *in vivo* gene modulation to the placenta of animal models. More recently, nanoparticles have also been tested for gene or drug delivery to the placenta, however, the number of studies utilizing placenta specific gene targeting is thus far limited.

While we will allude to a range of available methods in this review, this article will focus on transposase directed genetic modulation of the placenta and therefore will only offer a cursory view of other techniques in order to compare and contrast those methods to transposase based approaches. A full review of non-transposase methods of placental genetic modulation is outside the scope of this article.

Placenta-specific gene modulations present an unparalleled opportunity to address a wide range of pregnancy complications such as IUGR, FGR, or preeclampsia. At present, no specific clinical treatments are available. Hence, targeting the placenta *in vivo* via novel approaches may open new avenues of preventative and interventional medicine, including new models for testing drug treatments as well as gene therapy interventions. Since the placenta is a tissue with a finite life span, essential for fetal development but discarded after birth, we believe it is well suited for improving fetal well-being by gene modulation, without causing problems previously associated with gene transfer directly into the maternal or fetal genome. When choosing a vector for placental gene therapy, it is important that the vector is delivered safely with great efficiency and that the transgene is expressed at a high rate without any off-target expression in other tissues.

In order to develop better models for the investigation of placental function and dysfunction, as well as new methods that specifically target gene modulations to the placenta *in vivo* without interfering with the genome of mother and fetus, modifications can be made by several different methods. The approaches addressed in this review are transposase-mediated gene modulation methods, virus-mediated gene modification methods and other non-viral gene editing methods. A summary of these methods is depicted in Figure 1.

Methods

Transposases

It has been more than 60 years since Barbara McClintock first suggested transposable elements existed. Since that time, the field has grown and many of these elements have been discovered and harnessed for use in genetics [7]. Transposable elements fall into two categories, class I, which use a “copy and paste” mechanism for transposition and class II, which we will focus on in this review. Class II transposons are mobile genetic elements that are capable of self-directed excision and subsequent reintegration within the host genome. Several transposases catalyzing this cut-and paste mobilization (Figure 2A) have been described, including *piggyBac*, Sleeping Beauty and Tol2. Specifically, transposases recognize and bind to the inverted repeat elements flanking transposons, cut this DNA segment from the donor and reinsert it to the recipient genome at target sites. These properties are an invaluable tool for gene delivery in applications such as transgenesis, mutagenesis for cancer research and gene therapy [8].

One transposase that has been studied by our group for transgenic animal development as well as gene therapy applications is *piggyBac*, an insect transposase isolated from *Trichoplusia ni*. Genome insertion site preferences exist for transposases, with *piggyBac* inserting into the host genome at TTAA repeat sequences displaying a semi-random integration pattern. In contrast, many viruses used in gene therapy applications preferentially insert into active genes or near promoters [9] such as retroviruses, while adenoviral vectors are non-insertional [10].

We have improved our *piggyBac* plasmid (*pmGENIE-3*) constructs to contain the whole transpositional machinery on a single plasmid and have described a safety mechanism engineered into *pmGENIE-3* to prevent the *piggyBac*-driving promoter from maintaining its activity after excision of the transposon from the plasmid (Figure 3). Specifically, the CMV enhancer-chicken beta actin promoter-beta globin intron (CAG) promoter located in the transposon should not be able to contribute to aberrant gene activation, as the remaining fragment of the *piggyBac* transposase gene and the chimeric intron contain two stop codons (UAA) engineered into the transposon construct [11]. In comparison to virus-derived vectors, transposase-based non-viral vectors and specifically *piggyBac* vectors have several advantageous qualities for both the development of transgenic animals and *in vivo* therapeutics, mainly the safety of administration without severe side-effects, long term transgene expression *in vivo* [12] and the possibility of repeated administration. Additionally, *piggyBac* is capable of transposing transgenes of up to 100kb [13] allowing large transgene cassettes containing multiple genes, long genes or genes including their introns and up- or downstream regulatory elements to be delivered. This permits more extensive investigation of the potential influences of whole gene regions as compared to a single transcript variant when using cDNA.

In many instances, transposases and transgenes are being driven by high-expression, constitutive promoters, such as the human cytomegalovirus (CMV) immediate early enhancer and promoter or the above-mentioned CAG promoter. However, when using tissue specific promoters it is possible to confine transgene integration and expression to a specific

tissue, as for example different tissues in transgenic models of development [14]. Transposase based gene therapy methods have not currently been published for use in targeting trophoblast tissue but transposase based vectors have been used to target other tissues including those in the liver [15] and kidney [16].

One of the limitations of transposase-based methods is the non-targeted nature of the transposition as it only allows for gene knock-ins and untargeted, random gene knockouts but not for targeted gene knock-downs. Additionally, while transposase-based or other non-viral gene editing systems have been modified to target the nucleus once inside the target cell, the delivery to the target cell itself remains a challenge.

Other non-viral methods

Endonuclease-based gene modification approaches, in particular the CRISPR/Cas9 system are well-suited for gene editing and have been used to target other specific tissues, such as cardiac tissue [17], *ex vivo* and *in vitro* as they have demonstrated the ability to target gene modifications to specific host loci. However, while the generation of CRISPR/Cas9 knockout mutations via the non-homologous end joining (NHEJ) mechanism is highly efficient, knock-in mutation mediated by this system rely on the host's cell homology directed repair (HDR) and therefore remain inefficient [18]. Off target modifications are an issue with CRISPR/Cas9 editing that have yet to be resolved [19]. Still, the field of CRISPR/Cas9 technology is rapidly evolving at this time as multiple groups are working to decrease off target activity [20, 21].

Additionally, the Cre/LoxP system is widely used for generating conditional knock-out mice. In this system, excision of genomic regions, which have to be flanked by LoxP recognition sites is catalyzed by the Cre recombinase [22]. To create mutants with cell-specific mutations, two independent transgenic mice need to be crossed, the floxed mouse containing the LoxP-flanked region to be excised and the Cre mouse which contains the coding sequence for Cre under a tissue-specific promoter. At present, only a few transgenic mouse lines are available [23] that provide robust trophoblast-specific transgene expression using this method.

Viruses

Viruses intended for use in gene therapy are able to very efficiently infect cells and in this process transfer DNA or RNA to the host. For this reason, there are currently more virus-based, FDA approved gene therapy trials than there are for non-viral methods. Virus-based gene delivery approaches are associated with increased technical demands and may suffer from virus-associated toxicity, such as genotoxicity, hepatotoxicity and inflammatory response from the host animal or patient [24]. For these reasons, viral vector genomes have been engineered to improve safety by making them replication incompetent. Lentivirus and adenovirus derivatives are the most common vectors chosen for transgenic animal generation and gene therapy development, respectively. Lentiviral vectors are a subset of retroviral vectors that require cells to be dividing to transduce their DNA cassette into the host genome and are generated using an HIV-1 provirus pseudotyped with a VSV-G protein coat, which has been shown to be effective across a variety of mammalian cell types and throughout the

cell cycle [25]. By contrast, adenoviruses can transduce their DNA into both dividing and non-dividing cells but do not integrate the transgene into the host genome, an advantage when developing vectors for short-term *in vivo* gene therapy. Adenoviruses are considered the most effective viral means of gene therapy currently available [26].

Applications

Transgenic animals

A well-established method, the lentiviral gene transduction of mouse blastocysts followed by re-implanting of the transduced blastocyst into a surrogate dam [27, 28] has enabled persistent trophoblast-specific gene expression [29–31] in these animals. However, when using this ex-vivo approach, blastocysts must be transduced and implanted for each generation, the intended gene modulation will only modify the placenta for that pregnancy and is not passed down in the germline. Germline transgenesis, developed in a wide range of animal models has recently been improved by employing transposases such as *piggyBac* and *Sleeping Beauty* [32]. We have previously shown that we were able to achieve transgenesis efficiencies rivaling those of lentivirus-based methods using *piggyBac* enhanced-transgenesis [33]. Using this approach, the plasmid can be designed so that the trophoblast-specific promoters are positioned upstream of the transgene, driving it, while a constitutive promoter controls *piggyBac*'s cut-and paste-based, semi-random integration into the genome. This approach allows for the generation of a wide range of transgenic animals testing the influence of specific genes on placental function. Some of the promoters known to restrict expression to the placenta or part of the placenta (usually trophoblast cells) include the *adenosine deaminase* promoter, the CYP19 promoter [34], the Eomesodermin promoter and the PLAC promoter [34, 35], the growth hormone-releasing hormone promoter [36] and the human chorionic somato-mammotropin promoter [37].

A recent technique, the CRISPR/Cas9 [38] system offers advantages for generating knock-out transgenic animals and has demonstrated targeted gene integration into site-specific host loci. However, since the placenta is essential for fetal well-being and survival, it is difficult to attribute observed phenotypic changes in offspring from knock-out mice, as they may be due to genetic modification affecting placental function or fetal development *per se*. There has also been concern about off target effects [19], which are currently being addressed by modifications to the CRISPR/Cas9 systems [20,21]. By restricting the genetic manipulation to the placenta, it is possible to avoid some of these ambiguities and to more clearly define the effects on placental development and function. Furthermore, transgenesis rates for gene knock-in animals using CRISPR/Cas9 are significantly lower than those achieved with transposon-based transgenesis. For example, in one of the most recent reports on Cas9-mediated transgenesis, Chu and colleagues [39] reported an increase in efficiency compared to previous CRISPR/Cas9 techniques with a maximal rate of 10% transgenic animals. In contrast, we showed efficiencies of 26% using transposon enhanced-transgenesis using enhanced *piggyBac* more than 4 years ago [33] and have since achieved rates of more than 50% by improving the method [40].

Previously, both mice and pigs have been generated with targeted insertions using a Cre/LoxP system to modify genes in the placenta [41–44]. Another available system utilizes a

combination of an integrase-deficient lentivirus with a Cre/loxP mouse breeding scheme to allow for the selective activation and inactivation of genes in the placenta [23], effectively merging viral and non-viral techniques. However, this system is limited by the laborious, expensive and time-consuming task of multiple rounds of breeding, in particular when multiple knock-outs are intended. Finally, while non-viral methods for germline gene editing may offer translatable strategies for therapeutic interventions, the Cre/LoxP can only be utilized for the generation of transgenic animals.

***In vivo* and gene therapy applications**

Transposase-based systems have been applied to gene editing in several tissues [45, 46] as they have been shown to integrate transgene cassettes efficiently. Indeed, a clinical trial to treat non-Hodgkin lymphoma using the *Sleeping Beauty* transposase [47], sets a precedent for the possible future use of transposase-based systems in humans. While this technique has not yet been used for *in vivo* genome modifications of the placenta to date, it holds great potential for the following reasons: (1) applying transposase-based systems to the placenta allows for the possibility of integrating placenta specific transgene cassettes that do not elicit inflammatory reactions and (2) while showing a low risk of genotoxicity. Additionally, long term transgene expression can be achieved, potentially providing therapeutic transgene expression in the placenta throughout pregnancy while avoiding genetic modification in the mother or child.

Nanoparticles and polymer driven delivery systems have been an area of intense biomaterials research for some time with the hope that they could be targeted to specific organs, such as placenta, with minimal or no off-target effects and deliver DNA such as plasmids with an efficiency similar to viruses. At present, the successful application of this method seems to strongly depend on the nanoparticle formulation. For example, syncytiotrophoblasts and trophoblasts readily take up gold nanoparticles without passing them into fetal circulation in perfused human placentas as well as showing promising results in a BeWo cell culture model [48, 49]. Additionally, a British group has recently reported a successful delivery of drugs to the placenta utilizing tumor homing peptides coupled with nanoparticles [50]. In contrast, other nanoparticles have been shown to carry the risk of fetal toxicity, or cause inflammation [51].

Virus-mediated *in vivo* gene transfer in animals shows the potential to successfully alter placental function [52–54] as exemplified by attempts to treat IUGR in a murine model of placental insufficiency [52, 55] by delivering human insulin-like growth factor I (hIGF-1). Although birth weight was restored, unintended gene transfer to the pups and some maternal organs was observed in a few cases [53]. Fetal growth could also be improved by modifying the blood flow to the placenta *in vivo*. In both sheep and guinea pig models of FGR, using a modified adenoviral vector to deliver VEGF to the uterine artery proved successful [54, 56] and is currently moving towards a human clinical trial [57].

Summary

The ability to modulate gene expression in the placenta is critically important for a deeper understanding of maternal, fetal and offspring health and for developing early intervention

strategies for a wide range of pregnancy complications. At present, there are no existing methods for placental gene modulation that provide the high delivery efficiency of viruses while simultaneously offering the safety features of non-viral gene modulation strategies. We believe that transposase-based systems in combination with an efficient non-viral delivery system, such as nanoparticles, hold great promise to allow placenta-specific gene modulation in animal models. The translational aspects of the methodology may eventually lead to early intervention strategy to reduce placenta-associated pregnancy complications.

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Abbreviations

IUGR	Intra-uterine growth restriction
PNI	Pronuclear microinjection
ICSI	intracytoplasmic sperm injection
FGR	Fetal growth restriction
pB	<i>piggyBac</i>

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Figure 1.
Prospective methods for placenta-directed gene modifications.

Figure 2.

The cut-and-paste transposition mechanism of a class II transposon. Transposases recognize and bind to the inverted repeat elements flanking transposons, excise this DNA segment from the donor and reinsert it to the recipient genome at target sites.

Figure 3.

Features of the *pmGENIE-3* plasmid. The 3' inverted repeat sequence (IRS) of the transposon is located within a *piggyBac* (*pB*) intron. Transposition will therefore separate the promoter and the 5' end of the *pB* gene from the remainder of the gene.