

REVIEW

In utero stem cell transplantation and gene therapy: rationale, history, and recent advances toward clinical application

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Recent advances in high-throughput molecular testing have made it possible to diagnose most genetic disorders relatively early in gestation with minimal risk to the fetus. These advances should soon allow widespread prenatal screening for the majority of human genetic diseases, opening the door to the possibility of treatment/correction prior to birth. In addition to the obvious psychological and financial benefits of curing a disease *in utero*, and thereby enabling the birth of a healthy infant, there are multiple biological advantages unique to fetal development, which provide compelling rationale for performing potentially curative treatments, such as stem cell transplantation or gene therapy, prior to birth. Herein, we briefly review the fields of *in utero* transplantation (IUTx) and *in utero* gene therapy and discuss the biological hurdles that have thus far restricted success of IUTx to patients with immunodeficiencies. We then highlight several recent experimental breakthroughs in immunology, hematopoietic/marrow ontogeny, and *in utero* cell delivery, which have collectively provided means of overcoming these barriers, thus setting the stage for clinical application of these highly promising therapies in the near future.

Molecular Therapy — Methods & Clinical Development (2016) **5**, 16020; doi:10.1038/mtm.2016.20; published online 30 March 2016

INTRODUCTION

Since 1982, when Harrison *et al.*¹ described the first successful fetal surgery to treat congenital hydronephrosis, tremendous progress has been made in open fetal surgery and fetoscopic intervention, which now enable the treatment of a variety of anatomic anomalies that would otherwise place the fetus at a risk of death or lifelong disability.^{2,3} However, fetal surgical intervention is intrinsically limited to the correction of structural abnormalities. In contrast, *in utero* stem cell transplantation (IUTx) and *in utero* gene therapy (IUGT) offer the possibility of treating, and ideally curing, a wide range of genetic disorders. With the advent of high-resolution ultrasonography and exquisitely sensitive, high-throughput molecular techniques, the vast majority of congenital conditions can now be diagnosed early in gestation, often using fetal cells or cell-free fetal DNA present in the maternal blood,⁴ essentially eliminating any risk to the fetus.

Importantly, these remarkable advances in prenatal imaging, molecular diagnostics, and fetal surgical techniques have not only improved the ability to identify diseases early in development, they have also made it possible to safely deliver stem cells and/or gene therapy vectors to precise anatomic sites within the early gestation fetus. Preemptive treatment of the fetus by IUTx or IUGT would completely transform the paradigm for treating genetic disorders,² allowing physicians to intervene prior to clinical manifestations of disease, an approach that could promise the birth of a healthy infant who required no further treatment. In addition to the obvious psychological benefits of curing a disease *in utero*, the elimination of the need for lifelong, noncurative treatment would have a profound impact on the quality of life of the patient, and his/her

family, as well as dramatically reducing the cost burden for society. It is critical to note that there are also several biological advantages unique to fetal development, which provide compelling reasons to believe that stem cell transplantation and/or gene therapy would be far more efficient and effective if administered during fetal life rather than postnatally.

In this article, we present the therapeutic rationale for the use of IUTx and IUGT and review key experimental evidence to support their use. We discuss some of the unforeseen biological barriers that have thus far precluded more widespread clinical application/success of IUTx, and we provide an overview of IUGT, illustrating some of the unique advantages it possesses, as well as some of the potential risks that will need to be addressed prior to clinical implementation. We showcase the hemophilias as an archetype genetic disease for correction via IUTx and/or IUGT and conclude the review by highlighting several recent breakthroughs that promise to move these exciting therapeutic approaches into the clinic in the near future.

RATIONALE FOR IUTX

Although several cell types have been considered and explored in the context of IUTx, this review will focus on hematopoietic stem cells (HSC), since HSC were the first cell type tested in IUTx and are the cells that have been used in the vast majority of both experimental and clinical IUTx studies. The HSC is a multipotent stem cell that undergoes self-renewal and multilineage differentiation to generate all of the mature hematopoietic lineages and thereby maintain functional hematopoiesis throughout fetal and adult life.^{5,6}

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Received 17 December 2015; accepted 29 January 2016

As such, it is well suited for treating a broad range of hematopoietic disorders, and the successful transplantation of HSC to treat disease can result in lifelong correction. HSC are the most extensively characterized stem cells in the body, and much of what we know about the biology and behavior of stem cells in general is based on the paradigm established with studies on HSC. HSC are the only stem cell population that has been isolated to a high degree of purity.⁷⁻⁹ In addition, HSC and all of their mature progeny express class I and class II major histocompatibility (MHC) antigens¹⁰ and participate in self-antigen presentation during immune system development, which, as we will discuss in more detail shortly, makes induction of donor-specific tolerance possible following transplantation.^{6,11}

When considering how to most effectively treat genetic diseases, one must consider that many of these disorders exert a significant amount of irreversible damage during embryonic and fetal development. For example, substantial neuronal damage is associated with inherited metabolic diseases such as Lesch-Nyhan, Tay Sachs, and the acute neuropathic forms of Gaucher's. In these patients, even state-of-the-art treatment given postnatally only mediates a limited therapeutic benefit (or none at all), since it cannot reverse the damage that the disease has exerted during development. By far, the most compelling rationale for IUTx (and IUGT, as we will discuss later) is the ability it affords to treat these diseases early enough in development to prevent disease onset and thereby avoid the devastating manifestations that would otherwise occur before birth. It is important to note that, even in patients with diseases that can be cured postnatally, compelling psychological and financial benefits exist to argue for performing correction *in utero*, since it would allow the birth of a healthy infant, who, ideally, would require no further treatments.

There are also undeniable biological reasons for attempting to perform IUTx, rather than waiting until after birth to treat. It has long been appreciated that normal developmental events that occur within the nascent hematopoietic/immune system create several unique opportunities that may facilitate the engraftment of allogeneic (foreign) cells and avoid the complications and toxic myeloablative conditioning associated with postnatal bone marrow (BM) transplantation¹²⁻¹⁵ (reviewed in ref. 16).

During fetal development is the only time that large-scale migration of stem cells occurs to seed tissue compartments. Looking specifically at the hematopoietic system, definitive hematopoiesis commences in the yolk sac and/or aorto-gonadal-mesonephric region, migrates to the fetal liver, and finally shifts to the BM, where it then resides for the remainder of life.¹⁷⁻¹⁹ A large part of the initial rationale for trying to perform HSC transplantation *in utero* was based on the hope that these migrations and the development of new hematopoietic niches during development could provide opportunities to selectively engraft donor HSC without the need for cytotoxic myeloablation, which is one of the primary causes of the marked morbidity and mortality associated with postnatal BM transplantation. It was, therefore, the hope of investigators in the early days of IUTx that the normal biology of the fetus would allow the clinician to exploit hematopoietic ontogeny, such that the transplanted HSC could, in effect, piggyback on the naturally occurring processes of migration, engraftment, differentiation, and expansion, thereby allowing donor reconstitution of the defective hematopoietic compartment and correction of the disease.

Unfortunately, as will be discussed in detail in a later section, it has become apparent in recent years that this hope was naively optimistic. Because of the large numbers of circulating HSC and their relatively high proliferative and repopulating capacity compared

to their adult counterparts,²⁰⁻²² it is now recognized that the fetal hematopoietic system is highly competitive and represents a daunting barrier to engraftment of transplanted adult HSC. However, if the regulatory signals controlling the migrations of HSC and their seeding of nascent marrow niches were better understood, it is conceivable that these processes could ultimately be manipulated to drive the engraftment of donor cells.²³

From a logistical/technical standpoint, it also bears mentioning that the very small size of the fetus offers a distinct advantage over treating a pediatric or adult patient with HSC transplantation. At 12 weeks of gestation, which is during the period in which IUTx would ideally take place, the human fetus only weighs roughly 35 g.^{2-4,16,24,25} As such, it is possible to transplant much larger cell doses on a per-kilogram basis than could ever be achieved after birth. The sterile environment within the uterus provides another advantage of the fetal environment. Specifically, if one considers the treatment of an immunodeficiency *in utero*, the maternal womb functions as a sterile isolette, allowing fetal immune reconstitution prior to exposure to pathogens.²

Another aspect of fetal biology that provides what is perhaps the most compelling reason to perform HSC transplantation *in utero* is the possibility that IUTx could induce donor-specific immune tolerance.¹² Early in gestation, the nascent immune system undergoes a process of self-education. This occurs primarily in the fetal thymus, and it consists of two critical components: (i) the positive selection of pre-lymphocytes that recognize "self"-MHC and (ii) the negative selection (deletion) of any pre-lymphocytes that exhibit the ability to recognize, with high-affinity, any of the myriad self-antigens in association with self-MHC.^{26,27} Ideally, this process creates an immune system that is devoid of self-reactive lymphocytes (the presence of which could later lead to autoimmunity) and is populated with a diverse repertoire of lymphocytes that recognize foreign antigens in association with self-MHC.^{16,27} In theory, therefore, introduction of allogeneic cells by IUTx, with subsequent presentation of donor antigens in the thymus prior to the completion of this naturally occurring process of thymic education, should lead to deletion of alloreactive T cells, creating donor-specific immune tolerance.

Long before scientists ever contemplated performing IUTx, experiments of nature provided what is still considered to be the most compelling evidence for the ability of foreign hematopoietic cells to induce durable immune tolerance, if introduced early during fetal development. The seminal discovery that exposure to foreign antigens can lead to tolerance was first made by Owen in 1945,²⁸ who astutely observed that the shared placental circulation present in monozygotic dizygotic cattle enabled intrauterine exchange of circulating HSC, with resultant lifelong hematopoietic chimerism and donor-specific tolerance for the sibling twin.²⁸⁻³⁰ Of relevance to clinical application, since this remarkable discovery, natural chimerism has also been shown to occur in twins in both nonhuman primates and humans twins,³¹⁻³⁶ and this chimerism has been shown to lead to a lack of alloreactivity between the two siblings.^{31,36} Moreover, in the case of dizygotic human twins, the frequency of chimerism is relatively high (8% for twins and 21% for triplets), and levels of chimerism observed have often been sufficient that they would be predicted to exert a therapeutic effect in most hematologic diseases.¹⁶

Despite the immense promise these findings hold, there is an important caveat that must be considered, namely, that these natural chimeras result from placental vascular anastomoses, which allow for continual exchange of blood components and exposure to

allogeneic cells/antigens beginning very early in gestation.³⁷ Such a scenario obviously poses formidable challenges when one tries to envision recreating this process experimentally, using animal models of IUTx, or, ultimately, in a clinical setting to treat human patients.²⁴ Nevertheless, these exciting findings in nature have long provided the scientific basis for the promised therapeutic potential of IUTx, and for its ability to induce donor-specific immune tolerance, and have fueled efforts to bring this therapy to clinical fruition.

These findings are important in and of themselves, since they suggest that the induction of tolerance following IUTx should facilitate maintenance of the donor hematopoietic cells. Perhaps even more importantly, and of more immediate clinical applicability, they have led scientists to the exciting realization that, even if the levels of donor cell engraftment following IUTx are not sufficient to be curative/therapeutic, the antigen-specific tolerance induced by IUTx may make it possible to administer postnatal “booster” transplants of same-donor HSC to achieve therapeutic levels of engraftment without the need for toxic myeloablation, a possibility that has been explored in some detail by Peranteau *et al.*,^{38–40} or perhaps even same-donor solid organs.^{40–45}

EXPERIMENTAL STUDIES WITH IUTX

Seminal experiments conducted by Billingham *et al.*¹² in the 1950s provided the first experimental evidence that the *in utero* transplantation of allogeneic cells could, indeed, induce donor-specific tolerance to postnatal skin grafts in mice. However, despite a fairly extensive amount of work being done on naturally occurring chimeras and the potential for *in utero* chimerism to produce immune tolerance, it was not until the late 1970s that scientists began exploring the possibility of performing experimental IUTx with the goal of engrafting donor (allogeneic) HSC. Fleischman and Mintz were the first to report successful hematopoietic chimerism following IUTx and to provide proof that IUTx could reverse a genetic disorder. Using a line of mice with genetic anemia due to a stem cell deficiency based on the absence of c-Kit, Fleischman and Mintz showed that transplanting adult allogeneic BM stem cells into the placenta of E11 fetal mice reversed the genetic anemia.⁴⁶ These important studies also revealed another interesting aspect of donor cell engraftment following IUTx: the degree of erythroid replacement correlated with the degree of underlying anemia, such that the erythroid compartment of lethally anemic homozygous mice was rapidly and completely replaced with donor-derived erythropoiesis. Mintz then went on to make the remarkable discovery that erythroid reconstitution could be achieved in this model following transplantation of just a single HSC.⁴⁷

While these early studies were directed toward questions in stem cell biology rather than IUTx as a potential therapeutic approach, they were the first to identify host cell competition as a barrier to donor cell engraftment after IUTx, a finding that, as we will soon discuss, proved critical to clinical implementation of IUTx. Nearly 20 years would pass before Blazar *et al.*⁴⁸ confirmed the ability to achieve multilineage chimerism after IUTx in stem cell-deficient recipients, extending the findings of Fleischman and Mintz to the case of lineage deficiency, by demonstrating only lymphoid reconstitution (split chimerism) occurred when mice with a T-cell proliferation and survival defect due to severe combined immunodeficiency (SCID) were treated by IUTx.^{49,50} These studies were thus the first to provide evidence that host cell competition was unexpectedly able to limit donor cell engraftment following IUTx and solidified the notion that, in the presence of a lineage deficiency, IUTx is able to reconstitute the defective lineage, while contributing

minimally, if at all, to other hematopoietic lineages. These findings thus suggested that it would likely prove much more difficult to achieve meaningful levels of donor cell engraftment after IUTx in normal animals (and ultimately human patients) with a competitive hematopoietic compartment. Indeed, early studies of IUTx using immunocompetent, wild-type recipient mice were uniformly plagued by very low rates of engraftment, and it was soon noted that it was possible to engraft immunodeficient mice much more efficiently than their wild-type counterparts.^{46,48,49,51}

Since these initial ground-breaking studies in mice over 30 years ago, great progress has been made in the field. The sheep model was the first large animal model to demonstrate engraftment of allogeneic cells after fetal transplantation.⁵² Of the available animal models for the study of IUTx, sheep have been a particularly valuable preclinical tool. Fetal sheep provide a natural, unperturbed environment in which to study IUTx, and since xenogeneic cells are not rejected (if IUTx is performed early enough in gestation), it is possible to study the engraftment and differentiation capacity of a variety of human stem cells.^{53–62} Sheep share many important physiological and developmental characteristics with humans. As a result, they have been used extensively in the study of mammalian fetal physiology, and the results obtained with this model have been directly applicable to the understanding of human fetal growth and development.⁶³ In contrast to dogs, pigs, and many other large animals that tend to have large litters of offspring, sheep, like humans, typically give birth to only one or two offspring in each pregnancy. Importantly, sheep are similar in size/weight to humans, both at birth and as adults, making it possible to develop and test clinically relevant doses of cells/therapy vectors directly in this model prior to translating to the clinical arena. In addition, their immune system and hematopoietic development during fetal ontogeny has been thoroughly delineated and is quite similar to that of humans,^{64–70} making this model ideal for investigating the immune facets of IUTx (and IUGT). Also of note, their long life span allows the important issues of long-term efficacy and safety to be adequately addressed.

The fetal sheep model has also played a critical role in defining the phenotype of long-term engrafting human HSC, enabling the identification of several novel human stem cell markers/phenotypes (*e.g.*, CD34+CD38-, HLA-DR-, Thy-1+, CD133, KDR, and CD34-),^{71–80} many of which are now in clinical use, attesting to the high translational value of data obtained with this preclinical model system. This system has also proven valuable for defining the role of the marrow microenvironment in the engraftment of HSC following IUTx,^{63,81–83} and as a preclinical model in which to examine HSC mobilization, since engrafted human hematopoietic cells respond to human granulocyte colony-stimulating factor in a similar fashion to their native counterparts.^{82,84–87} These data collectively support the value of the fetal sheep model for developing/testing approaches to IUTx (and IUGT) and obtaining results of high clinical relevance.^{25,88,89}

In similarity to sheep, the canine model also accepts xenogeneic transplants, and low-level multilineage hematopoietic engraftment has been demonstrated in hematologically normal dogs.⁴⁰ More recently, Flake *et al.* showed that the levels of hematopoietic engraftment in canine recipients following IUTx are sufficient to: (i) ameliorate or cure the clinical phenotype of the canine analog of human leukocyte adhesion deficiency (canine leukocyte adhesion deficiency) and (ii) induce donor-specific tolerance in some animals that is adequate to facilitate postnatal “boosting” of chimerism using a low-dose busulfan conditioning regimen, followed by transplantation of same donor T-cell-depleted BM.^{15,40,90,91} IUTx has also

been successfully performed in goats^{92,93} and pigs,⁴⁰ and low levels of engraftment have been achieved in nonhuman primates.^{93–99} Subsequent studies in the pig model provided compelling evidence that induction of immune tolerance in the fetus is highly beneficial for postnatal solid organ transplantation, as IUTx of adult BM-derived HSC in fetal swine prolonged the survival of a kidney allograft.¹⁰⁰ These studies thus provide important experimental support for the possibility of using this strategy in fetuses with congenital abnormalities that require postnatal organ transplantation.

Due to their ease of genetic manipulation, mouse models have been used to interrogate various aspects of the immune system to gain an understanding of the mechanism(s) of tolerance induction following IUTx. Data from over the last decade from Flake, MacKenzie, Peranteau, Shaaban, Nijagal, and colleagues have collectively provided compelling evidence that clonal deletion, energy, and induction of donor-specific Tregs are all critical to the establishment of chimerism and the induction of immune tolerance.^{42,43,45,101–106} Interestingly, these studies in mice have demonstrated that stable engraftment of even low levels of allogeneic HSC (1–2% engraftment) can lead to postnatal tolerance across full MHC barriers and have revealed that tolerance induction depends upon achieving a threshold level of engraftment and on maintaining chimerism in the host.¹⁰⁷ Elegant recent work from Shaaban *et al.*^{108–111} has indicated that NK cell tolerance appears to play a key part in establishing this engraftment threshold.

While similar mechanistic studies are clearly needed in preclinical large animal models, as the human immune system may well present its own set of unique challenges, these collective results demonstrate the technical feasibility of IUTx, confirm its ability to induce donor-specific immune tolerance, and shed light on some of the requisite pathways to tolerance induction, establishing an essential foundation for ultimate clinical application of IUTx.

BARRIERS TO IUTX SUCCESS

Despite the preceding evidence, and the seemingly sound presumption that the fetus should be “preimmune” during the so-called “window of opportunity”¹³ prior to the appearance of mature T cells in the fetal thymus and peripheral circulation (~12–14 weeks of gestation in the human fetus),²⁷ with increasing experimental experiences, it became clear that there were significant barriers to successful engraftment after IUTx if the recipient did not possess a lineage-specific defect, such as anemia or SCID, to confer a competitive advantage to the donor cells.^{13,104,112–115} Important studies by Peranteau *et al.*¹¹⁶ shattered the notion that the fetus is truly “preimmune,” by showing that mice could more consistently be engrafted, and at higher levels, when cells from congenic donors were transplanted compared to phenotypically identical cells from allogeneic donors, with only ~30% of the recipients of allogeneic cells consistently exhibiting chimerism. These surprising findings thus suggested that the fetal immune system is one of the major barriers to engraftment following IUTx. Early tracking of donor cells and long-term assessment of donor chimerism then enabled the authors to demonstrate that 100% of allogeneic and congenic recipients maintained high levels of engraftment up to 3 weeks after IUTx. However, between 3 and 5 weeks, 70% of allogeneic animals lost their engraftment, while 100% of congenic animals remained chimeric.¹¹⁶

The authors confirmed the presence of an adaptive cellular and humoral alloresponse that was quantitatively higher in nonchimeric versus chimeric animals, which would logically lead one to conclude that the host (fetal) immune response was responsible for limiting donor cell engraftment. This finding was clearly at odds with a

wealth of prior data, from this same group and others, demonstrating long-term chimerism in a percentage of recipients (both mice and other animals) following IUTx, and the presence of deletional tolerance. The crucial observation explaining this inconsistency was that if transplanted pups were placed with surrogate mothers that had not been exposed to donor antigen, 100% of the recipients maintained their chimerism.^{101,117} These findings suggested that IUTx triggered maternal alloimmunization, with subsequent transfer of alloantibodies to the pup via breast milk, inducing an adaptive alloimmune response in the pup with a subsequent loss of chimerism. Perhaps most importantly, this study confirmed that in the absence of a maternal immune response, either via foster nursing or through the use of maternal donor cells, engraftment and tolerance were uniformly present via a mechanism of partial deletion of donor-reactive T cells and the induction of a potent T-regulatory cell response.

An independent series of murine IUTx studies, performed at around this same time by Nijagal, MacKenzie, and colleagues, corroborated the finding of maternal alloimmunization as a result of fetal intervention but found that subsequent maternal–fetal T-cell trafficking was the main factor responsible for the loss of chimerism.^{102,103,118–120} Obviously, murine placentation, maternal–fetal trafficking of antibodies and cells, and the time course of events after IUTx are considerably different in mice when compared to large animal models or during human pregnancy. Nonetheless, these findings raise the important question of whether maternal immunization is an issue in large animal models and clinical circumstances, and whether it is a limitation to engraftment after IUTx. Until this question is addressed, these findings suggest that it may be prudent to use maternal cells in any clinical application of IUTx to remove the possibility of triggering a maternal immune response.

Despite the rigor of these studies, Shaaban *et al.* have recently taken issue with the role of the maternal immune response in the context of human IUTx, astutely pointing out that the maternal immune system has been intact in all human patients who have thus far undergone IUTx for a variety of diseases, despite the nature of the clinical outcome (success or failure); data which they feel supports the conclusion that the maternal immune response cannot be a key determinant in IUTx-related engraftment failure.^{108,109} This group has, accordingly, focused its efforts on investigating the ontogeny of the fetal immune system to ascertain which cells/pathways are present at the time of IUTx that could account for the apparent immunological barrier to engraftment of allogeneic cells. These authors have homed in on the NK arm of the innate immune system and have identified a subset of early NK cells within the fetal liver that express adult levels of alloreactive receptors, suggesting that NK cells may pose a barrier to engraftment of transplanted cells as early as the end of the first trimester in humans. Furthermore, this same group has shown that depletion of NK cells from the fetus, but not from the mother, enables reliable engraftment of allogeneic cells following IUTx^{108,109,111} and that the levels of early chimerism required to induce NK cell tolerance agree exactly with the threshold levels discussed earlier. In a more recent study, this same group has provided data supporting a mechanistic link between the induction of prenatal NK cell tolerance and the process of trophoblast invasion, explaining how levels of engraftment of only 1–2% could result in exposure of donor antigens to a sufficient number of NK cells to reliably induce donor-specific tolerance.¹¹⁰ While these important findings will have to be reproduced in large animal models of IUTx, these elegant and highly mechanistic studies collectively provide a very persuasive argument for the importance of fetal NK

cells in the ability to achieve engraftment of allogeneic cells following IUTx, just as has been seen in postnatal HSC transplantation.¹²¹

In addition to the fetal and/or maternal immune response, perhaps the most important perceived barrier to engraftment of allogeneic HSC is host cell competition. In the setting of postnatal HSC transplantation, the recipient receives myeloablative conditioning prior to donor cell infusion, to suppress endogenous hematopoiesis and, perhaps, free spaces within the hematopoietic niches of the BM. In marked contrast, following IUTx, the donor cells must compete against the robust fetal hematopoietic compartment. The idea that donor (adult) cells may have a competitive disadvantage in the fetal environment is supported by the ease with which high levels of donor hematopoiesis can be achieved in *c-kit*-deficient mice, in which as few as one or two normal HSC can fully reconstitute the hematopoietic compartment after IUTx.⁴⁶ Studies of IUTx performed in SCID mice also illustrate the importance of host cell competition.^{49,122} In this model, in which donor lymphoid cells have a survival and proliferative advantage, IUTx results in complete reconstitution of the lymphoid compartment with minimal engraftment of other unaffected lineages.

These preceding experimental data illustrate just how effective this competitive advantage can be in the setting of a proliferative defect in one or more lineages. However, when no defect in host hematopoietic vigor is present, the scale tips in favor of the endogenous fetal HSC, which have a marked competitive advantage over their adult-derived counterparts,^{20,123–126} due to their accelerated/enhanced cycling and expansion kinetics. Data from the congenic mouse model of IUTx provide a striking example of the degree to which this competition limits long-term donor cell engraftment. In this setting, in which no immune barriers exist, even delivering massive doses of donor cells (2×10^{11} donor cells/kg), long-term donor cell engraftment levels remain below 10%.¹¹⁶

The limited number of available niches and the proliferative capacity of the fetal environment have also been implicated as a barrier to success with IUTx.⁴⁴ Favorable competition of transplanted HSC with the host cells for available hematopoietic niches is essential for successful engraftment, as evidenced by the enhanced success of IUTx when more competitive fetal donor cells or larger doses of adult cells are used.^{23,127} Improved competition for available host niches would obviously be predicted to lead to higher levels of early chimerism, as is seen in adult mice, in which selective depletion of host HSC before BM transplant results in high rates of engraftment.¹²⁸ However, no direct evidence exists to support the existence of quantitative or qualitative differences in the number of HSC or available niches between recipients with SCID and those with sickle cell disease or β -thalassemia (reviewed in refs. 129,130), or any of the range of other disorders that have proven refractory to correction by IUTx. As such, it is hard to envision how a competitive niche model could explain the conflicting observations for immunodeficient versus non-immunodeficient recipients, with respect to success of donor cell engraftment following IUTx.¹⁰⁹ Nevertheless, the possibility that there is a finite number of available hematopoietic niches for donor cell engraftment following IUTx is supported by the finding that increasing the dose of donor cells results in an eventual plateau of engraftment efficiency in an allogeneic and xenogeneic fetal sheep model.¹³¹ Moreover, maternal administration of busulfan 6 days prior to IUTx has been shown to significantly increase engraftment in fetal sheep.¹³² While informative, it is not clear whether the toxicities associated with the use of a myeloablative agent during pregnancy would be clinically acceptable. However, recent studies from MacKenzie *et al.* demonstrated that

selective *in utero* depletion of host HSC using an antibody against the *c-Kit* receptor (ACK2) results in therapeutic levels of engraftment after neonatal transplantation,¹³³ without any of the cytotoxic effects of an agent like busulfan. These two studies collectively support the notion that vacating host stem cell niches may be a viable means of improving chimerism after IUTx. Clearly, however, further studies are needed to better understand this important issue and develop methods of optimizing the benefits on donor HSC engraftment while minimizing risks to the fetus and mother.

CLINICAL EXPERIENCE WITH IUTX

The early success of IUTx in experimental animal systems generated a great deal of excitement and was followed by many attempts around the world to treat various hematologic disorders with IUTx. In humans, the first successful IUTx was performed for bare lymphocyte syndrome.¹³⁴ Following this seminal case, successful transplantation of fetuses with SCID was also achieved in a number of centers.^{135–138} In these cases, fetal liver, paternal BM, or maternal BM-derived CD34+ cells were transplanted between 16 and 26 weeks' gestation and resulted in engraftment of donor cells at birth and clinical improvement. To date, IUTx has been performed on 46 human patients for 14 different genetic disorders, including hemoglobinopathies, chronic granulomatous disease, Chediak-Higashi syndrome, and inborn errors of metabolism^{13,25,139–142} (reviewed in ⁴). These studies have collectively provided unassailable proof that the early human fetus can be accessed multiple times with an extremely low procedure-related risk, assuming that a minimally invasive, ultrasound-guided approach is employed.^{13,25,117,135,139,140,143}

Unfortunately, with the notable exception of patients with SCID, the clinical experience thus far with IUTx has been largely disappointing. However, SCID is a unique disorder that provides a survival and proliferative advantage for donor T-cells, and the engraftment achieved in these patients has only been documented to reconstitute the T-cell lineage (split chimerism),²⁵ just as was observed in the early experimental work in mice performed by Bazar *et al.*^{49,122} The results of the 46 clinical IUTx cases performed to-date have clearly demonstrated that IUTx, using currently employed methods, is not able to establish clinically relevant/therapeutic levels of engraftment in recipients whose hematopoietic system exhibits a normal level of competitiveness. The large number of variables among these reported clinical cases makes identifying common factors that are responsible for the observed poor engraftment very difficult. For example, transplantations occurred at different centers, donor cells were isolated from different sources, and the transplants were performed at variable gestational ages. The inherent inconsistency in these studies has made it impossible to blame the lack of success on one specific factor and has made it necessary to perform more carefully controlled experimental studies in animal models to gain insight into the barriers that limit engraftment after IUTx, as has just been discussed in detail.

Since the majority of the anticipated target disorders for treatment with IUTx, such as the hemoglobinopathies and the lysosomal storage diseases, are competitively normal during fetal development, methods must be developed in clinically relevant animal models to overcome host cell competition to improve clinical success with IUTx. A recent study performed by Flake *et al.* in the canine model¹⁵ showed that administering large numbers of highly enriched HSC via an ultrasound-guided intravascular (intracardiac) route resulted in significantly higher levels of engraftment than the intraperitoneal route that has been used in most clinical studies. Of note, the levels obtained via this new route would be predicted to

be therapeutic in most candidate diseases, generating enthusiasm in the field.^{144,145} However, other studies in sheep have produced contradictory results, showing that the intravascular route is no better than the intraperitoneal route, leading the authors of this other study to conclude that the markedly greater safety afforded by the intraperitoneal route will likely make this the clinical route of choice.¹⁴⁶

One area that has received a great deal of attention is the idea that the best clinical application for IUTx in the near future may be to use the strategy of prenatal tolerance induction to facilitate nontoxic postnatal BMT.^{3,16} This approach greatly significantly lowers the threshold of chimerism that would be required for clinical success, since, as discussed in the preceding section, stable levels of donor cell engraftment of only 1–2% are sufficient to reliably induce donor-specific immune tolerance. A very recent report by Peranteau, Flake, and colleagues has unequivocally validated the therapeutic merit of such an approach.³⁹ In this study, the authors demonstrate that low level hematopoietic engraftment to induce tolerance, followed by postnatal nonmyeloablative same donor “boosting” BM transplantation, results in high levels of donor cell engraftment and phenotypic correction in murine models of β -thalassemia and sickle cell disease. These exciting results led the authors to conclude that “if adequate engraftment can be achieved to consistently induce donor-specific tolerance without graft-versus-host disease in a pre-clinical model, then clinical trials of IUTx for treating genetic disorders that can be prenatally diagnosed and treated by mixed hematopoietic chimerism, such as the hemoglobinopathies and selected immunodeficiency disorders, should be initiated.”³⁹

IUGT: RATIONALE FOR APPROACH

Multiple outstanding reviews have been written over the last decade, discussing IUGT in detail.^{2,16,25,89,147–155} For this reason, we will endeavor to highlight some of the key advantages and risks to this, as yet, experimental therapeutic approach, focusing on the utility of this treatment modality for correcting hemophilia A (HA). It is important to note that many genetic diseases exert a significant amount of irreversible damage during embryonic and fetal development. As such, the same rationale exists for treating these diseases prior to birth by IUGT as was presented earlier for IUTx. Even in patients with diseases that can be cured postnatally, compelling psychological and financial benefits exist to argue for performing correction *in utero*, since it would allow the birth of a healthy infant, who, ideally, would require no further treatments. While IUTx can potentially treat many disorders, some genetic diseases may not be amenable to correction by the transplantation of “healthy” stem cells, and for some, it may be preferable to correct the genetic abnormality *in situ*.

In addition to the clinical and financial advantages of correcting a genetic disease prior to birth, numerous aspects of the fetus make it a more suitable gene therapy recipient than the adult. For example, due to their ability to integrate into the genome of the host cell, γ -retroviruses and lentiviruses have received a great deal of attention as gene delivery vectors, since transduction of a long-lived cell could provide lifelong therapy following a single administration. However, one of the main limiting factors to the successful application of these integrating vectors to *in vivo* gene therapy is the low level of initial transduction and the limited degree of expansion of transduced cells that occurs following gene therapy, since most stem cell populations in the adult are relatively quiescent and may be difficult to access because of tissue distribution and anatomic barriers. During specific developmental periods, however, stem and progenitor cell populations exist at high relative frequencies,

and may be accessible to gene transfer, providing a unique window of opportunity for gene transfer to these expanding nascent stem cell populations, which will be inaccessible later in life.^{3,16} In the fetus, the cells in all of the organs are actively cycling to support the continuous expansion that occurs throughout gestation. Thus, most cell types that are largely quiescent in the adult are far more mitotically active in the fetus. As such, these cells should be far more amenable to genetic correction with vectors requiring cell division. Furthermore, the active cycling of the cells in all of the organs to support the continuous expansion that occurs throughout gestation should result in expansion of the gene-corrected cells during the remainder of gestation, allowing initial transduction of even small numbers of target cells to result in significant levels of gene correction by birth. Clearly, being able to take full advantage of this fetal expansion will likely require the use of vectors that integrate into the host genome, since vectors based on nonintegrating viruses, such as adenovirus, or those that integrate only rarely, such as AAV, will largely be lost during cellular division, causing cessation of therapeutic effect.

In addition to the ability to access nascent stem cell populations, just as discussed in the context of IUTx, the immature immune system of the fetus should enable delivery of immunogenic transgenes or viral vectors that would be rejected by the intact immune system of a postnatal patient. For example, the majority of the world's population has been exposed to, and harbors antibodies to, the capsid proteins in various serotypes of AAV, making postnatal gene delivery with these valuable vectors difficult. Intervening *in utero*, prior to maturation of the immune system, would likely allow efficient gene transfer with these vectors, since these antibodies are not present. With respect to the transgene, many patients suffer from the genetic diseases currently being targeted with postnatal gene therapy because they have never produced a single specific protein. As a result, their immune system has never “seen” this protein, and following gene therapy, the cells of the immune system seek to eliminate any cells in the body that are expressing the very protein that could cure the patient of his/her disease. The low levels of gene delivery to the desired target cells and the immune response combine to yield very low levels of expression of the therapeutic protein, and even these small amounts are often only produced for a short time. Performing IUGT should induce a state of tolerance to the transgene and, perhaps, to the vector itself, which not only ensures long-term, stable transduction and expression but should also make it possible to administer postnatal “booster” treatments (if required) with the same vector and transgene without eliciting an immune response. An important caveat to inducing tolerance to the vector, however, is that such tolerance could potentially render the individual susceptible to postnatal infection with the wild-type virus on which the vector was based. Such an outcome is clearly not desirable, and preclinical animal studies will be needed to determine whether this risk exists or not.

Finally, in similarity to our discussion on IUTx, the extremely small size of the fetus at the proposed time of intervention offers distinct advantages over treating a child or adult patient. By virtue of the fetus' small size, it is possible to achieve much higher vector-to-cell ratios than would be possible later in life, which should greatly enhance the efficiency of transduction. In addition, the ability to administer a small volume of vector and achieve the desired rate of transduction is of additional benefit from a technical/logistical standpoint, since the large-scale production of certain vectors under GMP conditions is often extremely difficult.

EXPERIMENTAL STUDIES ON IUGT: HA AS A MODEL GENETIC DISEASE FOR CORRECTION BY IUGT

Gene transfer using viral vectors exploits the natural ability of the parent virus to efficiently attach to a target cell and transfer its genetic material to the host cell nucleus but are engineered to be devoid of most, if not all, viral genes, rendering the viral vector incapable of replication or expression of potentially immunogenic and/or toxic viral genes. Because the vector is ultimately responsible for the transfer of genes to the fetus, the choice of vector is of utmost importance in fetal gene therapy. While a complete discussion of viral vectorology is beyond the scope of this article, suffice it to say that the specific vector to be used for a given IUGT application will depend largely upon one's goals and the disease and/or cell type being targeted and should be selected after careful consideration of such factors as the ability to integrate into host genomic DNA, tissue tropism, packaging capacity, and potential immunogenicity. Most investigators in the field would likely agree that an ideal vector for curing a genetic disease via IUGT (or postnatal gene delivery as well) would possess the ability to selectively target a specific cell type/organ and be able to mediate sufficient levels of gene transfer to produce therapeutic effect with only a single application. The ideal vector tropism will, however, clearly depend upon the disease to be treated. As we will discuss in detail in a subsequent section, in diseases such as the hemophilias, in which tissue-specific expression of the corrective gene is not required, greater therapeutic benefit would clearly be obtained by using a vector that is capable of widespread transduction and gene expression within the developing fetus.

When one considers initial target diseases for exploring the therapeutic potential of IUGT, it stands to reason that the diseases that would be most amenable to treatment by IUGT are those caused by a mutation in a single gene. To contemplate *in utero* treatment, the testing for the target disease must be in place to allow diagnosis before birth, and there must be compelling reasons to pursue prenatal treatment rather than waiting until after birth. Using a variety of animal model systems and rodent models of human genetic diseases and a wide range of transduction methods, IUGT, using a wide variety of viral vectors, has been targeted to multiple organs,^{89,140,143,156–175} and in several disease models, phenotypic rescue has been accomplished.^{89,140,143,161–163,166–168,176–192} In the interest of space, and for the purpose of illustrating the profound therapeutic potential of IUGT, and the ease with which it could be implemented clinically to cure disease, the next section of this review will focus on HA, presenting HA as a paradigm for genetic diseases that could be corrected by IUGT, the rationale for pursuing its treatment prior to birth, the feasibility of doing such, and clinical, societal, and financial advantages IUGT could offer over existing treatments for this disease.

The need for better HA treatments

HA is the most commonly occurring inheritable deficiency of coagulation.¹⁹³ While the clinical severity of HA (based on FVIII plasma levels) can vary, up to 70% of patients with HA present with a severe, life-threatening phenotype,^{194–196} suffering frequent spontaneous hemorrhaging, which leads to hematomas, chronic painful and debilitating arthropathies, and potentially life-threatening internal bleeding.¹⁹⁴ The current standard of care for HA is prophylactic factor infusion, which is comprised of 2–3 intravenous infusions of recombinant or plasma-derived FVIII per week to maintain hemostasis. While this “protein-replacement therapy” has greatly improved quality of life and extended the life expectancy for many

patients with HA, it is far from an ideal therapy. Patients are sentenced to a lifetime of multiple intravenous infusions each week and are financially strapped with treatment costs that can exceed \$300,000/year. Even among the ~25% of HA patients worldwide who are fortunate enough to have access to FVIII prophylaxis, ~30% will mount an immune response (inhibitors) to the infused FVIII.¹⁹⁷ In the best-case scenario, these inhibitors simply reduce the effectiveness of subsequent infusions of FVIII; in the worst-case scenario, they can lead to treatment failure, putting the patient at risk of a life-threatening bleed. These significant shortcomings highlight the need for novel therapies that can promise longer-lasting correction, or permanent cure, of HA.

In contrast to current protein-based therapeutics, a single gene therapy treatment could promise lifelong correction of HA; indeed, several aspects of HA make it an ideal target disease for correction by gene therapy.^{173,198–206} First, FVIII does not need to be expressed in either a specific tissue or cell type to produce a therapeutic effect. Although the major site of FVIII production within the body is thought to be the liver,²⁰⁷ and its expression by endothelial cells that harbor Weibel Palade bodies ensures its appropriate processing and efficient secretion, FVIII can exert its appropriate clotting activity as long as it is produced by cells that can release the synthesized FVIII into the circulation. Second, even if FVIII levels could be restored to only 3–5% of normal, this seemingly minimal change could exert a marked clinical effect and greatly improve the quality of life of patients with severe HA, converting these patients to a moderate/mild phenotype. Conversely, even FVIII levels as high as 150% of normal are predicted to be safe.¹⁹⁶ Armed with this knowledge, the hemophilias were included in the most promising, “Target 10” diseases in the American Society of Gene and Cell Therapy (www.ASGCT.org) roadmap.

Preclinical animal models to study IUGT for HA

Fortunately, colonies of HA dogs in which spontaneous mutations occurred within the FVIII gene^{208,209} and FVIII-deficient mice produced via gene targeting/knockout²¹⁰ are both available to study the biology of FVIII and to explore/develop gene-based strategies to treat HA. Pronounced therapeutic benefit has been demonstrated in multiple postnatal gene therapy studies in murine models.^{201,203,211–217} Phenotypic correction has also been achieved with postnatal gene therapy in dogs with HA, but correction in this more clinically predictive model has proven much more difficult than that in mice.^{218,219} However, despite the promising results that have been obtained in both these models, no therapeutic benefit has yet been seen in any of the clinical gene therapy trials that have been conducted to-date for HA. This is in striking contrast to the recent successes that have been reported in clinical gene therapy trials treating patients with hemophilia B (HB),²²⁰ while difficulties packaging the large FVIII transgene into most viral vector backbones is likely at least partially to blame, the precise reasons for the marked difference in the ability of gene therapy to correct HA vs. HB thus far are not entirely clear. Nevertheless, as a result of the disappointing outcomes thus far, no active clinical trials are currently ongoing in which gene therapy is being used to treat HA. This is especially vexing when one considers that roughly 80% of all hemophilia cases are HA.

The difficulties seen thus far translating success in animal models into therapeutic benefit in human patients highlight the importance of preclinical animal models that both precisely mimic the disease process of HA and closely parallel normal human immunology and physiology. To this end, we used a variety of reproductive

technologies to successfully re-establish, and then clinically characterize, a line of sheep^{221–225} that possess a spontaneous frameshift mutation^{223,224} causing severe HA, which, if not treated at birth, is fatal within the first hours/days of life.^{226–228} All 10 affected animals born thus far have experienced multiple spontaneous episodes of severe bleeding, including muscle hematomas, hematuria, and hemarthroses, all of which have promptly responded to infusion of human FVIII. FVIII activity is undetectable in plasma of these sheep using a highly sensitive chromogenic assay, explaining their severe, life-threatening phenotype. Importantly, just like human patients with severe HA, these sheep experience frequent spontaneous bleeds into their “knees,” which, over time, produce crippling arthropathies that ultimately lead to decreased movement, difficulties walking, and eventually symptoms of pain even just to stand up. These recurring spontaneous joint bleeds make this line of sheep unique among animal models of HA. Also in similarity to human patients, some of these sheep developed inhibitors following administration of FVIII. However, since we had not yet cloned and sequenced ovine FVIII, we were restricted to treatment with human FVIII, leaving unanswered the question of whether these animals will also make inhibitors to the ovine protein. An ongoing collaboration with Drs Spencer and Doering at Emory University has recently resulted in the successful cloning and large-scale production of recombinant B domain-deleted ovine FVIII,²²⁹ making it possible to address this important question and to construct gene therapy vectors encoding ovine FVIII for testing in this valuable model.

As discussed in detail in the section on IUTx, sheep possess many characteristics that make them an ideal preclinical model for IUGT. An additional unique advantage to using sheep in the context of HA treatment is that, like humans, the majority of the FVIII carrier protein, vWF, is stored/located within their platelets. This is in contrast to dog, in which vWF circulates free in plasma.^{230,231} This key difference makes the sheep the most clinically relevant large animal model in which to test the efficacy of recently described platelet-targeted gene therapy approaches for treating HA.^{206,232–234} For these collective reasons, we feel that sheep are an especially fitting model in which to develop and test gene therapy treatments for HA.

Feasibility and justification for treating HA prior to birth

Even if FVIII costs were reduced to the point that most HA patients could afford prophylaxis, these patients would still require recurrent, intravenous infusions throughout their lives, and still have a significant risk of treatment failure due to inhibitor induction. These problems, as well as many of the obstacles that have precluded gene therapy from curing patients with HA (and many other diseases) to-date, could likely be overcome/eliminated by performing gene therapy prior to birth. For individuals with a family history of HA (~75% of HA cases), prenatal diagnosis for HA is feasible, available, and is both encouraged and cost-effective, even when considering developing third world countries.^{235–246} Moreover, another recent study has shown it is now possible to diagnose HA *in utero* by performing digital PCR on the small number of fetal cells present within the mother’s peripheral blood, making it possible to diagnose HA prenatally with essentially zero risk to the fetus or mother.²⁴⁵ Despite the availability of prenatal screening, still, ~1 in 5,000 boys born each year worldwide are affected with HA.¹⁹³ In the United States alone, correcting this disease prior to birth could benefit the ~240 patients/year born into families with a history of HA. IUGT could promise the birth of a healthy infant who required no further treatments, removing the heavy physical, psychological, and monetary burden on the patients, their families, and the healthcare

system. The current estimate for the lifetime cost of prophylactic treatment for one HA patient is \$20 million. Curative IUGT would thus save ~\$48 billion over the lifetime of the HA patients born just this year in the United States.

Importantly from a safety standpoint, during early fetal life, activation of FX occurs predominantly via tissue factor activity, making it largely independent of the FIXa/FVIIIa phospholipid complex.²⁴⁷ As a result, the fetus develops without hemorrhage, despite having little or no expression of FVIII and FIX.^{247–249} The unique homeostasis of the fetus should thus allow IUGT to be performed safely for HA; indeed, one of the 46 human patients that has thus far received IUTx^{140,141} was transplanted in the hopes of correcting HA or at least inducing immunological tolerance to FVIII.^{139,142} While only this one HA patient was treated, he suffered no untoward effects as a result of the *in utero* intervention, he has thus far exhibited reduced severity of disease compared to his siblings, and he (in contrast to his siblings) has not developed inhibitors with FVIII treatment (JL Touraine, personal communication and ref. 142). This remarkable case thus provides clinical validation for prior experimental studies demonstrating that exposure to vector-encoded proteins (including coagulation factors) during early immunologic development induces stable immune tolerance.^{170,171,250,251} The lifelong tolerance to FVIII induced by an IUGT-based HA treatment should therefore prevent the development of FVIII inhibitors that plague patients treated with replacement therapy.^{197,252–255} In this one clinical case, Dr Touraine relied on the ability of unpurified fetal liver cells to endogenously produce sufficient levels of FVIII, after transplant, to mediate correction. The only partial correction observed in this patient supports the approach of using gene transfer to ensure adequate levels of FVIII are obtained for full phenotypic correction.

Although the clinical and financial advantages of IUGT are compelling, in and of themselves, it is important to acknowledge that there are also features of the fetus that make it a far better gene therapy recipient than the adult.^{89,256,257} For instance, cell populations that are quiescent in the adult, and largely refractory to transduction with many commonly employed viral vectors, are actively cycling in the fetus and amenable to transduction at relatively high efficiencies. For example, we showed that by administering a single intraperitoneal injection of a small volume of γ -retroviral vector at the optimal stage of gestation (which we determined experimentally), it is possible to achieve gene transfer levels within the hematopoietic system of 5–6%,^{163,189,258} levels that would undoubtedly be beneficial in HA. Further studies involving antibody selection of CD34+ cells and serial transplantation/repopulation,^{163,189,259} provided compelling evidence that this approach successfully modified *bona fide* HSC, indicating this method could provide lifelong disease correction.

Our results also demonstrated that this approach successfully transduced hepatocytes and hepatic endothelium at levels that could well be therapeutic in HA, and defined the temporal window during gestation for optimal transduction of these cells.¹⁶¹ Concurrently, fetal gene delivery experiments conducted in sheep, rodent, and nonhuman primate models, by other investigators who employed a variety of viral-based vectors, produced similar results.^{89,140,143,156–175} The collective results of these studies clearly support the ability of this method to deliver a FVIII transgene to the nascent liver with sufficient efficiency to convert severe HA patients to a moderate or, perhaps, even mild phenotype.¹⁶¹

While the active cell cycling in the fetus enables efficient transduction with vectors that require mitosis, it is important to note that this ongoing proliferation in all of the fetal organs is also of benefit

when using vectors that do not have an absolute requirement for mitosis. Gene delivery early in gestation, regardless of the vector employed, also makes it possible to achieve subsequent expansion of these gene-corrected cells throughout the rest of gestation. As such, even if the initial gene transfer only transduces a small number of the desired target cells, this subsequent expansion could produce clinically useful levels of gene-correction by birth.

As mentioned earlier, one of the biggest obstacles/drawbacks to treating severe HA by repeated protein infusion is the formation of inhibitory antibodies in ~30% of patients. It is important to note that the distinct immunologic benefits to performing IUTx also apply to IUGT. We have spent the last two decades performing IUGT studies in the sheep model^{189,161–164,189,250,258,260–263} and have shown that it is possible to take advantage of this unique temporal window of relative immuno-naivete to efficiently deliver exogenous genes a variety of fetal tissues and induce durable tolerance to the vector-encoded gene product.²⁵⁰ This tolerance induction appears to involve both cellular and humoral mechanisms, since antibody and cellular responses to the transgene product were both significantly diminished in these animals, even several years after IUGT. Further mechanistic studies demonstrated that IUGT early in fetal development exploits both central and peripheral tolerogenic avenues in the fetus.²⁵¹ These results strongly imply that IUGT, even if not curative, would still be an ideal treatment modality for HA, since the induced immune tolerance would ensure that postnatal therapy, be it protein- or gene-based, could proceed safely without any of the immune-related problems that currently plague HA treatment.

Interestingly, although the incidence of HA is ~7 times that of HB, to-date, the only experimental studies to directly investigate IUGT for treating the hemophilias have targeted HB (factor IX (FIX) deficiency).^{156,157,169–171,173–175,264,265} The choice to target HB rather than HA most likely results from the greater ease with which FIX can be cloned into a variety of viral vectors, and efficiently expressed upon transduction of appropriate target cells; this is in marked contrast to the difficulties that were initially seen when attempting to express FVIII in the context of viral vectors.²⁶⁶ The treatment of HB by IUGT has been extensively studied in murine models with gene transfer performed at various gestational ages, via different routes of injection, and using different vector types. Schneider *et al.*¹⁷⁵ compared intraperitoneal, intramuscular, and intravenous injections of human FIX carried by adenovectors and AAV-2 into mouse fetuses and found that adenovectors resulted in initially higher levels of FIX. Interestingly, given their episomal nature, adenovector-injected mice maintained therapeutic levels of FIX for 6 months, and no antibodies developed against either vector or transgene. In other studies, Sabatino *et al.*²⁶⁷ reported low-level human FIX expression following intramuscular injection of fetal and neonatal mice with either AAV-1 or AAV-2. Curiously, the injection of AAV-1-induced tolerance and allowed the postnatal readministration of the FIX-encoding AAV-1 vector, increasing FIX levels sufficiently to reach the therapeutic range, while injection of AAV-2 did not induce immune tolerance.

Without a doubt, the most impressive and clinically promising results of IUGT in hemophilic mice were achieved by Waddington *et al.*¹⁷¹, who injected a FIX-encoding lentiviral vector into E15 mouse fetuses and demonstrated therapeutic levels of FIX (9–16% of normal) and improved coagulation for 14 months post-IUGT. Furthermore, no immune response developed to FIX, even when the protein was repeatedly injected postnatally.

Collectively, these murine studies have provided compelling evidence that IUGT can result in expression of FIX at levels that not only

have therapeutic significance but are often sufficient to induce tolerance, thus allowing postnatal administration of the same vector or the FIX protein without eliciting an immune response. Because HA patients have at least a 10-fold higher likelihood of developing inhibitors than HB patients,^{268,269} these studies, while encouraging, leave unanswered the critical question of whether fetal gene delivery's ability to induce immune tolerance to marker gene products and FIX will hold true for the induction of tolerance to FVIII, given FVIII's higher inherent immunogenicity. We are currently addressing this important question in the sheep model.

RISKS OF IUGT

Despite the great promise IUGT hold for the treatment of HA and the myriad other genetic diseases that can be diagnosed prenatally, several important safety concerns must be addressed prior to its clinical application. While the risks of postnatal gene therapy have been recognized and extensively discussed, specific risks may be higher for the fetus than for the postnatal recipient. There are two sets of potential safety concerns associated with IUGT: those associated with fetal intervention and those due to the gene transfer itself. As with any fetal intervention, infection, preterm labor, and fetal loss are all theoretically possible. In reality, however, a wealth of clinical data exist that provide unassailable proof that the early human fetus can be accessed multiple times with an extremely low procedure-related risk, assuming that a minimally invasive, ultrasound-guided approach is employed.^{13,25,117,135,139,140,143} However, one study has shown that AAV vectors administered to the fetus can cross the blood/placental barrier, enter the maternal circulation, and lead to transduction of multiple tissues within the mother.²⁶⁵ Since this study was performed in nonhuman primates, whose placentation is very similar to that of humans, this is an issue that will likely need to be explored in greater detail, and with other commonly employed vectors, to better define/quantitate the risk of inadvertent gene transfer to maternal tissues, and ascertain what risk, if any, this will pose to the mother.

The risks that cause the most concern regarding the use of IUGT include disruption of normal organ development, insertional mutagenesis, and germline transmission.²⁷⁰ Although IUGT holds great potential for restoring normal function, manipulating the fetus has the potential to alter normal organ development, and the possibility for deleterious effects due to the injection and from any inherent toxicity of the vector itself both need to be considered and carefully evaluated. Nonhuman primates injected with lentiviral vectors *in utero* via either the intrapulmonary or intracardiac route showed no adverse effects on postnatal heart and lung development.¹⁶⁷ In contrast, studies performed by Flake *et al.* found that expression of FGF-10 in the developing rat lung following IUGT leads to cystic adenomatoid malformations illustrating how forced expression of a specific transgene can lead to malformation.²⁷¹ These findings suggest that strategies involving expression of growth factors, transcription factors, or other regulatory molecules will need to be carefully examined, as they may have significant potential to alter normal organ development, particularly early in gestation.

Genomic integration-associated insertional mutagenesis

Insertional mutagenesis is a major concern with all of the integrating viral vectors and has been the subject of intense investigation since the clinical observation of four cases of T-cell leukemia, diagnosed 31–68 months after postnatal γ -retroviral-mediated gene transfer to autologous HSC to correct children with X-linked SCID. This concern was further heightened when linker-mediated

PCR analysis of lymphocytes from these patients revealed that insertional mutagenesis had occurred in all four cases and was at least partially responsible for the observed leukemogenesis^{272–274}; a subsequent study in which genotoxicity/leukemogenesis was also observed following γ -retroviral/HSC-based gene therapy to treat Wiskott–Aldrich syndrome²⁷⁵ further added to this concern. Importantly, in our long-term IUGT studies in fetal sheep, we also employed γ -retroviral vectors and achieved significant levels of gene transfer to hematopoietic cells, which persisted in these sheep throughout the 5-year course of study.^{163,189,258} Moreover, transgene-positive CD34+ cells could be detected in the marrow of these animals several years post IUGT,²⁵⁹ and gene-marked BM cells isolated from these IUGT recipients were able to serially engraft secondary fetal sheep recipients. These three pieces of data demonstrate that this approach resulted in gene transfer to *bona fide* HSC, yet we never observed leukemogenesis in any of these animals. Given that sheep have a life span of roughly 10 years, this study should more or less approximate a 35-year follow-up in “human years.” The difference between our study and the clinical trial (aside from the obvious species difference) that likely explains the differing outcome is the differing transgene. In our experimental proof-of-concept studies, we employed marker genes to facilitate tracking and quantitation of gene marking in various tissues. In the clinical trial for X-SCID, the vector encoded the therapeutic common gamma chain (γ C) gene (IL-2RG), as this was the gene defect causing X-SCID. Subsequent studies revealed that the observed leukemogenic event in these patients was likely the result of a combinatorial effect of both the insertion of the vector in close proximity to the LMO-2 gene (which has, itself, been associated with T-cell leukemias) and a growth advantage conferred on the transduced cells by the high expression levels of the therapeutic γ C gene.^{276–279} This clinical trial thus provides a valuable lesson in the complexities of risk assessment in gene therapy, which is still a relatively new and rapidly evolving field.

To-date, there has been only one report of oncogenesis after IUGT. In these studies, Themis, Waddington, Buckley, and colleagues reported a high incidence of postnatal liver tumors in mice following prenatal injection with a third-generation equine infectious anemia virus (a lentivirus) vector. These tumors were not seen in mice that received a very similar vector constructed on an HIV backbone.^{280–282} The authors did not identify the genomic insertion sites in these animals, so it remains unclear whether insertional mutagenesis was the cause of the observed tumor formation. Nevertheless, this important study demonstrates that the fetus may be particularly sensitive to tumorigenesis induced by certain vectors. The findings of Themis and the results of the clinical trial for X-SCID collectively suggest that preclinical assessment of the risk of insertional mutagenesis following IUGT will require very carefully designed studies with the actual vector to be employed for the pending clinical trial, in an animal model that has been thoroughly validated in the setting of the target disease.

Potential risk to fetal germline

While gene transfer to the vast majority of the fetal tissues would be desirable for correcting diseases, such as the hemophilias, that would benefit from widespread systemic release of a secreted transgene product, PCR of sheep that had received IUGT also revealed that the fetal reproductive tissues often contained the vector sequences, raising the troubling possibility that the vector may have reached the developing germline. Targeted gene therapy that occurs after the compartmentalization of primordial germ cells

should not affect the germline.¹⁵⁰ In the human fetus, the primordial germ cells are compartmentalized in the gonads at 7 weeks of gestation.¹⁵⁰ The germline should only be accessible through the vascular system, so targeted gene therapy that is administered after this time period should not affect the germline. Nevertheless, the possibility of inadvertent gene transfer to the germline is clearly a major safety concern and a bioethical issue, and our PCR results suggested that this critical issue needed to be investigated in greater detail.

Since prior studies had demonstrated that both the embryonic germline^{283–286} and isolated primordial germ cells²⁸⁷ can readily be infected with γ -retroviral vectors and pass the vector genetic material to subsequent generations in a Mendelian fashion as part of the permanent genome, we used three approaches to examine this important issue in detail: (i) immunohistochemical staining on tissue sections prepared from the *in utero* treated animals; (ii) genetic analysis on the sperm cells from the treated males; and (iii) breeding experiments in a limited number of animals.^{160,162,163,189} These studies indicated that although the fetal ovaries appeared to be largely unaffected by this approach to IUGT, numerous cells within the developing fetal testes were in fact modified, including interstitial cells, Sertoli cells, and small numbers of both immature germ cells within the forming sex cords and the resultant sperm cells.

Importantly, however, gene-modified germ cells were only observed in two of the six animals examined in our studies, and, in these two animals, the incidence of germ cell modification was roughly 1 in 6,250, a frequency that is well below the theoretical level of spontaneous mutation within the human genome.²⁸⁸ This low frequency of modification coupled with observations that genetic alterations to the germ cells may produce deleterious effects, placing them at a disadvantage during fertilization, suggest that the likelihood that any genetic alterations present would be passed to subsequent offspring would be extremely unlikely. In agreement with this supposition, we did not observe transfer of the vector sequences in any of the 10 offspring we studied, even when both the parents had received IUGT. This is clearly an issue that will need to be addressed in greater detail, nevertheless, prior to moving *in utero* gene therapy into clinical trials. This need for further investigation is underscored by the fact that, in other studies, employing lentiviral vectors in nonhuman primates, Tarantal *et al.* observed modification of the female germline, but no effect upon the male germ cells.¹⁵⁹ Thus, the issue of germline safety will likely have to be investigated in more than one preclinical model, employing the specific vector being considered for clinical use, in order to obtain an accurate assessment of the risk posed by the procedure.

While these studies in different animal models both suggest that the frequency of germline transduction is low and related to gestational age and mode of vector administration, they also suggest that low-level transduction of germ cells after systemic administration of integrating vector to the fetus may not be entirely avoidable. As such, when contemplating ultimate clinical application of IUGT, careful consideration may need to be given to determining what frequency of potential germline transduction is considered acceptable in the context of treating a severe, perhaps life-threatening genetic disorder.

Genome editing

The field of gene therapy is rapidly advancing, and the development of gene-editing technologies such as zinc finger nucleases, TAL effector nucleases, and CRISPR/Cas^{289,290} has the potential to revolutionize the whole way in which gene therapy is conceptualized. The ability to modify a chosen sequence in its native genomic

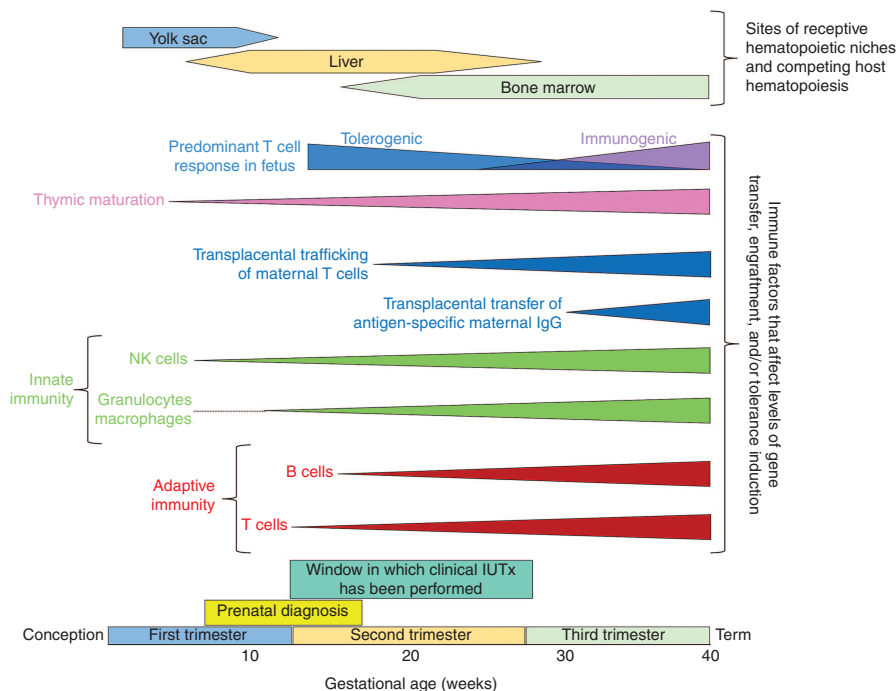


Figure 1 Overview of hematopoietic and immune events impacting IUTx and IUGT.

locus offers incredible advantages, in terms of both safety and efficacy, over current “gene-addition,” and should largely eliminate existing concerns related to random genomic integration, inappropriate levels, or tissue distribution of transgene expression, and inadvertent germline alteration. As such, one would anticipate that these newer gene-editing technologies are likely to be a key component of future IUGT studies/trials. These systems will, however, likely introduce their own unique set of risks/concerns, and further study will be required for us to define and fully understand what these risks may be and evaluate whether the benefits these systems can offer over “traditional” viral vectors outweighs these risks. In addition, well-designed experimental studies in suitable preclinical models will be required to determine whether these genome-editing systems should be administered directly to the recipient to mediate gene-correction (which will likely require the use of viral vectors to achieve sufficient efficiency), or if modifying suitable cell populations *in vitro*, followed by the infusion/transplantation of these gene-corrected cells into the recipient, will prove to be the safer approach to moving these technologies towards clinical application.

CONCLUSIONS AND FUTURE DIRECTIONS

Although great progress has been made, there are many remaining hurdles for IUTx and IUGT to overcome before they become mainstream clinical modalities. A graphical overview of some of the main factors during fetal hematopoietic/immune ontogeny that affect the levels of donor cell engraftment and gene transfer and, and govern whether IUTx and/or IUGT will successfully induce immune tolerance appears in Figure 1. Key developmental events are superimposed upon a gestational timeline that includes indications as to when prenatal diagnosis is possible and the developmental window during which clinical IUTx trials to-date have been performed. Challenges for IUTx are primarily related to overcoming the competitive barriers to engraftment in the fetus, and better defining the innate and adaptive immune limitations to engraftment in large animals and

humans. As our understanding of stem cell biology and the ontogeny of hematopoiesis and the hematopoietic niche ontogeny advance, the therapeutic applications of IUTx will likely expand from their current narrow focus to include the treatment of nonhematopoietic diseases. While the strategy of prenatal tolerance induction for facilitation of postnatal HSC transplantation is nearing clinical application and has great potential to benefit many patients, the development of an IUTx strategy that allowed a single-step treatment to achieve therapeutic levels of engraftment would be ideal and would likely propel this promising therapy into the clinic.

IUGT holds even greater promise for treating/curing essentially any inherited genetic disease. From our findings in the sheep model and those of other groups exploring IUGT in sheep, mice, and nonhuman primates, it is clear that the direct injection of viral vectors into the developing fetus can be an effective way of delivering an exogenous gene and achieving long-term expression in multiple tissues, suggesting that IUGT may one day be a viable therapeutic option for diseases affecting any of the major organ systems. Moreover, even if not curative, IUGT would be ideal for a disease like HA, since lifelong immunologic tolerance could be induced to FVIII, thus overcoming the immune-related hurdles that currently hinder postnatal treatment of this disease. Despite its great potential, however, it is important to realize that IUGT is still in the experimental stages and several important safety concerns need to be extensively investigated in appropriate preclinical animal models prior to commencing application in human patients.

At present, IUTx and IUGT stand at a critical juncture¹⁴⁵ and have vast potential for dramatically improving human healthcare. Many of the most daunting obstacles have recently been overcome in animal models, or are at least better understood, which has reinvigorated this exciting field. There is no doubt that surpassing the few remaining hurdles to allow clinical implementation of these therapies will dramatically change the whole paradigm for the way we perceive and treat many genetic disorders.

ACKNOWLEDGMENTS

The authors gratefully acknowledge support from grants R01-HL097623 and R21-HL117704 from National Institutes of Health (NIH), grant NNX13AB67G from National Aeronautics and Space Administration (NASA), the Intramural Pilot Program of Wake Forest School of Medicine, and Wake Forest University Health Sciences Translational Science Institute 20667 Clinical Pilot Award.

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