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Treatment of Hemophilia A in Utero and Postnatally using Sheep as a Model for Cell and Gene Delivery

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Abstract

Hemophilia A represents the most common inheritable deficiency of the coagulation proteins. Current state-of-the-art treatment consists of frequent prophylactic infusions of plasma-derived or recombinant FVIII protein to maintain hemostasis, and has greatly increased life expectancy and quality of life for many hemophilia A patients. This treatment approach is, however, far from ideal, due to the need for lifelong intravenous infusions, the high treatment cost, and the fact that it is unavailable to a large percentage of the world's hemophiliacs. There is thus a need for novel treatments that can promise long-term or permanent correction. In contrast to existing protein based therapeutics, gene therapy offers to provide a permanent cure following few, or even a single, treatment. In the present paper, we review ongoing work towards this end, focusing on studies we have performed in a large animal model. Some of the key topics covered in this review include the unique opportunities sheep offer as a model system, the re-establishment and clinical and molecular characterization of a line of sheep with severe hemophilia A, the advantages and feasibility of treating a disease like hemophilia A in utero, and the use of Mesenchymal Stem Cells (MSC) as cellular delivery vehicles for the FVIII gene. The review finishes with a brief discussion of our recent success correcting ovine hemophilia A with a postnatal transplant with gene-modified MSC, and the limitations of this approach that remain to be overcome.

Hemophilia A and the Need for Better Treatments

Hemophilia A represents the most common inheritable deficiency of the coagulation proteins [1]. The severity of hemophilia A is traditionally based on plasma levels of FVIII, with persons exhibiting less than 1% normal factor (< 0.01IU/mL) being considered to have severe hemophilia, persons with 1-5% normal factor moderately severe, and persons with 5%-40% of the normal FVIII levels mild [2-4]. Up to 70% of hemophilia A patients present with the severe form of the disease, and suffer from frequent hemorrhaging, leading to chronic debilitating arthropathy, hematomas of subcutaneous connective tissue/muscle, and internal bleeding. Over time, the collective complications of recurrent hemorrhaging result in chronic pain, absences from school and work, and permanent disability [2]. Current state-of-the-art treatment consists of frequent prophylactic infusions of plasma-derived or recombinant FVIII protein to maintain hemostasis, and has greatly increased life expectancy and quality of life for many hemophilia A patients.

This treatment approach is, however, far from ideal, due to the need for lifelong intravenous infusions and the high treatment cost. Moreover, this treatment is unavailable to a large percentage of the world's hemophiliacs, placing these patients at great risk of severe, permanent disabilities and life-threatening bleeds. Furthermore, even among the patients who are fortunate enough to have access to, and the financial means to afford, prophylactic FVIII infusions, approximately 30% will form FVIII inhibitors [5]. The formation of these inhibitors greatly reduces the efficacy of subsequent FVIII infusions, and can ultimately lead to treatment failure, placing the patient at risk of life-threatening hemorrhage. There is thus a significant need to develop novel, longer-lasting hemophilia A therapies.

In the past three decades, the remarkable progress in the understanding of the molecular basis of the disease, the identification and characterization of FVIII gene, structure, and biology has heightened the interest and feasibility of treating hemophilia A with gene therapy. In contrast to current protein-based therapeutics, lifelong improvement or permanent cure of hemophilia A is theoretically possible after only a single gene therapy treatment; indeed, several aspects of hemophilia A make it ideally suited for correction by gene therapy [6-14]. First, in contrast to many other genetic diseases, the missing protein (coagulation FVIII) does not need to be expressed in either a cell or tissue specific fashion to mediate correction. Although the liver is thought to be the primary natural site of synthesis of FVIII, expression of this factor in other tissues exerts no deleterious effects. As long as the protein is expressed in cells which have ready access to the circulation, it can be secreted into the bloodstream and exert its appropriate clotting activity. Second, even modest levels (3-5%) of FVIII-expressing cells would be expected to convert severe hemophilia A to a moderate/mild phenotype, reducing or eliminating episodes of spontaneous bleeding and greatly improving quality of life. Thus, even with the low levels of transduction that are routinely obtained with many of the current viral-based gene delivery systems, a marked clinical improvement would be anticipated in patients with hemophilia A. Conversely, even supra physiologic levels of FVIII as high as 150% of normal are predicted to be well tolerated, making the therapeutic window extremely wide [4]. Based on this knowledge, the American Society of Gene and Cell Therapy (www.ASGCT.org) recently provided NIH director, Dr. Francis Collins, with a roadmap of disease indications that it feels will be viable gene therapy products within the

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next 5-7 years. The hemophilias were identified as belonging to the most promising, "Target 10", group of diseases.

Sheep as a Preclinical Model of Hemophilia A

A number of animal models have been developed to evaluate new methods of not only treatment of coagulation disorders, but also the prevention and treatment of inhibitor formation. Transient hemophilic rabbit models induced by infusion of plasma containing inhibitors have been used to evaluate the effect of different bypass products to factor VIII [15], but these models, while valuable for inhibitor studies, do not accurately recapitulate the human disease, precluding their use for gene therapy studies. Fortunately, dog models of hemophilia A with congenital deficiency [16,17] and mouse models obtained by gene targeting and knockout technology [18] are available to study FVIII function and gene therapy approaches for treating hemophilia A. Therapeutic benefit has been obtained in numerous studies using a variety of vector systems in the murine model [9,10,19-25], and phenotypic correction of hemophilia A in the dog has been achieved, but has proven to be far more difficult than in mice [26,27]. Despite promising results in both canine and murine models, however, no clinical gene therapy trial has shown phenotypic/clinical improvement of hemophilia A in human patients. This is in marked contrast to the recent clinical successes with gene therapy for hemophilia B [28]. The reasons for the disparity in the efficacy of gene therapy for treating hemophilia A versus B is not presently clear. Nonetheless, based on the disappointing results to-date, there is currently no active hemophilia A clinical gene therapy trials, even though hemophilia A accounts for roughly 80% of all cases of hemophilia.

The difficulties seen thus far translating success in animal models into therapeutic benefit in human patients underscores the importance of preclinical animal models that both precisely mimic the disease process of hemophilia A, and closely parallel normal human immunology and physiology. To this end, between 1979 and 1982, a number of male offspring of a single white alpine ewe at the Swiss Federal Institute of Technology all died several hours post-partum due to severe bleeding from the umbilical cord [29-31]. Daughters and granddaughters of this ewe also gave birth to lambs exhibiting the same pathology. Investigation of the affected animals showed extensive subcutaneous and intramuscular hematomas. Spontaneous hemarthroses were also frequent, leading to reduced locomotion and symptoms of pain in standing up, restricting nursing activity. Stronger injuries that arose when animals were not placed in carefully controlled isolation resulted in heavy bleeding and intensive pain. Laboratory tests showed increased PTT, and FVIII levels (as assessed by aPTT) of about 1% of control animals. Replacement therapy with human FVIII (hFVIII) concentrate or fresh sheep plasma resulted in remission of disease and rapid clinical improvement.

Unfortunately, due to the expense and effort of maintaining these sheep, the Swiss investigators allowed the line to die out, saving only a few straws of semen prior to allowing this valuable resource to pass into extinction. We recently used a variety of reproductive technologies to successfully re-establish this line of hemophilia A sheep, we fully characterized both the clinical parameters and the precise molecular basis for their disease, and we developed a PCR-based screen for the disease-causing mutation that allows identification of affected animals in utero [32-37]. In similarity to mutations seen in many human patients [38], these animals possess a premature stop codon with a frame shift mutation. This is the only animal model of hemophilia A with this clinically relevant mutation-type, providing a unique opportunity to study therapies in this context. All ten animals to date have exhibited bleeding from the umbilical cord, prolonged tail and nail cuticle bleeding time, and multiple episodes of severe spontaneous bleeding including hemarthroses, muscle hematomas, and hematuria, all of which have responded to human FVIII concentrate. Just like human patients with severe hemophilia A, a hallmark symptom in these sheep is repeated spontaneous joint bleeds, which lead to chronic, debilitating arthropathies and reduced mobility. Importantly, chromogenic assays performed independently at the Blood Center of Wisconsin and Emory University revealed undetectable FVIII activity in the circulation of these sheep, explaining their severe phenotype.

In addition to the value of another large animal model of hemophilia A and the uniqueness of the mutation, sheep possess many characteristics that make them an ideal preclinical model for gene therapy. The first of these is the size. Sheep are fairly close in size to humans, weighing roughly 8lbs at birth and 150-200lbs as adults, likely obviating the need for scale-up of vector dose to move from experiments in sheep to trials in humans. This is in marked contrast to mice which are ~2800 times smaller than a typical human patient [39]. Of course, the large size of the sheep also carries with it the inherent cost to generate a sufficient volume/quantity of the novel therapeutic to be tested, making the sheep's size a double-edged sword. Secondly, sheep share many important physiological and developmental characteristics with humans; for example, the pattern of fetal to adult hemoglobin switching, and the naturally occurring changes in the primary sites of hematopoiesis from yolk sac to fetal liver and finally to the bone marrow near the end of gestation. In addition, in contrast to other large animal models such as dog and pig, sheep, like humans, typically have singleton or twin pregnancies rather than large litters of offspring. It is thus not surprising that fetal sheep have been used extensively in the study of mammalian fetal physiology, and results obtained with this model have been directly applicable to the understanding of human fetal growth and development. Thirdly, sheep are out bred, and thus represent a wide spectrum of genetic determinants of the immune response, as do humans. As the immune response to both the vector and the vector–encoded FVIII are likely to play a key role in FVIII inhibitor formation (or lack thereof), this represents an advantage not found in most other models, with the possible exception of the dog, which could conceivably be out bred as well to achieve a broader genetic spectrum. This wider genetic spectrum could, however, also introduce greater animal-to-animal variability, potentially necessitating the use of larger numbers of animals per group to achieve statistical significance. In addition, the development of the sheep immune system has been investigated in detail [40-46], making sheep well suited for studying the immunological aspects of gene therapy for hemophilia A. Importantly, the large size of the sheep, their long life span (9-12 years), and their relative ease of maintenance and breeding make it possible to conduct the long-term studies in large numbers of animals that are necessary to fully evaluate the efficacy and safety issues related to gene therapy. For these reasons, we feel that the sheep are a particularly relevant model in which to examine gene therapy for hemophilia A. An additional unique advantage to using sheep to study hemophilia A treatment is that in sheep, like human, a large percentage of the Von Willebrand Factor (VWF) is found within platelets rather than free in plasma. This is in contrast to dog (in which Von Willebrand Factor (VWF) circulates free in plasma [47,48]), and makes the sheep an ideal large animal model in which to explore the use of platelet-targeted gene therapy for hemophilia A [14,49-51].

Rationale for Treating Hemophilia A before Birth

Even if the cost of current hemophilia A therapies were reduced sufficiently that the majority of patients could afford prophylactic FVIII infusions, these protein-based therapies would still require frequent, lifelong intravenous infusions, and would still be plagued by a high rate of treatment failure due to inhibitors. These problems could likely be circumvented by performing gene therapy before birth. Similarly, many of the hurdles that have thus far prevented gene therapy from curing patients with hemophilia A, or many of the other diseases that have been investigated, could likely be circumvented by performing in utero gene therapy. At the present time, hemophilia A, and many of the other diseases considered as candidates for gene therapy, can be diagnosed relatively early in gestation, making it feasible to begin devising methods for performing gene therapy in utero rather than waiting until after birth. Methods for accessing both the sheep and the human fetus are well established and clinically viable. Indeed, fetal transfusions and in utero stem cell-based therapies have safely been performed clinically for decades [52,53]. To date, 46 in utero transplants have been performed in human patients [54,55], for 14 different genetic disorders [53], and have proven that accessing the early human fetus multiple times, using a minimally invasive, ultrasound guided approach, poses minimal procedure-related risk [52-54,56-59]. Importantly, experience and knowledge gained from studies performed in the fetal sheep model were used to design and perform the first curative human in utero transplantation for X-SCID [56], highlighting the value of the fetal sheep model for not only developing clinically viable methodology, but also for predicting clinical outcome. Using these established clinically applicable methodologies to perform gene therapy early in gestation could correct hemophilia A prior to parturition, promising the birth of a normal healthy baby who, ideally, would require no further treatments. While most individuals with a family history of hemophilia A are encouraged to have prenatal screening, parents currently have only 2 choices following prenatal diagnosis of hemophilia A: termination of pregnancy or the birth of an affected child. The availability of a safe and effective in utero treatment would provide parents with a much-needed 3rd option that could promise the birth of a healthy infant who required no further treatments. This opportunity would undoubtedly fuel much more widespread prenatal screening for HA. Although in vitro embryo screening and selection is a possible solution, this option is not widely available due to both its high cost and the lack of the required technology in developing countries. In utero gene therapy, in contrast, does not require any sophisticated equipment that would not already be in place for prenatal diagnosis. Indeed, several recent studies have now conclusively demonstrated the marked cost-effectiveness of prenatal screening for the hemophilias, even within developing third world countries [60-62]. Looking specifically at the US, according to the CDC, 320 babies are born with hemophilia A each year. The ability to correct this disease prior to birth could thus benefit the ~240 patients each year born into families with a history of hemophilia A. In addition to the clinical and financial advantages of performing gene therapy prior to birth, numerous aspects of the developing fetus make it a better recipient than the adult [63-65]. For example, due to their ability to integrate into the genome of the host cell, γ-retroviruses and lenti-viruses 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 cell populations in the adult are relatively quiescent unless injury is used to induce cell cycling. In the case of hemophilia A, the primary site of FVIII synthesis under normal physiologic conditions is the liver [66]. Yet, in a mature animal, it is estimated that only 1 in 10,000 hepatocytes is actively cycling at any given time [67], making it very difficult to obtain meaningful levels of gene transfer unless the gene delivery system mediates high efficiency transduction of quiescent cells, or injury such as partial hepatectomy is employed to induce cell division to enhance transduction and/or drive expansion of the limited numbers of transduced cells, as was done in dogs with hemophilia B [68]. In the fetus, the cells in all of the organs are actively cycling to support the continuous expansion that occurs throughout gestation. Thus, cells such as hepatocytes 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 offers the possibility of achieving expansion of the gene-corrected cells during the remainder of gestation, such that initial transduction of even small numbers of target cells should lead to significant levels of gene-correction by birth. Importantly, even in the event of extremely high levels of transduction, supraphysiologic levels of FVIII as high as 150% of normal are predicted to be well tolerated, making the therapeutic window extremely wide [4]. Nevertheless, the sheep model provides a unique system in which to determine the potential for toxicity as a result of high-level expression of FVIII and optimize the dosing levels prior to considering attempting in utero clinical trials for hemophilia A. Remaining cognizant of the immune-aspects of hemophilia A treatment, it is important to note that, in addition to the ability to target cells which are largely refractory to transduction in the adult, unique immunologic advantages also exist for performing gene therapy in utero. There is a window of time in early immunologic development, before thymic processing of mature lymphocytes, during which the fetus is largely tolerant of foreign antigens. Exposure to foreign antigens during this period often results in sustained tolerance, which can become permanent if the presence of the antigen is maintained [69]. Given these unique immunological advantages suggest that in utero gene therapy would be an ideal approach for treating hemophilia A, since lifelong tolerance could theoretically be induced to FVIII. This would thus ensure that, even if in utero gene therapy was not curative, postnatal gene therapy or protein replacement could proceed safely without the risk of inhibitor formation.

Experimental in Utero Gene Therapy Studies

With the knowledge that performing gene therapy in utero would provide these advantages over existing post-natal approaches, we have spent the last two decades using the sheep model to investigate whether it is possible to exploit the highly proliferative state and relative immuno-naïveté of the early gestational fetus to achieve significant levels of gene transfer following a single intraperitoneal injection of a γ-retroviral vector [63,70-79]. This approach to in utero gene therapy is safe and technically simple, involving only a single ultrasound-guided injection into the peritoneum of the fetus, as early as 54 days of gestation (term: 145 days). This approach resulted in gene transfer levels as high as 5-6% within the hematopoietic system [73,78-80], a level that would certainly be therapeutic in hemophilia A. Importantly, these gene-marked hematopoietic cells persisted in these sheep over the course of 5 years of study [78,79], transgene-positive $CD34^+$ cells could be detected in the bone marrow of these animals several years post in utero gene transfer [81], and bone marrow cells isolated from

these recipients successfully engrafted the hematopoietic system of secondary fetal sheep recipients upon re-transplantation. These three pieces of data provide compelling evidence that this approach enabled us to successfully insert genes into true hematopoietic stem cells, suggesting this method could provide lifelong genetic correction. While transduction of clinically significant levels of HSC within these sheep following a single injection of vector into the peritoneal cavity hinted at the therapeutic potential of this simple approach to in utero gene therapy, the retroviral vectors we employed in these studies did not possess any type of targeting moiety which would restrict transduction to cells of the hematopoietic system. Not surprisingly, examination of other tissues of the recipients revealed that gene transfer was not limited to cells of the hematopoietic system, but had occurred in essentially all of the organs examined, including numerous cell types within the liver, lung, and brain [76,78,79,82]. Our results also revealed transduction of hepatocytes and hepatic endothelial cells (the cells thought to be the natural site of FVIII synthesis/production within the body) at levels that would likely be therapeutic in hemophilia A, and delineated the time period during gestation when hepatic transduction is optimal [76]. Concomitantly, in utero gene transfer studies performed by other investigators in sheep, rodent, and non-human primate models employing a variety of viral-based gene delivery vectors produced similar results [11,63,76-78,83-100], supporting the notion that this method could be used to deliver FVIII to the developing liver at levels that would covert patients with severe hemophilia A to a moderate or even mild phenotype [76]. Moreover, since tissue-specific expression is not necessary for FVIII, the transfer of this gene into a wide range of tissues with ready access to the circulation, followed by long-term expression, would greatly enhance the therapeutic potential of this approach for treating/curing hemophilia A. As discussed previously, one of the major hurdles hindering treatment of hemophilia A by factor replacement therapy is the formation of inhibitory antibodies that can occur in roughly 30% of patients with repeated FVIII infusions. While analyzing the tissues from the sheep that received in utero gene transfer, we noted that the thymus frequently exhibited transgene positivity by PCR [78, 79]. Given the pivotal role of the thymus during the development of the fetal immune system's ability to distinguish self from non-self, we undertook studies to ascertain the immunologic significance of the presence of these transgene-positive cells within the thymus. In our first set of studies, [101] we demonstrated that in utero gene transfer successfully induced durable immune tolerance to the vector-encoded β-galactosidase. This tolerance induction appeared to involve both cellular and humoral mechanisms, since both antibody responses and cellular responses to the transgene product were blunted in these animals even several years after in utero gene transfer. Further mechanistic studies demonstrated that performing in utero gene transfer early in gestation takes advantage of multiple tolerogenic avenues present in the fetus, since it results in the transduction of both thymic epithelial cells, which may promote induction of central immune tolerance, and cells of Hassall's corpuscles, which can instruct dendritic cells to induce Tregs that can help maintain peripheral immune tolerance to the transgene products. These findings thus suggest that, even if not curative, in utero gene therapy would be ideal for hemophilia A, since lifelong tolerance could be induced to FVIII, thus overcoming the immune related hurdles that currently hinder post-natal treatment of this disease. Interestingly, the only studies that have thus far explored the possibility of performing in utero gene therapy for the treatment of the hemophilias have been aimed at correcting hemophilia B (factor IX deficiency) [11, 83, 84, 95-97, 99, 100, 102, 103], likely due to difficulties encountered in initial attempts to express FVIII as a transgene in the context of viral vectors [104]. Given the fact that patients with hemophilia A are >10x's as likely to develop inhibitory antibodies to the exogenous coagulation factor as patients with hemophilia B [105, 106], these studies thus leave unanswered the critical question of whether the ability to induce immune tolerance to marker gene products and FIX in utero will ultimately translate into the ability to induce tolerance to FVIII, given FVIII's higher inherent immunogenicity. Studies are ongoing in the sheep model to address this important issue. While gene transfer to multiple fetal tissues would be desirable for correcting a disease such as hemophilia A, that would benefit from widespread systemic release of a secreted transgene product, our analyses also revealed that the fetal reproductive tissues often contained proviral DNA, raising the troubling possibility that the developing germline had been modified as a result of in utero gene therapy. Since prior studies had demonstrated that both the embryonic germline [107-110] and isolated Primordial Germ Cells (PGC) [111] can readily be modified with murine retroviral vectors and pass the vector genetic material to subsequent generations in a Mendelian fashion, we rigorously addressed the risk to the germline in the fetal sheep model [70,77,87,112]. Although the fetal ovaries appeared to be unaffected by this approach to in utero gene transfer, we found that numerous cells within the developing testes were in fact modified, including small numbers of immature germ cells within the forming sex cords and the resultant sperm cells. Importantly, however, gene-modified germ cells were only observed in 2 of the 6 animals examined, and, in these two animals, the incidence of germ cell modification was roughly 1 in 6250, a frequency that is well below the theoretical level of spontaneous mutation within the human genome [113]. 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. Intriguingly, other studies employing lentiviral vectors in non-human primates revealed modification of the female germline, but no effect on male germ cells [86]. Thus, the issue of germline safety will likely have to be investigated in more than one preclinical model, and the specific vector being considered for clinical use will have to be employed, in order to obtain an accurate assessment of the risk posed by the procedure. This multi-species data could then be presented to the FDA to determine whether whatever risk exists to the developing germline is outweighed by the potential benefits of intervening in utero.

The ultimate development of vectors that can target specific cell types following in vivo administration will ultimately make it possible to eliminate the risk of inadvertently modifying non-target cells, like those of the germline, following a direct vector injection approach to in utero gene therapy. Given the current absence of such vectors, and our desire to develop safer means of correcting hemophilia A and other diseases prior to birth, we have been exploring the possibility that mesenchymal stem cells can be used as vehicles to deliver exogenous genes to the developing fetus or neonate.

Mesenchymal Stem Cells (MSC) as Hemophilia A Therapeutics

In pioneering studies [114,115] performed over 30 years ago, Friedenstein demonstrated that fibroblastoid cells obtained from the bone marrow were capable of transferring the hematopoietic microenvironment to ectopic sites, thus establishing the concept that the marrow microenvironment resided within the so-called marrow

stromal cells. Years later, scientists began to explore the full potential of these microenvironmental cells, and results of these studies led to the realization that this population harbored cells with properties of true stem cells, now alternately referred to as marrow stromal cells or Mesenchymal Stem Cells (MSC) [116]. MSConly comprise roughly 0.001 0.01% of cells within the marrow [117], but can be passaged extensively in vitro without a loss of differentiative potential, making it possible to readily generate clinically relevant numbers of these cells [118]. Although much of the work to date has focused on MSC isolated from adult bone marrow, cells that appear phenotypically and functionally to be MSC have now been isolated by our group and others from numerous tissues, including brain, liver, lung, fetal blood, umbilical cord blood, kidney, and even liposuction material [119-126]. MSC have also been isolated from the amniotic fluid and the chorionic villi, making it possible to begin envisioning the use of autologous MSC as cellular therapeutics or gene delivery vehicles for in utero therapy [127-132].

Importantly, although MSC from each of these tissues appear similar with respect to phenotype and overall differentiative potential, studies at the RNA and protein level have now revealed that subtle differences exist between MSC from these various tissues, with MSC from each tissue possessing a molecular fingerprint indicative of their tissue of origin [121,122,133-137]. Using the fetal sheep model, we showed that these differences result in a bias for human MSC to home to and give rise to cells of their tissue of origin in vivo [138,139], suggesting that utilizing the appropriate source of MSC may make it possible to tailor the site(s) of engraftment. We and others have devoted a great deal of energy to demonstrating the ability of MSC from various sources to serve as therapeutics for liver disease [139-169]. It is now clear that, not only do MSC have the ability to generate, in vitro and in vivo, cells which are indistinguishable from native hepatocytes, but transplantation of MSC in a range of model systems can result in fairly robust formation of hepatocytes which repair a variety of inborn genetic defects, toxin induced injuries, and even fibrosis. The fetal sheep model provides a unique system in which to explore the full differentiative potential of various stem cell populations, since the continuous need for new cells within all of the organs during fetal development provides a permissive milieu in which gene-modified donor cells can engraft, proliferate, and differentiate. Furthermore, by performing the transplant at a stage in gestation when the fetus is considered to be largely immuno-naïve, it is possible to engraft human cells at significant levels, which persist for the lifespan of the animal due to induction of donor-specific tolerance [144-146]. Indeed, in ongoing studies, we have found that, after transplantation into fetal sheep, human MSC engraft at levels of up to 12% within the recipient liver [140,145,146,170-175], and contribute to both the parenchyma and the perivascular zones of the engrafted organs, placing them ideally for delivering FVIII into the circulation. Since FVIII levels of 3-5% of normal would convert a patient with severe hemophilia A to a moderate or mild phenotype, these levels of engraftment should be highly therapeutic. Given that the liver is thought to be the primary site of FVIII within the body, these collective results suggest that MSC may represent an ideal cell type for treating hemophilia A. However, although MSC engrafted at significant levels within organs that are natural sites of FVIII synthesis, only a small percent expressed endogenous FVIII, suggesting that simply transplanting "healthy' MSC will not likely provide an effective means of treating hemophilia A. By using gene therapy to engineer MSC to express FVIII, however, it is highly probable that the levels of engrafted MSC we have thus far achieved in utero could provide therapeutic benefit in hemophilia A. Unlike hematopoietic stem cells which are difficult to efficiently modify with most viral vectors while preserving their in vivo potential, MSC can be readily transduced with all of the major clinically prevalent viral vector systems including those based upon adenovirus [177-179], the murine retroviruses [179-183], lentiviruses [183-188], and AAV [189,190], and efficiently produce a wide range of cytoplasmic, membrane-bound, and secreted protein products. Furthermore, human MSC are stable in culture, do not undergo transformation, and do not form tumors in vivo (in contrast to murine MSC). By transducing the MSC in vitro, rather than injecting the vector directly, there is no risk of off-target transduction, and the vector being employed simply needs a strong constitutively active promoter to ensure that all cells derived from the transplanted MSC continue to express the FVIII transgene and mediate a therapeutic effect. Importantly, the only documented cases of retroviral-induced insertional mutagenesis have been observed following genetic modification of hematopoietic stem cells [191-193]. There is no evidence that MSC transform or progress to clonal dominance following transduction, and recent studies have shown that even if genomic instability is intentionally induced, MSC undergo terminal differentiation rather than transformation [194]. Critical proof-of-principle studies have already shown that MSC can be transduced with FVIII-expressing viral vectors and secretes high levels of FVIII protein. FVIII purified from the conditioned medium of the transduced MSC was proven to have a specific activity, relative electrophoretic mobility, and proteolytic activation pattern that was virtually identical to that of FVIII produced by other commercial cell lines [195]. Given the widespread distribution and engraftment of MSC following their systemic infusion, the ability of MSC to give rise, in vivo, to cells of numerous tissue types, and their ability to efficiently process and secrete high amounts of biologically active FVIII, they are, not surprisingly, being viewed as ideal vehicles for delivering a FVIII transgene throughout the body and thus providing long-term/permanent correction of hemophilia A [195-197]. In addition to their widespread engraftment and their ability to serve as delivery vehicles for the FVIII gene, the rather unique immunological properties of MSC may further increase their utility for treating hemophilia A. MSC do not normally express MHC class II or the co stimulatory molecules CD80 and CD82, unless they are stimulated with IFN-γ, and are thus known to be relatively hypo-immunogenic. As such, they do not provoke the proliferation of allogeneic lymphocytes or serve as very effective targets for lysis by cytotoxic T cells or NK cells. In fact, a large body of evidence is now accumulating that MSC can be readily transplanted across allogeneic barriers without eliciting an immune response [198,199]. Thus, if one wished to use MSC to treat hemophilia A, off-the-shelf MSC from an unrelated donor could theoretically be used, greatly increasing the feasibility of obtaining and using these stem cells for therapy. This property may also be important in the context of in utero therapies, given recent studies by Mackenzie and Flake showing that, while the fetal immune system has been presumed to still be largely naïve at the time of in utero transplant, both the fetal and the maternal immune system appear, at least within the mouse, to have the ability to negatively impact upon the engraftment of allogeneic cells [200,201]. Perhaps even more important from the standpoint of their potential use as hemophilia A therapeutics, more recent studies have provided evidence that MSC also appear to have the ability to exert both immunosuppressive and anti-inflammatory properties both in vitro and in vivo. These properties appear to result from MSC's ability to intervene, at multiple levels, with the generation and propagation of an immune response. To name just a few examples,

MSC have been demonstrated to interfere with the generation and maturation of cytotoxic and helper T cells [202-211], dendritic cells [212-215], and B cells [216]. In addition to actively shutting down the generation of immune effector cells, MSC also have the ability to induce the formation of potent Tregs [118,217-219]. MSC are also known to express an arsenal of factors [118,205-207,217,220-224] that reduce local inflammation, blunt immune response, and counteract the chemotactic signals released to recruit immune cells to the site of injury/inflammation. It is thus tempting to speculate that these immune-dampening properties could enable the delivery of FVIII without eliciting an immune response and subsequent inhibitor formation, thus overcoming one of the major hurdles to plague current treatment/management of hemophilia A.

As will be discussed in the next section, however, our postnatal studies in the hemophilic sheep suggest that further work will be required to discover how to obtain these potential immune benefits in the context of the ongoing injury/inflammation present in animals/patients with clinically advanced hemophilia A.

In addition to the aforementioned properties, preclinical animal studies examining the potential of MSC isolated from adult tissues have also highlighted another interesting and clinically valuable characteristic of MSC; their ability to selectively navigate to sites of injury and/or inflammation within the body. Once reaching these specific sites, the MSC then mediate repair both by engrafting and generating tissue-specific cells within the injured tissue [225-227], and by releasing trophic factors that blunt the inflammatory response and often promote healing by activating the tissue's own endogenous repair mechanisms. While the mechanisms responsible for this trafficking to sites of injury are currently not well understood, this observation raises the exciting possibility that, following systemic infusion, FVIII-expressing MSC could efficiently migrate to sites of active bleeding/injury, thereby focusing the therapy where it is most needed. As will be discussed in the next section, our postnatal studies in the Hemophilia C sheep support this conclusion.

Need for Postnatal Strategies and Success with Novel Treatment

Despite the multiple advantages of early intervention, roughly 16,000 people within the US are already living with hemophilia A, and could thus not benefit from an in utero therapy. In addition, over 25% of hemophilia A-causing mutations arise *de novo* with no family history, making it unlikely this patient population would be screened prenatally. Even when diagnosis is made prenatally, the minimal risks and/or ethical issues associated with in utero therapy may be unacceptable to the parents. As such, the ability to treat postnatally is indispensible for a universal approach that could be applied to and benefit all hemophilia A patients. For this reason, we have begun exploring whether it is still possible to exploit the many advantages of MSC as a cellular vehicle for delivering a FVIII gene if these FVIII-expressing MSC are transplanted during early childhood, rather than in utero. To directly address this question, we recently tested a novel, non-ablative transplant based gene therapy in 2 pediatric hemophilia A lambs. During the first 3-5 months of life, both these animals had received frequent, on-demand infusions of human FVIII for multiple hematomas and chronic, progressive, debilitating hemarthroses of the leg joints which had resulted in severe defects in posture and gait, rendering them nearly immobile. In an ideal situation, one would use autologous cells to deliver a FVIII transgene, and thus avoid any complications due to MHC-mismatching. Unfortunately, the severe life-threatening phenotype of the hemophilia A sheep prevented us from collecting bone marrow aspirates to isolate autologous cells. We therefore elected to utilize haploidentical allogeneic cells from the ram that had sired the two hemophiliac lambs, hoping that, by using paternal (haploidentical) MSC, immunologic incompatibility between the donor and recipient should be minimized sufficiently to allow engraftment. Based on our prior work in the fetal sheep model, we knew that the intraperitoneal (IP) transplantation of MSC results in widespread engraftment throughout all of the major organs [140,145,174,228-230] and durable expression of vector-encoded genes [229-231], at least in the context of the developing fetus. We further reasoned that using the IP route would also have the advantage of enabling the cells to enter the circulation in an almost time-release fashion, after being engulfed by the omentum and absorbed through the peritoneal lymphatics. Importantly, the IP route also avoids the lung-trapping which hinders the efficient trafficking of MSC to desired target organs following IV administration, and also poses clinical risks due to emboli formation [232,233].

Following isolation, MSC were simultaneously transduced with 2 HIV-based lentivectors, the first of which encoded an expression/secretion optimized porcine FVIII (pFVIII) transgene [234]. A porcine FVIII transgene was selected for two reasons. First, we had not yet cloned the ovine FVIII cDNA and constructed a B domain-deleted cassette that would fit in a lentivector. Secondly, the pFVIII transgene had previously been shown to be expressed in/secreted from human cells at 10-100 times higher levels than human FVIII [8,9,235]. We reasoned that, with these very high levels of expression/secretion, even very low levels of engraftment of the transduced MSC might still be able to exert a therapeutic benefit. The second lentivector encoded an eGFP marker gene to facilitate tracking and identification of donor cells *in vivo*. Combining the 2 vectors in the same transduction unexpectedly resulted in preferential transduction with the eGFP vector, such that only about 15% of the MSC were transduced with the pFVIII-encoding vector, as assessed by qPCR. Once the transduced MSC had been sufficiently expanded, the first animal to be transplanted was treated with a dose of hFVIII calculated to correct the levels to 200%, to ensure no procedure-related bleeding occurred. The animal was then sedated, and 30x10^6 transduced MSC were transplanted into the peritoneal cavity under ultrasound guidance in the absence of any preconditioning. Following transplantation, FVIII activity (assessed by chromogenic assay) was undetectable in the circulation, but this animal's clinical picture improved dramatically. All spontaneous bleeding events ceased, and he enjoyed an event-free clinical course, devoid of spontaneous bleeds, obviating the need for hFVIII infusions. Existing hemarthroses resolved, the animal's joints recovered fully and resumed normal appearance (as assessed by two attending veterinarians), and he regained normal posture and gait, resuming a normal activity level. To our knowledge, this represents the first report of phenotypic correction of severe hemophilia A in large animal model following transplantation of cells engineered to produce FVIII, and the first time that reversal of chronic debilitating hemarthroses has been achieved.

Based on the remarkable clinical improvement we had achieved in this first animal, we transplanted a second animal with 120x10^6 paternal MSC which had been subjected to 2 additional rounds of transduction with the pFVIII vector, such that > 95% of these cells were transduced and expressing pFVIII. We anticipated that by transplanting 4x's the number of cells with roughly 6x's the transduction efficiency, we would achieve pronounced improvement and therapeutic levels

of FVIII in the circulation of this animal, given that the transplanted cells were producing ~24x's the levels of FVIII (720units/24hrs) as those transplanted into the first animal. In similarity to the first animal, hemarthroses present in this second animal at the time of transplant resolved, and he resumed normal activity shortly after transplantation. This second animal also became factor-independent following the transplant. These results thus confirm the ability of this MSC-based approach to provide phenotypic correction in this large animal model of hemophilia A. However, just as we had observed in the first animal, no FVIII was detectable in the circulation of this animal, making the mechanism by which this procedure produced such pronounced clinical improvement indeterminate.

Despite the pronounced clinical improvement we observed in the first animal, he mounted a rapid and fairly robust immune response to FVIII, in similarity to prior studies performed with hemophilia A mice [235]. Before transplant, this first animal had Bethesda titers against hFVIII of only ~3, yet this lifesaving procedure resulted in a rise in Bethesda titer to ~800 against the vector-encoded pFVIII and nearly 700 to hFVIII. The formation of such high titer inhibitors with cross-reactivity to the human protein was surprising, given the well established ability to successfully use porcine FVIII products in human patients to bypass existing anti-hFVIII inhibitors [236-239]. Similarly, despite having no detectable inhibitors prior to transplant, the second animal receiving the higher FVIII-expressing cell dose developed titers of ~150 Bethesda units against the vector-encoded pFVIII following transplantation which also exhibited cross-reactivity to the human protein. Following euthanasia of these animals, we performed a detailed tissue analysis of to begin deciphering the mechanism whereby this novel MSC-based gene delivery produced its pronounced therapeutic effect at a systemic level. PCR analysis demonstrated readily detectable levels of MSC engraftment in nearly all tissues analyzed, including liver, lymph nodes, intestine, lung, kidney, omentum, and thymus. These molecular analyses thereby proved our hypothesis that it is possible to achieve widespread durable engraftment of MSC following transplantation in a postnatal setting in a large animal model without the need for preconditioning/ablation, and in the absence of any selective advantage for the donor cells. Confocal immunofluorescence analysis on frozen tissue sections revealed large numbers of FVIII-expressing MSC within the synovium of the joints which exhibited hemarthrosis at the time of transplant, suggesting the transplanted MSC possessed the intrinsic ability to home to and persist within sites of ongoing injury/inflammation, releasing FVIII locally within the joint, providing an explanation for the dramatic improvement we observed in this animal's joints. This finding is in agreement with prior studies [240], showing that local delivery of FIX-AAV to the joints of mice with injury-induced hemarthroses led to resolution of the hemarthroses in the absence of any detectable FIX in the circulation. While this finding provides an explanation for the reversal of the joint pathology present in these animals at transplant, it cannot explain the observed systemic benefits such as the cessation of spontaneous bleeding events.

Thus far, confocal analysis has also revealed engrafted cells within the small intestine, demonstrating that MSC can still engraft within the intestine following postnatal transplantation, just as we had observed in our prior studies in fetal recipients [229]. Given the ease with which proteins secreted from cells within the intestine can enter the circulation, future studies aimed at improving the levels of engraftment within the intestine have the potential to greatly improve the systemic release of FVIII. In addition to the intestine and hemarthrotic joints, significant levels of engraftment were also seen within the thymus of this animal. While the ability of the transplanted MSC to traffic to the thymus could clearly have important implications for the likelihood of long-term correction with this approach to hemophilia A treatment, additional studies are required to determine with which cells within the thymus these MSC are interacting to ascertain the immunologic ramifications of thymic engraftment.

Conclusions

Current hemophilia A treatments allow many patients with hemophilia to live relatively normal lives, but their high cost, the need for lifelong therapy, and the inaccessibility of these therapies to the vast majority of patients with hemophilia A worldwide [5] highlight the need to develop novel therapies offering longer-lasting benefit or permanent cure [7,8,10-14]. Based on their developmental similarities to humans, we have employed sheep as a model system to study the potential of in utero gene therapy and in utero MSC transplantation to correct a disease like hemophilia A prior to birth, and have shown that direct vector injection results in levels of gene transfer that would likely be therapeutic in hemophilia A and induces durable immune tolerance to the vector-encoded genes, but possesses the inherent risk of off-target transduction.

Similarly, the transplantation of MSC in utero led to significant levels of widespread cell engraftment in both the parenchyma and the perivascular regions of multiple tissues, placing the cells ideally for release of FVIII into the circulation. Unfortunately, expression of the endogenous FVIII gene by these cells following engraftment and differentiation was too low to be of therapeutic value. It thus appears that a combination of these two approaches may represent the ideal means of treating hemophilia A prior to birth. Having recently re-established a line of sheep that accurately recapitulates both the genetics and the clinical symptoms of the severe form of human hemophilia A, we are now ideally poised to address this issue. In similarity to human patients, this line of sheep exhibits a severe bleeding phenotype with frequent spontaneous hemarthroses leading to reduced locomotion, muscular hematomas, and episodes of hematuria and internal bleeding leading to death. The nature of the mutation found in these animals further adds to the uniqueness of this model. Murine models have been generated through knockout/deletion technology, and the naturally occurring dog colonies exhibit gene inversions [241]. In similarity to mutations seen in many human patients [38], hemophilia A in sheep is caused by a premature stop codon with a frame shift mutation, making this sheep colony the first large animal model yet described to possesses a mutation of this kind. It is our hope that the availability of this animal model that closely parallels normal human weight and physiology, and in which the severity and symptoms of the disease clearly resemble that of humans, will provide researchers in the field with a valuable preclinical resource for developing novel therapies, such as those utilizing stem cell transplantation and gene therapy, for hemophilia A. In recent studies, we used this line of sheep to test a novel postnatal approach to treating hemophilia A in which MSC served as delivery vehicles for a pFVIII transgene. This approach was technically straightforward, involving a single injection into the peritoneal cavity under ultrasound guidance, in the absence of any prior cytoablation/conditioning, and thus posed minimal risk to the recipient. This MSC-based approach converted both animals treated to-date from a severe, life-threatening phenotype to a moderate phenotype, devoid of spontaneous bleeds, but still bleeding in response to accidental trauma. Remarkably, this approach also resulted in a complete reversal of the joint stiffness/swelling as well as the gait and postural defects that had

developed as a result of frequent hemarthroses during the first months of life.

To our knowledge, this represents the first time that reversal of the severe, crippling hemarthroses which plague human patients with hemophilia A has been achieved. These results thus open the door to the development of new MSC-based therapies for this debilitating condition, either systemically or, perhaps, administered directly into affected joints of hemophilia patients. Despite the marked clinical success we achieved, however, both animals developed high-titer inhibitors, likely limiting both the magnitude and duration of the therapeutic effect. Mechanistic studies are currently underway to understand and ultimately overcome the formation of inhibitors resultant from this procedure since, in their absence, this approach could result in even more pronounced clinical improvement. It is important to note that, were this same procedure implemented in utero, the possibility of inhibitor formation would be eliminated, possibly making this approach to hemophilia A treatment curative.

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