FETAL THERAPIES AND MATERNAL-FETAL TOLERANCE

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FETAL THERAPIES AND MATERNAL-FETAL TOLERANCE

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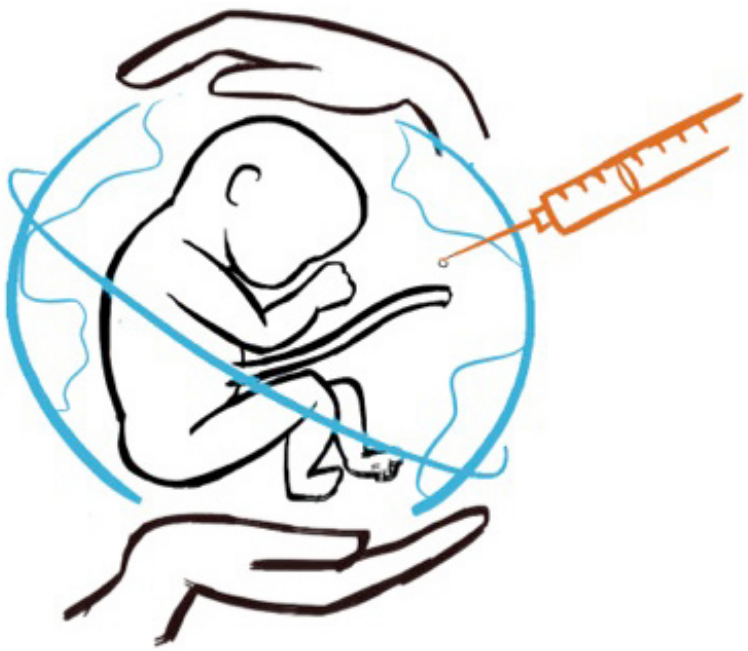

Image by Tippi MacKenzie

The ability to diagnose and treat genetic diseases before birth represents one of the foremost breakthroughs of modern medicine. While fetal surgery has advanced in the last several decades, the prospect of applying developments in stem cell biology and gene therapy to the fetal environment remains an open frontier. This issue represents the work of international experts in the field of fetal therapy, who came together at the first meeting of the International Fetal Transplantation and Immunology Society in 2014. This meeting was convened in an effort to provide a consensus for future applications of in utero transplantation and gene therapy, as well as form an international community of colleagues to nurture this field.

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Consensus statement from the first international conference for *in utero* stem cell transplantation and gene therapy

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On April 17–18, 2014, basic and translational scientists and clinicians convened in San Francisco, CA for a conference in fetal stem cell transplantation, stem cell biology, tolerance, and gene therapy.

The purpose of the meeting (http://pedsurglab.surgery.ucsf.edu/news–events/fetal-symposium-2014.aspx) was to outline the goals of *in utero* transplantation, review the barriers that have been encountered, and learn about new developments that can be applied to the field.

Information discussed at this conference will help pave the way for developing novel strategies to achieve therapeutic engraftment levels in the fetus, and identify ways to safely translate these strategies to a wide range of clinical applications.

We held a final consensus session to achieve an international agreement for future pre-clinical and clinical studies of *in utero* hematopoietic cell transplantation (IUHCT). We agreed on the following items:

- *In utero* transplantation is a viable strategy to treat fetuses with selective congenital disorders.
- Given recent publications that the maternal immune response can limit engraftment, the clinical strategy for IUHCT should involve transplantation of autologous or maternal-derived cells. The host immune response may be a limiting factor that might be circumvented with early cell delivery.
- The fetal microenvironment plays a primary role in supporting the engraftment and expansion of transplanted cells and requires further investigation.
- Recent data from large animal studies suggests that intravascular injection may be the delivery route of choice to achieve engraftment of hematopoietic stem cells in the fetus.
- Currently, there is no proven safe method of host conditioning in the fetus. Until specific, non-toxic conditioning methods (such as antibody-mediated depletion of host HSC) are optimized in pre-clinical models, large cell doses should be used to overcome host competitive barriers.
- Experimental model data are sufficient to warrant a phase 1 clinical trial of IUHCT for select fetuses. The most suitable hematological diseases are hemoglobinopathies such as sickle cell disease and thalassemia, given their high morbidity/mortality, the availability of reliable prenatal screening programs, and the paucity of optimum postnatal care options.
- The value of alternative cells, such as mesenchymal stromal cells (MSC) and amniotic fluid-derived cells, for other appropriate congenital pathologies warrants investigation.
- Reports of using MSC *in utero* to treat osteogenesis imperfecta (OI) in a limited number of patients are promising and suggest that, after optimization, MSC could be used to improve/treat OI.
- Treatment of the fetal patient using gene therapy and gene-modified cells have great future potential and should be fields of active investigation.
- A new society focused on fetal stem cell transplantation and gene therapy will be formed (FeTIS: Fetal Transplantation and Immunology Society), with the mission of accelerating clinical applications of stem cell transplantation and gene therapy approaches to treat fetuses with congenital anomalies.
- The society should develop and maintain an international registry of treated patients and their outcomes to facilitate reporting and sharing of results. This database will not be publicly accessible and data will be anonymized.
- The society will provide a forum for members to share best practice and clinical governance for *in utero* stem cell and gene therapy cases.

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Cell-based interventions *in utero*: time to reconsider

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INTRODUCTION

In 1999, the NIH Recombinant DNA Advisory Committee held a Gene Therapy Policy Conference on *in utero* gene transfer (NIH, 1999) and determined that it would be premature to undertake *in utero* gene transfer research in humans (RAC, 1999). Much has happened since then. Gene transfer research enrolling infants and very young children as patient-subjects has had results both beneficial and harmful in several conditions (Hacein-Bey-Abina et al., 2003; Aiuti et al., 2012; Corrigan-Curay et al., 2012). Some fetal surgical interventions have become accepted (Adzick et al., 2011). And much has been learned about the immune system and how pregnancy influences immune response.

We now know a lot more about how much more there is to learn. Researchers in cell- and gene-based interventions are eager to move to human trials in order to continue the learning process. Yet funders and oversight bodies are reluctant to support cell-based intervention research in human fetuses. In this commentary we address probable reasons for this hesitancy, reasons to move forward with caution, and issues to address in planning first-in-human (FIH) trials of cell-based interventions *in utero*.

REASONS FOR RELUCTANCE

First, there is concern that existing alternatives obviate the need for *in utero* interventions—that is, if *in utero* treatments are unnecessary, then *in utero* research is too. For couples known to be at risk of giving birth to offspring with serious genetic or metabolic anomalies, *in vitro* fertilization (IVF) and preimplantation genetic diagnosis (PGD) are available (Dresser, 2004). For couples without known risk factors, prenatal diagnosis and abortion are available. However, IVF and PGD are costly, burdensome, and thus unavailable for many couples, and abortion is morally unacceptable to many and increasingly difficult to obtain for many others. Thus, these alternatives by no means eliminate the need for or the value of *in utero* interventions, and cannot justify failure to support *in utero* research (Strong, 2011).

Second, the growing tendency to categorize unprecedented and untested stem cell interventions as innovation rather than research may be thought to offer investigators an alternate route to the clinic. However, the extensive cautionary literature on the problem of innovation makes clear that FIH *in utero* cell-based interventions should be regarded and treated as research (Chescheir and Socol, 2005; Hyun et al., 2008; Daley, 2012; Sugarman, 2012).

Finally, and most important, federal regulations restrict research involving pregnant women and fetuses. 45 CFR 46.204, in Subpart B, requires prior "scientifically appropriate preclinical and clinical studies" and permits only trials in which "[t]he risk to the fetus is caused solely by interventions or procedures that hold out the prospect of direct benefit for the woman or the fetus; or, if there is no such prospect of benefit, the risk to the fetus is not greater than minimal and the purpose of the research is the development of important biomedical knowledge which cannot be obtained by any other means" and "[a]ny risk is the least possible for achieving the objectives of the research" (DHHS, 2014a).

Satisfying these regulatory requirements means successfully addressing three definitional controversies in research ethics. The first is about what constitutes good prior research and persuasive data. The regulations assume that clinical studies in adults and children will precede research on pregnant women and fetuses, and that the resulting data will help to establish potential benefit and minimize risks of harm. This is the traditional model for pharmaceutical research. However, the translational research trajectory for novel biotechnologies has rarely applied that model, in large part because better data can often be obtained from younger patient-subjects, and older patients may not be suitable subjects.

Another definitional difficulty lies in defining minimal risk. The obligation to pursue studies that pose no more than minimal risk in the absence of potential direct benefit raises important questions about when risk can be considered minimal. The federal regulations define minimal risk in terms of daily life and routine tests and procedures (DHHS, 2014c). What are the daily life risks of fetuses and pregnant women? Should the risks of procedures like amniocentesis be considered in assessing risk (Iltis, 2011)?

Third, there is disagreement about when it is appropriate to regard a study as offering potential benefit. Wishful thinking notwithstanding, the prospect of direct benefit cannot reliably be held

out to patient-subjects in FIH trials. The focus of FIH research must therefore be on minimizing the risks of harm. Yet both research enrolling children under Subpart D (DHHS, 2014b) and research enrolling pregnant women and fetuses under Subpart B pose a significant risk of the therapeutic misconception, whereby potential subjects, investigators, funding agencies, the media, and research oversight bodies tend to view research as treatment, exaggerate the potential for benefit, and underestimate the risks of harm (Dresser, 2002; Henderson et al., 2005).

A related problem in research with pregnant women, fetuses, and children is "benefit creep," whereby investigators and IRBs exaggerate the prospect of direct benefit in order to meet the regulatory requirements for enrolling children and fetuses as patient-subjects (King, 2000). Unfortunately, overstating the potential for direct benefit in FIH research can easily both create the therapeutic misconception and end with the materialization of serious and unexpected risks of harm.

Addressing these challenges and making the argument that the time is right for FIH trials in pregnant women and fetuses is thus no easy feat. It requires clear and significant justification and persuasive data, and may be quite challenging under the current regulatory scheme.

REASONS TO PROCEED (WITH CAUTION)

Nonetheless, there are good reasons to move forward toward FIH trials of cell-based interventions *in utero*. First, animal models and other types of preclinical modeling have advanced considerably in the last 15 years and continue to improve. Thus, it is becoming easier to assemble scientifically relevant preclinical data, even when clinical data from adult patient-subjects is unavailable or uninformative (Chescheir and Socol, 2005; Coutelle and Ashcroft, 2012).

Second, the effects of early interventions may be easier to measure in treatment-naive patient-subjects, making it more feasible to demonstrate proof of concept in FIH studies (King and Cohen-Haguenauer, 2008). Thus, very young patient-subjects may be more likely to provide data demonstrating proof of principle or even surrogate measures suggestive of efficacy. In addition, in some disorders, earlier interventions may be more effective. Characteristics of the immune system in fetuses, their size, and the opportunity to intervene at an earlier stage of illness all may help increase the effects of cell- and gene-based interventions, though much remains unknown (Niyibizi and Li, 2009; Strong, 2011).

Finally, as has been demonstrated in preclinical and clinical research for a range of conditions and interventions (see the rest of this issue), cell-based FIH trials *in utero* will surely have another important outcome that is too often overlooked in the pressure to achieve clinical translation: Simply learning more about the complex immune relationship between pregnant woman and fetus. Despite profound societal desire for progress in treatment of specific diseases and conditions, translational research often yields important knowledge when it proceeds in unanticipated directions. As much (or more) can be learned from going sideways, or back to basics, as from pushing toward the clinic (Kimmelman, 2010).

MOVING TO HUMANS: QUESTIONS TO CONSIDER

It is therefore time to restart progress toward FIH trials in cell- and gene-based *in utero* interventions. When considering FIH trials, the following questions must be addressed:

- Has enough preclinical information been collected so that the only reasonable way to learn more is to move to humans?
- Has enough been done to reduce the risks of harm to humans, and to maximize the likelihood that the intervention will ultimately show benefit in humans?
- Has the point of irreducible uncertainty been reached?
- Is the amount of irreducible uncertainty small enough that it is fair to subjects to ask them to participate?

Affirmative answers, supported with reasoning and data, can provide both justification for moving to human trials and the basis for informed decision-making about participation. However, answering these questions is challenging for FIH *in utero* research. Following are several specific considerations for FIH *in utero* research that suggest the benefit of reconsidering Subpart B.

First, couples who have undertaken IVF and PGD may be willing to donate affected embryos for research rather than discarding them (Lyerly and Faden, 2007), and couples who have learned that their fetus is affected may be willing to participate in research prior to obtaining an abortion. It will be necessary to design trials to make fair and appropriate use of these subject populations (Dresser, 2004; Chervenak and McCullough, 2007; Strong, 2011; Coutelle and Ashcroft, 2012).

Second, to support informed decision-making about trial participation, clear and complete information must be provided about the risks of harm to both subjects, the unlikely prospect of direct benefit to the fetus, alternatives to participation, requirements for long-term follow-up, and the future possibility of autopsy. It must be emphasized that FIH trials represent proof of concept studies and are not designed or expected to offer direct benefit to the fetus (NIH, 1999; King, 2000; Dresser, 2004; King et al., 2005).

Although direct benefit is unlikely, the consequences of partial success should be addressed whenever relevant. If correction were to be partial, would that be a success—that is, better than no correction because it can be augmented by available treatments? Or would it be a failure—that is, worse because it promises impaired survival (NIH, 1999; Chescheir and Socol, 2005)? There are no easy answers to these questions; nonetheless, investigators must prepare to address them.

Third, important choices must be made about where to start—with what diseases and conditions—in these FIH trials. Concentrating effort where the need is greatest, where the most progress has already been made, and where funding is available are very different starting points (NIH, 1999; Dresser, 2001; King and Cohen-Haguenauer, 2008).

Finally, it is essential to consider whether there are appropriate ways to minimize the risks of harm and/or increase the prospect of direct benefit in FIH *in utero* research. Harm-benefit assessment must be detailed, and should distinguish between direct health benefits from the

experimental intervention and benefits to patient-subjects that arise from participating in research generally (such as the satisfaction of trying everything or the value of altruism), not from the intervention itself (King, 2000). The question is not only about what risks of harm and potential benefits exist, but also about how we measure, judge, and compare them (Iltis, 2011).

Some researchers have argued that it is unethical to conduct phase I gene transfer studies in any patient-subjects because there is no prospect of direct benefit. Instead, they argue, studies should begin at the phase II/III stage (Coutelle and Ashcroft, 2012). This argument appears to assume that an FIH trial of an *in utero* intervention would be justified if it were designed to provide doses calculated or expected to be therapeutic. This is a perfect example of unacceptable and potentially unsafe "benefit creep." No matter how the study is designed and what data precede it, someone has to be first, and what researchers believe will be safe and effective often fails to realize those hopes.

CONCLUSION

The benefit creep problem demonstrates the need to address the growing lack of fit between regulatory requirements for research with pregnant women, fetuses, and children and the realities of FIH and other early-stage research involving novel biotechnologies. Reconsidering Subpart B need not mean exposing vulnerable patient-subjects to excessive risk. FIH *in utero* cell- and gene-based intervention trials should require highly persuasive preclinical data, and the amount of irreducible uncertainty should be well-justified, but a prospect of direct benefit should not be required. Instead, researchers must do their best to identify and minimize all risks of harm, and provide clear and complete information to potential subjects. Then, if a well-informed pregnant couple views participation in the research as a reasonable choice, even if one of their reasons is "trying everything just in case," it may be time to move forward.

This step must be taken deliberately, with thorough oversight, care for patient-subjects, and respect for what we do and do not know. Ongoing public and professional discussion is essential, as best practices for the design, conduct, and oversight of *in utero* research continue to evolve.

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Immunological considerations in *in utero* hematopoietic stem cell transplantation (IUHCT)

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In utero hematopoietic stem cell transplantation (IUHCT) is an attractive approach and a potentially curative surgery for several congenital hematopoietic diseases. In practice, this application has succeeded only in the context of Severe Combined Immunodeficiency Disorders. Here, we review potential immunological hurdles for the long-term establishment of chimerism and discuss relevant models and findings from both postnatal hematopoietic stem cell transplantation and IUHCT.

Keywords: *in utero* hematopoetic stem cell transplantation, fetal alloresponse, maternal alloresponse, central tolerance, regulatory T cells (T-Regs)

TERMINOLOGY

Central tolerance: An immune mechanism for specificity of the immune response set up in the thymus. Functional central tolerance prevents T cells with a high affinity to "self" from exiting into the periphery.

Peripheral tolerance: Immune protocols that mediate specificity of the immune response, other than central tolerance. All the following can be considered peripheral tolerance:

Dominant tolerance: A tolerance mechanism that can override other tolerance mechanisms such as Tregs (regulatory T cells) or myeloid-derived suppressor cells with capacity to suppress effector mechanisms of other cells.

Regulatory T cells: Specialized CD4+FoxP3+ T cells that inhibit proliferation and effector functions of other immune cells via several mechanisms. Most studied for their effect on CD8+ T cells but potentially also important for regulation of other effector cell types.

Clonal deletion in reference to exhaustion: A type of terminal differentiation in T cells (CD4 and CD8) elicited by continuous presence of antigen. T cells gradually lose functionality in a stepwise, strictly controlled process and sometimes die as a function of continued antigen presence. Partial exhaustion is sometimes reversible.

Anergy: Unresponsiveness of T cells previously stimulated with their cognate antigen in the absence of an appropriate second signal. Anergic T cells do not execute normal effector function.

In utero hematopoietic stem cell (HSC) transplantation is not a standard clinical approach, but with greater understanding of the immune system and its development, as well as the disease processes that are optimally treated *in utero*, the procedure may become more widely used. Stem cell transplantation, as any other transplantation is subject to potential rejection reactions by the hosts' immune system that recognizes the tissue antigens of a different genetic makeup as "foreign" and elicits an immune attack. This attack, if not properly controlled by immune-suppressants, can lead to graft damage and ultimately graft loss. Transplantation of hematopoetic stem cells *in utero* seeks to take advantage of the early developmental stages of the fetal immune system and elicit a dampened immune rejection or perhaps achieve full graft tolerance. While attractive in theory, the practical outcomes of *in utero* hematopoetic stem cell transplantation have been disappointing for a multitude of reasons. Here we review the complex issues pertaining the immune system that have bearing on *in utero* hematopoetic stem cell transplantation for a general clinical audience.

CENTRAL AND PERIPHERAL TOLERANCE

The main function of the immune system is to fight off infection of a huge variety of pathogens. To successfully fight "foreign" invaders without damaging the host, the immune system has to sort through enormous antigenic diversity to recognize the difference between "self" vs. "non-self." Allogeneic transplants (solid-organ and cell transplants) are not "self" from the recipient perspective and hence are attacked by the recipient immune system (allorejection) necessitating the administration

of immunosuppressants. However, successful HSC transplants will actively participate in the immune system as they give rise to all white cells. Additionally, the special situation of HSC transplantation in the context of the fetal immune system during IUHCT warrants specific considerations of the developing recipient environment to ensure graft survival.

Several fundamentally different processes establish necessary self-tolerance and are also involved in (allogeneic) graft-tolerance: central tolerance, exhaustion/peripheral deletion and regulatory T cells (Treg).

Central tolerance results from deletion of self-reactive T cells in the thymus (Rothenberg, 1992). This form of tolerance prevents cells that are stimulated by a self-antigen from migrating into the periphery where they can cause autoimmune damage, and defines the immunological "self," resulting in a state of "ignorance" to self-antigens. *Ex vivo* stimulation of a central tolerant T cell population with self-antigens does not result in activation or proliferation. Central tolerance is effective but not complete and a few self-reactive T cells can migrate into the periphery despite a functioning central tolerance mechanism (Griesemer et al., 2010). Largely, these self-antigen-specific T cells do not exert autoimmune function in the periphery because they are suppressed by a T cell population which inhibits immune effector functions, the Tregs (Takahashi et al., 1998; Sakaguchi, 2003). A population of cells that is tolerant only because of Tregs *is* stimulated by self-antigen but so are the inhibitory Tregs that prevent self-reactive effector functions. Hence, Tregs establish a state of "active tolerance" toward an antigen. *Ex vivo* stimulation of this cell population with self-antigen also does not result in activation or proliferation. However, when Tregs are depleted in mixed lymphocyte culture systems, stimulation with self-antigen results in activation and proliferation of self-reactive T cells (essentially an "unmasking" of previously inhibited responses). While central tolerance and T regs both mediate non-reactivity to self, the mechanisms by which tolerance is established are very different. There is another distinction between the two mechanisms: Central tolerance is dependent on localization to the thymus while Tregs are mobile, and when transferred to another host, can and will perform appropriate suppressive function if properly stimulated and maintained (Asseman et al., 2000).

Another mechanism that results in a non-reactive T cell pool is T cell exhaustion. T cell exhaustion occurs when T cells are chronically exposed to antigen under inflammatory conditions. Over time, these T cells lose the ability to provide effector functions and die. T cell exhaustion has been observed in persistent infections and has been postulated as a mechanism by which alloreactive T cells decline in functionality and are deleted in solid-organ transplants.

In classical *in vitro* mixed lymphocyte reactions (MLR) all the mechanisms discussed above result in non-reactivity of the responder T cells (**Figure 1**). But that result is misleading in that additional experiments (some of which are technically challenging) can unmask activity of T cells in these "negative" MLR studies. These studies are really controls needed to interpret results of engraftment protocols with confidence, especially since the protocols are often not standardized, and use very different models, time points, cell sources etc. The absence of follow-up studies also highlights the problems in reproducing results or adapting protocols from one model system to another.

THE MECHANISM OF CENTRAL TOLERANCE

Hematopoietic progenitor cells mature in the thymus, and then are selected to generate a functional, self-tolerant peripheral T cell pool. The outer region (cortex) of the thymus supports the early stages of T cell development, when thymocytes are selected for ability to interact with self-MHC molecules. Cells that survive this positive selection process travel on to the inner thymus (medulla), where negative selection against self-tissue-specific antigen recognition takes place (self -peptide-tolerance).

THYMIC DEVELOPMENT

Lymphoid progenitor cells migrate during embryonic development in several discrete waves, coordinated with organogenesis (Le Douarin, 1973; Le Douarin and Joterau, 1975). The E11.5 murine embryonic thymus receives a first influx of a small number of lymphoid progenitor cells (Owen and Ritter, 1969; Fontaine-Perus et al., 1981; Liu et al., 2005; Foster et al., 2008). In humans, the thymus appears roughly 8 weeks after conception at which time the first T cell progenitors originating from the fetal liver and later from the bone marrow migrate into and colonize it (Haynes et al., 1989). By week 10, 95% of the human fetal thymus are lymphocytes (Fernandez and de Alarcon, 2013). In mice, a model that is widely applied in studies of immune development, only small numbers of T and B cells can be found at parturition and the division of the spleen into T-cell and B cell zones (red and white pulp) only occurs in the first week after birth (Burns-Naas et al., 2008). In contrast to this, in humans, small numbers of T and B cells can be found at the end of the first trimester while splenic demarcation occurs at the beginning of the third trimester.

The thymus is composed of cells of stromal and hematopoietic origin such as mesenchymal cells, thymic epithelial cells (TECs), endothelial cells, and dendritic cells. Thymic epithelial cells are the main cell type interacting with and providing selective clues to T cells (**Figures 2**, **3**). Based on location in the thymus, TEC are characterized as cortical TEC (cTEC) or medullary TEC (mTEC). Both arise from a common progenitor during thymic organogenesis (Rossi et al., 2006) and, if undisturbed, this progenitor pool continues to support TEC generation and homeostasis in postnatal life (Bleul et al., 2006).

HOW DO TECs SHAPE THE T CELL POOL?

Initial stages of T cell selection ensure that the resulting pool can successfully "communicate" with cells of the host. To ensure proper communication, T cells must be equipped to engage in productive interactions with proteins that constitute the communication system, the MHC proteins. The cortical thymus selects for this function in a process termed positive selection: T cells with low-affinity interaction of T cell receptors with peptide-MHC complexes receive survival signals while T cells with no or too low affinity engagement die by neglect (Starr et al., 2003). Importantly, T cells with too high an affinity for MHC are also deleted as potentially damaging self-reactive players. Thymocytes travel to the medulla where they interact with mTEC and dendritic cells that present self-antigens under the control of the

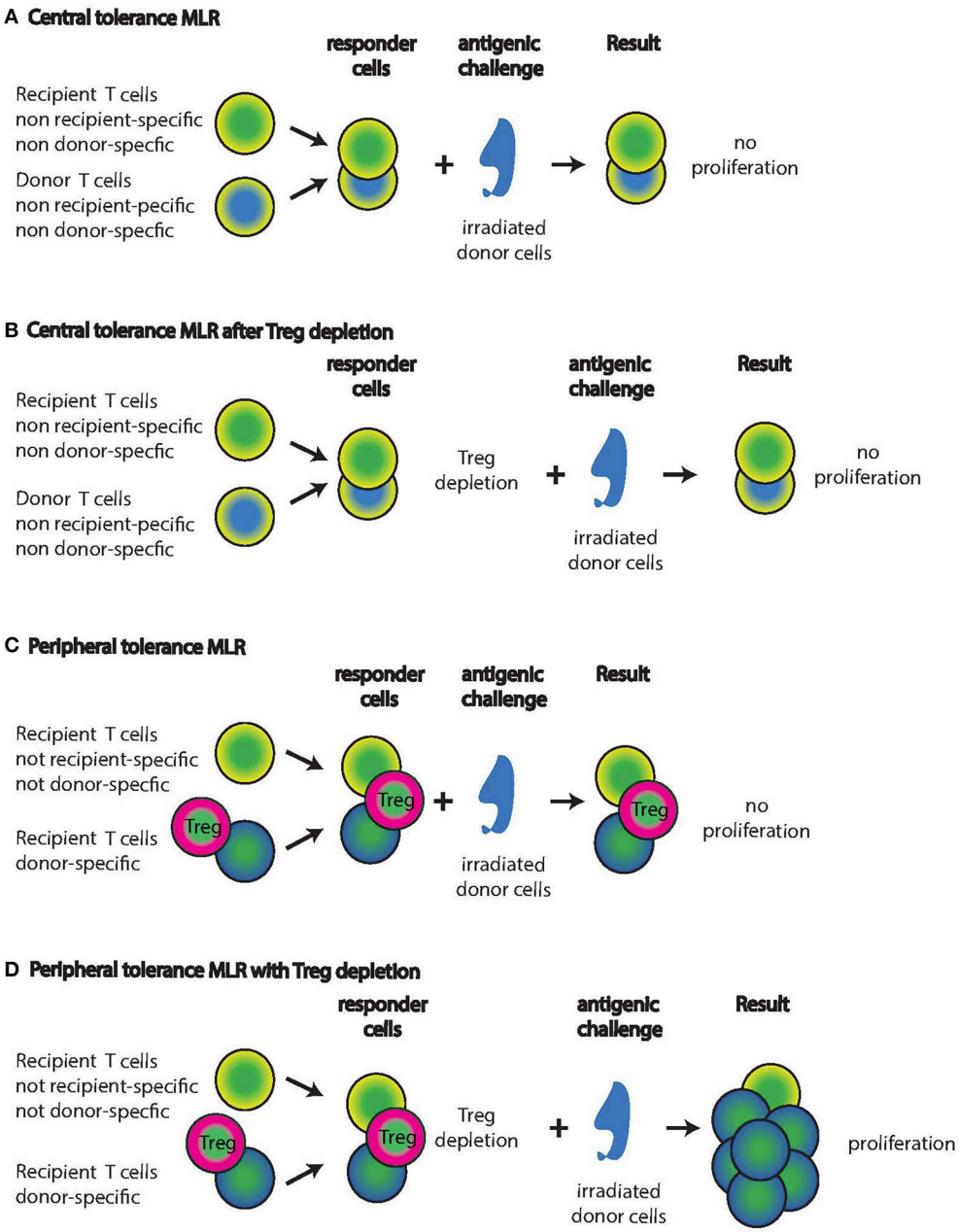

FIGURE 1 | Mixed Lymphocyte Reactions (MLR) with central tolerant or peripheral tolerant lymphocyte populations from IUHCT graft recipients. Recipients of IUHCT carry mixed hematopoietic chimerism. **(A,B)** In central tolerance, thymic selection will only allow recipient T cells that are non-recipient and non-donor specific to persist (yellow/green cells). Similarly, donor cells are only allowed to persist if they are non-recipient and non-donor specific (blue/yellow cells). Exposure of these two responder populations to irradiated donor cells will not elicit a proliferative response **(A)** even after the deletion of Tregs **(B)**. **(C,D)** In peripheral tolerance, recipient T cells with specificity for the donor are allowed to progress through thymic selection into the periphery (Green/blue cells) but effector functions and proliferation are inhibited by Tregs (Green/pink cells). Exposure of these three responder populations to donor cells will not result in proliferation **(C)**. After the deletion of Tregs, the recipient T cells with donor specificity will proliferate vigorously in response to donor cells **(D)**. Note: It is unknown whether the Tregs are donor-derived or recipient-derived or both.

Autoimmune regulator gene (Aire) (Laan and Peterson, 2013). Thymocytes reactive to presented self-antigens are deleted as potentially self-reactive T cells (also affinity dependent). The T cells reside in this environment for 4–5 days until they are released to the periphery.

THE THYMIC DEFINITION OF "SELF"

The repertoire of self-antigen available for thymic selection is of critical importance as it ultimately defines T cells as self-reactive or not self-reactive. The diversity of this peptide pool is ensured by a remarkable heterogeneity of thymic antigen-presenting cells

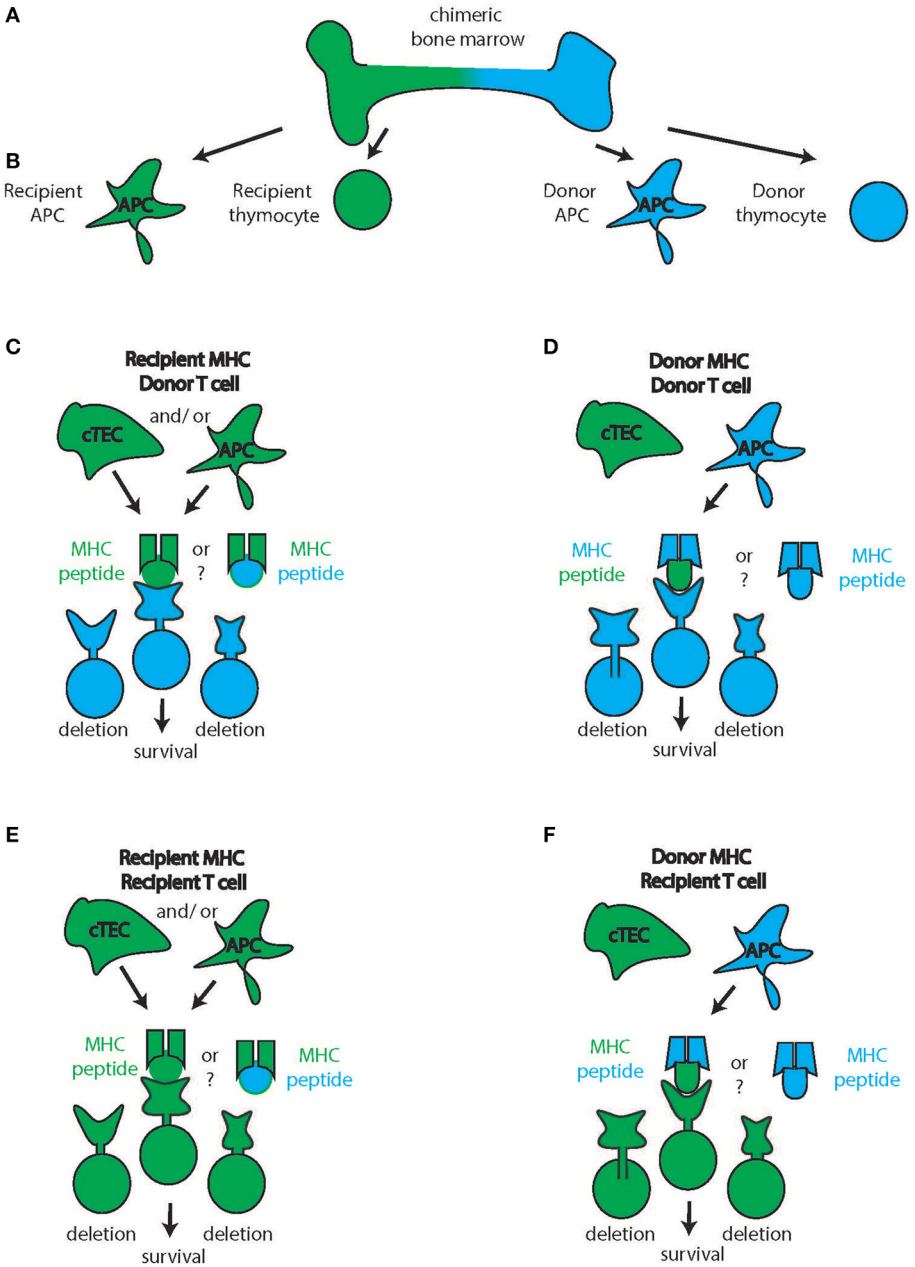

FIGURE 2 | Cortical thymic T cell selection. (A) Chimeric bone marrow can give rise to multiple blood products from the two stem cell sources (recipient: green, donor: blue) such as antigen-presenting cells (APC) and thymocytes **(B)** In the thymic cortex, productive interaction with the MHC takes place. Though knowledge is limited, cortical Thymic Epithelial Cells (cTEC) likely will be recipient-derived (**C–F**, green cTEC cell). In contrast, APC are constantly turned over and immigrate from the periphery theoretically allowing for the participation of donor-derived APC in the thymic selection process (**D,F**). Therefore, donor thymocytes can interact with recipient cTEC and recipient APC and hence recipient MHC to receive survival signals **(C)** or with recipient cTEC and donor APC hence interacting with donor APC **(D)** Conversely, recipient thymocytes can interact with recipient cTEC and recipient APC and hence recipient MHC to receive survival signals **(E)** or with recipient cTEC and donor APC hence interacting with donor APC **(D)** The source of the peptides during this process (recipient or donor) is unknown.

(APC) and the utilization of unconventional molecular pathways by APC. These pathways ensure unique self-peptide repertoires for both the negative and positive selection steps, with peptides derived from the thymus itself, from the periphery and different developmental stages, including some from stages other than the current developmental state. This remarkably diverse repertoire is ensured by (1) promiscuous gene expression by mTEC, (2) specific antigen processing features including proteasome composition and protease sets, (3) intracellular antigen sampling via autophagy of thymic epithelial cells and (4) extracellular antigen sampling provided by immigrating dendritic cells (DC) that sample the periphery. mTEC express hundreds

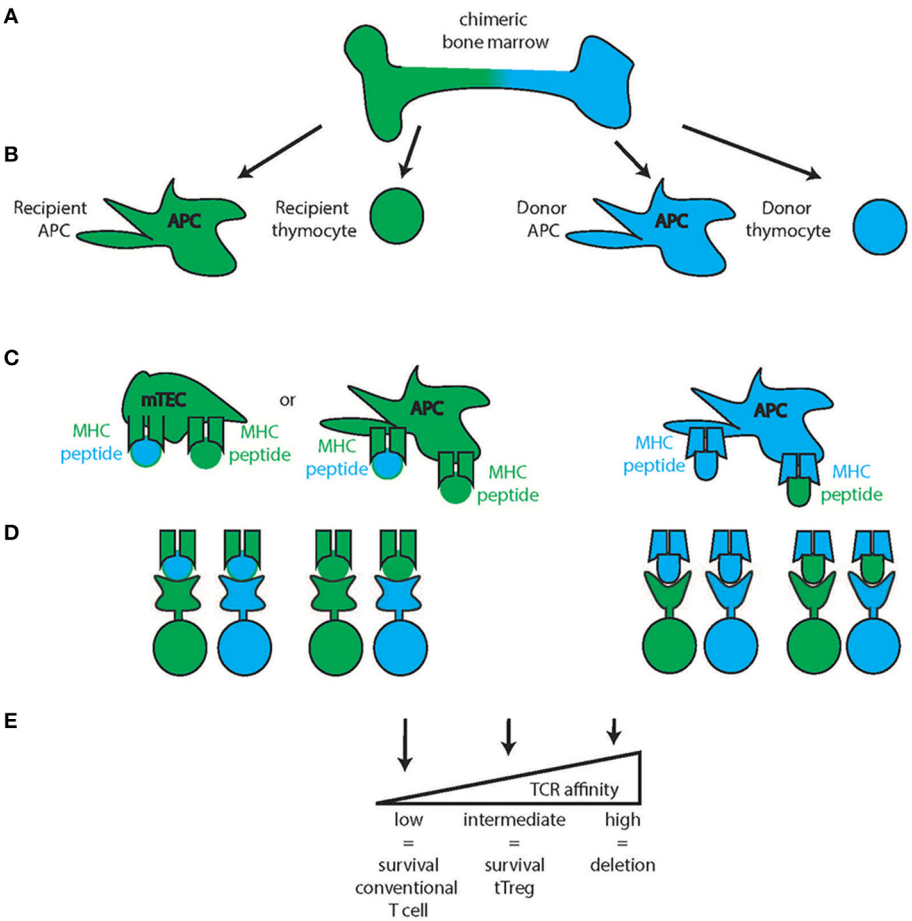

FIGURE 3 | Thymic medullary selection. In the second step of thymic selection, T cells are selected for the absence of high affinity self-peptide-self-MHC reactivity. Chimeric bone marrow **(A)** can give rise to multiple blood products from the two stem cell sources (recipient: green, donor: blue) such as antigen-presenting cells (APC) and thymocytes **(B)** While the Medullary Thymic Epithelial Cells (mTEC) are recipient derived (green mTEC, **C)**, medullary APC can be either recipient derived (green, left) or donor derived (blue, right) and all of these cells can either present recipient (green) or donor derived (blue) peptides on their respective MHC. Combined with thymocytes of either recipient or donor origin, several combinations of MHC-peptide-TCR reactivity are possible **(D)** of which those with high affinity will be deleted **(E)** intermediate reactivity will develop into tTregs and low affinity will develop into conventional T cells of the CD4 or CD8 lineage.

of self-antigens that are otherwise expressed only in strictly regulated spatial or temporal contexts. In mTEC, proteins are translated for the sole purpose of undergoing degradation, after which they are loaded onto MHC molecules. Regulation of this complex and unique process is still poorly understood but the recent discovery of the transcriptional regulator Aire has shed some light on this process (Anderson et al., 2002). Aire deficiency manifests with autosomal recessive inheritance pattern and causes a polyglandular disorder that classically manifests as spontaneous autoimmunity against the parathyroid and/or adrenal glands, and/or by a mucocutaneous candidiasis infection (termed Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), also known as autoimmune polyendocrine syndrome–type 1 (APS-1) (Bjorses et al., 1998; Rosatelli et al., 1998; Scott et al., 1998). Aire, amongst other functions, controls the expression of tissue antigens (as detailed above in 1) and regulates intrathymic DC positioning via Aire-dependent XCL1-expression (Lei et al., 2011). In mice, the first Aire + mTEC subsets can be observed at around embryonic day E16 and hence precede the formation of fully mature thymocytes (Zuklys et al., 2000; White et al., 2008). In humans, the kinetics by which APECED- associated autoantibodies appear in pediatric patients suggest onset of Aire-mediated self-tolerance at or before birth (Wolff et al., 2013).

Macro-autophagy enables mTECs to present intracellular cargo on MHC II molecules (Nedjic et al., 2009). Resident thymic DC's are known to sample and cross-present mTEC-derived antigens and blood-borne antigens (Klein et al., 2009). Additionally, about 50% of thymic DC are immigrating DC that already sampled the periphery and imported peripheral peptide cargo. Antigen presentation occurs in two important steps—processing of proteins into MHC-binding peptides and binding of a peptide to an MHC complex. Peptides destined for loading onto MHC class I are generated by the proteasome, a multiprotein complex that executes protein degradation processes dependent on the inflammatory milieu. The housekeeping proteasome

generates peptides from proteins under non-inflammatory conditions and the immunoproteasome under inflammatory conditions (Kloetzel and Ossendorp, 2004). Thymic DC and mTECs express a mixture of both types of proteasomes and thus present both partly non-overlapping peptide pools but with careful exclusion of pathogen-associated molecules (Nil et al., 2004; Bonasio et al., 2006; Hadeiba et al., 2012). MHC class II binding peptides are generated in the endosomal/lysosomal compartment by a mixture of proteases including those of the thymus, again mirroring the enormous complexity presented to the developing immune system (Gommeaux et al., 2009).

MECHANISMS OF PERIPHERAL TOLERANCE

REGULATORY T CELLS (TREGS)

Thymic selection is a highly efficient process but it is not perfect. It is now accepted that self-reactive T cells escape the deletion process and circulate in the periphery. The reason self-reactive T cells do not cause pathology is that they are suppressed by regulatory T cells (Tregs), a subset of CD4+ T cells defined by intranuclear expression of the transcription factor FoxP3. Thymic Tregs (tTregs) develop during thymic T cell selection from CD4+ clones with moderate-to high-affinity for self-antigens. Peripheral Tregs (pTregs) arise from mature, naïve CD4+ T cells in the periphery when they encounter antigen under certain conditions (Curotto de Lafaille and Lafaille, 2009). Peripheral induction of pTregs is thought to require TCR stimulation of naïve CD4+ T cells in the presence of TGF-β and IL-2 and occurs in the tissues (Chen et al., 2003; Mucida et al., 2007; Curotto de Lafaille and Lafaille, 2009) rather than the circulation. For example, most of the pTregs important for mucosal tolerance to environmental and food antigens are induced in the gut (Faria and Weiner, 2006a,b).

Despite the fact that tTregs essentially follow the same ontogeny as effector T cells, the TCR repertoires of Tregs and conventional T cells are distinct with only limited overlap (Hsieh et al., 2004; Pacholczyk et al., 2006). Although there are still many open questions about tTreg development, an amount of self-reactivity below the threshold for negative selection is likely an important factor driving Treg lineage commitment (Hsieh et al., 2012). The contribution of different populations of APC to tTreg formation was explored in a series of studies utilizing genetically engineered mice. Transfer of MHC II–sufficient or MHC II-deficient bone marrow cells into irradiated RAG-1 deficient mice resulted in comparable frequencies of tTregs generated, suggesting that bone-marrow derived APC are dispensable for tTreg induction (Liston et al., 2008). In turn, experiments in which irradiated mice genetically devoid of co-stimulatory molecules were reconstituted with wild-type bone marrow, tTreg frequency was again unaffected suggesting that bone-marrow derived APCs are capable of tTreg suppor (Proietto et al., 2008; Spence and Green, 2008). Of note, the expression of "normal" TCR diversity and function in tTreg populations generated under these artificial experimental conditions has not yet been fully investigated.

Human fetal tissues in the second trimester display a significantly increased frequency of Treg compared to other developmental stages and locations; the lymph nodes contain about 15–20% of these cells (compared to about 5% in other locales and time points) (Cupedo et al., 2005; Michaelsson et al., 2006). Interestingly, this Treg abundance is not true for the fetal thymus at the same time suggesting that the peripheral fetal Treg either originated from nTreg that expanded in the periphery or that they are iTreg (Burt, 2013). Elegant experiments by Mold et. al. demonstrated that these fetal Treg arise in response to Non-Inherited Maternal Antigens (as further discussed below) and unveiled a general propensity of the fetal immune system to react toward and antigenic challenge with the induction of Treg mediated tolerance (Mold et al., 2008). These observations have led to some speculation about a potential contribution to fetal and neonatal increased sensitivity to infections (increased severity and infections with pathogens that are usually considered commensal flora in adults) and less effective immunization by vaccines (Mold et al., 2008). Importantly, the emerging picture points toward the fact that the human fetal immune system is not inert, non-developed or immature but instead highly active generating tolerogenic responses as further detailed in the model of the layered immune system (Mold et al., 2008).

T CELL EXHAUSTION

T cell exhaustion has been observed in many chronic infections and cancer. It is a state in which T cells exhibit poor effector functions, and express inhibitory molecules and a transcriptome distinct from that of functional effector or memory T cells (Wherry, 2011). Exhaustion is best described for persistent infections with high levels of viral replication such as the human immunodeficiency virus (HIV), or hepatitis C virus (HCV). In those settings, CD8+ T cells lose effector functions in a distinctive hierarchical manner starting with proliferative capacity, IL-2 production, then killing capacity. Severely exhausted (end-stage) cells are unable to degranulate at all and are eventually deleted (Moskophidis et al., 1993; Zajac et al., 1998; Wherry et al., 2003). The precise features of exhaustion seem to vary between infections but in general, low CD4+ helper cells and high viral replication are correlated with greater exhaustion of the CD8+ T cell pool in humans (Wherry and Ahmed, 2004; Blattman et al., 2009; Virgin et al., 2009). In both solid-organ and HSC (including IUHCT) transplantation, the recipient and donor alloantigens are continuously present and hence can potentially induce exhaustion of alloantigen-specific T cells. In fetuses or neonates, exhaustion has thus far not been observed possibly due to the tolerogenic propensity in human fetuses (discussed above).

WHAT TYPES OF TOLERANCE MECHANISMS HAVE BEEN OBSERVED IN IUHCT TRANSPLANTATION?

In the case of iatrogenic solid organ allotransplants, tolerance is defined as donor-specific hyporesponsiveness in the absence of pharmacologic immunosuppression (Fung, 1999). Operational tolerance has been reported for different types of transplantation but the underlying mechanisms are not fully understood. In IUHCT and HSC transplantation, graft failure or diminished/disappearing chimerism can be due to two factors or a combination thereof: (1) loss of HSC or their differentiation products due to lack of engraftment or (2) immunorejection of otherwise established, healthy HSC or their differentiated products. Some mechanisms that were elucidated in solid organ or

HSC transplantation may apply to IUHCT and will be discussed in this context.

IN UTERO TOLERANCE ESTABLISHMENT, THE EXPERIMENTS OF BILLINGHAM, BRENT AND MEDAWAR

In 1953, Billingham, Brent, and Medawar conducted a series of experiments to test the hypothesis that "mammals and birds never develop, or develop to only a limited degree, the power to react immunologically against foreign homologous (as allografts were known then) tissue cells to which they have been exposed sufficiently early in fetal life" (Billingham et al., 1953). Skin grafts between adult mice of two different strains normally result in rejection after about 11 days. In the 1953 study, mouse pups age E15 or 16 (term is day 21) were inoculated with adult tissue from another strain. Five littermates were born to which skin from the same strain as the *in utero* inoculant was grafted. The grafts were rejected by two, accepted long-term in two and accepted only short-term in one recipient. This experiment demonstrates that transplant rejection can be ameliorated by *in utero* exposure to adult allogeneic tissues. However, grafting these mice tolerant to one strain with skin from yet another strain did result in rejection, demonstrating that the acquired tolerance was indeed specific. Lastly, they used the mice tolerant to the skin graft and transferred cells from a mouse of the same strain that was immunized against the same skin graft into these recipients. They found that these previously tolerant mice now rejected the graft, the tolerance was "broken." Despite much deeper understanding of immunoregulation today, it is still unclear which tolerance mechanism/s were at work in these experiments by Medawar and colleagues (**Figure 1**).

THE THYMUS IN IUHCT TRANSPLANTATION

Donor-derived APC transplanted as passenger leukocytes are known to play an important role in early graft recognition and graft loss in solid organ transplantation. This type of antigen presentation is termed direct presentation and will stimulate T cells that can productively interact with the donor-MHC-peptide combinations present on the donor APC. The role of direct presentation diminishes over time as the passenger APC die off. In contrast to solid organ transplants, HSC transplants will continuously generate donor APC over the lifetime of the graft. At later times after solid organ transplantation, rejection is triggered by a process termed indirect presentation and refers to the presentation of donor(allo)-peptides by recipient APC. This type of presentation will be continuously functional throughout the lifetime of the graft, as recipient APC circulate and pick up antigen. First observed in solid-organ transplantation, the kinetics and contribution of direct vs. indirect antigen presentation pathways are much less understood in HSC transplantation and IUHCT.

In animals or humans with mixed bone-marrow chimerism, both donor and recipient HSC can theoretically give rise to the different blood products of all blood lineages. To generate peripheral T cells, donor T cells must be positively and negatively selected in the thymus (**Figures 2**, **3**). The establishment of tTreg-mediated tolerance to the donor can follow different scenarios. As mentioned above, the APC compartment of both the thymic cortex and medulla is in constant turnover with peripheral APC migrating in and new resident APC differentiating locally. If the recipient has hematopoietic chimerism after IUHCT, both APC populations can be either donor-derived or recipient-derived (**Figures 2**, **3**). Additionally, these APC sample blood-borne antigens that can also be donor- or recipient-derived. Under these conditions, the selection of both effector T cells as well as tTregs from both sources of HSC that recognize both the host and the donor as "self" is theoretically possible. This scenario would establish a two-way tolerant state, utilizing mechanisms of both clonal deletion and tTreg generation. Indeed, in a mouse model of IUHCT, both donor (direct presentation) and recipient APC (indirect presentation) were found to participate in antigen presentation and both induced clonal effector cell deletion in the thymus (Nijagal et al., 2013). Interestingly, deletion was also detected in the spleens of animals that did not develop chimerism indicating that transient antigen presence is sufficient for the development of central tolerance.

In a SCID patient with a haploidentical fetal liver and thymus transplant, T cell clones were established after 11 years of healthy life of this patient (no indications of GVHD) (Roncarolo et al., 1988). B lymphocytes were found to be of recipient origin while the T cells were of donor origin. Of the 50 established T cell lines, 15 displayed recipient reactive proliferative and cytotoxic responses (6 CD4+ T cell lines and 9 CD8+ T cell lines) demonstrating that clonal deletion of host-reactive T cells is not complete in this patient. In subsequent SCID patients transplanted with either haploidentical bone marrow or fetal liver stem cells, donor-anti-recipient MLR were non-reactive but again, host-reactive donor T cell clones were isolated (Bacchetta et al., 1993, 1995). However, these effector cells, especially the CD4 T cell clones, displayed reduced cytokine production capabilities and reduced proliferative capacity compared to non-transplanted donor cells. These observations suggest a Treg-mediated suppression of T cell reactivity *in vivo* and in MLR accompanied by an exhaustion mechanism that limits reactivity of the recipient-reactive donor cells that escape thymic selection.

REGULATORY T CELLS IN IUCHT TOLERANCE

In a murine IUHCT model, a higher proportion of Tregs was observed in animals that retained chimerism vs. animals that did not retain chimerism or were not subject to IUHCT (Merianos et al., 2009). The authors speculate that a similar mechanism that leads to fetal Treg induction in response to trafficking maternal cells would be responsible for the observed increase in animals with chimerism. However, in this study, the origin of Tregs (donor or recipient derived) was not further addressed. That question was addressed in a study utilizing breeding schemes of T cell transgenic animals to explore the role of direct vs. indirect presentation after IUHCT (Merianos et al., 2009; Flutter et al., 2010). Similar to the finding of the previous study, both with direct and indirect presentation, the percentage of donor-derived Tregs in the thymus and spleen was significantly increased. However, the absolute numbers of Tregs were not increased but instead the skewed proportions were due to a decrease in effector T cells. While these studies indicate that the donor cells are converted to Tregs and contribute to the tolerogenic environment, whether there is an increase in conversion to Tregs after IUHCT is still somewhat unclear.

T CELL EXHAUSTION/CLONAL PERIPHERAL DELETION IN IUHCT TOLERANCE

Given that the key factors involved in T cell exhaustion—the continued presence of antigen and inflammation—applies to both solid organ and HSC/IUHCT transplantation, the role of T cell exhaustion needs to be critically evaluated in this context (Valujskikh and Li, 2012).

In IUHCT, the mechanism of T cell exhaustion would be in play if clonal deletion of recipient-reactive donor cells in the thymus is incomplete. In that case, exhaustion of recipient-reactive donor cells would limit GVHD damage that these cells would otherwise cause. Indeed, in SCID patients transplanted with either fetal liver stem cells or haploidentical bone marrow cells, recipient-reactive T cell clones displayed dysfunctional cytokine production demonstrating both incomplete thymic clonal deletion as well as T cell exhaustion of those reactive clones (Bacchetta et al., 1993, 1995). Similarly, in a mouse model of IUHCT, partial clonal deletion and absence of functional rejection of a donor skin graft were observed (Kim et al., 1999). In that model, functionality of the remaining donor-specific T cells could be re-established after the addition of IL-2, indicating that these cells were indeed partially exhausted, albeit this can only be determined with certainty in MLR with and without Treg depletion.

Information about the mechanism of T cell exhaustion can be gleaned from the study of HSC transplantation in the setting of chimerism with non-hematopoietic recipient cells serving as potential APC is similar to IUHCT. Indeed, the antigenic source responsible for donor T cell exhaustion was addressed in a mouse model of allogeneic bone marrow transplantation. Here, a clinical scenario of non-myeloablative transplantation and delayed lymphocyte infusion was modeled (Flutter et al., 2010). Murine recipients were lethally irradiated and reconstituted with mixed bone marrow cells that included T cell-depleted host marrow and T cell-depleted donor marrow with a partial MHC mismatch and a congenic marker allowing identification. After reconstitution and recovery from lymphopenia, donor T cells were infused which led to the elimination of host hematopoietic elements but not to GVHD, modeling a graft-vs.-tumor effect (**Figure 4**). The donor lymphocytes expanded vigorously up to day 12 and then declined but even after day 60, 5–10% of peak numbers of these alloantigen-primed cells persisted in lymph nodes. However, tests of donor-host cytotoxic activity *in vitro*, showed reduced T cell effector function, proliferative capacity, and IFN-γ production by day 60. This observation of continued presence of donor cells but reduced effector and proliferative capacity is strongly indicative of classical exhaustion. The authors next inquired whether persistent antigen expression by the recipient non-hematopoietic compartment was responsible for donor cell exhaustion. To address this, they performed a similar experiment as discussed above utilizing a transgenic mouse strain that does not express non-hematopoietic tissue antigen (minor HC) genes. In those mice, stimulation of allo-responses occurs only via professional APC while in wild-type mice, both professional APC and non-professional cells such as endothelial cells can present antigen. At day 60, cytotoxicity, recall responses, and memory formation were impaired in wild type mice experimentally. In contrast, in mice devoid of minor HC genes in the non-hematopoietic compartment, none of these features of exhaustion appeared and host-reactive T cells remained fully functional even after 60 days, suggesting that antigen presentation by non-professional APC is responsible for allo-specific T cell exhaustion. These observations strongly indicate that recipient-reactive donor T cells can escape thymic clonal deletion but will acquire an exhausted phenotype in the periphery due to continued presence of "their" antigen in the context of non-professional antigen-presenting cells. The extent to which this is the underlying explanation for T cell exhaustion in the context of IUHCT remains to be determined.

IMMUNOLOGICAL BARRIERS TO IUHCT

Successful, long-term engraftment of HSC requires successful initial engraftment overcoming the barriers of host-competition, immunological space availability, "receptivity" of appropriate niches to support stem cells and possibly active rejection mechanisms. At later times, graft maintenance and functionality requires both the continuous absence of active rejection mechanisms (or the presence of rejection that is low-level and does not delete the graft) and successful participation of graft-derived cells in the selection and maturation processes to form fully differentiated cells. Failure of any of these requirements may limit engraftment or lead to poor long-term graft function but the participation of each of these components in the final phenotype is sometimes difficult to assess. In IUHCT, the presence of the maternal immune system adds another layer of complexity, the effects of which are only partially understood.

FACILITATING HSC ENGRAFTMENT IN IUHCT

Current observations in IUHCT models suggest that successful engraftment starts in the fetal liver from which the cells migrate into the bone marrow. In mice, CXCR4 chemokines follow an SDF-1a chemoattractant gradient for fetal-liver to fetal bone marrow migration. Migrating HSC also express matrix metalloproteinases (MMP-2 and MMP-9) at their leading cellular edge, facilitating degradation of extracellular matrix during migration toward marrow, and they secrete collagen and metalloprotease inhibitors to modify the niche. Hematopoietic stem cells used for IUHCT should express the necessary migratory molecule repertoire and other factors of fetal liver HSC to allow engraftment.

Facilitation of engraftment was further addressed in an IUHCT model in which donor bone marrow was stimulated with vascular endothelial growth factor and stem cell factor (Shaaban et al., 2006). These cytokine-stimulations were chosen to provide a competitive advantage to the graft with regard to homing, establishing residence, proliferation, apoptosis, and bidirectional tolerance. This pre-treatment regimen resulted in higher initial chimerism rates and prolonged survival of skin allografts but long-term engraftment remained unchanged. Expression analysis of relevant homing receptors on the cytokine-treated graft cells did not indicate any changes and host liver homing was not impacted by the treatment. The authors suggest that early progenitor cell subsets did display enhanced homing which could have contributed to the temporary increase in chimerism. They further conclude that cytokine pre-treatment induced cell-cycle activation of normally quiescent progenitors

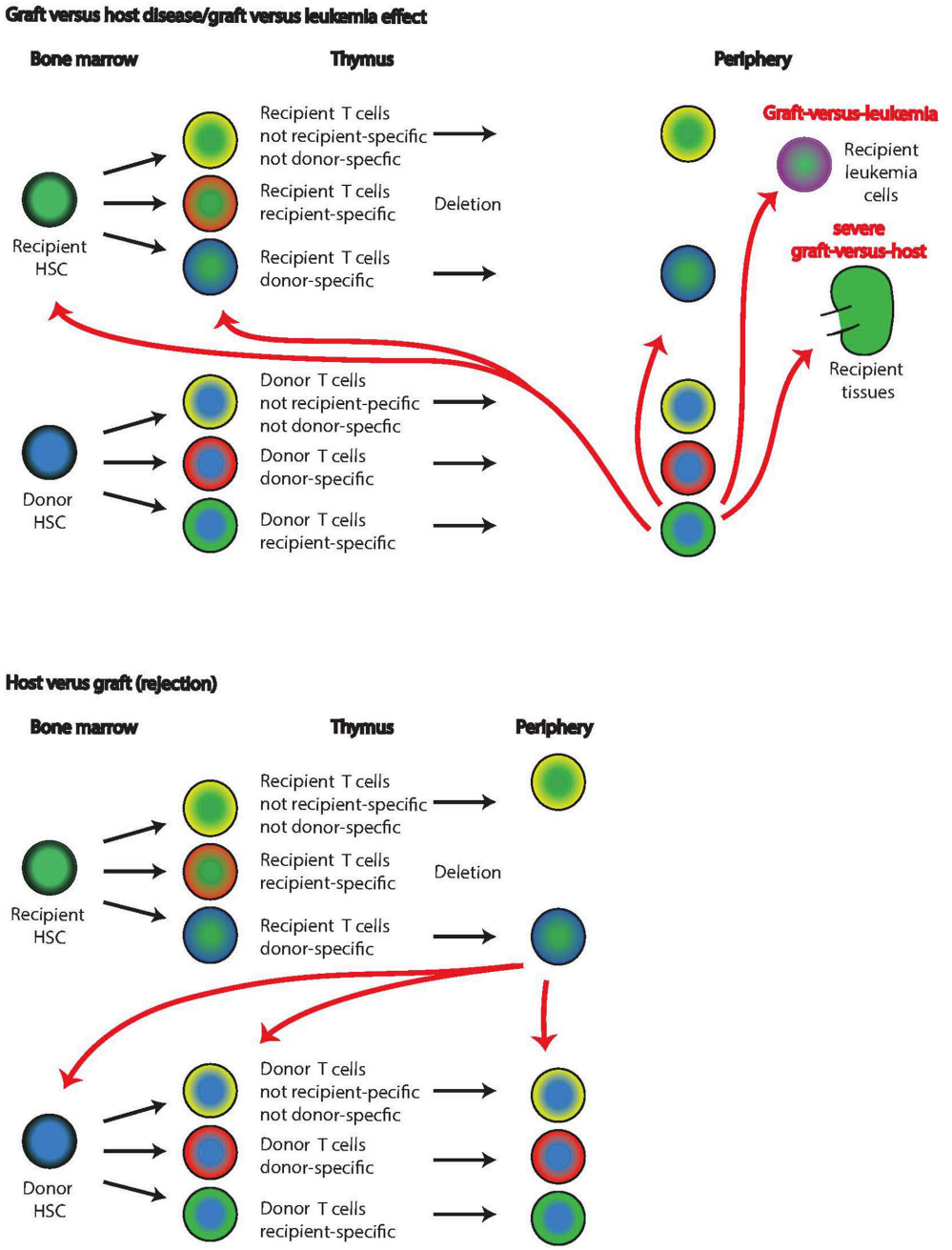

FIGURE 4 | Graft-vs. host and host-vs. graft disease. Graft-vs. host disease or graft-vs. leukemia effect is based on the same anti-host reactivity of the graft lymphocytes (top panels). Recipient (green centers) and donor (blue centers) cells undergo thymic selection, and donor T cells with recipient specificity escape into the periphery (lowest row of top panel). These recipient-reactive cells will delete leukemia cells and other host lymphocytes in the periphery. In severe cases, graft-vs. host disease develops and recipient tissues such as the kidney are attacked. The extent of recipient-specific effector cells attacking recipient T cells already in the thymus or HSC in the bone marrow is unknown. Host-vs. graft reactions (lower panels) are the basis of graft rejection in which recipient T cells with donor specificity escape thymic deletion and attack donor cells. While it is known that ultimately disappear from the bone marrow in this case, it is unclear whether this is due to direct deletion by donor-specific recipient cells or another mechanism.

leading to rapid expansion of donor hematopoiesis and increased early chimerism. These observations further underline the importance of long-term monitoring of chimerism in these models and the fundamental disconnect between early engraftment and chimerism and its long-term maintenance. In a series of follow-up experiments, competitive advantage of the graft was achieved by experimental inhibition of CD26 expression in a mouse model of IUHCT (Peranteau et al., 2006). CD26 is a peptidase that cleaves SDF-1a and thereby inhibits its chemoattractant effect on HSC. Blocking of CD26 in IUHCT of bone marrow or enriched HSC

resulted in increased homing to the fetal liver, and an increased frequency of animals that developed chimerism with increased levels and prolonged stability.

SELF-ANTIGEN AND T CELL HOMEOSTASIS IN DIFFERENT IUHCT APPLICATIONS

Homeostatic maintenance of T cells requires the interaction with self-MHC loaded with self-antigen and γ chain cytokines. T cells need to interact with self-MHC of the appropriate class loaded with self-peptide to maintain a tonal TCR signal that ensures T cell survival (Jameson, 2002). Under lymphopenic conditions such as in a SCID fetus or after anti-thymocyte treatment, naïve T cells not only survive but proliferate until the "immunologic space" is filled. This process of lymphopenia-induced expansion also necessitates self-MHC self-peptide interactions (Bender et al., 1999; Kieper and Jameson, 1999; Oehen and Brduscha-Riem, 1999). Lymphopenia-induced expansion is limited by the available "immunologic space," physical space with the appropriate microenvironment and resources such as APCs and cytokines for which T cells compete (Ernst et al., 1999; Surh et al., 2000). Restrictions of the immunological space ensure that the steady-state size of the lymphocyte pool is stable *and* diverse. T cells that expand in response to lymphopenia frequently change phenotype, and acquire the phenotypic and functional properties of memory cells without transitioning through the typical effector intermediates (Cho et al., 2000; Tchao and Turka, 2012). Further, memory autoimmune cells are more resistant to immunodepletion and will proliferate vigorously in response to lymphopenia, resulting in a disproportionate enrichment of memory autoimmune cells (Monti and Piemonti, 2013). Considerably less is known about the antigen sources used in self-peptide/self—MHC presentation during homeostatic T cell interactions than is known about the antigen sources used in thymic selection of T cells. Cell death via apoptosis, an orderly process and an integral part of normal development, does not prompt an immunologic explosion. In contrast, necrosis or the related necroptosis occurs in response to starvation or membrane disruption and, despite production of the same antigens as in apoptosis, does provoke the immune system (Walsh and Bell, 2010; Lu et al., 2014). Thus, the presence of antigen does not absolutely imply its accessibility for a given immunological process such as presentation of self-peptide self-MHC in the steady-state homeostatic context.

POTENTIAL FETAL ADAPTIVE IMMUNE MECHANISMS OF REJECTION

Current protocols for IUHCT in dog and non-human primate models use T-cell replete bone marrow for transplantation. In the absence of this repletion, engraftment efficiency is greatly reduced (Shields et al., 2003; Petersen et al., 2013; Vrecenak et al., 2014a). These observations suggest that functional T cells are necessary for efficient engraftment, possibly to suppress fetal rejection responses or to create hematopoietic space, but these speculations require further study.

Thymic T cell colonization begins 8–9 weeks after human conception and by 10 weeks, T cells constitute >95% of the cells in the thymus (Haynes et al., 1989). Similarly, B cells start to appear in the omentum and fetal liver at 8 weeks post-conception and in the fetal spleen at ∼11 weeks. By week 22, 70% of splenocytes are lymphocytes. NK cells appear in human fetal liver at 5 weeks after conception; at 6 weeks they constitute 5–8% and by 18 weeks 15–25% of fetal liver lymphocytes (Uksila et al., 1983; Thilaganathan et al., 1993). They also constitute 10–15% of cord blood lymphocytes. Alloreactive fetal lymphocytes (lymphocytes that responded to *in vitro* exposure to allogeneic cells by proliferating) are detectable in fetal livers as early as 7.5 weeks of gestation, in thymus at 12.5 weeks, and spleen and blood at 14.5 weeks (Stites et al., 1974). T cell lines generated from fetal livers demonstrate allo-reactivity (Renda et al., 2000a). Though limited numbers of relevant studies have been reported, the complexities of the human fetal immune system are becoming increasingly apparent from comparisons of transcriptional profiles of fetal cells vs. adult cells, revealing fundamental differences (Mold et al., 2010). These studies strongly suggest that phenotypic similarity between fetal and adult immune cells might not extend to functional similarity, and that functionality of fetal immune cells deserves direct investigation. Currently, human fetuses after 14 weeks of gestation are considered immunocompetent with regard to donor-rejection responses (Flake and Zanjani, 1999a,b; Renda et al., 2000b).

In an elegant study, Mold et al. demonstrated fetal regulatory T cell responses elicited by non-inherited maternal alloantigens (NIMAs, **Figure 5**) (Mold et al., 2008). NIMAs are components of the maternal genome, including MHC genes, not inherited by the fetus. Fetal exposure to these antigens occurs via the transplacental migration of maternal cells and can be detected in multiple fetal organs as early as week 14 (Jonsson et al., 2008). The presence of maternal cells raises the possibility of fetal anti-maternal immune rejection and implies parallel development of regulatory elements that limit rejection (reviewed in Burt, 2013). Indeed, Tregs are fairly abundant in the human fetus and the early fetal immune system seems skewed toward tolerogenic responses until effector functions increase and dominate at term (theory of the layered immune system in human fetuses) (Herzenberg et al., 1992). Fetal tolerance of NIMAs was postulated before it was demonstrated experimentally based on epidemiological data showing that maternal donor organs elicit less rejection than paternal donor organs (Burlingham and Benichou, 2012). The conditions under which (semi)allogeneic maternal cells induce fetal Tregs, are also not yet fully understood. The constant, low number of immigrating maternal cells is clearly different from IUHCT in which a large bolus of cells is delivered at once, disrupting the normal cell number ratios and relationships. Better understanding of this mechanism could be useful in the development of protocols to induce Treg tolerance to the IUHCT.

REJECTION BY FETAL INNATE IMMUNE MECHANISMS

The role of innate immunity in solid allograft and HSC rejection is poorly understood (Alhajjat et al., 2010; Loewendorf and Csete, 2013). Human umbilical cord blood contains NK cells with mature MHC responsive receptors suggesting that NK self-recognition is functional before birth (Grzywacz et al., 2006; Wang et al., 2007). Insights into the role of innate immunity in IUHCT were gleaned from recent mouse studies in which threshold levels of initial chimerism (>1.8%) were found to predict

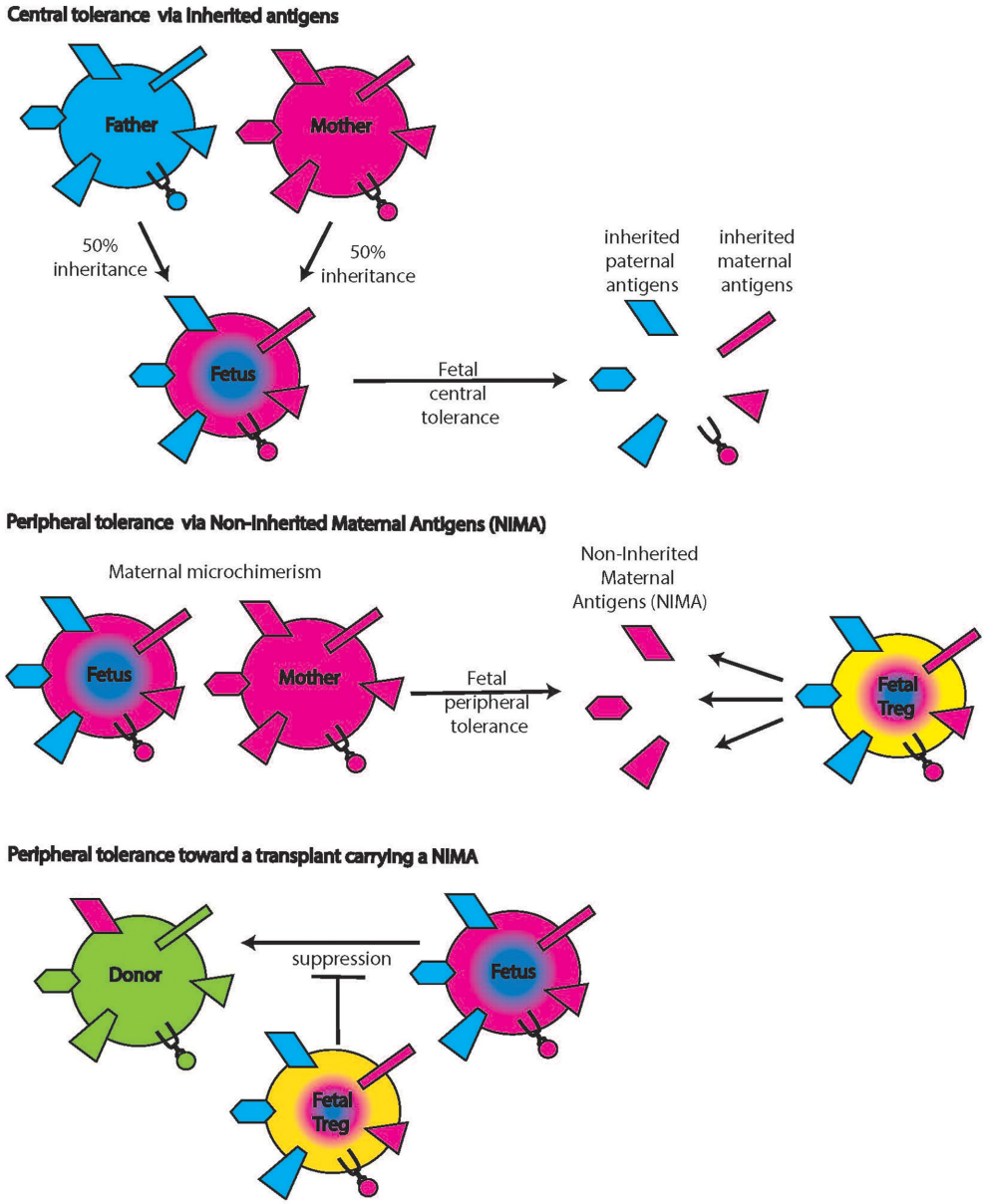

FIGURE 5 | Non-inherited maternal antigens (NIMAs). Central tolerance includes tolerance toward antigens that the fetus inherited from both mother and father (top panels). Maternal cells inhabiting the fetus bring along additional antigens that were not inherited by the fetus (middle panel, left hand side symbols on the maternal cell). The presence of these antigens induces fetal Tregs specific for these NIMAs (middle panels, yellow cell on the right). When donor cells carry NIMAs, these fetal Tregs will suppress allo-reactions (lower panels), an effect that can be either detrimental or desirable.

long-term engraftment and correlated with a donor-tolerant NK response (Durkin et al., 2008). NK depletion in animals with sub-threshold chimerism resulted in abrogation of allograft rejection and the establishment of chimeras. NK cell reactivity depends on a delicate balance of activating and inhibitory receptors and in stable chimeras, the expression of the inhibitory NK receptor Ly49A is down-regulated compared to animals in which chimerism was lost. Initially, Ly49A expression was reduced in all recipients of IUHCT albeit still higher in the animals that subsequently lost chimerism indicating that those animals experienced an inadequate degree of receptor-ligand interaction resulting in failed host NK-cell education during the time of receptor acquisition. In a subsequent study, the transfer of donor MHC to host NK cells (trogocytosis) was shown to correlate with NK cell tolerance (Alhajjat et al., 2013).

MICROCHIMERISM vs. MACROCHIMERISM AND ITS ROLE IN TRANSPLANT TOLERANCE AND IUHCT

Since the discovery of donor microchimerism in recipients of solid-organ transplants by Starzl et al. donor leukocytes that remain functional through the organ preservation process and enter the host bloodstream were suspected mediators of

transplant tolerance (Starzl et al., 1992; Hisanaga et al., 1995; Hundrieser et al., 1995; Schlitt et al., 1995). Chimerism that is not detectable by FACS but only with sensitive PCR methods is generally referred to as microchimerism while macrochimerism is chimerism at levels readily detectable by FACS. Donor leukocytes can persist in the recipient for many years and result in allogeneic microchimerism not unlike maternal-fetal microchimerism (Verdonk et al., 2011). Stable levels of donor microchimerism have been suggested as a marker of transplant tolerance allowing reduction of immunosuppressive drug doses (Ayala et al., 2009). One percentage microchimerism can be sufficient for transplant tolerance in xenogenic animal models (Ildstad and Sachs, 1984). These studies have prompted exploration of chimerism as a tolerogenic mechanism, clinically accomplished by transplantation of allogeneic bone marrow after appropriate host conditioning (Trivedi et al., 2005). Nonetheless, the mechanism of tolerance induced by microchimerism is not completely clear. Early interpretations such as the theory of reciprocal deletion and exhaustion of host and donor effector T cells were based on the absence of donor-reactivity in *in vitro* MLR or killing assays without taking the role of Tregs into account (Starzl et al., 1994). Clinically, various types of chimerism levels from none to high or temporary have been observed without clear correlation to clinical rejection or operational tolerance (Billingham et al., 2003).

In a mouse model of IUHCT, congenic adult bone marrow was used for IUHCT followed by booster injections on days 2, 4, and 7 of life (Milner et al., 1999). The congenic setting ensured that immunologic effects would not confound the observations. Postnatally boosted animals had significantly higher levels of donor cell engraftment as determined in the peripheral blood 6 weeks after birth and the increased levels were stable until the last time point analyzed, 6 months of age. In a subsequent study a booster transfer of allogeneic donor bone marrow was applied after non-myeloablative total body irradiation (Peranteau et al., 2002). In that study, low-level chimerism after IUHCT was enhanced to high-level chimerism depending on the irradiation dose; the mechanism was found to be donor tolerance by the host and a transient competitive advantage of the non-irradiated donor stem cells. Lastly, no evidence of GVHD was observed in these experiments, suggesting that this protocol of prenatal tolerance induction in preparation for subsequent postnatal treatments is a potential clinical strategy. Booster regimens were also found to be possible in canine models, in 2 of the 5 boosted animals the degree of chimerism increased from <1% after IUHCT to 35–45% after postnatal boost with busulfan conditioning (Peranteau et al., 2009). In contrast, postnatal HSC transplantation did not result in detectable chimerism in naïve controls, demonstrating that IUHCT conditioning is crucial to facilitate postnatal minimal conditioning HSCT. Importantly, the phenotype of canine leukocyte adhesion deficiency (CLAD) was ameliorated by the IUHCT serving as a proof of principle that this lethal phenotype can be rescued by IUHCT. The reason for the heterogeneous chimerism levels within one group of experimental animals is unclear. Possibly, a certain threshold microchimerism needs to be established via IUHCT to achieve central tolerance which subsequently allows for the effective postnatal boost but further studies are required to understand this phenomenon.

A study refining the delivery method of IUHCT in the haploidentical canine model recently achieved, for the first time, long-term stable therapeutic levels of chimerism in a large animal model without prior conditioning or GVHD (Vrecenak et al., 2014b). Intraperitoneal injection was compared to intracardiac delivery and the latter achieved far higher intravascular levels of circulating donor cells, higher levels of initial fetal liver engraftment, and subsequent higher long term donor chimerism. Donor-derived renal grafts were transplanted and monitored for rejection to assess the presence of donor specific chimerism vs. tolerance. All recipients with chimerism above 10% showed no acute or chronic rejection at any point and, importantly, no signs of GVHD were observed, further underlining that fetal recipients are less prone to the development of this serious complication. Taken together, these experiments provide important experimental justification to move forward with clinical trials of IUHCT for inherited hematologic diseases.

REJECTION BY MATERNAL MECHANISMS

Maternal antibodies in utero and in breast milk

Maternal immunoglobulin G (IgG) antibodies are transported to the placenta via an FcR-mediated process (Malek et al., 1996). The amount of maternal antibody present in the fetal bloodstream increases exponentially during gestation, reaching 50% of maternal blood levels at weeks 28–33, then continues to increase, resulting in higher amounts of IgG in fetal than maternal serum at term (Malek, 2003; Esposito et al., 2012). This maternal antibody protects the baby in the first months of life, and maternal vaccination during pregnancy with the goal of protecting the infant by transplacental IgG transfer has been demonstrated in clinical trials (Esposito et al., 2012). After birth, maternal antibodies are continuously transferred via breast milk and thus provide continuous protection. Potential danger posed to fetuses by maternal antibodies first became evident when fetal hemolytic anemia, resulting from maternal anti-Rhesus antibodies was discovered (Chown, 1954).

In IUHCT, maternal anti-donor antibodies have been shown to play an important role in long-term chimerism maintenance: Merianos et al. demonstrated that maternal sensitization during IUHCT and subsequent transfer of alloantibodies via maternal breast milk induces an adaptive immune response in mouse pups which leads to graft rejection (Merianos et al., 2009). This rejection was not observed if the pups were fostered by dams not subjected to the IUHCT procedure and thus did not produce alloantibodies. In contrast to traditional views that suggest maternal antibodies act by directly binding the target antigen, the authors convincingly demonstrate the elicitation of a fetal effector response, similar to observations by others in the setting of fetal antibody exposure (Greeley et al., 2002; Setiady et al., 2003). The maternal alloantibodies produced in this setting were both IgM and IgG (mostly IgG2a) classes. Although the specific mechanism by which maternal alloantibodies elicit an immune response in the fetus is not yet clear, these observations suggests that maternal sensitization during IUHCT in humans could pose an additional hurdle as the gestation time in humans is much longer, potentially

allowing for transplacental IgG alloantibody transport that cannot be avoided (in contrast to breast milk-related alloantibody exposure).

Maternal microchimerism

Many maternal cell types contribute to microchimerism of the fetus; cells of myeloid and lymphoid lineages have been identified as well as hematopoietic progenitors (Jonsson et al., 2008). Though maternal microchimerism represents a low percentage of total fetal immune cells, it is potentially relevant for IUHCT. In cord blood, 0.1–0.5% of total T cells are maternal (Hall et al., 1995; Mold et al., 2008). These maternal cells are enriched for cells specific for antigens inherited by the fetus from the father, and can enhance graft-vs.-leukemia effects if the cord-blood recipient shares them (Burlingham and Benichou, 2012). In turn, these maternal cells can elicit rejection responses if the HSC graft carries paternal antigen or other antigens to which maternal cells have been sensitized (**Figure 1**). To avoid graft rejection caused by maternal chimeric cells, a maternal transplant source or avoidance of paternal inherited antigens is advantageous. MLR detection of preexisting maternal reactivity to a potential HSC donor can help identify such potential rejection responses.

Maternal cells with anti-donor specificity can also directly inhibit the establishment of chimerism in IUHCT (Nijagal et al., 2011). In these experiments, T or B cell deficient dams were bred to wild-type fathers and the fetuses were subjected to IUHCT. Engraftment was significantly improved in dams lacking T cells but not B cells. This improvement was not observed if the graft matched the MHC of the mother suggesting that for initial engraftment, transplacental trafficking of maternal allospecific T cells poses a substantial barrier. Whether these maternal cells directly kill donor cells or whether the loss of chimerism is due to other mechanisms is currently unclear. Additionally, the authors demonstrated a marked increase in maternal T cell and also B cell trafficking to the fetus after IUHCT of allogeneic cells (compared to control pups receiving PBS). Potentially, the presence of the additional antigenic load from the donor cells or the induction of local inflammatory responses to cellular debris or transferred apoptotic cells that confer danger signals attracts more maternal cells.

Altered maternal fetal cellular trafficking in humans has been reported after fetal surgeries, preeclampsia and pregnancy termination but potential roles for these insults in the limitation of chimerism has not been determined (Jeanty et al., 2014).

CONCLUSION

The fetal immune system is a complex system that changes dramatically as a function of developmental stage, and to patterns of exposure to alloantigens. Given that direct query of the immune response after IUHCT is difficult, well-designed *in vitro* and *in vivo* experiments are needed to dissect the mechanisms of tolerance and rejection for this potentially important clinical procedure.

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NK cell tolerance as the final endorsement of prenatal tolerance after *in utero* hematopoietic cellular transplantation

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The primary benefits of *in utero* hematopoietic cellular transplantation (IUHCT) arise from transplanting curative cells prior to the immunologic maturation of the fetus. However, this approach has been routinely successful only in the treatment of congenital immunodeficiency diseases that include an inherent NK cell deficiency despite the existence of normal maternal immunity in either setting. These observations raise the possibility that fetal NK cells function as an early barrier to allogeneic IUHCT. Herein, we summarize the findings of previous studies of prenatal NK cell allospecific tolerance in mice and in humans. Cumulatively, this new information reveals the complexity of the fetal immune response in the setting of rejection or tolerance and illustrates the role for fetal NK cells in the final endorsement of allospecific prenatal tolerance.

Keywords: *In utero* transplantation, T cells, NK cells, fetus, tolerance, chimerism

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In utero hematopoietic cellular transplantation (IUHCT) remains a promising intervention for treatment of a wide variety of congenital disease (Merianos et al., 2008). A primary assumption in IUHCT is that the early-gestation fetus has an immature immune system that is incapable of rejecting a donor cell transplant. As a result, the introduction of donor antigen prior to the development of the adaptive immunity should lead to life-long donor-specific tolerance. Thus, current protocols for IUHCT favor that the initial transplant be delivered by 12 weeks gestation within a "therapeutic window" that opens shortly after prenatal diagnosis and closes with thymic maturation (Westgren, 2006). Observations of naturally occurring hematopoietic chimeras demonstrates that, in essence, this is feasible (Owen, 1945). However, repeated clinical failure of IUHCT in the setting of a non-defective immune system has forced a re-examination of this central dogma, i.e., the translation of bedside observations back to the bench for hypothesis-driven inquiry.

The Clinical Paradigm for Prenatal Transplantation

Two related observations arising from clinical experience with IUHCT are in need of a scientific explanation. First, clinical application of IUHCT has documented success in the treatment of severe combined immunodeficiency (SCID). Indeed, the greatest clinical success has been realized in the treatment of NK cell deficient SCID (xSCID or ADA-SCID) whereas the use of IUHCT for the treatment of congenital diseases in which the fetal immune system is not defective has been uniformly unsuccessful (Touraine et al., 1992; Flake et al., 1996; Wengler et al., 1996) This includes most of the clinical experience with IUHCT for hemoglobinopathies such as sickle cell disease

or thalassemia. Second, the maternal immune system has been intact for every case despite the nature of the clinical outcome (success or failure) suggesting no independent role for the maternal immune response in IUHCT-related engraftment failures. Taken together, these observations frame the clinical paradigm for IUHCT and serve as a template for translational study.

It has been postulated that competition between the donor and recipient cells for a limited number of available host hematopoietic stem cell (HSC) niches is responsible for the clinical failure of IUHCT in the treatment of hemoglobinopathy (Peranteau et al., 2004, 2006). Favorable competition with the host cells for available niches within the fetal liver or bone marrow is vital for successful engraftment and likely explains the enhanced clinical and experimental success of IUHCT with the use of more competitive fetal donor cells or larger doses of bone marrow cells (Shaaban and Flake, 1999; Peranteau et al., 2006; Shaaban et al., 2006). Improved competition for available host niches would logically lead to higher levels of early chimerism. Previous reports from our group illustrate that the early chimerism level (discussed below) is the major determinant of successful allogeneic engraftment and link this to the development of donor-specific NK cell tolerance (Shaaban et al., 2006; Durkin et al., 2008a,b; Alhajjat et al., 2013). However, a competitive-niche model struggles to explain the dichotomous observations for immunodeficient vs. non-immunodeficient cases and seems to disregard the obvious difference. More specifically, no direct evidence exists to support the existence of quantitative differences in the number of HSCs or available stem cell niches between the xSCID and sickle cell disease or β-thalassemia patients. To the contrary, the defects in SCID emerge following the lineage-specific differentiation of HSCs rather than during their maintenance or self-renewal (reviewed in Schmalstieg and Goldman, 2002; Kalman et al., 2004) As a result, the pre-thymic SCID fetal hematopoietic microenvironment should theoretically have the same frequency of available stem cell niches as in pre-thymic fetus with defective β-globin production and should engraft similarly if niche availability is the limiting factor. Therefore, a model in which donor cell competition for host niches solely determines the outcome of IUHCT does not adequately reconcile the clinical paradigm and prompts further study of early gestation fetal alloimmunity.

Studies in murine and primate models of IUHCT support the presence of an early gestation immune barrier to allotransplantation (Peranteau et al., 2007; Durkin et al., 2008a,b). In a murine study by Peranteau et al. (2007), both congenic and allogeneic transplant recipients demonstrated similar multi-lineage engraftment at 1 week of age. Thereafter, most allogeneic recipients lost engraftment (Peranteau et al., 2007). Similarly, we have also demonstrated that congenic recipients maintain long-term engraftment regardless of the chimerism level whereas allogeneic recipients require a minimum level of circulating chimerism to maintain stable engraftment and prevent a chronic form of rejection (Durkin et al., 2008b). Lastly, despite promising results in other large animal models (Lee et al., 2005; Vrecenak et al., 2014), numerous studies of allogeneic IUHCT in non-human primates have yield poor overall engraftment regardless of the gestational age (Cowan et al., 1996; Shields et al., 2003, 2004). In general, the use of fetal conditioning or mature T cell co-transplantation resulted in higher engraftment rates and chimerism levels overall (Peranteau et al., 2002; Hayashi et al., 2004; Ashizuka et al., 2006). Collectively, these findings point to the existence of a previously unrecognized immune barrier to IUHCT that resides within the fetal host potentially complicating the kinetics of early engraftment.

Fetal T Cells are Unlikely to Act Alone in Rejection after IUHCT

In the complexity of the developing fetus, successful prenatal engraftment likely requires that all components of the immune system develop tolerance. Similarly, failed engraftment in clinical cases that do not involve immunodeficiency likely results from a lack of tolerance in one or more components of the fetal immune response. In either case, an intrinsic immune barrier to prenatal allotransplantation exists within the fetal host and awaits further delineation. In the greater context, a better understanding of the critical parameters regulating the emergence of self-tolerance may explain the pattern of success and failure of IUHCT.

Previous studies reveal that T cell self-recognition is established before birth in most strains of mice including the C57BL6/J and Balb/c inbred strains. The first measurable indicator of self-recognition is the emergence of phenotypically mature T cells as early as E17 (Crispe et al., 1986). Phenotypically mature single-positive T cells have been found in the human thymus as early as 12 weeks gestation (Stites and Pavia, 1979; Haynes et al., 1989). Confirmation of the functional capacity to reject an allograft comes from transplant studies in newborn mice which do not accept fully allogeneic hematopoietic grafts without myeloablation or immunological preparation (Soper et al., 2003). Conversely, the capacity to reject allogeneic grafts does not exist earlier in gestation. The studies by Kim et al. (1998, 1999) first demonstrated that allospecific T cell tolerance can be reliably achieved by IUHCT to the murine fetus at E14 resulting in the deletion and anergy of donor-reactive T cells. Subsequent studies confirmed the allo-receptivity of C57BL/6 and Balb/c fetuses at E14 (Shaaban et al., 1999, 2006; Hayashi et al., 2002;Durkin et al., 2008a,b; Alhajjat et al., 2013; Nijagal et al., 2013). Collectively, these findings suggest that the potential for donor-specific tolerance to reliably develop following IUHCT is lost shortly after the appearance of mature single positive T cells.

Furthermore, in the studies by Kim et al. (1998, 1999), T cell and skin allograft tolerance could be seen with extremely low levels of *microchimerism* (<0.1%) following IUHCT between MHC class I-matched or mismatched strain combinations. These findings seem to conflict with subsequent studies (Ashizuka et al., 2006; Durkin et al., 2008b) demonstrating that tolerance to a hematopoietic graft tolerance requires *macrochimerism* (1–2%) but may be reconciled through an understanding of the different measures of tolerance. Skin-graft acceptance has been

shown to reflect T cell rather than NK cell tolerance whereas tolerance to a hematopoietic graft reflects both. NK cells fail to reject allogeneic skin grafts in the absence of IL-15 activation (Kroemer et al., 2008). Thus in sub-threshold microchimeric mice, host T cells may be tolerant to allogeneic skin grafts while host NK cells are not tolerant to allogeneic hematopoietic cells. Additionally, although NK maturation occurs in the early second trimester of human gestation, it continues for several weeks after birth in the B6 mouse (Dorfman and Raulet, 1998). In the aforementioned studies by Kim et al. (1998, 1999), the majority of skin grafts were placed on the microchimeric mice during the prolonged phase of chronic rejection that is typical of low-level prenatal chimerism. As such some degree of hyporesponsiveness to skin and possibly hematopoietic grafts likely exists during this rejection period. Comparative measurements of the allospecific response between T cells and NK cells in microchimeric or sub-threshold chimeric mice might reveal these differences. Thus, the existence of hematopoietic microchimerism during that period appears to be sufficient for donor-specific T cell and skin allograft tolerance to develop but insufficient for NK cell tolerance. As such, the potential for either T cell tolerance or rejection to develop following IUHCT seems to hinge on the timing of transplant rather than the level of chimerism. Perhaps these differences arise from the relatively high-affinity interactions between T cell receptors and peptide-MHC complexes (TCR-pMHC) that regulate much of thymic selection (reviewed in Moran and Hogquist, 2012; Morris and Allen, 2012). Given the high cell dose per kg of fetal body mass previously used for clinical IUHCT, it is likely that sufficient chimerism levels were present for the induction of T cell tolerance. As such, T cell-mediated rejection seems to be an inadequate stand-alone explanation for the failed engraftment seen in the treatment of hemoglobinopathy by IUHCT.

Fetal NK Cells as an Intrinsic Barrier to Prenatal Allotransplantation and a Target for Immunotherapy

The presence of a secondary barrier to prenatal engraftment mediated by fetal NK cells would explain the clinical pattern of enhanced success in NK cell deficient recipients (Flake et al., 1996; Wengler et al., 1996; Archer et al., 1997). An NK cell barrier would also explain the delayed experimental engraftment loss (chronic rejection) previously reported by multiple investigators (Carrier et al., 2000; Donahue et al., 2001; Peranteau et al., 2007; Durkin et al., 2008b) as this coincides temporally with the maturation of NK cell allorecognition (Roth et al., 2000). For these reasons, we queried the response of NK cells in a murine model and ultimately confirmed the existence of an NK cell-mediated barrier to the engraftment of prenatally transplanted allogeneic hematopoietic cells (**Figure 1**). Importantly, this barrier was found to be critically dependent on the level of circulating chimerism (Durkin et al., 2008b). With high levels of chimerism, recipients maintained stable engraftment and exhibited donor-specific NK cell tolerance. Conversely, recipients with low chimerism levels displayed NK cell-dependent chronic graft rejection.

The essence of this *quantitative* model for NK cell education is that the donor-specific tolerance requires a threshold level of

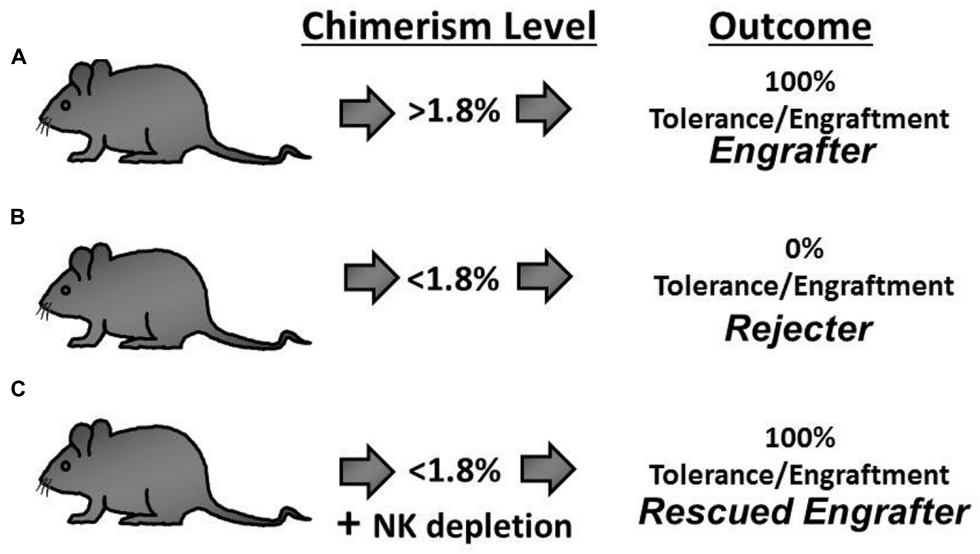

FIGURE 1 | The outcome of engraftment or rejection following allogeneic *In utero* hematopoietic cellular transplantation (IUHCT) is predicted by the early chimerism level and hinges on the development of NK cell tolerance. (A) Mice with an early chimerism level above the chimerism threshold (Engrafter mice; >1.8% circulating chimerism) exhibit durable long-term multi-lineage engraftment. **(B)** With early chimerism levels below the chimerism threshold, NK cell tolerance does not develop and universal rejection occurs (Rejecter mice; <1.8% circulating chimerism). **(C)** Rejection is abrogated when NK cells are depleted in rejecter mice thereby rescuing prenatal engraftment (Rescued Engrafter mice).

exposure to the donor ligands during development – a chimerism threshold (>1.8%). Remarkably, we found that the chimerism threshold reliably predicts the binary outcome of either engraftment or rejection which is the arguably most meaningful measure of prenatal tolerance induction. Furthermore, the chimerism threshold proved to be irrelevant in IUHCT between congenic strains of mice which are immunologically matched reducing the likelihood that limiting-dilution kinetics were the cause of failed engraftment below the chimerism threshold (Durkin et al., 2008b). Lastly, we demonstrated that the engraftment loss can be prevented in sub-threshold chimeras by early *in vivo* depletion of host NK cells (**Figure 1**). During the period of NK cell depletion, chimerism levels remained stable or increased slightly. When the host NK cells were allowed to return following withdrawal of the depleting antibody, abrupt rejection was observed in all of the animals. These findings defined a critical relationship between a threshold level of donor chimerism and the development of donor-specific NK cell tolerance. In a larger context, the significance of this threshold lies in the identification of the minimum level of antigen exposure during the T and NK cell education that is necessary for durable recognition as self.

Trogocytosis as a Mechanistic Link between the Chimerism Threshold and Prenatal NK Cell Tolerance

In order for prenatal tolerance to develop at such low levels of chimerism (1.8%), the process of self-education should include a mechanism to compensate for the low probability of effector cells to encounter appreciable levels of donor ligand. We have recently reported that donor-to-host MHC transfer (trogocytosis) might function in this role (Alhajjat et al., 2013). Trogocytosis of donor MHC may permit sustained *cis*-recognition of the donor ligands during development in the absence of *trans*-interaction directly with the donor cells or with host APC's. The *cis*-recognition of donor antigens by tolerant host NK cells may selectively prevent the apoptosis of "friendly" phenotypes during the selection of the mature NK cell repertoire. Support for this mechanism comes from the recent report by Brodin et al. (2012) who examined Ly49D+ NK cells in H-2D^d transgenic B6 mice and found that the co-expression of Ly49A conferred a resistance to apoptosis. Consistent with this postulate, high levels of trogocytosis were found on the surface of phenotypically friendly NK cells that express donor-specific inhibitory receptors when compared to the phenotypically hostile NK cells that do not express these inhibitory receptors. Subsequent to their developmental selection, the *cis*-recognition of transferred donor-MHC by phenotypically friendly NK cells might provide a continuous exposure to the donor ligands affecting their maturation, survival and function (Kim et al., 2005; Chalifour et al., 2009). In this manner, trogocytosis of donor ligands could provide an intrinsic mechanism for the development and maintenance of donor-specific NK cell tolerance.

Fetal NK Cells at the Interface with the Maternal Immune System

A maternal immune response toward the donor cells has been proposed as a barrier to prenatal allo-transplantation resulting in the delayed chronic rejection of the donor graft (Merianos et al., 2009; Nijagal et al., 2011). As discussed earlier, this postulate is inconsistent with the recurring clinical observation that IUHCT has been successful in cases where the maternal immune response was intact but the fetal immune system was defective (Flake et al., 1996; Wengler et al., 1996; Merianos et al., 2008). Also incompatible with this conclusion is the observation that engraftment or rejection after IUHCT occurs in littermates subjected to the same maternal influence (Durkin et al., 2008b; Merianos et al., 2009). Lastly, the *in vivo* elimination of host NK cells prevents the chronic rejection seen in sub-threshold chimeras and establishes that the effectors of this response reside within the fetal recipient. Collectively, these findings argue against the existence of a clinically relevant maternal immune barrier to IUHCT and should be reconciled with those of previous reports concluding that a maternal immune response toward the transplanted cells leads to engraftment loss in allogeneic IUHCT.

In the study by Merianos et al. (2009), prenatal allogeneic chimeras were noted to lose engraftment at a relatively high rate several weeks after birth. When naïve foster dams were used after delivery, no engraftment loss occurred. This observation was explained by the finding of donor-specific alloantibodies in the maternal breast milk that perhaps induced graft rejection after birth. The authors provided no data regarding the level of chimerism in the animals that rejected their graft. Additionally, the presence of maternal alloantibodies did not result in graft rejection in nearly 1/3 of the littermates of the pups that lost engraftment suggesting some heterogeneity in the effect of the maternal immune response.

These findings may relate to the experimental methodology employed in their study. The study by Merianos et al. (2009) used very large doses of adult donor bone marrow cells (20×10^6cells/fetus) which provided a large number of mature T cells to the fetus (approximately 10^9 mature T cells/kg). These large T cell doses may have resulted in a significant graft-vs-host reaction that may have diminished the maternal–fetal immune barrier leading to a greater exposure of the fetal cells to the maternal immune system. The high chimerism levels seen in this model likely compounded the exposure to the donor alloantigens leading to a functionally significant maternal immune response and precipitous fetal loss. Conversely, mature T cells are absent from the fetal liver donor cell populations used in other studies and chimerism levels are much more modest at the lower doses that were used (Rebel et al., 1996; Szilvassy et al., 2001; Taylor et al., 2002; Hayashi et al., 2003). Further study is necessary to confirm the potential to provoke a maternal humoral response with the use of large T cell doses or high early chimerism levels in IUHCT.

These possibilities are further supported by the study of Nijagal et al. (2011), which utilized fetal liver cells for prenatal transplantation and found that the use of naïve foster dams had no impact on delayed engraftment loss. Instead, the authors observed that a significant numbers of T cells traffic from the

mother into the fetus following prenatal transplantation and proposed that a maternal T cell response was responsible for the lower early engraftment rates in allogeneic vs. congenic IUHCT. Indeed, higher engraftment rates were seen with the use of T cell deficient mothers or by matching the donor cells with the maternal MHC antigens in wild-type matings thereby avoiding the potential for a maternal T cell response in either setting.

However, the reported early engraftment rate in this study is unusually low given the relatively large number of transplanted fetal liver cells. Using the same methodology to prepare and prenatally transplant a similar dose of MHC mismatched fetal liver donor cells (2×10^6 cells/fetus) in this strain combination, we have reliably detected engraftment in 100% of the offspring despite the use of immunologically normal wild-type dams (Durkin et al., 2008b). The reasons for the different engraftment rates between the two studies are unclear but may result from differences in the strain combination or technical variations in the transplant procedure. Additionally, the authors concluded that maternal T cells persisted in the chimeric offspring for months after birth and led to chronic rejection. However, no maternal cells could be found within the recipient at any point beyond the fetal period making it difficult to reconcile engraftment loss by this mechanism that occurred months later. Despite these unresolved issues, the finding of maternal cells in the fetal immune system illustrates the potential complexities involved in prenatal transplantation and clearly warrants further study.

Early Gestation Human Fetal NK Cells Possess the Capacity for Allorecognition and the Potential to Respond to Prenatal Allo-Transplantation

The gestational time frame in which human fetal NK cells develop the capacity for allorecognition has not been directly elucidated. The finding of mature cytotoxic NK cells in human cord blood provides clear evidence that this occurs prior to birth (Wang et al., 2007). It is conceivable that this coincides with acquisition of killer immunoglobulin-like receptors (KIR) similar to the acquisition of the homologous Ly49 NK cell receptors in mice (Roth et al., 2000). Acquisition of Ly49 receptors is commensurate with development of mature cytotoxic capacity and temporally coincides with rejection in sub-threshold chimeras or tolerance and engraftment in above threshold chimeras (Anfossi et al., 2006; Durkin et al., 2008b; Orr and Lanier, 2010). In a seminal report by Phillips et al. (1992), NK cells were found in human fetal liver as early as 6 weeks and in fetal spleen by 15 weeks of gestation. Similar to observations in fetal mice, these early human fetal liver NK cells were found to express high levels of the class Ib-specific CD94/NKG2 receptors. However, an analysis for the expression of the class Ia-specific KIR receptors was not included. The *in vitro* study of differentiating CD34+ hematopoietic progenitors indicates that CD94/NKG2A expression precedes the expression of HLA-specific KIR receptors by human NK cells (Grzywacz et al., 2006). For this reason, we examined the expression of KIR receptors by early gestation human fetal NK cells and found that small subsets of human fetal NK cells express adult levels of KIR receptor by 10 weeks of gestation with more appreciable levels identified by 14 weeks gestation (Alhajjat et al., 2010). A subsequent report by Ivarsson et al. (2013), demonstrated paradoxical hyporesponsivness to KIR-specific stimulation of second trimester human fetal NK cells. Hence, although early gestation human fetal NK cells possess the necessary machinery for allorecognition, a confirmation of their capacity for allospecific cytotoxicity requires future study.

Closing Remarks

Successful engraftment in IUHCT likely requires tolerance in all components of the host immune system. Our studies in the murine model have indicated that a minimum level of circulating ligand is necessary to induce and maintain tolerance. This level seems to be higher for NK cells than the level required for other components of the immune system. Thus, the chimerism threshold might represent the minimum qualification in the education of the developing NK cells and NK cell tolerance as the final endorsement of donor recognition. The study of NK cell repertoire formation, maturity, and trogocytosis in prenatal transplantation will not only facilitate understanding of the NK cell barrier, but will also contribute to the overall understanding of NK cell biology.

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In utero hematopoietic cell transplantation: induction of donor specific immune tolerance and postnatal transplants

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In utero hematopoietic cell transplantation (IUHCT) is a non-myeloablative non-immunosuppressive transplant approach that allows for donor cell engraftment across immunologic barriers. Successful engraftment is associated with donor-specific tolerance. IUHCT has the potential to treat a large number of congenital hematologic, immunologic, and genetic diseases either by achieving high enough engraftment levels following a single IUHCT or by inducing donor specific tolerance to allow for non-toxic same-donor postnatal transplants. This review evaluates donor specific tolerance induction achieved by IUHCT. Specifically it addresses the need to achieve threshold levels of donor cell engraftment following IUHCT to consistently obtain immunologic tolerance. The mechanisms of tolerance induction including partial deletion of donor reactive host T cells by direct and indirect antigen presentation and the role of regulatory T cells in maintaining tolerance are reviewed. Finally, this review highlights the promising clinical potential of *in utero* tolerance induction to provide a platform on which postnatal cellular and organ transplants can be performed without myeloablative or immunosuppressive conditioning.

Keywords: *in utero*, immune tolerance, postnatal transplant, fetus, hematopoietic stem cell, myeloablation, immunosuppression, *in utero* hematopoietic cell transplantation

INTRODUCTION

The fetal environment offers the unique opportunity to take advantage of the developing immune system to induce immunologic tolerance to foreign antigen. This was initially recognized in an experiment of nature in which Owen observed permanent red blood cell chimerism in dizygotic cattle twins that shared cross-placental circulation (Owen, 1945). Later studies by Billingham, Medawar, and others confirmed the ability to induce immunologic tolerance by early gestational exposure to foreign antigen (Anderson et al., 1951; Billingham et al., 1952; Simonsen, 1955). *In utero* hematopoietic cell transplantation (IUHCT) seeks to take advantage of this developmental phenomenon. In multiple animal models, IUHCT has been shown to be a non-myeloablative non-immunosuppressive transplant approach that allows for engraftment across immunologic barriers and is associated with the induction of donor specific tolerance (Flake and Zanjani, 1999; Kim et al., 1999; Peranteau et al., 2002). Clinically, IUHCT has the potential to treat any congenital hematologic, genetic or immunologic disorder which can be prenatally diagnosed and which is currently managed with a postnatal hematopoietic stem cell (HSC) transplantation requiring a matching donor and/or myeloabalative and immunosuppressive conditioning.

The clinical application of IUHCT could take one of two potential courses (**Figure 1**). A single *in utero* transplant may result in high enough levels of donor cell engraftment to treat the target disease. Alternatively, IUHCT may be used to induce donor specific tolerance which would allow for postnatal same-donor transplants with non-toxic conditioning regimens to increase donor cell engraft to clinically relevant levels. Tolerance achieved by IUHCT may also be used to permit postnatal same-donor organ transplants without immunosuppressive conditioning. To date, IUHCT has only been clinically successful in the treatment of severe combined immunodeficiency disorder (SCID; Flake et al., 1996; Wengler et al., 1996). Broader clinical application of IUHCT is limited by the ability to consistently achieve high enough levels of donor cell engraftment to treat the target disease. Thus, tolerance induction by IUHCT to allow for postnatal "booster" transplants may be instrumental to the future clinical application of IUHCT. In this review, we focus on the progress that has been made in understanding and achieving immunologic tolerance following IUHCT and how this tolerance can be used as a platform for non-myeloablative non-immunosuppressive postnatal transplants to either achieve clinically acceptable levels of engraftment or allow for solid organ transplants.

IUHCT AND ALLOGENEIC ENGRAFTMENT: FROM MICRO TO MACROCHIMERISM AND TOLERANCE INDUCTION

In utero hematopoietic cell transplantation has been studied in multiple animal models. Initial results in the sheep model were very encouraging demonstrating stable long-term hematopoietic chimerism in three of four sheep following IUHCT (Flake et al., 1986). Unfortunately, these findings did not translate into similar results in clinical studies. Successful engraftment following IUHCT in humans has been limited to circumstances of immunodeficiency and those in which a donor cell selective advantage exists (Flake et al., 1996; Wengler et al., 1996; Gil et al., 1999; Bartolome et al., 2002; Pirovano et al., 2004; Touraine et al., 2004;

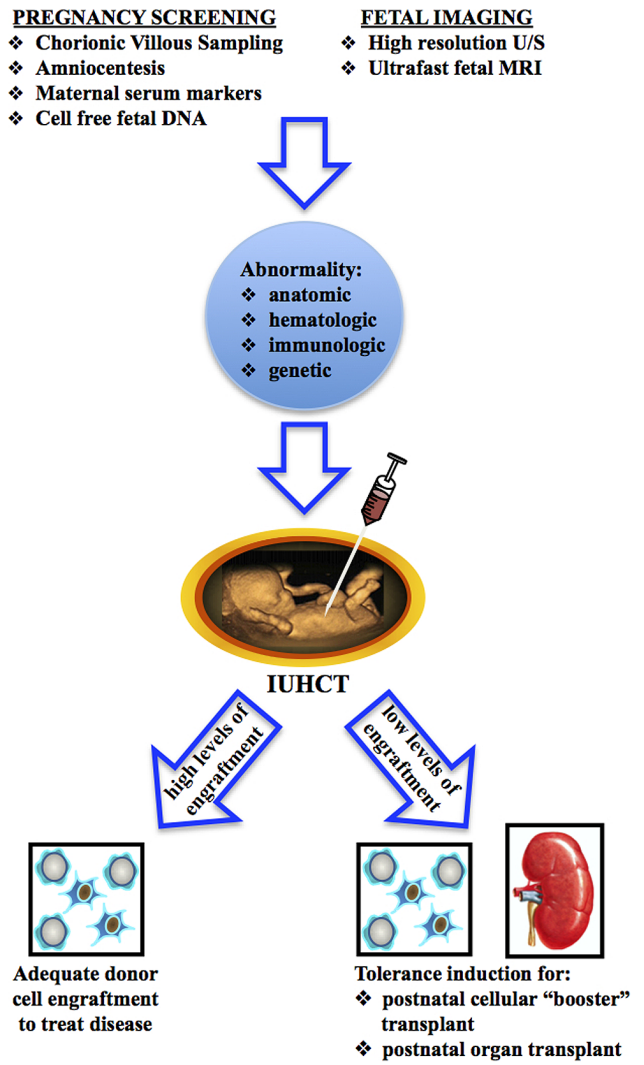

FIGURE 1 | Two approaches for the clinical application of IUHCT.

Muench, 2005; De Santis et al., 2011). These discouraging results highlighted the need for a more in depth study of the events following IUHCT including the induction of donor specific tolerance. To this aim, murine models of IUHCT have been developed. Studies in these models support an intimate relationship between the levels of donor cell chimerism following IUHCT and tolerance induction. In chimeric mice in which donor cell engraftment was only detectable by PCR and undetectable by flow cytometry (microchimerism), donor specific tolerance, as demonstrated by skin graft acceptance, response to postnatal boosting transplants, and *in vitro* proliferation assays, is inconsistent and occurs in only a subset of animals (Billingham et al., 1953; Carrier et al., 1995; Kim et al., 1999). Interestingly, studies in mice and large animals have found that tolerance following IUHCT may persistent even when peripheral blood chimerism levels are low if donor cells persist in tissues or the peritoneal cavity of recipients (Carrier et al., 1995; Mathes et al., 2001, 2005; Chen et al., 2004). Technical advances, including the ability to deliver higher doses of donor cells at the time of IUHCT via an intravenous injection, have allowed for the creation of mice with chimerism levels consistently greater than 1% (macrochimerism; Peranteau et al., 2006, 2007). The ability to achieve higher initial levels of donor cell engraftment has demonstrated that induction of donor specific tolerance can be consistently achieved in macrochimeric animals and tolerance correlates with donor chimerism levels (Hayashi et al., 2002; Ashizuka et al., 2006). Specifically, 60% of mice with peripheral blood chimerism levels of less than 1%, and 100% of mice with chimerism levels greater than 1% following IUHCT demonstrated successful enhancement of allogeneic engraftment following postnatal, same-donor, bone marrow (BM) transplants suggestive of the presence of donor specific tolerance. Decreased donor specific reactivity was demonstrated by MLR in those mice with <1% chimerism in which engraftment could be successfully enhanced following IUHCT compared to those in which engraftment could not be enhanced (Ashizuka et al., 2006). In another study, peripheral blood chimerism was noted to correlate with thymic chimerism and donor specific tolerance as measured by skin graft acceptance. In this study, chimerism levels greater than 3% at the time of skin graft placement were consistently associated with donor specific tolerance and graft acceptance (Chen et al., 2010). In this study, adequate levels of donor cell engraftment were needed for the induction of tolerance. However, peripheral blood chimerism was not required for the maintenance of tolerance as demonstrated by persistence of donor skin grafts despite the loss of peripheral blood chimerism in some mice.

IUHCT AND MECHANISM OF DONOR SPECIFIC TOLERANCE

Fetal immunologic tolerance is a phenomenon believed to be temporally related to thymic development (Billingham et al., 1953). The developing fetal thymic microenvironment plays a primary role in the positive and negative selection of pre-T cells resulting in the deletion of presumed auto-reactive T cell clones with a high affinity for self antigen in association with self MHC while maintaining a T cell repertoire for foreign antigen (Sprent, 1995; Goodnow, 1996; Goodnow et al., 2005). In the human fetus, TCR bearing, single positive lymphocytes can be identified as early as 13–14 weeks gestation. In the murine system, this stage of development corresponds to ∼17 days gestation. Thus, IUHCT attempts to introduce donor cells into the fetal thymic microenvironment prior to this time such that donor cells will be identified as "self" and donor antigens will undergo appropriate thymic antigen presentation resulting in clonal deletion of donor alloreactive host T cells.

Although donor specific tolerance following IUHCT is well accepted, the mechanisms underlying this tolerance have only recently begun to be understood. Early studies suggested tolerance was the result of partial deletion of donor specific host T cells combined with peripheral suppression of donor reactive T cells that escape deletion (Kim et al., 1999; Nijagal et al., 2011). Thymic deletion of donor reactive host T cells can occur via the direct pathway in which donor antigen is presented by donor antigen presenting cells (APCs) or the indirect pathway in which recipient APCs process donor derived allo-MHC molecules into peptides and then present those peptides to T-cells on self-class II

MHC molecules. Additionally, the "semidirect" pathway whereby intact donor MHC molecule-donor peptide complexes are taken up by host APCs and directly interact with reactive T cells may be involved (Herrera et al., 2004; Nijagal et al., 2013). Initial studies using the mammary tumor virus (*Mtv*) superantigen system demonstrated that partial deletion of donor reactive host lymphocytes occurs via both the indirect and direct route of antigen presentation following IUHCT (Shaaban et al., 2000; Peranteau et al., 2002). More recently, murine studies using TCR-transgenic systems that allow differentiation of direct vs. indirect antigen presentation with subsequent donor reactive T cell deletion confirm that deletional tolerance can occur via both pathways (Nijagal et al., 2013). In this study, expression of donor-derived class II antigens on host APCs was assessed to determine the possible contribution of the "semidirect" pathway to deletion of donor reactive T cells. No expression was seen suggesting that the "semidirect" pathway does not play a significant role in deletional tolerance following IUHCT. In addition to inducing immunologic tolerance of host cells to donor cells, IUHCT also results in partial deletion of host reactive donor T cells derived from hematopoietic stem or early progenitor cells at the time of IUHCT via the direct pathway (Bacchetta et al., 1993; Shaaban et al., 2000; Peranteau et al., 2002). In these studies, the direct route of antigen presentation was more efficient with respect to the degree of relevant clonal deletion, but neither route resulted in complete deletion of donor (or host) reactive lymphocytes. Remaining donor (or host) reactive lymphocytes are thought to be suppressed in the periphery by mechanisms that remain to be fully elucidated. This is similar to clinical experience in children who have undergone a successful IUHCT for SCID. These children were immunologically tolerant and were shown to have residual clones of donor reactive cells that were anergic in proliferative assays (Roncarolo et al., 1988; Sakaguchi et al., 1995; Touraine et al., 2005). The mechanism by which this occurs is hypothesized to be related to peripheral regulatory cells using the natural mechanisms of controlling autoreactive T cells that escape thymic deletion (Muench, 2005). In the murine model, the contribution of $CD4^+CD25^+Foxp3^+$ T regulatory cells to this process remains unclear. Studies have shown an increase in the percentage of Treg cells (as well as the Treg/Teff ratio) in the thymus and spleen of chimeric mice following IUHCT related to deletion of the Teff population but not an increase in the absolute number of Treg cells (Nijagal et al., 2013). Although this shift in the Treg/Teff ratio may play an important role in the establishment of engraftment, the contribution of Tregs to maintaining chimerism following IUHCT remains to be shown.

IN UTERO TOLERANCE INDUCTION AND POSTNATAL TRANSPLANTS

Technical improvements in injection techniques have highlighted the intravascular route as a promising alternative to the intraperitoneal route of injection. IUHCT via the intravascular route has achieved initial chimerism levels of 1–23 and 3–39% in the murine model and the preclinical canine model respectively (Peranteau et al., 2006; Vrecenak et al., 2014). These results are encouraging and those animals at the higher end of the engraftment spectrum have donor cell chimerism levels that may be adequate to treat the target disease. However, studies in murine models of Sickle cell anemia, a primary target disease for treatment by IUHCT, suggest that 70 and 40% donor cell myeloid engraftment is needed to eliminate peripheral RBC sickling and anemia respectively (Iannone et al., 2001). To obtain these and higher levels of engraftment in all recipients of IUHCT, alternative approaches must be explored. *Ex vivo* modification of donor HSCs or *in vivo* treatment of fetal recipients with agents which provide a competitive advantage to donor HSCs over endogenous fetal HSCs may be employed to increase donor cell engraftment to clinically relevant levels following a single IUHCT (Peranteau et al., 2006; Derderian et al., 2014). Alternatively, donor specific tolerance induction by IUHCT can be used as a platform on which postnatal transplants using the same prenatal donor source can be performed following non-myeloablative, non-immunosuppressive conditioning to increase engraftment levels.

A review of the literature reveals multiple studies demonstrating the feasibility of tolerance induction by IUHCT followed by postnatal same-donor "booster" transplants (**Table 1**). In the murine model, allogeneic donor cell engraftment was minimally increased when postnatal same-donor transplants were performed in the absence of any conditioning regimen (Carrier et al., 1995; Donahue et al., 2001). We performed additional studies in which non-myeloablative non-toxic conditioning regimens, including low dose total body irradiation (TBI) or busulfan, were administered to chimeric recipients prior to a postnatal same-donor transplant (Peranteau et al., 2002; Ashizuka et al., 2006). Engraftment enhancement directly correlated with the dose of TBI or busulfan administered with near complete donor cell chimerism achieved at the highest doses. The increase in donor cell chimerism resulted from the postnatal donor cell source as opposed to expansion of donor HSCs which had engrafted following IUHCT. Finally, chimeric mice in which engraftment was successfully enhanced demonstrated reduced donor cell reactivity of recipient cells by MLR following IUHCT and prior to the postnatal transplant. These studies highlight the potential to increase allogeneic donor cell engraftment to clinically relevant levels by a combination of tolerance induction by IUHCT and engraftment enhancement by a postnatal BMT using the same prenatal donor. They demonstrate the need for some conditioning regimen to provide a competitive advantage to the donor cell population to achieve the desired engraftment levels independent of preexisting immunologic tolerance. Studies in the preclinical canine model also support the feasibility of this approach with results that reflect similar findings to those achieved in the murine model. Specifically, we demonstrated the ability to successfully enhance peripheral blood donor cell chimerism in the canine model by a combination of IUHCT and a postnatal same-donor BMT using a low-dose busulfan conditioning regimen (Peranteau et al., 2009). In this study, donor chimerism levels were increased from <1 to 35–50% and remained stable up to 6 months to 1 year after transplant in two of six recipients. Control dogs which did not receive an IUHCT never demonstrated any donor cell engraftment following postnatal BMT. The 33% success rate of enhancing engraftment in dogs with initial chimerism levels <1% following IUHCT concurs with murine studies in which 60% of mice with chimerism levels <1%

Table 1 | Summary of studies using IUHCT to induce donor specific tolerance for postnatal allogeneic cellular or organ transplants.

Animal model	Postnatal transplant	Result	Study
Murine	BM (cellular)	Small increase in donor engraftment following unconditioned postnatal transplant (0.2–5% donor chimerism)	Carrier et al. (1995)
		Small increase in donor engraftment following unconditioned postnatal transplant (0.05–0.58 to 2.53%)	Donahue et al. (2001)
		Conversion to >90% donor cell engraftment following low dose non-myeloablative TBI and postnatal transplant	Peranteau et al. (2002)
		Conversion to near total donor cell chimerism following minimally myeloablative conditioning and postnatal transplant	Ashizuka et al. (2006)
Canine	BM (cellular)	Transient elevation of donor cell engraftment (<1–40% donor cell chimerism) in all recipients of an IUHCT and postnatal BM transplant following low-dose Busulfan conditioning. Sustained long-term enhancement of engraftment (donor cell chimerism: 35–50%) in two of six recipients	Peranteau et al. (2009)
Non-human primate	BM (cellular)	Persistent hyporesponsiveness to donor cells on mixed lymphocyte reaction but no significant increase in donor cell engraftment	Shields et al. (2004)
Murine	Skin graft	Prolonged skin graft acceptance in microchimeric mice	Carrier et al. (1995)
		Skin graft acceptance in 66% of microchimeric mice	Kim et al. (1998)
		Skin graft acceptance in 100% of macrochimeric mice	Hayashi et al. (2002, 2004)
		Donor cell chimerism levels >3% required to consistently accept postnatal skin grafts	Chen et al. (2010)
Ovine	Renal	Donor kidney rejected 10 days after transplant in sheep that had 3–5% donor cell engraftment following IUHCT	Hedrick et al. (1994)
Swine	Renal	Prolonged donor kidney survival after minimal immunosuppression for minor histocompatibility antigens	Mathes et al. (2001)
		Prolonged donor kidney survival with minimal or no immunosuppression and no evidence of anti-donor antibodies	Mathes et al. (2005)
		Renal allograft survival for >100 days without immunosuppression	Lee et al. (2005)
Canine	Renal	Long-term acceptance of donor kidney transplant without immunosuppression in four recipients; No evidence of rejection in three of four recipients (12–55% donor cell chimerism at transplant); Mild chronic rejection noted in recipient who had lowest donor cell chimerism (7%) at the time of transplant	Vrecenak et al. (2014)
Non-human primate	Renal	Prolonged survival of paternal kidney transplant in chimeric recipients (donor chimerism level <0.1%) of a paternal IUHCT vs. controls which did not receive an IUHCT (time to rejection: 1 vs. 4–7 weeks)	Mychaliska et al. (1997)

All studies evaluated the ability of IUHCT to provide a platform on which postnatal allogeneic transplants, using the same-donor source that was used to perform the IUHCT, could be performed to increase the levels of donor cell engraftment or allow for successful organ transplant with minimal or no myeloablation or immunosuppression.
BM, bone marrow; TBI, total body irradiation.

following IUHCT successfully enhanced donor cell engraftment using a similar postnatal transplant regimen (Ashizuka et al., 2006). In both studies, failure to achieve stable enhanced donor cell engraftment was associated with increased donor cell reactivity of recipient cells on MLR suggesting a lack of definitive tolerance. These studies support the need to achieve initial levels of donor cell engraftment >1% following IUHCT to reliably induce donor specific tolerance for postnatal cellular transplants. More recently, IUHCT via the intracardiac route in the canine model has more consistently resulted in donor cell engraftment at levels believed to be associated with donor specific tolerance (Vrecenak et al., 2014). These results highlight the potential to

more reliably enhance donor cell chimerism by the combination of IUHCT and postnatal same-donor transplants in the clinical setting.

In utero hematopoietic cell transplantation may also induce donor specific tolerance and allow for postnatal solid organ transplants without the requirement for immunosuppressive conditioning (**Table 1**). Acceptance of donor skin grafts, a classic method of assessing donor specific tolerance, has been repeatedly demonstrated in the murine model of IUHCT with success dependent on the levels of donor cell chimerism (Chen et al., 2010). A renal transplant is potentially the most clinically relevant solid organ transplant in the setting of tolerance induction by IUHCT. Studies in the swine and canine model support the ability of donor specific tolerance induction by IUHCT to allow for successful postnatal same-donor renal transplants without immunosuppression. Interestingly, in the canine model, clinically insignificant but histologically detected mild chronic rejection of one recipient of a postnatal renal transplant following IUHCT was noted. This recipient had the lowest levels of peripheral blood donor cell chimerism (7%) at the time of renal transplant. The other renal transplant recipients had chimerism levels of 12–55% at the time of transplant and demonstrated no clinical or histologic evidence of rejection (Vrecenak et al., 2014). Donor cell engraftment levels of 7% are above what would be expected to induce donor cell tolerance and allow for successful non-myeloablative postnatal cellular transplants suggesting that chimerism levels that allow for successful postnatal solid organ transplants without immunosuppression may be different than those required for postnatal cellular transplants. Finally, tolerance induction by IUHCT to allow for xenogeneic solid organ transplants has also been investigated. Results from these limited studies highlight the potential of this approach to overcome the immune limitation to xenogeneic transplantation (Tanaka et al., 1998).

CONCLUSION

In utero hematopoietic cell transplantation is a non-myeloablative non-immunosuppressive transplant approach that allows for donor cell engraftment and donor specific tolerance across immunologic barriers. It has the potential to treat a large number of congenital hematologic, genetic, and immunologic disorders which, because of advances in prenatal care, can be diagnosed before birth and before the maturation of the fetal immune system. Studies in murine and preclinical large animal models suggest that, in limited circumstances, a single IUHCT may result in high enough levels of donor cell engraftment to ameliorate the target disease. However, even in the absence of obtaining therapeutic levels of engraftment, the major benefit of IUHCT may be in the reliable induction of donor specific tolerance to allow for postnatal non-myeloablative same-donor cellular transplants to enhance engraftment to target levels with minimal treatment related toxicity. Although less clinically relevant at the current time, a similar approach of tolerance induction by IUHCT to allow for postnatal organ transplants without immunosuppression may hold promise in the future. In order to embrace the full potential of *in utero* tolerance induction for postnatal cellular and organ transplants, additional insights into the mechanisms involved in the induction and maintenance of tolerance including the role of peripheral regulatory cells as well as the barriers to engraftment that prevent the acquisition of donor specific tolerance in all recipients of IUHCT must be investigated.

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In utero hematopoietic cell transplantation for hemoglobinopathies

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In utero hematopoietic cell transplantation (IUHCTx) is a promising strategy to circumvent the challenges of postnatal hematopoietic stem cell (HSC) transplantation. The goal of IUHCTx is to introduce donor cells into a naïve host prior to immune maturation, thereby inducing donor–specific tolerance. Thus, this technique has the potential of avoiding host myeloablative conditioning with cytotoxic agents. Over the past two decades, several attempts at IUHCTx have been made to cure numerous underlying congenital anomalies with limited success. In this review, we will briefly review the history of IUHCTx and give a perspective on alpha thalassemia major, one target disease for its clinical application.

Keywords: in utero transplantation, fetal therapy, alpha thalassemia, chimerism, tolerance

HISTORY OF IUHCTx

In utero hematopoietic cell transplantation offers the benefit of treating congenital stem cell disorders prior to birth while avoiding host myeloablative conditioning with cytotoxic agents (Golombeck et al., 2006; Vrecenak et al., 2014). The idea that exposure to foreign antigens can lead to tolerance was first recognized by Owen (1945), with the discovery that monochorionic dizygotic cattle were tolerant of long-lived chimeric cells from their siblings. Since then, natural chimerism has been described in both human and non-human primates (Picus et al., 1985; van Dijk et al., 1996), although it was not until the late 1970s that Fleischman and Mintz reported the first successful chimerism resulting from IUHCTx. Using a c-Kit deficient mouse which resulted in genetic anemia, they were able to reverse the anemia by transplanting adult allogeneic bone marrow stem cells into the placenta (Fleischman and Mintz, 1979). Since then, IUHCTx has proven to be successful in many animal models including mice (Carrier et al., 1995), goats (Pearce et al., 1989), dogs (Blakemore et al., 2004; Peranteau et al., 2009; Vrecenak et al., 2014), sheep (Flake et al., 1986), and non-human primates (Harrison et al., 1989; Tarantal et al., 2000). Mouse models have been used to manipulate various aspects of the maternal (Merianos et al., 2009; Nijagal et al., 2011) and fetal (Misra et al., 2009; Nijagal et al., 2013) immune systems to understand the mechanism of tolerance induction. In the large animal models, which are a necessary step to understanding the effects of immune ontogeny of human fetuses, high dose transplantation has enabled achieving clinically relevant levels of chimerism (Vrecenak et al., 2014).

In humans, the first successful IUHCTx was performed for bare lymphocyte syndrome (Touraine et al., 1989). Successful transplantation of fetuses with severe combined immunodeficiency (SCID) was also achieved by several groups (Flake et al., 1996; Wengler et al., 1996). However, subsequent attempts into fetuses with various disease processes including hemoglobinopathies, chronic granulomatous disease, Chediak–Higashi syndrome and inborn errors of metabolism were met with limited success (reviewed in Vrecenak and Flake, 2013). These limitation have led several groups to explore barriers to engraftment which include the fetal and maternal immune systems, the competitive disadvantage of donor cells when transplanted into an intact fetal host, and a lack of space within hematopoietic niches (reviewed in Nijagal et al., 2012). Since it has been shown that the maternal immune system (both T cells and B cells) is a critical barrier to engraftment (Merianos et al., 2009; Nijagal et al., 2011), clinical efforts should focus on transplantation of maternal (or maternally matched) hematopoietic cells. The levels of engraftment can also be increased by transplanting a high number of CD34 enriched, CD3 depleted bone marrow cells using an intravascular (as opposed to intraperitoneal) approach (Vrecenak et al., 2014). Further efforts to improve the competitive advantage of the transplanted cells and to create space for their engraftment in the hematopoietic niche will likely be necessary. For example, we have recently demonstrated that selective in utero depletion of host HSCs using an antibody against the c-Kit receptor (ACK2) results in therapeutic levels of engraftment after neonatal transplantation (Derderian et al., 2014), providing a proof of concept for such a conditioning approach in the fetal environment. This approach may also avoid the need for conventional myeloablative drugs such as busulfan that could cause tissue cytotoxicity in utero. Finally, transplantation prior to the development of circulating T cells is likely critical and further measures to promote fetal tolerance induction for example, by co-transplantation of regulatory T cells, should be explored.

THERAPEUTIC POTENTIAL OF IUHCTx FOR ALPHA-THALASSEMIA

In utero hematopoietic cell transplantation has excellent potential to treat common hemoglobinopathies such as sickle cell disease and thalassemias. In particular, alpha thalassemia major can be diagnosed early in gestation and poses risks to the developing fetus including hydrops fetalis, which may provide further justification for an in utero intervention.

Alpha-thalassemia is one of the most common single-gene disorders, affecting approximately 5% of people worldwide (Lau et al., 1997; Chui and Waye, 1998; Leung et al., 2008). It is an autosomal recessive disease, resulting from DNA sequence deletions on chromosome 16. At least 40 deletions are known (reviewed in Vichinsky, 2009), the most common of which is the Southeast Asian deletion ($-^{SEA}$; Chui and Waye, 1998; Hoppe, 2009). Since there are 4 alleles coding for the alpha-globin protein, the disease can present as a spectrum. The homozygous form ($-/-$), often referred to as Hb Bart's, results in the absence of all alpha-globin production. Unaffected chains accumulate and form tetramers unable to transport oxygen, ultimately leading to hypoxia, non-immune fetal hydrops, and in utero demise (Leung et al., 2008).

EARLY DIAGNOSIS IN UTERO

Advancements in prenatal diagnostic tools have provided means for early diagnosis of many congenital anomalies, including alpha-thalassemia. Anemia caused by alpha-thalassemia can be detected on ultrasound by an increase in the cardiothoracic ratio, an increase in middle cerebral artery peak systolic velocities, and the presence of non-immune hydrops. These changes have been detected as early as 12 weeks' gestation (Lam et al., 1999; Li et al., 2007), which is well within the window of optimal timing for IUHCTx. Once anemia is suggested on ultrasound, the diagnosis of alpha-thalassemia requires fetal DNA for genetic sequencing. Currently, the most common modalities to obtain fetal DNA for analysis are amniocentesis, which can be performed as early as 16 weeks' gestation with only a 0.5% risk of fetal demise (No authors, 1976), or chorionic villus sampling, which is performed as early as 10 weeks' gestation (Nicolaides et al., 1994; Sundberg et al., 1997). More recently, genetic disorders have been diagnosed using cell-free fetal DNA, which is detectable in maternal serum as early as 7 weeks' gestation (Lo et al., 1998). Advances in laboratory technology have increased the likelihood that we will soon be able to reliably diagnose alpha thalassemia major prenatally with maternal plasma (Sirichotiyakul et al., 2012; Ge et al., 2013). Although this strategy has great potential, detecting complex mutations of alpha-thalassemia major remains a challenge. It appears likely soon women at risk for carrying a fetus with Hb Bart's will have the opportunity to undergo cell-free fetal DNA testing not only before the onset of fetal hydrops but at a time when the fetus is still in an immune tolerant state (Ge et al., 2013).

IN UTERO MANIFESTATION

Fetuses with Hb Bart's produce aberrant alpha-globin, which results in accumulation of dysfunctional hemoglobin tetramers, and impaired oxygen transportation. Definitive erythrocytes, composed predominantly of fetal hemoglobin ($\alpha_2\gamma_2$), begin circulating at 10 weeks' gestation (Migliaccio and Papayannopoulou, 2001). In utero, Hb Bart's leads to anemia, heart failure, fetal growth restriction, oligohydramnios, and non-immune hydrops (Fucharoen et al., 1991), which historically was considered to be a harbinger of fetal demise (Laros, 1994). More recently, in utero exchange transfusion, which removes the dysfunctional hemoglobin, has been shown to reverse anemia, fetal growth restriction, and oligohydramnios (Dwinnell et al., 2011). However, this temporizing therapy is directed toward symptom relief and not curing the underlying disorder. An alternative strategy would be to offer IUHCTx to cure the genetic anemia even before the onset of any symptoms.

Table 1 | In utero transplantation for alpha-thalassemia.

Case	GA at diagnosis (weeks)	GA at transplant (weeks)	Cell source	Cell number	Route	Engraftment	Reference
1	10	13, 19, and 24	Paternal $CD34^+$ BM cells	3×10^6/kg	i.p., i.v., i.v.	CB at Birth and BM at 3 months – detectable alpha globin but no donor cell engraftment detected	Hayward et al. (1998)
2	N/A	18	Maternal *T*-cell depleted BM	6.3×10^8	i.p.	CB at 20, 22, and 24 weeks – no engraftment Extramedullary engraftment on autopsy	Cowan and Golbus (1994)
3	13	15, 31	Cryopreserved FL from abortions between 5 and 10 weeks' gestation	2.2×10^{10}, 2.7×10^{10}	i.p., i.v.	CB at 29 weeks GA – no donor DNA or alpha globin No postnatal donor cell engraftment detected	Westgren et al. (1996)

GA, gestational age; BM, bone marrow; kg, kilogram; i.p., intraperitoneal; i.v., intravenous; CB, cord blood; FL, fetal liver. N/A, not available.

CURRENT IN UTERO THERAPY

Nearly 20 documented cases of Hb Bart's have been treated with in utero transfusion and outcomes have been generally favorable (Carr et al., 1995; Singer et al., 2000; Zhou et al., 2001; Lucke et al., 2005; Weisz et al., 2009; Yi et al., 2009; Dwinnell et al., 2011). However, these children are transfusion dependent and require iron chelators to prevent complications resulting from iron overload such as cirrhosis and insulin dependent diabetes. Neonatal complications include cognitive and limb reduction defects (Dwinnell et al., 2011). Among fetuses who do not undergo blood transfusions and survive to birth, 25–50% are affected by neurological or developmental shortcomings (Lucke et al., 2005; Lee et al., 2007), presumably from prolonged in utero hypoxemia. However, fetuses transfused early in gestation have a much lower incidence of cognitive and limb reduction defects. Despite our awareness that this process begins in utero, the only prenatal therapy available is in utero transfusions, which is merely directed at symptom relief.

CLINICAL EXPERIENCE WITH IUHCTx FOR ALPHA-THALASSEMIA

There have been three attempts to treat alpha-thalassemia with IUHCTx (**Table 1**) and only one has demonstrated donor cell chimerism on autopsy. Each case used various strategies, making them difficult to compare. The timing of in utero transplantation differed, with cases #1 and #3 performed earlier in gestation (13 and 15 weeks, respectively) while case #2 was performed later (18 weeks). The source of donor cells differed as well. Case #2 used maternally derived bone marrow HSCs and was the only one with evidence of microchimerism on autopsy (termination was pursued at 24 weeks' gestation after no evidence of engraftment was demonstrated by cord blood sampling). This observation is supported by experiments in mice demonstrating that maternally derived HSCs engraft better than paternally derived HSCs (Merianos et al., 2009; Nijagal et al., 2011).

While we cannot draw any definitive conclusions from these attempts, strategies to improve engraftment are necessary. In each case, the first series of transplanted cells were injected into the peritoneal cavity, whereas evidence in animal models now supports that intravascular infusion is more likely to establish stable donor engraftment. Since fetuses with Hb Bart's will be transfusion dependent, transplantation may be performed at the same time as an intrauterine transfusion. Based on animal models, transplantation of T-cell depleted, CD34 enriched maternal-derived HSCs should avoid a maternal immune response against the graft as well as taking advantage of pre-existing fetal tolerance to maternal cells. Additional areas to explore to improve engraftment are *ex vivo* manipulation to increase HSCs proliferative ability and homing potential [reviewed in Peranteau et al. (2009) in this issue] as well as fetal conditioning with non-myeloablative agents such as antibodies against the c-Kit receptor.

In summary, IUHCTx has only been successful in fetuses with SCID and the subsequent lack of success in other diseases has left the field undervalued. With advancements in technical strategies and a new repertoire of therapies, it is time to revisit the idea of IUHCTx for hemoglobinopathies. As with all fetal treatment endeavors, careful patient selection, meticulous attention to technical details, and accurate reporting of results will be critical to the success of future clinical trials.

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Hemophilia A: an ideal disease to correct *in utero*

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Hemophilia A (HA) is the most frequent inheritable defect of the coagulation proteins. The current standard of care for patients with HA is prophylactic factor infusion, which is comprised of regular (2–3 times per week) intravenous infusions of recombinant or plasma-derived FVIII to maintain hemostasis. While this treatment has greatly increased the quality of life and lengthened the life expectancy for many HA patients, its high cost, the need for lifelong infusions, and the fact that it is unavailable to roughly 75% of the world's HA patients make this type of treatment far from ideal. In addition, this lifesaving therapy suffers from a high risk of treatment failure due to immune response to the infused FVIII. There is thus a need for novel treatments, such as those using stem cells and/or gene therapy, which have the potential to mediate long-term correction or permanent cure following a single intervention. In the present review, we discuss the clinical feasibility and unique advantages that an *in utero* approach to treating HA could offer, placing special emphasis on a new sheep model of HA we have developed and on the use of mesenchymal stromal cells (MSC) as cellular vehicles for delivering the FVIII gene.

Keywords: hemophilia, *in utero* transplantation, *in utero* gene therapy, fetal intervention, immune tolerance, mesenchymal stromal cells, sheep model

HEMOPHILIA A AND THE NEED FOR BETTER TREATMENTS

Hemophilia A (HA) is the most commonly occurring inheritable deficiency of coagulation (Mannucci and Tuddenham, 2001). 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, due to having less than 1% of the normal plasma levels of FVIII activity (Kay and High, 1999; High, 2003; Agaliotis et al., 2006). These patients suffer frequent spontaneous hemorrhaging, which leads to hematomas, chronic painful and debilitating arthropathies, and potentially life-threatening internal bleeding (Agaliotis et al., 2006). The current standard of care for HA is prophylactic factor infusion, which is comprised of regular (2–3 times per week) intravenous infusions of recombinant or plasma-derived FVIII to maintain hemostasis. While the availability of this protein-based treatment has greatly improved the quality of life and extended the life expectancy for many patients with HA, it is far from and 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, approximately 30% will mount an immune response to the infused FVIII, forming inhibitory antibodies (inhibitors) to FVIII (Kaveri et al., 2007). 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, precluding restoration of hemostasis and putting the patient at risk of a life-threatening bleed. These significant shortcomings highlight the urgent unmet need for novel therapies that could promise longer-lasting correction, or permanent cure, of HA.

In contrast to current protein-based therapeutics, a single gene therapy treatment could promise lifelong improvement or permanent cure of HA; indeed, several aspects of HA make it an ideal target disease for correction by gene therapy (Lipshutz et al., 1999; Arruda, 2006; Ponder, 2006; Doering et al., 2007, 2009; Ide et al., 2007; Shi et al., 2008; Nichols et al., 2009; Tellez et al., 2010; High, 2011). First, FVIII, unlike the proteins that are missing/defective in many other genetic diseases, does not need to be expressed in either a specific tissue or cell type to produce a therapeutic effect. Although the majority of FVIII produced within the body is thought to be synthesized within the liver (Fahs et al., 2014), as long as FVIII is produced by cells that are close enough to the vasculature to secrete the synthesized FVIII into the circulation, FVIII can exert its appropriate clotting activity. Second, even if FVIII levels could be restored to only 3–5% of normal, this seemingly minimal change would be predicted to exert a marked clinical improvement and greatly improve the quality of life of patients with severe HA, since it would convert these patients to a moderate/mild phenotype. Conversely, even FVIII levels as high as 150% of normal should be safe. As such, FVIII has a very wide therapeutic window (Kay and High, 1999). Armed with this knowledge, the hemophilias were among the most promising, "Target 10," group of diseases in the roadmap the American Society of Gene and Cell Therapy (www.ASGCT.org) recently provided to NIH director, Dr. Francis Collins.

SHEEP AS A PRECLINICAL MODEL OF HEMOPHILIA A

A number of animal models have been developed to evaluate new methods of treating coagulation disorders, and also for preventing and devising way to overcome inhibitor formation. Fortunately, colonies of HA dogs in which spontaneous mutations occurred within the FVIII gene (Hough et al., 2002; Lozier et al., 2002) and FVIII-deficient mice produced via gene targeting/knockout (Bi et al., 1995) are both available to study the biology of FVIII and to begin developing/exploring gene-based strategies for treating HA. Pronounced therapeutic benefit has been demonstrated in multiple studies in the murine models (Gallo-Penn et al., 1999; Garcia-Martin et al., 2002; Reddy et al., 2002; Moayeri et al., 2004, 2005; Sarkar et al., 2004; Doering et al., 2007; Ide et al., 2007, 2010). Phenotypic correction has also been achieved in dogs with HA, but correction in this more clinically predictive model has proven to be much more difficult than in mice (Gallo-Penn et al., 2001; Scallan et al., 2003). However, despite the promising results that have been obtained in both these models, no clinical benefit has yet been seen in any of the clinical gene therapy trials that have been conducted to-date in human patients with 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) (Nathwani et al., 2011); the reasons for the marked difference in the ability of gene therapy to correct HA vs. HB are not, at present, 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 a line of sheep (Bormann et al., 2006, 2007; Almeida-Porada et al., 2007; Sanada et al., 2008; Porada et al., 2010), originally described by investigators at the Swiss Federal Institute of Technology (Neuenschwander et al., 1992; Backfisch et al., 1994; Neuenschwander and Pliska, 1994), that possess a spontaneous mutation causing severe HA, which, if not treated immediately at birth, is fatal within the first hours/days of life. Upon re-establishing this line, we fully characterized the clinical parameters of this new model (Bormann et al., 2006, 2007; Almeida-Porada et al., 2007; Sanada et al., 2008; Porada et al., 2010). All 10 affected animals born thus far have presented with prolonged umbilical cord bleeding, protracted nail (hoof) cuticle bleeding time, bleeding following routine tail docking, and they have all experienced multiple spontaneous episodes of severe bleeding, including muscle hematomas, hematuria, and hemarthroses, all of which have promptly responded to infusion of human FVIII. Since aPTT can be fairly inaccurate when FVIII levels are very low, we had our collaborators at the BloodCenter of Wisconsin and at Emory University independently run a highly sensitive chromogenic assay to accurately quantitate the level of FVIII activity present in the circulation of these animals. The results of these assays quickly explained the severe, life-threatening phenotype we observed in this line of sheep, as FVIII activity was undetectable. 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 were restricted to treatment with human FVIII (we had not yet cloned and sequenced ovine FVIII), it is not yet clear whether these animals will also make inhibitors to the ovine protein. An ongoing collaboration with investigators at Emory University has recently resulted in the successful cloning and large scale production of recombinant B domain-deleted ovine FVIII (Zakas et al., 2012), making it possible to address this important question and to construct gene therapy vectors encoding the native ovine sequence for testing in this valuable model.

In addition to studying the clinical picture of these animals, we also sequenced the entire coding region of the ovine FVIII gene to define the precise molecular basis for their disease. This knowledge of the nature of the disease-causing mutation enabled us to then design a PCR-based RFLP that allows us to unequivocally identify affected animals at birth and even *in utero*, using amniotic fluid-derived cells (Bormann et al., 2006, 2007; Almeida-Porada et al., 2007; Sanada et al., 2008; Porada et al., 2010). These studies revealed that HA in this line of sheep is caused by a frame shift mutation that introduces a premature stop codon part way through the FVIII coding region. Importantly, this type of mutation has also been reported in many human HA patients (Park et al., 2004). Since this line of sheep is, to our knowledge, the only animal model of HA yet described that possesses this type of mutation, these sheep provide a unique system in which to study therapies in this context.

While another large animal model of HA would already be of value, and the nature of the mutation present in these sheep makes them unique as an HA model, sheep possess many characteristics that make them an ideal preclinical model for gene therapy, especially in the context of HA. Firstly, 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 (Jeanblanc et al., 2014). In contrast to dogs, pigs, and many other large animals which tend to have large litters of offspring, sheep, like humans, typically give birth to only one or two offspring in each pregnancy. Secondly, 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 vector/cells directly in this model prior to translating to the clinical arena. Thirdly, the development of the immune system during fetal ontogeny has been thoroughly delineated in sheep (Silverstein et al., 1966; Sawyer et al., 1978; Osburn, 1981; Tuboly et al., 1984; Maddox et al., 1987a,b,c), making this model ideal for investigating the immune facets of treating HA via gene therapy. An additional unique advantage to using sheep to study HA treatment is that in sheep, like human, 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 (McCarroll et al., 1988; Parker et al., 1991). This key difference makes the sheep the most clinically relevant large animal model in which to test the efficacy of platelet-targeted gene therapy approaches for treating HA (Shi et al., 2006, 2008; Shi and Montgomery, 2010; Montgomery and Shi, 2012). 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. At the present time, HA can be diagnosed relatively early in gestation (10–12 weeks), just like many other genetic diseases. The ability to diagnose HA early in development makes it feasible to begin devising methods to try to correct this disease prior to birth. Fetal transfusions and *in utero* stem cell-based therapies have safely been performed clinically for decades (Flake and Zanjani, 1999; Troeger et al., 2006). Indeed, to date, 46 *in utero* transplants have been performed in human patients (Tiblad and Westgren, 2008; Tarantal and Lee, 2010), for 14 different genetic disorders, including 1 case of HA (Troeger et al., 2006; Touraine, 2013). 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 (Flake et al., 1996; Flake and Zanjani, 1999; Tarantal et al., 2006; Troeger et al., 2006; Merianos et al., 2008; Roybal et al., 2010; Tarantal and Lee, 2010). It is important to note that it was studies performed in the fetal sheep model that provided the experience and knowledge that led to the first curative *in utero* transplant in a human patient (Flake et al., 1996), emphasizing the value and importance of the fetal sheep model for developing clinically viable approaches to therapy, and for predicting clinical outcome. Using these established, clinically proven methods to deliver a corrective FVIII gene early in gestation could cure HA *in utero*, enabling the birth of a normal healthy baby requiring no further treatments. Such a treatment, if successful, would clearly represent a major advance, both from an economic standpoint (one treatment rather than a lifetime of expensive treatments several times each week), and with respect to the quality of life of the patient.

While most individuals with a family history of HA are encouraged to have prenatal screening (∼70–75% of new HA cases arise in families with a history of HA), parents presented with a prenatal diagnosis of HA currently have only 2 possible choices: pregnancy termination or the birth of a child with HA. The availability of a safe and effective *in utero* treatment would provide parents a much-needed 3rd option, which would certainly provide the needed impetus for much more widespread prenatal HA screening. In contrast to *in vitro* embryo screening and selection, which has been proposed as a possible solution in families with a history of HA and other genetic diseases, *in utero* gene therapy requires only minimal equipment that would already be in place for prenatal diagnosis, and should not be prohibitively expensive. Several recent studies have provided conclusive evidence that prenatal screening for the hemophilias can be cost-effective, even when considering developing third world countries (Klein et al., 2001; Sasanakul et al., 2003; Peyvandi, 2005). 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 (Tsui et al., 2011).

Although the clinical and financial advantages of *in utero* gene therapy are compelling, in and of themselves, it is important to realize that there are also features of the fetus that make it a better gene therapy recipient than the adult (Matzinger, 2002; Porada et al., 2004a,b). 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 a single intraperitoneal injection of a small volume of γ-retroviral vector resulted in gene transfer levels within the hematopoietic system of 5–6% (Porada et al., 1998, 2001a, 2002a; Tran et al., 2000); levels that would undoubtedly be beneficial in HA. Further studies involving antibody selection of $CD34^+$ cells and serial transplantation/repopulation (Porada et al., 1998, 2008; Tran et al., 2000), provided compelling evidence that this approach successfully modified bona fide hematopoietic stem cells, 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 within the liver (Porada et al., 2005a). Concurrently, fetal gene delivery experiments conducted in sheep, rodent, and non-human primate models, by other investigators who employed a variety of viral-based vectors, produced similar results (Porada et al., 1998, 2002b, 2004a, 2005a,b; Lipshutz et al., 1999, 2000; Schneider et al., 1999, 2002; Themis et al., 1999; Tarantal et al., 2001a,b,c, 2005, 2006; David et al., 2003; Waddington et al., 2003, 2004; Chen et al., 2004a; Jimenez et al., 2005; Lee et al., 2005; Park et al., 2009; Tarantal and Lee, 2010). 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 (Porada et al., 2005a).

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 infusion of purified or recombinant FVIII protein is the formation of inhibitory antibodies in ∼30% of patients. It is important to note that there are also distinct immunologic benefits to performing gene therapy in the developing fetus. Early in immunologic development, before thymic processing of mature lymphocytes, the fetus appears to be highly receptive to foreign antigens. Indeed, exposure to foreign antigens during this period often results in sustained tolerance, which can become permanent if the presence of the antigen is maintained (Billingham et al., 1954). We have spent the last two decades performing *in utero* gene transfer studies in the sheep model (Porada et al., 1998, 2001a,b, 2004a, 2005a; Tran et al., 2000; Park et al., 2003a,b, 2004), and have shown that it is possible to take advantage of this unique temporal window of immunonaïveté to deliver exogenous genes during this period of gestation and induce durable tolerance to the vector-encoded gene product (Tran et al., 2001). 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 fetal gene transfer. Indeed, further mechanistic studies demonstrated that gene delivery early in fetal development exploits several central and peripheral tolerogenic avenues that exist in the fetus (Colletti et al., 2008). These results strongly imply that fetal gene therapy, even if it does not cure HA, would still be an ideal treatment modality for this disease, since permanent immune tolerance to FVIII could be induced. This would thus 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.

To-date, the only experimental studies to directly investigate fetal gene therapy for the treatment of the hemophilias have targeted hemophilia B (factor IX deficiency) (Lipshutz et al., 1999; Schneider et al., 1999, 2002; Themis et al., 1999; David et al., 2003, 2011; Waddington et al., 2003, 2004; Chen et al., 2004a; Mattar et al., 2011). This is most likely a result of 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 (Ponder, 2011). Because HA patients have at least a tenfold higher likelihood of developing inhibitors than hemophilia B patients (Ehrenforth et al., 1992; Chitlur et al., 2009), 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 is important question in the sheep model.

All of the afore-referenced studies demonstrated 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 and confirmed the therapeutic potential of an *in utero* approach to gene therapy. However, for this direct vector injection method of fetal gene delivery to move forward into the clinical arena, vectors that can target specific cell types will likely need to be developed, to eliminate the risk of off-target modification of undesirable non-target cells, like those of the germline (Park et al., 2004, 2009; Lee et al., 2005). Since such vectors are currently not available, we have been testing the ability of mesenchymal stromal cells (MSC) to serve as vehicles for delivering genes to the developing fetus to safely correct HA and other diseases prior to birth. In the next section of this chapter, we will discuss our rationale for using these cells as therapeutics and summarize results to-date following *in utero* delivery of MSC.

MESENCHYMAL STROMAL CELLS (MSC) AS HA THERAPEUTICS

Decades after the pioneering studies of Friedenstein on the marrow microenvironment (Friedenstein et al., 1974; Friedenstein, 1991), results of studies from various labs around the world have revealed that mesenchymal stromal cells (MSC) possess a very broad differentiation potential, both *in vitro* and *in vivo*, and exhibit properties that suggest that at least some of the cells contained within this population may be stem cells (Caplan, 1991; Liechty et al., 2000; Mackenzie and Flake, 2001; Fukuda, 2002; Jiang et al., 2002; Airey et al., 2004; Chen et al., 2004b; Kassem, 2004; Porada et al., 2006; Banas et al., 2007; Chamberlain et al., 2007; Colletti et al., 2009a; Porada and Almeida-Porada, 2010). MSC are very rare, only comprising roughly 0.001–0.01% of cells within the marrow (Galotto et al., 1999). However, they can be passaged extensively *in vitro* without a loss of differentiative potential, making it possible to readily generate clinically relevant numbers of these cells (Crop et al., 2009). Since MSC were first discovered within the bone marrow, many of the studies performed thus far have utilized MSC isolated from this tissue. However, we and others have now shown that cells with the phenotype and functionality of MSC can also readily be isolated from a variety of different tissues, including umbilical cord blood, kidney, liver, lung, brain, fetal blood, and even adipose tissue collected via liposuction (Zuk et al., 2001, 2002; Almeida-Porada et al., 2002; Morizono et al., 2003; in 't Anker et al., 2003; Lee et al., 2004a; Fan et al., 2005; Gotherstrom et al., 2005). Importantly from the standpoint of *in utero* therapies, MSC have also been isolated from the amniotic fluid and the chorionic villi, raising the exciting possibility that autologous MSC could be used as cellular therapeutics or gene delivery vehicles for *in utero* therapy (Poloni et al., 2011; Shaw et al., 2011a,b; Fernandes et al., 2012; Karlsson et al., 2012; Weber et al., 2012).

As discussed earlier, the liver is thought to be the body's main source of FVIII. Studies from our group and others over the past decade have provided compelling evidence that MSC from various sources can give rise, *in vitro* and *in vivo*, to cells which appear identical to native hepatocytes, and have shown that transplanting MSC in a range of model systems results in the generation of substantial numbers of hepatocytes, with resultant repair/correction in a variety of inborn genetic defects and injuries (Almeida-Porada et al., 2001a, 2003a, 2004; Schwartz et al., 2002; Theise and Krause, 2002; Almeida-Porada and Zanjani, 2004; Fang et al., 2004; Sakaida et al., 2004; Lee et al., 2004b; Luk et al., 2005; Sato

et al., 2005; Zhao et al., 2005; Ishikawa et al., 2006; Oyagi et al., 2006; Popp et al., 2006; Talens-Visconti et al., 2006; Aurich et al., 2007, 2008; Banas et al., 2007, 2008, 2009; Chamberlain et al., 2007; Colletti et al., 2007, 2009b; Higashiyama et al., 2007; Muraca et al., 2007; Sgodda et al., 2007; di Bonzo et al., 2008; Enns and Millan, 2008; Lysy et al., 2008; Zheng and Liang, 2008). We have shown, in the fetal sheep model, that, by performing the transplant at a stage in early gestation when the fetal immune system is still relatively immature, it is possible to achieve significant levels of human cell engraftment. Moreover, because this approach induces donor-specific tolerance, these xenogeneic human cells persist for the whole life of the transplanted animals (Almeida-Porada et al., 2001a, 2004; Almeida-Porada and Zanjani, 2004). Of direct relevance to HA treatment, we have demonstrated that, after transplantation into fetal sheep, human MSC engraft at levels of up to 12% within the recipient liver (Almeida-Porada et al., 2000, 2001b, 2003b, 2004; Almeida-Porada and Zanjani, 2004; Chamberlain et al., 2004, 2007; Porada and Almeida-Porada, 2006, 2010), and contribute to both the parenchyma and the perivascular zones, placing them in an ideal location to deliver FVIII into the circulation. Since FVIII levels of only 3–5% of normal would convert a patient with severe HA to a moderate or mild phenotype, it seems reasonable to conclude that these levels of engraftment should be highly therapeutic. In other recent studies, we have demonstrated that MSC from various tissues throughout the body endogenously produce and secrete biologically active FVIII (Soland et al., 2014). Collectively, these results support the notion that MSC are uniquely and ideally suited for treating HA.

However, upon further analysis, we found that, although MSC engrafted at significant levels within the natural sites of FVIII synthesis, the levels of FVIII production were too low to effectively treat HA. If, however, one were to use gene transfer to engineer MSC to express FVIII, it is likely that the levels of MSC engraftment we routinely achieve following transplantation *in utero* would be beneficial/therapeutic in HA, especially if newer, expression-optimized FVIII variants were used in the gene therapy vectors (Gangadharan et al., 2006; Doering et al., 2009; Dooriss et al., 2009; Ide et al., 2010). Importantly, MSC can be efficiently transduced with all of the major viral vector systems that are in clinical use, including adenovirus (Bosch et al., 2006; Bosch and Stice, 2007; Roelants et al., 2008), murine retroviruses (Meyerrose et al., 2007; Sales et al., 2007; Piccoli et al., 2008; Roelants et al., 2008; Gnecchi and Melo, 2009), lentiviruses (Zhang et al., 2002, 2004; Meyerrose et al., 2008; Fan et al., 2009; Wang et al., 2009; Xiang et al., 2009), and AAV (Kumar et al., 2004; Stender et al., 2007). Furthermore, in contrast to studies with hematopoietic stem cells (Racine et al., 1995; Fox and Chowdhury, 2004; Muraca and Burlina, 2005), human MSC are stable in culture, do not form tumors *in vivo* (unlike murine MSC, Tasso et al., 2009), and there is no evidence that transduction can cause human MSC to undergo transformation or progression to clonal dominance. Instead, recent studies have shown that, even following intentional induction of genomic instability, human MSC undergo terminal differentiation rather than transformation (Altanerova et al., 2009), with very rare transformants only arising after very extended *in vitro* propagation, and being easily identifiable (and removable) based on their altered cell surface marker profile (Pan et al., 2014). As such, MSC appear to represent very safe cellular vehicles for delivering a therapeutic gene.

Looking specifically at using MSC to treat HA, multiple studies have already proven that MSC can be efficiently transduced with murine retroviral and lentiviral vectors with gene cassettes encoding FVIII from a variety of species and produce/release high levels of functional FVIII protein. Importantly, when FVIII was purified from the tissue culture medium of transduced MSC, its specific activity, electrophoretic mobility, and proteolytic activation pattern were all identical to commercially produced FVIII (Doering, 2008). Given the widespread distribution and engraftment of MSC following their transplantation, the ability of MSC to give rise, *in vivo*, to cells of numerous tissue types, and their ability to efficiently process and secrete significant quantities of biologically active FVIII, it is not surprising that we and others feel that MSC represent ideal vehicles for delivering a FVIII transgene throughout the body, and thereby providing long-term/permanent correction of HA (Van Damme et al., 2003; Doering, 2008; Pipe et al., 2008; Porada et al., 2011).

In addition to their widespread engraftment and their ability to serve as delivery vehicles for the FVIII gene, MSC have rather unique immunological properties that may further increase their utility for treating HA. MSC do not normally express MHC class II or the co-stimulatory molecules CD80 and CD82. As a result, they do not induce allogeneic lymphocytes to proliferate, nor do they serve as very effective targets for cytotoxic T cells or NK cells. Actually, a growing body of evidence exists to support the conclusion that MSC can be transplanted across allogeneic barriers without eliciting a pronounced immune response (Bartholomew et al., 2001; Devine et al., 2001). Thus, it is theoretically possible that HA (and other diseases as well) could be treated using "off-the-shelf" MSC from an unrelated donor, which would greatly facilitate the use of these cells for therapy. It has long been presumed that the immune system is immature/absent in the fetus at the time when *in utero* transplant is performed. However, recent studies conducted in mice by Mackenzie and Flake have challenged this assumption by showing that the engraftment rate of allogeneic hematopoietic cells can be negatively affected by not only the developing fetal immune system, but also by that of the mother (Peranteau et al., 2007; Nijagal et al., 2011). As such, the success of *in utero* therapies may also benefit from the hypoimmunogenic state of MSC.

In addition to their unique immune properties, MSC also possess another interesting characteristic that is potentially of great clinical value; the ability to selectively migrate to sites within the body where injury/inflammation exist. Upon arriving at these sites, the MSC then repair the damaged/diseased tissue by: (1) releasing trophic factors that dampen inflammation and stimulate the local tissue's endogenous repair mechanisms; and (2) engrafting within the target tissue and reprogramming to produce tissue-specific cells (Jiang et al., 2005, 2006a,b). This property raises the exciting possibility that, following infusion, FVIII-expressing MSC might have the ability to selectively traffic to active bleeds/sites of injury, thus directing the therapy to regions of the body that are in greatest need of help.

PRECLINICAL SUCCESS WITH MSC-BASED TREATMENT FOR HA

Despite the multiple advantages of early intervention, there are already roughly 1.6 × 10^4 individuals with HA, in the US alone, who obviously could not benefit from the development of a therapy that would be administered prior to birth. Furthermore, over 25% of the mutations that cause HA arise *de novo*; as such, it is highly improbable that this group of patients would undergo prenatal screening for HA. We therefore began investigating, in two pediatric HA lambs, whether some of the afore-mentioned properties that make MSC ideally suited for delivering FVIII could still be realized if the MSC engineered to express FVIII are delivered early in childhood. During their first months of life, both HA lambs in this study were given frequent human FVIII treatments in an effort to control multiple hematomas and recurring bleeds in the leg joints, that had given rise to chronic, progressive, debilitating hemarthroses. As a result of these joint bleeds, the two sheep developed extreme postural and gait defects, which made it difficult for them to even stand upright, and precluded them from walking. Given the severe, life-threatening phenotype of the HA sheep, we chose to use cells from the "father" of the two HA lambs (haploidentical), rather than attempting to collect marrow from the two HA lambs to isolate autologous MSC.

Based on our prior *in utero* studies, we knew that MSC should engraft throughout all of the major organs (Feldmann et al., 1992; Almeida-Porada et al., 2004; Aurich et al., 2006; Russo et al., 2006; Chamberlain et al., 2007; Colletti et al., 2009a) and durably express the vector-encoded genes (Feldmann et al., 1992; Zanjani et al., 1993; Colletti et al., 2009a) following intraperitoneal (IP) injection. The IP route also enabled the MSC to gradually enter the circulation over an extended period of time, as they were absorbed through the peritoneal lymphatics, rather than as a single large bolus, as would occur via IV injection. The IP route also allowed us to avoid the extensive lung-trapping which occurs following IV administration of MSC, promising more efficient delivery of the MSC to the desired target tissues, and eliminating the clinical risk of emboli formation (Traas et al., 2007; Mancuso et al., 2009).

MSC were simultaneously transduced with 2 lentiviral-based vectors; one of which contained a cassette coding for an expression/secretion optimized porcine FVIII (pFVIII) transgene (Yamagami et al., 2006), and the second of which encoded eGFP, to enable us to follow/trace the donor cells *in vivo* following injection. Two factors drove our choice to use a pFVIII transgene: (1) the cDNA for ovine FVIII had not yet been cloned; and (2) prior studies had shown that, even when expressed in human cells, the pFVIII transgene was produced/secreted at levels that were 10–100 times higher than those seen with hFVIII (Gangadharan et al., 2006; Doering et al., 2007, 2009). As such, we anticipated that even if the transplanted MSC only engrafted/persisted at relatively low levels, they should still be able to exert a clinical benefit. Once the transduced MSC had been sufficiently expanded, transduced MSC were transplanted into the peritoneal cavity of the first animal under ultrasound guidance, without any prior preconditioning of the recipient.

At various time points after transplantation, a highly sensitive chromogenic assay was unable to detect any FVIII activity in the circulation of this animal, but his clinical picture was markedly improved within only days of the transplant. The animal stopped experiencing spontaneous bleeds, and he enjoyed an event-free clinical course, no longer requiring hFVIII infusions. What was most remarkable, however, was that the animal's joints recovered fully. His existing hemarthroses resolved, restoring normal posture and gait, and allowing him to resume a normal activity level. This is the first report describing phenotypic correction of severe HA in a large animal model after transplanting cells modified to express FVIII. It is also the first time that chronic debilitating hemarthroses have been reversed as a result of any type of therapeutic intervention.

Encouraged by this first animal's marked improvement, we performed an identical transplant procedure on a second animal, using a four-fold higher cell dose, in the hopes of achieving detectable FVIII activity in the circulation. Just as had occurred in the first animal, this straightforward procedure resolved existing hemarthroses in this second animal, and he promptly regained normal activity. The transplant also enabled this second animal to achieve factor-independence. These results thus confirm the ability of MSC to serve as highly effective cellular vehicles for delivery a FVIII transgene, and establish their ability to exert a pronounced clinical benefit in this large animal HA model. Nonetheless, the plasma of this second animal, just like that of the first animal, was completely devoid of FVIII activity. As such, the question of the mechanism(s) whereby this procedure produced such clear therapeutic benefit remains unanswered.

Following euthanasia, PCR analysis of tissues collected from these animals confirmed widespread engraftment of significant levels of MSC in all of the tissues we analyzed, including liver, lymph nodes, intestine, lung, kidney, omentum, and thymus. Subsequent analysis of frozen tissue sections by confocal microscopy revealed large numbers of MSC had engrafted within the synovia of the joints that were experiencing hemarthrosis at the time of transplant. Moreover, these MSC were still expressing the vector-encoded FVIII transgene. These analyses thus confirmed the intrinsic ability of transplanted MSC to home to and persist within sites of ongoing injury/inflammation. Furthermore, the continued production/release of FVIII by the engrafted cells, locally within the joint, provided a mechanistic explanation for the pronounced improvement this procedure exerted on the animals' joints. However, the long-term presence of FVIII-expressing MSC within the animals' joints cannot really account for the clear systemic benefits we observed in these animals, the most striking of which was their complete cessation of spontaneous bleeding events.

Confocal microscopy also demonstrated that transplanted MSC had engrafted within the small intestine, in agreement with what we had seen in our prior *in utero* studies (Feldmann et al., 1992). Since proteins secreted from cells within the intestine should have fairly easy access to the circulation, future studies designed to enhance the levels of intestinal engraftment could likely produce a dramatic improvement in the amount of FVIII that is released into the systemic circulation. Aside from the intestine and injured/diseased joints, MSC engraftment also occurred in the liver, the lungs, and the thymus of the treated animals. Collectively, the results of the PCR and confocal analyses

strongly support the conclusion that widespread durable engraftment of MSC can be achieved in a large animal model following transplantation in a postnatal setting, without the need for preconditioning/ablation. However, the levels of engraftment seen in these pediatric animals were substantially lower than those obtained in our prior *in utero* studies. Moreover, despite the marked clinical improvement and the widespread engraftment of the transplanted MSC, both animals mounted a strong immune response to pFVIII, which agrees with prior studies conducted in HA mice (Gangadharan et al., 2006). These inhibitors exhibited cross-reactivity to hFVIII, which was unanticipated, since a good deal of clinical data exist to support the continued efficacy of pFVIII products in human patients that have developed anti-hFVIII inhibitors (VandenDriessche et al., 1999; Brown and Lillicrap, 2002; Bhakta et al., 2006; Son et al., 2006).

Therefore, while this postnatal approach proved that MSC can serve as cellular vehicles to deliver a FVIII transgene and produce a therapeutic benefit, we feel it is safe to conclude that administering this same treatment *in utero* would have a more pronounced and more durable effect, since higher levels of donor MSC engraftment could be achieved, and because inhibitor formation could be avoided due to the induction of immune tolerance to the FVIII transgene.

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In utero therapy for congenital disorders using amniotic fluid stem cells

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Congenital diseases are responsible for over a third of all pediatric hospital admissions. Advances in prenatal screening and molecular diagnosis have allowed the detection of many life-threatening genetic diseases early in gestation. *In utero* transplantation (IUT) with stem cells could cure affected fetuses but so far in humans, successful IUT using allogeneic hematopoietic stem cells (HSCs), has been limited to fetuses with severe immunologic defects and more recently IUT with allogeneic mesenchymal stem cell transplantation, has improved phenotype in osteogenesis imperfecta. The options of preemptive treatment of congenital diseases *in utero* by stem cell or gene therapy changes the perspective of congenital diseases since it may avoid the need for postnatal treatment and reduce future costs. Amniotic fluid stem (AFS) cells have been isolated and characterized in human, mice, rodents, rabbit, and sheep and are a potential source of cells for therapeutic applications in disorders for treatment prenatally or postnatally. Gene transfer to the cells with long-term transgenic protein expression is feasible. Recently, pre-clinical autologous transplantation of transduced cells has been achieved in fetal sheep using minimally invasive ultrasound guided injection techniques. Clinically relevant levels of transgenic protein were expressed in the blood of transplanted lambs for at least 6 months. The cells have also demonstrated the potential of repair in a range of pre-clinical disease models such as neurological disorders, tracheal repair, bladder injury, and diaphragmatic hernia repair in neonates or adults. These results have been encouraging, and bring personalized tissue engineering for prenatal treatment of genetic disorders closer to the clinic.

Keywords: congenital disease, *in utero* therapy, stem cells, gene therapy, amniotic fluid

INTRODUCTION

Congenital diseases attributed to about 510,000 deaths globally in 2010 (Lozano et al., 2012), and are estimated to contribute to over a third of pediatric admissions to the hospital and up to 50% of the total costs of pediatric hospital treatment (McCandless et al., 2004). Prenatal diagnosis of many congenital diseases are performed using traditional invasive techniques such as amniocentesis or chorionic villus sampling (CVS), but increasingly non-invasive methods using circulating fetal DNA in the maternal blood are feasible and available for prenatal diagnosis early in gestation (Danzer et al., 2012; Danzer and Johnson, 2014). The current options for most parents facing congenital diseases following prenatal diagnosis are either to terminate or continue with a known affected pregnancy.

Progress over the last two decades have resulted in fetal therapy being available for a small number of congenital structural anomalies such as spina bifida, identical twin placental complications, and congenital diaphragmatic hernia, using open surgical or fetoscopic interventions (Pearson and Flake, 2013). These options are currently restricted to the treatment of fetal pathophysiology and are usually performed in the second half of gestation, when pathology is already evident. There are almost no therapeutic options however for life-threatening genetic disorders which have pathology beginning *in utero*. Success with *in utero* transplantation (IUT) using allogeneic hematopoietic stem cells (HSCs), has been limited to fetuses with severe immunologic defects where there is an effective lack of immune response to allogeneic cells, and transplanted genetically normal cells have a proliferative advantage (Tiblad and Westgren, 2008). Mesenchymal stem cells (MSCs) appear to be less immunogenic than their hematopoietic counterparts (O'Donoghue and Fisk, 2004) and have shown to reduce fracture rate in a mouse model (Guillot et al., 2008) and engraft in human fetuses with osteogenesis imperfecta in an allogeneic setting (Horwitz et al., 2002). Attempts to treat diseases such as sickle cell disease (Westgren et al., 1996) with *in utero* HSC transplantation, have been

unsuccessful, even where a suitably matched donor has been available. Mouse studies suggest that the immune barrier to allogeneic *in utero* HSC transplantation may be stronger than previously thought (Peranteau et al., 2007). Transplantation of autologous progenitor cells, which have been corrected for the disease, could avoid the fetal immune barrier and may prove more successful than allogenic progenitors.

Autologous progenitors can be obtained from the fetus itself. Both proliferative and differentiation potentials of amniotic fluid stem (AFS) cells has been demonstrated *in vitro* and *in vivo* (De Coppi et al., 2007; Ditadi et al., 2009). Studies exploring the potential of this stem cell source for the use in autologous or allogenic prenatal therapy of congenital diseases have been conducted in large animal models (Shaw et al., 2014). In this review, we explore the latest developments in the field of *in utero* therapy for congenital disorders such as stem cell transplantation and gene transfer using AFS and their potential clinical applications.

AMNIOTIC FLUID AS A FETAL CELL SOURCE FOR IN UTERO THERAPY

Amniotic fluid (AF) consists of cells of fetal origin such as the amnion, skin, and respiratory system (Prusa and Hengstschläger, 2002; Tsai et al., 2004) and it can be obtained by routine clinical amniocentesis during pregnancy, a minimally invasive procedure used for prenatal diagnosis that usually takes place from 15 weeks of gestation (Gosden, 1983; Prusa and Hengstschläger, 2002; Delo et al., 2006). AF can also be collected during therapeutic amniodrainage procedures or even at cesarean section surgeries. Other fetal stem cell sources include the placenta, which can be accessed via ultrasound-guided CVS from 11 weeks of gestation or after birth yields epithelial, hematopoietic, and MSC types (Pipino et al., 2013; Jones et al., 2014). Fetal blood and the HSCs therein can also be collected from the umbilical cord in the first trimester of pregnancy by thin-gauge embryo fetoscopic-directed or ultrasound-guided blood sampling, although the long-term outcome following this procedure is not known (Chan et al., 2008).

In recent years, AFS cells have been explored in many clinical applications such as tissue engineering, cell transplantation, and gene therapy (Kaviani et al., 2001, 2003; Fauza, 2004; Tsai et al., 2004). C-Kit$^+$ cells can be successfully isolated from AF, expanded with good population doublings and possess a very well characterized phenotype (Tsai et al., 2004; Delo et al., 2006; De Coppi et al., 2007; Ghionzoli et al., 2010). The surface antigen c-Kit (CD117) is known to be the receptor of stem cell factor and plays an essential role in gametogenesis, melanogenesis, and hematopoiesis (Fleischman, 1993; De Coppi et al., 2007). The successful expansion of AFS c-Kit$^+$ cells has led to the finding of unique cell types such as mesenchymal (AFMSCs) and hematopoietic progenitors (Prusa and Hengstschläger, 2002; Delo et al., 2006; De Coppi et al., 2007; Ditadi et al., 2009).

Human AFS cells give rise to a variety of cell types such as osteogenic, myogenic, adipogenic, endothelial, hepatic, and neuronal origin; differentiation which has been be validated by the expression of mRNAs in lineage specific genes (Tsai et al., 2004; De Coppi et al., 2007). Rodent and murine AFS cells, like human AFS cells share similar growth properties and differentiation potential *in vitro* as well as the expression of embryonic and adult stem cell markers, respectively (De Coppi et al., 2007). AFS cells derived from human, mice, and sheep can be easily transduced without losing their characteristics (De Coppi et al., 2007; Ditadi et al., 2009; Mehta et al., 2011; Shaw and Bollini, 2011), and they possess privileged immunological characteristics that make them an ideal and reliable source for therapeutic transplantation (Ditadi et al., 2009).

The immune modulatory properties of AFS have shown resistance to natural killer (NK) cytotoxicity by inflammatory priming of AFS with interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and its ability to modulate lymphocyte proliferation according to its gestational age (Di Trapani et al., 2014). For instance, IFN-γ increases both MHC Class I and MHC Class II expression, indicating that like MSCs, they may not strongly contribute to rejection responses in allogeneic hosts (Moorefield et al., 2011). Moreover, AFS have shown to release high levels of cytokines including IL-6, MCP-2, MIP-3α, and MIP-1α when activated suggesting that they possess alternative molecular mechanisms to modulate immune response and regulation (Perin et al., 2010; Moorefield et al., 2011).

GROWTH AND CHARACTERIZATION OF AMNIOTIC FLUID-DERIVED STEM CELLS

AFS cells have an estimated doubling time of 36 h and are grown without feeder layers (De Coppi et al., 2007). MSC subpopulations of AFS cells (AFMSCs), like other MSCs, maintain their spindle-shaped fibroblast-like morphology, their proliferation rate as well as their differentiation potential. Growth kinetics assays have shown that AFMSCs have a higher proliferation rate with an average doubling time of 25–38 h compared to bone marrow (BM) derived-MSCs that have an average doubling time of 30–90 h (Kaviani et al., 2001; Roubelakis et al., 2007). In addition, AFMSCs have a greater clonogenic potential compared to BM-MSCs (86 ± 4.3 versus 70 ± 5.1 colonies; Nadri and Soleimani, 2007). Despite the high proliferative rate of AFMSCs, they are still able to retain a normal karyotype with no evidence of tumorigenicity (Roubelakis et al., 2007). Human AFS cells express both markers of mesenchymal and pluripotent stem cells origin, such as stage-specific embryonic antigen (SSEA)-4 and Oct-4 (De Coppi et al., 2007). Once cultured in adherence however, they do not express markers of hematopoietic lineage such as CD45, CD34, and CD133 and express CD29, CD44, CD73, CD90, and CD105 (De Coppi, 2013). Interestingly, their plasticity, which is superior to adult stem cells, allow reprogramming into AFS derived induced pluripotent stem cells (iPS) with the change of the culture conditions they are exposed to (Lu et al., 2012; Moschidou et al., 2012, 2013; Pipino et al., 2014). This is particularly relevant as AFS cells can be utilized for cell banking of patient-specific pluripotent cells for potential applications in allogeneic cellular replacement therapies, pharmaceutical screening, and disease modeling (Moschidou et al., 2012, 2013).

In addition AFS cells, similarly to other fetal cells may represent the ideal source for therapy because, similarly to ES cells, they are easy to expand, and, in common with the adult counterparts, they are less controversial, not tumorigenic, readily cryopreserved for cell banking and their use can be accomplished

on an autologous setting (De Coppi, 2013). The latter is particularly important in neonatal surgery, in the context of congenital malformations.

GENE TRANSFER TO AMNIOTIC FLUID STEM CELLS

To be a successful autologous therapeutic resource for correcting genetic disease, AFS cells must be easily transduced to give high levels of therapeutic transgenic protein expression. The transduction of human AFS cells with vectors was explored with recombinant adenovirus vectors containing reporter genes such as AdHCMVsp1LacZ and AdCMV.eGFP (Grisafi et al., 2008). Human AFS cells presented a transduction efficiency of 100% when infected with 50 pfu/cell. Transduced human AFS cells maintained stemness features such as the expression of stem cell markers (SSEA4 and OCT4), adhesion and stromal molecules (CD29 and CD73) as well as adipogenic and osteogenic differentiation potential after infection (Grisafi et al., 2008). However, a decrease in SSEA4 expression, no expression of lipoprotein lipase (Lpl), an important adipogenic gene during differentiation and the expression of transcription factors Cbfa1 and PPaRγ detected only during early stages of differentiation suggests a slowdown in the differentiative progression and pluripotency after transduction.

Recently, we have shown that sheep AFS cells have the ability to be transduced using a lentivirus vector encoding the HIV-1 central polypurine tract element, the spleen focus forming virus LTR promoter, and the marker gene eGFP (63.2% efficiency). They have the ability to maintain the expression of MSC markers (CD44, CD58, and CD166) but were negative for hematopoietic, and endothelial markers (CD14, CD31, and CD45) as well as differentiate into adipogenic and osteogenic lineages (Shaw and Bollini, 2011; Shaw et al., 2014).

IN UTERO TRANSPLANTATION

IUT involves the transplantation of cells to the fetus *in utero* (Muench, 2005) with the aim of treating congenital disorders by providing the correct stem cells (IUSCT) or gene corrected stem cells (IUSCGT). The benefits of an *in utero* approach to correcting genetic disease includes the prevention of pathology when it arises antenatally in those genetic diseases that cause irreversible damage to organs *in utero*, targeting of stem cell progenitors that are abundant and accessible in the fetus as well as dose scaling where the small size of the fetus allows relatively high doses of cells to be delivered. There is an opportunity for engraftment of donor cells without the need for myeloablation due to the immature status of the fetal immune system prior to thymic processing of self-antigen, a normal event in hematopoietic ontogeny (Waddington et al., 2007; David and Peebles, 2008; Shaw et al., 2011). Furthermore, immunological tolerance would allow postnatal reinfusion of cells to boost the effect after birth, as demonstrated recently in two children with osteogenesis imperfecta treated by IUT using MSCs (Götherström et al., 2014). Postnatal reinfusion with more MSCs from the original infusion source resulted in improved growth rate after birth.

As with any new therapeutic modality, the risks of IUT are not well characterized and the efficacy is still to be determined for some diseases. For *in utero* gene therapy, where vectors are given directly to the fetus for correction of genetic disease there has been direct guidance given by the NIH Recombinant DNA advisory committee report (RAC, 2000) on a pre-proposal for the initial application. The recommendations included that treatment should be limited only to: diseases that carry serious morbidity and mortality risks for the fetus either *in utero* or postnatally, do not have an effective postnatal therapy, or have a poor outcome using available postnatal therapies, can be definitively diagnosed *in utero* and have a well-defined genotype/phenotype relationship, have an animal model for *in utero* gene transfer that recapitulates the human disease and that the therapy would correct all serious abnormalities. It was recognized that a direct fetal vector injection approach would be difficult to justify given the above.

A combination IUSCGT approach however seems more likely to be acceptable. The UK Gene Therapy Advisory Committee (GTAC) considered this in their broader judgments about gene therapy *in utero* (Eckstein, 2003). The New and Emerging Technologies subgroup of GTAC found that the use of genetically modified stem cells in stem cell transplantation to the fetus was a possibility stating "such *ex vivo* modification would be unlikely to carry with it any higher risk to the germ line than the trials of postnatal somatic gene therapy which have already been approved."

DEVELOPMENT OF THE FETAL IMMUNE SYSTEM AND BARRIERS TO ENGRAFTMENT AFTER IUT

The fetal immune system is commonly regarded as immature and unresponsive despite reports showing its functional immune response (Mold and McCune, 2012). IUT relies crucially on the concept that the developing fetal immune system might accept a foreign cell or antigen and become tolerant to it. The presence of human NK cells have been detected as early as gestational week 6 in the fetal liver and in the fetal spleen at gestational week 15 (Phillips et al., 1992). Fetal NK cells have the ability to differentiate early *in utero* and are highly responsive to cytokines and antibody-mediated stimulation, and have shown to be functionally immature compared to adult NK cells (Phillips et al., 1992; Ivarsson et al., 2013).

The immune system during early gestation undergoes a process of self-education that occurs in the thymus. The positive and negative selection of pre-lymphocytes for the recognition of "self" major histocompatibility complex (MHC) antigen allows a repertoire of lymphocytes to be capable of direct and indirect antigen presentation which results in the deletion of alloreactive T-cells, regulatory T cells (Tregs) enrichment and creates donor-specific immune tolerance (Nijagal et al., 2013). Thus, to prevent limited engraftment, transplants should be introduced prior to the appearance of mature T-cells in the fetal thymus (Peranteau et al., 2006; Roybal et al., 2010; Nijagal et al., 2013).

In the human fetus, the immune system develops from 12 to 14 weeks of gestation, when profound increases in circulating T lymphocytes can be observed (Darrasse-Jèze et al., 2005; Takahama, 2006). Delivery of gene therapy may be required before this gestational age, which currently could limit the routes of application that can be safely used, although advances in engineering and imaging is leading to large improvements in fetal imaging and injection systems. It was demonstrated that the human fetus

may have developed a functional immune system during the second trimester of gestation (Tse et al., 2005). Hematological compositions of human fetal blood and liver between 8 and 17 weeks gestation showed an increase in fetal red blood cell, white blood cell, and platelet counts with advancing gestation reflecting hematologic development (Pahal et al., 2000). An increase in circulating and hepatic T lymphocytes showed the presence of thymic maturation before the 13th week of gestation while the proportion of circulating primitive hematopoietic stem and progenitor cells decreased after each successive gestational week. These findings support the concept of introducing IUT before the 13th week of gestation to induce actively acquired specific tolerance to the foreign antigen (Pahal et al., 2000). Thus, IUT could be performed to the corresponding hematopoietic compartments or systemically depending on the gestational age the transplantation occurs (Tavian and Peault, 2005).

Studies in mice strongly support there being an immune barrier to allogeneic engraftment after IUHCT. Transplantation of allogenic HSCs at day 14 post conception gave initially similar results to IUT with congenic HSCs at 1 week of age (100%) but after 6 months, engraftment dropped rapidly (19% allogenic versus 100% congenic; Muench, 2005; Tse et al., 2005; Peranteau et al., 2007; Shaw and Bollini, 2011). Strategies to improve engraftment of allogenic HSC *in utero* have include the use of busulfan, cotransplantation of LLME-treated, MHC-sensitized donor lymphocytes, CD26 inhibition and using haploidentical HSC sources (Hayashi et al., 2004; Ashizuka et al., 2006; Vrecenak et al., 2014). For instance, low-levels of allogeneic chimerism could be enhanced to near-complete donor chimerism in murine models by postnatal minimally myeloablative total body irradiation (TBI) followed by same-donor BM transplantation (Peranteau et al., 2002). Due to the concerns with toxicity, minimally toxic postnatal regimens such as busulfan conditioning have been studied and shown to improve therapeutic levels of allogeneic engraftment (Ashizuka et al., 2006). Mice with <1 and >1% chimerism, had 60 and 100% enhanced engraftment, respectively (Ashizuka et al., 2006).

Maternal T cells play a key role in the success *in utero* therapy by being a barrier to engraftment (Nijagal et al., 2011). There were no differences observed in engraftment of syngeneic and allogeneic fetal recipients when cells were matched to the mother in a murine model. It is believed that the immune barrier may result from maternal pathogenic immune responses as a result of pro-inflammatory signals released during fetal intervention (Nijagal et al., 2011). Recent studies of canine IUT with HSCs *in utero* were encouraging. A time of 40 days gestation (term 63 days) was chosen for these experiments since it was at the initiation of thymic selection, and prior to BM hematopoiesis, therefore being optimal for engraftment. Intracardiac injection was the most efficient delivery method giving much higher levels of donor cell engraftment than intraperitoneal injection. The authors achieved stable long-term multilineage engraftment in 21 of 24 surviving recipients with an average level of initial chimerism of 11.7% (range 3–39%) without conditioning and with no evidence of graft versus host disease (GVHD). Donor cell chimerism remained stable for up to 2 years and was associated with donor specific tolerance for renal transplantation (Vrecenak et al., 2014). Intracardiac injection early in gestation currently carries an increased risk of miscarriage in clinical practice compared to intraperitoneal injection, but these findings suggest that clinically relevant levels of engraftment might be achievable using this approach and research is underway to evaluate safety and feasibility in relevant pre-clinical animal models prior to the first human studies. Using stem cells that are matched to the fetus, i.e., autologous cells, is an alternative approach which is discussed further on in this review.

SEVERE COMBINED IMMUNODEFICIENCY

Severe combined immunodeficiency (SCID) has been successfully corrected by ultrasound guided intraperitoneal or intravenous fetal injection of HSCs derived from the paternal BM or an allogeneic fetal liver (Flake et al., 1996; Westgren et al., 2002). X-linked SCID is an immunodeficiency caused by the mutation of *IL2RG,* which encodes the cytokine-receptor γ chain that results in a block in T-cell development and a severe deficiency of mature T cells (Flake et al., 1996). After IUSCT, stable split chimerism with the T-cell lineage of donor origin and all other lineages of host origin was seen postnatally in treated individuals as evidence of immune system reconstitution (Flake, 2004). For most patients, the diagnosis of SCID is only made in the neonatal period meaning that postnatal treatment is the only option. Rapid advances in fetal medicine, such as the availability of non-invasive prenatal diagnosis in the first trimester is likely to make prenatal screening a reality. IUSCT currently is an option for affected families that have a one in four risk of recurrence, where first trimester prenatal diagnosis can be made by CVS leaving time to perform stem cell transplantation using allogeneic stem cells. The most common treatment for SCID patients is a postnatal BM transplant where a matched donor is required. More recently, where a suitable donor is not available, a stem cell gene therapy approach has used gene corrected autologous BM transplantation with great success (Demaison et al., 2002; Gaspar and Thrasher, 2005; Thrasher et al., 2006; Gaspar et al., 2011; Montiel-Equihua et al., 2012). For instance, in 2012, around 30 patients had been treated most of whom had experienced clinical benefit with the absence of any vector-related complications (Gaspar and Thrasher, 2005). There is a chance of insertional mutagenesis occurring during retroviral and lentiviral vector integration into host-cell chromosomes as well as the development of lymphoproliferative disease in individuals with SCID (Gaspar and Thrasher, 2005; Yáñez-Muñoz et al., 2006; Howe et al., 2008). It is important for clinical therapies to achieve stable transgene expression while minimizing insertional mutagenesis (Baum et al., 2003). Integration-deficient lentiviral vectors and self-inactivating (SIN) gammaretroviral vectors have a low risk and in cellular and *in vivo* models of SCID can mediate stable transduction (Yáñez-Muñoz et al., 2006; Thornhill et al., 2008).

CONGENITAL BLOOD DISORDERS

Inherited blood disorders such as the hemoglobinopathies or clotting disorders would be a relatively simple target for IUT as the fetal circulation can be reached through the umbilical vein (UV) at the placental cord insertion or the intrahepatic UV, or even via the peritoneal cavity, a route used successfully to transfuse anemic fetuses.

Prenatal screening and diagnostic services for congenital hemoglobinopathies are available in many countries making them an attractive option for an *in utero* therapeutic approach (David and Waddington, 2012). Prenatal diagnosis can be achieved currently from 11 weeks of gestation using CVS, or amniocentesis from 15 weeks, but increasingly there are advances in non-invasive prenatal screening and diagnosis using circulating fetal DNA detected in the maternal plasma allows the diagnosis of congenital disorders as early as 7 weeks (Lo et al., 1998). Since AF or chorionic villus samples are accessible relatively easily and early in pregnancy, they would provide the potential for therapeutic use after clinical prenatal diagnosis have been performed.

Inherited abnormalities of hemoglobin (Hb), a tetramer of two α-like and two β-like globin chains, are a common and global problem. Over 330,000 affected infants are born annually worldwide, 83% with sickle cell disorders and 17% with thalassemias (Modell and Darlison, 2008). Current treatment of β-thalassemia is by postnatal allogeneic hematopoietic stem cell transplantation (HSCT) which can cure the condition with recent results of 90% survival and 80% thalassemia-free survival (Angelucci et al., 2000). However, this option is only available in approximately 30% of cases due to the lack of a suitable matched donor (Lucarelli, 2002), and it is associated with complications such as GVHD. For children where HSCT is unavailable, they are dependent on blood transfusions that result in iron overload, and the need for iron chelation therapy. In alpha-thalassemia, some individuals who make very little or no α globin chains, have severe anemia, termed Hb Bart's hydrops fetalis syndrome which is commonly diagnosed prenatally and if untreated causes death in the neonatal period (Harteveld and Higgs, 2010). Current treatment of sickle cell relies on a number of strategies such as the use of prophylactic antibiotics, pneumococcal vaccination and good hydration, and effective crisis management such as using oxygen and pain-relief (Meremikwu and Okomo, 2011).

Attempts to cure thalassemia and sickle cell disease using gene therapy have been hampered by the large globin gene and globin promoters that are difficult to accommodate within vector systems. Amelioration or even cure of mouse models of human sickle cell disease (Pawliuk et al., 2001) and β-thalassemia major (Pawliuk et al., 2001; Persons et al., 2003; Puthenveetil et al., 2004) has been achieved using lentivirus vectors that contain complex regulatory sequences from the LCR region. Recent advances in vector design have improved gene transfer for the hemoglobinopathies such as the ubiquitous chromatin opening element (UCOE) augmented spleen focus forming virus (SFFV) promoter/enhancer which provides lentivirus vectors with a natural tropism for the hematopoietic system (Antoniou et al., 2003; Williams et al., 2005) resulting in reproducible and stable function in BM and all differentiated peripheral hematopoietic cell lineages (Zhang et al., 2007).

Clotting disorders are caused by deficiencies in coagulation factors, for example, hemophilia B, which is due to mutations in the factor IX (F9) gene resulting in a deficiency in the blood clotting protein human factor IX (hFIX; Waddington et al., 2004b). The current treatment offered to patients with inherited coagulopathies includes lifelong recombinant protein infusions, which is required to avoid major pathology, and it is an expensive and limited resource. In some patients, protein infusions can also lead to the formation of antibodies to the infused product, which prevents its use. Gene therapy cure of inherited coagulopathies has come closer to reality with the use of adeno-associated virus vectors (AAV). Animal experiments have shown AAV to be a promising vector system and this has led to the first human trials for this disease by applying AAV-hFIX intramuscularly to eight adult patients with severe hemophilia B which showed a small increase in hFIX plasma levels and a reduction in exogenous protein requirement (Kay et al., 2000; Manno et al., 2003). More recently, one trial used a self-complimentary AAV-hFIX vector that gives higher levels of transgenic protein expression *in vivo* than earlier single-stranded vectors. A single peripheral vein infusion of a serotype-8-pseudotyped, self-complementary AAV vector expressing a codon-optimized hFIX transgene in six patients with severe hemophilia B (FIX activity, <1% of normal values) gave FIX expression at 2–11% of normal levels in all participants. A short course of glucocorticoid therapy normalized raised liver enzyme levels that were observed in two patients (Nathwani et al., 2011). AAV vectors with hFVIII and hFVII are becoming available and are being tested in pre-clinical studies (Binny et al., 2012; McIntosh et al., 2013).

Proof of principle studies have shown long-term expression of hFIX proteins at therapeutic levels and induction of immune tolerance (Waddington et al., 2007) after *in utero* gene therapy using lentiviral vectors in mice (Waddington et al., 2004a,b). More recently, using the same self-complementary AAV8 vector expressing the human factor IX (hFIX) gene used for the clinical trials, long-term hFIX expression was observed after ultrasound guided intraperitoneal injection of fetal sheep in early and late gestation (Nathwani et al., 2006; David et al., 2011). No functional antibodies could be detected against the vector or transgene product and no liver toxicity was observed. Antibodies to the therapeutic gene were detectable when the animals were challenged at 6 months of age postnatally with the hFIX recombinant protein, showing that induction of immune tolerance was not achieved. This was probably due to the fall in hFIX expression that was undetectable by 1 year after birth. UV delivery in fetal non-human primates of a 10-fold higher dose of the same self-complementary AAV system in late gestation produced clinically relevant levels of hFIX sustained for over a year, with liver-specific expression and a non-neutralizing immune response (Mattar et al., 2011).

In utero transplantation for congenital blood disorders

In comparison to the relative success of postnatal transplantation for blood disorders, results of clinical cases of IUT to cure blood disorders have been disappointing (Nijagal et al., 2012; Pearson and Flake, 2013). For instance, the transplantation of $CD34^+$ cells from either fetal liver or adult BM in cases of hemoglobinopathies, showed no evidence of engraftment in all 22 cases with a clinical outcome of lifelong blood transfusion or disease-related mortality (Tiblad and Westgren, 2008). Attempts to treat other diseases such as sickle cell or metabolic storage disorders have been unsuccessful after fetal liver-derived stem cell transplantation, even wherein a suitably matched donor has been available (D'Azzo, 2003; Westgren, 2006).

Transplantation of autologous derived fetal liver stem cells has been attempted in the fetal sheep. Fetal liver stem cells collected from first trimester preimmune sheep fetuses using ultrasound-guided hepatic sampling were labeled with PKH26 and then transplanted intraperitoneally into allogeneic and autologous fetal recipients. Engraftment of donor cells was equivalent after autologous or allogeneic transplantation (up to 4.7% in fetal liver, spleen, BM, blood, and thymus) but the fetal loss rate was high (29% allogeneic and 73% autologous transplantation) making this technique difficult to justify in clinical practice (Schoeberlein et al., 2004).

For coagulopathies, transplantation of MSCs may be a feasible therapeutic option. In a sheep model of hemophilia A that recapitulates the human condition (spontaneous bleeds and debilitating hemarthroses), encouraging results were found after postnatal intraperitoneal infusion of paternally derived MSCs transduced with a porcine FVIII-encoding lentiviral vector. Infusions of factor VIII were no longer required and damaged joints were fully recovered. However, a sharp increase in pre-existent antibodies occurred with time following transplantation which decreased the effectiveness and limited the duration of therapy (Porada et al., 2011). This emphasizes the need for an IUSCT approach for this condition.

In utero stem cell gene therapy for congenital blood disorders using AF-derived stem cells

Given the concerns around *in utero* application of gene therapy directly to the fetus, our group have been studying whether a combination of autologous transplantation with gene corrected AFS might be a potential therapeutic approach. We have studied the functional hematopoietic potential of transduced green fluorescent protein $(GFP)^+$ sheep AF-derived stem cells, before and after autologous IUSCT. First trimester sheep AF was collected by ultrasound-guided amniocentesis or at post mortem examination. We used a novel sheep $CD34^+$ antibody that allows flow cytometric detection of sheep HSC/progenitors present within BM, cord blood, and mobilized peripheral blood. This antibody also enriches for HSC/progenitors with enhanced *in vitro* colony-forming potential (Porada et al., 2008). Sheep $CD34^+$AF or adult BM cells were selected and transduced overnight with an HIV lentivirus vector containing eGFP. Transduced fresh or frozen $CD34^+$AF, or BM cells, were injected intravenously into NOD-SCID-gamma (NSG) mice. GFP^+ cells were detected in the hematopoietic organs and peripheral blood of NSG mice primary and secondary recipients 3 months later (**Figure 1**). Autologous IUSCT was performed in fetal sheep using ultrasound-guided intraperitoneal injection of fresh transduced GFP^+CD34^+AF cells. GFP^+ cells were detected in the peripheral blood of injected lambs up to 6 months postnatally (**Figure 2**) and 3 months after secondary transplantation of BM from autologous IUSCT lambs into NSG mice, GFP^+ cells were detected in hematopoietic organs. This demonstration of autologous IUSCT of $CD34^+$AF cells in a large animal model supports the concept for clinical translation to treat congenital hematopoietic diseases *in utero* (Shaw et al., 2014).

Human β-thalassemia iPS have now been generated from AFS using a single excisable lentiviral stem cell cassette vector. AFS from the prenatal diagnosis of a β-thalassemia patient were reprogrammed by expression of the four human reprogramming factors Oct4, KLF4, SOX2, and c-MYC using a doxycycline lentiviral system and demonstrated teratoma formation (Fan et al., 2012). There are concerns that these iPS cells may be more likely to develop teratomas than AFS cells that have a low risk of this complication. This type of cell manipulation however, may provide clinicians with corrected autologous patient-specific iPS cells to use in a combination IUSCGT approach for the treatment of thalassemia (Fan et al., 2012).

IN UTERO TRANSPLANTATION FOR OTHER CONGENITAL DISORDERS

IUT is a possible treatment strategy for congenital disorders that affect organ systems other than the blood. These include myelomeningocele (MMC; Danzer et al., 2012) which represents the most severe form of spina bifida, cystic fibrosis (CF), lysosomal storage diseases such as acute neuronopathic (type II) Gaucher disease, neuronal ceroid lipofuscinoses, and Niemann–Pick disease type C, ornithine transcarbamylase deficiency (OTC), as well as muscular dystrophy (David et al., 2003). Many of these diseases and organ systems would be amenable to IUT using MSCs, which compared to HSCs, are less immunologically competent and may result in less transplantation related rejection (O'Donoghue and Fisk, 2004).

In utero transplantation of mesenchymal stem cells

Human BM-MSCs have shown to have long-term engraftment and have the ability to differentiate into various tissues when transplanted into fetal sheep (Mackenzie and Flake, 2001). The therapeutic potential for combining surgical repair and transplantation of MSCs *in utero* has been demonstrated recently for the treatment of spina bifida in a rat model (Li et al., 2012). IUT of first trimester human fetal blood MSC ameliorates the skeletal disorder in a mouse model of osteogenesis imperfecta (Guillot et al., 2008). IUT of fetal MSCs reduced fracture rates and skeletal abnormalities (Guillot et al., 2008). Two cases of IUT using allogenic fetal liver MSC in the third trimester had encouraging results with a successful engraftment which demonstrated 7.4% chimerism at 9 months of age in one case and good long-term outcomes (Le Blanc et al., 2005; Götherström et al., 2014).

In utero stem cell gene therapy using amniotic fluid-derived MSCs

High fetal survival was found after intraperitoneal injection of autologous AFMSCs in the sheep (Mehta et al., 2011; Shaw and Bollini, 2011). AF was collected under ultrasound-guided amniocentesis in early gestation pregnant sheep ($n = 9$, 58 days of gestation, term = 145 days) and AFMSCs were isolated, expanded, and transduced using an HIV vector encoding enhanced GFP with 63.2% (range 38.3–96.2%) transduction efficiency rate (**Figure 1**). Transduced AFMSCs were injected into the peritoneal cavity of each donor fetal sheep at 76 days under ultrasound guidance with a 78% overall survival rate for the full procedure. After 2 weeks, GFP^+ cells and protein was detected in fetal tissues including liver, heart, placenta, membrane, umbilical cord, adrenal gland, and muscle and this was further confirmed by cytofluorimetric and immunofluorescence analysis (**Figure 3**).

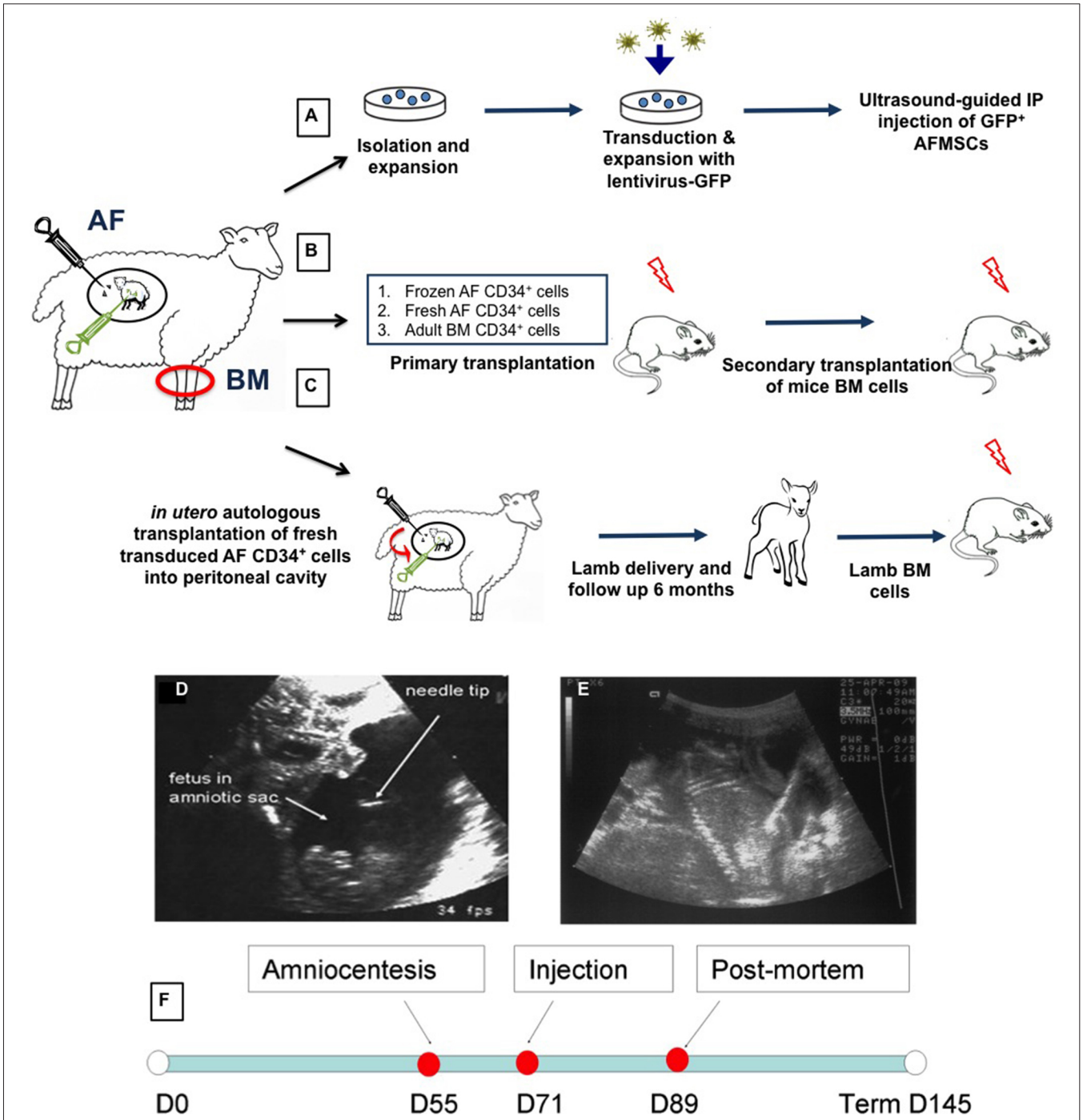

FIGURE 1 | Experimental design and injection procedure. After amniocentesis collection, amniotic fluid mesenchymal stem cells (AFMSCs) were cultured in adherence in defined conditions. Cells were transfected with lentivirus GFP and re-injected into the peritoneal cavity of the fetal donor **(A)**. Transduced sheep eGFP$^+$ CD34$^+$ selected from fresh or frozen AF and adult BM cells were transplanted into immunocompromised NSG mice (primary and secondary xenogeneic transplantation) **(B)**. Transduced sheep eGFP$^+$CD34$^+$ fresh AF were also injected into donor sheep fetuses (*in utero* autologous transplantation) that were subsequently delivered and followed for up to 3 months of age. Bone marrow from these primary sheep recipients was then used to perform xenogeneic secondary transplantation into NSG mice **(C)**. AF, amniotic fluid; BM, bone marrow. Sonograms showing ultrasound guided amniocentesis **(D)** and intraperitoneal injection **(E)**. Amniocentesis was performed using a 22 gauge needle to collect 10 ml amniotic fluid from the amniotic cavity around a fetal sheep at 58 days of gestation. For intraperitoneal injection of transduced expanded amniotic fluid cells we used a 20 gauge needle inserted through the anterior abdominal wall of a sheep fetus at 76 days of gestation. Echogenicity can be seen throughout the peritoneal cavity after injection of cells. Scale bars: 5 cm. **(F)** Timeline of the experiment.

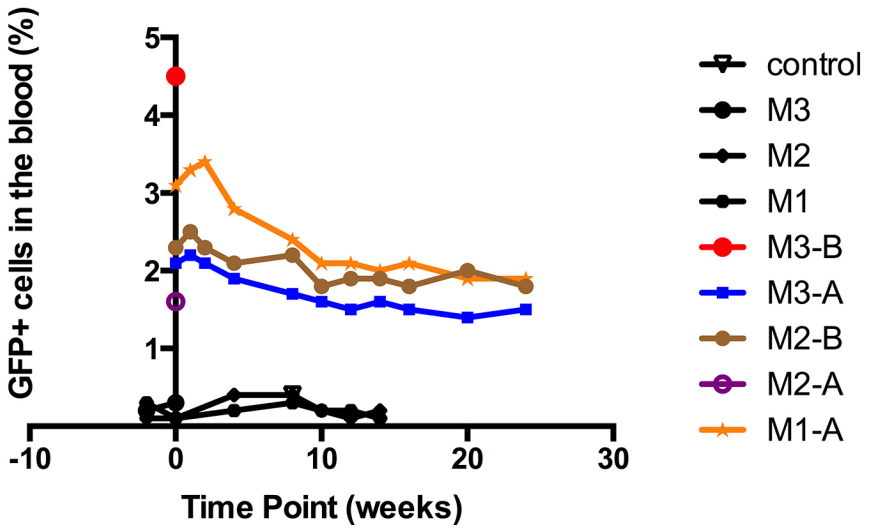

FIGURE 2 | *In utero* autologous intraperitoneal transplantation of sheep amniotic fluid CD34$^+$ cells in fetal sheep with long-term follow up. Engraftment in the peripheral blood after *in utero* transplantation of autologous sheep CD34$^+$eGFP$^+$ AF cells. All five born lambs showed eGFP$^+$ cells in the peripheral blood at birth (M1-A, M2-A, M3-A, M2-B, and M3-B), and all three survivors revealed persistent levels of engraftment of around 2% that persisted up to the last sampling point at 6 months of age (M1-A, M2-B, and M3-A). Negative control: peripheral blood from uninjected sheep. M1, M2, and M3: the three ewes that showed negativity for eGFP signal.

AFS cells intraperitoneally injected in a necrotizing enterocolitis rat model had shown improved survival, clinical status, gut structure, and function (Eaton et al., 2013; Zani et al., 2014). These findings suggest that the use of AFS cells as a source of cells for *in utero* therapy could be an alternative way of ameliorating prenatal congenital disease.

ROUTE TO THE CLINIC

Preclinical testing in animal models of disease will be an important step before clinical translation is realized. There is no ideal animal model and a balance is needed, taking into consideration the gestational development of the organ to be targeted and how that relates to its development in the human, the type of placentation, fetal size, number and lifespan, parturition, and the fetal and maternal immune response (Mehta et al., 2012; Mehta and Abi-Nader, 2012). An assessment of the safety, accessibility, transduction efficiency, and behavior of various stem cells (i.e., cord blood, placenta, AF, fetal tissue) *in vitro* as well as in the fetal environment are required to evaluate proof-of-principle strategies based on gene transfer or cell transplantation into the fetus to ensure accurate organ-directed manipulation and delivery (Moreno et al., 2012). Thus, the efficacy of treatment can be evaluated from murine models to large animal models such as sheep and primates (Mehta et al., 2012; Mehta and Abi-Nader, 2012).

Toxicology studies will be needed using animals such as the pregnant rabbit, in which reproductive toxicology is commonly performed, with good historical datasets and a model that is understood by the regulators. A variety of guidelines and regulations such as those described by the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency will need to be taken into consideration when planning preclinical study protocols. These could include for example, the guidelines on the non-clinical testing for inadvertent germline transmission of gene transfer vectors (EU, 2006) or on the non-clinical studies required before first clinical use of gene therapy medicinal products (European Medicines Agency, 2007).

In addition to animal studies, the safety of gene therapy vectors has to be evaluated. Integration site analysis has become a critical tool to measure the "vector-on-host" and "host-on-vector" effects in gene therapy (Biasco et al., 2011). Also, models such as the human placenta can be utilized *in vitro* as it would provide a wealth of data on the physiology of normal and pathological human placentae and may be useful in measuring the spread of vector from the fetus to the mother or vice versa.

CONCLUSION

Advances in prenatal screening and molecular diagnosis have provided the ability of detecting the majority of genetic diseases early in gestation. Early diagnosis provides the option of possible treatment options that can be explored either at the prenatal or postnatal period depending on the condition. The option of preemptive treatment of congenital diseases *in utero* by stem cell or gene therapy are encouraging as it changes the perspective of congenital diseases. However, further work focusing on the safety and ethical issues need to be addressed before clinical applications can be considered.

AUTHOR CONTRIBUTIONS

Durrgah L. Ramachandra, Steven S. W. Shaw, Panicos Shangaris, Stavros Loukogeorgakis, Pascale V. Guillot, Paolo De Coppi, and Anna L. David made substantial contributions to the conception and design of the work. Durrgah L. Ramachandra and Panicos

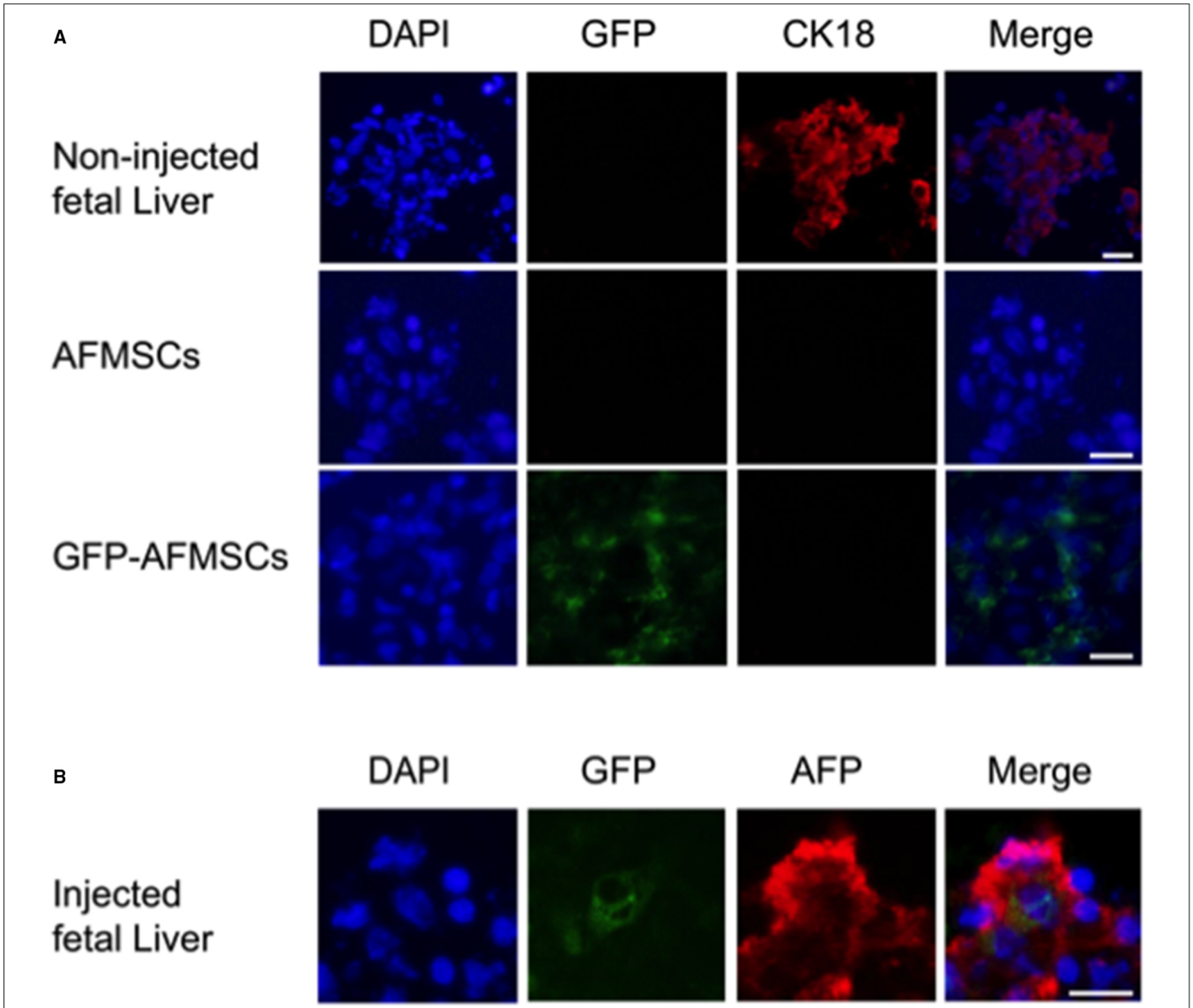

FIGURE 3 | Immunofluorescence for CK18 and AFP expression in fetal liver and amniotic fluid stem cells. Panel **(A)** shows positive CK18 expression in cells cultured from a control fetal sheep liver but no expression in amniotic fluid mesenchymal stem cells (AFMSCs) or transduced cells (GFP-AFMSCs) before injection. An uninjected sheep fetus of comparable gestational age was used as the control. Panel **(B)** shows co-expression of GFP with expression of Alpha fetoprotein (AFP) another liver specific marker in the fetal liver after transplantation of transduced AFMSCs. Scale bars: 20 μm.

Shangaris drafted the work and Paolo De Coppi and Anna L. David revised it critically for important intellectual content. Durrgah L. Ramachandra, Steven S. W. Shaw, Panicos Shangaris, Stavros Loukogeorgakis, Pascale V. Guillot, Paolo De Coppi, and Anna L. David gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Corrigendum: *In utero* therapy for congenital disorders using amniotic fluid stem cells

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I am writing this manuscript to correct the second author information. My name is correctly spelt, S.W. Steven Shaw. But "S.W. Steven" is the first name, "Shaw" is the last name. My publications are always searched by "Shaw SW" in pubmed. However, in this article, my name is searched by "Shaw SS."

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Prenatal transplantation of mesenchymal stem cells to treat osteogenesis imperfecta

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Osteogenesis imperfecta (OI) can be a severe disorder that can be diagnosed before birth. Transplantation of mesenchymal stem cells (MSC) has the potential to improve the bone structure, growth, and fracture healing. In this review, we give an introduction to OI and MSC, and the basis for pre- and postnatal transplantation in OI. We also summarize the two patients with OI who have received pre- and postnatal transplantation of MSC. The findings suggest that prenatal transplantation of allogeneic MSC in OI is safe. The cell therapy is of likely clinical benefit with improved linear growth, mobility, and reduced fracture incidence. Unfortunately, the effect is transient. For this reason, postnatal booster infusions using same-donor MSC have been performed with clinical benefit, and without any adverse events. So far there is limited experience in this specific field and proper studies are required to accurately conclude on clinical benefits of MSC transplantation to treat OI.

Keywords: prenatal transplantation, *in utero* transplantation, intrauterine transplantation mesenchymal stem cells, fetal stem cells, osteogenesis imperfecta

OSTEOGENESIS IMPERFECTA

Osteogenesis imperfecta (OI), or brittle bone disease, is a group of genetic disorders caused mainly by defects in collagen synthesis (Forlino and Marini, 2000). The majority of OI cases are caused by some 1500 different dominant mutations in the COL1A1 or COL1A2 gene, resulting in abnormal assembly of the protein (Canty and Kadler, 2005). More recently, recessive forms for OI has been described, where defects in proteins involved in post-translational modifications or transport leading to perturbations of the collagen 3-hydroxylation complex (Barnes et al., 2010), and collagen chaperone pathways (Alanay et al., 2010; Christiansen et al., 2010). OI presents in a clinically heterogenous manner, ranging from the mild type I, to the progressively deforming type III and the perinatally lethal type II according to the original Sillence classification (Sillence et al., 1979). An evolving understanding of the genetics has now made it possible to consider refining and adding to the original Sillence classification (Forlino et al., 2011).

Currently, the goal of clinical management is to optimize the patient's gross motor abilities and to achieve a level of independence during childhood life. This is largely accomplished empirically through physical rehabilitation and life-long orthopedic interventions in correcting bony deformities of the long bones and vertebra (Laron and Pandya, 2013). Pharmacological intervention is underpinned by the use of bisphosphonates in an effort to reduce bone resorption and increase bone mineralization (Rauch and Glorieux, 2006). While mineralization has been shown to improve with bisphosphonate treatment, a recent meta-analysis of randomized trials failed to demonstrate consistent benefits in fracture rates, reduction of pain, or functional mobility (Dwan et al., 2014). Moreover, there is a growing concern on the role of bisphosphonates in impairing bone remodeling in these children (Marini, 2009). Growth hormones have also been evaluated, and have shown encouraging benefits in the increase in linear growth (Antoniazzi et al., 1996), and are being considered for treatment in combination with bisphosphonates (Antoniazzi et al., 2010).

Due to the lack of effectiveness in current modalities of treatment, which does not address the underlying molecular defect, alternative approaches are currently being explored. Some of these experimental treatments include allogeneic cell transplantation (Horwitz et al., 1999, 2001, 2002; Otsuru et al., 2012). The genetic defect may be corrected through homologous recombination of the patient's stem cells (Chamberlain et al., 2004), or through the degradation of abnormal COL1A1/2 transcripts (Millington-Ward et al., 1997, 2002). In this paper, we will focus on the use of allogeneic mesenchymal stem cells (MSC) in pre- and postnatal treatment of OI.

MESENCHYMAL STEM CELLS AS DONOR CELLS IN OI

Mesenchymal stem cells are stromal cells that have been originally identified from the adherent portion of bone marrow

(Friedenstein et al., 1966, 1968). They grow as spindle shaped cells displaying colony-forming capacity in low density cultures and are non-hematopoietic and non-endothelial. MSC can be propagated through multiple passages in cell culture and differentiate down the standard osteogenic, adipogenic and chondrogenic lineages under permissive conditions (Pittenger et al., 1999). MSC do not express human leukocyte antigen (HLA) class II antigens, and are generally considered to be non-immunogenic in nature and possess immune-modulatory properties (Le Blanc, 2003). Although attempts at standardizing the nomenclature of what constitute a MSC has been proposed by the International Society of Cellular Therapy (Dominici et al., 2006), evidence of bona fide stem cell properties had only been demonstrated in CD146 positive perivascular cells in human bone marrow with *in vivo* self-renewal properties (Sacchetti et al., 2007). Over the past couple of decades, the isolation of MSC-like cells has been reported from multiple organs and tissues (da Silva Meirelles et al., 2006), which may originate from NG2 (neuron-glial antigen 2) and CD146 positive pericytes (Crisan et al., 2008), although non-bone marrow-derived MSC have yet to be validated for stemness.

As MSC are readily isolated and expanded, are non-immunogenic and have multilineage differentiation capacity, they have been studied and indeed trialed clinically in a diverse range of clinical scenarios ranging from the treatment of graft versus host disease (Le Blanc et al., 2008), autoimmune diseases such as Crohn's disease (Uccelli et al., 2008; Uccelli and Prockop, 2010), cardiovascular diseases like acute myocardial infarction and stroke and orthopedic applications including bone and cartilage repair (Zhang et al., 2012). However, MSC have limited ability to be expanded under standard conditions especially with increasing age (Siegel et al., 2013; Choudhery et al., 2014), rapidly senescing in culture and with restrictions in multilineage differentiation capacity (Digirolamo et al., 1999; Muraglia et al., 2000). In addition, MSC yield, growth kinetics and differentiation capabilities vary significantly between individuals, restricting their clinical utility (Javazon et al., 2004).

Another class of MSC has been characterized in fetal and perinatal life, initially from first trimester tissues (Campagnoli et al., 2001; Gotherstrom et al., 2003; Chan et al., 2008), and have now been found in multiple tissue types including the umbilical cord, placenta, and amniotic fluid (O'Donoghue and Chan, 2006; De Coppi et al., 2007). These primitive MSC types have been found in higher frequencies with a higher colony-forming capacity, having longer telomeres and a higher proliferative potential (Chan et al., 2005; Guillot et al., 2007), and differentiate more readily into bone and muscle (Chan et al., 2006, 2007; De Coppi et al., 2007; Zhang et al., 2009), and non-mesenchymal lineages such as neural and hepatic cells (De Coppi et al., 2007; Kennea et al., 2009). Like their adult counterparts, human fetal MSC (hfMSC) are also non-immunogenic, and have the ability to modulate immune responses (Gotherstrom et al., 2003, 2004; Gotherstrom, 2007; Di Trapani et al., 2014). Given the ability of hfMSC to be expanded several fold more efficiently than adult MSC, their enhanced colony-forming capacity and increased bone differentiation capacity, they may be the ideal cell type for treatment of OI.

The rationale for the use of allogeneic MSC for the treatment of OI is underpinned by the ability of MSC to home to bone (Pereira et al., 1995; Guillot et al., 2008) and indeed to regions of active remodeling as found in fracture sites and in patients with OI. MSC secretes both paracrine growth factors and normal type I collagen conducive for generating normal bone tissues and thus ameliorating the bone fragility phenotype in OI.

PRENATAL TRANSPLANTATION

Skeletal dysplasia occurs in fetal life, which can be diagnosed readily through mid-trimester fetal anomaly scans (Schramm et al., 2009; Barkova et al., 2014). Affected fetuses may present with shortened long bones and the occurrence of multiple fractures, alerting the clinician toward a diagnosis of OI. Definitive prenatal genetic diagnosis may be achieved through standard amniocentesis or fetal blood sampling, both of which are established fetal medicine procedures largely available in most developed countries. This opens up the possibility of offering prenatal treatment.

In the context of OI, the most convincing argument for prenatal transplantation would be to ameliorate the disease process at a time of rapid skeletal development where spontaneous fractures are occurring. Other arguments in favor of a prenatal approach includes (i) the relatively smaller cell doses required due to the size of the fetus, (ii) the shunting of the intravenously delivered cells to the arterial circulation through the patent foramen ovale in fetal life rather than being trapped in the lungs in postnatal life, and (iii) possibly the lower risks of immune rejection in the developing immune system of the recipient (Lee et al., 2009; Mattar et al., 2012).

PRENATAL TRANSPLANTATION IN ANIMAL MODELS OF OSTEOGENESIS IMPERFECTA

Experimental evidence of the efficacy of prenatal MSC transplantation was provided by Guillot and colleagues, who tested the ability of first trimester fetal blood-derived MSC to ameliorate OI in the *oim* mouse, a naturally occurring recessive mouse model approximating human type III OI, with progressive deformities and skeletal fractures (Chipman et al., 1993). In this model, 10^6 culture expanded hfMSC were injected intraperitoneally at E14 gestation (a high dose of around 10^9/kg fetal weight) and allowed to litter naturally in this xenogeneic transplantation model in a fully immune-competent recipient (Guillot et al., 2008). Donor cells engrafted in a wide range of tissues such as the skin, heart, lung, brain, and thymus, but were found in greater quantities in skeletal tissues where up to 5% of cells were of donor origin. Human donor cells expressing the bone marker osteopontin tended to cluster around areas of active bone formation and at fracture sites. Transplanted mice demonstrated improved bone strength, length, and cortical thickness, with a two-third reduction in fractures (Guillot et al., 2008).

More recently, Panaroni et al. (2009) investigated the ability of prenatal allogeneic bone marrow transplantation in the BrtlIV mouse, a dominant model of OI more reflective of human type II OI. Here, 5×10^6 unmanipulated bone marrow from adult donors was transplanted intraperitoneally to E13.5–E14.5 fetuses where wild type females were mated with heterozygous BrtlIV males, which should produce affected fetuses in half the litter.

The transplantation resulted in rescue of perinatal lethality, as transplanted mice had a higher proportion of surviving BrtlIV offspring. At 2 months of age, only 64% of transplanted mice were chimeric for donor cells in multiple hematopoietic tissues including bone marrow where donor cells accounted for 1–2% of all cells, and produced up to 20% of the bone collagen. Donor cells were found in clusters in long bones, with accompanying improvement in bone mineral density and cortical thickness in treated compared to untreated BrtlIV mice. Thus, these two models provide evidence supporting a prenatal approach to treat of OI, leading to higher engraftment rates, amelioration of disease phenotype and rescue of perinatal lethality. In addition, data from the BrtlIV mouse study suggests that significant amount of normal collagen can be deposited by a relatively small population of chimeric donor cells. This would explain the marked improvements in mineralization and growth seen in the clinical transplantation cohort where engraftment levels are generally around 1%.

CLINICAL EXPERIENCE OF POSTNATAL CELL TRANSPLANTATION IN OSTEOGENESIS IMPERFECTA

The first clinical proof of principle of an allogeneic stem cell transplantation approach came from Horwitz and colleagues where children affected with type III OI underwent transplantation with unmanipulated bone marrow from HLA-identical or single-antigen-mismatched siblings after ablative conditioning therapy. The treated children exhibited increased linear growth velocities and reduced fracture frequencies in spite of the low frequency (<2%) of donor osteoblast engraftment in the bone (Horwitz et al., 1999, 2001). The same group carried out further clinical studies with MSC isolated from the same bone marrow donors. This study included six children at age 2–4 years that received two infusions of $1-5\times10^6$/kg HLA-matched gene marked adult MSC. It resulted in MSC engraftment and an increase in linear growth velocities (Horwitz et al., 2002). Thus, it was established here that allogeneic MSC infusion is safe in the context of OI, with donor cells engrafting in bone and results in an increase in mineralization and growth velocity, albeit only for a limited time. Although the same group later showed that the origin of donor blasts might be from the non-adherent fraction of bone marrow, i.e., the hematopoietic stem cells, rather than the adherent fraction from where MSC are generally isolated (Otsuru et al., 2012). Not withstanding that, MSC can contribute to bone growth and mineralization, possibly through secreted growth factors (Otsuru et al., 2012). Moreover, it would be inconceivable to put a child through a bone marrow transplantation procedure with all its attendant morbidity and mortality when the use of non-matched MSC would obviate the need for conditioning therapy, and have shown an excellent safety profile through thousands of clinical infusions (Aguilar et al., 2007; Zhang et al., 2012).

CLINICAL EXPERIENCE OF PRENATAL MSC TRANSPLANTATION IN OSTEOGENESIS IMPERFECTA

Promising results of tissue repair in animal studies have led to numerous clinical studies using MSC to treat severe disorders and several reports indicate a role for MSC therapy in the treatment of OI both pre- and postnatally. As described above, Horwitz et al. (2002) performed the first study using HLA-matched MSC to treat OI postnatally. Results were promising showing low toxicity, engrafted donor cells and accelerated growth. Encouraged by this study, prenatal transplantation using hfMSC has since then been reported in two cases of OI.

The first case presented at gestational week 15 and was later diagnosed as OI type III, which postnatally was confirmed with genetic analysis (COL1A2 c.3008G>A; p.Gly1003Asp; Gly913Asp in the triple helical domain; Le Blanc et al., 2005; Gotherstrom et al., 2014). At week 24, all limbs were −5 SD and angulated, with femoral fractures noted. The baby was infused with 6.5×10^6/kg HLA-unmatched hfMSC at gestational week 31. At 4 months of age, bisphosphonate treatment was initiated due to presence of vertebral compression fractures. Until 8 years of age, she was doing acceptably well with little more than one fracture and one compression fracture per year (5 femoral, 2 clavicular, 1 shoulder and 1 skull fracture and 11 vertebral compression fractures). Remarkably she continued growing and followed her own height and weight growth curve at −5 SD until the age of 6, when it had deteriorated to −6.5 SD at the age of 8 years. Due to the increased fracture rate and declined growth, the patient was transplanted with 2.8×10^6/kg same-donor cells at the age of 8 years. The subsequent 2 years after the re-transplantation the patient did not suffer from any new fractures and the linear growth and mobility improved (she was able to walk 1000 m without difficulties, started dance classes, increased her participation in gymnastics at school). Donor osteoblastic cells were detected in the bone, but not in any other tissues, at 9 months and 9 years of age. The level of engraftment was varying, between 0.003 and 16.6%. Only one other patient is currently known to have an identical COL1A2 mutation and presented with a very severe phenotype of OI. This patient did not receive MSC therapy and succumbed at 5 months of age despite postnatal bisphosphonate therapy.

The second case was a baby with OI type IV who presented with short long bones (<5th centile) and multiple fresh and healing fractures at 26 weeks of gestation (Gotherstrom et al., 2014). The baby was transplanted with 30×10^6/kg HLA-unmatched hfMSC at 31 weeks of gestation, and did not suffer any new fractures for the remainder of the pregnancy or during infancy. The patient's family had a history of short stature and multiple fractures and genotyping of the patient and family members identified an autosomal dominant mode of inheritance (c.659G>A; p.Gly220Asp, Gly130Asp in the triple helical domain). No donor cells were detected in umbilical cord blood, umbilical cord, and placenta. There have been no opportunities to obtain bone samples for analysis in this case. Bisphosphonate therapy was initiated from 1 month of age due to poor mineralization. The patient followed her own growth curve until 12 months of age (just below the 3rd centile), where longitudinal length plateaued. A postnatal infusion of 10×10^6/kg MSC from the same donor was performed at 19 months of age, resulting in resumption of her growth trajectory and she continued to grow just below the 3rd centile. She started to walk shortly after the transplantation.

Similarly as described in the study by Horwitz and colleagues, the above described pre- and postnatal transplantations report a transient clinical effect after hfMSC infusion (Horwitz et al., 2002; Le Blanc et al., 2005; Gotherstrom et al., 2014), and several

repeated transplantation might be required during the patients lifetime, especially in childhood. Nevertheless, intravenous infusion of same-donor hfMSC pre- and postnatally appears safe. The reported follow-up period is 3–10 years after prenatal transplantation and 2–2.5 years after postnatal transplantation. There were no signs of any adverse early or late reactions. There was no alloreactivity of the patient's lymphocytes detected toward the donor hfMSC. Before the re-transplantations, analysis showed the absence of antibodies directed toward HLA class I and II, IgG and IgM, or fetal bovine serum (FBS).

The cell dose is a critical parameter in cell transplantation since it may relate to efficacy but a high cell dose may cause toxicity. In the reported hfMSC transplantations, the cell dose varied from 5×10^6 to 30×10^6/kg at prenatal transplantation and from 2.8×10^6 to 10×10^6/kg at postnatal transplantation. All doses were well tolerated, however, it is unclear from this data on two patients or from data on adult MSC transplantation for other disorders if a high cell dose is more efficacious. This remains to be investigated.

SUMMARY

The two cases described here suggest the safety and feasibility of prenatal transplantation using HLA-mismatched hfMSC. Furthermore, it suggests a potential benefit to children with OI. However, the benefit from a single transplant before birth was transient and subsequent boosters with same-donor cells were performed with good effect. This is in line with the results from the study on postnatal MSC therapy in OI by Horwitz et al. (2002). The summarized data highlights the need to modify the pre- and postnatal transplant strategies, and possibly the cells, in order to improve MSC homing and engraftment for better long-term outcomes.

To accurately evaluate if pre- and postnatal MSC therapy in OI is an effective treatment, coordinated studies and joint efforts are necessary. Development of common programs, registries and guidelines (cell source, isolation and expansion and release criteria, patient inclusion criteria, transplantation strategies, follow-up measures, etc.), as well as coherence between investigators within the field is of significant importance. Considering the complexity of the disease and procedure, we see this as the only realistic way to proceed if we are to accurately evaluate the potential of MSC therapy in OI.

The summarized cases demonstrate that prenatal transplantation of allogeneic hfMSC and postnatal boosters using same-donor cells in OI is safe. The MSC infusions appear to give clinical benefit, although transiently. However, so far we have limited experience and further studies are required.

AUTHOR CONTRIBUTIONS

Jerry K. Y. Chan and Cecilia Götherström drafted, wrote, and revised the manuscript. Both authors approved the final version of the manuscript.

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REVIEW
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Engineering muscle tissue for the fetus: getting ready for a strong life

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Congenital malformations frequently involve either skeletal, smooth or cardiac tissues. When large parts of those tissues are damaged, the repair of the malformations is challenged by the fact that so much autologous tissue is missing. Current treatments require the use of prostheses or other therapies and are associated with a significant morbidity and mortality. Nonetheless, affected children have generally good survival rates and mostly normal schooling. As such, new therapeutic modalities need to represent significant improvements with clear safety profiles. Regenerative medicine and tissue engineering technologies have the potential to dramatically improve the treatment of any disease or disorder involving a lack of viable tissue. With respect to congenital soft tissue anomalies, the development of, for example, implantable muscle constructs would provide not only the usual desired elasticity and contractile proprieties, but should also be able to grow with the fetus and/or in the postnatal life. Such an approach would eliminate the need for multiple surgeries. However, the more widespread clinical applications of regenerative medicine and tissue engineering technologies require identification of the optimal indications, as well as further elucidation of the precise mechanisms and best methods (cells, scaffolds/biomaterials) for achieving large functional tissue regeneration in those clinical indications. In short, despite some amazing scientific progress, significant safety and efficacy hurdles remain. However, the rapid preclinical advances in the field bode well for future applications. As such, translational researchers and clinicians alike need be informed and prepared to utilize these new techniques for the benefit of their patients, as soon as they are available. To this end, we review herein, the clinical need(s), potential applications, and the relevant preclinical studies that are currently guiding the field toward novel therapeutics.

Keywords: skeletal muscle, tissue engineering, stem cells, biomaterials, regenerative medicine, congenital abnormalities, functional regeneration, animal models

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Introduction

The majority of children affected by congenital malformations have a defect involving either skeletal, smooth or cardiac tissues. When large parts of those tissues are damaged, the repair of the malformations can be often challenged by the fact that autologous tissue is missing (de Coppi, 2013). Major cardiac anomalies, bladder exstrophy, omphaloceles, diaphragmatic hernia or long gap oesophageal atresia are only some of the situations in which we have to use prostheses or adopt

solutions associated with a significant degree of morbidity and mortality. Since these children have, overall, good survival rates and mostly normal schooling, it is important to avoid using solutions that are not optimal, as this may limit their options in the future. Ideally, besides the usual elasticity and contractility proprieties, the implanted muscle, in contrast to implantation in post-adolescent individuals, should be able to grow with the fetus and/or into the postnatal life. To avoid a planned re-do surgery, absorbable materials should therefore be considered.

One classical example is congenital diaphragmatic hernia. Fetuses diagnosed with this malformation mostly receive a synthetic prosthesis at birth which allows the repair of the diaphragmatic defect. However, this patch will neither grow with the child, nor integrate with the native tissue, so there's more chance of muscle contraction with possible scoliosis, hernia recurrence or patch infections resulting in a poor quality of life for the child and his or her family (de Coppi and Deprest, 2012). For all these reasons many surgeons dealing with congenital malformations have been interested in tissue regeneration (Grikscheit et al., 2003; Atala et al., 2006; Kunisaki et al., 2006).

The possibility of making new tissue *in vitro* would indeed completely change the way we treat these children and transform their lives. The recent progress in tissue engineering (TE) and regenerative medicine (RM) has been possible due to improved understanding and utilization of the stem cells and biomaterials that are cornerstones for this field of medical science. Due their overt importance to the more widespread clinical applications of TE and RM technologies, both will be considered herein. We will begin with consideration of stem cells.

Stem Cells for TE and RM Applications

Stem cells have developmental potentials varying from the totipotency of cells derived from the first few divisions of the fertilized egg to the unipotency of somatic cells present in peripheral tissue (Thomson et al., 1998; Pittenger et al., 1999). To regenerate large amounts of tissues, pluripotent cells would be ideal because they can be expanded and are able to generate any tissue (Thomson et al., 1998). However, they are still limited in their clinical use because, besides ethical concerns and immunogenicity, which have been partially overcome with the discovery of induced pluripotent stem (iPS) cells, they are so powerful that they can be tumorigenic (Takahashi and Yamanaka, 2006). On the other side we have multipotent cells, which are limited to the generation of tissues within the same germ layer but they are safer and indeed they have already been adopted to correct some of these malformations (Pittenger et al., 1999; Elliott et al., 2012).

Embryonic stem cells would be ideally positioned to build muscle tissues for children with congenital malformations (Thomson et al., 1998). However, besides their tumorigenic potential and the ethical issues, immunosuppressive treatment should also be adopted to avoid their rejection by the transplanted patient. It is believed that embryonic stem cells are less immunogenic, but this is only true if you consider them prior to differentiation. Once they are terminally differentiated and express all the major histocompatibility complexes, they would be rejected if immunosuppression therapies were not adopted. ES cells have been demonstrated to differentiate reliably to cardiomyocytes (Burridge et al., 2012), which have been successfully engineered to obtained cardiac microtissues (Thavandiran et al., 2013). However, the fully formed heart is composed of diverse cell lineages including myocytes, endothelial cells, vascular smooth muscle cells (SMC), and fibroblasts that derive from distinct subsets of mesoderm during embryonic development. As a consequence engineering of functional cardiac muscle for clinical application is still a major challenge. In this regard, ES cells can also be differentiated into distinct populations of SMC subtypes under chemically defined conditions (Cheung et al., 2012). As such, their ability to derive an unlimited supply of human cell types, including SMCs, could further accelerate applications of stem cells to regenerative medicine as well as disease modeling (e.g., patient-specific stem cells for exploring mechanisms of disease) (Cheung et al., 2014). PAX7-positive skeletal muscle progenitors can also be obtained from human and mouse ES cells opening the possibility of engineering autologous skeletal muscle in large quantity through the direct reprogramming of cells from children born with a malformation (Shelton et al., 2014).

On the opposite side of the picture there are the adult stem cells (Pittenger et al., 1999). Somatic stem cells can be expanded from different postnatal tissues and could be useful for therapy particularly in neonates and children where they are generally more abundant and probably more potent than in adults (Fulle et al., 2012). Classically the bone marrow contains, besides haematopoietic stem cells, mesenchymal stem cells, but somatic cells with different potentials can also be isolated and grown in good quantities. These cells can be used in an autologous setting avoiding immunogenic problems. As far as we know they are not tumorigenic, and their use does not raise any ethical issues (Bianco et al., 2008).

Proof of Concept for Clinical Applicability of RM

As an example of the potential of TE and RM technologies, 15 years ago a cover of Nature Biotechnology celebrated the first artificial bladder taking shape in dogs (Oberpenning et al., 1999). In those studies, the whole dome of the bladder was successfully replaced using smooth muscle and urothelial cells expanded from the recipient and this established the basis for treating the first patients affected by bladder exstrophy. The group, coordinated by Dr. Atala, described in 2006 in The Lancet a pilot study of seven patients who had received implanted tissue engineered bladders from 1998 onwards (Atala et al., 2006). Similar to the animal model, they reported the use of either collagen scaffolds seeded with cells or a combined polyglycolic acid (PGA)-collagen scaffold seeded with cells for bladder replacement. These engineered tissues were implanted with or without omental coverage. Patients reconstructed with engineered bladder tissue created with cell-seeded PGA-collagen scaffolds and omental coverage showed increased compliance, decreased end-filling pressures, increased capacities and longer dry periods over time (Atala et al., 2006). More recently, the same group showed that in 5 boys who had urethral defects, tubularised urethras could be engineered and remain functional in a clinical setting for up to 6 years (Raya-Rivera et al., 2011). A tissue biopsy was taken from each patient,

and the muscle and epithelial cells were expanded and seeded onto tubularised polyglycolic acid:poly(lactide-coglycolide acid) scaffolds. Patients (range 10–14 years old), who had surgery between March 2004, and July 2007 were followed up until July 2010 showing maintenance of normal function and tissue architecture after biopsy (Raya-Rivera et al., 2011). However, as recently noted by Andersson (2014), despite encouraging proof of concept results, the more widespread applications of the bladder repair technologies awaits further preclinical investigation. Another example of the utility of somatic cells for TE and RM applications derives from the use of adult cardiomyocytes. In contrast to what was initially thought, cardiomyocytes can also be expanded from adult tissue and they have been used in in patients with ischaemic cardiomyopathy (Bolli et al., 2011). However, their numbers are limited and expansion may not be efficient enough to generate sufficient cell populations for engineering functional tissue. In addition, satellite cells, the skeletal muscle precursors, can be easily isolated and expanded. In fact, satellite cells have been used for cellular therapy and tissue engineering purposes in both synthetic and decellularised polymers in small and large animal models. Freshly isolated SCs showed a higher regenerative potential, with implemented proliferation and migration. They retain a high myogenic potential *in vitro* and more interestingly *in vivo* during the first few passages but they are unable to be expanded for longer in culture (Rossi et al., 2010). Within the muscle there are at least two other cell types, muscle associated but not somite-derived, that present a high myogenic potential. The mesoangioblasts, vessel-associated stem cells, express early endothelial markers, such as Flk-1, CD34, stem cell antigen 1 and VE (vascular-endothelial)-cadherin, but not late markers, like Von Willebrand factor (Cossu and Bianco, 2003). They can be expanded for several passages, are not tumorigenic and, even if they do not express the transcription factors Myf5 and MyoD, they can be easily induced toward myogenesis upon co-culture with myoblasts. Similarly, pericytes have also shown myogenic potential. They are, as the mesoangioblasts, vessel-associated progenitors, they do not express endothelial markers but they do express NG2 proteoglycan and alkaline phosphatase (ALP). Unlike the canonical myogenic precursors (SCs), pericyte-derived cells express myogenic markers only in differentiated myotubes, which they form spontaneously with high efficiency (Mitchell et al., 2010).

Given these initial successes and the possibilities they portend, why don't we always use adult stem cells? First, because the numbers of cells are small and they decrease with age. Second, these cells are multipotent not pluripotent, so they cannot give rise to all lineages. Finally, they can be exposed to virus and toxins during their lifetime. (Pittenger et al., 1999). That means that we have cells in our body that continuously accumulate deletions and mutations (Bianco et al., 2001). Our immune system normally destroys them, however if they are replicated in large numbers in the laboratory and transplanted back in the recipient they may be able to fight against our immune system and generate a tumor.

In 2006 a seminal paper published by Shinya Yamanaka described how some of the limitations of both embryonic and adult stem cells might be overcome (Takahashi and Yamanaka, 2006). His group found, first in mice and subsequently in humans, that pluripotent stem cells could be generated from their adult counterpart using defined transcription factors (Takahashi et al., 2007). The findings were confirmed by independent groups and it is now possible to derive induced pluripotent stem (iPS) cells using different methodologies (Zhao and Daley, 2008). iPS cells, when compared to ES cells, eliminate the immunogenic problem, so you can use them in an autologous setting, and they also reduce the ethical concerns. However, iPS are still tumorogenic and their clinical use has still not been adopted.

Amniotic fluid stem (AFS) cells should also be considered. They are distinct both from adult and embryonic stem cells, can be used in an autologous setting, their use is not controversial and they are not tumorogenic (Pozzobon et al., 2010). Moreover, they are more naïve than adult stem cells and can be superior both in terms of proliferation and differentiation. Isolation of stem cells from amniotic fluid is easy to perform, there's a low risk for the mother and the fetus and it is a widely accepted method for prenatal diagnosis. So, AFS cells are ideal for pre-natal and neo natal applications (Moschidou et al., 2013a). AFS cells, are immunoselected by the stem cell factor receptor c-kit (CD117) and give rise to lineages representing the three germ layers both *in vitro* and *in vivo* (de Coppi et al., 2007a). The cells express markers of all three germ layers, and endogenously express the important transcription factor OCT4, which maintains the pluripotency of ESCs. AFS cells are easily reprogrammed not only by DNA-integrating systems (Wolfrum et al., 2010), but also without any genetic manipulation by means of the histone deacetylase inhibitor, valproic acid (VPA) (Moschidou et al., 2012, 2013b). Both human and rodent AFS cells display multi-lineage potential (Ditadi et al., 2009) and can exert a beneficial paracrine action in models of bladder (de Coppi et al., 2007b), heart (Bollini et al., 2011), kidney (Sedrakyan et al., 2012), and lung (Grisafi et al., 2013) disease.

AFS cells could also have a role for *In utero* stem cell therapy (IUSCT) (Surbek et al., 2008). IUSCT in humans have been successful only for the treatment of congenital severe combined immunodeficiency (SCID). (Tiblad and Westgren, 2008) Rejection of allogeneic cells *in utero* could be at least partially explained by the migration of the *in utero* injected cells into maternal circulation and mounting of a rejection response, which could diminish the engraftment. This is most likely due in mice to activated maternal T cells which can cross the placenta in mice and destroy engrafted allogeneic cells (Nijagal et al., 2011). In order to avoid this response, stem cells matched to the mother could be used. Alternatively, in monogenic disease, AFS cells derived from the fetus could be used for therapy after genetic modification since they would not trigger an immunogenic response from either the fetus or the mother.

Regarding the application of AFS cells for the treatment of acquired muscle conditions, we and others have tested various disease models. In a mouse model of Spinal Muscular Atrophy with a muscular dystrophy appearance of the skeletal muscle (*HSA-Cre, SmnF7/F7* mice) receiving intravenous injection of a small number of AFS cells were able to survive with drastic improvement of their muscle force (Piccoli et al., 2012). Histopathological evaluation of the treated animals revealed integration of AFS cells not only in the skeletal muscle fibers, but also in the stem cell compartment of the muscle. Indeed secondary

transplants of satellite cells (SCs) derived from treated mice indicated that AFS cells integrate into the muscle stem cell compartment and have long-term muscle regeneration capacity **indistinguishable** from that of wild-type-derived SC (Piccoli et al., 2012).

AFS cells however, have not been used clinically yet. Therefore, attempts to generate tissues or organs in the laboratory for the correction of congenital malformations has only been tried thus far using adult somatic cells.

Developing TE Strategies for the Fetus and Newborn

As summarized above, there has been a lot of research directed toward identifying cell source(s) with potential applications for improving TE and RM technologies. Another critical component of TE/RM approaches is the biomaterial component. Although, as noted in a recent review (Wolf et al., 2014), biomaterials/scaffolds alone are being actively pursued both pre-clinically and clinically for restoration of volumetric muscle loss (VML; injuries of sufficient magnitude to result in permanent functional and cosmetic deficits) injuries in adults, the focus in this review will be on TE and RM solutions for the fetus/neonate. In addition, several other excellent recent reviews are available that address more general aspects of TE/RM for skeletal muscle repair (Rossi et al., 2010; Juhas and Bursac, 2013; Mertens et al., 2014).

With respect to the explicit purpose of this report, any contemplated TE implant for the fetus or neonate would need to grow with the patient, we direct the remainder of this report to consideration of biomaterials/scaffolds that are being designed for the presence of a cellular component. This emphasis seems especially applicable to the large volume muscle tissue replacement required for correction of congenital anomalies in fetuses and newborns, as discussed herein. In this scenario, the biomaterial serves as a cellular delivery vehicle that can provide structure and appropriate environmental context and/or instructional cues for improved regeneration. Recent research in this area has begun to address the enormous possibilities this approach portends.

Biomaterials for Skeletal Muscle TE and RM for the Fetus and Newborn

As noted in the discussion thus far, the vast majority of preclinical studies conducted to date for skeletal muscle tissue repair—that might eventually be applicable to fetal/neonatal tissue repair and replacement—have used adult somatic cells, and in particular, the focus has been on myogenic progenitor cells (i.e., satellite cells, myoblasts, myotubes). This is true for studies conducted both *in vitro* and *in vivo*. In that regard, much progress has been made in engineering skeletal muscle since the seminal work of Vandenburgh and colleagues on avian myocytes in 1988 (Vandenburgh et al., 1988). The pertinent literature in this area is still actively growing. Below we provide a thorough, though not exhaustive, review of the recent PubMed database related to cell-based skeletal muscle tissue engineering approaches. The goal was to review the literature and identify the major sources of tested biomaterials/scaffolds for TE-based skeletal muscle repair/replacement.

As noted in **Table 1** and schematically depicted in **Figure 1**, synthetic and naturally-derived biomaterials have been used with similar frequency for TE purposes. Of the studies reviewed, a naturally-derived biomaterial was used in roughly half of all studies conducted. Only a minority of studies have combined natural and synthetic biomaterials as part of the preferred scaffold configuration. Also of note, roughly 1/3 of the studies reviewed (25/78) have been conducted using C2C12 cells, which while more convenient to work with for a variety of reasons, lack clinical applicability. Thus, the discussion below emphasizes the use of primary cultures. So, how have these biomaterials been combined with myogenic cells to yield TE skeletal muscle?

From an experimental perspective, three general approaches to *in vitro* TE skeletal muscle have been utilized thus far: (1) Cells embedded in a hydrogel, (2) Cells placed on or within a more structured/patterned scaffold, and (3) Cells placed in culture and allowed to develop their own extracellular matrix *in vitro* (scaffold-free, but resulting in a naturally-derived extracellular matrix). These cell-seeded scaffolds have been subjected to a variety of strategies including different culture media, incorporation of mechanical forces and electrical cues, as well as incubation times of distinct durations. The end result has been to produce myotubes and myofibers of varying lengths and diameters *in vitro*. A host of histological, immunochemical and molecular evaluations have been conducted to assess the phenotype of the TE skeletal muscle produced. However, only ≈30% of all studies conducted (either *in vitro* or *in vivo*) actually evaluated the contractile function of the resultant constructs (see **Table 1** and **Figure 1**). Because the functional status of TE skeletal muscle, at all stages of the TE process, is critical to evaluating their potential clinical applications and experimental utility (e.g., as a screening tool for drugs *in vitro*), we will focus going forward on a discussion of those studies that measured function, especially those that did so *in vivo* or following implantation *in vivo*.

Characteristics of TE Skeletal Muscle *In Vitro*

One school of thought for development of TE skeletal muscle for *in vivo* implantation is to create constructs that are as similar to native skeletal muscle as feasible prior to implantation. An intrinsic benefit of this approach is that these same technologies may be applicable to drug screening for muscle toxins, as well as for treatment of muscle diseases and disorders. Thus far, however, all attempts to create TE skeletal muscle *in vitro* still result in a relatively immature/neonatal muscle phenotype, with respect to fiber diameter (generally $<20\,\mu M$) and functionality (the degree of measured force following stimulation), as well as expression of embryonic myosin isoforms. In fact, absolute forces for TE skeletal muscle *in vitro* have typically ranged from as little as ≈1–30 μN (Borschel et al., 2004; Fujita et al., 2010), to 400–800 μN (Dennis and Kosnik, 2000; Dennis et al., 2001; Huang et al., 2005; Borschel et al., 2006; Lam et al., 2009). The specific force, when it has been measured (Dennis et al., 2001), has only been a

TABLE 1 | Summary of literature on cell and biomaterial combinations used for tissue engineered skeletal muscle.

References	Scaffold material	Natural/Synthetic	Cells and implantation model/site
Juhas and Bursac, 2014	Fibrin hydrogel	Natural	Rat Myoblasts, satellite cells
Juhas et al., 2014	Fibrin hydrogel	Natural	Rat Myoblasts (Murine SCI window)
Kikuchi et al., 2014	Collagen hydrogel	Natural	Myoblasts
Hosseinzadeh et al., 2014	PCL	Synthetic	Satellite cells
Fuoco et al., 2014	PEG hydrogel	Synthetic	Muscle-derived pericytes (Porcine SCI)
Ostrovidov et al., 2014	Gelatin, carbon nanotubes	Synthetic, Natural	C2C12 cell line
Cei et al., 2014	PLLA, PCL, PLGA films or scaffolds	Synthetic	Human myoblasts, fibroblasts
Corona et al., 2014	Bladder acellular matrix (BAM)	Natural	Rat myoblasts (TA defect)
Salimath and Garcia, 2014	PEG-maleimide hydrogel	Synthetic	C2C12 cell line
Wang et al., 2014	Alginate	Synthetic	Murine myoblasts (TA SCI)
VanDusen et al., 2014	Naturally-derived, scaffold-free ECM	Natural	Rat MPCs (TA defect implantation)
Ahadian et al., 2014	CNT/Methacrylated gelatin hydrogel	Synthetic	C2C12 cell line
Zhang et al., 2014	PLGA hexagonal patterned	Synthetic	C2C12 cell line
Ye et al., 2013	PGS	Synthetic	Human skeletal muscle, ECs (SCI and IPI)
Corona et al., 2013	Muscle ECM	Natural	Rat BM-derived Stem Cells (TA defect)
Wang et al., 2013	PLGA grooved films, RGD or YIGSR peptides	Synthetic	C2C12 cell line
Shah et al., 2013	Collagen coated glass fibers	Synthetic, Natural	Human muscle progenitor cells
Tamaki et al., 2013	3-D nerve-vascular gel patch	Synthetic, Natural	Murine muscle-derived cells
Martin et al., 2013	Fibrin hydrogel	Natural	Human MPCs
Jana et al., 2013	Chitosan	Natural	Murine MPCs
Guex et al., 2013	Poly(epsilon-caprolactone)	Synthetic	C2C12 cell line
Greco et al., 2013	PEDOT/PSS	Synthetic	C2C12 cell line
Du et al., 2013	Calcium alginate gel	Synthetic	Rat BM-MSCs (LPP; urethra defect)
Criswell et al., 2013	Bladder acellular matrix (BAM)	Natural	Murine ECs, MPCs, pericytes (SCI)
Bandyopadhyay et al., 2013	PLC sponge	Synthetic	Human myoblasts (SCI)
Williams et al., 2013	Naturally-derived, scaffold-free ECM	Natural	Rat MPCs (Hindlimb implantation)
Bayati et al., 2013	Polycaprolactone/polycarbonate-urethane	Synthetic	Human adipose-derived stem cells
Wang et al., 2012	Alginate, RGD peptides	Synthetic	Mouse myoblasts
Shah et al., 2014	Phosphate Glass	Synthetic	Human MPCs
Sengupta et al., 2012	2-D RGD-peptide micropatterned film	Synthetic	Human Myoblasts
Ku et al., 2012	PCL/PANi	Synthetic	C2C12 cell line
Fujie et al., 2012	PLL/PMMA microarray films, fibronectin	Synthetic	C2C12 cell line
Corona et al., 2012	Bladder acellular matrix (BAM)	Natural	Rat myoblasts (Rat LD defect)
Fernandes et al., 2012	Maltodextrin-derivative	Synthetic	C2C12 cell line (SCI)
Hinds et al., 2011	Fibrin hydrogel	Natural	Rat myoblasts
Sirivisoot and Harrison, 2011	Carbon nanotube/Polyurethane	Synthetic	C2C12 cell line
Page et al., 2011	Fibrin microthread	Natural	Human MPCs (Murine TA defect)
Ladd et al., 2011	PCL/collagen, PLLA/collagen	Synthetic	C2C12 cell line
Ker et al., 2011	Polystyrene fiber	Synthetic	C2C12 cell line
Machingal et al., 2011	Bladder acellular matrix (BAM)	Natural	Rat myoblasts (LD Defect)
Falco et al., 2011	EHD, EH, EH-PEG	Synthetic	Human myoblasts
Rossi et al., 2011	Hyaluronic acid-photoinitiator	Synthetic	Murine MPCs or satellite cells (TA defect)
Borselli et al., 2011	Alginate gel, RGD peptides	Synthetic	Murine myoblasts (Murine TA defect)
Yang et al., 2010	poly(β- amino esters)/DNA nanoparticles	Synthetic	Human MSCs, ESCs (Murine SCI)
Fujita et al., 2010	Magnetite-incorporated C2C12 constructs	Synthetic	C2C12 cell line
Merritt et al., 2010	Muscle-derived ECM	Natural	Rat MSCs (Gastrocnemius defect)
Ayele et al., 2010	Bovine tunica vaginalis	Natural	Rabbit myoblasts (abdominal wall defect)
Singh et al., 2010	pHEMA-gelatin cryogel	Synthetic	C2C12 cell line
Moon et al., 2008	Collagen acellular matrix	Natural	Human MPCs (SCI)
Kim et al., 2010	MPEG-PCL gel	Synthetic	Human ADSCs (SCI)
Stern et al., 2009	Muscle-derived extracellular matrix (M-ECM)	Natural	C2C12 cell line, rat myoblasts

(Continued)

TABLE 1 | Continued

References	Scaffold material	Natural/Synthetic	Cells and implantation model/site
Lam et al., 2009	PDMS, Fibrin gel	Synthetic, Natural	Rat satellite cells and myoblasts
Beier et al., 2009	Collagen-fibrin gels, collagen sponges	Natural	Rat myoblasts
Bian and Bursac, 2009	Fibrin/Collagen I gel	Natural	C2C12 cell line, rat myoblasts
Riboldi et al., 2008	DegraPol	Synthetic	C2C12 cell line
Stern-Straeter et al., 2008	Gelatin	Natural	Human myoblasts
Kroehne et al., 2008	Collagen sponge	Natural	C2C12 cell line (Murine TA defect)
Falco et al., 2008	EH	Synthetic	Rat myoblasts
Boldrin et al., 2008	PLGA	Synthetic	Human MPCs (Murine TA defect)
Matsumoto et al., 2007	Fibrin gel	Natural	C2C12 cell line
Boontheekul et al., 2007	Alginate gel/G4RGDSP peptide	Synthetic	C2C12 cell line
Yan et al., 2007	Collagen	Natural	Rat Satellite cells
Huang et al., 2006	PLLA	Synthetic	C2C12 cell line
Borschel et al., 2006	Fibrin Gel	Natural	Rat myoblasts
Hill et al., 2006	Alginate	Synthetic	Murine myoblasts
Larkin et al., 2006	Naturally-derived, scaffold-free ECM	Natural	Rat MPCs
Conconi et al., 2005	Muscle matrix	Natural	Rat myoblasts (Abdominal wall defect)
Shah et al., 2005	Phosphate glass	Synthetic	Human MPCs
Huang et al., 2005	Fibrin gel	Natural	Rat myoblasts
Riboldi et al., 2005	DegraPol	Synthetic	C2C12 cell line, human satellite cells
Borschel et al., 2004	Muscle-derived extracellular matrix (M-ECM)	Natural	C2C12 cell line
Kamelger et al., 2004	PGA, alginate, or hyaluronic acid gels	Synthetic, natural	Rat myoblasts (SCI)
Kim et al., 2003	Collagen gel	Natural	Rat myoblasts (Tongue defect)
Lai et al., 2003	Collagen gel	Natural	Rat myoblasts (Abdominal wall defect
Dennis et al., 2001	Naturally-derived, scaffold-free ECM	Natural	Rat, mouse MPCs, C2C12, 10T1/2
Dennis and Kosnik, 2000	Naturally-derived, scaffold-free ECM	Natural	Mouse MPCs
Saxena et al., 1999	PGA fiber mesh	Synthetic	Rat myoblasts
Mulder et al., 1998	Polyurethane thin films	Synthetic	Mouse G8 skeletal myoblasts cell line

Red lettering indicates functional studies were performed on TE constructs implanted in vivo (i.e., contraction of engineered/retrieved constructs were evaluated. Highlighted in yellow are the engineered skeletal muscle tissues that were implanted, as well as the site and nature of implantation. Abbreviations: EH network: polymeric scaffolds (EH network) made from the cyclic acetal monomer, 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl- 1,3-dioxane-2-ethanol diacrylate (EHD); PEG, polyethylene glycol; Poly-hydroxyethyl methacrylate (pHEMA)-gelatin cryogel scaffold; MPEG–PCL, methoxy poly(ethylene glycol) poly(3-caprolactone); PDMS, polydimethylsiloxane; PGA, poly glycolic acid; PLGA, poly-lactic-glycolic acid; PLC, L-lactide/epsilon-caprolactone copolymer; PLA, poly lactic acid; PLLA, poly (L-lactic acid); PLL, poly (L-Lysine); PMM, Poly (methyl methacrylate); PANi, polyaniline; PEDOT:PSS, poly(3,4-ethylenedioxythiophene):poly(styrene sulfonate); TA, tibialis anterior muscle; LD, latissimus dorsi muscle; SCI, subcutaneous implantation; MPCs, muscle progenitor/precursor cells; ADSCs, adipose-derived stem cells; MSCs, mesenchymal stem cells; ECs, endothelial cells.

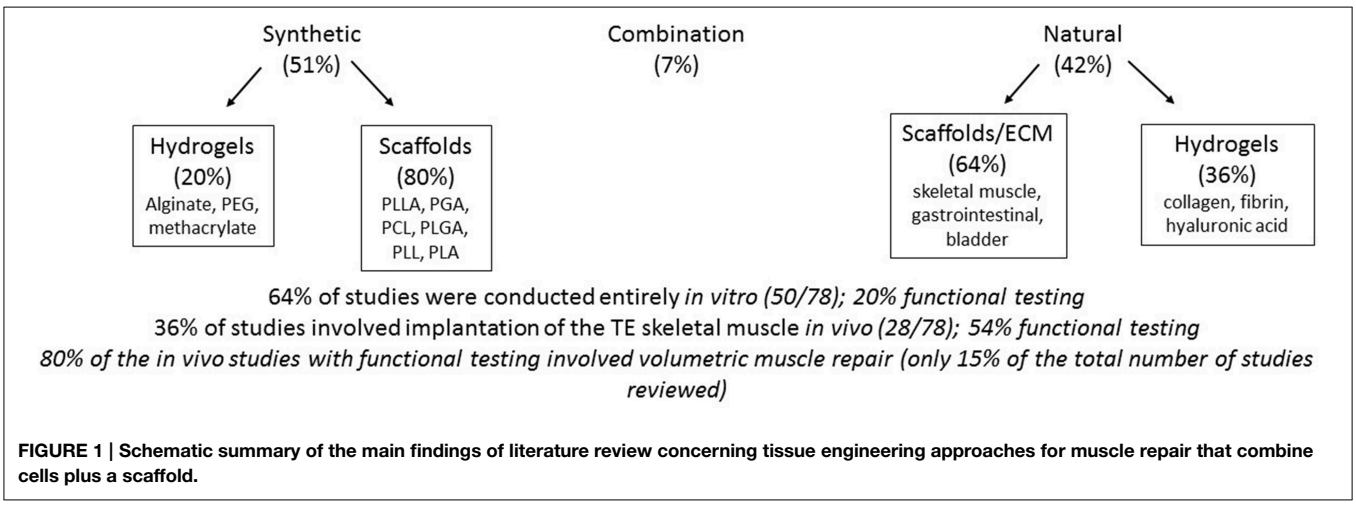

FIGURE 1 | Schematic summary of the main findings of literature review concerning tissue engineering approaches for muscle repair that combine cells plus a scaffold.

fraction (<10%) of what might be considered normal for a mammalian/rodent skeletal muscle (250 kN/M^2). The most complete functional analysis of force generation *per se* on TE skeletal muscle is that of Dennis and colleagues (Dennis et al., 2001). More recently, Larkin and colleagues (Williams et al., 2013; Mertens et al., 2014; VanDusen et al., 2014), as well as Bursac and colleagues (Perniconi et al., 2011; Juhas and Bursac, 2013, 2014; Juhas et al., 2014) have made significant improvements in both the phenotype and function (contractility) of TE skeletal muscle *in vitro*. Bursac, in particular, has shown that key aspects of excitation-contraction coupling (calcium transients) are intact, and moreover, that the constructs maintain the ability for myogenesis and regeneration *in vitro*. Both Bursac's biomimetic scaffolds (Juhas et al., 2014), as well as the SMUs (skeletal muscle units) of Larkin and colleagues (VanDusen et al., 2014) showed significant improvements in phenotype and contractility, as well as vascularization, following implantation *in vivo* for 1–4 weeks. These latter observations clearly point to the importance of the *in vivo* environment for enhanced maturation and function of TE skeletal muscle, even when TE muscle begins to more closely approximate native muscle with respect to excitation-contraction coupling and force generation. However, when thinking about this approach more broadly, it seems plausible that these more immature phenotypes may not be as large a barrier to TE skeletal muscle repair and replacement in the fetus and newborn, as they may be for skeletal muscle repair and replacement of VML injuries in adult mammals.

Implantation of TE Skeletal Muscle

As detailed in **Table 1**, fewer than 36% (28/78) of all the studies we reviewed involved implantation of TE skeletal muscle constructs *in vivo*. These implantations were either subcutaneous or in a model of VML injury. The remaining 16 (57%) *in vivo* implantations were placed in a model of VML injury to assess restoration of muscle tissue volume and/or function. These are each briefly described below.

In Situ Implantation of TE Skeletal Muscle

Of the 28 studies that included *in vivo* implantation, 12 (43%) were implanted subcutaneously or otherwise *in situ* (e.g., rat hindlimb), essentially using the body as a "bioreactor" to evaluate the impact of the *in vivo* environment on TE muscle maturation. However, only 3/12 studies actually evaluated contractile function (Moon et al., 2008; Williams et al., 2013; Juhas et al., 2014). Importantly, as alluded to above, in all of those (3) studies *in vivo* implantation was found to enhance muscle maturation and function.

Skeletal Muscle TE for Improved Regeneration of VML Defects *In Vivo*

The explicit goal of TE skeletal muscle for the fetus/neonate is to develop strategies that can repair or regenerate congenital anomalies. Importantly, the magnitude of muscle regeneration required in the VML rodent models is a reasonable approximation of the requirement of any TE/RM strategy in the fetus/neonate that would also be sufficient to accommodate growth of the fetus/neonate. Thus, another approach to TE skeletal muscle for repair and replacement *in vivo,* is to develop constructs that mainly mature *in vivo*. In contrast to *in vitro* TE approaches, many of these constructs lack the functional characteristics of even immature skeletal skeletal muscle (i.e., contraction; see above for details), but contain various combinations of satellite cells, myoblasts, myotubes, etc., on a cell delivery vehicle that will subsequently leverage the existing *in vivo* environment to provide the required key components for accelerated and/or enhanced functional regeneration in the scenario of VML injury.

In this regard, 16 studies evaluated implantation of a TE muscle construct in a VML defect *in vivo* (most commonly, surgically created defects), where by definition, there was no improvement expected in the absence of repair. In 75% of those studies functional outcomes were evaluated. Interestingly, with respect to surgically-created VML injuries to the legs (eight different studies), despite the distinct approaches that have been tried thus far (implantation of fibers in a hydrogel (Rossi et al., 2011), implantation of myoblasts on a fibrin microthread (Page et al., 2011), scaffold implantation with subsequent stem cell injection (Merritt et al., 2010; Corona et al., 2013), implantation of SMUs (VanDusen et al., 2014), bioreactor preconditioned myoblasts and myotubes (Machingal et al., 2011; Corona et al., 2012, 2014) in all cases, there were residual functional deficits, generally in the 20–30% range. Such an observation, albeit on a very small sample size with significant differences in models, muscles and measures, points out both the incredible promise of TE/RM for VML injury, as well as the limitations of current technologies and the need for standardized animal models and physiological measures.

Potential Clinical Applications to Neonates

We are not aware of any current or proposed clinical trial for the use of an RM/TE technology in the treatment of a craniofacial VML injury in the fetus or newborn. However, our group has been pursuing a tissue engineered muscle repair (TEMR) technology for clinical applications to craniofacial reconstruction and repair. We have been using the rodent latissimus dorsi (LD) muscle as a model system. The LD muscle has long-standing clinical utility (surgical reconstruction, heart wrap, etc.,) and further, is a relatively thin, sheet-like muscle that is morphologically analogous to the muscles in the face (i.e., muscles of mastication). The TEMR constructs have been implanted in a surgically created VML injury (i.e., excision of 50% of the LD muscle). These constructs are created by seeding myoblasts on a bladder acellular matrix (BAM), and subjecting the construct to cyclic mechanical preconditioning (10% stretch) in a bioreactor prior to implantation of a construct containing myoblasts and myotubes in a unidirectionally organized monolayer into the LD VML injury. As alluded to above, TEMR implantation is associated with restoration of significant functional capacity (60–70% recovery of contractile force) in athymic nude mice within 2 months of implantation (Machingal et al., 2011; Corona et al., 2012). This recovery appears to involve, at least to some extent, regeneration of a portion of the muscle fibers that were surgically removed.

Thus, building on the preclinical development of the TEMR technology, Christ and colleagues at UT-Houston (Drs. Mark Wong and Phil Freeman) have identified a craniofacial muscle-only defect (secondary revision of cleft lip), which represents a VML injury that might be effectively treated by this first generation TEMR technology. The study that will be proposed would be to address secondary revision of unilateral cleft lip (UCL) in adults. If successful though, these studies could have important implications for neonates as well. In fact, clefts of lip and the palate are among the most common congenital defects observed, with a frequency of about 1.7 per 1000 liveborn babies (Mossey et al., 2009), in which the orbicularis oris muscle has been shown to be deficient in both volume and function. In addition, secondary repair of UCL is necessary in a significant percentage of patients for correction of both functional and cosmetic deformities. It is conceivable, that TE approaches, such as the TEMR technology, may find utility for this clinical application, as implantation will occur in a fresh surgical wound bed in healthy subjects and is readily scalable to construct tested in rodents. Discussions are currently ongoing with the US Federal Drug Administration (FDA) to this end.

Summary

Clearly there is certainly much to be excited about with respect to the potential applications of TE skeletal muscle for clinical applications to the fetus/newborn. Nonetheless, there is much work still to be done. In short, overall, too few functional assessments are being performed, as is too little work in relevant animal models. In addition, there are numerous biomaterials, animal models, muscles, time points, cell types, etc., that have been utilized thus far, and therefore, there is a need for standardization of animal models and functional measures to permit more direct comparisons of different approaches in similar VML injuries.

Conclusion

In conclusion regenerative medicine and tissue engineering are changing the way we think about how we might 1 day treat patients born with serious congenital malformations involving muscle tissue. However, the science still needs time to better understand mechanism of action(s) responsible for improved functional regeneration, as well as the best cell(s) and biomaterial(s) and/or their combinations for maximizing the rate and magnitude of functional regeneration. In addition, we still must determine the safety profile of stem cell products and biomaterials prior to clinical applications. Nonetheless, the advances are coming rapidly along, and we need to be informed, educated and prepared to utilize these techniques for the benefit of patients, as soon as they are available.

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