Regenerative Medicine - From Gene to Cell and Tissue therapies (I): Prenatal Approaches

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Abstract

This series of two review articles focuses on recent advances and applications of regenerative medicine which could benefit paediatric patients. Innovations in genomic-, stem cell- and tissue-based technologies bring success in disease modelling and new therapies for congenital and incurable paediatric diseases. Prenatal approaches present unique opportunities associated with substantial biotechnical, medical and ethical obstacles. Maternal plasma fetal DNA analysis is increasingly adopted as a noninvasive prenatal screening or diagnostic test for chromosomal and monogenic disorders respectively. The molecular basis for cell-free DNA detection stimulated development of circulating tumour DNA testing for adult cancers. *In utero* stem cell-, gene-, gene-modified cell-, and to a lesser extent tissue-based therapies have shown early clinical promise in a wide range of pediatric disorders. Fetal cells for postnatal treatment and artificial placenta for *ex-utero* fetal therapies are new frontiers in this exciting field.

Key Messages

- Recent advances in regenerative medicine are ideally suited to solve congenital diseases which currently do not have effective therapies.
- Understanding and success in disease modelling and novel gene-, cell-, cell-free- and tissue-based therapies of rare pediatric diseases have led to first-in-human breakthroughs, laying the foundation for clinical trials.
- Advances in pediatric regenerative medicine have inspired applications for some adult conditions.
- Prenatal intervention provides multiple advantages but also presents biomedical, technological and ethical challenges.

Search Strategy

We searched Web of Science and PubMed for reports in English from October 1, 2011 to September 1, 2021 using the search terms “congenital diseases”, “paediatric diseases”, “ regenerative medicine“, “stem cell therapy”, “gene therapy”, “gene editing”, “cell-free therapy”, “*in utero* therapy”, “prenatal diagnosis”, “fetal therapy” “tissue engineering”, and “artificial placenta”. Some older references were also included owing to their importance. Because of restrictions in the number of references allowed, review articles were chosen where appropriate to provide readers with more details and further references to some worthy, but older, original articles.

Introduction

Recent advances in stem cell biology and tissue engineering technologies are ideally suited to solve congenital diseases which do not have alternative therapeutic options[1]. An example of paediatric application of regenerative medicine technology is the use of patient-specific organoids derived from rectal biopsies of patients with cystic fibrosis to quantitate individual drug response *in vitro*, allowing prospective choice of more efficacious treatments for the patient[2]. On the other hand, while laboratory discoveries for regenerative medicine hold great promise, clinical translation remains a major challenge. Understanding and success in disease modelling and novel gene-, cell- and tissue-based therapies of rare congenital diseases can advance the entire field of regenerative medicine with first-in-human breakthroughs. Examples of clinical trials of regenerative medicine for pediatric diseases will be discussed in this duet of review articels. There are also areas in which paediatric regenerative medicine is actually leading the way with approaches in adult regenerative medicine closely following its progress. An example is noninvasive prenatal testing by analysis of fetal DNA in maternal plasma which has helped the development of liquid diagnosis for adult cancers[3,4].

Technological Platforms

Stem/Progenitor Cells

Pluripotent/multipotent stem cells with the capacity to self-renew and differentiate into target cell types are a cornerstone for regenerative medicine. Stem cell therapy represents the most established of the treatment modalities under the regenerative medicine title. The first successful haematopoietic cell transplantations were reported in the late 1950s, and in the intervening 60 years advances in understanding of immune modulation have drastically improved outcomes for patients with current survival exceeding 90% in cases of non-malignant haematological disorders. Indeed, haematopoietic stem/progenitor cells (HSC) were the first cell type successfully used in a fetal setting to cure patients with immunodeficiencies[5,6], and form the focus of several recent high-profile trials of autologous ex vivo gene-correction for haemoglobinopathies[7,8]. Additionally, mesenchymal stem/progenitor cells (MSC) are multipotent cells that have the potential to differentiate into the osteogenic, chondrogenic, myogenic and adipogenic lineages, and they have a minimal oncogenic risk. MSC display a non-immunogenic profile allowing transplantation across major histocompatibility barriers without immunosuppression. Due to these favourable characteristics, they are tested in clinical trials for many disorders[9]. Finally, advances in embryonic stem cell biology have been pivotal in the field, but the greatest preclinical/clinical impact has arisen from the success of human induced pluripotent stem cell (iPSC) applications for disease modelling and new therapeutics[10]. Although it is unlikely that iPSC will be transplanted directly to the fetus, targeted differentiation to multipotent stem/progenitor cells (such as HSC and MSC) could extend their utility to prenatal therapy. Stem cells (embryonic, iPSC or adult organ-specific) can be grown into self-organizing 3D organoids in vitro. Human fetal hepatocytes have been used successfully to overcome difficulties of expansion of primary human hepatocytes[11]. However, organoid technologies are generally more applicable for postnatal appilcations (see accompanying article in this duet of regenerative medicine reviews).

Gene Engineering

Gene engineering is a broad term encompassing gene addition, knockdown and, more recently, gene editing. Further diversity exists around the vectors for the delivery of these platforms which can be separated into non-viral vectors and engineered viruses which may integrate into the genome, as in lentiviruses, or may exist outside the nucleus, such as adeno-associated viruses (AAV)[12]. The Nobel-winning discovery of CRISPR (Clustered-Regularly-Interspaced-Short-Palindromic-Repeats)-Cas9 technology by Charpentier and Doudna as a precise gene-editing tool has provided unprecedented opportunities to improve understanding and to cure both genetic and non-genetic diseases[13-15]. The ability

to precisely 'nick' and repair/replace a defective genetic sequence opens a therapeutic window for thousands of monogenic diseases. Cells or tissues may also be altered/generated/regenerated specifically by manipulation and engineering of the cell genome either ex vivo or in situ to restore normal physiology for patients suffering from non-genetic diseases.

Ongoing concerns persist around safety (specifically, immunogenicity of transgenes and vectors and potential for off-target effects) and efficacy (non-specific targeting and inefficient transduction or lack of durable effect). Technologies addressing many of these are currently being researched extensively in various preclinical models and have been reviewed extensively recently, and while a viral vector/ex vivo approach will likely represent the first application of gene therapy to a fetal subject, it is likely that non-viral vectors and in vivo gene editing will represent the prevailing future direction of the field[16].

Cell Signalling Manipulation

During fetal development, morphogenesis is hierarchical and involves a variety of stem/progenitor cells that are tightly regulated spatially and temporally by paracrine mediators. To recapitulate this highly complex developmental process for regenerative medicine, a cell-based, cell-free, or combined therapeutic approach is often employed. Paracrine factors can be directly introduced as small molecules/small-molecule drugs, recombinant proteins, synthetic modified mRNA, small non-coding RNA (such as microRNA), antisense oligonucleotides, and extracellular vesicles (EV; such as exosomes)[17]. A recent example is the use of cardiosphere-derived exosomal microRNA for myocardial repair in pediatric dilated cardiomyopathy[18]. Following encouraging preclinical investigations, a prospective phase 1a study was conducted in 5 patients and showed safety and improved cardiac function, thus laying the foundation for further randomized trials.

Delivery of Therapy to the Fetus

Delivery of any treatment in utero is particularly challenging as it needs to take into account the fragile nature of the fetus and the risk of premature delivery or spontaneous abortion. The latter is of significant importance when regenerative medicine-based therapies need to be delivered during early gestation. Fine-needle injections can be useful to deliver in utero stem cell-, gene-, or gene-modified cell-therapy. In order to increase precision, accurate identification of the needle tip is a major challenge, and ultrasonic needle tracking systems have been developed to address this[19]. Moreover, multimodal navigation systems can combine detailed prenatal imaging with accurate image-guided instrumentation. The latter can provide clear advantages for some prenatal interventions, and has been recently tested in fetoscopic laser photocoagulation for the treatment of twin-to-twin transfusion syndrome (TTTS). Similar to other in utero interventions, this procedure is particularly challenging due to the limited field of view, poor visibility, and poor image quality. Fetoscopic "mosaicking" can help create an image with an expanded field of view which could aid clinicians performing minimally-invasive fetal interventions[20]. Moreover, the development of smaller instruments will help decrease the risks of prenatal intervention to the pregnancy and allow early delivery of regenerative medicine-based therapies to the fetus, both of which are key for improved outcomes. An example of the latter is single-access fetal endoscopy for the management of myelomeningocele (MMC), which has been tested successfully in sheep[21]. Ultimately robot-assisted technology coupled with artificial intelligence/machine learning could address some of the challenges related to early fetal intervention. Such platforms could augment accuracy and dexterity, enhance efficacy and safety, and ultimately improve outcomes of fetal intervention[22].

Indications, Limitations and Ethical Considerations of Fetal Therapy

The unique fetal physiology provides multiple advantages for treating congenital diseases prenatally. The average weight of a 20 week fetus is over 10 times lower than the average term birthweight, allowing a high cell and/or viral particle number-to-weight dosage compared to treating the neonate[10]. This brings a possibility to administer higher doses resulting in a better efficacy, and also at a cost advantage. The foramen ovale and the ductus arteriosus permit systemic infusions into the umbilical vein to bypass the fetal lungs, avoiding stem cell sequestration in the lung microvasculature which occurs in postnatal infusion[23]. The natural stem cell proliferation and migration to different anatomic compartments allows for wider engraftment of donor cells[24]. In addition, stem cell proliferation facilitates integration of therapeutic transgenes delivered through viral vectors or gene editing technologies. Donor specific immune tolerance may be facilitated, as during fetal life the immune system undergoes self-education, and depending on the timing of transplantation, foreign cells may be recognized as self. Not only does this permit acceptance of the graft without myeloablation or immunosuppression which is required for postnatal transplantation, it may also enable postnatal 'booster' transplantation with the same donor cells[25]. Psychologically, *in utero* treatment may offer an advantage for prospective parents of an affected fetus, as instead of having the only options of terminating the pregnancy or awaiting the delivery of a severely affected child, there is the prospect of an active fetal treatment and potential cure.

On the other hand, safely trialing and introducing fetal therapy into clinical practice has a number of challenges, not least the emotive environment in which parents and healthcare practitioners will need to make rapid judgements/decisions. Involving patient groups and parents with experience of the condition to be treated can overcome potential ethical hurdles of when to approach trial participation, inclusion/exclusion criteria and primary outcome measures[26,27]. The International Fetal Transplantation and Immunology Society (IFeTIS, https://www.fetaltherapies.org) recently facilitated a panel discussion to define best practice and to consider safety aspects, patient monitoring and managing ethical dilemmas[28]. Safety evaluations must consider the risks of both the mode of administration and the product itself to the fetus and to the mother. Risks include fetal bleeding and fetal loss although large case series of fetal blood transfusion for anemia provide reassurance that minimally invasive, ultrasound-guided injection into the umbilical vein is safe[29]. Fetal interventions may need to be delivered in the first trimester to avoid a competent fetal immune response. Techniques such as intracardiac injection have been evaluated in non-human primates with some success[30]. Adverse events are most likely to occur short-term after fetal therapy and can now be defined and graded using MFAET, the first systematic Maternal and Fetal Adverse Event Terminology[31]. Fetal monitoring remains a challenge, particularly at preterm gestations below 32 weeks where interpretation of cardiotocography (CTG) is compromised by the physiological immaturity of the cardiovascular and neurological systems[32]. At extreme preterm gestations for example <26 weeks, fetal heart rate decelerations are common and not considered to be abnormal.The decision to perform an emergency Caesarean section in the event of a life-threatening fetal complication will need careful discussion between the parents and healthcare providers, taking into consideration the potential quality of life at the gestational age of the intervention.

Noninvasive Prenatal Testing: Fetal Genome, Epigenome and Transcriptome in Maternal Plasma

Noninvasive prenatal testing (NIPT) has come a long way since the first report of the presence of cell-free fetal DNA in maternal plasma or serum in 1997[33]. The technique of massively parallel sequencing has enabled high-throughput deep sequencing and analysis of fetal genome from chromosomal abnormalities to single-gene disorders[34,35]. In the clinical setting, NIPT is currently used to screen for trisomy 21 (detection rate over 98%) and aneuploidies, particularly in ultrasound-screen positive pregnancies such as those with increased nuchal translucency. The implementation of NIPT for chromosomal aneuploidies has led to a dramatic reduction in the number of invasive tests performed for prenatal diagnosis. NIPT is now being applied clinically to diagnose fetal single gene disorders such as fetal FGFR mutations associated with skeletal dysplasias and craniosynostosis, ß-thalassaemia and for fetal RhD genotype in RhD negative

pregnant persons[3,36]. NIPT for multiple Mendelian monogenic disorders has been reported with high accuracy[4]. However, it must be noted that the limited amount of fetal DNA during early pregnancy and high maternal BMI may give rise to false negative result. Thus, it is vital to determine the fetal DNA fraction accurately. In addition to DNA sequence variations, epigenetic changes are also implicated in gene regulation and fetal development, DNA methylation being one of the best-known epigenetic modifications. By identifying and studying placenta-specific methylation markers serially, any methylation status change may help to monitor for obstetric disease such as intrauterine growth restriction[37].

As well as DNA, fetal RNAs are also released into the mother's bloodstream: the measurement of these may reflect changes in the fetoplacental transcriptome and offer an insight into alterations in placental function, which might then be used to detect fetal hypoxia and to predict obstetric disease such as pre-eclampsia [38].

With further advances in whole-genome haplotype-phasing techniques and newer sequencing platforms that enable deeper sequencing and fewer sequencing errors, it is envisioned that fetal genomic, transcriptomic, and methylomic analysis could become part of routine prenatal care in the future, providing more clinically meaningful data on disease severity, prevalence, and prognosis. Nonetheless, for many conditions, expectant parents should be clearly informed that NIPT when used as a screening tool is not diagnostic, and that any high-risk result should be followed up with confirmatory invasive testing such as amniocentesis or chorionic villus sampling, supported by the appropriate counseling.

While the existence of circulating tumour DNA (ctDNA) had been known for more than two decades, ctDNA constitutes only a small proportion of total plasma DNA and hence highly sensitive and specific assays are required for meaningful analysis. The development of molecular methods that permit the accurate detection of circulating fetal DNA and success of its clinical application have inspired scientists-physicians in prenatal testing as well as adult cancer scientists-physicians to adopt and develop liquid diagnosis for cancer screening. A proof-of-principle study for a virus-associated cancer showed that plasma Epstein–Barr virus (EBV) DNA-based screening of 20,174 men provides an 11% positive predictive value (PPV) for nasopharyngeal carcinoma[39,40]. In a recent prospective study involving 10,006 asymptomatic women, the use of circulating DNA and protein markers for multiple cancer screening resulted in 1.35% positive cancer detection[39].

In Utero Stem Cell Therapy

Since the late 70's, preclinical studies in small and large animal models including non-human primates demonstrated the feasibility of using *in utero* stem cell transplantation (IUT) to correct a wide variety of genetic disorders. Studies of IUT of HSC showed that successful chimerism was achieved, but that gestation days, route of administration, host cell competition, niche immaturity, and fetal and/or maternal immunity limited engraftment, reducing donor-derived hematopoiesis[41] (Figure 1). Nevertheless, other studies also indicated that even low levels of allogeneic hematopoietic chimerism led to postnatal tolerance across major histocompatibility barriers[42,43]. In the mid-90's, attempts to increase levels of HSC engraftment by co-transplanting MSC indicated that MSC engrafted and promoted HSC differentiation after IUT[44]. Successful fetal-derived MSC engraftment in bone after IUT in a patient with severe osteogenesis imperfecta (OI) established the ability of MSC to integrate and differentiate into bone[44], opening the doors to new approaches to treat OI. However, the use of MSC in utero in general has provided little evidence of clinically meaningful engraftment and most effects appear to be paracrine.

Human IUT has been performed on 46 fetal recipients for 14 different genetic disorders, including primary immune deficiencies, hemoglobinopathies, inborn errors of metabolism, lysosomal storage diseases, and hemophilia A (reviewed in[45,46]) (Figure 1).. Unfortunately, these studies demonstrated that IUT, , was not able to establish clinically relevant levels of HSC engraftment except in primary immune deficiencies. This is due to the fact that these attempts have used access methods during early gestation that were inefficient

(such as intra-peritoneal injection), or they were performed too late in gestation for likely success (due to maturation of the fetal immune system; see “Immunological Considerations” below)[47].

Current developments in this field have focused on identifying the optimal modality of administration of IUT (such as intravenous injection into the vitelline or umbilical veins[48]) and more importantly on new strategies for providing donor cells a competitive advantage over the fetal recipient’s endogenous stem cells. The latter has been identified as the major barrier to achieving therapeutic levels of engraftment following IUT. HSC derived from adult bone marrow have been used extensively in experimental IUT, but engraftment levels have been subtherapeutic due to the fact that they are “outcompeted” for space in the fetal haematopoietic stem cell niche by host equivalents. Attempts to modulate HSC proliferation kinetics prior to transplantation[49], or to ablate the fetal haematopoietic stem cell niche prior to IUT[50], have had limited success. Two strategies have been proposed recently that may have significant translational potential. The first strategy involves the use of fetal donor cells for IUT such as amniotic fluid stem cells (AFSC)[51]. AFSC have been shown to have better engraftment potential compared to bone-marrow derived HSC in murine models of IUT and could be used in the autologous setting (with ex-vivo gene engineering)[51], but significant challenges remain regarding their isolation from human amniotic fluid, as well as the requirement for expansion prior to IUT. The second strategy involves therapeutic cell engineering of bone marrow-derived HSC using growth factor/small molecule drug-loaded nanoparticles. Release of these growth factors/small molecule drugs from nanoparticles attached on the cell surface of donor HSC allows prolonged and targeted modulation of donor HSC proliferation kinetics in vivo (via a “pseudo-autocrine” mechanism and may ameliorate the competitive advantage the fetal host equivalents. A recent proof of principle study has demonstrated that remarkable levels of long-term engraftment post experimental IUT can be achieved by “decorating” hematopoietic cells with GSK3 inhibitor-loaded nanoparticles with the hope of allowing future single-step prenatal treatment of congenital hematological and other inherited disorders[52].

There are currently two ongoing IUT clinical trials using HSC (ClinicalTrials.org ID: NCT02986698) or MSC (ClinicalTrials.gov ID: NCT03706482) to treat alpha-thalassemia major (a-thal) and severe OI, respectively [28]. Without intervention, alpha-thalassemia is fatal *in utero*, and the phase I clinical trial investigates the safety, feasibility and efficacy of administering one dose of CD34+ enriched HSC derived from maternal bone marrow to 10 fetuses diagnosed with a-thal. The HSC are administered into the umbilical vein between gestational weeks 18–25 at the same time as the intrauterine transfusion of red blood cells indicated for fetal anemia treatment. The Boost Brittle Bones before Birth (BOOSTB4) trial uses first-trimester fetal liver-derived MSC IUT as a therapy for severe forms of OI (type III and severe type IV). Previous case studies suggest that prenatal and postnatal transplantation of fetal MSC is safe and efficient in this patient group [53] (albeit with limited engraftment and a paracrine mechanism of action). In this phase I/II multicenter trial both IUTand transplantation after birth are evaluated, and endpoints include safety, tolerability (mother, fetus and infant) as well as efficacy.

In Utero Gene Therapy

A wide range of genetic disorders would be amenable to treatment by in utero gene therapy (IUGT)[41] (Figure 2). Although results obtained with current postnatal gene therapy trials are encouraging[7,8], barriers to effective therapy include immune responses to the delivery vector or therapeutic protein which pose a notable problem in those cases where repeated therapy might be required, and initiation of treatment after disease onset in fetal life. Furthermore, the limited access to relevant numbers of fetal stem cells mean that autologous ex vivo therapy is likely to be of limited use in those conditions where a fetal treatment is desired. Delivery of an in vivo platform to the developing fetus poses additional requirements of safe fetal access, appropriate biodistribution and targeted delivery of the therapeutic platform. Specific to integrating platforms (predominantly lentiviruses) there are very real concerns regarding insertional mutagenesis[54]. Furthermore, off-target effects of base-editing in vivo will require ongoing work assuring the specificity of CRISPR-Cas9 platforms[55-57], or by employing alternative means of editing such as adenine-base editing which inherently has a reduced rate of mutagenesis[58,59].

Specifically regarding in vivo approaches, a number of preclinical models have been published utilising a variety of different approaches. Earlier prominent studies have explored the use of in utero gene addition. For example, in both murine and non-human primate models, prenatal delivery of human factor IX (hFIX) or hFX using direct administration of AAV vectors gave long-term curative plasma concentrations of hFIX or hFX, immune tolerance, and no evidence of clinical toxicity[60]. Similarly, a lentiviral approach has also been used in a humanised mouse model of beta-thalassaemia. Here, the heterozygous mice, injected in utero with beta-globin expressing lentivirus (GLOBE) demonstrated near normal outcomes measured by postnatal cardiac imaging, spleen size and haemoglobin levels[54].

More recently, gene editing in vivo has been a focus of a number of studies. Bose et al. have published an elegant set of experiments demonstrating phenotypic rescue of a mouse model of Hurler syndrome (mucopolysaccharidosis type I) by systemic prenatal delivery of a paired AAV2/9 vector bearing a CRISPR-Cas9 construct which successfully base-edited the causative mutation[59]. A similar AAV-based gene editing approach was used for IUGT of hereditary tyrosinaemia type I[61]. The utility of non-viral (nanoparticle-based) platforms for delivery of IUGT has also been demonstrated. A seminal study by Ricciardi et al. reported delivery of a mutation-specific peptide-nucleic acid (PNA) in a poly lactic-co-glycolic acid (PLGA) nanoparticle to a mouse model of beta-thalassaemia, and demonstrated phenotypic near-normalisation[16].

Preemptive fetal treatment by IUGT may one day allow disease correction before irreversible tissue damage and clinical manifestations occur in a broad spectrum of conditions[62]. This might include diseases such as neurometabolic disorders, cystic fibrosis, and the hemoglobinopathies (all causing severe perinatal morbidity), as well as those in which early exposure to a missing protein would result in immunological tolerance (e.g. hemophilia A). Gene delivery to treat genetic disorders before birth has been intensely discussed for decades. Reporter gene expression in multiple fetal tissues during gestation, at the levels required for therapeutic efficacy was demonstrated in the late 1990s[63]. Simultaneously the NIH Recombinant DNA Advisory Committee issued a statement on IUGT[64], outlining the additional preclinical work that would be required for safe clinical translation. Ultimately, maternal and fetal safety are priorities[28]; therefore, minimizing the risk of trafficking to the maternal subject and reducing off-target events have to be considered prior to allow for clinical utilisation.

In Utero Tissue-Based Therapy

There are a limited number of fetal structural defects for which anatomical repair in the prenatal period is offered. The best example is myelomeningocele, for which there is level I evidence that the same operation as is done after birth, improves outcome[65]. The wide acceptance of this procedure boosted the interest in this condition, but also defined its limitation. Outcomes could be improved by better surgical technique (e.g. to reduce tethering or prevent inclusion cysts) as well as an earlier intervention. Indeed the effects of fetal surgery seem to be time sensitive with earlier spinal closure associated with superior walking ability [66]. Very early in pregnancy surgical repair is difficult with current instrumentation, so that alternative strategies to cover the lesion have been proposed. These can be simple barriers such as the application of amniotic fluid or placental-derived MSC which a recent systematic review of preclinical application found to be safe and effective[67]. Bioactive approaches to promote neuronal repair or an engineered functional tissue to cover large lesions have also been tested. The latter has included cell-free approaches such as alginate microparticles loaded with basic fibroblast growth factor which successfully induced tissue coverage in a rat model of myelomeningocele[68], or novel bioadhesive which facilitate the delivery and attachment of alginate-polyacrylamide hydrogels to cover the spina bifida defect in a fetal rabbit model [69]. Similarly, collagen scaffolds embedded with VEGF and FGF2 could be used to treat full-thickness replace fetal skin defects in sheep. More complex constructs aimed at repairing and regenerating the neuronal placode have also been designed to include stromal cells for modulation of immune-mediated local damage to the spinal cord, or to include neurons that would be integrated in vivo. Finally, complex tissue engineering tissues such as fetal skin biodegradable collagen scaffolds can be used to treat full-thickness fetal skin defects[70], and facilitate skin closure in fetuses undergoing prenatal repair in small and large animal models. The latter

may become useful to treat other prenatal defects such as gastroschisis which could be treated before birth[71].

Though it is very tempting to address congenital malformations by an anatomical repair, which is often feasible, it is not necessarily the optimal approach. One such example is congenital diaphragmatic hernia (CDH), in which anatomical prenatal repair was quickly abandoned for a procedure that focuses on the actual life-threatening factor in CDH, i.e. pulmonary hypoplasia. Lung growth is stimulated by fetal endoluminal tracheal occlusion, which has now been shown to be beneficial in fetuses with severe pulmonary hypoplasia, either right or left sided [72-74]. Though it improves outcome, results are still suboptimal, so that adjuncts are being considered to further stimulate lung development, e.g. by transplacental drug administration [75]. Along the same lines, adding cell therapy to a surgical intervention with proven benefit has been considered as well (Figure 3). The administration of EV derived from AFSCs have shown the ability to regenerate underdeveloped fetal lungs when delivered in established preclinical animal models[76]. In particular, when delivered intratracheally AFSC EV administration promoted branching morphogenesis and alveolarization, rescued tissue homeostasis, and stimulated epithelial cell and fibroblast differentiation. This is in keeping with previous observations that intra-tracheal injection of AFSC improved pulmonary development combined with FETO in a rabbit model for CDH[77]. Moreover, intravenous infusion of MSC-derived EV in a rodent CDH model attenuated pathological extra-cellular matrix (ECM) and vasculature remodelling in the CDH pulmonary vasculature[78,79]. Similarly, transamniotic delivery of stem cells can positively influence both lung maturation[80,81] and lung vascular development of animal induced to CDH [82,83]. Finally, stem cell technology can also help modelling the disease and finding new therapeutic options. In the context of CDH, *in vitro* models have been recently described by using transgene-free human induced pluripotent stem cells generated from fetuses and infants affected by CDH[84].

Immunological Considerations

IUT was initially proposed as a way to transplant allogeneic stem cells that can engraft and where immune tolerance towards the donor cells in the primitive fetal immune system could be achieved without myeloablation, especially when transplanted during a “window of opportunity” in early gestation – prior to the completion of thymic maturation. However, a number of reports have since demonstrated the presence of mature T-cells (both effector and regulatory), functioning NK-cells and a fully developed antigen-presenting network at 12–14 weeks’ gestation[85-87], which can recognize and reject foreign cells.

Accumulating data proposes that donor derived T-cells can support in achieving clinically significant levels of donor cell engraftment in the fetus. Also, data from studies in animals suggest that low-level donor cell engraftment can induce central fetal tolerance, which can be exploited in booster transplantation with minimal myeloablation post-natally. Additionally, the use of fetal donor cells may offer some benefit, i.e. a fetal-to-fetal approach may result in higher engraftment and lower risk for graft-versus-host disease. The permissive immunological status at this gestation would also likely mean a more permissive environment for the delivery of a transgene product or gene therapy vector with immunologenic potential.

The poor engraftment reported after clinical IUT, except where the fetus has an immunodeficiency, may be due to other reasons aside from immune rejection. These include the lack of an ideal composition of the graft, and the previously unappreciated competitiveness of the fetus’s own cells (see “In Utero Stem/Progenitor Cell Transplantation”)[49,52]. The fetal immunological barrier is evident by the fact that clinical IUT using HSC has been most successful in fetuses affected by immunodeficiency disorders who cannot reject the donor cells[41]. However, using MSC that exhibit a low immunogenic profile, long-term low-level engraftment has been achieved after IUT for OI. The fetal barrier hampers the development of IUT, and new strategies must be developed alongside studies to further understand the fetal immune system[67], including manipulation of peripheral tolerance mechanisms that may extend the immunological window of opportunity[88].

A final immunological consideration in prenatal therapy is that of the maternal immune system. Maternal alloimmunization triggered by the transplanted donor cells with subsequent transfer of alloantibodies to the fetus across the placenta can impact the success of IUT[89]. However, this originates from studies in mice, and it remains to be determined if the reported data is true also in humans – but has led to early translational attempts of allogeneic IUT matching donor cells to the mother. Of note, all successful IUTs in humans occurred in patients with an intact maternal immune system. Pregnancy in itself poses an immunological challenge because a genetically different fetus must be supported throughout gestation. This is recognised to be a delicate balance of mutual tolerance which maintains a healthy pregnancy, with a infection, inflammation or fetal stress likely resulting in a common pathway of pre-term labour, with evidence of fetal alloreactivity to the mother[90]. It is challenging to find a suitable animal model to study maternofetal tolerance aside from non-human primates due to significant differences in placentation, maternal tolerance mechanisms and gestation length[91].

Fetal Cells for Postnatal Treatment

Advancement in early and specific prenatal diagnosis can also help tailor postnatal regenerative medicine treatments[92]. Fetal cells can be derived from several sources; they have the advantage to be broadly multipotent; they can expanded in large number and can be fully reprogrammed to pluripotent stem cells[93]. This approach can be particularly valuable for conditions which do not need necessarily to be treated before birth but require surgical repair at birth. Cardiac malformations are the classical example with engineered constructs prepared using fetal cells harvested by direct biopsy of the fetus[94], or derived from pluripotent cells reprogrammed from amniotic fluid and undergoing functional cardiomyocyte differentiation[95-98]. The latter is particularly exciting because of the potential to generate disease models that could help develop innovative treatments. Beside cardiac tissue, human AFSC could be reprogrammed into vascular endothelial cells without transitioning through a pluripotent state[99], and be engineered *in vitro* into functional heart valves[100]. Moreover, sheep AFSC were seeded in trileaflet heart valves fabricated from biodegradable PGA-P4HB composite matrices and implanted orthotopically into the pulmonary position using an in-utero closed-heart hybrid approach. The engineered valves showed *in vivo* functionality with intact valvular integrity and absence of thrombus formation and could open the way for future clinical applications[101]. Similarly AFSC could be used to engineer skeletal muscles which could help a functional repair of the diaphragm at surgery. AFSC can be induced to skeletal muscle diffentiation through full reprogramming, using MyoD[102], or by defined media[103]. These technologies, particularly when associated with full or partial reprogramming, are going to be particularly useful for development of efficient therapies. AFSC or human muscle progenitors can be engineered in diaphragm-derived ECM and used in small animal models to repair surgically-created diaphragmatic defects, and they have been seen to promote the generation of new blood vessels, boost long-term muscle regeneration, and recover host diaphragmatic function[104,105]. While, cells from different origins such as lung[106], kidney[107], and liver have been isolated from amniotic fluid and could be used for therapy, significant problems remain (including small numbers *in vivo*, difficulties in expansion without loss of “stemness”, and low efficiency of functional differentiation) that limit clinical aplication associated to their low number in vivo, the difficulties to expand them maintaining stemness or to efficiently obtain functional differentiation. Alternatively, AFSC or their vesicles have also shown immunomodulatory potential and have been proposed to rescue clinical features of necrotizing enterocolitis[108], renal failure[109], hepatic failure[110], and lung fibrosis[111].

Artificial Placenta for *Ex-Utero* Application of Therapies to the Fetus

Extreme prematurity remains the leading cause of infant morbidity and mortality worldwide. Advances in neonatal intensive care have increased survival of extremely premature infants but interventions are associated with long-term morbidity[112]. Despite this, the limit of viability of extreme prematurity sits at around 22 weeks due to incomplete development of most major organ systems, especially the lungs[113]. Thus, the

concept of an extra-uterine system to mimic the placental environment to support ongoing fetal growth and development would challenge our current boundaries of survival. This system could support extreme premature infants that are currently being supported in our NICUs and provide an extra-uterine environment for fetuses needing therapeutic intervention.

There are obvious obstacles to overcome in the design of an 'artificial placenta' (AP), such as the need of a circuit (either pumpless arteriovenous or pump-assisted venous-venous) with low resistance and surface area oxygenator[114], whilst maintaining fluid-filled lungs. The provision of sterile fluid submersion and supply of nutrition for fetal development are also important considerations[115]. Some systems are based on a veno-venous extracorporeal membrane oxygenation (VV ECMO) with a tracheal plug whereas others employ a pumpless arterio-venous (AV) circuit and a fluid environment that maintains fetal physiology. A few research groups have been working to develop an AP system to various degrees of success using prematurity animal models[116-118] (Figure 4). Despite this, there are still significant challenges to be overcome, such as the need for swift transfer from the uterus into the system, risks of haemorrhage, infection and the optimal duration of support, before clinical application can become a reality. For eventual clinical use of AP, it should be able to support fetuses with early preterm premature rupture of the membranes (PPROM) or early onset fetal growth restriction by delivering to an *ex-utero* setting. Furthermore, these systems can also provide complete access to the fetal circulation independent of the mother for a sustained period of time, so that earlier repair of severe congenital defects or correction of genetic disorders via gene transfer or autologous stem cell transplantation (with genetically corrected stem cells) can be achieved.

With regard to the concept of using AP to challenge the current threshold of extreme prematurity, it is still at present, an issue full of ethical controversies. Nonetheless, we can foresee that the development and use of AP will be rapid and additional regulation will be needed to ensure that any potential clinical applications are safe and ethical.

Looking Into the Near Future

The current scientific advances and clinical applications of regenerative medicine make it an exciting, rapidly evolving field with great potential for treatment of congenital disease before birth. Increased knowledge on prognosis and long-term outcomes, coupled with the rapid evolution of diagnostic tools and therapeutic instrumentations have made the safe and effective prenatal delivery of regenerative therapies possible. Administration of treatment prior to development of permanent tissue injury/damage, as well as fetal size, immunological immaturity and healing potential are some of the clear advantages of intervention prior to the neonatal period. While major technical and ethical limitations remain, including the risks of treatment complications in both the mother and the fetus, the evolution of platforms such as the artificial placenta could facilitate the in utero application of regenerative therapies. Many breakthroughs are still to be made within the preclinical arena before the full potential of regenerative medicine can be realised for prenatal therapy, but some of these therapeutic platforms will be deployed to the fetus in the very near future.

Contributors

PKHT & PDC designed and orchestrated the review. Sections were written respectively by KKYW & PKHT (Prenatal testing, Artifical Placenta), PDC, PKHT, CG, SL (Introduction, Technological Platforms, Fetal cells), SL, ALD, GA-P, JKYC (IUGT), SL, CG, GA-P (IUT), SL, CG, JC (Immunological Considerations), PDC, JD (IUTT), CG, ALD, SL (Indications and Limitations). PKHT, PDC critically reviewed and redrafted the whole report. All authors approved the final version of the report for publication.

Declaration of interests

We declare no competing interests.

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Figure 1

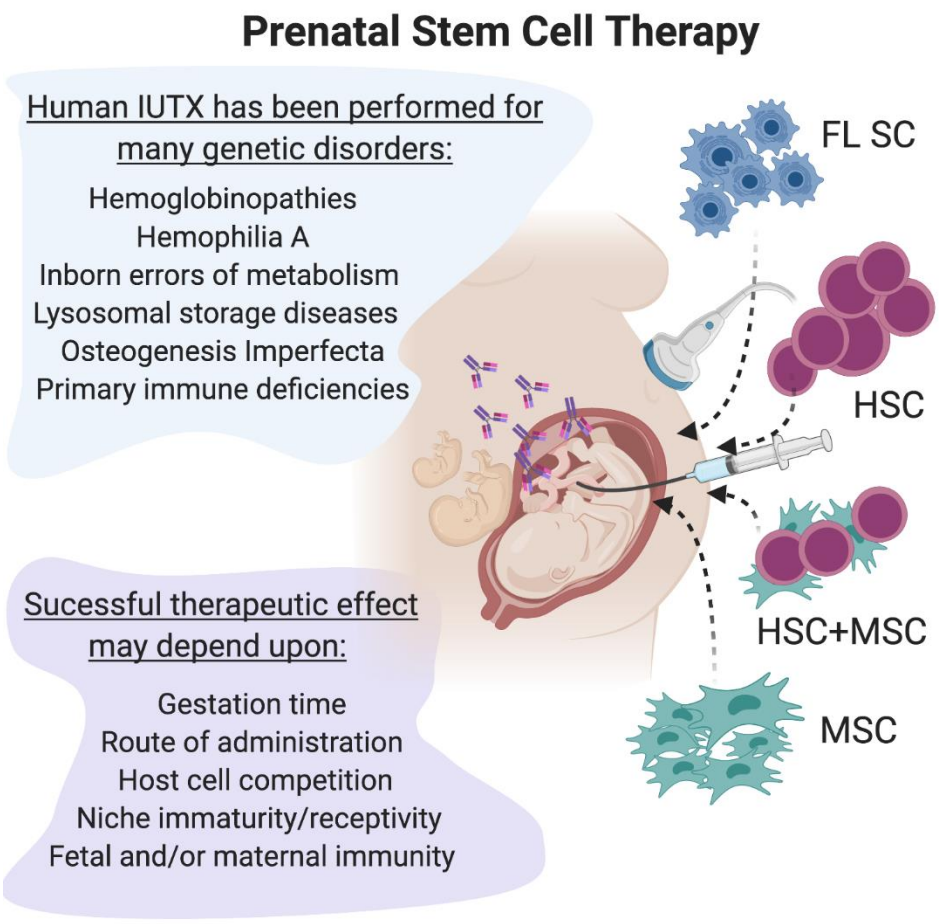

Prenatal Stem Cell Therapy
Human IUTX has been performed for many genetic disorders:
Hemoglobinopathies
Hemophilia A
Inborn errors of metabolism
Lysosomal storage diseases
Osteogenesis Imperfecta
Primary immune deficiencies
Sucessful therapeutic effect may depend upon:
Gestation time
Route of administration
Host cell competition
Niche immaturity/receptivity
Fetal and/or maternal immunity
FL SC
HSC
HSC+MSC
MSC

Figure 2

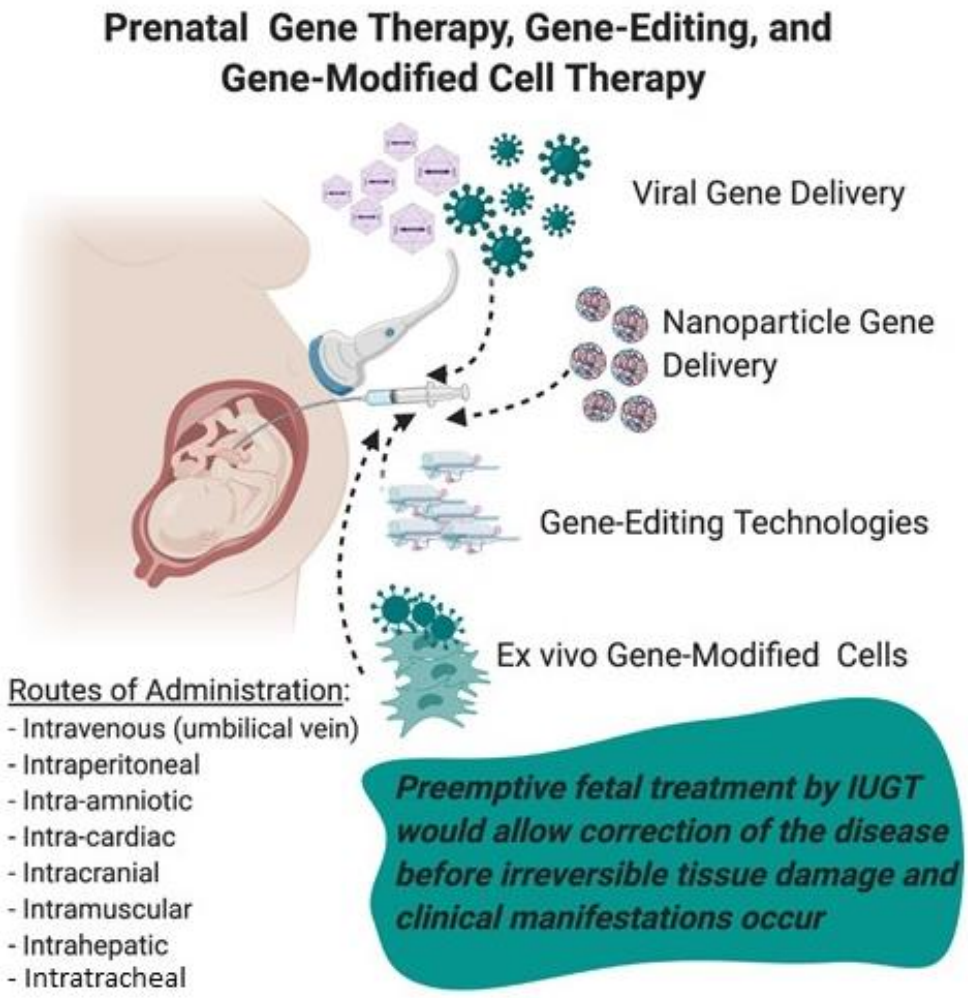

Prenatal Gene Therapy, Gene-Editing, and Gene-Modified Cell Therapy
Viral Gene Delivery
Nanoparticle Gene Delivery
Gene-Editing Technologies
Ex vivo Gene-Modified Cells
Routes of Administration:
- Intravenous (umbilical vein)
- Intraperitoneal
- Intra-amniotic
- Intra-cardiac
- Intracranial
- Intramuscular
- Intrahepatic
- Intratracheal
Preemptive fetal treatment by IUGT would allow correction of the disease before irreversible tissue damage and clinical manifestations occur

Figure 3

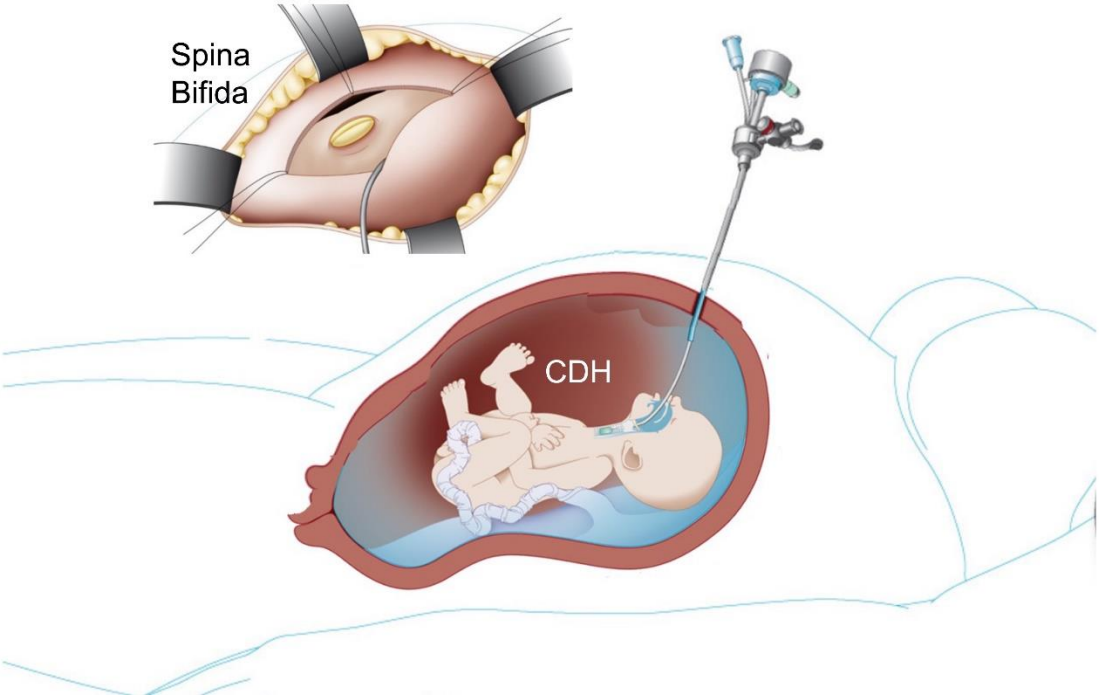
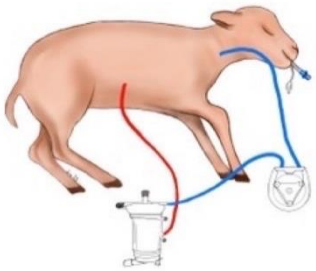
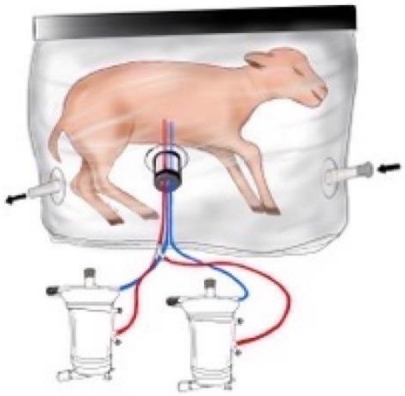


Figure 4

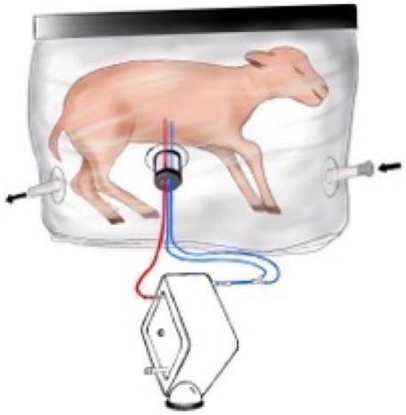
A



B



C



Legend to figures

Figure 1. *In utero* stem cell therapy has been performed in several disorders using different types and sources of cells. Successful therapeutic effect may depend upon the gestational age (depicted as fetuses at different gestational ages), the route of administration, host cell competition, niche receptivity, and fetal and/or maternal immunity, such as the presence of maternal antibodies crossing the placenta (depicted as the antibodies on the mother and fetus). Abbreviations: IUT=*in utero* stem cell transplantation; HSC=hematopoietic stem cell; MSC=mesenchymal stem/stromal cell; FLSC=fetal liver stem cell.

Figure 2. *In utero* gene therapy, gene editing, and gene-modified cell therapies are all viable options to provide a cure for monogenic diseases. Correcting the disease prior to birth has multiple potential advantages over postnatal treatment, including the ability to induce tolerance to foreign cells or proteins, prevent irreversible tissue damage, and deliver to multiple organs by using different routes of administration.

Figure 3. *In utero* tissue therapies. Examples of prenatal diagnoses that would benefit from prenatal repair using regenerative medicine approaches. Congenital diaphragmatic hernia (CDH) associated pulmonary complications can be addressed with fetoscopic endoluminal tracheal occlusion (FETO), but may also benefit from autologous tissue engineered lung or diaphragmatic tissue. Similarly, myelomeningocele, which is repaired in utero, may take advantage of skin or neuronal engineered tissue to cover the defect and/or repair damage to the spinal cord respectively.

Figure 4. Current successful artificial placenta (AP) systems to support fetal growth in animal models. The basic charateristics of artificial placenta comprise of: an extra-corporeal circuit; the maintanenece of fetal circulation; fluid-filled lungs; womb-like environment for organ protection and development. (A) A venous-venous extra-corporeal circuit with a pump [91]. (B&C) These two systems utilise the umbilical vessels for access and have pumpless circuit. They also provide an extra-uterine environment with continuous excange of “amniotic fluid” [92,93].

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