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Cellular Therapy for Fanconi Anemia: The Past, Present, and Future

Margaret L. MacMillan,[1] Mark R. Hughes,[2] Suneet Agarwal,[3] George Q. Daley[3]

Allogeneic hematopoietic cell transplantation (HCT) remains the only proven curative therapy for the hematologic manifestation of Fanconi anemia (FA). Over the past 2 decades, major advances have been made such that transplant outcomes have markedly improved. With the development of in vitro fertilization and preimplantation genetic diagnosis, HLA-matched sibling donor umbilical blood transplantation may be an option for more patients with FA. Recently, the use of pluripotent stem cells has been explored as a novel approach to model the hematopoietic developmental defects in FA, and to provide a potential source of autologous stem cells that can be genetically manipulated and used to generate corrected hematopoietic progenitors.

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INTRODUCTION

Fanconi anemia (FA) is a genetically and phenotypically heterogeneous disorder characterized by congenital malformations, progressive marrow failure, and a marked predisposition to malignancy [1]. Hematologic abnormalities occur in FA patients at a median of 7 years (range: birth to 31 years) [2]. By 40 years of age, the risk of developing bone marrow failure is 90%, advanced myelodysplastic syndrome (MDS). or acute leukemia 33%, and nonhematologic malignancies 28% [3]. To date, allogeneic hematopoietic cell transplantation (HCT) remains the only proven therapy to potentially cure the hematologic abnormalities of FA.

From the [1]The Division of Pediatric Hematology, Oncology, and Blood and Marrow Transplantation, University of Minnesota Blood and Marrow Transplant Program, Minneapolis, Minnesota; [2]The Genesis Genetics Institute, Applied Genomics Technology Center of Michigan, Detroit, Michigan; and [3]Stem Cell Transplantation Program, Division of Pediatric Hematology/Oncology, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Children's Hospital Boston, and Dana Farber Cancer Institute; Division of Hematology, Brigham and Women's Hospital; Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School; Harvard Stem Cell Institute, Boston, Massachusetts.

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Correspondence and reprint requests: Margaret L. MacMillan, MD, MSc, Department of Pediatrics, University of Minnesota, MMC 484, 420 Delaware Street SE, Minneapolis, MN 55455 (e-mail: macmi002@umn.edu).

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THE PAST

HCT for FA patients is uniquely challenging for a number of reasons. FA patients are hypersensitive to DNA alkylating agents [4,5] and irradiation [6], and therefore can only tolerate substantially lower doses of chemotherapy and radiation compared to non-FA patients. In 1984, Gluckman [7] developed the first successful preparative regimen for FA patients consisting of low-dose cyclophosphamide (CY) and single-fraction irradiation. This novel approach led to a marked reduction in regimen-related toxicity (RRT) and enhanced survival, particularly after HLA-matched sibling donor HCT. Nevertheless, high rates of graft failure, acute and chronic graft-versus-host disease (aGVHD, cGVHD), and opportunistic infections still led to significant morbidity and mortality, especially after alternative (ie, an HLA-mismatched related or unrelated) donor HCT.

In 1985, Gluckman et al. [8] reported on 48 FA patients who underwent alternative donor HCT between 1978 and 1994 and reported to the International Bone Marrow Transplant Registry (IBMTR) database. Probabilities of aGVHD and cGVHD were 51% and 46%, respectively. The probability of survival at 2 years was 29%. Notably, graft failure occurred in 24% patients, suggesting the reduced-intensity conditioning was insufficient to eradicate host cells. In 2000, MacMillan et al. [9] observed a particularly high rate of graft failure in patients with T cell mosaicism, suggesting that the presence of diepoxybutane (DEB)-resistant T cells increased the risk of rejection. It was hypothesized that the standard preparative therapy consisting of CY, total-body irradiation (TBI), and antithymocyte globulin (ATG) was insufficient to eradicate DEB-resistant T cells [9]. Historically, high rates

of GVHD and opportunistic infections also contributed to poor survival after alternative donor HCT for FA patients.

THE PRESENT

Many of the initial HCT obstacles have been overcome in the last 2 decades translating into improved survival rates. Most centers have added fludarabine (FLU), an antimetabolite and immunosuppressive agent, to the preparative regimen. As FLU is not a crosslinking agent, it had the potential to enhance immunosuppression and overcome the risk of graft failure without increasing RRT. Additionally, the use of T cell depletion (TCD) has greatly reduced the risk of GVHD. This reduction of GVHD has not only reduced the morbidity and mortality associated with this complication, but potentially the risk for late malignancies that have been linked to the development of GVHD [10,11]. The major remaining HCT goals are to decrease the risks for opportunistic infections and for late complications including malignancies, endocrinopathies, and sterility.

HLA-Identical Sibling Donor HCT

At present, HCT from HLA-identical sibling donors is usually associated with an excellent outcome if performed prior to the development of advanced MDS or acute leukemia, particularly within the first decade of life. Historically, the Gluckman approach of low-dose CY and limited field irradiation (LFI) has been the standard HCT preparative regimen [7]. Factors associated with higher survival after HLA-identical sibling HCT have included age at transplant, pretransplant platelet count, conditioning regimen, and GVHD prophylaxis [8,12].

Over this past decade, a major focus has been the elimination of irradiation from the preparative regimen prior to HLA-identical sibling donor HCT as a means to potentially reduce the risk of late malignancies. Several centers have been successful by either increasing the amount of CY [13,14], adding busulfan (BU) to CY [15,16], or adding FLU to CY [16-19]. In 2008, Pasquini et al. [20], on behalf of the Center for International Blood and Marrow Transplant Research (CIBMTR) compared the early HCT outcomes using nonirradiation containing regimens (n = 71) to outcomes of regimens with irradiation (n = 77) for FA patients transplanted with HLA-matched sibling donors. Hematopoietic recovery, aGVHD, cGVHD, and survival were similar after the 2 types of regimens. With a median follow-up of >5 years, the 5-year probability of survival was 78% after irradiation-containing regimens and 81% after nonirradiationcontaining regimens.

At the University of Minnesota, MacMillan et al. [18] developed a nonirradiation-based regimen using low-dose CY (20 mg/kg), FLU (175 mg/m^2), and ATG (150 mg/kg) [18]. To date, 24 patients with FA have undergone HLA-genotypic identical TCD bone marrow (BM) or umbilical cord blood (UCB) HCT using this regimen. All patients achieved neutrophil engraftment, and none developed aGVHD or cGVHD. Of the 22 FA patients with aplastic anemia prior to HCT, 20 are alive and well. Longer follow-up of these patients and others who received chemotherapy-only-based regimens is necessary to determine whether the elimination of irradiation has reduced late effects particularly the development of malignancies.

Alternative Donor HCT

The majority of FA patients do not have an unaffected (ie, FA negative) HLA-identical sibling donor. Until recently, alternative donor HCT for patients with FA was less successful than after HLA-identical donors because of high rates of graft failure, GVHD, and opportunistic infections. As with HLA-identical sibling HCT, the addition of FLU to the preparative regimen has greatly enhanced engraftment, and TCD has markedly decreased the risk of GVHD after alternative donor HCT. Factors associated with improved survival after alternative donor HCT for FA include younger age, good organ function, negative cytomegalovirus (CMV) serostatus, adequate stem cell dose, and good Karnofsky performance status [9,21,22]. Poorer survival has been associated with the number of malformations (≥3 sites), prior exposure to androgens and absence of FLU in the preparative therapy [9,21,22].

In 2007, Wagner et al. [22], on behalf of the CIBMTR, reported the results of 98 FA patients who underwent unrelated donor BM transplantation between 1990 and 2003. Nearly all recipients (44 of 36) of a FLU-based preparative regimen received T cell-depleted grafts versus half (26 of 52) of the recipients of non-FLU regimens. Probabilities of neutrophil recovery (89% versus 69%) and platelet recovery (74% versus 23%) were higher after FLU-containing regimens than non-FLU containing regimens. Among recipients of non-FLU-containing regimens, engraftment tended to be poorer in those with evidence of DEB T cell mosaicism. Risk of aGVHD was 4 times higher in recipients of unmanipulated grafts compared to recipients of TCD grafts. For recipients of a non-FLU-containing regimen, the probability of grade II-IV aGVHD at day 100 after HCT was 70% for non-TCD BM recipients and 21% for TCD BM recipients. The probability of aGVHD after a FLU-containing regimen was 16%. The use of a FLU-containing regimen was also associated with a higher probability of survival. Three-year adjusted overall survival (OS) rates were 13% versus

52% for recipients of FLU-containing regimens [22]. Other factors associated with higher survival were younger recipient age (<10 years), CMV seronegativity in the recipient, and a history of fewer than 20 blood product transfusions [22].

Similar to HLA-identical sibling HCT, efforts are now underway to decrease or eliminate irradiation in the conditioning regimen for alternative donor HCT. A TBI dose deescalation trial was conducted at the University of Minnesota to determine the lowest dose of TBI required to achieve engraftment in FA patients undergoing alternative donor HCT [23]. All patients received CY 40 mg/kg, FLU 140 mg/m^2, ATG 150 mg/kg, and a single fraction of TBI with CT guided thymic shielding (TS, previously shown to decrease the risk of opportunistic infections, possibly by enhancing immune recovery [24]). TBI 300 cGy with TS along with CY/FLU/ATG was determined to be the lowest possible dose to achieve consistent engraftment, whereas TBI 150 cGy with TS was not adequate. Other centers have eliminated irradiation by increasing the dose of CY. Long-term follow-up studies are needed to determine whether these efforts will lead to a lower rates of late malignancies.

HCT for FA patients with advanced MDS (≥5% blasts) or acute leukemia is more challenging. Although there is some evidence that such patients can tolerate chemotherapy prior to HCT [25], there is no evidence it is necessary. Ideally FA patients are monitored closely after diagnosis such that HCT occurs before the development of advanced MDS or leukemia. All FA patients should undergo a bone marrow aspirate and biopsy with cytogenetic evaluation annually and more frequently if dysplastic changes or cytogenetic abnormalities are noted. FA patients should be referred to a transplant center with an expertise in FA prior to receiving transfusions to help facilitate a timely HCT.

In vitro Fertilization and Preimplantation Genetic Diagnosis

Until recently, bone marrow was the preferred stem cell source as further cells would be available in the event of graft failure. However, as graft failure is no longer a major obstacle, UCB is being utilized more frequently as a stem cell source for patients with FA [26]. Recently, in vitro fertilization and preimplantation genetic diagnosis (IVF-PGD) has been employed as a possible means for parents of an affected FA child to have an unaffected offspring [27]. In addition, HLA screening can also be performed on the embryos to identify potential HLA-matches to an already affected child.

Couples undergo conventional IVF in their home area as though they were infertile and 1 totipotent blastomere is biopsied from each of their embryos on day 3 of development. The embryos are returned to culture and the biopsy is couriered to a molecular genetics reference center where the gene mutation(s) of concern are analyzed and the HLA haplotypes are determined overnight in 1 cell. The following day, embryos found to be genetically unaffected and HLA-identical to the affected child are transferred to the uterus. Scientists at Genesis Genetics first began performing this technology in 1998 for severe combined immunodeficiency syndrome (SCID), and by 2000, PGD for disease and HLA had been expanded successfully to include FA, Diamond Blackfan, Schwachman Diamond, and Wiskott-Aldrich. Interacting with some 230 fertility centers, Genesis Genetics has successfully treated 154 couples for FA, including 83 families for *FANCA*, 41 families for *FANCC*, and 14 for *FANCF*.

Importantly, for IVF-PGD to be a possibility for a couple to have an unaffected child, the specific mutation of the affected child must be known in order to select nonaffected embryos. Chorionic villus sampling or amniocentesis must be performed to confirm that the fetus is healthy and HLA identical to the affected sibling if desired. Prior to HCT, repeat testing is then performed again once the UCB is collected.

It is imperative that couples contemplating IVF-PGD have a team of experts to facilitate the process including a PGD team, a transplant center, genetic counselor, and an obstetrician. As IVF-PGD is a time-consuming process, it can only be as an UCB cell source if the affected sibling is stable enough to allow for sufficient time for the delivery of a healthy HLA-identical sibling donor.

THE FUTURE: INDUCED PLURIPOTENT STEM CELLS

Stem Cell Models to Understand FA Pathophysiology

In the past 15 years, 13 genes have been found to be mutated in FA, and all appear to function in a common pathway regulating DNA repair. However, the mechanism underlying bone marrow failure and hematologic malignancy in patients with these mutations remains largely unexplained. FA exemplifies many of the difficulties with studying human genetic diseases in mouse models or primary patient tissues. Cells from patients with FA display a characteristic hypersensitivity to DNA crosslinking agents such as mitomycin C, which is also evident in mice with deletions in *Fanca*, *Fancc*, *Fancg*, and *Fancd2*. However, single mutant mice do not develop marrow hypoplasia or leukemia [28], but recent data shows that double mutants in *Fancc* and *Fancg* reflect the marrow failure and myelodysplasia seen in human disease, suggesting critical differences between the functions of the mouse and

human Fanconi pathway [29]. Direct analysis of primary hematopoietic cells from FA patients is restricted by their limited numbers and poor proliferation, which also hinders prospects for gene therapy. Pluripotent stem cells offer a novel approach to model the hematopoietic developmental defects in FA, and as importantly, provide a potential source of patient-identical stem cells that can be genetically manipulated and used to generate corrected hematopoietic progenitors.

Human Embryonic Stem (ES) Cell Models of FA

ES cells are derived from blastocyst stage embryos and possess several key attributes that make them amenable to human disease modeling: (1) the ability to divide and replicated endlessly (*self-renewal*); (2) amenability to genetic manipulation (eg, gene knockdown and gene targeting via homologous recombination); (3) and the ability to give rise to any tissue in the body (*pluripotency*). To overcome the limitation of obtaining hematopoietic progenitors from FA patients, Tulpule et al. [30] recently exploited ES cells to model the effects of FA gene insufficiency on hematopoietic development in vitro. The authors depleted *FANCA* and *FANCD2* in human ES cells by stable lentiviral-mediated RNA interference (RNAi), and showed that the ES cells displayed the characteristic DNA repair defects found in FA. *FANCD2*-depleted ES cells showed a significant reduction in hematopoietic gene expression and progenitors upon in vitro hematopoietic differentiation, whereas *FANCA* depleted ES cells showed a similar but less severe phenotype. The hematopoietic deficiencies in the knockdown cell lines could be compensated by FA gene complementation. The results point to the importance of an intact FA pathway in the earliest stages of hematopoietic development, which are recapitulated in human ES directed differentiation protocols, and provide a new experimental platform to explore pathogenesis in FA.

Direct Reprogramming and Induced Pluripotency

ES cell modeling of human genetic diseases by lentiviral knockdown is limited by difficulties recapitulating hypomorphic and qualitatively (rather than quantitatively) defective disease alleles. *Direct reprogramming* technology offers the remarkable opportunity to generate ES-like cells directly from patient tissue samples, which carry the patient's naturally occurring disease alleles on their own genetic background. Following the landmark report by Takahashi and Yamanaka in 2006 [31], several groups showed that ectopic expression of a combination of transcription factors (eg, *OCT4*, *SOX2*, *KLF4*, *MYC*, *NANOG*, and *LIN28*) in adult human somatic cells, followed by culture under ES cell conditions, permitted the identification and isolation of cells that had reverted to an embryonic-like state, so-called *induced pluripotent stem* (iPS) cells [32-34]. Human iPS technology thus permits the creation of highly tractable and versatile cell lines carrying our patients' genetic lesions [35], ushering in new possibilities for disease modeling and autologous cellular therapy.

Modeling FA Using iPS Technology

Several groups have attempted to generate iPS cells from the somatic tissues of FA patients. As recently reported by Raya et al. [36], however, reprogramming primary fibroblasts from FA patients from a variety of complementation groups has proven extremely difficult. Rather, if the FA lesion is complemented in the cells by introduction of a wild-type transgene, reprogramming efficiency is restored and iPS cells carrying the endogenous FA mutation can be generated. Raya et al. [36] found that subsequent knockdown of the correcting transgene led to rapid loss of self-renewal. These results suggest, unexpectedly, that an intact FA pathway is required for induction and maintenance of pluripotency. Why might this be? One possibility is that because cells carrying FA mutations exhibit premature senescence, they are unable to sustain sufficient cell divisions to undergo reprogramming and/or they are unable to induce self-renewal mechanisms while transitioning to a pluripotent state. Moreover, conventional reprogramming strategies such as those employed by Raya et al. [36] depend on numerous retroviral or lentiviral integration events. It is possible that disruption of the DNA repair machinery in FA cells precludes resolution of the DNA breaks associated with viral integration, resulting in cell death. Supporting this possibility is a recent report by Mitalipov and colleagues [37] that using nonintegrating transgenes for reprogramming permits the generation of FA iPS cells without prior correction of the underlying genetic lesion. These iPS cells retained the characteristic FA-associated hypersensitivity to DNA crosslinking agents. However, in contrast to the results of Raya et al. [36], these disease-carrying FA cells can be propagated continuously. Clearly, the FA-associated lesions hinder reprogramming, but further studies will be required to elucidate the precise role of the FA pathway in induction and maintenance of pluripotency.

Like ES cells, human iPS cells have the potential to be differentiated to any cell lineage in the body. Importantly, Raya et al. [36] showed that transgene-corrected FA iPS cells were able to give rise to hematopoietic progenitors by directed differentiation in vitro. Given that hematopoietic stem cell failure is a primary cause of death in FA, these studies set the framework for gene correction in patient-identical pluripotent stem cells, followed by derivation of

progenitor cells to replace those in the affected lineage. Importantly, molecular analysis of blood progenitors derived under current iPS differentiation protocols suggests a primitive or embryonic phenotype, and it is unclear what hematopoietic reconstitution potential these derivatives possess. Generating definitive blood progenitors from human ES and iPS cells that are capable of long-term hematopoietic reconstitution is an area of intense investigation [38].

Therapeutic Potential of iPS Cells for FA and Other BM Failure Syndromes

iPS technology offers a potentially limitless source of autologous, genetically tractable, tissue-specific progenitors, which could circumvent the significant complications of allogeneic HCT including RRT and GVHD. We face several challenges on the road to patient-specific iPS-based cellular therapy. First, the original reprogramming strategies using retroviral transgenes cause insertional mutagenesis, and reexpression of oncogenic factors such as MYC cause tumors in iPS-derived mice [39]. Several strategies to circumvent this problem have been reported, including transient or nonintegrating DNA expression systems, transduction of proteins or RNA, and/or small molecules [40-43]. Second, gene-targeting efficiency is relatively poor in human pluripotent stem cells. Tools such as zinc-finger nucleases and adeno-associated viral vectors to increase homologous recombination efficiency [44,45], and methods to convert human iPS cells to a more mouse-like pluripotent state [46], are being pursued to address this issue. Third, and probably the most significant set of obstacles facing the field, is how to derive definitive tissue progenitors of interest from iPS cells, at a scale suitable for human transplantation, and without partially differentiated or undifferentiated cells in the final transfusion/transplantation product. The genetic and epigenetic integrity of the iPS derivatives will also need to be ensured. Translating human iPS technology to cellular therapy faces formidable challenges, but if the recent history of the reprogramming field is any indication, we may continue to expect the unexpected.

SUMMARY

Successful HCT for FA patients is challenging. Many initial obstacles have been overcome, particularly graft failure with the use of FLU, and GVHD with the use of T cell depletion. The major remaining HCT goals are to decrease the risks for opportunistic infections, and for late complications including malignancies, endocrinopathies, and sterility. With the development of IVF-PGD, there is a greater opportunity for parents to have further unaffected children who may also be an HLA-matched sibling donor for the affected child. Patient-specific iPS-based cellular therapy is being developed and may further reduce the morbidity and mortality of HCT in the near future.

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