Gene Replacement Therapy for Sickle Cell Disease and Other Blood Disorders

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Previous studies have demonstrated that sickle cell disease (SCD) can be corrected in mouse models by transduction of hematopoietic stem cells with lentiviral vectors containing anti-sickling globin genes followed by transplantation of these cells into syngeneic recipients. Although self-inactivating (SIN) lentiviral vectors with or without insulator elements should provide a safe and effective treatment in humans, some concerns about insertional mutagenesis persist. An ideal correction would involve replacement of the sickle globin gene (β^S) with a normal copy of the gene (β^A). We recently derived embryonic stem (ES) cells from a novel knock-in mouse model of SCD and tested a protocol for correcting the sickle mutation by homologous recombination. Animals derived after gene replacement produced high levels of normal human hemoglobin (HbA), and the pathology associated with SCD was corrected. These experiments provided a foundation for similar studies in which our group collaborated with Rudolf Jaenisch's laboratory to correct SCD by gene replacement in iPS (induced pluripotent stem) cells derived by direct reprogramming of sickle skin fibroblasts. Corrected iPS cells were differentiated into hematopoeitic progenitors that were transplanted into irradiated sickle recipients. The transplanted animals produced high levels of normal human HbA, and the pathology of SCD was corrected. These proof-of-principle studies provide a foundation for the development of gene replacement therapy for human patients with SCD and other blood disorders.

Sickle cell disease (SCD) is an autosomal recessive disorder that affects a significant proportion (approximately 1 in 500 individuals) of the African-American population. Hispanic, Arabic, Mediterranean and some Asian populations are also affected. Estimates from gene frequencies worldwide suggest that approximately 250,000 children are born each year with SCD. Over 70,000 individuals in the U.S. suffer from the disease. The molecular basis for sickle cell anemia is an A to T transversion in the 6th codon of the human β-globin gene.[1,2] This simple transversion changes a polar glutamic acid residue to a non-polar valine in the β^S-globin chain on the surface of HbS ($\alpha_2\beta^S_2$) tetramers. The valine creates a hydrophobic projection that fits into a natural hydrophobic pocket formed on Hb tetramers after deoxygenation.[3,4] The interaction of tetramers results in the formation of HbS polymers/fibers that cause a multitude of changes in RBCs.[5-16] On one hand, HbS polymers cause RBCs to become rigid and non-deformable, to adhere to WBCs, endothelial cells and platelets and, consequently, to occlude small capillaries. On the other hand, HbS polymerization results in RBC fragility, hemolysis and consequent tissue damage mediated by cell free hemoglobin and other red cell components. The end result of these pleiotropic effects is severe tissue damage that can result in strokes, splenic infarction, kidney failure, liver and lung disorders, painful crises and other complications.

Mouse Models of Sickle Cell Disease

During the first few months after birth, SCD is normally a relatively benign disorder because human fetal hemoglobin (HbF) has potent anti-sickling properties. HbF, which comprises approximately 70% of total hemoglobin at birth, is gradually replaced by HbS. Rising HbS levels result in the onset of disease between 3 and 6 months of age. We recently produced a knockout/transgenic mouse model that mimics this switch from HbF to HbS. The locus control region (LCR) γ-β^S transgene in these animals was designed to switch hemoglobins after birth[17-19] rather than before birth[20-25] as observed in animals produced with cosmid, BAC (bacterial artificial chromosome) or YAC (yeast artificial chromosome) transgenes. The LCR γ-β^S transgenic animals are relatively healthy at birth and then develop severe anemia when the switch to HbS is completed at approximately 3 weeks of age. More recently, we used the same γ-β^S configuration to produce a knock-in mouse model of SCD.[3] Mouse β-globin genes were replaced with human γ- and β^S-globin genes and mouse α-globin genes were replaced with human α-globin genes. These animals switch human hemoglobins (HbF to HbS) after birth and develop the same severe anemia as the knockout/transgenic mice at approximately 3 weeks of age.

Gene Addition Therapy

Two groups have corrected SCD in mouse models by transduction of hematopoietic stem cells with lentiviral vectors containing anti-sickling globin genes followed by transplantation of these cells into syngeneic recipients.[26,27] Although self-inactivating (SIN) lentiviral vectors with or without insulator elements should provide a safe and effective treatment for hemoglobinopathies,[28] some concerns about insertional mutagenesis persist.[29] If viral integration inhibits a tumor suppressor gene or activates an oncogene, leukemia can result. Although relatively few insertional mutations have been observed in viral gene therapy studies, the SCID gene therapy trials in France demonstrated that leukemic cell clones can arise from insertional activation of LMO-2,[30] and experiments in non-human primates suggested that insertional inactivation of BCL-2A1 can result in acute myeloid leukemia.[31] The risk of mutagenesis is a consequence of random insertion of one or more copies of the viral vector in a large number of cells. If 2 to 3 million $CD34^+$ cells per kilogram of body weight are transduced and transplanted, a 50 kg patient would receive 100 million cells and, potentially, 100 million different viral insertions. Although the number of reported insertional mutations after viral gene therapy has been low, the large number of insertion sites remains a concern.

Gene Replacement Therapy

An approach that bypasses the problem of insertional mutagenesis is replacement of the sickle globin gene (β^S) with a normal copy of the gene (β^A). We produced the knock-in sickle mice described above in order to test gene replacement therapies for the disease.[32] Our original plan was to derive nuclear transfer ES (ntES) cells from skin fibroblasts of these mice, and we initiated a collaboration with Rudolf Jaenisch's laboratory at the Massachusetts Institute of Technology to perform the nuclear transfer. While the sickle ntES cells were being produced, we derived ES cells from blastocysts of the mice, constructed a gene replacement vector, and tested the feasibility of the approach. One β^S allele was replaced with β^A in these knock-in sickle ES cells, and the cells were transplanted into blastocysts. Hematopoietic stem cells (HSC) derived from these cells in vivo produced corrected red blood cells that did not sickle in recipients; consequently, the anemia and organ pathology of the disease was cured.[32]

Gene Replacement in Murine iPS Cells

While sickle ntES were being produced, Yamanaka's group published their landmark results demonstrating that primary skin fibroblasts of mice could be reprogrammed into ES-like cells termed induced pluripotent stem (iPS) cells.[33] Amazingly, the delivery of only four transcription factors (Oct4, Sox2, Klf4 and c-Myc) were required to reprogram skin fibroblasts to pluripotent stem cells that could form most, if not all, cell types. The Jaenisch laboratory and others quickly reproduced and extended these results,[34-38] and we decided to switch our emphasis from ntES cells in favor of correcting the sickle mutation in iPS cells. Tail tip fibroblasts from the knock-in sickle mice were reprogrammed into sickle iPS cells, and the construct described above was used to replace one β^S allele with βA.[39] Successful gene replacement in the iPS cells was important because the experiment demonstrated for the first time that homologous recombination was possible in iPS cells. The corrected iPS cells were differentiated into hematopoietic progenitors in vitro (GEMM is illustrated in **Figure 1;** see Color Figures, page 500), and these cells were transplanted into irradiated sickle recipients. Erythroid cells derived from these progenitors synthesized high levels of human HbA and corrected the hemolytic anemia and organ pathology that characterize SCD in humans.[39]

Gene Replacement in Human iPS Cells?

In the fall of 2007, human iPS cells were derived from primary skin fibroblasts by Yamanaka's group[40] and Thomson's laboratory,[41] and later by Daley's group[42] and Plath's laboratory.[43] These cells are similar to human ES cells and can be differentiated into cells derived from all three germ layers. Based on these initial results, it is reasonable to expect that protocols used to differentiate human ES cells into transplantable progenitors of many cell types will soon be possible.[44] Homologous recombination in human ES cells has been reported;[45] however, pure colonies of genetically modified cells are more difficult to obtain than pure colonies of mouse ES or iPS cells because the cells must be subcultured as clumps. One approach to circumvent this problem is to perform homologous recombination in primary somatic cells before reprogramming into iPS cells. **Figure 2** (see Color Figures, page 500) illustrates the DNA sequence derived from human sickle skin fibroblasts in which one endogenous β^S gene has been replaced with a β^A gene (Wu, Sun, Pawlik and Townes, unpublished). After conversion to iPS cells, corrected hematopoietic progenitors can be derived for transplantation.

Prospects for Gene Replacement Therapy in Humans

As mentioned above, gene replacement therapy avoids the problem of insertional mutagenesis that can result from gene addition therapy. However, to date the production of human iPS cells from skin fibroblasts has been reported only by the insertion of reprogramming factor genes into the genome. Clearly, alternative methods must be developed to transiently deliver the reprogramming factor genes to somatic cells with non-integrating viral vectors or to induce the expression of endogenous reprogramming fac-

tors with growth factors and/or chemical compounds[46] before clinical applications in humans can be tested.

Another challenge is the efficient differentiation of human iPS cells into transplantable hematopoietic stem cells (HSC). Presently, murine iPS cells are induced to form embryoid bodies, and overexpression of HoxB4 in mesodermal cells is required for efficient differentiation into HSC. However, HoxB4-directed differentiation of mesodermal progenitors into HSC is not as effective in human cells; therefore, additional factors will be required for efficient human iPS to HSC differentiation. These factors must also be delivered transiently to cells before clinical trials can be initiated.

Differentiation of iPS cells into HSC will never be 100% efficient; therefore, purification of HSC from undifferentiated iPS cells before transplantation is essential for the prevention of teratomas. Fluorescent activated cell sorting (FACS) for HSC markers and against iPS cell antigens is one option. Clinical grade sorters have recently become available, and these instruments will be important for the successful translation of this technology to humans.

Finally, some myeloablative conditioning of patients may be required to "create space" in the marrow for transplanted cells. Although the corrected HSC are genetically identical to the patient, except for the correction of the sickle mutation, the conditions for efficient engraftment of HSC derived in culture may be slightly different from HSC derived from bone marrow. Therefore, clinical studies will be required to determine whether conditioning is necessary and, if so, to determine the dose and intensity of an optimal regimen.

Summary

The recent correction of SCD in a humanized mouse model by gene replacement therapy in iPS cells[39] and the recent derivation of human iPS cells that can be differentiated into erythroid cells[40-43] suggest that an effective patient-specific cell therapy for this disease can be developed in the next 5 to 7 years. These results also provide a foundation for the development of effective gene replacement therapies for other heritable and acquired diseases.

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