2. **SCIENTIFIC ABSTRACT**

Asklepios BioPharmaceutical Inc. (AskBio) is a biotechnology company engaged in the development and delivery of novel protein and cellular based therapies. AskBio, which is located in Chapel Hill, NC, was formed as a spin-out from the University of North Carolina (UNC) in 2003. The company received exclusive licenses to several AAV-based platform technologies originally developed by Dr. R. Jude Samulski, Director of Gene Therapy at UNC. AskBio is developing a gene therapy for the treatment of hemophilia B.

Hemophilia B is a genetic X-linked bleeding disorder caused by a deficiency in blood clotting factor IX (FIX) activity. FIX is synthesized in the liver and circulates in the blood as a proenzyme. In the coagulation cascade, FIX is activated to FIXa by contact activation via FXIa or as a consequence of vascular wall injury via tissue factor–FVIIa complex. FIXa forms a complex with activated FVIII (FVIIIa), and this complex activates factor X (FX) to FXa, which ultimately leads to the formation of a stable fibrin clot, as depicted in Figure 1 below.

![Figure 1: Role of coagulation factor IX (FIX) in the clotting cascade](image)

**Figure 1:** Role of coagulation factor IX (FIX) in the clotting cascade

FIX is activated to FIXa by FXIa or tissue factor–FVIIa complex (step 1). FIXa forms a complex with FVIIIa (step 2) to activate FX to FXa (step 3), which ultimately leads to the formation of a fibrin clot. TF, tissue factor.

Hemophilia B affects 1 in 25,000 male births with 46% of those affected having severe phenotype. Disease severity correlates directly with the concentration of functional FIX protein in the plasma. Severe disease is characterized as having < 1% of normal plasma levels of FIX (100% = 1 IU activity/ml or approximately 5000 ng/ml plasma). Patients with severe disease...
typically have weekly to bi-weekly soft tissue or intra-articular joint bleeding episodes requiring treatment with either plasma-derived or recombinant FIX products. Chronic intra-articular bleeding leads to arthropathy with eventual joint degeneration, while intracranial and soft tissue bleeds can be life threatening. Patients with mild disease maintain > 5%-30% of normal FIX levels. Patients with mild disease rarely have spontaneous bleeds, however, they are at risk for trauma-induced bleeding (Schulman 2006).

Current treatment for hemophilia B is based on replacement of the deficient factor IX with intravenous injections of recombinant protein IX on a prophylactic basis or as needed to treat bleeding episodes. FIX replacement therapy has been shown to be effective in limiting acute bleeds and reducing mortality and morbidity (Manco-Johnson et al., 1994; Lofqvist et al., 1997). To prevent a bleeding crisis, people with hemophilia and their families are taught to administer factor IX concentrates at home at the first signs of bleeding. People with severe forms of the disease may need ongoing, preventive infusions. Replacement therapy can cost from $60,000 to $150,000 per year (National Hemophilia Foundation 2006).

Although protein replacement therapy has improved the quality of life and prolonged the life expectancy of patients with hemophilia, there are several significant limitations associated with this therapy. Because it is not curative, patients remain at risk for life-threatening bleeding episodes and chronic joint damage. In addition, FIX replacement therapy is constrained by the high cost, limited availability and short half-life (~ 19 hours) of the clotting factors (Petrus et al. 2010; Roth et al. 2001).

Another important limitation of replacement therapy is that up to 5% of patients with hemophilia B develop neutralizing antibodies, clinically referred to as inhibitors, against the administered FIX protein, which may make further therapy ineffective and make bleeding episodes extremely difficult to manage (Franchini and Mannucci 2010). Depending on the specific genetic defect, the administered protein may present neoantigens to the immune system, inducing formation of antibodies, or inhibitors (Ragni 2004).

Hemophilia is an ideal target disease for gene therapy since it is caused by a well-known single gene defect and has a broad therapeutic window (Petrus et al. 2010). Tight regulation of FIX protein production is not required: an increase in plasma clotting factor levels above 1% of normal is sufficient to prevent most of the morbidity associated with the disease, whereas higher than normal concentrations of clotting factors (up to 150%) are not associated with thrombotic side-effects. Given the drawbacks of protein replacement therapy, gene therapy may provide an alternative for patients with hemophilia B. By introducing a functional FIX gene copy into the target hepatic cells, gene therapy may obviate the need for frequent infusions.

In recent years, gene therapies using adeno-associated virus (AAV) vectors have shown potential for the treatment of hemophilia B. Phase 1 clinical trials involving administration of AAV2 vectors through intramuscular (Manno et al. 2003) or hepatic artery injection (Manno et al. 2006) have been carried out in patients with severe hemophilia B and have demonstrated the feasibility of FIX gene transfer. In a Phase 1 ascending dose trial of single stranded AAV2-hFIX administered into the hepatic artery, patients who received doses of $8 \times 10^{10}$ and $4 \times 10^{11}$ vector genomes (vg)/kg of body weight showed no vector-related toxicity. However, these doses failed
to achieve therapeutically relevant FIX levels. Doses of $2 \times 10^{12}$ vg/kg resulted in transient therapeutic circulating FIX activity in 7 patients, followed by immune recognition and CTL-mediated elimination of AAV FIX gene-corrected hepatocytes (Manno et al., 2006). Transient FIX expression at 5–12% of normal levels was detected in one patient receiving the highest dose ($5 \times 10^{12}$ vg/kg), but this was followed by evidence of hepatic parenchymal inflammation, manifested by a transient elevation of liver transaminases and a decrease in FIX levels to less than 1% of normal. Because most humans have previously been exposed to wild-type AAV2 viruses, pre-existing humoral or cellular immunity may make it difficult to avoid safety issues with this capsid, including the transaminitis.

Subsequently, an AAV8 vector, derived from a serotype with reduced pre-existing immunity, has been used for delivery of the FIX transgene (Nathwani et al. 2010). Also, hepatic transduction of AAV8 makes it more efficient at transgene expression compared to AAV2 (Graham et al. 2008). Superior hepatic transduction efficiency of AAV8 also allows for systemic administration of the gene therapy, which is less invasive than intrahepatic infusion.

An AAV8 vector that incorporates a self-complementing AAV genome form and optimized codon usage of human FIX (AAV8.sc-LP1-FIXco) is currently being tested by St. Jude Children’s Research Hospital (SJCRH) in patients with severe hemophilia B (Nathwani et al. 2010). Careful ex vivo examination of T lymphocyte responses from asymptomatic patients receiving intermediate doses of AAV8.sc-LP1-FIXco have demonstrated T cell activation, nonetheless, persistent expression of FIX has been reported in the ongoing clinical trial with vector doses much lower than the dose associated with immune recognition in the previous trial (Nathwani et al. 2010). It should be noted, however, that a higher titer estimate (approximately 7.5-fold) for AAV8.sc-LP1-FIXco was reported based on a dot-blot assay compared to estimates utilizing a QPCR assay (Allay et al. 2011). Thus, comparisons of various AAV.FIX vectors must consider the assay used to determine the doses administered. The undesirable effects of the AAV.FIX vectors (liver inflammation, immune stimulation and vector clearance) are attributed to the AAV capsid rather than to the FIX transgene (Ponder 2006; Nienhuis and High 2011), thus the dose of AAV capsid must also be considered when comparing AAV.FIX vectors.

AskBio is pursuing strategies to reduce the dose of vector required to achieve safe and effective FIX protein levels based upon the results obtained to date with AAV.FIX vectors in humans. One strategy is to improve FIX transgene expression in the liver, and the second strategy is to increase the potency of the FIX protein produced by the transgene. AskBio has identified FIX variants with increased activity relative to wild-type FIX. In addition, they have developed a self-complementary AAV8 vector that allows fewer vector genomes to be administered for a given level of transgene protein expression.

In summary, the product that AskBio plans to study in the clinic, AskBio009, combines the following features designed to minimize the dose of vector needed to produce therapeutically relevant levels of FIX activity:

- Self-complementary genomic form that enhances gene expression
• Optimized FIX codon (FIX.opt) that improves transcription/translation and mRNA stability

• Liver-specific transthyretin (TTR) enhancer-promoter that increases hepatic transgene expression

• Use of an AAV8 capsid that, when compared to AAV2 capsid vectors used in previous hemophilia B trials, has several potentially advantageous properties:
  1. Strong AAV8 tropism for liver results in low relative biodistribution and expression in off-target tissues
  2. Onset of gene expression is immediate due to rapid AAV8 uncoating at the host cell nucleus
  3. Reduced exposure to wtAAV8 as reflected in lower prevalence of anti-AAV8 in humans is potentially associated with a lower risk of antibody-mediated vector neutralization and lower risk of memory lymphocyte reactivation in response to the vector

• Variant FIX transgene (R338L) that produces a FIX protein with higher potency than wild-type factor IX

AskBio009 is an intravenous formulation containing the vector, AAV8.sc-TTR-FIX338Lopt. Preclinical studies conducted by AskBio scientists and other investigators support the potential improvements that were designed into AAV8.sc-TTR-FIX338Lopt. AAV8.sc-TTR-FIX338Lopt has been tested in a mouse model of hemophilia B (FIX-/- mice) and dose-dependent prolonged FIX expression and activity have been demonstrated.

The FIX R338L variant exists as a spontaneous mutation in at least one human family in Padua, Italy (Simioni et al. 2009). The index case was a 23 year old male who was identified due an episode of deep vein thrombosis in the leg. Thrombophilia workup revealed very high levels of FIX activity (776% of normal) with normal concentrations of FIX protein. DNA analysis of the index case and his 11 year old brother, who had elevated FIX activity of 551% of normal, revealed a point mutation in the FIX gene that caused a substitution of leucine for arginine at position 338. The 46 year old mother, with 337% of normal FIX levels, was heterozygous for the variant gene with no history of bleeding or clotting (thrombotic) abnormality. Another brother and the father had normal FIX genes and activity levels.

Several studies conducted by AskBio scientists and other investigators support these strategies, leading to the current design of AAV8.sc-TTR-FIX338Lopt. In mice, self-complementary AAV, optimized FIX codon and the liver-specific promoter, TTR, have been shown to enhance FIX transduction efficiency (Wu et al. 2008). The superiority of AAV8 liver transduction and FIX expression and liver tropism of AAV8 has also been demonstrated in animal models of hemophilia (Wu et al. 2005; Graham et al. 2008).
In *in vitro* assays and following *in vivo* delivery of adenoviral vectors in hemophilia B mice and dogs, single amino acid substitutions at arginine 338 in the catalytic domain of the FIX protein have been shown to increase clotting activity of FIX relative to wild-type (Chang *et al.* 1998; Brunetti-Pierri *et al.* 2009; Finn *et al.* 2009). The substitution of arginine to alanine at amino acid 338 (R338A.FIX) in the heavy chain of FIX results in a 3-fold increase in the kinetics ($k_{\text{cat}}$) of factor X activation to factor Xa generation (Step 3, Figure 1) (Brunelli-Pierri *et al.* 2009). AskBio has shown improvements in FIX specific activity as high as 10 times wild-type FIX with an R338L variant that substitutes arginine with leucine (Monahan *et al.* 2011).

Studies conducted by AskBio in hemophilia B (FIX-/-) mice have shown that the AAV8.sc-TTR-FIXR338Lopt vector achieves dose-dependent and sustained levels of human FIX expression (> 100%) for 42 weeks without evidence of toxicity or inhibitor antibody formation. The safety and biodistribution of AskBio009 is being tested in an ongoing study in normal mice receiving a single intravenous dose of AskBio009.

The planned first-in-humans trial of AskBio009 is a Phase 1 single ascending dose study in up to 15 adult subjects with severe hemophilia B and no evidence of FIX inhibitors. The primary endpoint will be safety, based on adverse events and standard laboratory safety evaluations. The secondary endpoints will include biodistribution and the pharmacodynamic endpoints of blood plasma factor IX protein concentration and activity. Clinically apparent bleeding episodes and any use of factor IX protein concentrate replacement therapy will be recorded. Evidence of humoral or cell mediated immune (CMI) responses to the vector and therapeutic gene product will be monitored.

In the absence of dose limiting toxicities (DLTs), it is planned that 2 subjects per dose level will be enrolled in the Phase 1 study until both subjects in the cohort demonstrate FIX activity in blood of >10% and ≤ 40% normal values at 42 days post dose or until evidence of antibody or CMI responses are observed. At least four additional subjects will then be enrolled in that highest cohort and followed for ≥ 24 weeks before final Data Safety Monitoring Board (DSMB) review and a recommendation to proceed to a Phase 2/3 study.