Chapter 1 : Project Description

1.1 Specific Aims

Hemophilia B is a bleeding disorder that affects approximately 70,000 individuals worldwide.[1] Hemophilia B is caused by a deficiency in or dysfunction of factor IX (FIX), a complex plasma glycoprotein required for the formation and maintenance of blood clots. Complications from hemophilia include bleeding into joints, swelling of joints, hemorrhage in the gastrointestinal and urinary tracts, surgical complications, and prolonged bleeding of cuts. Treatment for hemophilia B involves intra-venous infusion of exogenous FIX, either purified from pools of human plasma (pd-FIX) or recombinant FIX (rFIX) made in Chinese hamster ovary (CHO) cells. Safety issues with pd-FIX products coupled with the high cost and low availability of these current FIX therapies make treatment for most of the world's hemophiliacs infeasible. Some 80% of hemophiliacs remain untreated[1] because of the high cost of clotting factor, an average of $130,000 per patient per year of life,[2] with even fewer receiving the most ideal form of treatment, a prophylactic regimen with rFIX. Current FIX production methods are insufficient and as a result, a cheaper, more abundant source of FIX could drastically improve the life prospects of the world's hemophiliac population.

The long-term goal of this research is to make a recombinant FIX product in the milk of transgenic pigs (tg-FIX) at a low cost and high abundance that can be used for the prophylactic treatment of hemophilia B patients. The objective of *this* project was to determine both the structure and function of tg-FIX produced in the transgenic pig bioreactor at 1-3 mg/ml. In contrast to other transgenic livestock, the transgenic pig has been shown to have the capacity to produce active FIX at a level of 100-200 μg/ml without obvious rate limitations in effecting the post-translational modifications (PTMs) required for FIX activity.[3] It was hypothesized that production of recombinant FIX at 1-3 mg/ml in the porcine mammary epithelium would result in unique FIX isoforms with respect to post-translational modification structure, and thus yield unique functional properties. The *rationale* for this project was that in order to progress to human clinical trials, the structural isoforms that are produced must be fully characterized. This research

was a collaborative effort with ProGenetics LLC (contributing transgenic pig milk) and Professor Paul Monahan at the University of North Carolina School of Medicine (preclinical evaluation in hemophilia B mouse models).

The specific aims of this project were the following:

Specific aim # 1. ***Determine the PTM structure of tg-FIX when expressed at high levels in the transgenic pig to see if unique isoforms are being produced.*** The working hypothesis for this aim, based on preliminary data, was that unique tg-FIX structures are produced as the result of rate limitations and/or species-specific differences in post-translational processing enzymes in the transgenic pig bioreactor. To test this hypothesis, a process for purifying and fractionating tg-FIX from pig milk was developed and the resulting purified tg-FIX was then subjected to amino acid, propeptide processing, and phosphorylation analysis.

Specific aim # 2. ***Characterize the in vitro and in vivo function of tg-FIX to determine if these structures result in unique or advantageous properties.*** The working hypothesis for this aim was that tg-FIX produced at high levels in the transgenic pig has different properties with respect to enzymatic activity and pharmacokinetics. The approach used to evaluate this hypothesis was the isolation of tg-FIX isoforms followed by testing *in vitro* clotting activity, *in vitro* activation, and injection into animal models to determine *in vivo* activity and pharmacokinetics.

This research is necessary for evaluating the potential of the porcine mammary epithelium as an expression apparatus for large quantities of biologically active FIX, and it represents a significant step in the process of reaching human clinical trials. The results from this research indicate that the transgenic pig has the potential to generate active FIX with potentially advantageous pharmacokinetic properties. By using the proficient transgenic pig bioreactor and taking advantage of the pig's demonstrated capabilities for effecting that the necessary PTMs required for FIX function, we expect to make significant progress in addressing the needs of the world's hemophiliac population.

1.2 Significance of Research

If untreated, hemophilia B is a debilitating and eventually fatal disease. Current treatment of patients with exogenous FIX greatly improves the life prospects for hemophiliacs, especially on prophylactic regimens where bleeds are prevented, not just treated, with weekly injections of clotting factor. On prophylaxis, the length and quality of life are almost identical to the unaffected individual.[4] However, the needs of the world's hemophiliac population are not being met by the current methods of FIX production. Both pd-FIX and rFIX produced in CHO cells are prohibitively expensive, with the average cost per dose for a 70 kg (~150 lb) person being over $7,000 for pd-FIX and over $8,000 for rFIX.[5] Thus prophylaxis is impractical in most developed countries, and impossible in lesser developed countries. In contrast to mammalian cell culture, where only micrograms per liter are expressed, the transgenic pig bioreactor is producing recombinant FIX at *gram/liter/hour* rates.[6] However, before the potential of the transgenic animal bioreactor can be realized and clinical trials initiated, both the structure and function of tg-FIX must be characterized in detail. *The reported research is significant, therefore, because we have determined the structure of tg-FIX with respect to multiple post-translational modifications when produced at high expression levels in the milk of transgenic pigs and the subsequent in vitro and in vivo functions.* The determined structure/function relationships may lead to future "designer" FIX molecules with improved function. The abundance of the transgenic pig bioreactor coupled with the low cost of transgenic animal production should significantly reduce the expense of FIX clotting factor from the current $1.18 / IU[7] for CHO cell rFIX. In addition, because the transgenic animal bioreactor is so proficient, other delivery mechanisms, including oral administration, of FIX can now be investigated in animal models. These achievements will revolutionize how hemophilia B is treated in infants, children, and in developing countries. Aside from the production of FIX, this research also represents a further characterization of the transgenic pig bioreactor which will help determine its potential use for production of other recombinant proteins.

[1] Prepared by the World Federation of Hemophilia (WFH). 1998. Facts and figures monograph series: Key issues in hemophilia treatment. WFH, Montreal.

[2] Globe DR, Curtis RG, Koerper MA. 2004. Utilization of care in hemophilia: a resource-based method for cost analysis from the Haemophilia Utilization Group Study (HUGS). *Haemophilia* 10(Suppl. 1): 63-70.

[3] Van Cott KE, Butler SP, Russell CG, Subramanian A, Lubon H, Gwazdauskas FC, Knight J, Drohan WN, Velander WH. 1999. Transgenic pigs and bioreactors: a comparison of gamma-carboxylation of glutamic acid in recombinant human protein C and factor IX by the mammary gland. *Genet Anal-Biomol E* 15: 155-160.

[4] Jones PJ. 2002. *Living with Haemophilia*. Oxford University Press, New York.

[5] Shord SS, Lindley CM. 2000. Coagulation products and their uses. *Am J Health-Syst Ph* 57: 1403-1417.

[6] Lindsay M, Gil GC, Cadiz A, Velander WH, Zhang C, Van Cott KE. 2004. Purification of recombinant DNA-derived factor IX produced in transgenic pig milk and fractionation of active and inactive subpopulations. *J Chromatogr A* 1026: 149-157.

[7] *Drug Topics Red Book*. 2003. Medical Economics, Montvale, NJ.