Children's Hospital of Philadelphia

Annual Progress Report: 2007 Nonformula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Nonformula Grant Overview

The Children's Hospital of Philadelphia received $2,110,362 in nonformula funds for the grant award period June 1, 2008 through May 31, 2012. Accomplishments for the reporting period are described below.

Research Project: Project Title and Purpose

Prenatal Stem Cell Therapy for Sickle Cell Disease - We are proposing a new and novel approach to cure Sickle Cell Disease. In utero hematopoietic stem cell transplantation is the transplantation of blood forming stem cells into the fetus. Because of unique aspects of fetal biology, these cells can engraft and produce a fraction of the blood cells in the recipient. While this fraction is too small to treat Sickle Cell Disease, it results in permanent immunologic tolerance for the specific donor. This tolerance allows a non-toxic bone marrow transplant from the same donor to be performed after birth, resulting in high enough levels of donor cells to cure Sickle Cell Disease. If the aims of this proposal are achieved, it will lead to clinical trials that may change the way Sickle Cell Disease is currently treated, and will provide a non-toxic therapy that will prevent children from ever experiencing the manifestations of this disease.

Anticipated Duration of Project

6/1/2008 - 5/31/2012

Project Overview

In utero hematopoietic stem cell transplantation (IUHCT) is a method of achieving allogeneic mixed hematopoietic chimerism with associated donor specific tolerance without myeloablation or immunosuppression. Individuals made tolerant by IUHCT can undergo a minimal conditioning postnatal hematopoietic stem cell transplant (HSCT) from the same donor without toxicity and improvement of donor cell chimerism to levels that are potentially therapeutic for Sickle Cell Disease (SCD). The broad objective of this proposal is to develop the strategy of IUHCT combined with minimal conditioning postnatal HSCT to cure patients that are prenatally diagnosed with Sickle Cell Disease. The Specific Aims of this proposal are:
Specific Aim #1. To perform pre-clinical studies in the canine model designed to optimize the safety of the IUHCT followed by postnatal minimal conditioning HSCT strategy. Further studies will be performed in the established pre-clinical canine model to confirm the optimal T-cell dose

for facilitation of engraftment with minimal risk of GVHD. These studies are a priority and will be completed prior to initiation of the Phase I clinical trial.
Specific Aim #2. To experimentally test novel strategies to enhance engraftment and donor specific tolerance after IUHCT. We have developed a protocol in the canine model that can achieve levels of donor cell chimerism that would be anticipated to be curative for SCD patients. However, additional strategies designed to improve the consistency of tolerance induction that are translatable to clinical application will be evaluated.
Specific Aim #3. To establish a model of community based participatory research inclusive of the SCD and African American community and to lay the groundwork for a future clinical trial of IUHCT for treatment of SCD. This aim will be coordinated through the Sickle Cell Center at the Children's Hospital of Philadelphia, Cheyney University, and the Philadelphia/Delaware Valley Chapter of the Sickle Cell Disease Association of America. It will include: 1) a social awareness program designed for education of African American couples and others at risk for an affected child with SCD for assessment of their views and opinions regarding a trial of this therapy and 2) research training of undergraduate students and mentoring of junior faculty from Cheyney University through direct involvement in the scientific and social components of this proposal.

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Expected Research Outcomes and Benefits

Sickle Cell Disease (SCD) is a major cause of morbidity, mortality, shortened life-span, and health care costs among African Americans. It is the most common genetic disorder among African Americans with 1 in 8 African Americans carrying a mutation and over 50,000 homozygous, affected individuals in the US alone. On a worldwide basis, hemoglobinopathies, i.e., SCD and Thalassemia are among the most common of all genetic disorders causing untold human suffering and cost to society. At present there is no curative therapy for SCD that can be widely applied. While hematopoietic stem cell transplantation (HSCT) is curative, the morbidity and mortality of standard HSCT, except in the few individuals with a sibling matched donor, is prohibitive. The goal of this proposal, if successful, would be the development of a therapy that would result in the complete correction of SCD in prenatally diagnosed patients. Because of

treatment early in life, affected children and adults would never experience the manifestations of the disease and would be expected to have a normal life span without the sequelae of sickling red cells and the associated complications related to microvascular occlusion. Moreover, the treatment would be anticipated to be non-toxic with few short or long-term complications allowing the beneficiaries of the treatment to live normal lives. The approach could be applied to most patients born with SCD in this country and in many areas of the world if population screening for carrier status were instituted and carrier couples underwent early prenatal diagnosis on all pregnancies. Currently, population screening for SCD is incomplete, and early prenatal diagnosis has not been embraced by the African American community due to lack of a therapeutic endpoint. We would predict if this therapy is proven successful, that African American attitudes toward these procedures would change, with institution of widespread prenatal diagnosis for couples at risk. Finally, if successful for SCD, the same approach would be predicted to be applicable to many other disorders, including Thalassemia and many immunodeficiency diseases.

Summary of Research Completed

Research progress is summarized in the order of the original proposed specific aims.

Specific Aim #1. To perform pre-clinical studies in the canine model designed to optimize the safety of the IUHCT followed by postnatal minimal conditioning HSCT strategy.

As justified in the last annual progress report, our results in Specific Aim #1 required the addition of a set of tracking experiments to better understand the reason for the minimal engraftment achieved in the initial canine studies. These studies were completed in the past fiscal year with findings that have fundamentally changed the approach to IUHCT in large animal systems; the researchers anticipate the same for humans.

Experiment #1a.
The researchers extended the tracking studies described last year by adding a 42 day gestational age analysis (to 30, 34, and 38 days) and time points of 48 and 96 hours (to 4 and 24 hours). Each gestational age and time point was performed by intraperitoneal injection of fluorescently labeled cells (PKH-26) under ultrasound guidance. The animals were analyzed by fluorescent stereomicroscopy, flow cytometry, and immunohistochemistry. The researchers had two objectives with the tracking studies which were determination of the hematologic and immunologic ontogeny of the dog fetus in the context of IUHCT and determination of the early homing and engraftment kinetics after IUHCT. The results of the first objective suggested an optimal time for IUHCT of between 34 and 38 days gestation. This was based on predominant hematopoiesis in the fetal liver (with no BM hematopoiesis present and hematopoiesis just beginning in the spleen), and absence of mature thymic architecture or single positive lymphocytes in the thymus or peripheral blood. However, on tracking, the early engraftment results were disappointing and in line with the levels of chimerism after birth with approximately 1% of injected cells engrafting the fetal liver and minimal levels of engraftment in all hematopoietic tissues at all time points. In addition, levels of donor cells circulating in the PB were under 2 to 3% at all time points. This is in contrast to the murine model where approximately 5% of injected donor cells are engrafted in the fetal liver at 4 hours and levels of

donor cells are as high as 70% in the circulation at 4 hours (with intraperitoneal injection of 5 million donor cells). The most striking finding was that donor cells stayed in the peritoneal cavity or leaked out of the fetal liver (after intrahepatic injection) into the peritoneal cavity and were not absorbed into the circulation. The researchers therefore decided to attempt intravascular injection in the canine model by injecting cells into the cardiac chambers under ultrasound guidance. The results were striking as seen in Figure 1. The levels of donor cells in the fetal liver at 24 hours after injection were on average over 30% and at 96 hours maintained at over 10%. Circulating donor cells persisted for the duration of the experiment and fell from 50% at 4 hours to 3 to 5% at 96 hours. These studies strongly supported intravascular injection as a superior mode of injection that based on the researchers' experience in the murine model, should increase levels of donor cell engraftment into a range that will consistently exceed the threshold required for achievement of donor specific tolerance (1 – 2%).

Experiment # 1b

In view of the dramatic improvement in early engraftment achieved by intravascular injection in the tracking studies the researchers have initiated long term survival and engraftment studies using intracardiac injection of donor cells. The researchers anticipate that direct intravascular injections under fetoscopic guidance are completely feasible at 12 – 14 weeks of human gestation but because of the small size of the canine pups, the researchers are forced to use intracardiac injection as a surrogate for direct intravascular injection. The researchers have had some technical issues to overcome with this protocol. The first approach was to maximize the number of cells injected per fetus and to attempt to perform the injections as early as technically possible at around 34 days. Because of limitations of cell number on the maternal BM harvests the researchers could only inject 1 to 3 fetuses/litter with high doses of CD34 enriched donor cells. The researchers used a contrast agent to inject the non-injected fetuses for marking. Unfortunately, the contrast agent was inconsistently observed after birth complicating identification of injected fetuses. Thus, particularly when fewer fetuses were delivered than injected (i.e. procedural loss or normal fetal loss in the canine model) the researchers could not determine with certainty, whether non-chimeric dogs were failures of engraftment, missed injections, non-injections, or engrafted below the sensitivity of VNTR detection (Approx. 2.5%). There were also a number of neonatal or early deaths, some clearly related to the injection (a diaphragmatic hernia for instance) or some that were related to other causes (midgut volvulus in one animal). The summation of these early injections are shown in Table 1.

Thus far, the researchers have performed IUHCT on a total of 6 litters representing 40 total pups, 20 of which underwent intra-cardiac injection with donor cells, and the rest intraperitoneal contrast injection. The gestational ages of the litters have been 33 to 35 days except one animal who was mis-dated at the breeders and was injected at 43 days gestation. Survival for at least one analysis has been 50% (20 pups) and only 3 of these could be excluded by the visualization of contrast after birth. Of interest are the 3 animals that had engraftment prior to boosting that was far above any of the intraperitoneal injected animals (10.9, 24, and 7%). Unfortunately, 2 of these died, one at 13 days of age of an intestinal volvulus, and one of an apparent intestinal enteritis after the busulfan conditioning and boosting with maternal cells. It is of interest that two of these animals were from the litter injected late in gestation. The third injected survivor of this litter was not chimeric pre-boosting by VNTR, but boosted successfully suggesting engraftment below the threshold of detection with associated tolerance. The third animal with

evidence of chimerism prior to boosting was the lone survivor of a litter of three injected at 34 days in a litter where 3/3 fetuses received donor cells. An additional 3 animals have been boosted and are pending analysis at the time of this report, 3 had no detectable chimerism pre-boost and are awaiting boosting, and 2 have just been delivered and are awaiting their first analysis of chimerism. Thus at the present preliminary stage of these studies, the researchers are hopeful but have not definitively demonstrated that intravascular injection can consistently achieve levels of engraftment above the threshold for induction of tolerance. These studies need to be expanded upon and suggest that more consistent delivery of cells at later gestational ages to all members of the litter need to be performed.

Specific Aim #2. To experimentally test novel strategies to enhance engraftment and donor specific tolerance after IUHCT.

Work has been completed by Dr. Edwin Horwitz's group regarding the generation of a transplantable population of canine mesenchymal stromal cells to facilitate the performance of Experiment #2a. Using these cells, the researchers co-transplanted 1 million canine MSC with 1-5 X 10^8 fluorescently labeled CD34 enriched BM cells by intraperitoneal injection, and tracked the cells as described above. No increase in donor cell engraftment, or on levels of circulating donor cells was observed, and the vast majority of both labeled BM cells and MSC stayed in the peritoneal cavity up to 96 hours after injection. Based on these studies, the researchers have not performed additional long-term studies but may again visit this strategy once the intravascular data is clarified.

Experiment 2b Design of an in situ gelation system for sustained cell delivery

Drs. Levy and Chorny have continued to pursue this strategy for maintaining injected cells at their site of delivery. In the past year, optimized pluronic gels and fluorescently labeled BM cells have been tested in vivo in the murine IUHCT model and in neonatal postnatal model. In both systems GFP+ cells suspended in 15% Pluronic F-127 solution at $1x10^6$/cells µl, were driven through a pre-cooled injection needle with a diameter of 100 µm. In the IUHCT model, the cells were injected into the fetal liver at E14 under ultrasound guidance. Volumes of 2 to 5 ul were injected with visible disruption of the liver at volumes greater than 2 ul. While a space could be expanded in the fetal liver, considerable leakage of the injectate into the peritoneal cavity along the needle tract was observed and procedure related mortality approached 100% at volumes greater than 2 ul. While tracking studies suggested that the gel might delay entry into the circulation, the numbers of cells injected were limiting with respect to short, and long term analysis. In the postnatal model, neonates were cooled on ice for hypothermic anesthesia and injected under ultrasound guidance with the same suspension. In this model, the suspension did not make an expanding space in the liver but leaked out along the needle track into the peritoneal cavity, likely due to the increased integrity of the liver tissue and the liquid state of the pluronic gel at hypothermic temperatures. The researchers have concluded from these studies that the murine system is not ideal for testing this strategy and plan to test the approach in dogs once the intravascular injection model is optimized.

Experiment 2c To selectively enhance the competitive capacity of the donor cell population

Over the past year Dr. Brazelton has developed an optimized in vitro culture system by which enriched HSC can be cultured in hypoxic conditions (confirmed by up regulation of hypoxia inducible factors by

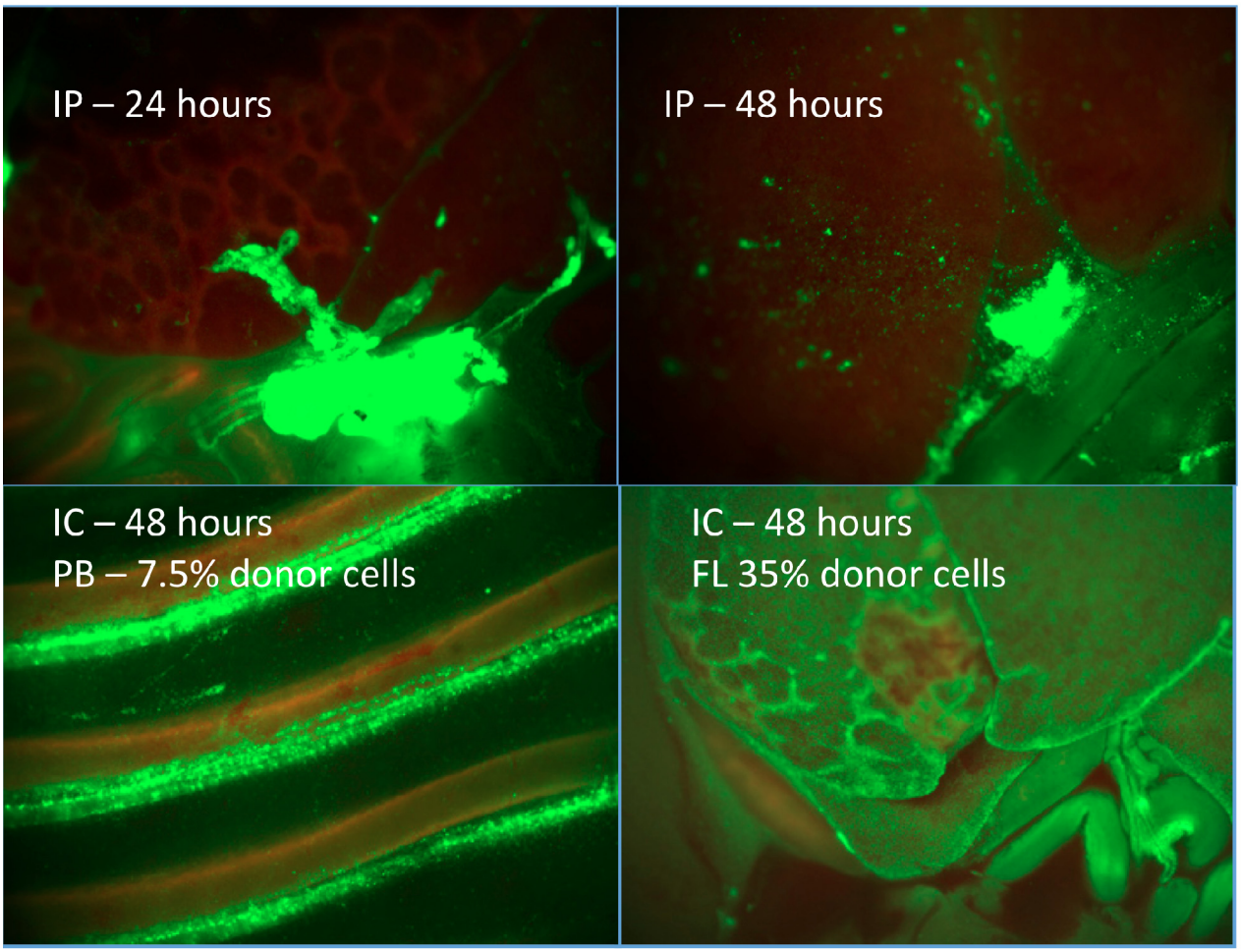
PCR) with factors hypothesized to increase donor cell competitive capacity by a number of established pathways. Dose responses have been determined for all factors to be tested by a number of criteria and experimental dosages for *in vivo* experiments have been determined (see Table 2). To date two factors have been tested: A small molecule inhibitor of GSK-3 (SB216763, which mimics Wnt signaling thereby inhibiting GSK-3), was added to the standard HSC culture. However, no change in short term engraftment was observed. A second factor, Tat-Bcl-xl, was added to the HSC culture but again no change in engraftment was observed. Both factors are being further optimized for concentration (SB216763) or to improve delivery (Tat-Bcl-xl) and the effect of treating hypoxic cultures with these factors is being determined. Thus, at this time the majority of preliminary studies have been completed and testing of the influence of the proposed factors is underway.

Specific Aim #3. To establish a model of community based participatory research inclusive of the SCD and African American community and to lay the groundwork for a future clinical trial of IUHCT for treatment of SCD.

The first year of the Applied Biological Methods course was completed and the second year has been initiated as designed in collaboration with Cheyney University. Each year 8 selected Cheyney undergraduates have participated in an 8 week long, 4 hr/week, hands-on training course at CHOP on basic biological research methods. Dr. Brazelton and laboratory personnel are still holding the 8 week sessions in Dr. Flake's laboratory. Of the 8 participants in the first year, seven wished to participate in the summer internship program and 3 were selected and completed the internship. Of these two, one has applied to medical school and one to Bioscience PhD programs. In addition to the undergraduate summer internship program Dr. Adayiah, an associate professor of Biochemistry at Cheyney completed the Faculty Summer Research Sabbatical at CHOP.

Sub Aim #3a. Social awareness program designed for education of African American couples and others at risk for an effected child with SCD for assessment of their views and opinions regarding a trial of this therapy. This cooperative effort headed by Dr. Ohene Frempong between the CHOP Sickle Cell Center, the Delaware Valley Chapter of the Sickle Cell Disease Association, and Cheyney University was scheduled to begin this summer (June). Questionnaires are being designed and the program organized but it is too early for any results to be available.

Figure 1. Tracking studies comparing IP and IC injection



IP = Intraperitoneal; IC = Intracardiac. Images are taken by stereoscopic Fluorescent microscopy, Percent values obtained by flow cytometry

Table 1. Summary of Intracardiac IUHCT

Mom	# cells/kg per pup	GA	#Injected	# Live Births	Puppy	Gender	Dye	Chimerism	Outcome
Charlie	3.7x10^10	34d	(2/5)	2	Raul	Male	yes	n/a	Adopted
		34d	(2/5)	2	Chase	Female	No	0	boosted
Bella	1.58x10^10	43d	(3/4)	3	Jacob	Male	No	Pre- 0 Post-16.8%	boosted
		43d	(3/4)	3	Alice	Female	No	Pre- 10.9%	died shortly after boosting
		43d	(3/4)	3	DOL 13	Male	No	Pre - 24%	died at DOL13
Noel	6.33x10^10	34d	(1/7)	5	Rudolf	Male	No	Pre-0	Adopted
		34d	(1/7)	5	Donner	Male	No	Pre-0	Adopted
		34d	(1/7)	5	Blizten	Female	YES	**n/a**	Adopted
		34d	(1/7)	5	Dasher	Female	No	Pre-0	For adoption
		34d	(1/7)	5	Comet	Female	No	Pre-0	Adopted
Candy	2.86x10^10	35d	(4/6)	5	Reese	Male	No	Pre-0 Post- pending	boosted
		35d	(4/6)	5	Snickers	Male	No	Pre-0 Post- pending	boosted
		35d	(4/6)	5	Rolo	Male	No	Pre-0	For adoption
		35d	(4/6)	5	Nestle	Female	No	Pre-0 Post-Pending	boosted
		35d	(4/6)	5	Skittles	Female	YES	n/a	Adopted
Mystery	4.9x10^10	34.5d	(3/3)	1	Scooby	Female	No	Pre-6.95 Post-12.5%	boosted
Monica	2.85x10^10	34.6	(4/6)	4	Chandler	Male	YES	n/a	Adopted
		34.6	(4/6)	4	Joey	Male	No	0	boosted
		34.6	(4/6)	4	Phoebe	Female	No	Pre-0 Post – 17%	boosted
		34.6	(4/6)	4	Rachel	Female	No	Pre-0 Post - pending	on protocol
Misty	7.09X10^10	33.8	(4/8)	4	Johnny	Male	No	Pre- 0 Post-pending	on protocol
		33.8	(4/8)	4	Apolo	Male	No	Pre-0 Post-0%	boosted
		33.8	(4/8)	4	Shani	Male	No	Pre-0 Post-pending	on protocol
		33.8	(4/8)	4	Bode	Male	No	Pre-0 Post -0%	boosted
Liberty	3.19x10^11	32	(7/7)	2	Rocky	Male	n/a	n/a	alive, not weaned
		32	(7/7)	2	Betsy	Female	n/a	n/a	alive, not weaned

Table 2. Status of dose response studies and experimental doses for the factors to be tested in Specific Aim 2c.

Agent:	Status	Target genes	Optimal dose by Q-PCR	Viability	Prolif	Apoptosis	Western	In vivo Test Dose(s)
Tat-HoxB4	Construction completed						X	
SU5402	DONE	X	X	X	X	X	20, 40 μM	40 μM
Wnt3a	DONE	c-fos, CL100, myc, cdx4	fos: 100, CL100: 200, myc: 100	200 ng/mL	100 ng/mL	X	X	100 ng/mL
Wnt5a	DONE	Siah2, cdkn1b,c-fos, CL100, myc, cdx4	cdkn1b: 500, fos: 300, myc: 500, CL100: 1	1 μg/mLL	500 ng/mL	X	X	500ng/mL
BIO	DONE	Siah2, cdkn1b,c-fos, CL100, myc, cdx4	Siah2: 2, cdkn1b: 2, myc: 0.5	1.0 μM	1.0 μM	X	X	1.0 μM
IQ-1	MTA pending					X	X	
SCF	DONE	X	X	50 ng/mL	X	50 ng/mL	X	50 ng/mL
SDF-1a	DONE	X	X	30 ng/mL	X	30 ng/mL	X	30 ng/mL
VEGF-A	DONE	flt, kdr mapk1,	flt: 50, Tie2: 300, Mapk1: 300	300 pg/mL	300 pg/mL	X	X	300 pg/mL
Ang-1	DONE	itgb1, cdh2	itgb1: 1, gata2: 500	2 μg/mL	2 μg/mL	X	X	1 μg/mL
PGE2	DONE	runx1, cmyb, gata2	Runx1: 2	2 μM	2 μM	X	X	2 μM
Diprotin A	DONE	X	X	X	X	X	X	5 mM
SB21	DONE	Siah2, cdkn1b,c-fos, CL100, myc, cdx4	cdkn1b: 4, CL100: 4, myc: 1-4			X	X	4 μM

X = Not needed