Brief Synopsis of Research:

Although islet transplantation has shown great progress in the treatment of diabetes mellitus, broader application of the technology is limited by the scarcity of human donor tissue and the massive islet loss in the peri-transplant period. Human embryonic stem cell (h-ESC)-derived islets hold great promise as a robust option for clinical application, providing an unlimited source of beta cells with the ability to recover cell mass over time and more durable response to hypoxia.[1-4] However, these h-ESC-derived islets remain susceptible to primary graft failure (PGF), with death of 60-80% of islets in the first 3-5 days prior to revascularization.[5-7] Early clinical trials with h-ESC islets will take place within an encapsulation device in a subcutaneous location, providing immunoprotective barrier function and protection against malignant transformation. The Tang lab at UCSF has observed that the high density packing required for encapsulation further exacerbates PGF. Optimizing graft survival remains the central challenge to establishing islet transplantation as a feasible therapeutic option. As PGF mainly occurs in the early transplant period prior to revascularization, I hypothesize that nutrient deprivation and hypoxia are two independent primary triggers of PGF. I will test this hypothesis with h-ESC-derived islets in vitro and in a humanized mouse model. Successful completion of this study will help to optimize early h-ESC islet survival, decrease encapsulation device size, and make h-ESC islet transplantation a viable clinical therapeutic option on the human scale.

Narrative:

Reason for Applying for ASTS Scientist Scholarship:

I am applying to the ASTS Scientist Scholarship to provide financial support for a two-year, interdisciplinary research plan which will serve as a foundation for a fellowship and career as a surgeon-scientist in Transplant Surgery. This project, which investigates the effects of nutrient and oxygen supply on h-ESC-derived islet survival, allows me to combine my background in Biomaterials Engineering and interest in transplantation across multiple disciplines important to transplant research: islet transplantation, stem cell therapeutics, bioengineering, and device design. I have been fortunate to develop a multidisciplinary team of leaders in the field which is suited to address all aspects of human embryonic stem cell-derived islet transplantation. I worked closely with my mentor Dr. Peter Stock, current President of the ASTS and Surgical Director of the Kidney and Pancreas Transplant programs at UCSF, to ensure that this grant will yield a productive research training experience. He has been an unparalleled advocate throughout my residency, and will continue to contribute as an advisor on on all surgical matters and mentor on research and professional aspirations. Dr. Qizhi Tang, Director of the Transplantation Research Laboratory at UCSF, has a reputation as a successful and insightful researcher, supportive and experienced mentor, and has a proven record of success in collaboration. Her expertise in the metabolic response of islets and stem cells to ischemic conditions will be essential to the success of this project. Dr. Matthias Hebrok, Director of the Diabetes Center at UCSF and pioneer in embryonic stem cell research, will be an invaluable resource in experimental design and execution related to embryonic stem cell-derived islets. Dr. Tejal Desai, Director of the UCSF Therapeutic Microtechnology and Nanotechnology Laboratory, has extensive expertise in Bioengineering with a specific focus in device design for application in transplantation. I have been fortunate to develop outstanding rapport with each of these sponsors in coordinating this project, and I have especially enjoyed their universal willingness to collaborate. These investigators have helped focus this study to design a feasible research plan, productive from a training perspective and of scientific merit which takes advantage of each of their strengths. Collectively, these sponsors provide an optimal environment for success of this project and in my development as a surgeon-scientist.

Specific Objectives for Duration of Grant:

Islet transplantation has shown great improvement at achieving insulin independence since its inception, with one year insulin independence improving from 10 to 80%, and 5-year insulin independence approaching whole organ pancreas transplant.[8,9] However, donor graft supply is insufficient to meet the large disease burden of patients eligible and wait-listed for transplant, thus limiting broader application. Human embryonic stem cell (h-ESC)-derived islets hold the promise of alleviating donor shortage with a renewable, unlimited source of islets for transplantation. h-ESCs can be driven to differentiate in vitro into insulin-positive pancreatic endoderm cells (referred to as pro-islets), with final maturation occurring in vivo following transplant. While h-ESC-derived islets hold great promise for clinical application, the Tang lab at UCSF has preliminary shown that h-ESC-derived islets remain susceptible to primary graft failure (PGF), with significant loss of pro-islets in the peri-transplant period. Thus, optimizing conditions for h-ESC-derived islet survival remains a key challenge in stem cell-based islet transplantation. Unlike solid organ transplant, where organs are vascularized immediately after transplant, revascularization of transplanted islets takes several days. During this period, pro-islets are dependent on imbibition of nutrients and oxygen from the surrounding interstitium at the transplant site. Until vascular supply is established, islets remain susceptible to ischemia-related stress and inflammation.[10] Pro-inflammatory mediators further damage islets directly or indirectly by recruiting immune cells. **I hypothesize that the nutrient and oxygen deprivation prior to revascularization are major drivers of islet stress and PGF (Fig 1).** Each of these factors alone is sufficient to induce cell death; therefore effective intervention will need to address both nutrient and oxygen deprivation during the peri-transplant period.

Encapsulation presents an additional challenge in overcoming PGF. Optimally differentiated h-ESC islets still retain a low malignant potential; thus, first human trials will require a containment barrier and easy removal of the graft in case of malignant degeneration. While encapsulation may provide some benefit as an immunoprotective barrier via blockade of host inflammatory cells, past studies in the Tang lab using murine and h-ESC-derived islets in mouse models have demonstrated density-dependent islet survival, with high islet loss at increasing density (Fig 2). **To deliver a sufficient dose of islets for human application, h-ESC-derived islets must survive the high density packing conditions within an encapsulation device**. I hypothesize that identification of optimal oxygen conditions within an encapsulation device and slow-release of essential nutrients through bioengineering design modifications will lead to improved h-ESC-derived islet survival at higher densities - making h-ESC-derived islet transplantation a clinical reality.

Aim 1. Determine the optimal nutrient supply for h-ESC-derived islet survival.

Rationale: Experience from clinical islet transplantation shows that at least 5,000 islet equivalents (IEQ) per kilogram are required for insulin independence in humans. If islets are to be transplanted in an iPhone-sized device, with a 400um thickness to the subcutaneous space in humans, islets will require packaging at a density of ~250 islets per uL. Achieving h-ESC-islet transplantation on the human scale will therefore require h-ESC-derived islet survival in high-density conditions. To determine islet tolerance to high-density packing, the Tang lab has cultured mouse islets in vitro at various densities and observed density dependent islet death. At a density of 1 islet/uL, 80% of the islets died within 12 hours of culture (Fig 2). Islet death occurred independent of oxygen concentration or glucose supplementation, which suggests nutrient deprivation as a cause of cell death. Islet cell death can be prevented in vitro following supplementation of glutamine to the culture medium, highlighting glutamine as a limiting nutrient in these cultures (Fig 3). The impact of nutrient deprivation on h-ESC-derived islets is not widely recognized and has not been investigated in depth. Experiments proposed in this aim will identify nutritional substrates most effective at promoting islet survival and function in high-density packing scenarios.

Experimental Design: Studies in Aim 1 will focus on comprehensive dose response and kinetic response profiles of all 20 amino acids to determine the optimal amino acids, concentrations, and durations of exposure for islet survival. Subsequent analysis will further include candidate metabolites as suggested by known biochemical pathways. Candidate nutrients will be added to h-ESC islet culture medium at multiple concentrations. Effects of nutrient supplementation will be assessed by:

a. Islet cell survival using propridium iodide (PI) and fluorescein diacetate (FDA) staining, followed by microscopy and flow cytometry;
b.Beta cell function by glucose stimulated insulin secretion (GSIS), percentage of GFP+ cells using the insulin reporter h-ESC line, and flow cytometric analysis of C-peptide production; and
c. Differentiation state of the cells, which will be assessed by qPCR analysis of insulin, Glut-2, Pdx1, Mafa, NKx6.1, Ngn3, and Sox9 gene expression, in addition to immunofluorescent analysis of insulin and Sox9 protein expression.[10]

Expected Results: For Aim 1, I expect to observe glutamine, in addition to several other candidate amino acids, as limiting nutritional reagents in culture. I expect to see a cluster of amino acids, known to play a central role in aerobic metabolism and the TCA cycle, as effective nutritional supplements to prevent cell death. Alternatively, glutamine is known to confer protective benefit to islets through an antioxidant mechanism.[11,12] If control of oxidative stress is the mechanism of action, I would expect sulfur-containing amino acids other metabolites such as glutathione, taurine, and N-acetylcysteine to confer protective benefit.[13]

Pitfalls and Alternative Strategies: h-ESC- derived islets may behave differently than mouse islets, although preliminary data in the Tang lab shows that glutamine supplementation can rescue h-ESC islets cultured at high density. It is possible that other amino acids do not have the same effect on overall survival. With this in mind, I will evaluate wider concentration ranges and include metabolic intermediates, such as pyruvate, acetyl-CoA, and TCA cycle in my analyses. Additional experiments may be continued with metabolites playing a role in antioxidant pathways.

Aim 2. Investigate the optimal oxygen tension for h-ESC-derived islet adaptation.

Rationale: Oxygen tension in islets changes dramatically during the peri-transplant period. Atmospheric pO_2 at room air is 159 mmHg, while the native islet capillary pO_2 within the pancreas is ~40 mmHg (equivalent to 5.6% O_2), and the pO_2 in the graft one day after transplantation is 5-15mmHg (<2% O_2).[14] During transplantation, h-ESC-derived islets will transit from supraphysiologic pO_2 in culture to a severely hypoxic environment over the course of minutes. Normally, cells can adapt to gradual or transient reduction of pO_2 through induction of factors such as hypoxia-inducible factor-1 (HIF-1) and heme oxygenase 1, which make the cells more resistant to subsequent hypoxia exposure. Human beta cells have been shown to survive and function in settings of restricted blood supply.[15] However, the sudden and persistent drop in pO_2 imposed on islets during transplantation does not allow adequate adaptation, imposing oxidative and ER stress on the islets and leading to cell death. The Tang lab has shown that modified conditioning with 10 cycles of oscillating pO2 between 40mmHg and 159mmHg every 6 minutes completely protected mouse islets against a 3-hr challenge pO2 at 15mmHg (Fig 4). For preconditioning to protect h-ESC-derived islets after transplant, the

protective adaptation needs to be robust and lasting for at least 5 days in hypoxic conditions before revascularization. I hypothesize that h-ESC- derived islet survival is directly proportional to pO_2, and that pre-conditioning of islets to partial pO_2 prior to transplantation will allow cells to induce protective mechanisms for adaptation to low O_2 states.

Experimental Design: Preliminary data from the Tang lab has shown that h-ESC-derived islets can persist longer under 15 mmHg pO_2 challenge than mature islets, but most of the cells died after 24 hr exposure. In the first set of experiments I will first establish the relationship between pO_2, duration of exposure, and h-ESC-derived islets survival, function, and differentiation by culturing pro-islets at variable incubator pO_2 concentrations from 15 mmHg to 159 mmHg. In a second set of experiments, I will determine if cyclic preconditioning can protect h-ESC-derived islets against hypoxic conditions. The experiments will proceed in three phases: 1) Oscillatory preconditioning down to 40mmHg pO2; 2) Hypoxic challenge at 15mmHg for up to 5 days; followed by 3) "Revascularization" for up to 5 more days with rise of pO2 to 40 mmHg. After preconditioning, on days 1, 3, and 5 during hypoxic challenge and revascularization, islets will be assessed using the same assays described above in Aim 1.

Expected Results: I expect an inverse relationship between pO_2 and pro-islet survival. It is possible that this inverse relationship is linear, or that there is an inflection point identifying a critical pO_2 for h-ESC-islet survival. I also expect that islets surviving hypoxic challenge without preconditioning will exhibit a less differentiated phenotype, with low insulin, Glut-2, and Mafa expression as well as increased Sox9 expression. I hypothesize that partial hypoxia pre-conditioning of h-ESC-derived islets will protect them from cell death against severe hypoxic challenge without triggering dedifferentiation. I expect that beta cell function, as identified by GSIS, will be inhibited during hypoxic challenge, as GSIS relies on mitochondrial respiration. However, the islets should remain viable during hypoxia and recover GSIS response with increased pO_2 during the revascularization phase. Further application of this data will allow for optimal preconditioning of h-ESC-derived islets in transplantation.

Pitfalls and Alternative Strategies: The goal of the above experiments is to 1) identify a critical oxygen tension required for h-ESC-derived islet survival, and 2) to identify a preconditioning regimen that will preserve islet viability and function. It is possible that preconditioning may have a transient effect and will not be robust enough to sustain h-ESC-derived islets until revascularization. In this event, I will consider alternate preconditioning regimens and/or pharmaceutical inducers of hypoxia, including diazoxide or carbon monoxide-releasing molecules (CORM).[16,17] Additionally, it is also possible that preconditioning may alter islet gene expression and compromise long-term insulin production. Deletion of VHL, which stabilizes HIF, has been shown to compromise glucose homeostasis in mice at 6-8 weeks following VHL ablation.[18] I will design and analyze our experiments with this potential caveat in mind.

In conclusion, this project offers a novel research approach toward h-ESC-derived islet survival. Conceptually, contribution of nutrient deprivation to islet PGF is not widely recognized or studied. Preliminary data in the Tang lab has not only defined its independent contribution to islet PGF in mouse models, but also suggests approaches to mitigate the impacts of PGF. My approach using hypoxia preconditioning to prevent hypoxia-induced islet death is distinct from other approaches that provide supplemental oxygen to the islets - which may ultimately prevent islet adaptation to hypoxic conditions. Clearer definition of their role in graft survival during high-density conditions will mark a significant advance toward h-ESC-derived islet transplantation as a curative therapy for millions of patients with diabetes.

Future Plans and Goals:
Encapsulation of h-ESC-derived islets will change the localized nutrient and oxygen supply, with high density packing creating potential for compounding PGF. This two-year research project aims to investigate the effects of encapsulation on h-ESC-derived islets, with device design modifications to overcome existing constraints. Completion of Aims 1 and 2 will allow for better characterization of essential nutrient and oxygen supply for h-ESC-derived islets to optimize islet survival in high density scenarios. I expect to complete the above experiments within the first year of research. Using those results, in the second year of research I plan to study h-ESC-derived islets within the setting of a thin-film, nanoporous polychromolactone (PCL) encapsulation device in conjunction with the Desai lab at UCSF. I will characterize the nutrient and oxygen conditions within the encapsulation device, and subsequently modify the PCL polymer to serve as a nutrient delivery vehicle to promote h-ESC-derived islet survival in the peri-transplant period.

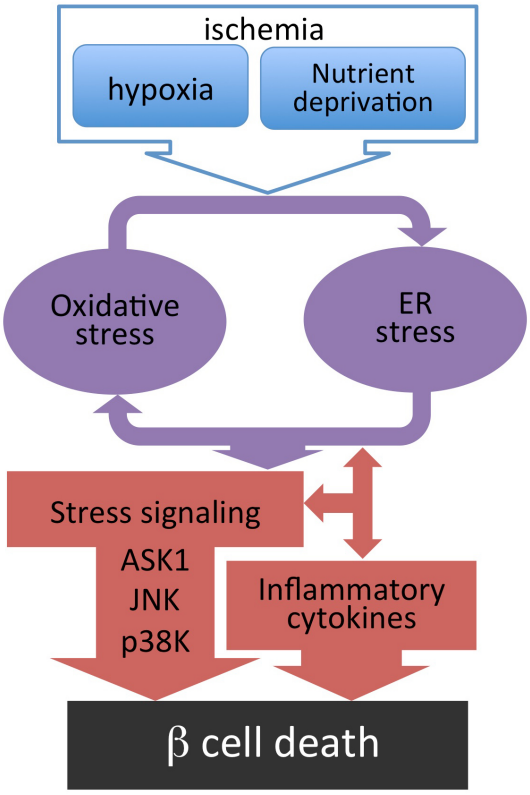

Fig 1. A model of ischemia, stress responses, and stress signaling in islet PGF.

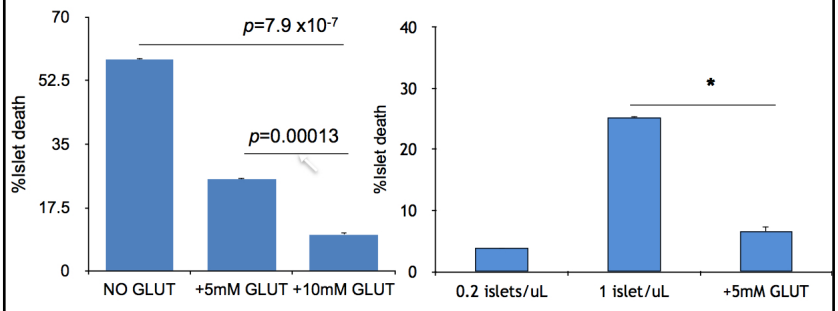

Fig 3. (A) High-density packing of mouse islets show dose-dependent improvement in *in vitro* survival with glutamine supplementation. **(B)** h-ESC-derived islets show improved *in vitro* survival with glutamine supplementation.

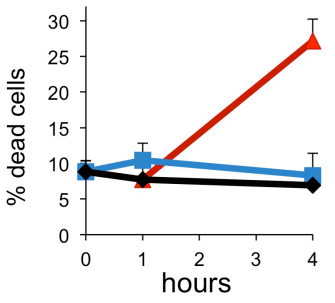

Fig 4. Preconditioning by pulsatile exposure to sub-hypoxic pO_2 protects islets from killing by severe hypoxic challenge. Mouse islets are exposed to pulsatile pO_2 oscillating from 159mmHg pO_2 to 40mmHg pO_2 every 6 minutes for 1 hour (**blue**). Control islets (**red**) were maintained at 159mmHg for 1hr. The islets are then challenged at 15mmHg pO_2 for 3 hours. A second control group of islets are cultured at 159mmHg pO_2 for the whole duration of the experiment (**black**).

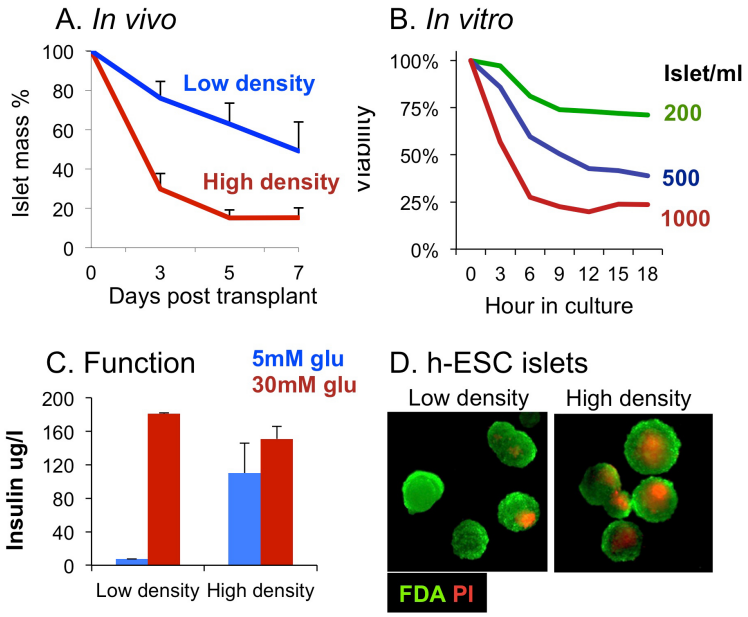

Fig 2. Impact of high-density packing on mature islet viability in vivo (**A**), in vitro (**B**), and function (**C**). **D**. Viability of h-ESC-derived islets after low and high density cultures assessed by FDA and PI staining.

1. Singh RP, Franke K, and Wielockx B. 2012. Hypoxia-mediated regulation of stem cell fate. High Alt Med Biol 13:162-8.
2. Heinis M, et. al. 2010. Oxygen tension regulates pancreatic beta-cell differentiation through hypoxia-inducible factor 1alpha. Diabetes 59:662-9.
3. Puri S, Akiyama H, and Hebrok M. 2013. VHL-mediated disruption of Sox9 activity compromises beta-cell identity and results in diabetes mellitus. Genes Dev 27:2563-75.
4. Puri S, Garcia-Nunez A, Hebrok M, and Cano DA. 2013. Elimination of von Hippel-Lindau function perturbs pancreas endocrine homeostasis in mice. PLoS One 8:e72213.
5. Biarnes M, et. al. 2002. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 51:66-72.
6. Eich T, et. al. 2007. Positron emission tomography: a real-time tool to quantify early islet engraftment in a preclinical large animal model. Transplantation 84:893-8.
7. Eriksson O, et. al. 2009. Positron emission tomography in clinical islet transplantation. Am J Transplant 9:2816-24.
8. Gerber PA, et. al. 2008. Simultaneous islet-kidney vs pancreas-kidney transplantation in type 1 diabetes mellitus: a 5 year single centre follow-up. Diabetologia 51:110-9.
9. Vantyghem MC, et. al. 2011. Diabetes cell therapy: a decade later. Minerva Endocrinol 36:23-39.
10. Pepper AR, Gala-Lopez B, Ziff O, and Shapiro AM. 2013. Revascularization of transplanted pancreatic islets and role of the transplantation site. Clin Dev Immunol 2013:352315.
11. do Amaral AS, et. al. 2013. Glutathione ethyl ester supplementation during pancreatic islet isolation improves viability and transplant outcomes in a murine marginal islet mass model. PLoS One 8:e55288.
12. Ko YG, et. al. 2001. Glutamine-dependent antiapoptotic interaction of human glutaminyl-tRNA synthetase with apoptosis signal-regulating kinase 1. J Biol Chem 276:6030-6.
13. Atmaca G. 2004. Antioxidant effects of Sulfur-Containing Amino Acids. Yonsei Med J 45:776-88.
14. Carlsson PO and Mattsson G. 2002. Oxygen tension and blood flow in relation to revascularization in transplanted adult and fetal rat pancreatic cells. Cell Transplant 11:813-20.
15. D'Hoker J, et. al. 2013. Conditional hypovascularization and hypoxia in islets do not overtly influence adult beta-cell mass or function. Diabetes 62:4165-73.
16. Ma Z, et. al. 2013. Preconditioning with associated blocking of Ca2+ inflow alleviates hypoxia-induced damage to pancreatic beta-cells. PLoS One 8:e67498.
17. Choi YK, et. al. 2010. Carbon monoxide promotes VEGF expresion by increasing HIF-1alpha protein level via two distinct mechanisms, translational activation and stabilization of HIF-1alpha protein. J Biol Chem 285:32116-25.
18. Puri S, Cano DA, and Hebrok M. 2009. A role for von Hippel-Lindau protein in pancreatic beta-cell function. Diabetes 58:433-41.

Budget:

Reagents and supplies - $9,600: Includes various biochemicals, solutions and media, serum, lab plastics, assay kits
$800/month.

Mouse colony maintenance - $5,840: Maintenance of 20 cages of NSG mice including four breeding cages and 16 experimental cages for in vivo analysis of hESC islet survival and function
$0.80/cage/day.

hESC-islet production - $12,500: One batch of hESC islets will be produced every week for in vitro and in vivo experiments.
$250/batch x 50 batches/year.

UCSF islet core recharge for human islets – $5,000: Human islets will be used occasionally to compare with hESC-derived islets
$1,000/prep, 5 preps/year.

Recharge for Xenogen IVIS use - $2,400: IVIS will be used to monitor hESC islet survival in vivo. Each transplanted mice will have an accumulated imaging time of 3 hrs over a course of 30 days.
20 mice/year, $40/hr.

Travel: $ 2,660: Travel expenses will help to subsidize airfare and lodging costs for attendance and presentation at annual society conferences, including the American Transplant Congress (ATC) and the International Pancreas and Islet Transplantation (IPITA) World Congress.

Publication cost: $2,000

Total budget: $40,000

Autobiography:
Upon completion of my General Surgery residency, I plan to pursue a fellowship in Transplant Surgery toward a career as a surgeon-scientist in the field of transplantation. Throughout several rotations on the Transplant service as a resident, I have found an ideal match for my clinical skills and professional interests. I have seen the dedication required of transplant surgeons - in last-minute flights and drives for organ procurements, bleary overnight calls to ensure optimal patient care, hours of lightning-quick technical focus in the operating room, and day-to-day clinical precision with no room for error. And I have seen the results of this labor, as patients are brought back from the brink of critical illness and given a vibrant new life in the mere days following an operation. As astounding and humbling as these outcomes are to me as a clinician, none of this would be possible without the decades of essential research which have helped to overcome the major immunologic obstacles to transplantation. To truly seek the best for my patients throughout a career in transplantation, I will dedicate my practice to improving patient outcomes through surgical expertise and research excellence.

As an undergraduate, I chose to study Materials Science and Engineering, based on my interest in math and science with a focus on real-world application. By specializing in Biomaterials, my coursework began to focus heavily on biology - and I became fascinated with the human body and chose to pursue graduate study in Medicine. Prior to matriculation in medical school, I worked under Dr. M. Eileen Dolan in the Cancer Research Center at the University of Chicago. Her lab focuses on the pharmacogenetic factors related to cancer chemotherapeutics. The Dolan Lab helped pioneer genome wide association (GWA) as a technique to identify genetic variants contributing to population-specific and inter-individual disparities in chemotherapeutic toxicity. Through investigation of well-characterized lymphoblastoid cell lines from parent-child trios of distinct global populations, we described 570 genes with differential gene splicing attributable to single nucleotide polymorphisms, some of which are implicated in genetic diseases. Our results suggest a potential genetic basis for differential health disparities and medication responses between individuals and ethnicities - all based on variance of a single nucleotide. While conducting this research, I was responsible for maintaining over 600 immortalized lymphoblastoid cell lines for distribution to 11 laboratories, designing all RT-PCR probes, and performing most RT-PCR assays for the lab during my research time. This productive year yielded two publications in peer-reviewed journals (Huang RS, et. al., 2009 and Zhang W, et. al., 2009).

In medical school, I had the opportunity to participate in clinical research investigating plastic surgery techniques for breast reduction. Reduction mammaplasty relies on preservation of a vascular pedicle to ensure adequate blood supply to the remaining breast tissue and the nipple-areolar complex. Traditionally, the reduction mammaplasty has been based off an inferior pedicle, which can result in a large, inverted-T (Wise pattern) shaped scar and breast ptosis in long-term outcomes. Alternative pedicles had been proposed, including the superomedial pedicle, commonly paired with a shorter, more aesthetic vertical scar and resulting in greater projection and improved long-term aesthetic results. These superomedial pedicle results had previously been limited to application for small-volume breast reductions. Our study (Antony et. al., 2013) was the first to directly compare the superomedial pedicle/vertical scar technique to the traditional inferior pedicle/ Wise pattern technique across reductions of all volume without restriction on pre- and post- reduction breast size. We were able to demonstrate no difference in overall outcomes between matched cohorts with respect to survival of the nipple-areolar complex, complication rates, or patient satisfaction. These findings support broader application of a more aesthetically refined technique to all patients interested in reduction mammaplasty. The resulting publication was awarded Best Breast Paper published in 2013 at the Plastic and Reconstructive Surgery annual meeting held in 2014. These research experiences, diverse in subject matter and required skill sets, have all brought unique challenges and reinforced the need for adaptability and critical thinking necessary to produce meaningful research. I will carry these lessons forward through a career as a surgeon-scientist.

I see my future career as a transplant surgeon deeply rooted in teaching. I find great joy in teaching junior residents and medical students, and I think this is an essential component for any academic surgeon. I will continue this focus on surgical education as an attending surgeon, and would be interested in directing a surgical residency program. To do so effectively requires a deep understanding of all facets of surgical practice, with the ability to serve as a mentor to the spectrum of career aspirations contained in the surgical field. This project will enhance my skills as a researcher, an educator in research, and in the administrative finesse required to nurture an idea into a functioning project and clinically applicable results. These abilities will be valuable not only to my career development, but to the development of future trainee's careers as a mentor.