Decreased Expression of Immune Related Genes in the Intrauterine Growth Restricted Fetal Sheep Pancreas

By

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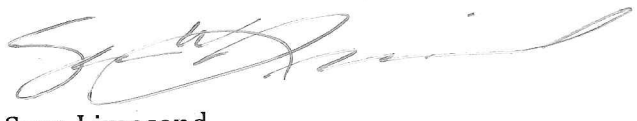

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Abstract

Type 2 diabetes (T2D) is an ever-increasing problem in the United States that causes many health problems. There is strong evidence that a poor fetal environment affects the metabolic health in adulthood. A specific and common example of this is intrauterine growth restriction (IUGR), which can be induced by placental insufficiency resulting in fetal hypoglycemia, hypoxia, and hypoinsulinemia. Sequencing of the transcriptomes of islets isolated from IUGR fetuses at 90% gestation showed decreased expression of cytokines and immune markers. This evidence led to the hypotheses that chemokines are reduced in IUGR fetuses because macrophage number and function is less or other cells in the pancreas secrete chemokines and act as paracrine cells. In order to test the hypotheses, we measured RNA markers for macrophages and T cells using real time quantitative polymerase chain reactions (RT-qPCR) and immunohistochemistry (IHC). RT-qPCR indicated macrophage genes were present in the fetal pancreas and that regulatory T cell marker TRDV1 was differentially expressed ($p<0.01$) between IUGR and control pancreases. IHC techniques are currently being used to determine whether these immune molecules co-localize with insulin producing cells or if they represent a population of immune cells within the fetal pancreas.

Introduction

Intrauterine growth restriction (IUGR) is a severe yet widespread complication affecting 10% of pregnancies in the U.S. The mechanisms underlying the cause of IUGR remain undefined, however, IUGR is associated with many conditions including: maternal malnutrition, severe maternal obesity and Type 2 Diabetes, autoimmune disease, and placental malformation. In all cases, an IUGR fetus does not obtain the necessary nutrients that it needs to support appropriate growth and development. Hales et al., proposed a hypothesis to explain what happens when a fetus does not receive adequate nutrients; Hales titled this the "thrifty gene" hypothesis. (Hales et al., 1992) The "thrifty gene" hypothesis states that when a fetus is deprived of nutrients it learns to store nutrients and allocate them to vital organ systems, like the nervous system. This means that physiological systems like the lymphatic system, which controls immune responses, do not receive as much nutrition, as it is not yet required. This action explains the asymmetric phenotype of IUGR neonates, with larger head to abdomen circumference measurements compared to healthy babies.

In humans and animal models, IUGR fetuses are subject to significantly lower birth weights, hypoglycemia, hypoxia, and reduced plasma insulin levels. Additionally, in severe IUGR fetal pancreatic beta cell mass is reduced. (Hayward, 1983) Pancreatic beta cells are endocrine cells that are responsible for secreting insulin. Insulin is a hormone that promotes growth and metabolism by signaling muscle and fat cells to use glucose. Decreased beta cell mass in IUGR fetuses leads to decreased insulin production and can eventually lead to adult onset type 2 diabetes.

Sheep are utilized in these experiments to model IUGR because the developmental progress, especially of the pancreas, closely resembles that of human development. (Cole, 2009) Sheep have singleton pregnancies and are able to be chronically catheterized. Decades of physiological data collection have provided the framework to study fetal programming. The IUGR fetal sheep captures many aspects of human IUGR. Additionally, previous work has shown there are pancreas specific defects in the IUGR fetal sheep with a 76% reduction of beta cell mass. (Limesand, 2005) RNAseq data identified over 1000 differentially expressed genes in the IUGR pancreatic islet. Functional enrichment of large gene lists gives us information on what might be happening. Functional enrichment of genes differentially expressed in IUGR identified numerous immunological pathways and functions. Using a public database we found that 10 pathways were significantly enriched. We see that of these 10 pathways, 4 of them are directly related to immune system activity. However despite this data, little is known concerning the role of the immune system action on the pancreas. Studies in humans are evolving and have shown new roles for the immune system in fetal life, but the direct role in the development of the fetal pancreas remains undefined.

KEGG Pathway	Genes	P-Value	Benjamini
ECM-receptor interaction	20	3.3×10^{-6}	5.3×10^{-4}
Glycine, serine and threonine metabolism	11	2.9×10^{-5}	2.3×10^{-3}
Complement and coagulation cascades	16	5.9×10^{-5}	3.2×10^{-3}
Proteasome	13	6.2×10^{-5}	2.5×10^{-3}
Prion diseases	9	2.3×10^{-3}	7.2×10^{-2}
Cell adhesion molecules	19	4.4×10^{-3}	1.1×10^{-1}
Cytokine-cytokine receptor interaction	31	4.4×10^{-3}	9.8×10^{-2}
Intestinal immune network for IgA production	10	6.0×10^{-3}	1.2×10^{-1}
ABC transporters	9	1.0×10^{-2}	1.7×10^{-1}
Porphyrin and chlorophyll metabolism	7	1.0×10^{-2}	4.3×10^{-1}

Figure 1: Pathways found significantly enriched in IUGR islets after analysis in the online databank DAVID (http://david.abcc.ncifcrf.gov/)

The immune system, in humans, begins to develop and mature early in the life of the fetus such that T cells, both helper and killer T cells, are leaving the thymus at around 14 weeks of gestation and moving towards the spleen. In addition, monocytes, the precursor to macrophages, (see figure 1 for immune cell formation lineage) have been determined to be mature and functional at the time of human birth. (Hayward, 1983) Previous experiments in rodents showed that tissue resident macrophages are important for proper organ development in the fetus. (Dahlén, 1998) These data suggest a role for the fetal immune system in the proper development of fetal structures. Recently, a population of T cells were shown to predominate the human fetal circulation and are denoted as γδ T cells. (Dimova et al., 2015) γδ T cells are named on the basis that they have a gamma (γ) and a

delta (δ) glycoprotein T cell receptor chains. Most T cells are alpha-beta T cells, however, γδ T cells are the most abundant type of T cell during fetal development. It is still unknown exactly what the role of γδ T cells is. In adults ruminants, like the sheep or cow, these T cells appear have a larger role. (Herzig, 2006)

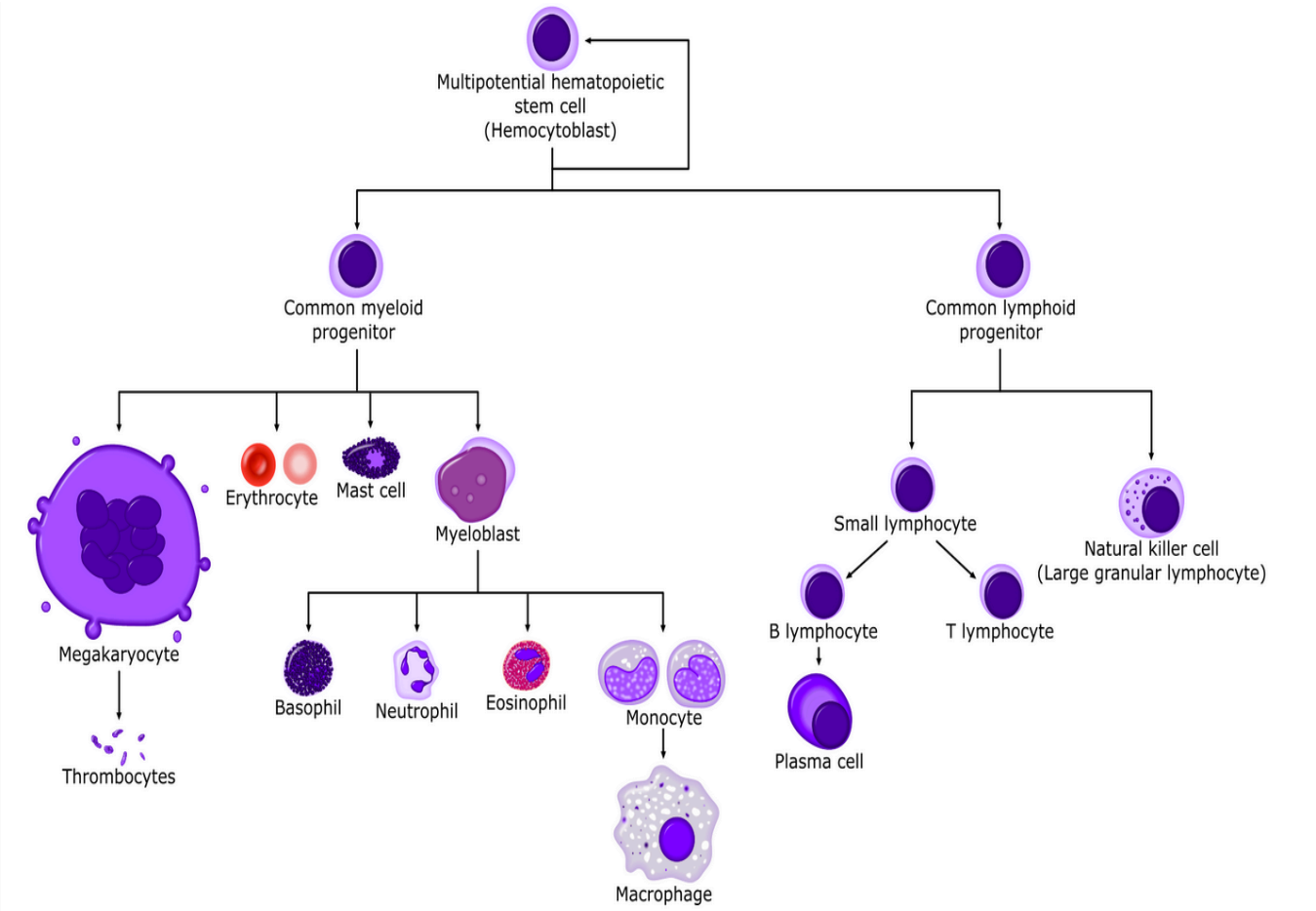

Figure 2: Hematopoietic stem cell lineage. Image of how different immunological cells come about. (Regenerative Medicine, 2006)

Thus the current work aims to first, identify the immune molecules, or cells using specific protein expression, in the fetal sheep pancreas and second, determine which of these immune molecules are affected buy IUGR. This was completed with RT-qPCR. To determine whether these immune molecules were present in the pancreatic beta cells, we

conducted preliminary immunohistochemistry. This allows us to see if cells that are producing insulin are the same cells producing some of the immune molecules we identified using RNAseq.

Materials & Methods

Animal Model Preparation

Ewes pregnant with singletons were obtained and the size of their litter was determined using ultrasound to confirm that each ewe only carried one fetal sheep. Placental insufficiency IUGR fetuses were created by placing pregnant ewes in moderate humidity (dew point 22˚C) and elevated ambient temperatures (35˚C for 12h; 40˚C for 12h) from 39±1 days gestational age to 119±2 days gestational age. Control sheep fetuses were obtained from healthy pregnant ewes that were placed in rooms at 25˚C. The control and placental insufficiency fetuses were pair-fed so that both groups received the same amount of food.

cDNA Library Generation

A cDNA library was created from the mRNA of tissue so that it contained the genes that were specifically expressed by that tissue/organism.

Real Time PCR

A master mix for qPCR was created so each tube contained 18μL of the mix (total of 720μL of master mix created). The master mix solution contained 272μL RNase water, 24μL primer 1, 24μL primer 2 (primers used were oCD4, oTRDV1, oS15, and oCXCR4), and

400µL of SYBR Green. 18µL of mix added to each of 40 tubes and then cDNA was placed in tubes. Plate spun at 4500rpm for 10 minutes at 4˚C on rotor JS5.3. Plate was then cycled at 95˚C for 15 minutes, then at 96˚C for 30 sec., 60˚C for 30 sec., and 72˚C for 10 sec. 45 times. Finally, plate was cycled from 60˚C to 96˚C (changing 0.2˚C every 2 sec.) 181 times and the data was analyzed. All of the cDNA utilized for the RT-qPCR was run at the same time and in duplicate.

ABC Staining

Slides were stained using DAB Rabbit Anti-CD68. Slides dried at 37˚C for 30 minutes and rehydrates in water. 0.3% Hydrogen Peroxide in water was used to remove endogenous peroxidases. Slides were then placed in 10mM Citric Acid (pH 6.0) and heated in microwave at 60% power for 10 minutes. Slides washed in PBS, outlined in wax pen, and blocked in ABC Rabbit Block. Slides were incubated in primary antibody (CD-68) overnight. Following day the slides were rinsed in PBS, incubated in Biotinylated Secondary Antibody at room temperature and rinsed again in PBS. Finally the slides were incubated in ABC reaction, washed in PBS, incubated in DAB, and dehydrated. The image below demonstrates this technique.

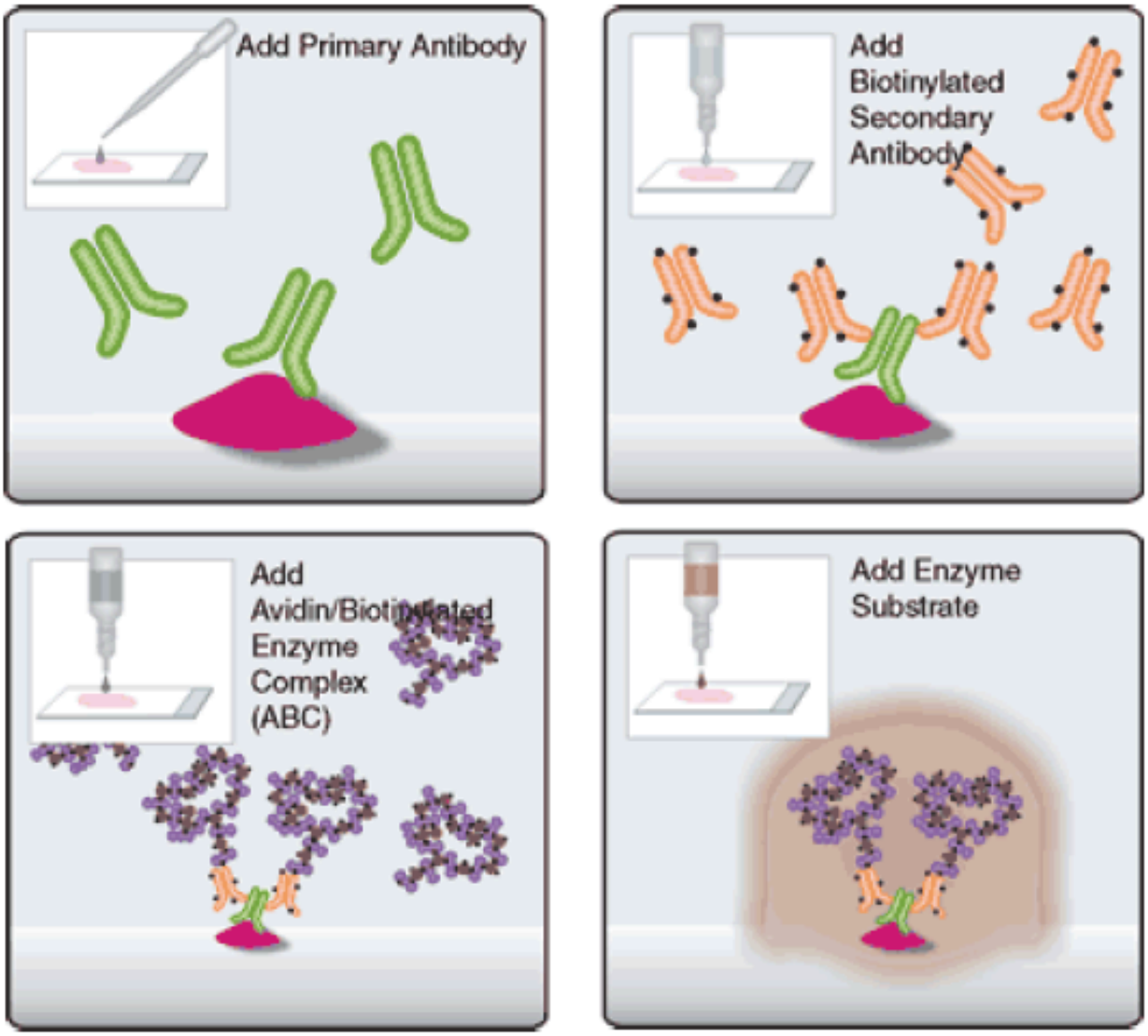

Figure 3: ABC Vector Staining Protocol using Vectastain Universal Elite ABC kit from Merck (Vector Laboratories, United Kingdom)

Results

RT-qPCR identified immune genes in the IUGR pancreas

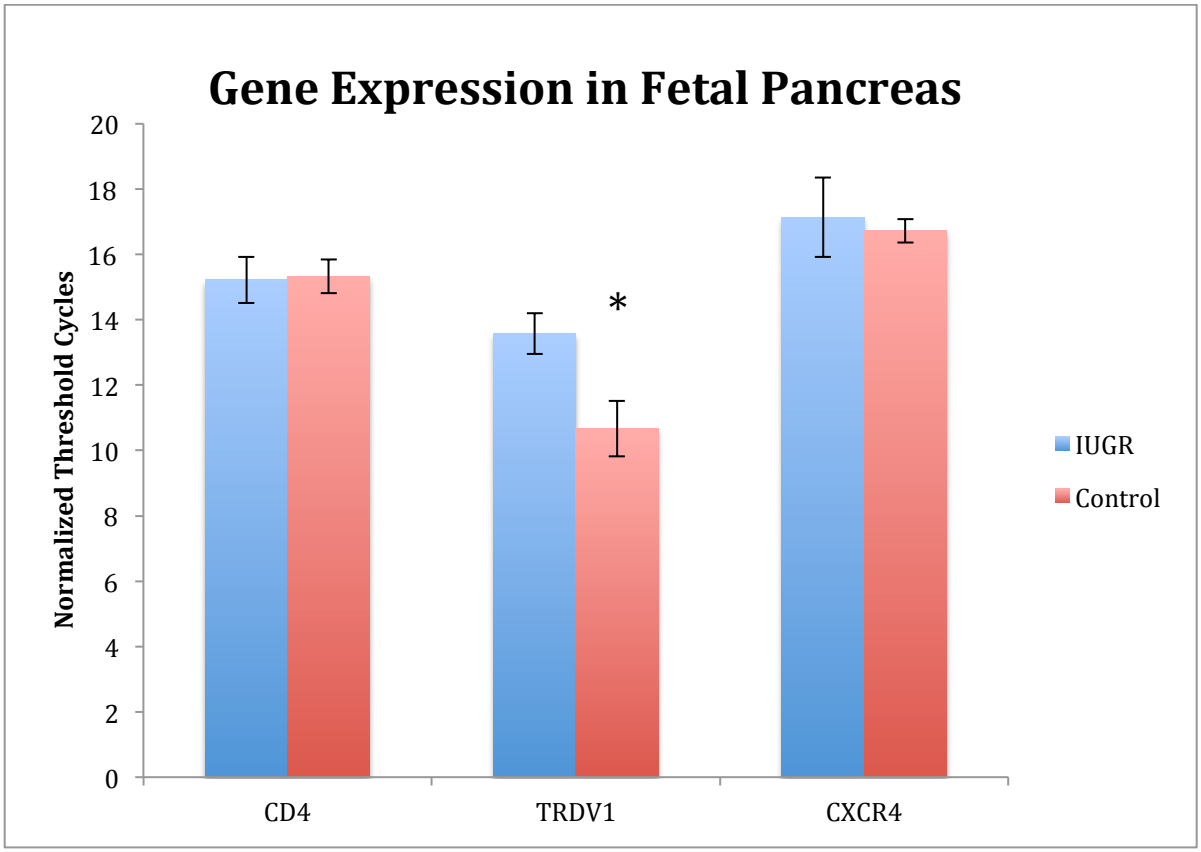

Figure 4: Demonstrates the differential expression of the genes CD4, TRDV1, and CXCR4 between control and placental insufficiency induced IUGR fetuses. The genes were normalized to the housekeeping gene ribosomal protein S15.

*$p<0.05$

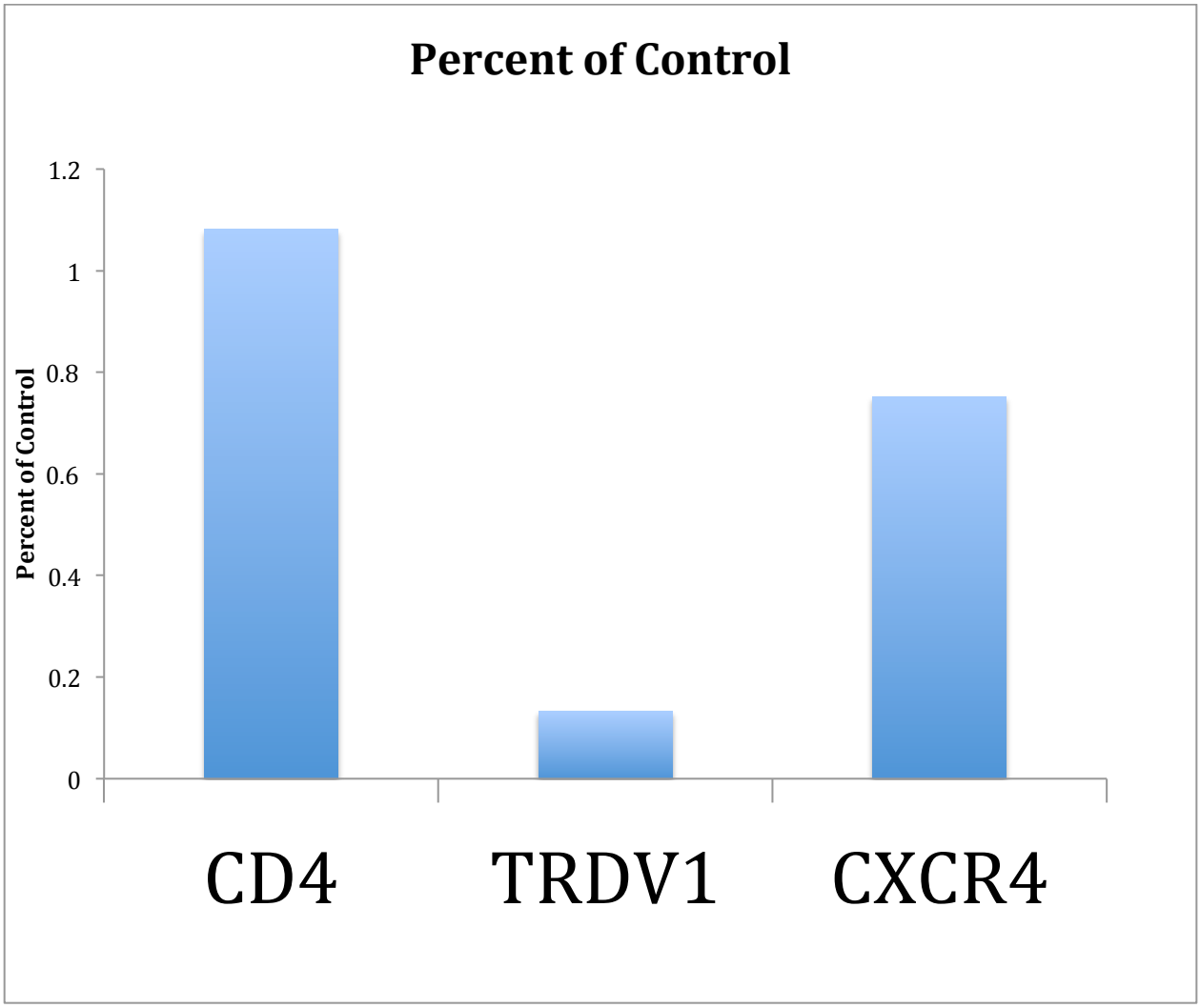

Figure 5: Demonstrates the percent of the control that the marker displayed. CD4 was 108.1% of the control, CXCR4 was 75.2 % of the control, and TRDV1 had the greatest decrease in gene expression with 13.3% of gene expression showing compared to the control.

Discussion

For this experiment, we looked at immune molecules in the fetal sheep pancreas at 90% gestation to identify changes stemming from IUGR. Gene expression indicates that immune related genes CD4, CXCR4, and TRDV1 are present in the fetal pancreas, but that

TRDV1 is the only gene tested that is differentially expressed. The gene CD4, a macrophage marker, presented with gene expression that was 108% of control and was not different between the control and IUGR fetal pancreas. The results for CD4 suggest that either CD4 is not an appropriate macrophage marker to monitor in the fetal pancreas or potentially there are distinct splice variants for CD4 that make it hard to capture. It shows that CD4 is present, but presents at the same levels in both control and IUGR fetal sheep. When using the same primers for CD4 in the spleen, the resulting DNA gel has two bands. This provides evidence that there may be novel splice variants of this gene in sheep.

CXCR4, a common chemokine receptor, displayed a decrease in expression that was 75.2% of the control, which is not statistically significant. CXCR4 was differentially expressed in the RNAseq data but not in the quantitative real time PCR data. CXCR4 has been shown to be involved in the function of insulin producing cells and thus may have a role in the dysfunction seen in IUGR. In contrast, TRDV1 displayed a significant decrease in which gene expression in the IUGR for TRDV1 was 13.3% (7.5 fold) of the control fetuses. A study by Dimova et al. might explain why the T cell marker, TRDV1, showed such significant differential expression. The study shows that high levels of the T cell type $V\gamma9^{-}V\delta1^{+}$ were present in the blood at term delivery and had increased throughout gestation. (Dimova et al., 2015) Since T cell variant $V\gamma9^{-}V\delta1^{+}$ is the γδ T cell subset that is highly expressed at term delivery, it would suggest that TRDV1, which would encompass T cell variant $V\gamma9^{-}V\delta1^{+}$, has decreased expression in IUGR fetal sheep, due to IUGR fetuses experiencing the “thrifty” effect. This means that energy and nutrients available to the fetus are decreased due to placental insufficiency so what little energy is available to the fetus is allocated to the most important body system like the brain and heart, instead of to

proliferating the growth and development of the immune system and this subset of T cells. Therefore, fewer nutrients are provided for the development of the immune system leading to a decrease in T cells, including the T cell variant $V\gamma9^{-}V\delta1^{+}$, which would explain the decreased expression of TRDV1 in the RT-qPCR.

Currently stains for CD-68, a common macrophage marker, have been inconclusive in the fetal pancreas, but have been functional in the fetal spleen and lamb pancreas. More staining is being performed in order to visualize the known immune related genes in the pancreas. However, the inconclusiveness of the fetal pancreatic staining and ability of the staining for CD-68 to work in the fetal spleen suggests that this macrophage marker that is common in humans and rats may not be present in the fetal sheep pancreas. (Holness, 1993)

The next step in determining which of our hypotheses is supported with the data is to stain for CD-68 in human fetal pancreas tissues. Since CD-68 is known to be present in the human, staining for it in the fetal human pancreas might yield more conclusive results than continuing to stain for CD-68 in the fetal sheep since it is unknown whether or not CD-68 is present. Another option would be to perform in-situ hybridization. In-situ hybridization is a technique used to both quantify and localize mRNA sequences in tissue. In order to perform in-situ hybridization, fetal sheep pancreatic tissue would be incubated with a probe, either cDNA or cRNA, that would hybridize to the target sequence of mRNA. Once the probe hybridizes to its target, antigen labeling would be used to indicate the location of the probe. If the probe for CD-68 were localized to the islets, then the in-situ technique would support the second hypothesis.

The goal of this study was to determine the function of the immune system in the development of the fetal pancreas. More specifically, we tried to determine which of our hypotheses regarding the immune system in relation to the fetal pancreas in sheep held true. We found that the three immune markers CD4, CXCR4, and TRDV1 were all present in the fetal sheep pancreas, but that only TRDV1 demonstrated differential expression so that the IUGR fetuses expressed TRDV1 at 13.3% of the level of the control fetuses. Current and future experiments will involve staining the fetal pancreas and co-staining with insulin to determine where the immune cells localize. The data gathered so far suggests that staining for T cells might yield the best stain results since TRDV1 was the only immune marker to be differentially expressed.

References

1. C. N. Hales, D. J. P. Barker. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia*, 1992, Volume 35, Number 7, Page 595
2. Limesand, S. W., Jensen, J., Hutton, J.C., Hay, W.W.. Diminished β-cell replication contributes to reduced β-cell mass in fetal sheep with intrauterine growth restriction. *American Journal of Physiology- Regulatory, Integrative and Comparative Physiology*. May 2005, 288
3. Hayward AR. The human fetus and newborn: Development of the immune response. Birth Defects Orig Artic Ser. 1983;19:289–294.
4. Dahlén, Eva, Kim Dawe, Lennart Ohlsson, and Gunnar Hedlund. "Dendritic Cells and Macrophages Are the First and Major Producers of TNF-α in Pancreatic Islets in the Nonobese Diabetic Mouse." *Journal of Immunology*. 160.7 (1998): 3585-3593. Print. <http://www.jimmunol.org/content/160/7/3585.short>.
5. Cole, L., Anderson, M., & Antin, P.B., Limesand, S.W. (2009). One process for pancreatic beta cell coalescence into islets involves an epithelial-mesenchymal transition. *Journal of Endocrinology* (203) , 19-31.
6. Melville JM and Moss TJM (2013) The immune consequences of preterm birth. *Frontiers Neuroscience*. **7**:79. doi: 10.3389/fnins.2013.00079
7. "Crude and Age-Adjusted Incidence of Diagnosed Diabetes per 1,000 Population Aged 18–79 Years, United States, 1980–2011." *Diabetes Public Health Resource*. Centers for Disease Control and Prevention, 02 Oct 2014. Web. 20 March 2015. <http://www.cdc.gov/diabetes/statistics/incidence/fig2.htm>.

8. *Regenerative Medicine.* Department of Health and Human Services. August 2006. </info/scireport/2006report.htm>
9. Dimova et al. "Effector Vγ9Vδ2 T cells dominate the human fetal γδ T-cell repertoire." *Proceedings of the National Academy of Sciences*. (2015): n. page. Print.
10. Herzig, Carolyn, Blumerman, S.L., Baldwin, C.L.. "Identification of three new bovine T-cell receptor delta variable gene subgroups expressed by peripheral blood T cells."*Immunogenetics*. 58. (2006): 746-757. Print.
11. Holness, C.L., Simmons, D.L.. "Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins." *Blood*. 81. (1993): 1607-1613. Print.

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Reflection

Over the past year, I have spent time in the lab of Dr. Sean Limesand working on this honors thesis for the completion of my degree in honors physiology. Prior to entering this lab, I had no experience with research and the only laboratory techniques that I was familiar with were ones that I had learned in basic science lab courses. Through the course of this year I have been able to learn a lot about different laboratory techniques like PCR, ABC staining, and performing western blots among other things. In addition, to learning

valuable laboratory techniques, I was able to gain insight into the hierarchy that exists inside of a lab. This experience was very valuable and has taught me so much more than I could have imagined it would have at the start of the process.