Development of a Fibroblast Growth Factor 21 Assay

A Thesis

Presented to

the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

by

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July 2017

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ACKNOWLEDGEMENTS

“That which we manifest is before us; we are the creators of our own destiny. Be it through intention or ignorance, our success and our failures have been brought on by none other than ourselves.”- Garth Stein

I would like to take a moment and thank Dr. Green and Amanda Schmelzle for everything that they have done for me in the last few months. Words can't express how grateful I am to have them as support and guidance through some very unconventional situations and for giving me the chance to prove myself and helping me along the way.

I would like to thank my family for believing in me and being my source of inspiration regardless of distance. You have given me everything and I owe it all to you. You have shown me the endless possibilities of what I can achieve and that is a priceless gift. I hope to someday inspire people the way you inspire me.

To my fellow graduate students, thank you for being my friend and teaching me so much along the way.

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Development of a Fibroblast Growth Factor 21 Assay

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ABSTRACT

Obesity and diabetes play a major role in many diseases prevalent in our current society. Fibroblast Growth Factor 21 (FGF21) is a recently discovered hormone that has potential for therapeutic treatment of obesity and diabetes. It acts as a hormone in times of metabolic stress and is shown to stimulate hepatic fatty acid oxidation, ketogenesis, induce weight loss, allow for sustained decrease in plasma glucose and triglycerides, and decrease the growth hormone response. Obese or diabetic individuals often have higher than normal amounts of circulating FGF21; consequently, they may be considered FGF21 resistant. It is then advantageous to have an assay to measure FGF21 that will allow us to study FGF21 further. It is also important to study FGF21 in various livestock species for potential biomarkers, or as indicators of carcass quality that could prove to advance further research. We have developed a working FGF21 assay using an amplified luminescent proximity homogeneous assay (AlphaLISA). It is a homogeneous, no-wash immunoassay with high sensitivity and wide dynamic range. It is a bead based technology that brings two antibodies near each other allowing for an unstable singlet oxygen to trigger a downstream cascade of chemical events leading to a sharp intense chemiluminescent emission that can be read on an EnVision machine. The assay was set up using a 'sandwich' design with guinea pig or rabbit anti-bovine FGF21 antibodies attached to the acceptor beads and biotinylated rabbit anti-bovine FGF21 antibodies

attached to a streptavidin coated donor bead. The assay has a working range from 0.2-200 ng/ml. Standard curves in serum from bovine, porcine, rabbit, rat, ewe, fetal bovine has been conducted and assay conditions were optimized for each. Manipulations of the sera were necessary in order to get functional curves. These included diluting it 2 fold or more, depending on the species, and increasing incubation temperatures. The assay can function using both serum and plasma.

CHAPTER 1

INTRODUCTION

Fibroblast growth factor 21 (FGF21) is a novel peptide hormone that has potential as a therapeutic treatment of metabolic diseases including obesity and diabetes. Because FGF21 is an endocrine FGF it has potential for both being a risk factor and biomarker for these diseases.

According to the Journal of American Medicine, the United States of America has more than 78.6 million people living with obesity, which is approximately 35% of the population (Mokdad et al., 2017). Obesity is correlated with increased risks of heart disease, diabetes, and high blood pressure. It is a complex disorder specifically defined as when a person or animal possesses excess body fat. This criterion differs from merely being overweight, which is defined as an excess of body weight, which can come from multiple sources (muscles, bone, fat, and water). The causes of obesity are many times explained as a simple energy imbalance. If the amount of energy (calorie) intake is equal to the amount of energy used by the body, then body weight tends to remain the same. But when the intake in calories is greater than the amount that is used then there is an energy imbalance that tends to lead towards weight gain, overweight, and obesity. Obesity can also be of genetic origin where genes could affect efficiency in fat storage and conversion of fat into energy. Obesity can also be caused by diseases such as Prader-Willi syndrome, Cushing's syndrome and other conditions although they only account for a small percentage. Lifestyle also impacts an individual's tendency to be obese. Obesity can lead to other diseases such as depression, disability, sexual problems, social isolation,

and lower work achievement. Treatments for obesity include weight loss, diet changes, increase in physical activity, medical treatment (e.g. insulin injection), and surgery (EASO, 2017).

Diabetes mellitus, commonly known as diabetes, is also another cause for concern in a significant amount of the American population. In 2015 the International Diabetes Foundation estimated that over 425 million people worldwide were living with diabetes. From 2012 to 2015 estimated deaths caused by diabetes resulted in 1.5 to 5 million deaths each year. Diabetes is a disease where blood-glucose levels are abnormally high in the blood due to inadequate production and/or sensitivity to insulin. Insulin, produced by the pancreas, is a key hormone involved in glucose transportation. In humans, diabetes mellitus has three typical presentations: type 1, type 2, and gestational diabetes. Atypical presentations are rare - accounting for only 1-5% of total cases. Individuals with type 1 diabetes, also called insulin-dependent diabetes, do not produce insulin in their body. Individuals with type 2 diabetes, also called non-insulin-dependent diabetes, usually produce some insulin but are unable to process it correctly or the insulin is produced in insufficient quantities for the body's needs. Obese individuals are at particularly high risk of developing type 2 diabetes and its related medical problems. Gestational diabetes occurs when women without a previous history of diabetes develops high blood-sugar levels due to pregnancy. The reported rate of gestational diabetes is between 2-10% of pregnancies. Gestational diabetes usually resolves itself after pregnancy. Having too much blood glucose can cause damage to the eyes, kidneys, and nerves; it can also lead to heart disease, and strokes. Treatments for diabetics include healthy diets, physical

exercise, maintenance of body weight, as well as oral medications or injectable insulin (Joslin Diabetes Center, 2017).

A significant amount of the American population is affected by both obesity and diabetes. Both diseases have metabolic disorder components and endocrinological manipulations may open opportunities for novel treatments, specifically as they relate to FGF21.

FGF21 is a fibroblast growth factor that acts more as a classical circulating hormone rather in a paracrine manner, as is typical of other fibroblast growth factors. It is mainly secreted from the liver in times of metabolic stress, e.g. starvation. Metabolic actions of FGF21 include stimulating hepatic fatty acid oxidation, ketogenesis, blocking the growth hormone signaling pathway, inducing thermogenesis in brown adipose tissue (BAT) and promotion of glucose uptake in white adipose tissue (WAT) (A Planavila et al., 2013). FGF21 has also been seen to cause weight loss, reduction in insulin resistance, and a sustained decrease of plasma glucose and triglycerides (Yu et al., 2011). But individuals that are obese, have type 2 diabetes, insulin resistance, or nonalcoholic fatty acid disease; have a hindrance in the FGF21 pathway that causes them to have increased circulating FGF21 levels (Yu et al., 2011). FGF21 can provide therapeutic benefits by attenuating apoptosis, oxidative stress and inflammation, and improving energy supply (Tanajak, Chattipakorn, & Chattipakorn, 2015). Multiple studies have also shown that pharmacological induction of FGF21, or an analogue, reduced plasma glucose levels, insulin levels, and body weight (Véniant et al., 2012 & Adams et al., 2013).

FGF21 shows great potential for diabetes and obesity treatment. There is still a lot of research and work to be done for this to be accomplished. My project will provide

a platform to measure FGF21 in livestock animals through immunoassays to help improve our knowledge of this hormone leading to better therapeutic treatments of obesity and diabetes.

CHAPTER 2

REVIEW OF LITERATURE

INTRODUCTION

Obesity and diabetes are two diseases that affect many Americans. Treatments for patients vary greatly with various rates of success. The recently discovered growth factor, FGF21, has potential in therapeutic treatments for ameliorating such diseases. It is also important to research FGF21 in livestock animals as it has many functions relating to growth and health that could affect carcass characteristics and more. For further progress to be made, a thorough understanding of its metabolic effects and mechanisms is needed. There must then be an assay platform available that allows accurate measurements of biological FGF21. There are many options available for assay development and our lab was presented with the opportunity to use AlphaLISA technology developed by PerkinElmer. Development of an assay is challenging in that there is the need to understand the technology being used as well as understanding the molecule being measured. It is also necessary to understand the protocols for assay development, optimization, and validation to provide an accurate and replicable assay that can be further used for further research.

IMMUNOLOGY

The development of successful immunoassays depends greatly on the reactivity of the antibodies used. (Al-Tubuly, 2000)(Ritter, 2000)(Crowther, 1995). Antibodies belong to the gamma-globulin family of proteins. They are typically referred to as ‘immunoglobulins’ (Ig) (**Figure 2.1**). Monomeric antibodies are composed of two heavy

chains and two light chains that are covalently linked together through disulfide bonds. The structure of the tetrameric protein is roughly Y-shaped and two functionally distinct regions exist: 1) the 'arms' of the Y structure represent two antigen binding fragments (Fab), and 2) the fragment crystallizable (Fc) region represented by the base of the Y structure that interacts with immune cells and other proteins; the Fc region acts to initiate/modulate host defense mechanisms. The arms of the Y structure represent the 'variable' region (consisting of both portions of the heavy and light chains). The base of the Y shaped immunoglobulins represents the 'constant' region. The target to which the Ig binds is the *antigen* and the antibody-antigen interaction is dictated by specific contacts between the variable region (both its light and heavy chains) and the bound region of the antigen. Antibodies are specifically produced in the body to recognize antigens on foreign micro-molecules or on microorganisms themselves. The part of the antigen that the antibody binds to is called an *epitope* and the part of the antibody that binds through non-covalent interactions to the antigen is called a *paratope*.

The Fc region determines the antibody class (or **isotype**). These classes are IgM, IgG, IgA, IgE, and IgD. These different classes mediate different physiological functions, have different half-lives, and are present in different amounts in serum. For example, **IgM** is a pentamer that is effective against invading microorganisms. It is produced in the early phases of an immune response. **IgG** is the most prevalent antibody in the blood and one of its primary roles is to promote activation of macrophages. It is the main antibody class that provides passive immunity to a fetus/neonate by its ability to cross the placenta or the small intestine, depending on the species. IgG production and quality increases with each exposure to antigen, up to a point (after three to five exposures to an antigen,

any increases in the IgG response are usually negligible). Even so, the ability to improve the IgG response with multiple exposures to an antigen is a characteristic that can be manipulated in order to increase circulating IgG amounts. Such manipulations are the basis behind vaccines and for the harvesting of IgGs for use as reagents for antibody-dependent methods. **IgA** can exist as a monomer, dimer, or trimer and act as a protective barrier at several points of entry and is the main antibody in milk as well as sweat, tears, and saliva. **IgE** is a monomer that is present in blood in very low concentrations and is activated primarily by helminth parasites and during allergic atopic conditions. Finally, **IgD** is a monomer present at low concentrations; its functions are not well understood (Ritter, 2000).

B lymphocytes, once they have matured into plasma cells, secrete specific antibodies to combat invading pathogens. This is known as a polyclonal response, where different B cells collectively provide a range of antibodies that can recognize multiple epitopes on the same antigen. However, in clinical and experimental settings it is sometimes advantageous to produce monoclonal antibodies. Monoclonal antibodies can be obtained by the isolation, immortalization and propagation of a single B lymphocyte lineage that produces only a single immunoglobulin form. The ability to expand the modified B cell line in culture permits essentially an unlimited supply of its antibody (Ritter, 2000).

Monoclonal production was devised by Köhler and Milstein who won the Nobel Prize in Medicine in 1984 for this work. Monoclonal antibody production requires the growth of a clone of B lymphocytes and the collection of the antibodies produced by those cells. The difficulty arises because B lymphocytes are differentiated cells that

possess a finite lifespan. This problem is solved by fusing the B cell with an immortalized myeloma cell line to create a hybrid cell that secretes antibodies and can be propagated almost indefinitely. The myeloma cells are usually produced from mouse or rat origin and are genetically selected for an inability to produce their own antibodies ensuring that the production of antibody after the fusion only comes from the original B cell. Monoclonal antibody production is labor intensive and can take up to 6 months from initial immunization for sufficient antibody production (Ritter, 2000).

If working with polyclonal antibodies, it is advisable to immunize animals that are large enough to provide a high yield of antibodies from the serum. The most common animals used are rabbits, goats, and sheep as opposed to mice, rats, or hamsters that are better suited for monoclonal collection. It is also advisable to immunize female recipients since they usually have better immune responses (Crowther, 1995).

For polyclonal production, the best results are usually achieved with pure immunogen. Immunizations are administered with an adjuvant, a substance added to the antigen that is used to enhance the immune response. Some commonly used adjuvants include TiterMax, RIBI, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA).

In small animals, the immunogen can be administered intravenously (iv) or intraperitoneal (ip), whereas intramuscular (im), subcutaneous (sc), and intradermal methods are more common for larger animals. The route of immunization is important to consider for monoclonal production because the site of antigen entry will affect the anatomical location of the immune response. A response in the spleen is generated by iv entry while a response in lymph nodes are generated by ip, im, and sc routes. For

polyclonal antibodies, the site of injection is less important since the antibodies accumulate in the circulation. Affinity purification can then be used to separate the specific antibody from the serum. If not purified, serum may contain antibodies with specificities for different antigens in the serum not directed towards the intended target. Depending on the intended use of the polyclonal antiserum, the presence of non-specific antibodies may not be a concern.

The choice between using polyclonal versus monoclonal antibodies is dependent on intended use. Polyclonal antibodies are useful for recognition of more than one epitope on a target antigen. They are commonly used in ELISAs, western blotting, immunohistochemistry, and flow cytometry where the binding of antibodies to multiple sites on an antigen can increase signal from reporter systems. The disadvantage with the use of polyclonal antibodies is that the quantity is limited to the number of animals immunized. In addition, animals react differently to antigens and different antibody production attempts will produce antibodies with different ranges of specificities and affinities toward a given target. With monoclonal antibodies, there is a nearly unlimited source of homogenous antibodies that is predictable in its behavior. The disadvantage though is that they cannot provide signal amplification and there is limited choice for epitope selection due to limited species production by rodent, rabbit, and even 'humanized monoclonal antibodies (Ritter, 2000).

RADIOIMMUNOASSAY

To understand the AlphaLISA technology used in later experiments it is crucial to follow the progression of immunoassays starting with radioimmunoassays. The use of radioimmunoassay (RIA) as a method for measuring compounds in biological fluids was

first introduced by Yalow and Berson in 1959 to measure insulin (Parker & Ab, 1981) (Praither, 2016). In 1977 Yalow won a Nobel Prize for Medicine. In 1961, Unger et al. used this same method to assay serum glucagon. A couple of years later Greenwood et al. 1963, discovered the use of chloramine-T as a method to radioiodinate proteins; they used this approach to measure human growth hormone (hGH). The RIA technique combines both sensitivity and specificity, which have different connotations in diagnostic and analytical applications. In a diagnostic application, sensitivity is defined as the probability of a positive result occurring when in fact the samples are truly positive; $P(T|S) = \frac{\text{true positive}}{\text{true positive} + \text{false negative}}$ where T represents test results, and S represents true status of the measured sample. Specificity is defined as the probability of a negative result occurring when in fact the sample is negative; $P(T|S) = \frac{\text{true negative}}{\text{true negative} + \text{false positive}}$. In an analytical laboratory setting, sensitivity is often defined as the lowest value in which an analyte in an assay can be confidently detected; specificity is the confirmation that the signal generated represents only that derived from the intended target and is not due to binding to off-target interactions. From this point forward references to sensitivity and specificity will be presented mainly in the context of analytical diagnostics.

Although initially designed for measurement of circulating hormones, the RIA technique has vastly expanded to other fields such as pharmacology, microbiology, and biochemistry. The technique was initially designed for serum and plasma, but it has also been applied to other biological fluids (e.g. urine, spinal fluid, milk, etc.).

Competitive RIAs are based on the principle of competition between a radio-labeled target and unlabeled target for binding to an antibody. When the concentration of the unlabeled antigen is elevated in a sample, the amount of bound radioactivity (e.g.

labeled antigen) will be less due to competition for the limited number of available antibody binding sites. Likewise, when there is little unlabeled antigen present, the amount of bound radioactive antigen will be high (Radiobioassays. Volume I, Ashkar).

There are four main components that comprise a radioimmunoassay: sample, standard, label, and primary binder (antibody). The first part is the sample being analyzed that contains an unknown amount of the unlabeled target that will serve to compete with the labeled antigen. The second part is the *standard* which is a constituent that can be extracted and purified from tissue source, obtained as a recombinant protein or synthesized (e.g. a peptide known to be bound by the antibody). Ideally this constituent can be weighted out in the appropriate amounts and diluted for use in the assay. There are readily available purified forms of many hormones that are relatively inexpensive. The standard can be diluted to create a standard curve that, when compared to the signal obtained from the samples, can be used to quantify the amount of target present in the sample.

The third component is the *label*, which is also referred to as the ‘tracer’, ‘labeled antigen’, ‘tagged material’, the ‘tag’, etc. This component contains an attached radioactive atom to the target. In most RIAs, the antigen itself is labeled but in certain cases, such as with ‘sandwich assays’, the antibody itself is labeled. Labeling can be done with ^{131}I (8 day $\frac{1}{2}$ life), ^{125}I (56 day $\frac{1}{2}$ life), ^{14}C (5730 year $\frac{1}{2}$ life), and ^3H (12.3 year $\frac{1}{2}$ life). Most RIAs use ^{125}I as the radio-label mainly because of its relative availability and counting efficiency (Parker & Ab, 1981). Iodination of most proteins can be performed by using the chloramine T method, which was first described in 1962 by Hunter & Greenwood. This method directly attaches the iodine to the aromatic group of

tyrosine through a cationic iodine intermediate (Parker & Ab, 1981). The advantages of this method are its reproducibility, rapidity, efficiency, and avoidance of extreme pH or organic solvents. Despite these advantages, there are some proteins that undergo substantial denaturation with the chloramine T process. An alternative method is to iodinate with lactoperoxidase, where the iodine is introduced to the protein through a low molecular weight carrier, such as 3-(p-hydroxyphenyl) propionic acid-N-hydroxysuccinimide (HPPS).

Lactoperoxidase, in the presence of hydrogen peroxide, promotes the iodination of proteins - especially to those that possess sterically accessible residues such as tyrosine. This method is often preferred over the chloramine T method for hormones such as glucagon, insulin, growth hormone, LH, TSH, and FSH. One problem with this method is that it can fail to provide effective iodination if tyrosine are inaccessible to the enzyme. There also exists an indirect iodination, or two-stage labeling, first introduced in 1973 by Bolton & Hunter using HPPS where the iodine is attached to a low molecular weight carrier intermediate prior to it being bound to the target protein itself. The advantage of this method is that some proteins now can be labeled that couldn't be labeled with the direct method. This method can also iodinate polypeptides, proteins, and low molecular weight chemicals that lack tyrosine. The draw back to this procedure is that relatively large amounts of iodine need to be used for a lower specific activity of the final iodinated product. Labeling may also be introduced to the antibody rather than the antigen, especially if the antigen cannot be iodinated. Regardless of the method used it is undesirable to label the antigens too heavily. This could cause antigen denaturation, decrease immunoreactivity and stability, and increase the variability in the immunoassay.

In the direct method, it is often desirable to achieve a average substitution of less than one atom of iodine per molecule of antigen. Once iodination is complete, the labeled antigen is usually subjected to a short molecular sieve or column chromatography (Sephadex® G-50 or G-25) to remove unconjugated radioisotope.

The fourth component of RIAs, the primary binder, is usually an antibody specific for a target. Antibody production is accomplished by immunizing an animal (rabbits, guinea pigs, sheep, goats, monkeys, mice, rats, chicken, and donkeys) with the purified antigen or immunogen. Immunizations require multiple subsequent injections (boosters) at various intervals (2-6 weeks are common) to provide sufficient antibody titers. In most RIAs, the amount of tracer in any given antibody concentration should provide ~50% binding (i.e. it should produce counts that represent about 50% of total binding capacity for a given antibody concentration). Assay characteristics can be altered to help improve sensitivity such as by altering antibody concentrations to achieve optimal binding. In addition, antibodies with the highest affinity bind the antigen most effectively. Another aspect to consider is the specificity of the antibody. Specificity, in an analytical sense, is defined as how well the antibody detects what it is designed to detect. This is determined by its cross-reactivity to other compounds, meaning that the antibody is evaluated for its ability to bind to other compounds as well as to the desired one. High cross-reactivity represents low specificity. In cases such as these, extra steps such as extraction, chromatography, and purification can be done prior to antibody usage. Monoclonal antibodies are also a widely-used reagent. They offer the advantage of high specificity and the ability to be maintained and propagated in cell culture indefinitely, decreasing

problems that are often associated with lot to lot variations in polyclonal antibody production.

In conventional RIAs, the sample or standard competes with the tracer for a limited number of antibody binding sites. When these components are introduced together an equilibrium is eventually reached between the free fraction and already bound antibody. The time it takes to reach equilibrium is dependent on the affinity and specificity of the antibody and varies from assay to assay. RIAs are continually being developed in efforts to provide faster assays without sacrificing quality.

The last step in the RIA is the separation step where antibody bound fractions are removed from the unbound or free fraction for the radioactivity to be counted. Separation can be accomplished in a multitude ways including the use of a secondary antibody (an antibody to the target antibody), salt precipitation, or two-phase systems in which the antigen or the antibody is attached to a solid phase. There are two general categories in which separation techniques go under, either a liquid phase separation, where the primary antibody is part of the supernatant (e.g. the dextran coated charcoal method), or a solid phase separation, where the primary antibody is in a solid form, where it can either be a liquid reagent resulting in pellets (Secondary antibody) or solid reagents where only a wash is required (coated tubes). The specific method used is influenced more on preference and experience of the individual. Double antibody immunoprecipitation is the most common and broadly used despite its prolonged incubation times that range from 2-24 hours. It involves the immunization of an animal against the immunoglobulin class to which the first antibody belongs. Absorption assays involving charcoal are used as stripping agents for serum. When it is added to the RIA

mixture it absorbs the tracer or patient analyte but not those bound to the antibody. The disadvantage with this method is that it may increase intraassay variation as well as being sensitive to the protein content in the medium. Salt precipitation with ammonium sulfate can only be used with radioiodinated antigens that are soluble in 40 to 50% ammonium sulfate.

ELISA

The establishment of the RIA was a revolutionary technique which further inspired the development of both EIA (Enzyme Immunoassay) and ELISA (Enzyme-Linked Immunosorbent Assay). Both techniques use a similar concept as an RIA but use enzymes rather than radioactivity to label antigen or antibodies. These techniques arose out of concern for laboratory personnel safety, radioactive waste problems, the necessity for special facilities, and expensive radioisotope counting equipment involved in RIAs. Both techniques were developed simultaneously but in different labs. The ELISA technique was developed by Peter Perlmann and Eva Engvall and the EIA technique by Anton Schuurs and Dauke van Weemen. The idea of using enzymes was at first met with skepticism but Engvall and Perlmann published their first paper in 1971 successfully using alkaline phosphatase as the label and quantifying IgG in rabbit serum. In the same year, Van Weemen and Schuurs were able to quantify human chorionic gonadotropin concentrations in urine using an EIA (Perlmann et al., 1980)(Mendoza et al., 1999)(Rudolf, 2005).

ELISAs can be categorized as: direct, indirect, competitive, or sandwich (**Figure 2.2**). The choice in assay to use depends greatly on the purpose of the assay, the reagents, antigens, and antibodies available.

The competitive based ELISA uses the same principles as a competitive based RIA where two reactants are competing to bind to a single binding site. (**Figure 2.3**). The first step is to physically or chemically attach the appropriate amount of antibody to a solid phase followed by incubation, to equilibrium, of a mixture containing both known concentrations of enzyme-labeled antigen and unlabeled antigen. After washing the plate, incubation with substrate will yield product concentrations inversely proportional to the concentrations of standard. One of the disadvantages with this method is that it requires a large amount of purified antigen.

ELISAs can also be performed in a 'sandwich' format (**Figure 2.4**). Here, the antibody is attached to a solid phase and antigen or sample is then incubated with the immobilized antibody. After washing, enzyme labeled antibody (this antibody also is reactive toward the antigen or target) is added to the plate and left to incubate. In this case, the enzyme reaction is proportional to the concentration of antigen. This technique offers the advantage of enzyme-labeling antibodies that can be used across multiple assays instead of the necessity of having to create separate antigen-enzyme conjugates for each assay. It also allows for the binding of several enzyme-labeled antibodies to a single polyvalent molecule, allowing for amplification. The difficulty that arises with competitive ELISA lies in the need to incubate enzyme labeled antigen or antibody in biological fluids (serum, urine, tissue extract, etc.) that contain proteases that may alter the activity of the enzymes. The situation is avoided in a non-competitive ELISA due to separate incubations of test sample and enzyme-labeled antigen or antibody.

The enzyme chosen for labeling should have high specific activity and high sensitivity and should be detectable at or below the nanogram level. There are many

methods available to use for labeling with enzymes that include Glutaraldehyde, Toluene diisocyanate, Carbodiimides, *p*-Benzoquinone, *N, N'*-*o*-Phenylenedimaleimide, and *m*-Periodate.

ELISAs can be broken down into several steps (shown in no specific order) that are common across most ELISAs, although the specific details will often vary between assays.

- 1) The solid-phase step where the antigen or antibody are absorbed onto the plastic solid phase. The most commonly used solid-phase is the 96-well microtiter plate manufactured from polyvinyl chloride (flexible plates) or polystyrene (rigid plates). Flat bottomed plates generally work better for spectrophotometric reading while round bottomed wells seem to work better for simple visual assessments. Most proteins absorb nonspecifically to the plastic surfaces and in turn 'coat' the plate. It is important to determine the optimal concentration of antibody for coating. Coating time and incubations will vary depending on the assay.
- 2) Washing steps are necessary for the separation of bound and unbound reagents. Typically, wells are washed a minimum of three times with a buffer, most commonly PBS, to maintain isotonicity.
- 3) The addition of reagents is in volumes typically ranging from 50-200 μ l/well.
- 4) Incubation for the reaction between antigen and antibodies is dependent on time, temperature, and pH at which the incubation takes place. Incubations can be done on stationary plates or with shaking.
- 5) Prevention of non-specific absorption from samples added after coating of solid phase. There is a variety of blocking agents available including normal rabbit serum,

- normal horse serum, human serum albumin, bovine serum albumin, gelatin, detergents, etc.
- 6) The addition of reagents conjugated to enzymes. Enzymes are usually conjugated to antibodies but they can also be bound to other reactants including protein A and protein G, or various avidins (for use with the biotin-avidin system). The four most commonly used enzymes are Horseradish Peroxidase (HRPO) plus hydrogen peroxide substrate, Alkaline Phosphatase plus p-Nitrophenylphosphate, β -Galactosidase plus O-Nitrophenyl- β -Galactopyranoside, and Urease, pH change, and Bromocresol Purple Indicator.
- 7) The visual reading of the assay. There are two ways of reading an ELISA one is by performing a simple visual assessment of relative color development. The other is by using a spectrophotometer to quantify the amount of colored product that is produced. Visual assessments are qualitative and usually are set up to have samples with high concentrations of target exhibiting an intense color, weakly positive samples with partial color, and negative samples with no color. Interpretations of color from operator to operator as well as distinguishing weak positives from negatives can vary and result in a more subjective reading. Spectrophotometric reading on the other hand is measured by emitting light of a specific wavelength and measuring the absorption of that light at wavelengths associated with the absorbance maximum of the product being generated (Id, Kks, & Library, 2016)(Crowther, 1995).

ALPHALISA

The AlphaLISA[®] (amplified luminescent proximity homogeneous assay) is a homogeneous, no-wash immunoassay with high sensitivity and wide dynamic ranges. It

is a bead based technology developed by PerkinElmer as a more efficient and sensitive alternative to the ELISA (Beaudet et al., 2008). The AlphaLISA[®] addresses certain shortcomings of ELISAs including narrow dynamic range often requiring large sample volumes and wide dilution series as well as relative insensitivity. It facilitates measurements of a wide variety of samples including cell culture supernatants, crude cell lysates, serum, and plasma that allow for the quantification of secreted and intracellular proteins. The procedures permit the use of very small sample volumes (1-5ul) while still maintaining a wide range of detection (100-fold analytical range).

Protocols for the AlphaLISA[®] can be set up as a competition assay or as a sandwich assay (**Figure 2.5**). There are three components to the assay: acceptor beads, donor beads, and analyte. The antibody against the specific antigen or analyte is covalently conjugated to an AlphaLISA[®] acceptor beads. A secondary antibody, preferably against a distinct, non-overlapping epitope, is biotinylated and bound to a streptavidin-coated donor bead. The binding of each antibody to the analyte brings the two antibodies near one another (within approximately 200 nm) generating an unstable singlet oxygen due to a laser irradiation of the donor beads at 680 nm activating a photosensitizer. The singlet oxygen triggers a cascade of chemical reactions and energy transfer events that result in a sharp and intense chemiluminescent emission at 615 nm, due to Europium contained in the beads (**Figure 2.6**). The assay can produce high sensitivity because it occurs in a dark background and the light emitted can be detected easily. For the method to work, the beads must be in close proximity to each other (which happens when the antibodies simultaneously bind the analyte) to get signal from the singlet oxygen. Conversely, unbound beads are not sufficiently close enough for the

acceptor beads to react with the singlet oxygen to produce a signal. The beads contain reactive aldehydes due to the latex-based hydrogels coating the surface. This provides two functions; it allows for a variety of different ligands or receptor binding patterns to be attached to it and, although it is not obvious why, it reduces non-specific binding (Eglen et al., 2008).

ASSAY VALIDATION

Validation of an assay confirms, through experiments, that an assay fulfills its intended use and that it will produce reliable and reproducible results. Three different steps categorize the process of assay development: assay optimization, assay qualification, and assay validation. Assay optimization includes experiments that determine assay conditions, range of matrix and sample elements, and parameters for assay performance. Assay qualification is an experimental approach that provides evidence for meaningful data under specific conditions. This step does not require sensitivity but rather simply specificity to verify the protocol. The third step is assay validation where a series of experiments evaluate the quantitative performance of the assay including robustness, precision, trueness, limits of quantification, dilution linearity, parallelism, recovery, selectivity, and sample stability (Andreasson et al., 2015). While it may not be necessary to perform these analyses to have an assay that generates reliable results, each type of assay assessment contributes to increasing the confidence that one can have in an assay.

Robustness (ruggedness) of an assay is its ability to remain unchanged by variations in method parameter (incubation time, temperature, sample preparation, buffer pH, etc.). Ways to measure this include determining the critical parameters and then

perform the assay with systemic changes in those parameters.

Precision is defined as the variability in the data obtained from the same sample under normal assay conditions. Three different types of precision exist: repeatability (r), intermediate precision (Rw), and reproducibility (R). Repeatability, also called within-day or within-run precision, is the variability observed when as many variables as possible are held constant within a short period. Intermediate precision accounts for all variability except for change in laboratory. Reproducibility, also called between-run or between-day repeatability, accounts for all varied factors over several days. Precision is more commonly reported as its inverse, imprecision, using the standard deviation and coefficient of variation (%CV).

Trueness is the comparison of the average values obtained in large samples to the acceptable reference value. This is measured by using the bias (b), which measures the systemic difference between the acceptable values and the test results.

Limits of quantification is used to determine the working range of the assay by using the lower and upper limits of quantification.

Dilution linearity provides evidence that a sample can be diluted without hindering accuracy or precision. If a sample is spiked, then it can be diluted to a working range for accurate results.

Parallelism is similar to dilution linearity but instead of spiking a sample with analyte, parallelism uses samples which contain high endogenous concentrations of the analyte to ascertain that, upon dilution, the binding characteristics are the same as the calibrator or standard.

Recovery is used to compare the detected response from analytes added to a

biological matrix versus in a solvent. Signals in a biological matrix can be affected by the drastic difference in environment compared to a solvent and this test could help find an appropriate solution that would mimic the results in a solvent.

Selectivity is the ability to differentiate between the analytes and other biological components that may be present. Knowledge of the analyte is crucial in this step to be able to identify other potential interferences. Known substances that are physiologically similar can be spiked into a sample and the degree in which interference occurs can be investigated.

Sample stability simply refers to the potential that storage conditions, freeze-thawing, etc. can introduce systemic errors and affect results. Continuous thawing and freezing aliquot tests and seeing how they are affected is an easy way of testing for sample stability.

The standard for assay validation is to do the experiments in the time period of a month to provide a snapshot of the assay method characterization.

FIBROBLAST GROWTH FACTOR 21

History of FGF21

Fibroblast growth factor 21 (FGF21) is a 209 amino acid polypeptide; in its mature protein form, it has 181 amino acids (~22,3 kDa molecular mass). It was first identified in mice embryos by using RT-qPCR and degenerate FGF19 primers from humans (Nishimura T et al., 2000). In humans, the gene coding for it is found on chromosome 19 and in mice on chromosome 7. The FGF21 gene is highly conserved among mammals; for example, there is approximately 80% identity between the human and rodent orthologs (F. M. Fisher & Maratos-Flier, 2016). FGF21 is a member of the

FGF superfamily (Sa-nguanmoo, Chattipakorn, & Chattipakorn, 2016). The family is composed of 22 members classified together by their structure although they incorporate a wide range of functions that include cell growth, development, angiogenesis and wound healing (Zhang et al., 2008)(**Figure 2.7**). FGF21 along with FGF19 ('FGF15' in mice) and FGF23 are atypical to the FGF superfamily because they act as metabolic regulators. Due to their lack of a heparin-binding capacity (Zhang et al., 2008), they can circulate in the blood after secretion. They still retain structural homology with FGFs that function in a paracrine manner. Most notably, they retain the capacity to bind to FGF-receptors (Kilkenny & Rocheleau, 2016)(**Figure 2.8**). FGF21 acts as an endocrine hormone that is synthesized and secreted mainly from the liver, although it has also been proven to be secreted from white and brown adipose tissues, skeletal muscle, the brain, and the heart. It has multiple target tissues with a half-life of around 1 hour in the blood of rodents and monkeys. (Kilkenny & Rocheleau, 2016).

Molecular Mechanism

Unlike other members of the FGF family, the endocrine FGFs (FGF19, FGF21 and FGF23) are unique due to their low-affinity to heparin sulfate proteoglycan (HSPG) co-receptors (Degirolamo, 2015). This low affinity allows the endocrine ligands to escape the extracellular matrix to enter the blood circulation to bind target tissues away from their secretion source (Kilkenny & Rocheleau, 2016). FGF21 binds to three types of tyrosine kinase receptors FGFR1, FGFR3, and FGFR4, with FGFR1 having the highest affinity (F. M. Fisher & Maratos-Flier, 2016). FGF receptors are considered low-capacity, high-affinity receptors and at the amino acid level, exhibit a significant degree of sequence homology. FGFRs are expressed throughout the body and tissues can

express multiple receptor isoforms. FGF21 also has a critical co-receptor β -Klotho, a single pass trans-membrane protein (Sa-Nguanmoo, Chattipakorn et al. 2016) (**Figure 2.9**). β -Klotho has a significantly greater extracellular sequence of 996 amino acids than cytoplasmic sequence of 27 amino acids. The small cytoplasmic domain suggests that β -Klotho has a limited role in activating signaling but rather facilitates interactions between FGF21 and its receptors (Kilkenny & Rocheleau, 2016). β -Klotho is expressed in the liver, pancreas, brown and white adipose tissue, and to a lesser degree in the hypothalamus and brainstem. The C-terminus of the FGF21 molecule binds to the FGF receptor and the N-terminus binds to the co-receptor β -Klotho leading to the auto-phosphorylation of the fibroblast substrate 2 (FRS2), the first step in downstream regulation (Tanajak et al., 2015). The phosphorylation of FRS2 activates components of the downstream pathway including RAS_RAF-mitogen activation protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)-serine threonine protein kinase (AKT), signal transducer and activator of transcription (STAT) and phosphoinositide phospholipase c (PLC) γ (Sa-nguanmoo et al., 2016).

In a fasting state FGF21 is induced by the nuclear receptor peroxisome proliferator-activated receptor- α (PPAR α) that is involved in lipid metabolism (Lundåsen et al., 2007)(Gälman et al., 2008). Mice that were on ketogenic diets or fasting, had elevated levels of PPAR α and FGF21 and PPAR α deficient mice had lower basal expressions of FGF21 than normal mice (Woo, Xu, Wang, & Lam, 2013). Increased PPAR activity is also observed in ob/ob mice. In a fed state on the other hand FGF21 regulates PPAR γ through a feed-forward loop mechanism, acting as an autocrine factor (Woo et al., 2013). FGF21 induces peroxisome proliferator-activated receptor γ co-

activator protein-1 α (PCG-1 α) a transcriptional coactivator protein (Potthoff et al., 2009). It regulates fatty acid oxidation through the enhancement of mitochondrial function and biogenesis (F. M. Fisher & Maratos-Flier, 2016). PCG-1 α is induced by nutritional status changes, and other physiological stimuli such as cold and exercise (Potthoff et al., 2009). During fasting, the liver induces PCG-1 α by stimulating mitochondrial metabolic capacity through DNA-binding transcription factors. The genes transcribed are involved in fatty acid oxidation, TCA cycle flux, mitochondrial oxidative phosphorylation, and gluconeogenesis (Potthoff et al., 2009)(**Figure 2.10**).

FGF21 has also been shown to inhibit growth hormone and Insulin Growth Factor-1 (IGF-1) in mice through a negative feedback loop (Ge, Wang, Lam, & Xu, 2012) (Inagaki et al., 2008). In a normal pathway, GH would bind to its receptor (GHR) activating JAK2 leading to the phosphorylation of signal transducer and activator of transcription 5 (STAT5) inducing IGF-1. But FGF21 is capable of suppressing STAT5 leading to the reduction of IGF-1 expression (Ge et al., 2012).

The Akt-1 pathway in skeletal muscle regulates FGF21. Akt-1, Serine-threonine protein kinase is activated by a variety of stimuli through a phosphatidylinositol 3-kinase (PI3-kinase) pathway. Akt-1 was seen to reduce white adipose tissue accumulation and improvements in metabolic parameters in obese mice. The activation of AKT-1 leads to release of myokines, small proteins secreted from muscle cells responsive to muscle contraction that have paracrine and endocrine functions. Akt-1 overexpression or insulin stimulation in mice skeletal muscle was seen to induce FGF-21 mRNA and protein (Izumiya et al., 2008).

Adipose Tissue

Most fat reserves in the body are comprised of both white and brown adipose tissue. White adipose tissue is mainly made of connective tissue and adipocytes containing large lipid droplets giving it a white or yellow appearance. Brown adipose tissue is mainly composed of iron containing mitochondria and dissipates chemical energy as a method to protect against obesity and hypothermia. It actively burns lipids to produce heat resulting in non-shivering thermogenesis (M. Fisher et al., 2012). In neonates, thermogenesis and sustained physical muscle activity are necessary for survival. After birth, the first appearance of these processes is due to increase in metabolic activity and brown fat non-shivering thermogenesis, which requires high metabolic fuel oxidation. Neonates are exposed to a large amount of fatty acids due to their presence in milk requiring PPAR α to mediate fatty acid catabolism and ketogenesis. Research done by Hondares et al., 2010 showed that in mice high levels of FGF21 were seen 2-6 days after birth and then slowly reduced over time. They concluded that postnatal feeding is required for the induction of FGF21 by comparing two experimental groups that were administered either glucose or lipid to mimic milk feeding. Those that were given the lipid produced FGF21 while those that were given glucose did not. FGF21 was also seen to cause thermogenic activation of brown adipose tissue. Brown adipose tissue already expresses β -Klotho, required for FGF21 action, in neonates (Ito et al., 2000).

Exercise and Nutrition

Nutrition plays a major role in FGF21 secretion (**Figure 2.11**). Starvation and ketogenic diets proved to increase circulating FGF21 levels, specifically protein intake,

irrelevant of energy intake (Morrison & Laeger, 2015). Laeger et al. arrived at this conclusion by exposing rats to 4 different dietary conditions for four days: low protein; normal energy, normal protein; low energy, low protein; low energy, and normal protein; normal energy. Low protein; normal energy rats saw significant increase in FGF21 levels as well as hepatic FGF21 mRNA expression. Rats that were exposed to normal protein; high energy saw the opposite effect and had a decrease in FGF21. It was hypothesized that ketogenic diets alter FGF21 due to the protein restriction in the diet. To test the hypothesis, they placed mice on four diets: control, ketogenic diet alone, ketogenic diet supplemented with carbohydrates, and ketogenic diet supplemented with an equal amount of protein. As seen previously, mice on the ketogenic diet and those supplemented with protein showed increased levels of FGF21. In contrast, mice on the ketogenic diet with carbohydrate supplementation had no effect on FGF21 (Laeger et al., 2014).

Exercise also plays a role in the induction of FGF21 due to its involvement in metabolism. Exercise helps prevent metabolic disorders due to the release of cytokines following exercise (myokines). They act as paracrine and endocrine factors, and have beneficial metabolic and physiological responses to exercise (Pedersen BK et al., 2012). FGF21 was seen to act as a myokine in metabolic disorders and FGF21 gene expression increased in mice after acute exercise. In mice and healthy men, a single bout of acute exercise increased serum levels of FGF21. FGF21 levels were higher in subjects that had high-intensity exercise than those of mild exercise suggesting that FGF21 increased is proportional to intensity of exercise. It was also noted that FGF21 expression after exercise was found to increase in the liver but not in white adipose tissue or skeletal muscle of mice.(Kook Hwan Kim et al., 2013)

Research showed that administration of FGF21 reduced sweet and alcohol preference in mice and sweet preferences in cynomolgus monkeys. FGF21's effects were directly on the brain and administration of FGF21 for 2 weeks lowered dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA) and 3-methoxytyramine (3-MT) concentrations, except in the caudate putamen (Talukdar et al., 2015). Loss of FGF21 increased sugar preferences in heterozygous and knockout mice in the two-bottle test. It was concluded that FGF21 couldn't act directly on the taste buds due to the lack of β -Klotho receptors in the taste epithelium. Sugar ingestion stimulates FGF 21 in the liver through the transcription factor carbohydrate response element binding protein (ChREBP) and the loss of that signal increases sugar consumption (von Holstein-Rathlou et al., 2015).

Recent research found that fructose ingestion acutely stimulates FGF21 levels in humans. Subjects fasted overnight and were either given 75 g of fructose, 75 g of glucose, or a combination of 37.5 g of fructose and 37.5 g of glucose all dissolved in 225 ml of water. Blood samples were collected every 30 min to 1 hour. Subjects given fructose exhibited elevated levels of FGF21 at two hours between 1.5 and 6.6-fold. By three hours subjects returned to baseline FGF21 levels. Subjects given glucose on the other hand saw a decrease in FGF21 within the first hour and rising gradually peaking at 4 hours followed by a decrease to baseline levels. Although the mechanisms to explain this difference are still uncertain there is speculation that it is due to ChREBP. ChREBP is highly expressed in the liver and is activated by products of carbohydrate metabolism. The liver effectively extracts and metabolizes fructose leading to speculation that the fructose challenge might activate ChREBP and stimulate FGF21 production. Although

FGF21 has a historical role in fasting metabolism the research done here shows that FGF21 may play an imperative role in fructose metabolism in humans (Dushay, Toschi, Mitten, Fisher, & Herman, 2015).

Obesity and Diabetes

Obese individuals, type 2 diabetic patients, and people with dyslipidemia, insulin resistant diabetes and nonalcoholic fatty liver disease all show higher circulating FGF21 expressed from the liver and adipose tissue than normal individuals (Yu et al., 2011)(M. Fisher et al., 2010). The increase in FGF21 level can be explained by the increased in free fatty acids (FFAs), which can stimulate FGF21 (Yu et al., 2011). Transgenic mice with over expression of FGF21 were resistant to diet induced obesity and metabolic disturbance (Zhang et al., 2008). In research done by Zhang (Zhang et al., 2008), serum FGF21 levels were measured in 232 human subjects and the levels ranged from 45.3 to 795.2 ng/L. FGF21 in obese individuals had a median of 292.8 ng/L and individuals with hypertension showed a median 289.9 ng/L. Lean individuals had a median of 208.7 ng/l and individuals without hypertension had a median of 156.5 ng/l. There was no difference between sexes and no significant difference between diabetic and non-diabetic. They also found that there was a positive association with serum FGF21 levels with age, adiposity (BMI, waist circumference, waist hip ratio, and fat percentage), and insulin resistance. Despite having high levels of FGF21, obese individuals fail to exert the regular effects on glucose homeostasis and lipid oxidation due to the disrupted FGF21-FGFR1- β -klotho pathway leading to the decrease in ERK1/2, Akt, and AMPK phosphorylation (Tanajak et al., 2015)(**Figure 2.12**). Administration of pharmacologic doses of FGF21 to ob/ob mice leads to improved glucose tolerance and reduced serum

triglycerides (M. Fisher et al., 2010). It was also found that autophagy deficiency and subsequent mitochondrial dysfunction promote FGF21 expression and promotes the protection from diet-induced obesity and insulin resistance (K H Kim et al., 2013).

The Heart

FGF21 also plays a critical role in the heart. Mice lacking FGF21 were more prone to developing cardiac hypertrophy (A Planavila et al., 2013). Features of cardiac hypertrophy include increase in cardiomyocytes, enhanced protein synthesis, and higher degree of sarcomere organization (Ana Planavila, Iglesias, Giralt, & Villarroya, 2011). FGF21 treatment has shown to protect against hypertrophic responses, exert cardio protective effects in myocardial infarction, cardiac ischemia reperfusion injury, and diabetic cardiomyopathy. FGF21 also protects the heart from apoptosis (Tanajak et al., 2015). The heart expresses high amounts of FGF receptors as well as significant levels of β -klotho protein and FGF21 is released by cardiomyocytes, cardiac microvascular endothelial cells (CMECs) and cardiac cells (both a target and a source of FGF21). In cardiac cells FGF21 activates the ERK1/2-CREB-Sirt1-PGC1 α signaling pathway. This leads to the activation of PPAR α , which stimulates FGF21 expression as well increasing mitochondrial fatty acid oxidation preventing hypertrophy (Ana Planavila et al., 2011).

The Brain

Although FGF21 is mainly secreted from the liver the brain is also capable of producing its own FGF21 by glial cells as well as being able to diffuse it through the blood brain barrier (BBB). FGF21 in the brain has been detected in specific regions of the midbrain that include dopaminergic regions. Receptors for FGF21 are also expressed through the nervous system. β -Klotho, FGF21's co-receptor, has also been seen in the

suprachiasmatic nucleus (SCN), the dorsal vagal complex (DVC) of the hind brain, the area postrema (AP), the nucleus tractus solitarius (NTS), and the paraventricular nucleus (PVN). During starvation, cross talk mediated by FGF21 between the liver and the brain helps maintain homeostasis. Starvation increases the circulating levels of FGF21 allowing it to cross the BBB. Increased binding in the brain occurs, leading to the phosphorylation and activation of Erk1/2. Corticotrophin releasing hormone (CRH) is then secreted, stimulating the secretion of adrenocorticotrophic hormone (ACTH) releasing adrenal corticosterone from the adrenal cortex. The adrenal corticosterone binds to receptors in the liver that induce PCG-1 α expression which mediates expression of glucose 6 phosphate (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes that increase hepatic gluconeogenesis during fasting to prevent hypoglycemia. Higher levels of FGF21 also affect the SCN, in the hypothalamus, leading to infertility. The neuroendocrine axis is disrupted leading to a decrease in both AVP and Kiss 1 gene expression. This ultimately leads to irregular ovulation because of the reduction in kisspeptin, followed by reduced GnRH production, and in turn a lower LH surge (Sanguanmoo et al., 2016).

FGF21 also demonstrate a circadian rhythm with a nocturnal pattern that rises past midnight. Other metabolic hormones that have nocturnal rises include: leptin, GH, vasopressin and cortisol. FGF21 follows a similar pattern as cortisol and FFAs and the opposite patterns of insulin and glucose. Clinical studies were done by Yu et al., 2011, where 19 subjects were given 3 standardized meals a day (breakfast 08:00, lunch at 13:00, and dinner at 18:00). Blood samples were drawn by an indwelling venous catheter in the forearm every 30 min for 24 hours. Insulin, glucose, cortisol and Free Fatty Acids

were all measured. As expected there were spikes in glucose and insulin 30-60 min after meals. The lowest times for insulin and glucose were between midnight and early morning when FGF21 levels were highest along with cortisol (**Figure 2.13**). The FFA peak occurred 4 hours prior to the FGF21 peak supporting that FFAs stimulate FGF21 levels through PPAR α (**Figure 2.14**). Because both cortisol and FGF21 have similar onset and peak times, it is speculated that similar circadian clock genes might control both them.

It was also observed that the magnitude of the nocturnal rise in FGF21 observed in obese individuals is reduced compared to lean individuals. Obese individuals were shown to have a higher circulating FGF21 in the day and an impaired nocturnal rise and reduced circadian rhythmicity. Ten individuals were measured every 30 minutes for their FGF21 levels. The patterns for both obese and lean individuals were relatively similar only that the obese levels were higher. But the peak levels on both obese and lean individuals were the same, making the peak for obese individuals less drastic than that of lean individuals (Yu et al., 2011).

Figure 2.1. Representation of the basic structure of an IgG molecule where two light chains are bound to two heavy chains by sulfide bonds creating a Y shape. The arms of the Y represent the variable region (where the antigen binds) and the base represents the constant region (Crowther, 1995).

Figure 2.2. The four basic Elisa methods A) Direct, B) Indirect, C) Sandwich (direct) D) Sandwich (indirect) (*Crowther, 1995*).

1. Attach antibody to solid phase

2. Incubate with enzyme-labeled antigen in presence or absence of standard antigen or unknown sample

3. Incubate with enzyme substrate

Figure 2.3. Schematic representation of a competitive solid phase ELISA. The first step is to attach desired antibody to a solid phase, followed by incubation of enzyme-labeled antigen and unknown sample. The third step is to incubate with an enzyme substrate and measure the absorbance arising from product formation (*Perimann et al., 1980*).

1. Attach antibody to solid phase

2. Incubate with sample

3. Incubate with enzyme-labeled antibody

Figure 2.4. Schematic representation of a sandwich ELISA where the first step is to attach the desired antibody to a solid phase, followed by incubations with sample. The third step is then incubating with enzyme-labeled antibody and measuring the absorbance (*Perimann et al., 1980*).

Figure 2.5. AlphaLISA assay protocol provided by PerkinElmer. The first step is a one hour incubation at room temperature of the standard, buffer, biotinylated antibody, and AlphaLISA acceptor beads. The streptavidin coated donor beads are then added and are incubated at room temperature for 30 minutes. The plate is then read on the EnVision (Beaudet *et al.*, 2008).

Figure 2.6. The basic principle behind the AlphaLisa technology. A biotinylated antibody binds to the streptavidin-coated donor beads while a second antibody is directly conjugated onto an AlphaLisa acceptor bead. The presence of the analyte brings the two beads in close proximity. Excitation of the donor bead at 680 nm generates a singlet oxygen molecule. This triggers a series of chemical reactions in the acceptor bead resulting in an emission of light at 615 nm (*Beaudet et al., 2008*)

Figure 2.7. Phylogenetic representation of the Fibroblast Growth Factors Family comprised of 22 members that can be divided into three distinct subfamilies: intracrine, paracrine, and endocrine. FGF21 along with 15, 19, and 23, belong to the endocrine FGFs and are unique and act as metabolic regulators (Tanajak *et al.*, 2015).

Figure 2.8. Critical domains of paracrine and endocrine FGFs. Despite variability in biological function, paracrine and endocrine FGF peptides share a conserved core sequence of around 120 amino acids and present as a class of proteins of 18–24 kDa molecular mass. An N-terminal signal peptide (SP) and FGFR binding site (FGFR; directs interaction with high-affinity FGFRs) are also common sequence features. The C-terminal sequence displays some variability and is required for association with low-affinity HSPGs (HB; paracrine ligands) or klotho co-receptors (KL; endocrine ligands) (*Kilkenny & Rocheleau, 2016*).

Figure 2.9. Structure of KLB contains a large extracellular domain of 996 amino acids and a cytoplasmic domain of 27 amino acids. The extracellular region contains putative N-glycosylation sites (indicated by stick and ball markings) and two inactive glycosyl-hydrolase domains (D1, D2) maintained for binding galactoside sugars. (*Kilkenny & Rocheleau, 2016*).

Figure 2.10. Free fatty acids (FFA) released from adipocytes activate PPAR α leading to the induction of FGF21 via transcriptional activation. FGF21 secretion can act in an endocrine or autocrine/paracrine manner affecting ketogenesis, gluconeogenesis, fatty acid oxidation, and GH resistance (*Ge et al., 2012*)

Figure 2.11. Mechanism of FGF21 secretion in both fed and fasting states. During fasting or from a ketogenic diet free fatty acid levels activate PPAR α increasing FGF21 expression. In a fasting state glucagon is released binding to glucagon receptors in the liver. cAMP is activated affecting other downstream targets such as protein kinase a (PKA) and exchange proteins activated by cAMP (EPAC) leading to the activation of AMPK and p38MAPK, resulting in increased FGF21 (*Sa-nguanmoo et al., 2016*).

Figure 2.12. Normal mechanism of FGF21 in adipocyte. Obese individuals have decreased ERK1/2 and AMPK in the FGF21 pathway, diverging from normal (*Ge et al., 2012*). Such decreases in this branch of the pathway may contribute to the FGF21 *resistance* that has been described in the obese.

Figure 2.13. 24-hour metabolic profile of serum FGF21 overlaid with (A) insulin, (B) glucose, and (C) cortisol metabolic profiles. Serum samples were collected every 30 minutes from 19 volunteers (Yu *et al.*, 2011).

Figure 2.14. Serum samples collected every 30 minutes from 19 volunteers comparing a 24 hour profile of serum Free Fatty Acids and FGF21 (*Yu et al., 2011*).

CHAPTER 3

DEVELOPMENT OF AN FGF21 ASSAY

ABSTRACT

Obesity and diabetes play a major role in many diseases prevalent in our current society. Fibroblast Growth Factor 21 (FGF21) is a recently discovered hormone that has potential for therapeutic treatment of obesity and diabetes. It acts as a hormone in times of metabolic stress and is shown to stimulate hepatic fatty acid oxidation, ketogenesis, induce weight loss, allow for sustained decrease in plasma glucose and triglycerides, and decrease the growth hormone response. Obese or diabetic individuals often have higher than normal amounts of circulating FGF21; consequently, they may be considered FGF21 resistant. It is then advantageous to have an assay to measure FGF21 that will allow us to study FGF21 further. It is also important to study FGF21 in various livestock species for potential biomarkers, or as indicators of carcass quality that could prove to advance further research. We have developed a working FGF21 assay using an amplified luminescent proximity homogeneous assay (AlphaLISA). It is a homogeneous, no-wash immunoassay with high sensitivity and wide dynamic range. It is a bead based technology that brings two antibodies near each other allowing for an unstable singlet oxygen to trigger a downstream cascade of chemical events leading to a sharp intense chemiluminescent emission that can be read on an EnVision machine. The assay was set up using a sandwich model with guinea pig or rabbit anti-bovine FGF21 antibodies attached to the acceptor beads and biotinylated rabbit anti-bovine FGF21 antibodies attached to a streptavidin coated donor bead. The assay has a working range from .2-200

ng/ml. Standard curves in serum from bovine, porcine, rabbit, rat, ewe, fetal bovine has been conducted and have optimized assay conditions. Manipulation to serum were necessary in order to get functional curves that included diluting it 2 fold or further depending on the species and increasing incubation temperatures. The assay can function using both serum and plasma.

INTRODUCTION

The ability to measure target proteins and other compounds in biological fluids is critical for understanding the biological role of such molecules. The ability to quantify target molecules was advanced dramatically by the development of the radioimmunoassay (RIA) in the late 1950s. The radioimmunoassay was the first of its kind to be both sensitive and specific using radioactivity as the label to be measured. It started to be used across all fields including endocrinology, pharmacology, microbiology, and biochemistry. However, it was clear that there were problems with the routine use of RIAs. There were concerns related to radioisotope exposure, the handling of radioactive waste, the need for special facilities, and the use of expensive counting equipment. Efforts to establish alternative techniques were ongoing. One that was created was the Enzyme-Linked Immunosorbent Assay (ELISA). The ELISA uses a similar concept as an RIA but instead of using radioactivity labeling the measurement of a target is performed indirectly by measurement of an enzyme. Kits for both assay formats are available commercially for many different hormones and proteins. The limitation of the ELISA is its narrow dynamic range, the need for large volumes, wash steps, and lengthy assays. Amplified luminescent proximity homogeneous assay (AlphaLISA), developed by PerkinElmer, addresses many of the shortcomings of classical ELISAs. The AlphaLISA is a no-wash immunoassay that uses bead based technology with high sensitivity and a wide dynamic range. The protocol can be applied to small volumes of sample (1-5 μ l) while still maintaining a wide range of detection. The analyte brings an acceptor bead directly conjugated to an antibody in close proximity to a biotinylated antibody bound to a streptavidin covered donor bead. At approximately 200 nm, excitation of the donor

beads with a light beam of 680 nm elicits the generation and unstable singlet oxygen that triggers a cascade of chemical reactions and energy transfer events that result in a sharp chemiluminescent emission at 615 nm from the acceptor beads.

This technology is ideal for measuring FGF21 due to its sensitivity and ability to measure small quantities in biological samples. FGF21 has potential as a therapeutic treatment for individuals suffering from obesity or diabetes. There is much yet to understand about this unique fibroblast growth factor before any clinical use can occur. The development of a reliable FGF21 assay is a small step towards that goal.

MATERIALS AND METHODS

ANTIBODY PRODUCTION

Bovine, human and mouse FGF21 were donated by Arie Gertler from Protein Laboratories Rehovot in exchange for created antibodies. Both guinea pigs and rabbits were immunized against bovine FGF21. FGF21 was diluted with 1X Phosphate-buffered saline (10X PBS: 80 g NaCl, 2 g KCl, 6.1 g Na₂HPO₄ anhydrous) to create a final concentration of 1 mg/ml. The FGF21 antigen was then mixed extensively with equal amounts of Freund's complete adjuvant to create an emulsion. Guinea pigs were given 1 ml of antigen-adjuvant emulsion spread over 5 to 8 subcutaneous injections in the flank. The rabbits were given 2 ml of the emulsion as subcutaneous injections in several sites along the flank. Animals were boosted once a month and blood collection occurred 12 days after the booster injection.

After successful immunization and boosts, blood was collected for serum use. Acepromazine (1mg/kg) was given to sedate the rabbits. Blood was collected from the central ear artery by using a 19 gauge 3/4-inch butterfly needle. Guinea pigs were given telazol to anesthetize them; blood was collected from the anterior vena cava by using a 20 gauge Bd precision glide needles, usually yielding ~7 ml. Blood was allowed to clot overnight at 4°C and then centrifuged at a speed of 2800 rpm to separate serum from blood solids.

ANTIBODY PURIFICATION

Antibody was purified by using Caprylic Acid according to protocols found in Chapter 8 page 300 of *Antibodies: A laboratory manual* by Ed Harlow and David Lane. Two volumes of Sodium Acetate Buffer (consisting of: 60mM Sodium Acetate and 40mM Acetic Acid at a pH of 4.6) were added to pooled serum. The pH of the solution

was adjusted to 4.8 if necessary. Caprylic acid was then added slowly to the serum (0.75 ml of caprylic acid for every 10 mL of the original volume). The solution was then centrifuged for 10 min at 5,000 RPM. The supernatant was then filtered using standard coffee filters and put in a dialysis tubing with a pore size of 50K molecular weight cutoff (MWCO) and dialyzed against PBS overnight.

Ammonium sulfate precipitation at 50% saturation was then used to isolate the antibodies. The precipitation was allowed to proceed at 4 C overnight.

ELISA PROTOCOL

Plates were trapped with rabbit anti-FGF21 antibody in 0.1M Sodium Bicarbonate NaHCO_3 , pH 9.5 at a concentration of $5\mu\text{g/ml}$ and stored at 4 C overnight. The plate was then blocked with 1.5% non-fat dried milk (NFDM) in NaHCO_3 solution and left on rocker at room temperature for one hour. Standards ($50\mu\text{L/well}$) were then added and $100\mu\text{l/well}$ of diluent, either serum or buffer TBST block, comprised of 0.5% NFDM in 1X TBST (1 mM Tris pH 8, .75 M NaCl, .25% Tween 20, and .25 mM Thimerosal). The plate was then incubated with shaking for two hours at 37°C . The plate was washed twice with 1X ELISA plate wash solution (.154 M NaCl, .05% Tween 20, and .25 mM Thimerosal). Biotinylated guinea pig anti-FGF21 antibody ($100\mu\text{l/well}$) at a concentration of ($10\mu\text{g/ml}$) in 0.1x TBST block was added to each well and rocked gently at room temperature for one hour. The plate was then washed and $100\mu\text{l/well}$ of Alkaline Phosphatase-conjugated AffiniPure Goat Anti-guinea pig IgG ordered from Jackson Immuno Research (1:2000 dilution) in 10X Alkaline Phosphatase buffer (1X solution: .01 M Tris, .01 M NaCl, .5 mM MgCl_2) was added and left on rocker at room temperature for 30 minutes. Plates were washed with ELISA plate wash solution and 100

μ l/well of a 1 mg/ml solution of p-Nitrophenyl phosphate (PNPP) was then added to each well. Once product color was sufficiently developed (typically about 15 minutes) the plate was measured at 405 nm in a plate reader (ELx808 from Bio-Tek).

WESTERN AND STAIN PROTOCOL

Protocols were taken from Molecular Cloning: A laboratory Manual 4th Ed. (Green MR, Sambrook J. 2012). FGF21 from different species were resolved on 15% SDS-PAGE gels. 15% acrylamide resolving gel was made using: 36 mL 40% Acrylamide, 24 ml resolving gel, 40.5 ml DDH₂O, 480 μ L 20% Sodium Dodecyl Sulfate (SDS), 480 μ l 10% Ammonium per sulfate (APS), 40 μ l tetramethylethylenediamine (TEMED), and left in fridge overnight. The stacker gel (600 μ l 40% Acrylamide, 1.2 ml Stacker Buffer, 3ml DDH₂O, 24 μ l 20% SDS, 18 μ l 10% APS, 2-4 μ l TEMED) gel solution was pipetted on top of gel and a gel comb was inserted to create wells for loading the samples. Bovine, human, and mouse FGF21 with added dye (house made 6X SDS page dye) were denatured by boiling water. 30-35 μ l of sample (2 μ g of protein in sample for western and 10 μ g of protein for stain) was added to each well and 10 μ l of standard (BenchMarkTM Prestained Protein Ladder). Bath was filled with SDS and electrodes were set at 15 mA and left to run for 3-4 hours.

Western Blotting

Immobilon-P paper was rehydrated in methanol and left to soak in transfer buffer along with 6 pieces of chromatography paper. 3 pieces of chromatography paper, then immobilon-P paper was set up followed by the gel and 3 more chromatography papers. These were all put in the transfer cage that is filled with transfer buffer. Transfer was set at 120 mA and left over night. Immobilon-P paper was then left in blocking solution made up of 3% NFD, 3% bovine serum albumin (BSA) in TBST for one hour then

washed twice with TBST. It is then probed with a 1:2000 dilution of our rabbit anti-FGF21 antibody in TBST block and left on rocker for one hour. It is washed twice with TBST and then probed with a 1:2000 dilution of Alkaline Phosphatase-Conjugate AffiniPure mouse anti-Rabbit IgG in TBST block for one hour.

Stain

Some SDS-PAGE gels were stained to reveal total protein present. After the proteins were resolved, the gel was put in Tupperware and rinsed with DDH₂O. It is then left on rocker overnight in GelCode Blue Stain Reagent. Stain reagent is then dumped out and stain is left to soak in DDH₂O to de-stain for several hours.

ALPHALISA PROTOCOL

Antibody Biotinylation

Biotinylation was done at a 5:1 molar ratio of biotin to IgG using the biotinylation reagent, EZ-Link™ NHS-LC-Biotin at 3 mg/ml dilution in 100 % Dimethyl-Formamide (DMSO). The mixture was left to mix on a rotating mixture at 4°C for 3 hours or overnight. The solution was then dialyzed against PBS to stop the reaction. A Bradford was run from Protocol from Molecular Cloning: A laboratory Manual 4th Ed. (Green MR, Sambrook J. 2012) to determine the concentration of biotinylated antibody (**Appendix**).

Acceptor Bead Conjugation

The desired amount of AlphaLisa Acceptor Beads (Perkin Elmer) was acquired and prepared by transferring them into a 1.5 mL Eppendorf tube adding equal amounts of 1X PBS and centrifuged for 15 minutes 16,000 G force. The supernatant was carefully removed from the tube. Antibodies (concentration 1mg/mL), 1.25 µL of 10% Tween-20 were added together creating a total reaction of 40 µl per mg of beads. In a separate Eppendorf tube, NaBH₃CN was prepared in water to reach a concentration of 25mg/ml.

5 μ l of diluted NaBH₃CN was added to the antibody-bead mixture. It was then completed with enough sodium phosphate buffer (130 mM Na₂HPO₄ pH 8.0); the general rule being 200 μ l total volume for 5 mg of beads. Beads were left to incubate at 37°C overnight.

The next step involved blocking the acceptor beads. The first step was to prepare fresh CMO (65 mg/ml) in 800 mM NaOH. 10 μ l of CMO was added per 200 μ l of beads. Beads were left to incubate at 37°C for 1 hour then centrifuged at maximum speed for 15 minutes and the supernatant was discarded. The bead pellet was resuspended in 500 μ l of 0.1 M Tris-HCL pH 8.0 (1 ml per 5 mg of bead). Beads were centrifuged at maximum speed for 15 min and repeat resuspension 2 more times. The last suspension should be done in 200 μ l per mg of beads in PBS and 0.05% ProClin 300 (optional).

AlphaLISA Protocol

$\frac{1}{2}$ AreaPlate™-96 (Perkin Elmer) was utilized. 5 μ l of standard was pipetted per well in a 1:5 dilution series. Then, 5 μ l of the conjugated acceptor beads were added in 1 mL of AlphaLisa 1X Immunoassay buffer (AL000C Lot:2106504) diluted in water to a 10X solution. Following the addition of 20 μ l of the AlphaLisa conjugated acceptor bead solution to each well. Plate was left to incubate for 1 hour at room temperature.

The next step was to prepare the AlphaScreen Streptavidin-coated Donor Beads (catalogue #676002S Lot:2177463) Perkin Elmer (light sensitive). 32 μ l donor beads were added to 2 mL of AlphaLisa buffer. 25 μ l of donor beads was then added per well. Plate incubated for 30 minutes and then read on the PerkinELmer EnVison machine at a wavelength of 615 nm.

PRECISION STATISTICS

Inter- and intra-assay coefficient of variation were calculated to evaluate assay precision. The CV was calculated by dividing the standard deviation of reference sample replicates (6 replicates) by the average of the replicates and multiplied by 100.

VALIDATION PROCEDURES

Validation for the assay was done by testing the standard AlphaLISA protocol stated above under various conditions. Multiple experiments were done testing various antibody combinations, various combinations of assay conditions (e.g. incubation time, incubation temperature, shaking, and rocking), different sera (bovine, porcine, ovine, rabbit, rat, fetal bovine), plasma (bovine and porcine), cross-reactivity between bovine, human, and mouse FGF21, selectivity by spiking known amounts of FGF21 in serum and removing it via protein A or anti-FGF21 avidin affinity column, and running multiple biological samples of animals under different physiological conditions.

EXPERIMENTS

Experiment 1: Antibody Selection

The first step in establishing and optimizing the FGF21 assay was to identify the ideal antibody combination to use. Both rabbits and guinea pigs were immunized against bovine FGF21 and both were conjugated to acceptor beads and biotinylated. Four different standard curves were then run examining different combinations of antibodies. The combinations used included only rabbit antibodies, only guinea pig antibodies, rabbit conjugated to acceptor beads and biotinylated guinea pig antibodies and the reverse (**Figure 3.1**).

All four combinations proved to be successful. The curve that used guinea pig only antibodies had a greater dynamic range with the highest counts reaching just over 10,000 counts where the others fell in a range of 1,500-2,500 counts.

Experiment 2: Serum Effect

After successful standard curves created in buffer with our desired antibodies, we evaluated its effectiveness in bovine serum. Three standard curves in serum were run with either 50%, 25%, or 12.5% serum (**Figure 3.2**).

Serum that was diluted 50% did not produce a visible curve but showed random counts ranging from around 6000 to a little over 9000 counts. These counts were overall significantly higher than the other two curves. Standard curves with diluted serum of 25% or 12.5% both yielded visible curves. The highest counts for both curves fell between 1,500 and 1,700 counts, but the lowest range for serum diluted 25% was around 800 where serum diluted 12.5% was in the mid 300s.

This series of standard curves showed that serum contributes to interference in the assay, which can be dealt with by diluting serum.

Experiment 3: Species Variety

We then tested a variety of different species serum to assess the versatility of the assay. Standard curves using rabbit, rat, ewe, fetal bovine, porcine, and bovine serum diluted at 50% were run in standard AlphaLISA conditions (**Figure 3.4**).

Under normal conditions, the AlphaLISA assay successfully worked with rabbit, rat, ewe, and fetal bovine serum diluted at 50% with relatively low background. The diluted bovine and porcine serum on the contrary did not yield any clear curves under these parameter conditions. Both sera were then diluted further to 25% and 12.5% as well as altering incubation temperatures from room temperature to 37°C. Under these conditions, bovine serum diluted to 25% yielded a clear standard curve while porcine serum still yielded no curve (**Figure 3.2**). Porcine serum was then diluted further to 10% and incubation temperatures were elevated to 42°C. Under these conditions, porcine serum yielded a successful standard curve (**Figure 3.3**). Background in the assay though increased significantly compared to the other species serum with the counts starting at 8,000 where the others started on average in the 100s.

Experiment 4: Endogenous FGF21(Selectivity)

The next experiment done was to run serum with spiked FGF21 and samples that had spiked and followed by the removal FGF21. In 1 mL of ovine serum, 100 ng of FGF21 standard was spiked, along with 10 μ g of rabbit anti-FGF21 or anti-PAG (pregnancy associated glycoproteins) antibody, which would not be expected to bind the spiked FGF21. 10 μ l of protein A matrix was then added to all sera, including the control, and left to rotate overnight. The protein A matrix was isolated by centrifugation and the serum supernatant was used in the AlphaLISA FGF21 assay. Different standard curves were performed using four different sera: control serum, serum spiked with 100 ng of

FGF21, serum spiked with FGF21 and rabbit anti-FGF21 antibody, and serum spiked with FGF21 and rabbit anti-PAG antibody (**Figure 3.5**).

The control standard curve had a range of 426 counts with the highest counts in the mid 500s and lowest counts in the low 100s. Serum that was treated by spiking 100 ng FGF21 showed a standard curve that was vertically shifted, as expected, with highest detected counts reaching 900 and lowest counts reaching 600 but with a lower dynamic range of 305 counts. Serum that was treated by spiking FGF21 and rabbit-anti FGF21 had a dynamic range of 245 counts, 2-fold lower than the control range, but curve itself was shown to overlap the control curve. Serum spiked with FGF21 and rabbit anti-PAG antibodies showed a curve with a dynamic range of 246 counts ranging from 663 counts to 417 counts.

In this experiment, we showed that we could successfully detect FGF21. We were also able to remove FGF21 from the serum, showing that our antibodies are specific to FGF21. The non-specific antibody did not result in the removal of any signal. The range of the standard curve that was spiked with FGF21 and rabbit-anti FGF21 had a diminished range indicating that we very likely did not successfully remove all the antibodies from the serum. The presence of unbiotinylated anti-FGF21 probably acted as a competitor for binding to FGF21 in the standard curve; this is probably the reason for the lower curve signal in that sample.

To further evaluate if we had removed all the FGF21 that we had spiked in, we ran our pig serum through an anti-FGF21 avidin affinity column. An ELISA was run where six different samples were analyzed in triplicates: normal serum, normal serum run through column, serum spiked with 500 ng/ml of FGF21, serum spiked with 500 ng/ml of

FGF21 and run through column, serum spiked with 500 ng/ml of PAG, and serum spiked with 500 ng/ml of PAG and run through column.

Results (**Table 3.1**) showed a standard curve with an absorbance range from 1.2 to .2. The normal pig pool not run through the affinity column and the pig pool not run through the affinity column spiked with PAG both showed 0 ng/ml. The normal pool and sera spiked with FGF21 or PAG showed concentrations between 9-17 ng/ml after being passed through the column. The pig pool spiked with 500 ng/ml of FGF21 had an average concentration of 3,278 ng/ml with a recovery percent of 655. This increased amount of FGF21 detected in the sample is due to some leaching of FGF21 antibodies. This can also be seen with the non-treated control sera that was run through the column having a little bit of FGF21 detected.

With this experiment, we could show that we successfully spiked FGF21 detectable by the ELISA and able to remove it through an immuno-affinity matrix column and that little other non-specific binding was occurring because samples that had PAG in it read results similar to our normal serum.

Experiment 5: Cross-species reactivity and Parallelism

To continue the evaluation and versatility of the assay, a variety of assays were run to identify any parallelism between bovine, human, and mouse FGF21 with our working assay. A western blot and a stain were run to examine the functionality of our anti-bovine FGF21 antibodies against human and mouse FGF21 (**Figure 3.6**). Both the western and the stain showed bands for all three FGF21s that were between 19-26 kDa verifying that the rabbit-anti bovine FGF21 successfully bound to the human and mouse FGF21.

We then ran three standard curves using identical amounts of either bovine, human, or mouse FGF21 on both the AlphaLISA (**Figure 3.7**) and ELISA (**Figure 3.8**). The bovine FGF21 standard curve showed a successful curve but the human and mouse FGF21 showed no signs of binding. This could be due to the human and mouse FGF21 having different protein conformations that could be hiding or blocking the epitopes that our antibodies bind to. To address this problem three identical ELISA plates were run with different conditions. One plate was set to incubate overnight at 4°C, giving the antibodies time to recognize epitopes and reach equilibrium, the second plate was incubated for 2 hours at 42°C, and the third plate had .1% SDS added to the standards, in attempts to slightly denature the protein to potentially increase binding. These altering conditions did not yield any improvement in the results.

Experiment 6: Correlations between AlphaLISA and ELISA

We also decided to do calculate the correlation between the results we got from both the AlphaLISA and the ELISA of the bovine, human, and mouse FGF21 standard curves using the data analysis package in Excel.

Between the ELISA results for the bovine, human, and mouse FGF21, there was a 0.8 correlation between the bovine and human results. However, no correlation existed between bovine and mouse FGF21 or human and mouse FGF21.

Between the Alpha results for the bovine, human, and mouse FGF21, there was a 0.6 correlation between bovine and human FGF21, 0.9 correlation between bovine and mouse FGF21, and 0.5 between human and mouse FGF21.

Comparing the AlphaLISA results and the AlphaLISA results we see a 0.96 correlation between the bovine results, 0.4 correlation between the human results, and .2 between the mouse results.

Experiment 7: Plasma

We also tested the assay parameters by evaluating its efficacy in plasma rather than serum. The standard curve was set up using 12.5% serum in buffer and all incubations for steer serum and plasma were at room temperature while all incubations for pig serum were at 42°C, which were the optimal conditions for performing the assay in pig sera.

Both serum and plasma proved to work in this assay. The steer serum curve had a higher background compared to the plasma curve but it also had a steeper curve with a wider dynamic range. (**Figure 3.9a**).

Similarly, pig serum had higher background than the plasma as well as higher variation but with a wider dynamic range than that of the plasma curve with almost 2-fold difference. (**Figure 3.9b**).

Although plasma in both steer and pig serum had a lower range than serum, its ability to detect lower counts and have lowered background may prove to be a better alternative to using serum when measuring FGF21 that has previously shown high background in the regular assay such as with steer and pig serum.

Experiment 8: Precision

Once we had our working assay we wanted to evaluate its precision. To achieve this, we calculated the coefficient of variation (CV), used to identify the variability relative to the mean, between different standard curves.

For the inter-assay, CV was calculated from 4 different standard curves run by the same individual throughout three different time points within the same day. The CV for a sample containing 200 ng of FGF21 is 8.2%, 50 ng is 4.6%, and 0.195 ng is 10.1%.

For the intra-assay, CV was calculated from 4 different standard curves run by two different individuals done at the same time of day. The CV for a sample containing 200 ng of FGF21 is 12.9%, 50 ng is 20.23%, and 0.195 ng is 10.57%.

Experiment 9: Physiological FGF21

Having successfully defined the parameters for an AlphaLISA assay in pig serum, we now wanted to test the assay further in detecting FGF21 in biological samples that might be predicted to have endogenous FGF21 levels. Samples were collected from a previous study evaluating heat stress in barrows. Eighty barrows that had reached 42 kg were either fed 7.4 ppm Paylean® the final 30 days of finishing or received 110% of normal lysine requirement. Paylean is a feed additive that contains ractopamine hydrochloride that increases protein growth. It increases fat-free lean gain and allows for pigs to have high lean growth rates with reduced energy intake (A.P. Schinckel and B. T. Richert). We therefore wanted to evaluate the effect that a Paylean diet might have on FGF21 levels.

The pigs in the study were divided into four different groups: 100% Lysine recommendation; ractopamine included, 100% Lysine recommendation; no ractopamine, 110% Lysine recommendation; ractopamine included, and 110% Lysine recommendation; ractopamine not included.

FGF21 ELISA and AlphaLISA plates were run to evaluate these samples. From the results, the AlphaLISA assay could detect FGF21 in the samples but there was no

obvious pattern. This is understandable because from previous studies, protein restriction increased FGF21(Laeger et al., 2014) but ractopamine increases lean gain while reducing energy intake.

Sample	Affinity Column	Absorbance	Average Absorbance	Measured Concentration (ng/ml)	Average Concentration (ng/ml)	Recover %
Normal Pig pool	-	0.148 0.155 0.156	0.153	<0.000 <0.000 <0.000	0	-
Normal pig pool	+	0.171 0.203 0.191	.188	6.19 26.42 18.77	17.13	-
Spiked FGF21 pig pool (500 ng/ml)	-	1.203 1.231 1.198	1.211	3,136.37 3,639.06 3,058.64	3,278.02	655
Spiked FGF21 pig pool (500 ng/ml)	+	0.151 0.182 0.184	0.172	<0.000 13.09 14.34	9.14	1.83
Spiked PAG pig pool (500 ng/ml)	-	0.157 0.133 0.149	0.146	<0.000 <0.000 <0.000	0	-
Spiked PAG pig pool (500 ng/ml)	+	0.17 0.19 0.171	.177	5.56 18.13 6.19	9.96	1.99

Table 3.1. ELISA results for pig pool sera that were either run through an anti-FGF21 column or not, and that were either normal (control), spiked with FGF21 (500 ng/ml) or spiked with PAG (500 ng/ml). Each serum was run in triplicate samples.

Figure 3.1. Evaluating different antibody (AB) combinations. Both guinea pig (GP) and rabbit (R) anti-FGF21 antibodies were conjugated on acceptor beads and biotinylated. Standard curves were run to evaluate the different combinations of antibodies.

Figure 3.2. Evaluating serum dilutions. AlphaLisa assay standard curves set up using different bovine serum quantities to create a successful standard curve using 50% serum, 25% serum, and 12.5 % serum.

Figure 3.3. Testing different assay conditions using a 10% porcine serum. Three standard curves with equal amounts of FGF21 standard were either incubated at room temperature, 42°C, or 45°C.

Figure 3.4. Species Variation. Standard AlphaLISA assay was run using 50% rat, fetal bovine, ewe, and rabbit serum for standard curves with graph corrected for background.

Figure 3.5. Evaluation of the effects of endogenous FGF21 in pig serum. Normal AlphaLISA assay protocol was used to evaluate 4 different standard curve: control serum, serum spiked with 100 ng FGF21, serum spiked with 100 ng FGF21 and 10 μ g rabbit anti-FGF21 antibody, and serum spiked with 100 ng FGF21 and 10 μ g rabbit anti-PAG antibody. All four sera were then treated with 10 μ L of protein A matrix and the spun down for removal.

Figure 3.6. Cross reactivity between bovine, human, and mouse FGF21. (a) Western blot (2 μ g of protein per lane) and (b) stain (10 μ g of protein per lane) were run to identify protein identity between bovine, human, and mouse FGF21. Not shown above is a run with a control protein (BSA) that did not react with the antibody.

Figure 3.7. AlphaLISA results of three different standard curves using equal amounts of either bovine, human, or mouse FGF21.

Figure 3.8. ELISA results of three different standard curves using equal amounts of either bovine, human, or mouse FGF21.

	<i>ELISA-Bovine</i>	<i>ELISA-Human</i>	<i>ELISA-Mouse</i>	<i>Alpha-Bovine</i>	<i>Alpha-Human</i>	<i>Alpha-Mouse</i>
<i>ELISA-Bovine</i>	1					
<i>ELISA-Human</i>	0.765	1				
<i>ELISA-Mouse</i>	-0.027	-0.319	1			
<i>Alpha-Bovine</i>	0.962	0.661	-0.008	1		
<i>Alpha-Human</i>	0.589	0.393	0.303	0.610	1	
<i>Alpha-Mouse</i>	0.765	0.391	0.199	0.902	0.506	1

Table 3.2. Correlations between bovine, human, and mouse FGF21 ELISA and AlphaLISA results

Figure 3.9. Serum versus Plasma. Standard curves were run with 12.5% steer serum and plasma with all incubations at room temperature and 12.5% pig serum and plasma with all incubations at 42°C. Graphs are corrected for blanks.

DISCUSSION

Fibroblast growth factor 21 has been a novel peptide hormone that could lead to potential break through as a therapeutic treatment for patients with diabetes and obesity. These two conditions are becoming an epidemic in the United States and many western countries and the best solutions given to treat them at this point are exercise and diet. FGF21 acts as an endocrine hormone in times of metabolic stress and is shown to stimulate hepatic fatty acid oxidation, ketogenesis, induce weight loss, allow for sustained decreases in plasma glucose and triglycerides, and decrease the growth hormone response. It also provides therapeutic benefits by attenuating apoptosis, oxidative stress and inflammation, making it a good candidate to be used as an indicator of irregular metabolism (Tanajak et al., 2015). Multiple studies have also shown that induction of FGF21 or an analogue reduced plasma glucose levels, insulin levels, and body weight (Véniant et al., 2012 & Adams et al., 2013). The goal of this thesis was to provide an assay platform that could further investigate FGF21 in livestock animals.

For this thesis, we used amplified luminescent proximity homogeneous assay (AlphaLISA) as our assay platform. It is a bead based technology that brings two antibodies near each other allowing for an unstable singlet oxygen to trigger downstream cascade of chemical events leading to a sharp intense chemiluminescent emission that can be read on an EnVision machine.

The assay was set up using a sandwich design with guinea pig or rabbit anti-bovine FGF21 antibodies attached to the acceptor beads and biotinylated rabbit anti-bovine FGF21 antibodies. The assay has a working range from 2-200 ng/ml with an inter

assay variation at 200 ng of 8.2%, 50 ng of 4.6% and 0.195 ng of 10.1%. The intra-assay precision at 200 ng of 12.9%, 50 ng of 20.23%, and 0.195 ng of 10.57%.

A variety of sera (bovine, porcine, rat, ewe, fetal bovine, and rabbit) were tested and optimized assay conditions were established for them independently when necessary. It was determined that sera for rat, ewe, fetal bovine, and rabbit needs to be diluted to 50% to obtain a successful standard curve. Bovine serum needs to be diluted to 12.5%, and porcine serum also needs to be diluted 12.5% as well as changing incubation conditions to 42°C.

We did multiple experiments to identify the source of elevated background levels caused by porcine serum - one of which was the possibility of high concentrations of endogenous FGF21. We ran an assay where we successfully spike and remove FGF21 as a way to confirm the selectivity of the assay. But running the serum that had removed FGF21 did not show any improvement on the assay. The increased background level of porcine serum could be due to the pool that was used where certain individuals had elevated the FGF21 levels and was skewing our results. We were able to lower the background by using serum of individual pigs that had low FGF21 results but still had to use 42°C as our incubation temperature. We also obtained successful standard curves using bovine and porcine plasma rather than serum yielding curves that were lower in background but also had a lower dynamic range.

We also tested to see if our antibodies were compatible with human and mouse FGF21. We did not get successful standard curves using the different FGF21s with either an AlphaLISA (**Figure 3.7**) or an ELISA (**Figure 3.8**). We did run western blots and a stain to compare properties of the different FGF21 (**Figure 3.6**). The immunolocalized

bands corresponded to those on a parallel gel. The staining also exhibited similar intensity. The assumption from these results was that the antibodies were able to recognize common epitopes among the denatured proteins separated on the SDS-PAGE gels, but surface epitopes (i.e. ones available for binding to antibody in the plate assays) were insufficiently conserved. We then took some steps to try to improve antibody binding to the proteins in solution. These included increased incubation times, increased incubation temperatures and mildly denaturing conditions (data not shown). However, none of these modifications were successful in getting the anti-bovine FGF21 antibodies to recognize the mouse and human forms. If we had the time and necessary material, the next step would have been to try the assay with different species antibodies against FGF21 and identify those that are successful against human and mouse FGF21 in the AlphaLISA assay format.

The last thing we did was use the assay to identify FGF21 in biological samples. We used pig serum from a study that had barrows on a Paylean diet the last 30 days before finishing. Four different groups were measured either on the Paylean diet or not and either given 100% or 110% of the lysine requirement needed. AlphaLISA assay could successfully detect FGF21 in these samples but there was no indication that barrows fed a Paylean diet or not had any influence on FGF21 levels. Ractopamine chloride, the element included in the Paylean diet, is a small molecule beta adrenergic receptor agonists that increases high lean growth while decreasing energy. Previous studies have shown that protein restriction increases FGF21 levels and energy intake has no influence (Laeger et al., 2014).

In summary, we have a successful working assay that can be used across multiple livestock species. The AlphaLISA technology allows for fast results with the total assay time only taking 2 hours as well as providing increased sensitivity while using minimal sample volumes. Limitations we faced in the process included the expensive nature of the beads for the AlphaLISA technology and insufficient time to complete testing of biological samples of animals that were fed a low protein diet to have increased serum FGF21 to validate our assay further. There are still some unresolved questions including the cause of background in porcine serum and the unsuccessful assays using human and mouse FGF21 that we wish we could have examined further.

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APPENDIX

AlphaLisa Antibody Preparations

Ab Biotinylation

1. Add 100ug of desired antibody to a 1.5mL Eppendorf tube and set aside.

$$\frac{100 \text{ ug}}{\text{AB Concentration (ug/uL)}} = X \text{ ul AB}$$

2. To a tube of Solulink ChromaLink Biotin (1mg B-1001-105) add 100uL of Dimethyl-Formamide and mix by pipetting up and down until the biotin is completely dissolved.
3. Take a 10uL aliquot of the biotin and add it to a 1.5mL tube. Add 40uL of 1x PBS (pH 7.4) to obtain a 1:5 dilution.
4. The undiluted biotin can be stored at -20°C for up to 1 month.
5. To the tube of antibodies add enough 1x PBS to reach a total volume of 192uL.
 $192 - (X \text{ ul AB}) = Y$
6. To the Ab solution add 7.6uL of the diluted biotin and mix lightly by pipetting up and down.
7. Incubate at RT for 2 hr.

Washing Biotinated-AB

Washing Column:

1. Spin off liquid in spin column (Zeba™ Spin Desatting columns 7k MWCO .5mL 89882 Fisher) into 1.5 mL tube at 1500 g/ref for 1 min. (Break off end)
2. Dump flow through
3. Add 300 uL PBS to spin column for 1 min
4. repeat 4 times

Removal of Free biotin:

5. Add B-AB to column and put column in new 1.5 Ml tube
6. Spin 2 min at 1500 g
7. Flow through is final B-AB
8. Store at 4°C

Conjugation to Acceptor Beads (2mg)

1. Take 2 tubes (1mg each) of AlphaLisa Acceptor Beads (Perkin Elmer) and briefly spin to remove any liquid that may be stuck on the lid.
2. Pull out the bead solution from each tube (50uL each) and put into a single 1.5mL tube.
3. Add an equal volume of 1x PBS and mix up and down by pipetting.
4. Spin the beads in a microcentrifuge at full speed for 15 minutes.
5. Discard the supernatant.

6. To the beads add 40ug of desired Ab and mix by pipetting
 - a. General rule is 100ug of Ab per 5mg of beads so make adjustments as needed
$$\frac{40 \text{ ug}}{\text{AB concentration (ug/uL)}} = X \text{ ul AB}$$
7. To the Ab-bead mixture add 1.25uL of 10% Tween-20
8. In a separate 1.5mL tube dilute some NaBH₃CN in water to reach a concentration of 25mg/mL.
9. Add 5uL of the diluted NaBH₃CN to the Ab-bead mixture and mix by pipetting.
10. Add in enough sodium phosphate buffer (130mM Na₂HPO₄ pH 8.0) to reach a total volume of 80uL and mix by pipetting.
 - a. General rule is 200uL total volume for 5mg of beads so make adjustments as needed
11. Incubate at 37°C overnight

Blocking Acceptor Beads

1. Weight out some CMO powder then add enough 800mM NaOH to reach 65 mg/mL

$$X \text{ mg of CMO} \times \frac{\text{ml}}{65 \text{ mg}} = Y \text{ ml NaOH}$$

2. Mix until dissolved
3. Add 10 uL of CMO per 200 uL of beads
4. Incubate at 37°C for 1 hour
5. Spin at 16000 g for 15 min
6. Discard supernatant
7. Resuspend with 400 uL of Tris (1 mL per 5 mg of bead)
8. Spin for 15 min
9. Resuspend with 400 uL of Tris
10. Spin for 15 min
11. Resuspend with 400 uL of PBS
12. Optional add ProClin-300 to reach .05% concentration (.5uL)
13. Acceptor beads are now ready to use (enough for 20-40 plates)
14. Store at 4 °C

Assay Protocol

1. Measure B-AB in nano drop

Select 'Protein and Labels'
measure 2 peaks: 280 and 354

2. Calculate concentration

$$\text{Absorbance @ 354 nm} \times \text{Correction factor (0.23)} = X$$

$$\text{Absorbance @280 nm} \times X = \text{Concentration}$$

3. Make buffer: dilute 10X stock to 1 X with H₂O
4. Pipet 100 uL of buffer into 1st well.

1/2 Log Dilution	1	2	3	4	5	6	7	8	9	10	11	12
Concentration (ug/mL)	100	30	10	3	1	.3	.1	.03	.01	.003	.001	Blank
Buffer (uL)	100	70	60	70	60	70	60	70	60	70	60	Blank
Standard (ul)	16.7											

$$\text{Standard prep: } 100 \text{ uL} \times \frac{100 \text{ ng}}{\text{mL}} \times \frac{\text{mL}}{600 \text{ ng}} = 16.7 \text{ uL}$$

5. Take 30 ul from 1st well and put in next and continue serial dilution
6. Add 5 ul per well of standards in triplicates
7. Calculate how many wells will be used

20 uL of acceptor beads per well #well X 20 ul = X ul needed

5ul of beads per ml of assay buffer

$$X \text{ ml needed} \times \frac{5 \text{ ul}}{\text{ml}} = Y \text{ ul of beads in } X \text{ ml of buffer}$$

8. Incubate for 1 hour at room temperature
9. 5ul of beads per mL of dilutes assay buffer (want 2.5 nmol of B-AB)

$$(20 \times \# \text{wells}) \text{ ul} \times \frac{2.5 \text{ nmol}}{L} \times \frac{L}{\text{Concentration of B - AB (Umol)}} \times \frac{\text{umol}}{1000 \text{ nmol}} = Y \text{ uL of B - AB}$$

10. Use same dilution scheme for samples
11. Add samples to plate 5 uL per well in triplicates
12. 20 uL of Ab in each well
13. mix plate lightly
14. put plate cover tape over plate
15. Incubate for 1 hour at room temperature
16. Prepare streptavidin donor bead solution

32 uL Donor beads

2 mL of AlphaLISA buffer

17. Add 25 uL of donor beads per well
18. Incubate for 30 min
19. Read plate