CLONING, EXPRESSION AND PURIFICATION OF HUMAN FIBROBLAST GROWTH FACTOR 21 IN *Escherichia coli* AND *Pichia pastoris*

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Cloning, Expression and Purification of Human Fibroblast Growth Factor 21 in *Escherichia coli* and *Pichia pastoris*

ABSTRACT

Fibroblast Growth Factor 21 (FGF21) is a novel target with potential anti diabetic properties that are useful for treatment of hyperglycemia, insulin resistance, hyperlipidemia and metabolic disease. Producing recombinant FGF21 by *E. coli* without using fusion proteins is time consuming and will produce low quantity products. In this study, to establish and test the efficiency of other expression methods, the complete *fgf21* gene was constructed by overlapping PCR. The recombinant *fgf21* genes were expressed successfully in *E. coli* (*TB1*) and in yeast (*Pichia pastoris*) under the control of maltose binding promoter and alcohol oxidase I promoter. The degree of success in terms of yield and functionality of the produced recombinant proteins *in vivo* were compared by using animal models. The result demonstrated that both expression systems can promote more soluble FGF21 levels, with less purification steps while preserving the bioactivity of the protein *in vivo.* The FGF21 produced in *P. pastoris* underwent post translation modification and was more active in lowering blood glucose compared with that in *E. coli.* The histologic effects of recombinant FGF21 (rFGF21) on Sprague Dawley adult rats reproductive systems were also investigated by focusing on the pattern changes and occurring differences after 28 days of treatment with rFGF21. A significant increase in the weight and size of organs in both genders were observed. Spermatogenesis in male and number of follicles and corpora lutea in female also in the treated group increased significantly compared to the controls. Further studies are needed to clarify the mechanism of action and the components responsible for these pharmacological effects.

Keywords: fibroblast growth factor 21, anti diabetic, *E. coli, Pichia pastoris*

Pengklonan, Pengekspresan dan Penulenan *Human Fibroblast Growth Factor 21* di dalam *Escherichia coli* dan *Pichia pastoris*

ABSTRAK

Fibroblast growth factor 21 (FGF21) adalah sasaran baharu yang mempunyai ciri-ciri anti diabetik yang berguna untuk rawatan hiperglisemia, rintangan terhadap insulin, hiperlipidemia dan penyakit-penyakit metabolik. Penghasilan FGF21 rekombinan menggunakan *E. coli* tanpa menggunakan protein-protein tertaup mengambil masa yang lama dan amaun yang rendah. Dalam kajian ini, untuk menguji dan mengesahkan kecekapan kaedah pengekspresan yang berlainan, gen FGF21 yang lengkap telah dibina dengan kaedah PCR bertindih. FGF21 rekombinan telah diekspres dengan jayanya dalam *E. coli* dan *P. pastoris* di bawah kawalan promoter protein pengikat maltose (MBP) dan promoter alcohol oxidase 1 (AOX1). Tahap kejayaan dari segi amaun hasil yang diperolehi dan juga keberkesanan fungsi protein-protein rekombinan yang dihasilkan dalam sistem *in vivo* dibandingkan menggunakan model-model haiwan. Keputusan menunjukkan kedua-dua sistem pengekspresan mampu menghasil FGF21 terlarut pada kadar yang lebih tinggi, tanpa memerlukan langkah penulenan yang banyak, disamping mengekalkan tahap bioaktif protein tersebut dalam keadaan *in vivo*. FGF21 yang dihasilkan dalam *P. pastoris* menjalani pengubahsuaian pasca tranlasi, dan didapati lebih aktif dalam menurunkan kadar gula di dalam darah berbanding protein yang dihasilkan dalam *E. coli.* Kesan histologi protein rekombinan FGF21 ke atas sistem pembiakan tikus Sprague Dawley dewasa juga telah dikaji dengan menumpukan pemerhatian kepada perubahan corak dan tahap kejadian selepas rawatan selama 28 hari dengan FGF21 rekombinan. Pertambahan signifikan dalam berat dan saiz organ, pertambahan signifikan pada kadar spermatogenesis dalam tikus jantan, serta pertambahan bilangan folikel dan korpora lutea telah diperhatikan dalam kumpulan yang dirawat, berbanding kumpulan tikus kawalan. Kajian lanjut diperlukan diperlukan

untuk mendapat penjelasan lanjut mengenai mekanisma tindakan dan juga komponen-komponen yang terlibat dalam menghasilkan kesan-kesan farmakologikal yang diperhatikan.

Katakunci: fibroblast growth factor 21, anti diabetik, *E. coli, Pichia pastoris*

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
$CaCl_2$	:	Calcium Chloride
g/L	:	Gram per litre
kb	:	Kilo bases
kDa	:	Kilo Dalton
M	:	Molar
Mg^{2+}	:	Magnesium ion
mg/L	:	Milligram per Litre
mg/ml	:	Milligram per mililitre
mM	:	Milli Molar
µg	:	Microgram
µg/ml	:	Microgram per millilitre
µM	:	Micromolar
µl	:	Microlitre
O_2	:	Oxygen
s	:	Second
U	:	Unit
V	:	Volume
AOX	:	Alcohol oxidase
BMMY	:	Buffered Methanol-complex Medium
BMGY	:	Buffered Glycerol-complex Medium
bp	:	Base pair
BSA	:	Bovine serum albumin

cDNA	:	Complementary DNA
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxyribonucleoside triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
e.g	:	For example
et al.	:	Et alii (and other people)
EtBr	:	Ethidium bromide
FGF	:	Fibroblast growth factor
FGF21	:	Fibroblast growth factor 21
GH	:	Growth Hormone
Glu	:	Glucose
His	:	Histidine
hr	:	Hours
HRP	:	Horseradish peroxidase
i.e.	:	That is
LSLB	:	Low salt Luria-Bertani
min	:	Minute
mRNA	:	Messenger RNA
Mut^+	:	Methanol utilization plus
Mut^-	:	Methanol utilization minus
Mut^S	:	Methanol utilization slow
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
Ni	:	Nickel
OD_{600}	:	Optical density at 600nm

PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
Pfu	:	Pyrococcus furiosus
pI	:	Isoelectric point
rcf(Xg)	:	Relative centrifugal force
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
sdH_2O	:	Sterile distilled water
SDS	:	Sodium dodecyl sulphate
Ser	:	Serine
Taq	:	*Thermus aquaticus*
TBE	:	Tris Borate EDTA
TBS	:	Tris buffered saline
TCA	:	Trichloroacetic acid
UV	:	Ultraviolet
YPD	:	Yeast Extract Peptone Dextrose

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CHAPTER 1: INTRODUCTION

1.1 Fibroblast growth factor (FGF)

Gospodarowicz in 1975 reported that he had found a potent agent in the pituitary that could induce fibroblast cell growth and titled it as fibroblast growth factor (FGF) although its activity was not restricted to fibroblast and it could stimulate many cell types, including endothelial cells and chondrocytes (Gospodarowicz, 1975; Gospodarowicz *et al.*, 1978). Later Shing and his colleagues isolated and purified the first factor that specifically stimulated the growth of endothelial cells from different source by heparin affinity column in 1984, and classified these two prototypic members of the FGF family as acidic FGF (aFGF), and basic FGF (bFGF) which were very similar to what Gospodarowicz had reported earlier (Esch *et al.*, 1985, Gospodarowicz *et al.*, 1984; Shing *et al.*, 1984).

1.1.1 Different types of fibroblast growth factor (FGF)

Since the identification of the first FGF (FGF1 or αFGF), this family has grown steadily (Itoh, 2010). To date, fibroblast growth factors (FGFs) refer to a group of structurally related family of twenty-three polypeptides participates in many developmental and repair processes of several mammalian tissues, which are divided into seven subfamilies based on phylogeny, sequence identity and similar biological activities (Itoh *et al.*, 2008, Itoh & Ornitz, 2004, Mu *et al.*, 2008, Ornitz & Itoh, 2001; Reuss *et al.*, 2003). Currently, FGF family orthologs are conserved between human and mouse genomes (Algul *et al.*, 2009). This family of heparin-binding proteins are considered as hormonal factors with diverse biological functions, which are usually involved in cellular growth, angiogenesis, wound repair, cell differentiation, proliferation, morphogenesis, metabolic regulation and repair of tissue injury (Van der Walt *et al.,* 2004, Burgess & Maciag, 1989, Dostalalova *et al*., 2009, Evans *et al.,* 2004, Hutley *et al*., 2004, Itoh, 2010, Kelleher *et al*., 2013, Martin, 1998, McKeehan *et al.,*

1998, Mu *et al.*, 2008, Remmers *et al*., 1991, Sano *et al*., 1990, 1993; Yamagata *et al.,* 2004).

While most of FGFs act as local regulators of cell growth and differentiation, recent studies indicated that FGF19 subfamily members including FGF19 (human orthologue of mouse FGF15), FGF21 and FGF23 are different and exert their metabolic effects by an endocrine fashion (Dostalalova *et al.,* 2009). They regulate diverse physiological processes that are not affected by classical FGFs. These endocrine factors have wide metabolic activities including regulation of the bile acid, carbohydrate and lipid metabolism as well as phosphate, calcium and vitamin D homeostasis (Holt *et al.,* 2003, Inagaki *et al.,* 2005, Kharitonenkov *et al.,* 2005, Lundasen *et al.,* 2006, Shimada *et al.,* 2004; Tomlinson *et al.,* 2002).

Table 1.1: Different Fibroblast Growth Factors and their roles.

Name	Description
FGF1	Potential inducer of neovascularization in angiogenic diseases including atherosclerosis, cancer and inflammatory autoimmune diseases and stimulates proliferation of mesoderm and neuroectoderm-derived cells, as well as prostatic epithelial cells. (Basilico & Moscatelli, 1992, Brogi *et al.,* 1993, Chiu *et al.,* 1990, Crabb *et al.,* 1986, Folkman and Klagsbrun, 1987, Myers *et al.,* 1995, Sano *et al.,* 1990; Takahashi *et al.,* 1990)
FGF2	Stimulates smooth muscle cell growth, wound healing, tissue repair, hematopoiesis, plays a critical role in nervous system development, the eye, and the skeleton. It also works as an inhibitor of apoptosis, starter of spermatogenesis and as a promoter of follicle growth. (Basilico & Moscatelli, 1992, Bikfalvi & Han, 1994, Van dissel-Emiliani *et al.,* 1996, Eckenstein, 1994, Logan *et al.,* 1991, McAvoy *et al.,* 1991, Schwartz *et al.,* 1993, Unsicker *et al.,* 1992; Santos *et al.,* 2014)

Table 1.1, continued.

Name	Description
FGF3	Serve as a negative regulator of bone growth during ossification (Deng *et al.,* 1996)
FGF4	FGF4 functions as a signaling molecule, facilitates the survival and growth of the inner cell mass (Yuan *et al.,* 1995; Feldman *et al.,* 1995)
FGF5	Performs a critical role in the hair cycle (Higgins *et al., 2014)*
FGF6	Displayed oncogenic transforming activity when transfected into mammalian cells (Coulier *et al.,* 1991, Dickson, 1991, Goldfarb *et al.,* 1991; Yoshida *et al.,* 1991)
FGF7	As a mitogen for cultured keratinocytes (Aaronson *et al.,* 1991)
FGF8	As an androgen-induced growth factor and a glia-activating factor (Miyamoto *et al.,* 1993; Tanaka *et al.,* 1992)
FGF9	Stimulates proliferation of mesenchymal cells and migration of mesonephric cells into the testis and also is involved in ovarian progesterone production (Colvin *et al.,* 2001; Drummond *et al.,* 2007)
FGF10	Implicated in the differentiation processes in white adipose tissue and pancreas and also is involved in the initial follicular development stages in humans. (Bhushan *et al.,* 2001, Ohuchi *et al.,* 2000, Oron *et al.,* 2012; Sakaue *et al.,* 2002)
FGF11 FGF12 FGF14	Involved in nervous system development and function (Goldfarb *et al.,* 2007, Liu *et al.,* 1997; Smallwood *et al.,* 1996)
FGF13	Involved in nervous system development and function and induces cell growth of lung fibroblasts and aortic smooth muscle cells (Leung *et al.,* 1998; Smallwood *et al.,* 1996)
FGF15	Identified as a downstream target of the chimeric homeodomain onco-protein E2A-Pbx1 (McWhirter *et al.,* 1997)
FGF16	Specific factor in brown adipocytes (Konishi *et al.,* 2000)

Table 1.1, continued.

Name	Description
FGF17	Important role in the regulation of embryonic development and in the induction and patterning of the embryonic brain, required for normal brain development (Zhang *et al.,* 2006)
FGF18	Involved in bone development and repair and stimulates hepatic and intestinal proliferation (Davidson *et al.,* 2005, Hu *et al.,* 1998; Moore *et al.,* 2005)
FGF19	Regulates biosynthesis of biliary acids and adipogenesis and is a regulator of energy expenditure (Fu *et al.,* 2004, Inagaki *et al.,* 2005; Tomlinson *et al.,* 2002)
FGF20	Acts on proximal cells, significantly enhances the survival of cultured dopaminergic neurons by activating the mitogen-activated protein kinase (MAPK) (Ornitz & Itoh, 2015)
FGF22	Involved in epidermal homeostasis and/or cutaneous wound repair (Beyer *et al.,* 2003)
FGF23	Regulates calcium and phosphate homeostasis, controlling phosphaturia and vitamin D synthesis (Shimada *et al.,* 2004)

1.1.2 Fibroblast growth factor 21 (FGF21)

The 21st documented fibroblast growth factor is called FGF21, which was discovered in 2000 by Nishimura *and his colleagues* and belongs to superfamily of fibroblast growth factor nineteen (Itoh & Ornitz, 2004; Naderali *et al.,* 2009). The majority of the FGF family members are mitogens that stimulate cell proliferation and differentiation, like FGF19, where its metabolic activity is similar to FGF21. However, since FGF21 is free of the proliferative and tumorigenic effects, it became an interesting protein in the research area.

FGF21 was firstly isolated from mouse embryos. Its gene is located in chromosome

19 at the 5′region of the 1,2- fucosyltransferase human gene (Nishimura *et al.,* 2000). It consists of three coding exons like other *FGF* family member genes that are induced and secreted from liver, skeletal muscles and beta cells as a 181-amino acid polypeptide (Izumiya *et al.,* 2008, Kharitonenkov & Shanafelt, 2008, Nishimura *et al.,* 2000; Wente *et al.,* 2006). FGF21 mRNA was most abundantly expressed in the liver, and to lesser extent in the thymus. Its cDNA encodes a 209 amino acid nucleotide sequence, with a hydrophobic amino terminus in rodent, which was identified by genomic studies (Nishimura *et al.,* 2000). The amino terminus in rodent is approximately 30 amino acids that represent a typical signal sequence. The human DNA sequence of FGF21 is highly identical (75%) to mouse FGF21 and is most similar (35%) to FGF19 (Nishimura *et al.,* 2000).

1.1.3 Metabolic effects of FGF21

The role of FGF21 in regulating metabolism was first described by Kharitonenkov *and his colleagues* in mouse adipocytes. FGF21 has been initially identified as a novel metabolic regulator and since then this protein has become the focus of intense research in the area of glucose and lipid homeostasis since it plays an important role in the regulation of glucose, lipid, and energy homeostasis (Bottcher & Niehrs, 2005, Goetz *et al.,* 2007, Inagaki *et al.,* 2007, Kharitonenkov & Larsen, 2011, Kharitonenkov *et al.,* 2005; Szebenyi & Fallon, 1998).

Systemic administration of FGF21 resulted in striking improvement in metabolic parameters, including a sustained decrease in circulating glucose, fructosamine and lipid levels. It activates glucose uptake in mouse adipocytes as well as in differentiated human adipocytes, which causes a mild weight loss and improve glucose tolerance, insulin sensitivity and diabetic dyslipidemia (Inagaki *et al.,* 2007, Kharitonenkov *et al.,* 2005, 2007; Xu *et al.,* 2009). FGF21 unlike any other anti-diabetic marketed

compounds like the PPARγ activators, or thiazolidinediones was devoid of unwanted side effects such as hypoglycemia and weight gain or edema upon chronic FGF21 treatment, issues that are commonly associated with traditional insulin therapy (Boden & Zhang, 2006; Hermansen & Davies, 2007). Its role could be related with activation of the Akt signaling pathway since its activation is related to beta cells survival and growth (Bernal-Mizrachi *et al.,* 2004, Eswarakumar *et al.,* 2005, Jetton *et al.,* 2005, Huang *et al.,* 2006, Kharitonenkov *et al.,* 2005, Nicholes *et al.,* 2002, Ornitz & Itoh 2001; Wente *et al.,* 2006). In beta cells, the signaling pathway is usually related with glucose-induced activation and nuclear translocation of ERK1/2, which in turn stimulates insulin gene transcription (Wente *et al.,* 2006).

In fact, administration of FGF21 reduced plasma glucose and triglycerides to near normal levels in both ob/ob and db/db mice for >24 h post FGF21 administration. Similarly, systemic administration of FGF21 for 2 weeks in diet-induced obese and ob/ob mice reduces adiposity and total body weight by 20%, predominantly an effect which was not associated with total caloric intake or increased physical activity (Naderali *et al.,* 2009). Mice that overexpressed FGF21 were also reported to be lean and protected from age-associated or diet-induced obesity and insulin resistance (Xu *et al.,* 2009). The administration of FGF21 to obese leptin-deficient *ob/ob* and leptin receptor-deficient *db/db* mice and obese ZDF rats has also significantly lowered blood glucose and triglycerides, decreased fasting insulin levels and improved glucose clearance during an oral glucose tolerance test (Dostalova *et al.,* 2009).

In addition to the effects mentioned above, FGF21 also has the capacity to stimulate lipolysis in adipose tissue, inhibit fatty acid and triglyceride synthesis in liver, preserve β-cell mass and restore their function in the pancreas. FGF21 does not influence the mRNA expression of genes involved in regulating lipolysis, but instead it significantly

reduces the expression of the lipid droplet-associated phosphoprotein perilipin (Inagaki *et al.,* 2007, Naderali, *et al.,* 2009, Wente *et al.,* 2006; Xu *et al.,* 2009). An increase in total adiponectin and reduction of leptin, and inflammatory markers were also reported as FGF21 effects (Kharitonenkov *et al.,* 2007). Its administration to high-fat-diet–induced obese mice, increased fat utilization and energy expenditure to modulate energy metabolism by reducing hepatosteatosis, ameliorated glycaemia, plasma glucose, lipid concentrations and leptin resistance, increasing in fat oxidation and suppression of de novo lipogenesis in liver (Coskun *et al.,* 2008, Kharitonenkov *et al.,* 2005; Xu *et al.,* 2009).

FGF21 significantly reduces the low-density lipoproteins (LDL) cholesterol (with significant reduction in dense and small particles), and has 80% increases in high-density lipoproteins (HDL) cholesterol (Kharitonenkov *et al.,* 2007).

FGF21 also induces expression of hormone-sensitive lipase and adipocyte triacylglycerol lipase in White Adipose Tissue (WAT) in mice that caused fatty acids oxidation to produce acetyl-CoA by transferring them from WAT to liver during fasting. The acetyl-CoA synthesis ketone bodies, which in other tissues would reconvert to acetyl-CoA and oxidized in the tricarboxylic acid cycle to produce energy would survive animal in extended periods of nutrient deprivation (Coskun *et al.,* 2008, Fukao *et al.,* 2004; Inagaki *et al.,* 2007). During prolonged fasts, ketone bodies provide nearly half of the body's total energy and up to 70% of the energy required by the brain (Cahill, 2006).

In addition to altering the fuel sources, many small mammals conserve energy when food is scarce by undergoing to torpor, a short-term hibernation state in which animals can save energy by reducing body temperature and physical activity, in which lipolysis is promoted and caused increasing of FGF21 expression, followed by increasing the

synthesis of ketone bodies and changes in lipase gene expression and metabolism (Figure 1.1) (Badman *et al.,* 2007, Geiser, 2004, Inagaki *et al.,* 2007; Kliewer *et al.*, 2010). In fact FGF21-transgenic mice entered torpor during a 24-h fast whereas wild-type mice did not (Inagaki *et al.,* 2007).

FGF21 also has anti-apoptotic effects on germ cells (Cancilla *et al.,* 2000, Tagashira *et al.,* 1997; Yamamoto *et al.,* 2000). FGF21 did not induce mitogenicity, hypoglycemia, or weight gain at any dose tested in diabetic or healthy animals or when overexpressed in transgenic mice (Kharitonenkov *et al.,* 2005). These metabolic actions are unusual among FGFs; hence, FGF21 should be considered like metabolic hormone rather than a traditional growth factor that exhibits the therapeutic characteristics essential for an effective treatment of several components of metabolic syndrome including type 2 diabetes mellitus, dyslipidemia and obesity, hyperglycemia and insulin resistance and thus is an important novel target for drug discovery initiatives (Cuevas-Ramos *et al.,* 2009, Dostalova *et al.,* 2009, Kharitonenkov *et al.,* 2005, Kharitonenkov & Larsen, 2011, Mai *et al.,* 2011; Wente, 2006).

1.1.4 FGF21 mechanism of action

FGFs modulate their cellular activity via at least 5 distinct subfamilies of high-affinity FGF receptors (FGFRs) (Kharitonenkov *et al.,* 2005). It has been reported that FGF21 can act through all 4 FGFR isotypes (Kliewer *et al.,* 2010). FGF receptors have an extracellular ligand-binding domain formed by three-immunoglobulin type of domains including; a trans-membrane helix, intracellular domain with a tyrosine kinase (TK) activity and an extracellular ligand-binding domain (Figure 1.1) (Cuevas-Ramos, 2009). In contrast to other FGFs, which require heparan sulphate for high affinity receptor binding and activation, FGF21 binds heparan sulphate with low affinity (Goetz *et al.,* 2007). The weak heparan sulphate binding affinity keeps FGF21 away from being

captured in extracellular matrices and hence it can function as an endocrine factor (Mohammadi *et al.,* 2005). In addition, FGF21 contains intra-molecular disulfide bonds, which may increase its stability in plasma and thus allows it to function as a hormone (Harmer *et al.,* 2004).

FGF21 activity in the tissue depends on a very short cytoplasmic domain cofactor to activate FGF signaling, called beta-Klotho (Dostalova *et al.,* 2009; Naderali *et al.,* 2009). Beta-Klotho is a recently characterized single-pass membrane-spanning protein with 2 glycosidase domains that activate FGF signaling pathway in the tissue by making a complex with tyrosine kinases, which produces a metabolic but not mitogenic response (Figure 1.1) (Dostalova *et al.,* 2009, Kharitonenkov & shanafelt, 2008, Kurosu *et al.,* 2007, Micanovic *et al.,* 2009, Ogawa *et al.,* 2007, Suzuki *et al.,* 2008; Yie *et al.,* 2009).

The expression of βKlotho, in combination with particular FGFR isoforms, determines the tissue- specific metabolic activities of FGF21 (Kurosu *et al.,* 2007). βKlotho is expressed in adipose tissue, liver, and pancreas and thus the major actions of FGF21 are located in these tissues (Ito *et al.,* 2000). Cells lacking βKlotho do not respond to FGF21 and the introduction of βKlotho to these cells confers FGF21-responsiveness and recapitulates (Kharitonenkov *et al.,* 2007).

After stimulation of FGF-R by FGF21, the tyrosine kinase-signaling pathway was activated and stimulated phosphorylation of FGFR substrate 2α (FRS2α), MAPK, SHP-2, PI3K and p70S6K, Raf, Stat that induces stimulation of divergent downstream pathways like activation of extracellular response kinase 1/2 (ERK1/2) and Akt signaling pathways (protein kinase B) (Figure 1.1) (Carballada *et al.,* 2001, Deo *et al.,* 2002, Ibrahimi *et al.,* 2004, Izumiya *et al.,* 2008, Kharitonenkov *et al.,* 2005, Kontaridis *et al.,* 2002, Mohammadi *et al.,* 2005, Pelech *et al.,* 1986, Wente *et al*., 2006; Zhang *et*

al., 2006).

Moyers and colleagues (2007) reported that rapid accumulation of intracellular calcium and phosphorylation of Akt, GSK-3, p70(S6K), SHP-2, MEK1/2, and Stat3 is the early actions of FGF21 on 3T3-L1 adipocytes while in prolonged fasting for 72 h mice is activated through the PPAR- receptor, such as PPARγ and PPARα , that motivate the expression of fatty acid oxidative enzymes and synthesis of acetyl-coenzyme A (acetyl-CoA), which are used in the brain as a source of energy in prolonged fasting situation (Cuevas-Ramos *et al.,* 2009, Gälman *et al.,* 2008, Mraz *et al.,* 2009, Muise *et al.,* 2008; Naderali *et al.,* 2009). PPAR gamma (PPARγ) causes glucose transportation in adipocyte, which regulates FGF21 production by PPARα (Lundåsen *et al.,* 2007; Wente *et al.,* 2006).

PPARα, a nuclear receptor activated by fatty acids, binds to DNA response elements as a heterodimer with the retinoid X receptor (RXR), that regulate the transcription of numerous genes involved in fatty acid transport and oxidation (Kersten *et al.,* 2000). Obese ob/ob mice have increased PPARα levels and display 12-fold increased hepatic FGF21 mRNA levels (Naderali *et al.,* 2009). In fact after stimulation of peroxisome proliferator-activated receptor alpha (PPAR-), vita- min D receptor (VDR) and farnesoid receptor X (FXR), the expression of the FGF21, FGF23 and FGF19, will be increased, respectively (Figure 1.1)(Cuevas-Ramos *et al.,* 2009).

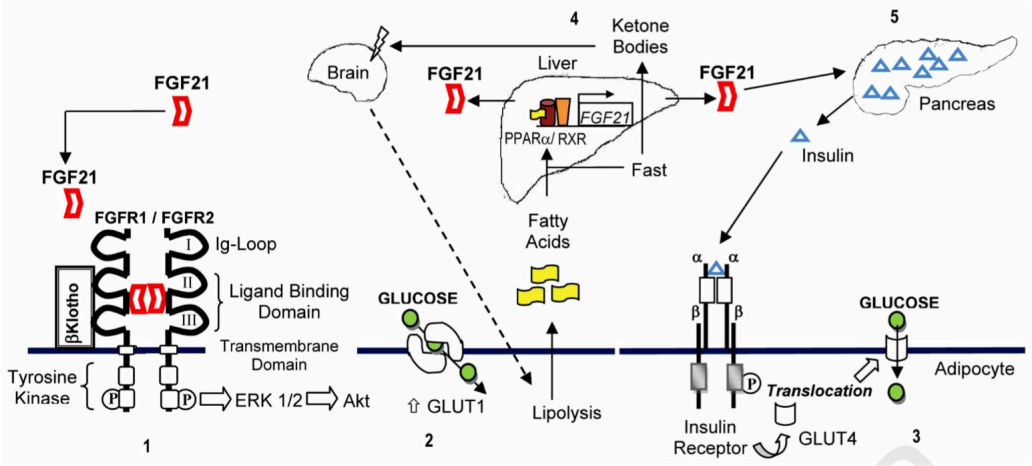


Figure 1.1: Mechanisms of action and metabolic activities of FGF21.

1. FGF21 is released by the liver into the bloodstream. Once in adipose tissue, it binds to its receptor (FGFR) forming a complex with its co-receptor Klotho. **2.** The main result is an increment of GLUT1 at the cellular membrane. **3.** This mechanism is independent of insulin- induced glucose uptake, which causes translocation to plasma membrane of GLUT4. Acting through different mechanisms, an additive effect is observed when FGF21 and insulin are administered together. **4.** After prolonged fast in mice, the brain induces lipolysis with the subsequent increment in blood fatty acids. Fatty acids bind to their receptor PPAR which in turn, increment FGF21 expression and ketone bodies formation that are in brain are used as a source of energy. **5.** Finally, FGF21 increases islet insulin content and glucose-induced insulin secretion in diabetic rodents (Cuevas-Ramos *et al.,* 2009).

1.1.5 FGF21 and liver

In the early stage of fasting, FGF21 does not have important role, but in prolonged fasting and starvation, after depleting glycogen stores, it starts gluconeogenesis through a mechanism requiring peroxisome proliferator-activated receptor α (PPARα) while its expression and circulating levels in mice are rapidly suppressed by re-feeding. In fact, the expression of FGF21 in the liver was very low in the fed state (Badman *et al.,* 2007, Dostalova *et al.,* 2009, Inagaki *et al.,* 2007, Kliewer *et al.,* 2010; Lundasen *et al.,* 2007).

FGF21 is an important mediator of the metabolic effects of peroxisome proliferator–activated receptors α agonists (PGC-1α), which is a transcriptional co-activator protein induced after FGF21 administration (Badman *et al.,* 2007, Inagaki *et al.,* 2007; Lundasen *et al.,* 2007). PGC-1α regulates metabolism in response to changes in nutritional status and other physiologic stimuli such as cold and exercise (Galman *et al*., 2008, Potthoff *et al.,* 2009; Puigserver & Spiegelman, 2003). Fasting also induces PGC-1α that stimulates the transcription of genes involved in gluconeogenesis, fatty acid oxidation, and ketogenesis (Burgess *et al.,* 2006, Rhee *et al.,* 2003; Yoon *et al.,* 2001). Hence, the metabolic actions of FGF21 are mediated in part through PGC-1α (Kliewer *et al.,* 2010).

Transgenic mice over-expressing FGF21 in liver had improved insulin sensitivity and glucose clearance, reduced plasma triglyceride concentrations, and were resistant to weight gain when fed a high-fat diet (Kharitonenkov *et al.,* 2005). In fact FGF21 overexpression reduced physical activity and promoted torpor in small mammals, while down-regulation or *in vivo* suppression of FGF21 in the liver resulted in the development of fatty liver disease and dyslipidemia like fatty liver, lipemia, and reduced serum ketones (Badman *et al.,* 2007; Inagaki *et al.,* 2007).

1.1.6 FGF21 in adipose tissue

Liver is the candidate for the paracrine and autocrine actions of FGF21, but FGF21 effects have been found dramatically in adipose tissues although adipose tissue does not express FGF21 as much as liver, while its expression in adipose tissue is positively correlated with circulating FGF21 levels (Kharitonenkov *et al.,* 2005, Moyers *et al.,* 2007; Zhang *et al.,* 2008). In fact, adipose tissue is now recognized as an important endocrine organ producing numerous factors involved in energy homeostasis and metabolic regulations (Haluzík *et al.,* 2004, Havel, 2002; Housa *et al.,* 2006).

1.1.7 FGF21 and insulin

Insulin and glucagon are well known in controlling substrate utilization responses. Recently fibroblast growth factor21 (FGF21) also has been identified as a metabolic regulator in the body (Kliewer *et al.,* 2010). Insulin and FGF21, reduce glucagon and triglyceride levels, increase HDL and induce lipogenesis in humans (Arner *et al.,* 2008, Kharitonenkov *et al.,* 2007; Moyers *et al.,* 2007).

FGF21 is an important modulator of insulin and glucose metabolism while its effects are insulin-independent and when FGF21 and insulin are administrated together in obese mice, a superior glucose clearance with less insulin levels (25 to 35%) and an additive effect between insulin and the growth factor are observed although FGF21 and insulin act through different mechanisms while in a prolonged fasting state, glucose levels are progressively reduced with suppression of insulin secretion whilst FGF21 levels progressively increased and induce ketogenesis (Cuevas-Ramos *et al.,* 2009, Dostalova *et al.,* 2009; Kharitonenkov *et al.,* 2005).

FGF21 in pancreatic beta cells increases islet insulin content by increasing insulin mRNA and protein levels. During high metabolic demand it increases insulin effects by suppressing glucagon secretion from pancreatic α-cells, reducing plasma glucagon

concentrations and stimulation of glucose uptake in adipocytes (Fig. 1.1) (Kharitonenkov *et al.,* 2005; Wente *et al.,* 2006).

The mechanism of action of FGF21 has similarities and differences with insulin (Table 1.1). FGF21 increases glucose uptake in adipose tissue by increasing expression of GLUT-1 and enhanced glucose transportation (Cuevas-Ramos *et al.,* 2009, Kharitonenkov *et al.,* 2005, Moyers *et al.,* 2007; Ogawa *et al.,* 2007). It would change expression of genes that take several hours; while, insulin induces translocation of GLUT-4 to plasma membrane in minutes (Epstein *et al.,* 1999). Opposite to insulin, the glucose lowering effect of FGF21 is moderate but sustained effect in nature lasting up to 24 h post-injection, whereas insulin has a potent but short action one (Cuevas-Ramos *et al.,* 2009; Naderali *et al.,* 2009). Importantly, while insulin induced occasional hypoglycemia in normal animals, no signs of plasma glucose below the basal level were observed with FGF21 in diabetic or lean mice, in the fed or fasted state, even at suprapharmacologic doses (Kharitonenkov & Adams, 2014).

Table 1.2: Comparing the effects of insulin and FGF21

Effects (Reference)	Insulin	FGF21
Glucose uptake (Kharitonenkov *et al.,* 2005)	↑	↑
Triglycerides Level (Kharitonenkov *et al.,* 2005; 2007)	↓	↓
LDL-c Level (Kharitonenkov *et al.,* 2007)	↓	↓
HDL-c Level (Kharitonenkov *et al.,* 2007)	↑	↑
GLUT-4 Expression (Kharitonenkov *et al.,* 2005)	↑	-
GLUT-1 Expression (Kharitonenkov *et al.,* 2005)	-	↑
Lipogenesis (Moyers *et al.,* 2007; Arner *et al.,* 2008)	↑	↑
Ketogenesis (Badman *et al.,* 2007; Inagaki *et al.,* 2007)	↓	↑
Weight (Kharitonenkov *et al.,* 2005; Coskun *et al.,* 2008)	↑	↓
Level with Insulin Resistance (Zhang *et al.,* 2008)	↑	↑
Level with Metabolic Syndrome (Zhang *et al.,* 2008)	↑	↑
Level in Diabetes Melitus (Chen *et al.,* 2008)	↑	↑

1.1.8 FGF21 and growth factor

Chronic exposure to FGF21 markedly inhibits growth in mice by promoting resistance to GH in order to conserve energy by reducing the active form of signal transducer and activator of transcription 5 (STAT5) concentrations, major mediators of GH actions that reduce circulating concentrations of GH/insulin-like growth factor I (IGF-I) signaling pathway (IGF-I) by 50% during starvation (Inagaki *et al.,* 2008, Thissen *et al.,* 1999; Naderali *et al.,* 2009). Moreover, FGF21 directly induces hepatic expression of IGF-1 binding protein 1 and suppressor of cytokine signaling 2, which blunts growth hormone (GH) signaling (Inagaki *et al.,* 2008).

1.1.9 FGF21 in pancreas

FGF21 was detected in human, rat and mouse pancreatic islets as well as in purified rat β-cells and INS-1E cells (Wente *et al.,* 2006). It increased insulin mRNA and protein levels that promote β-cell survival and protect mice from glucolipotoxicity and cytokine-induced apoptosis without any effect on glucose-induced insulin secretion in pancreatic islets isolated from healthy rats, while it increased islet insulin content and glucose-induced insulin secretion in islets isolated from diabetic rodents (Dostalova *et al.,* 2009; Kharitonenkov *et al.,* 2007).

Plasma insulin levels were reported to be lower in short-term treatment of FGF21 in normal or diabetic db/db mice while in long-term (constant infusion for 8 weeks) administration of FGF21, a dramatic reduction in glucose levels and increased in number of islets and β-cells are observed and the amount of insulin per islet in db/db mice were reported without any effects on islet cell proliferation due to the activation of p44/42 mitogen-activated protein kinase (Dostalova *et al.,* 2009; Wente *et al.,* 2006). FGF21 induces a variety of pancreatic lipases, which include pancreatic lipase, pancreatic lipase–related protein 2, and carboxyl ester lipase in liver, that are likely to contribute to the increase in hepatic fatty acid oxidation which efficiently hydrolyze triglycerides over a broad range of temperatures (Andrews *et al.,* 1998; Inagaki *et al.,* 2007).

1.1.10 Factors influencing expression of FGF21

FGF21 was considered as a novel protein associated with obesity-related metabolic complications in humans (Zhang *et al.,* 2008). FGF21 is nutritionally-regulated in animal models (Dostalova *et al.,* 2009). Fasting and high fat diet feeding caused in transcriptional up-regulation and higher serum FGF21 levels in adipose tissue of overweight/ obese subjects, while in transgenic animals, a resistance to diet-induced

obesity is observed and these animals are 40–50% smaller than wild-type mice (Badman *et al.,* 2007, Inagaki *et al.,* 2008; Muise *et al.,* 2008).

Similarly, in type 2 diabetic patients, fasting FGF21 levels were significantly increased since they are negatively correlated with fasting plasma glucose levels and this might represent a missing link in the adaptive response to long- term nutritional deprivation (Chen *et al.,* 2008; Dostalova *et al.,* 2009).

In fact, serum FGF21 has been reported to have positive correlation with adiposity, fasting insulin, triglycerides and serum leptin and a negative correlation with HDL cholesterol and adiponectin (Dostalova *et al.,* 2009; Zhang *et al.,* 2008).

Positive correlation was found between FGF21 levels and many parameters of obesity such as BMI (r=0.22), waist circumference (r=0.24), waist to hip ratio (r=0.23) and fat percentage (r=0.23), all adjusted for age (Cuevas-Ramos *et al.,* 2009). Indeed plasma FGF-21 levels were positively correlated with body mass index, serum leptin and insulin (Dostalova *et al.,* 2009).

FGF21 has been linked with lipid metabolism. Increasing of FGF21 synthesis on liver was identified after stimulation with pharmacologic agonists of the PPAR- administrated on mice and after fibrates administration in humans (Badman *et al.,* 2007, Gälman *et al.,* 2008; Inagaki *et al.,* 2007).

1.2 Recombinant protein therapeutics

Since the first bacterial expression system was used as a biological factory in 1978, recombinant protein production became an invaluable tool for supplying proteins for various purposes within life sciences, from basic research to drug discovery (Vernet *et al.*, 2011).

Protein therapeutics played a prominent role in the treatment of diabetes mellitus since the discovery of insulin in 1991 by Banting and Best. It has been extended over the years by introduction of human recombinant insulin, Humulin, the first engineered human insulin, insulin lispro (Humalog), and the recent introduction of the glucagon-like peptide-1 receptor agonist, exenatide IV (Byetta) (Kharitonenkov *et al.,* 2007).

Protein production is a complex process influenced by numerous genetic factors, such as gene stability, transcription initiation, mRNA stability, aminoacyl-tRNA availability, translation initiation, elongation and termination, and protein degradation machineries (Vernet *et al.*, 2011). During the last few years, significant efforts were made to facilitate the identification and production of therapeutic proteins and developing these technologies to produce proteins in quantities previously impossible to obtain (Lesley, 2001). New expression vectors, new or revitalized fusion tags for expression of more soluble protein, new micro-expression/solubility screening protocols and expression and purification in different host cells are updated everyday for rapid cloning systems (Card & Gardner, 2005, Dümmler *et al.*, 2005, Gräslund *et al.*, 2002, Jeon *et al.,* 2005, Kim *et al.,* 2004, Klock *et al.,* 2005, Knaust & Nordlund, 2001; Page *et al., 2004*). The choice of an expression system depends on cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications, the size, structure, biological activity and the final use of the expressed protein (Goeddel, 1990; Hodgson, 1993). In addition, the selection of a particular expression system requires a cost breakdown in terms of process, design, and other economic considerations (Makrides, 1996). These hosts range from simple prokaryotic organisms like bacteria to multicellular organisms such as transgenic plants and animals, and including unicellular eukaryotic organisms such as yeast and the more complex eukaryotic insect and mammalian cells (Jonasson *et al.,* 2002).

1.2.1 *E.coli* as a heterologous expression vector of eukaryotic protein

Among the various expression systems, bacterium is the first organism to be chosen as host strain. The most commonly used bacteria species is *Escherichia coli. E. coli* has been successfully used for production of proteins for years and the progress over recent years has widened its use even further (Jonasson *et al.,* 2002; Rudolph, 1996). *E. coli* is the preferred host for recombinant protein expression because it is rather easy to genetically manipulate. Its expression is fast, typically producing protein in a single day with an acceptable cost (Braun & LaBaer, 2003, Jonasson *et al.,* 2002; Studier & Moffatt, 1986).

These advantages have ensured that E. *coli* remains a valuable organism for the high-level production of recombinant proteins. However, in spite of the extensive knowledge on its genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism and it has some disadvantages as an expression host. *E. coli* is not capable of producing eukaryotic post-translational modifications especially glycosylation, which plays an essential role in secretion, stability and bioactivity. Bacteria lack extensive disulfide bond formation, which can be critical for the production of folded, active protein (Makrides, 1996; Peti & Page, 2007).

Typically, heterologous proteins synthesized in bacteria are not secreted into the culture medium. The proteins either form inclusion bodies in the cytoplasm or accumulate in the periplasm if it fused with a secretion signal. Inclusion body can be solubilized and refolded (Singh & Panda, 2005) but this additional step hampers the purification process. Proteins secreted to periplasm may degrade under proteolysis by envelope proteases (Jonasson *et al.,* 2002).

A major type of gene regulation in prokaryotic cells utilizes through inducible operons that can bind to either activate or repress transcription. Several promoter

systems, such as the lac promoter, T7 RNA polymerase, *trc* and *tac* promoter, have been developed and used to regulate and increase the production level of recombinant proteins (Terpe, 2006). For laboratory-scale production, the isopropyl *β*-D-thiogalactopyranoside (IPTG)-inducible promoters, which are regulated by the product of the *lacI* gene, the lac repressor, are widely used (figure 1.2)(Gronenborn, 1976).

1.2.2 Yeast as a heterologous expression vector of eukaryotic protein

In recombinant protein technology, normally the protein of interest would firstly be expressed in *E. coli*, and if the product is not biologically active in *E. coli* due to lack of essential post-translational modifications, incorrect folding or low recovery percentage of the native protein alternative expression systems such as yeast (eg: *Saccharomyces cerevisiae* or *Pichia pastoris)* are used (Georgiou, 1996; Jonasson *et al.,* 2002).

Pichia pastoris is a methylotrophic yeast and is one of the most important expression hosts for high-level production of recombinant proteins (Cereghino, 2002, Cereghino & Cregg, 2000; Higgins & Cregg, 1998). In the past ten years, over seven hundred proteins from human endostatin to spider dragline silk protein have been produced in this yeast (Li *et al.,* 2010).

The use of *P. pastoris* as a host offers many advantages compared to other expression systems (Cereghino & Cregg, 2000). This genetically engineered yeast is capable of performing many eukaryotic posttranslational modifications, such as glycosylation, disulfide bond formation, and proteolytic processing. These chemical modifications play an important role in protein structure, protein stability and also protein bioactivity and secrete properly folded and fully active recombinant proteins into the culture medium, valued for industrial, pharmaceutical and basic research purposes (Cregg *et al.,* 1998, Cregg & Madden, 1988; Higgins & Cregg, 1998).

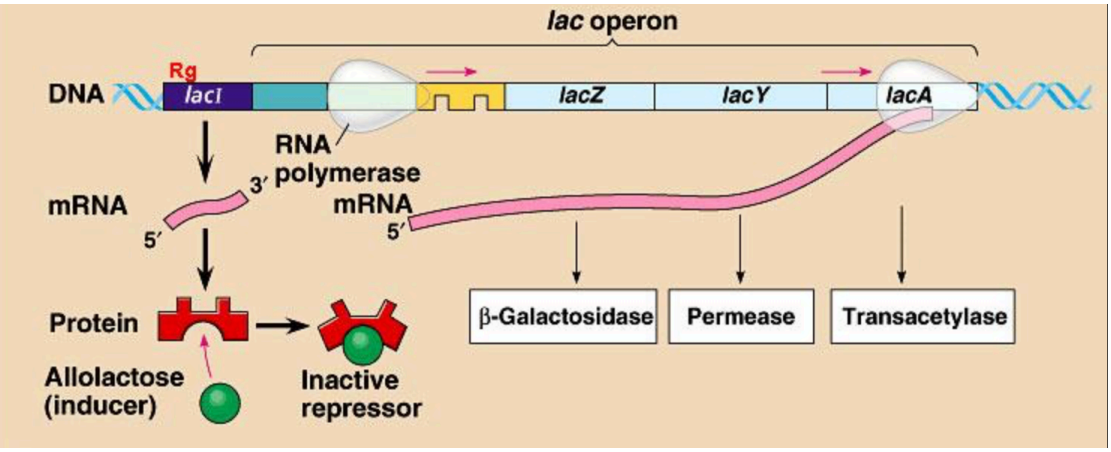


Figure 1.2: Lac Z operon

The *lac* operon (lactose operon) is required for the transport and metabolism of lactose in *Escherichia coli*. When lactose is required as a sugar source for the bacterium, the three genes of the lac operon is expressed and their subsequent proteins translated: *lacZ*, *lacY*, and *lacA*. The gene product of *lacZ* is β-galactosidase which cleaves lactose, a disaccharide, into glucose and galactose. *lacY* encodes Beta-galactoside permease, a protein which becomes embedded in the cytoplasmic membrane to enable transport of lactose into the cell. Finally, *lacA* encodes β-galactoside transacetylase. The gene for *β*-galactosidase is normally switched off, excepts in the presence of lactose and is commonly used in molecular biology as a reporter marker to monitor gene expression (Gronenborn, 1976).

P. pastoris does not have the endotoxin problem associated with bacteria, or the viral contamination problem of proteins produced in animal cell cultures. Its manipulation is easy and it can express proteins at high levels, intracellularly or extracellularly, at fairly low costs. Generally, recombinant proteins can be produced in higher amounts in the cytoplasm than if secreted into the culture medium. *P. pastoris* secretes only few of its native proteins into the medium. Secretion of recombinant proteins into culture medium can omit the cell-lysis step and reduces the amounts of redundant proteins from host cells. Indirectly, it also reduces the number of proteases that are released during cell lysis. The presence of proteases decreases the stability of recombinant proteins and promotes protein degradation. Due to these advantages, recovery of the recombinant proteins from the culture medium is easier since more than 80% of the total secreted proteins are recombinant proteins, making it a convenient host in terms of time and resources to purify the protein (Cregg *et al.,* 1998, Lin-Cereghino, 2007, Cereghino & Cregg, 2000; Romanos, 1995).

Several secretion signal sequences, including the native signal of foreign proteins (Soden *et al.,* 2002), the *S. cerevisiae* α-factor signal sequence (Kjeldsen *et al.,* 1999) and the *P. pastoris* acid phosphatase signal (PHO1) (Heimo *et al.,* 1997) have been constructed in *Pichia* vectors for the purpose of directing recombinant proteins into culture medium. Among these signal sequences, the *S. cerevisiae* α-factor signal sequence was found to be the most efficient in driving the secretion of heterogolous proteins into culture medium (Ilgen *et al.,* 2005).

P. pastoris can grow on methanol as a sole carbon and energy source by using methanol utilization pathway, the simple induction system under its efficient and tightly regulated promoter, the alcohol oxidase I gene (AOX1), which is used to drive the expression of the foreign gene. The AOX1 promoter is strongly repressed in cells grown on glucose and most other carbon sources, and induced over 1000-fold when cells are

shifted to a medium-containing methanol as a sole carbon source (Cregg & Madden, 1988; Cereghino *et al.,* 2002).

Because of these characteristics, some proteins that cannot be expressed efficiently in bacteria, *Saccharomyces cerevisiae* or baculovirus have been successfully produced in functionally active form in *P. pastoris* with genetic stability and scale-up without loss of yield (Cereghino *et al.,* 2002; Romanos, 1995). Such an expression system provides greater flexibility and offers the potential for shorter expression periods, lower manufacturing costs, and a more scalable expression process compared to even mammalian cell-based processes and provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications required for the protein functionality (Cereghino & Cregg, 1999; Wang *et al.,* 2007).

1.2.3 FGF21 Production in yeast and *Escherichia coli*

Since the isolation of FGF21 from mouse embryo by Nishimura in 2000, many researches have continued to produce and purify recombinant FGF21 in large amounts as this protein is only secreted in specific cellular conditions like long fasting periods, and since this product shows great potential to treat Type 2 diabetic patients, finding a cost effective method to produce and subsequently purify this protein is warranted.

The first production of recombinant FGF21 in *E. coli* was reported by Kharitonenkov in 2005 in which the product was found to accumulate and forms inclusion bodies. Multiple denaturation and purification steps were essential to produce pure bioactive recombinant protein but these steps, while effective, involved considerably larger costs. Furthermore, having to go through several purification steps inevitably results in reduced yield. Although Kharitonenkov's methods to produce recombinant FGF21 are still widely used by many researches, finding a cost effectively method to produce a

more soluble rFGF21 with high yield is important to facilitate development of this novel therapeutic agents.

In 2010, Wang and colleges used affinity tag modification technology and fused rFGF21 with SUMO tag that resulted in the expression of a more soluble rFGF21 in *E. coli*. In addition, the advent of various technological advances has enabled the production of recombinant proteins of high quality and more similar to human proteins, produced by eukaryotic cells like *Pichia pastoris.* (Wang *et al.,* 2007), but initial attempts to express FGF21 in *Pichia pastoris* resulted in low productivity (Kharitonenkov *et al.,* 2014).

1.3 Reproductive system

The reproductive system or genital system is a system of sex organs within an organism, which work together for the purpose of sexual reproduction. The role of reproduction is to provide the continued existence of a species; processes by which living organisms duplicate themselves.

1.3.1 Female reproductive system

The female rat reproductive system mainly composed of the vagina, ovaries, uterus, and mammary glands. It is specialized to produce ova (eggs), transport the egg cells to the site of fertilization, to provide a favorable environment for developing embryos, and to move offspring outside of the body at the appropriate time. Sexual maturity in female rats usually occurs between 30 and 50 days of age (approximately 7 weeks) (Goldman *et al.*, 2000, Kennedy & Mitra, 1963, Kim *et al.*, 2002; Kohn & Clifford, 2002).

The ovaries of the rat are grape-like structures, which are located at the distal end of the uterine horns near the kidneys. The oviducts connect the ovaries to each horn of the uterus. The surface of the ovary is covered by a single layer of modified peritoneal mesothelium; the ovarian surface epithelium (OSE), which supports the ovary and

completely encloses each ovary and oviduct. The ovaries produce the ova (egg) and certain hormones (Suckow *et al.*, 2005).

In sexually mature rats, the cortex of ovaries contains numerous follicles at various stages of development (Figure 1.3). The five stages of folliculogenesis consist of:

-Primordial follicle (earliest stage of follicular development); consists of a primary oocyteand located within the peripheral cortex, just beneath the tunica albuginea, covered with follicular cells so that their development stop at the first meiotic division. During each oestrous cycle, a cohort of "resting" primordial follicles starts to develop into primary follicles.

-Primary follicle; in this stage follicular cells surrounding the primordial follicle differentiate into a single layer of columnar cells, resulting in the formation of a primary follicle.

-Secondary follicle: Multilayered zone of granulosa cells around the oocyte formed a secondary follicle, which consists of thick glycoprotein and acid proteoglycan coat, between the oocyte and the zona granulosa. As the secondary follicle continues to grow, multiple fluid- filled spaces form within the zona granulosa.

-Tertiary follicle; the cystic spaces within the zona granulosa coalesce with a large central cavity (the follicular antrum). This cavity is filled with fluid (the liquor folliculi). The primary oocyte is eccentrically positioned within the tertiary follicle and resides within a mound of granulosa cells.

-Tertiary follicle; which has two zones: a theca interna and theca externa. The theca interna consists of polygonal cells with vacuolated cytoplasm and open faced, vesicular nuclei, which synthesises androstenedione (a sex steroid intermediate) while the cells of

the theca externa are spindle-shaped and merged with the surrounding ovarian stroma and they do not have any endocrine function.

-Preovulatory (Graafian) follicle – A small number of tertiary follicles enter a preovulatory stage and undergo further morphological changes. The follicular antrum continues to enlarge causing attenuation of the surrounding zona granulosa. Degeneration of the granulosa cells of the cumulus oophorus causes the primary oocyte to detach from the zona granulosa and float freely within the follicular antrum. The primary oocyte completes the first meiotic division just prior to ovulation and forms the secondary oocyte.

1.3.2 Male reproductive system

The male reproductive system consists of a pair of testes that produce sperm (or spermatozoa), ducts that transport the sperm to the penis and glands that add secretions to the sperm. The primary functions of the male reproductive system are the production of sperm, the transportation of sperm from the testes out of the male body, the placement of sperm into the female's vagina, and the production of glandular secretions and hormones like testosterone and multiple steroid hormones.

The testes contained in two separate membranes called scrotal sacs. Throughout the rat's life, the testes are able to move up into the rat's abdominal cavity. The testes consist of a mass of coiled tubes (the seminiferous or sperm producing tubules) in which Spermatogenesis occurs within them by meiosis. In a cross section of a seminiferous tubule, the germ cells are arranged in discrete layers (Figure 1.4). The seminiferous tubules are lined by Sertoli cells, which support germ cells in varying stages of development and produce the male sex hormone, testosterone. Spermatogonia

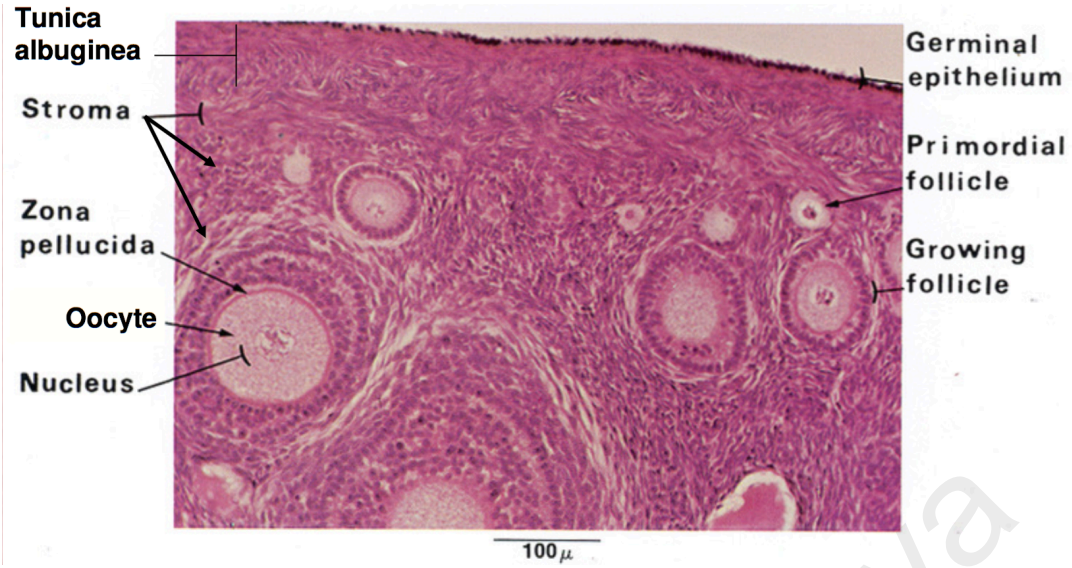


Figure 1.3: Picture showing the different types of follicular stages in the ovary (Bergman & Afifi, 1974).

lie on the basal lamina, spermatocytes are arranged above them and then one or two layers of spermatids above them. In any given normal tubule, four generations of cells develop simultaneously and in precise synchrony with each other. As each generation develops, it moves up through the epithelium, continuously supported by Sertoli cells, until the fully formed sperm are released into the tubular lumen (spermiation).

Spermatogenesis is the process whereby primitive, diploid, stem cell spermatogonia give rise to highly differentiated, haploid spermatozoa (sperm). In fact, the process comprises of a series of mitotic divisions of the spermatogonia, which the final divison gives rise to the spermatocyte; which undergoes the long process of meiosis beginning with duplication of its DNA during preleptotene, pairing and condensing of the chromosomes during pachytene and finally culminating in two reductive divisions to produce the haploid spermatid. The spermatid rapidly undergoes a series of complex morphological changes, where its nuclear DNA becomes highly condensed and elongated into a head region, which is covered by a glycoprotein acrosome coat while the cytoplasm becomes a whip-like tail enclosing a flagellum and tightly-packed mitochondria.

1.3.3 FGF21 in reproductive system

Fibroblast growth factors (FGFs) involved in many developmental and repair processes of several types of mammalian tissues (Reuss *et al*., 2003; Itoh *et al*., 2008) as well as the growth and development of reproductive organs including the testis and ovary. In the testis, FGFs works as mitogenic factors in Sertoli, Leydig, and germ cells that stimulate both DNA synthesis and cell multiplication (Jiang *et al*., 2013). On the other hand FGF2 in the ovary has been identified as an inhibitor of apoptosis and promoter of follicle growth (Santos *et al.,* 2014).

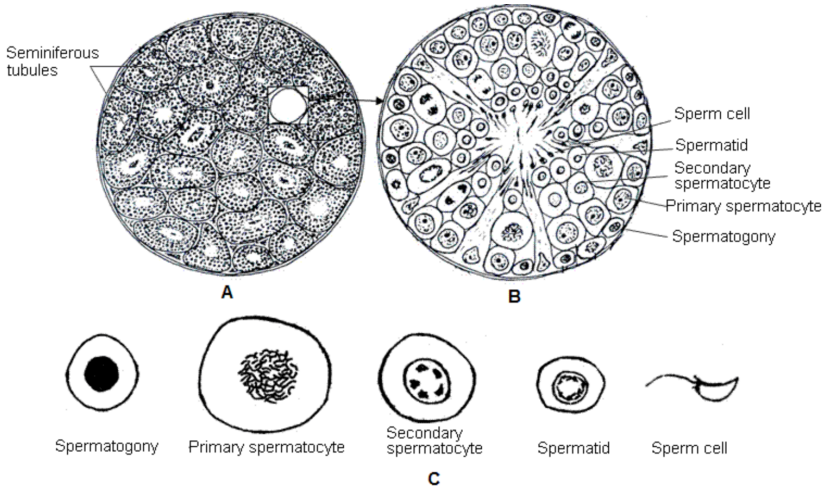


Figure 1.4: Schematic overview of testis and sperm production.

A) Rat testes with seminiferous tubules, B) Seminiferous tubule with different spermiogenesis phases, C) Developmental stages of spermiogenesis (Bergman & Afifi, 1974).

FGF21 has fairly recently been recognized as a novel therapeutic target with potential anti-diabetic properties, useful in treatment of hyperglycemia, insulin resistance, and hyperlipidemia (Mai *et al*., 2011). Although the involvement of FGF21 in various organs has been studied, little is known on its influence in the development of the reproductive organs. However, there were reports that insulin-sensitizing agents had been proposed as the therapy of choice for PCOS, since important pathogenic factors of this syndrome include insulin resistance and hyperinsulinemia (Gambineri *et al.*, 2002).

1.4 Objectives

Type 2 diabetes continues to be a growing global health concern, and as such more effective, safer therapies other than conventional strategies like insulin therapy is needed. Since FGF21 is recognized as a novel metabolic protein that plays a unique role in the regulation of carbohydrate and lipid metabolism, it would be interesting to produce FGF21 for commercial purposes. Till now artificial FGF21 production in large amounts was laborious. The use of *Pichia pastoris* expression system seems to be helpful in this case because of its various advantages mentioned above. Prior to introduction of *fgf21* gene into *Pichia pastoris* cells, a full length of the gene is required. Normally the entire gene without introns can be achieved via RT-PCR of the mRNA from human cells. Alternatively, to overcome the laborious step of mRNA extraction, the entire gene can be constructed *in vitro* via splicing by overlap extension PCR. The specific objectives include:

1-To design and construct fgf21 expression cassette for the purpose of producing the FGF21 protein in both the bacterial (*E.coli*) and yeast (*P.pastoris*) expression systems.

2-To compare and determine the efficacy of expression of the recombinant protein, followed by the determination of the recombinant proteins bioactivity and their effects on the cellular development of reproductive organs in rats.

CHAPTER 2: MATERIAL AND METHODS

2.1 *In vitro* construction of *rfgf21* gene by overlap-extension PCR

2.1.1 DNA extraction

DNA was extracted from human blood sample using QIAamp DNA mini kit (QIAgene, USA) following the manufacturer′s protocol. Briefly, the blood was first thawed to room temperature. Then 20 µl QIAGEN proteinase K was added to every 200 µl of blood and later 4 µl RNaseA (100 mg/µl) was added to each sample. 200 µl AL buffer was then added to the mixture, following incubation at 56 °C for ten minutes. Then the mixture was centrifuged at 13700 X*g* for 15 seconds and 200 µl ethanol 100% was added to the sample and centrifuged again for 15 seconds at 13700 X*g*. The mixture was then transferred to QIAamp spin column and centrifuged at 6000 X*g* for one minute. 500 µl AW1 was then added and the mixture was centrifuged at 6000 X*g* for three minutes. This step was repeated using 500 µl AW2 buffer following centrifugation at 20000 X*g* for one minute. Finally 200 µl AE buffer was added and the mixture was kept in room temperature for one minute following centrifugation at 6000 X*g* for one minute.

2.1.2 DNA Analysis

2.1.2.1 Quantification of DNA samples

Double-stranded DNA (dsDNA) samples were quantified using UV absorbance measurement. The samples' purity were checked by a measurement of absorbance in 230/260nm and 260/280nm with spectrophotometer. Extracted DNA was transferred to a quartz cuvette and placed inside the chamber. The dilution ratios were set to the photometer prior to absorbance read. The absorbance value of each sample was determined against distilled water as blank.

2.1.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was prepared in a final concentration of 1% (w/v) in 1X EDTA. The mixture was heated in a microwave for 80 seconds to dissolve the powder. After the gel was cooled enough, 1 µl ethidium bromide was added to the gel mixture and the whole mixture was poured into the gel casting tray and the comb was inserted to create wells. After the gel was solidified, it was transferred to an electrophoretic tank filled with 1X TBE buffer. 5 µl of each DNA sample was mixed with 1 µl of 6X loading dye and loaded into the wells. 3 µl of 100 bp ladders was loaded into a well in order to estimate fragment size. After loading was completed, the apparatus was connected to a power supply and the gel was run at 120 V until the loading dye reached the end of the gel. The gel bands were visualized by UV transillumination.

2.1.3 Construction of human *fgf21* gene

2.1.3.1 Polymerase chain reaction (PCR)

FGF21 exons were prepared in series of three different Polymerase chain reaction (PCR) amplification using different specifically designed primers. Table 2.1 shows the sequences of the primers used to amplify three exons of the *fgf21* gene. Primers were designed to have complimentary sequencing (overhangs) with each other to facilitate and manage the correct gene direction attachments in overlap extension PCRs. Restriction endonuclease sites were also incorporated into the primers at both ends of the FGF21 fragment to generate sticky ends in order to conduct the correct fusion of genes to plasmids. For this purpose, cleavage sites *EcoR*I and *Not*I for rFGF21-*Pichia Pastoris* construct were used meanwhile for rFGF21-*E. coli* construct, the *EcoR*I and *Hind*III sites were introduced into the N and C terminus of the fragments.

Table 2.1: Sequences of primers used in FGF21-PCR.

Primers	Sequences
FGF21-*EcoR*I-Ex1F	F5′GTCGAGAATTCCACCCCATCCCTGACTC3′
FGF21-Ex1R	R5′GGAGACTTTCGGGGCTCTGG3′
FGF21-Ex2F	F5′CCGAAAGTCTCCTGCAGCTG3′
FGF21-Ex2R	R5′GTGGAGCGATCCATACAG3′
FGF21-Ex3F	F5′GGATCGCTCCACTTTGACCCT3′
FGF21-*Hind*III-Ex3R	R5′AATCTGAAGCTTGGAAGCGTAG3′
FGF21-NotI-Ex3R	R3′ATCACAGCGGCCGCGGAAGCGTAGCTGGGGCTT3′

The underlined sequences are *EcoR*I, *Hind*III and *Not*I sites.

In the first reaction, a pair of primers was designed to produce a sequence containing a *Not*I / *Hind*III cloning site, region of human FGF21 exon 1 DNA sequence, and a linker sequence. In the second reaction, a pair of primers was designed to produce a sequence containing an overlapping portion of the first linker, a portion of the FGF21 coding sequence exon 2, and second linker sequence at its end. Respectively, in the third reaction, a pair of primers was designed to produce a sequence containing the second overlapping linker sequence exon three and an *EcoRI* cloning site.

PCR reactions were performed followed by Sambrook protocol (2000). PCR reactions were prepared in 0.2 ml tubes, consisting of 100 ng of human genomic DNA; 0.4 µM of each primer, 200 µM of each dNTPs, 1X PCR reaction buffer (EUR_X, Poland), 2 mM $MgCl_2$ and 1 U of *Pfu* DNA polymerase (Fermentas, USA). Optimization was performed by testing different annealing temperatures (T_m) and the concentration of magnesium ions to obtain the best condition for each exon. Magnesium concentration was subjected to vary from 0.5 to 2.5 mM and annealing temperatures from 45 °C to 64 °C were tested for each exon.

In all PCR experiments, a negative sample (containing all components except DNA) was used in order to make sure that there was not any contamination in the mixture to produce false results. PCR was performed with a PELTIER thermal cycler model PTC-200.

Amplifications achieved by initial denaturation at 95 °C for 3 minutes, followed by 36 cycles repeat of; 95 °C for 45 seconds as denaturation step, 45 seconds at different annealing temperature for each exon obtained from optimization step and extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 5 minutes. Agarose gel electrophoresis was performed as described in 2.1.2.2.

2.1.3.2 Gel purification of PCR products

Gel extraction was performed using QIAquick Gel Extraction Kit (QIAgene, USA) following the manufacturers procedure. The PCR products were run on agarose gel and then the desired bands were excised from the gel under UV light. The excised gels were then transferred into 1.5 ml tubes and their weights were measured. Buffer QG was added three times of each gel slice weight. After that, the tubes were incubated at 50 °C for ten minutes to dissolve the agarose. In the next step, isopropanol was added equally to each gel slice volume. The mixture was transferred to QIAquick spin column and centrifuged at 10000 Xg for one minute. 0.5 ml buffer QG was added to the spin column followed by centrifugation at 10000 Xg for one minute. 0.75 ml PE buffer was then added to the tubes followed by centrifugation. After transferring the spin column into new tubes, 50 µl EB buffer was added to the column and the pure DNA was collected by centrifugation at 10000 Xg for one minute. The extracted samples were run on an agarose gel again and checked for their quality and their fragment size.

2.1.3.3 Overlap-extension PCR

Three amplified exons of *fgf21* gene were assembled together by overlap-extension PCR. Overlap extension PCR is a bi-directional PCR amplification of specific alleles that conduct the reaction rapidly in One-Tube (Liu *et al*., 1997). Each overlapping reaction consisted of two different series of PCR. As FGF21 has three exons to be overlapped, in the first overlapping reaction, exon one or three were joined to exon two. In the second reaction, all three exons (1+2+3) were joined together.

The composition of the first PCR mixture was the same as initial PCR amplification mentioned above without using the primers. In this step, the two overlapping linker sites of the exons were joined together to link the products of the first PCR reactions produced in section 2.1.3.1. The first round PCR consisted of denaturation step at 95 °C for three minutes, followed by 15 repeating cycles of; 45 seconds at 95 °C, 45 seconds at 60 °C and one minute at 72 °C followed by final extension at 72 °C for five minutes. In the second PCR reaction, the flanking primers exon 1 F and 3 R were used to amplify the whole gene. The second PCR was done as previously described in section 2.1.3.1.

2.2 Production of FGF21 in *Escherichia coli* and *Pichia pastoris*

2.2.1 Preparation of culture media and plates

2.2.1.1 Preparation of Luria-Bertani (LB) media

To prepare the LB media 20g Luria-Bertani (LB), broth powder was dissolved in one liter of dH_2O and autoclaved for 20 minutes at 121 °C. For agar plates, 35 g LB agar powder was dissolved in one liter of dH_2O and autoclaved for 20 minutes at 121 °C. After the temperature decreased, ampicillin, IPTG and X-gal were added to the final concentration of 100 μg/ml for ampicillin, 0.5 mM for IPTG and 80 μg/ml for X-gal in agar plate's media. The mixture was then poured to a petri dish and solidified.

2.2.1.2 Preparation of Low Salt LB (LSLB) medium and agar plates

1% tryptone, 0.5% yeast extract, 0.5% NaCl were mixed with distilled water and PH was adjusted to 7.0 with 1 N sodium hydroxide (NaOH) to prepare the Low Salt Luria-Bertani broth medium. For the agar plates, 1.5% agar powder was added to the above medium and the mixtures were autoclaved for 20 minutes at 121 °C. After the temperature reached to 55 °C, Zeocin^{TM} was added to agar medium to a final concentration of 25 μg/ml. Agar medium was poured in petri dishes and stored in 4 °C.

2.2.1.3 Preparation of YPD and YPDS+ Zeocin^{TM} Agar

To prepare yeast extract peptone dextrose (YPD) broth media 1% yeast extract and 2% peptone were mixed with dH_2O and autoclaved for 20 minutes at 121 °C. For agar plates 2% agar powder was added to the mixture before autoclaving. After autoclaving the agar medium was cooled down and 2% dextrose and 100 μg/ml Zeocin^{TM} was added to the mixture and poured in petri dishes and stored in - 4°C.

2.2.1.4 Preparation of BMGY and BMMY

To prepare buffered glycerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY), 1% yeast extract and 2% peptone were added to dH_2O and autoclaved for 20 minutes at 121 °C. After autoclaving, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, 100m*M* potassium phosphate buffer, pH 6.0 and 1% glycerol for BMGY or 0.5% methanol for BMMY were added to the media.

2.2.1.5 Preparation of rich medium

10g tryptone, 5 g yeast extract, 5 g NaCl and 2 g glucose per each liter were added to dH_2O and the mixture was autoclaved. Ampicillin was added to the final concentration of 100 μg/ml after autoclaving.

2.2.2 Preparation of competent cells

2.2.2.1 Preparation of *Escherichia coli* JM109, TOP 10F' and TB1

Single colonies from overnight cultured competent cells (JM109, TOP 10F' (Invitrogen, Netherlands) or TB1) plate were picked and inoculated in 10 ml LB bottles overnight at 37 °C with shaking at 220 rpm. Each growing bottle was transferred to fresh LB bottles and incubated for two hours at 37 °C with shaking to prepare sub-cultures.

The bacterial cultures were then incubated on ice for 20 minutes and centrifuged at 4 °C at 2400 X*g* for 20 minutes. The bacterial pellets were re-suspended in 0.5 V of 0.1 $CaCl_2$ cold and incubated on ice for another 20 minutes. After centrifugation at 2500 X*g* at 4 °C for 20 minutes, 0.1 M $CaCl_2$ was added to the pellets again and cells were aliquot and kept in -70 °C.

2.2.2.2 Preparation of competent cell X33

Single colony of X33 was added to 10 ml YPD media and the mixture was incubated at 28 °C overnight with shaking at 220 rpm. The overnight culture was diluted in 10 ml fresh YPD media and incubated in the same condition for four hours until OD_{600} reached to 0.6-1. Then, the cultures were centrifuged at 500 X*g* for 5 minutes and the pellets were re-suspended in 5 ml solution I provided by the manufacturer (Invitrogen, USA) followed by second centrifugation. The pellets were re-suspended again in 500 µl sol I, aliquot in fresh tubes and incubated in -80 °C.

2.2.3 Digestion of products and construction vectors

In order to clone rFGF21 in *Escherichia coli* JM109, both rFGF21 and pGEMT not easy vector were digested by restriction endonuclelases (*EcoR*I and *Not*I*)* to create sticky ends for ligation. 2X rapid ligation buffer, digested pGEMT vector, digested

whole *fgf21* gene and *T4 DNA ligase* were then mixed slowly by pipetting and incubated at 4 °C overnight.

To construct the yeast vector, synthetic *fgf21* gene and pPICZαA vector were digested with *EcoR*I and *Not*I with Tango buffered separately and then mixed with 1 µl *T4 DNA ligase* (New England Biolabs, UK) and 1X ligase reaction buffered in 0.5 ml microcentrifuge tube and incubated at 4 °C overnight.

For *E. coli*, C4X and P4X vectors and *fgf21* gene were digested using mixture of *Hind*III, *EcoR*I with Tango buffer for two hours. After confirming the digestion by running the samples on gel, the ligation mixture was proceed by using 7 µl DNA sample, 1.6 µl vectors, 10 X ligase buffer and *T4 ligase* at 4 °C overnight.

The purified recombinant plasmids (pPICZαA-*rFGF21*) with the correct sequence of the *fgf21* gene, were linearized by *BstX* I (Fermentas, USA). 1 µgr of the plasmids was added to the mixture of 1 X buffer and 1 unit of enzyme in 55 °C overnight. To inactivate the enzyme, the mixture was incubated fifteen minutes in 80 °C. To confirm the digestion, 5µl of the mixture was run in agarose gel as previously described.

2.2.4 Transformation

Transformation Ligated products (*fgf21*-pGEMT) were transformed into 100 µl thawed competent cells (JM109, TOP 10F' or TB1) on ice. The mixture was incubated for 20 minutes (30 minutes for TOP 10F') on ice with gentle hand mix up, and kept for 45 seconds in 42 °C. Then, the mixture was immediately transferred and incubated in ice for five minutes. 900 µl of LB medium (or LSLB medium for TOP 10F') was added to the mixture and incubated for three hours at 37 °C while shaking. The cells were centrifuged and plated in LB agar plates containing ampicillin (100 µg/ml) or LSLB agar plates Zeocin^{TM} (25 µg/ml) and incubated at 37 °C overnight.

For EasyComb™ Transformation, 3 µg linearized vector was added to *P. pastoris* competant cells, strain X33 (wildtype) using an easy comb method followed by manufacturer's instructions; briefly, the mixture was incubated for one hour with 1ml solution II at 30 °C, followed by a heat shock in 42 °C for 10 minutes. Then, 1ml of YPD (yeast peptone dextrose) was added to the mixture and incubated for two hour at 30 °C without shaking. After the incubation, the cells were collected by centrifuge at 3000 X*g* for 5 minutes and the pellet was re-suspended in 500 µl solution III followed by second centrifugation. 150 µl Sol III was added to the pellet and the mixture was plated on yeast extract peptone dextrose (YPDS) medium containing zeocin (100 mg/ml). Plates were incubated at 30 °C for 3 to 10 days at 30 °C.

2.2.5 Colony selection

The recombinant colonies were selected by scoring for ampicillin or Zeocin™ on the plates. White colonies were picked from the overnight-cultured plates by toothpick to make a library of transformants and then, then transferred into 50 µl dH_2O in 0.5 ml tubes. After boiling the tubes at 99 °C for ten minutes, the samples were centrifuged and used as DNA source to amplify by colony-PCR using primer m13 forward and m13 reverse for JM109 competent cell samples, using primer 3R FGF21 and α factor primer sequence or 1F and AOX1 reverse primer for TOP 10 samples, using primers mal E and 3R FGF21 for TB1 samples and using primers AOXI reverse primer and α factor primer for X33 samples. Colony PCRs were achieved by amplifying the samples for three minutes at 95 °C for initial denaturation step, followed by 35 cycles of; 45 second at 95 °C, 45 second at 60 °C and 90 seconds at 72 °C followed by a final extension step at 72 °C for five minutes using *Taq* DNA polymerase (EUR_X, Poland). The amplified products were run on agarose gel to check the colonies with correct insertion. Sequence of positive purified transformants were confirmed by DNA sequencing.

2.2.6 Plasmid extraction

Single positive recombinant colonies were picked from library plates and cultured in 10ml LB medium with ampicilline (100 µg/ml) or LSLB medium with 25 µg/ml Zeocin^{TM} overnight at 37 °C with 220 rpm shaking. The next day, cultures were centrifuged at 6000 rpm for 15 minutes and then the pellets were re-suspended in 200 µl of solution I by vortex and transferred to 1.5 ml tubes. 200 µl solution II was added and the mixture was mixed by hand shaking for four minutes in room temperature.

After that, 200 µl solution III was added and the tubes were incubated at 0 °C for 15 minutes. The mixtures were then centrifuged for 10 minutes at 18000 X*g*, and the supernatants were transferred in new tubes. *RNaseA* (10 µg/µl) was then added to the supernatants and the mixtures were incubated for three hours at 37 °C, after which 600 µl phenol was added to the tubes and centrifuged at 18000 X*g* for three minutes. Again, the supernatants were transferred to new tubes and 600 µl chloroform was added to the tubes followed by vortex and centrifugation. After transferring the supernatants to new tubes, 0.1 volumes 5 M NaCl and 2.5 Volume isopropanol were added to the mixture and incubated on ice for 20 minutes. The tubes were then centrifuged at 18000 X*g* for 15 mintues and the pallets was washed with 70% ethanol and dried. After that, 50 µl dH_2O were added to the pallets and the tubes were kept in -20 °C. DNA sequencing was carried out to confirm the existence of the correct recombinant *fgf21* gene in the plasmids.

2.3 Protein expression

2.3.1 Induced expression of rFGF21 in yeast

Single large colonies of the confirmed yeast transformants from the YPDS plates were picked and initially inoculated into 125 ml conical flasks containing 10 ml BMGY medium at 30 °C for 24 hours at 220 rpm. When the initial culture OD_{600} reached to 2.0-

6.0, the culture was centrifuged for eight minutes at 3000 X*g*, and the pellets were collected and re-suspended by 2 ml YP medium and transferred to BMMY medium to induce expression under aseptic conditions. The cells were allowed to grow for 96 hours at 30 °C or 20 °C. Methanol was added every 24 hours to a final concentration of 0.5%-2.5% to maintain the best induction time during the incubation. Every 24 hours one of the samples was collected to study the time course.

2.3.2 Induced expression of rFGF21 in *E. coli*

Single large positive *E. coli* transformants from the LB plates were picked and initially inoculated in normal LB for overnight at 37 °C. Then, 800 µl of the overnight culture was sub-cultured into the 125 ml conical flasks containing Rich medium, glucose and ampicillin until the cell concentration OD_{600} reached to 0.5.

At this point, IPTG was added to the media to induce the cells. Samples were collected after the induction in different times and kept in column buffer. The control plasmids were subjected to the same treatment procedures as well.

2.4 Optimization

2.4.1 Optimization in Yeast

Three different parameters were compared for yeast expression. Different harvest time (24h, 48h, 72h, 96h), different methanol concentration (0.5%, 1%, 1.5%, 2%, 2.5%) and different culture temperature (20 °C and 30 °C) were tested.

2.4.2 Optimization in *E. coli*

Different IPTG concentration from 0.3 mM to 1 mM and different sampling time point after IPTG induction (one, two, three and four hours after induction) were tested.

2.5 Protein analysis

2.5.1 Preparation of proteins from supernatants

Harvested samples from yeast samples were centrifuged at 4000 X*g* for eight minutes to collect the cells supernatants and cells' debris. One volume of supernatants was mixed with three volumes of 100% ice-cold acetone for six hour in -20 °C. The mixture was then centrifuged at 4 °C 11000 X*g* for thirty minutes. The pellets were rinsed with dH_2O and re-suspended in PBS.

The *E. coli* samples were subjected to freeze and thaw for thirty seconds for five times, followed by centrifugation at 11000 X*g* for thirty minutes, to separate the cells' debris.

2.5.2 Preparation of proteins from cell pellets

Cell pellets from culture media were re-suspended in breaking buffer. Equal amount of acid-washed 0.5 mm glass beads (Sigma, USA) was added to the mixture. The mixtures were then vortexed and chilled on ice for eight times followed by centrifugation at 12000 X*g* for 10 minutes at 4 °C. The supernatants were kept and the pellets were again mixed with breaking buffer and 2% SDS followed by centrifugation at 2300 X*g* for five minutes at 4 °C. The supernatants were kept for further analysis.

2.5.3 Protein purification

2.5.3.1 Protein purification using Ni column

Acetone precipitated proteins derived from expression experiments were applied to the His GraviTrap™ column containing Ni Sepharose™ 6 fast flow (Amersham Biosciences, USA). The column was first equilibrated with phosphate buffer, and then the samples were added to the column. Binding buffer (20 mM Sodium phosphate buffer, 500 mM *NaCl* and 20 mM Imidazole) was then applied to the column followed

by elution buffer. Binding buffer washed the non-bound proteins. The bound materials were collected with 200 mM Imidazole and phosphate buffer (elution buffer).

2.5.3.2 Protein purification using amylose column

The *E. coli* samples were then purified using classic amylose purification column. The column was first equilibrated with column buffer, and then the samples were added to the column. Binding buffer was then applied to the column followed by elution buffer. The non-bound protein fraction was washed by column buffer (20ml 1.0*M* Tris-Hcl, pH=7.4, 11gr *NaCl* and 2ml 0.5*M* EDTA, 0.7ml *β*-mercaptoethanol), meanwhile the bound materials were collected with 10*mM* maltose and column buffer (elution buffer).

2.5.4 Sodium Dodecyl Sulphate Poly Acrylamide gel Electrophoresis

2.5.4.1 Poly- Acrylamide gel preparation

First, the gel apparatus was assembled. The gel mixture was prepared between two glass plates washed with absolute ethanol. 12.5% poly-acryl amide gel (separating gel) was prepared by combining all the reagents and poured gently to the cassette. Then, butanol was poured to the surface of the separating gel. After the gel dried completely, the butanol was discarded and 4.5% stacking gel was prepared and poured to the cassette, followed by placing the comb on top of the gel.

2.5.4.2 Sample preparation

Centrifuged samples were dissolved in denaturing buffer (0.5 M Tris-Hcl (PH=6.8), glycerol, 10% SDS, *2B*-mercaptoethanol, 0.05% bromophenol blue) and heated at 100 °C for four minutes.

2.5.4.3 Electrophoresis

After the gels were solidified, the cassettes were transferred into the tank and filled with 1 X electrophoresis buffer. Samples and pre-stained ladder (marker) were loaded into the wells. SDS PAGE analysis was performed according at 180 V for one hour in mini trans blot electrophoretic transfer cell (Bio Rad, Hercules, CA). Once the loading dye reached to the end of the plates, the connections were unplugged and the plates were separated.

2.5.5 Silver staining

The gel was transferred to a tray filled with Fixation solution and placed on a shaker at 160 rpm for thirty minutes. After that, the gel was transferred to infiltrating solution and shook for another thirty minutes. After rinsing the gel with distilled water for three rounds of five minutes, the gel stained with silver solution for twenty minutes on the shaker. Then the staining solution was added. Once all bands appeared with an acceptable contrast, stopping solution was added.

2.5.6 Western blot analysis

2.5.6.1 Transfer of proteins to the membrane

Separated proteins by polyacrylamide gel electrophoresis (PAGE) transferred from the electrophoresis gel to nitrocellulose membrane (Amersham Bioscienses, USA) by assembling gels and nitrocellulose membrane to sandwich apparatus and run at 100 V for one hour in transfer buffer.

2.5.6.2 Staining

The membrane was incubated in blocking buffer for one hour with 1 XTBS+5% milk powder in room temperature, and then incubated for two hours at room temperature with diluted anti FGF21 antibody (1:1000) from Abnova. Then, the membrane was washed five times with 1 XTBS+tween 0.02%, followed by second incubation with goat

anti mouse IgG conjugated to horseradish peroxidase (1:10000) (Promega, USA) for two hours. The washing step was repeated for five times, and then the western blue stabilized substrate for alkaline phosphatase (Promega, USA) was added to visualize the bands on the membrane.

2.5.7 Bradford assay

Protein concentration was determined by Bradford method (Bio Rad, USA). One volume of dye reagent was diluted with four volumes of sdH_2O and filtered through Whatman filter paper (Whatman, UK). Six different dilutions of BSA from 0 mg/ml to 0.5 mg/ml were prepared as standard solutions. 10 µl from purified samples and standards were transferred into separate microtiter plate wells (Greiner Bio-one, Germany). 200 µl diluted-dye was added to each well and mixed by pipetting. The plate was incubated at room temperature for five minutes and then the absorbance was read by ELISA reader at 595 nm.

2.5.8 Deglycosylation and dissection of MBP

2.5.8.1 Deglycosylation of the protein

Purified Yeast protein in PBS was digested with 0.1 U *endoglycosidase H* and *PNGase F* (New England BioLabs, UK) according to manufacturers' protocol. The sample was denatured with denaturing buffer at 100 °C for ten minutes. 1 µl of *PNGase F* or *endoglycosidase H* was added and the mixture was incubated at 37 °C for one to twenty four hours. The result was analyzed by SDS-PAGE and western blotting.

2.5.8.2 Cleavage of MBP

Fifty µg purified *E. coli* protein in column buffer+maltose was digested with 1 µg FXa at room temperature for one to twenty four hours, followed by SDS-PAGE and western blot analysis.

2.6 *In vivo* experiments

To evaluate the efficiency and the bioactivity of FGF21 expressed in *E. coli* and *P. pastoris*, *in vivo* examinations were done. Based on literature review, rats are excellent models for this type of study compared to other animal model. The rats are easy to handle, available, resistant to infection, they have short breeding duration, fast growing up and high similarity in biological activity to human body and the most important is their affordable price (Zhang *et al.,* 2012, Mu *et al.,* 2012, Sarruf *et al.,* 2010, Kubicky *et al.,* 2012; Xu *et al.,* 2009). For each experimental group three rats were investigated in respect to animal ethic committee as replicates. Studies with small numbers of measurements are rare, but they used to be common and remain plentiful in specialist clinical journals (Bland & Altman, 2009) and since our population is normal, and have almost identical genetically and the function of FGF21 had been investigated before, we don't need to have large number of replicates. First, toxicity study (acute and sub-chronic) was done to investigate the interrelation between toxicity and dosage as well as to determine the safe dose for further studies. For this purpose, Sprague Dawley adult rats were chosen since insulin resistancy will develop in this species rapidly by feeding them with diets high in sucrose (Pagliassotti *et al.,* 1994,1995,1996; Storlien *et al.,* 1988).

For toxicity tests sixty healthy Sprague Dawley adult rats aged 6-8 weeks (initial weight 180-200 g) were obtained from animal house, Faculty of Medicine, University of Malaya. The rats were divided to three groups; 24 were assigned for investigating the products obtained from bacteria and 24 were assigned for investigating the products obtained from yeast, and 12 were assigned as control.

Twelve Sprague Dawley rats in each group were used to study the acute or sub-chronic study of the *Ecoli* products and 12 Sprague Dawley rats were used to study the

acute or sub-chronic study of the Yeast products; from each, 12 males and 12 females SDs were divided equally and randomly. Two doses of the proteins were prepared for each experiment based on different literature reviews (Kharitonenkov *et al.,* 2007); high dose and low dose. All rats were housed individually and maintained under standard conditions of humidity (50-60%), temperature (22 ± 3 °C) and light (12h light: dark cycle) and fed with standard chow diet (Gold Coin Feedmills Pte. Ltd, Malaysia). Normal tap water was available throughout the experiment. Animals were kept in accordance with the ethical guidelines for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences, defined in the "guide for the care and use of laboratory animals" (Clark *et al.*, 1997). This experiment was approved by University Malaya animal ethics committee (Ethics No. ISB/19/07/2013/FE(R)).

Body weight, behavior and food intake were observed and monitored every day during the course of the study. Rats were anaesthetized prior to harvesting of blood using a combination of ketamine (conc. 100mg/ml) and Xylazine (conc. 20mg/ml) according to body weight with the ratio of 1/10, administered intra-peritoneally by using 1 ml syringe with 25-gauge needle. Blood samples were obtained from heart (cardiac puncture) into heparin vacutainer tubes, kept instantly at 4 °C and sent to Clinical Diagnostic Laboratory University Malaya Medical Center for the analysis of biochemical factors. The liver and kidney were removed and preserved in 10% formalin for histological section studies mentioned in 2.6.7.1 and 2.6.7.2 to investigate any general structural changes in the organs, degenerative alterations, necrosis evidence and signs of inflammation.

2.6.1 Acute test

A total of 30 healthy Sprague Dawley adult rats (15 females, 15 males) were divided to five groups (n=3): six were assigned for investigating the toxicity of *E. coli*-produced

FGF21 and six were assigned for investigating the toxicity of *P.pastoris*-produced FGF21, and three were assigned as control. Controls were not treated with any recombinant FGF21. FGF21 was injected to the rats' intra-peritoneal cavity once by 25G syringe with two different concentrations of 100 μg/kg body weight of FGF21 dissolved in dH_2O (Low-dose injection) and 300 μg/kg body weight of FGF21 dissolved in dH_2O (High-does injection) in different groups. The observation was done on mortality, such as death in which number of rats exited from the study before the study period was completed and the behavioral changes of rats following treatment for 14 days (Scott & Robert, 2006). All animals were sacrificed after 15 days.

2.6.2 Sub-chronic toxicity test

A total of 30 healthy Sprague Dawley adult rats (15 females, 15 males) were divided to five groups (n=3): six were assigned for investigating the toxicity of *E. coli*-produced FGF21 and six were assigned for investigating the toxicity of *P.pastoris*-produced FGF21, and three were assigned as control. Controls were not treated with any recombinant FGF21. FGF21 was injected to the rats' intra-peritoneal by 25 G syringe with two different concentrations of 100 μg/kg body weight of FGF21 dissolved in dH_2O (Low-dose injection) and 300 μg/kg body weight of FGF21 dissolved in dH_2O (High-does injection) in different groups. For the sub-chronic experiment, animals were dosed daily for 27 consecutive days. All animals were sacrificed after 27 days.

2.6.3 Effects of rFGF21 on blood glucose and lipids in diabetic model

Nine male Sprague Dawley rats (three groups, n = 3 each) were gavaged daily with 0.1 % body weight fresh high-sucrose mixed with 15% (w/w) of palm oil (Unilever Co. Malaysia) dissolved in water for four weeks to produce insulin resistance rats. High-sucrose high-fat diet was prepared daily.

After producing mimic type 2 diabetic rats, each rat received 200 μg/kg body weight rFGF21-*E. coli* or 200 μg/kg body weight rFGF21-Yeast or normal saline as control daily for duration of two weeks following previous diet. Another three male Sprague Dawley were kept without any injection nor any high-sucrose high-fat diet as negative control. Body weights were recorded weekly. Blood glucose levels were recorded daily with an Accu-check Instant Plus glucometer (Accu-Check, Roche Diagnostic Corporation, USA) during rFGF21 administration. Blood lipids were measured at the end of experiment.

2.6.4 Effects of treatment of FGF21 on blood glucose and lipid profile

Fifteen Sprague Dawley female rats (5 groups, n = 3 each) were fasted overnight and gavaged (V=0.1% BW) with high-sucrose high-fat diet. rFGF21 produced in *E. coli* and yeast was injected intra-peritoneally at 100 μg/kg as low dose, 300 μg/kg as high dose after feeding the animals. One group was injected with saline as control. Blood was collected by tail vein incision, and blood glucose was measured at 60,120,180 minutes after rFGF21 administration. Blood concentration of triglyceride, cholesterol, low-density lipoprotein and high-density lipoprotein were measured at the end of the experiment after sacrificing the animals.

2.6.5 Oral glucose tolerance test

Three groups (n=3) of female Sprague Dawley rats were fasted overnight and then subjected to oral glucose challenge (2 g/kg BW, with gavaging)(Sigma Aldrich, St. Louis, USA). At the same time, 200 mg/kg BW of rFGF21 protein produced in *E. coli* or yeast was injected and glucose (obtained by tail vein incision) was measured by Accu-check Instant Plus glucometer (Accu-Check, Roche Diagnostic Corporation, USA) at 0 (before glucose intake), 30, 60, 120, 180 minutes after the rFGF21 protein injection.

2.6.6 Effects of FGF21 protein on the reproduction system

Twelve healthy Sprague Dawley adult rats (six female and six male) aged 6-8 weeks were randomly divided into two groups; treated and untreated group (n=3 each). Female and male rats in treatment groups were treated with 300 μg/kg BW rFGF21 produced in yeast daily for one month. During the treatment period, clinical signs were recorded daily. Behavior and food intakes were observed and monitored every day and body weights were measured weekly during the experiment. rFGF21 produced in yeast were used because based on our experiments yeast produced the product faster in more amounts and it was easier to provide the sample.

2.6.7 Histological procedures

2.6.7.1 Collection of organs

24 hours after the last treatment, rats were anaesthetized prior to harvesting of blood using combination of ketamine (100 mg/ml) and Xylazine (20 mg/ml) according to their body weight with the ratio of 1/10 intraperitoneally by using 1 ml syringe 25 gauges. Rats weighted alive, before sacrificed, using a weighing balancer (Ohaus scale crop). Blood samples were obtained from heart (Cardiac punctur) and sent to CDL (Hospital) for biochemical test. The animals were then placed on a dorsal recumbency and a midline incision was made. The organs were examined *in situ*. The sizes of the reproductive male organs were measured using ruler. Reproductive organs were excised and weighed using a balancer P1210 (Mettler Instruments AG, Switzerland).

The following organs were measured and recorded: ovaries (paired), uterus, and adrenal gland (paired) for female organ and for male, testis, epididymis and seminal vesicle were weighed. Relative organ weights were calculated on the basis of the final body weight. After measuring the organs' weight, the organs were transferred in plastic cassettes and fixed in 10% formaldehyde overnight (Ajax Finechem, Taren Point,

Australia). The right testis was used for analyzing. Liver, kidney and reproduction system were removed for histopathological examination. At the end of the experiment, the carcasses were placed in plastic bags and stored in deep freezer to be disposed by the UM Registered Bio-disposable Contractor.

2.6.7.2 Tissue preparation

Histological analysis was done as described by Akpantah (2003). After organs were embedded in paraffin block, they were de-hydrated in ascending grades in an automated tissue-processing machine (Leica Microsystems, Nussloch, Germany). The tissues were immersed in 70% ethanol for one hour, 95% ethanol for one hour, first absolute ethanol for one hour, second absolute ethanol for ninety minutes, third absolute ethanol for ninety minutes, fourth absolute ethanol for two hours, first Xylene for one hour, second for Xylene one hour, first paraffin (Paraplast, USA) at 58°C for one hour, and second paraffin at 58°C for one hour. The tissues were passed through 90% absolute alcohol and xylene to become clearer. The processed tissues were embedded in paraffin to prepare them for sectioning.

Blocks to be sectioned were kept in ice for ten minutes before sectioning. A microtome (Leica Microsystems, Nussloch, Germany) was used to cut the tissues into sections of 5 μm to prepare them for staining. Excised sections were floated on the surface of the 37 ºC water bath and transferred to surface of glass slides. The slides with paraffin sections were incubated in 65 °C oven for overnight. These tissues were then de-paraffinized with xylene (BDH, England) and hydrated in absolute alcohol; 70% alcohol, 50% alcohol and then to water for five minutes. Subsequently, the slides were stained with hematoxylin and eosin (H & E) according to the standard protocol (Suzuki *et al*., 2012) and viewed under a light microscope (Olympus CH-BI45-2) and photographed using digital camera connected to a computer**.** The stained slices of the

tissues were subjected to the microscopic evaluation to observe any structural changes compared to the control groups. The samples were analyzed for general structural changes in the organs, degenerative alterations, necrosis evidence and signs of inflammation.

2.6.8 Statistical analysis

The data were analyzed as a completely randomized experimental design using the General Linear Model. Each molecular experiment was performed at least three times. Values were expressed as means ± S.E.M. As the efficiency and bioactivity of produced recombinant FGF21 in all our comparisons experiments were demanded, we used mean instead of median. Mean is preferred as the best measure of central tendency because it is the measure that include all the values in the data set for calculation and any changes in each scores will affect the value of the mean. The mean value comparisons between groups were performed by an analysis of variance (ANOVA) for repeated measurements, one-way ANOVA, post hoc test, Tukey HSD. Differences with $p < 0.05$ were considered significant.

CHAPTER 3: RESULTS

3.1 *In vitro* construction of *rfgf21* gene

3.1.1 Primer design

Overlap extension by PCR was applied to join the three exons (exons I, II, and III) of the *fgf21* gene. Eight pairs of specific primers were designed and they were made based on the published sequences of the *fgf21* gene to have complementary sequences with the adjacent exons at both ends in order to facilitate fragment joining. Additional nucleotides were inserted into primers to generate the derived gene fragments according to the aims of this project.

3.1.2 Construction of *fgf21* gene

Exons I (151 bp) and II (104 bp) were successfully amplified at annealing temperature 64 °C, 2mM of Mg^{2+} and 1U of *Pfu* DNA polymerase, while exon III (291 bp) was successfully amplified at annealing temperature 64 °C, 2mM of Mg^{2+} and 1U of *Pfu* DNA polymerase (Figure 3.1, 3.2 and 3.3). After successful gel purification (Figure 3.4), the correct sequences of these three coding exons were confirmed by DNA sequencing (appendix C).

The whole *fgf21* gene (546bp) successfully obtained by joining the linker sites of these three gel-purified fragments by running two different overlapping PCR amplification reactions (Figure 3.5). In the first overlapping PCR reaction, purified exon 1 and two or exon two and three linked together (Figure 3.6) and in the second overlapping PCR, exon 1+2 was successfully attached to exon 3, or exon 1 was attached to exon 2+3 respectively. The correct sequences of each reaction confirmed by comparing their DNA sequencing with reference gene sequence; GenBank accession number: NM_019113.2 after gel purification (Figure 3.7).

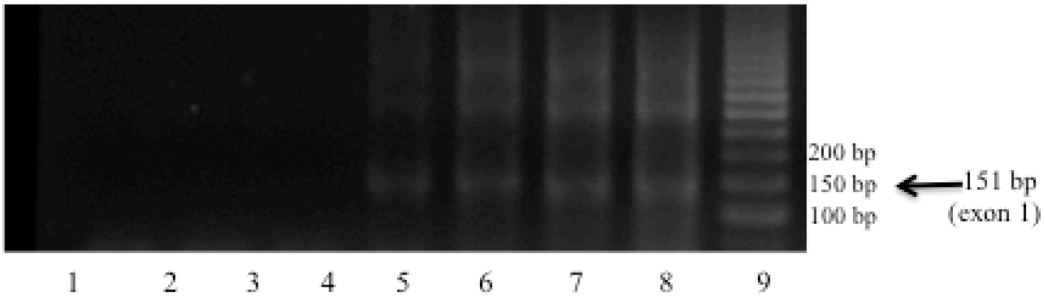


Figure 3.1: Example of PCR optimization using variable concentration of magnesium from 0.5 to 2.5mM. The expected amplicon size in exon one is 151bp. Magnesium concentration of 2mM was chosen as the most suitable condition for amplification of exon one.

Lane 1: 0.5 mM magnesium
Lane 2: 0.75 mM magnesium
Lane 3: 1 mM magnesium
Lane 4: 1.25 mM magnesium
Lane 5: 1.5 mM magnesium
Lane 6: 1.75 mM magnesium
Lane 7: 2 mM magnesium
Lane 8: 2.5 mM magnesium
Lane 9: 50bp DNA ladder (M)

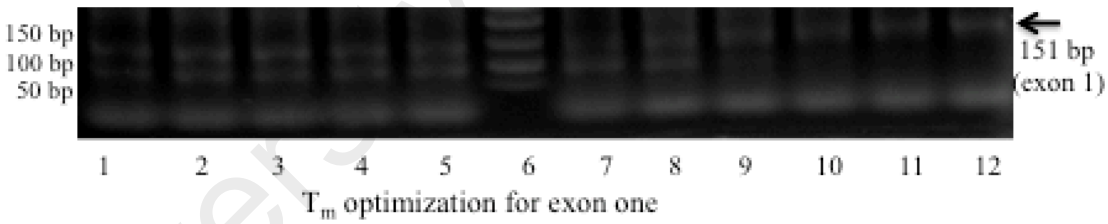
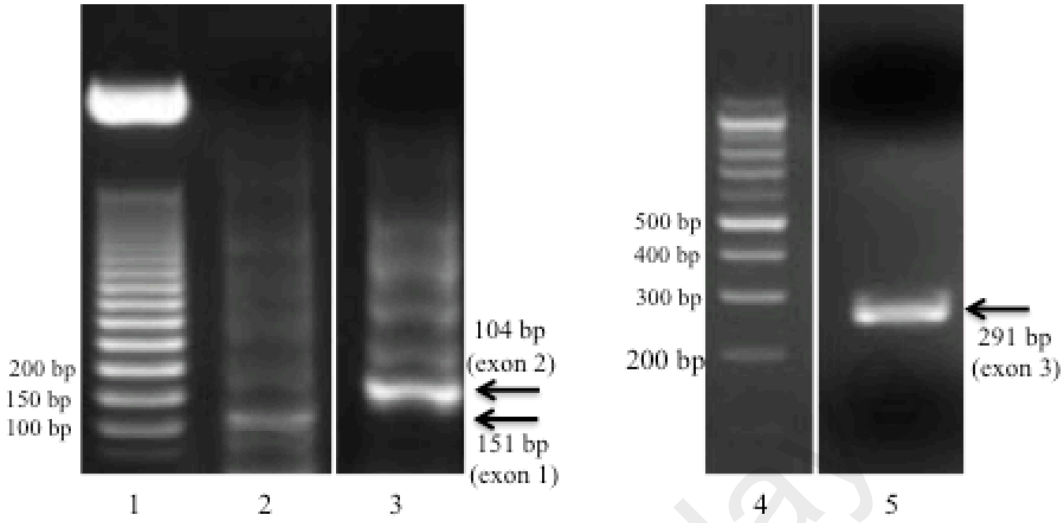


Figure 3.2: Example of PCR optimization using variable annealing temperature from 45 °C to 64 °C. The expected amplicon size in exon one is 151bp. T_m optimization 64 °C was chosen as the most suitable condition for amplification of exon one.

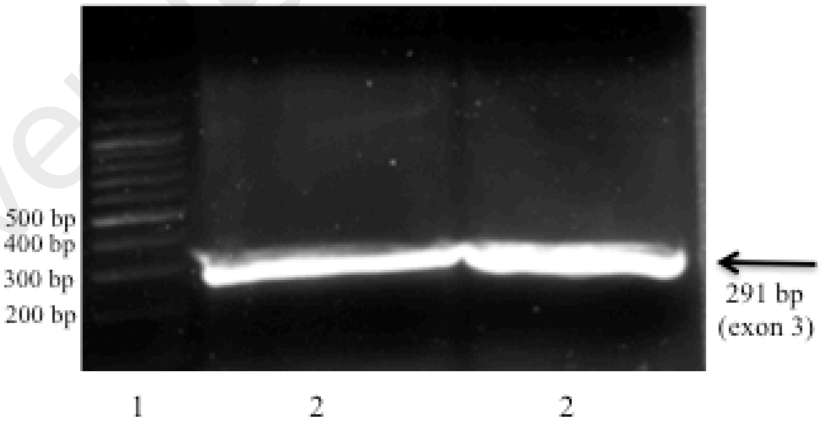
Lane 1: annealing temperature at 45 °C
Lane 2: annealing temperature at 46 °C
Lane 3: annealing temperature at 47 °C
Lane 4: annealing temperature at 48 °C
Lane 5: annealing temperature at 50 °C
Lane 6: 50bp DNA ladder (M)
Lane 7: annealing temperature at 53 °C
Lane 8: annealing temperature at 56 °C
Lane 9: annealing temperature at 59 °C
Lane 10: annealing temperature at 61 °C
Lane 11: annealing temperature at 63 °C
Lane 12: annealing temperature at 64 °C



PCR amplification of three exons of *fgf21* gene

Figure 3.3: PCR amplification of three exons of *fgf21* gene after first PCR. Exons one and two were successfully amplified using annealing temperature at 64 °C while exon three was at 55 °C as annealing temperature. The expected amplicon size for exon one is 151bp, exon two is 104 bp and for exon three is 291 bp.

Lane 1: 50bp DNA ladder
Lane 2: PCR product of exon 2
Lane 3: PCR product of exon 1
Lane 4: 100bp DNA ladder
Lane 5: PCR product of exon 3



Gel purification of exon three

Figure 3.4: Example of gel purification. The expected amplicon size in exon three is 291bp.

Lane 1: 100bp DNA ladder
Lane 2: PCR product of exon 3

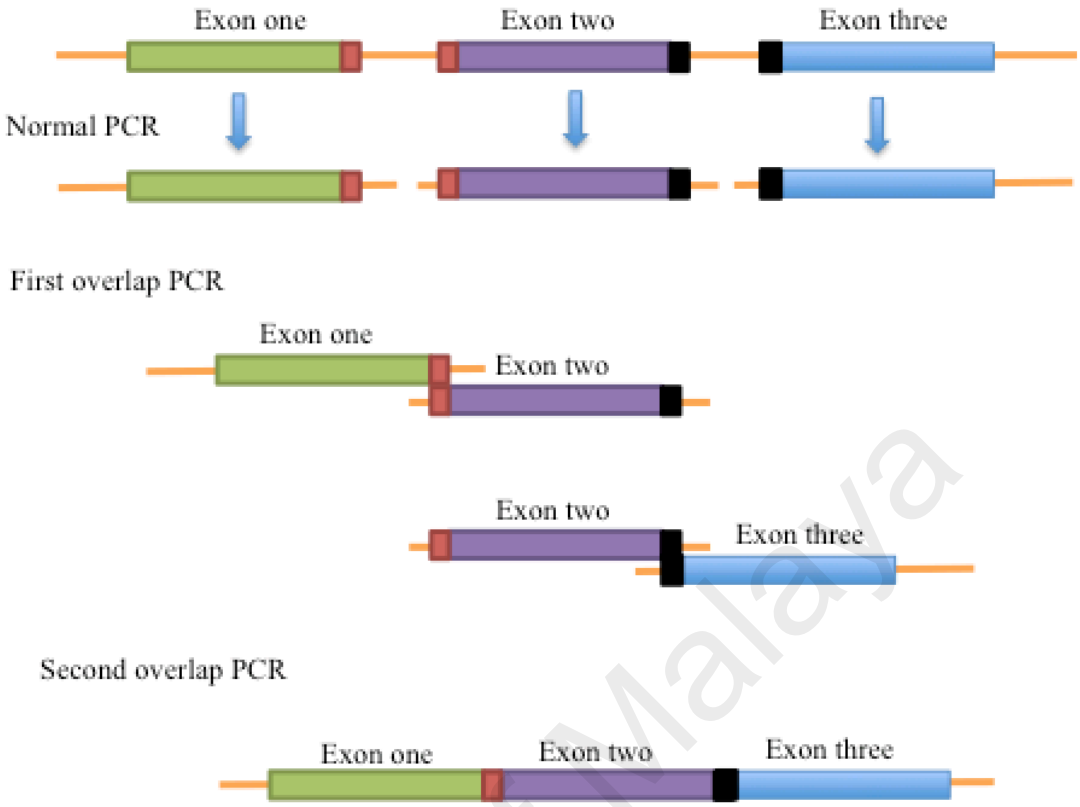


Figure 3.5: Schematic overview of constructing the entire *fgf21* gene. Three amplified exons of *fgf21* gene assemble together by overlap-extension PCR. Each overlapping reaction consist of two different series of PCR. As FGF21 has three exons to be overlapped, in the first overlapping reaction, exon one or three joins to exon two. In the second reaction, all three exons (1+2+3) join together.

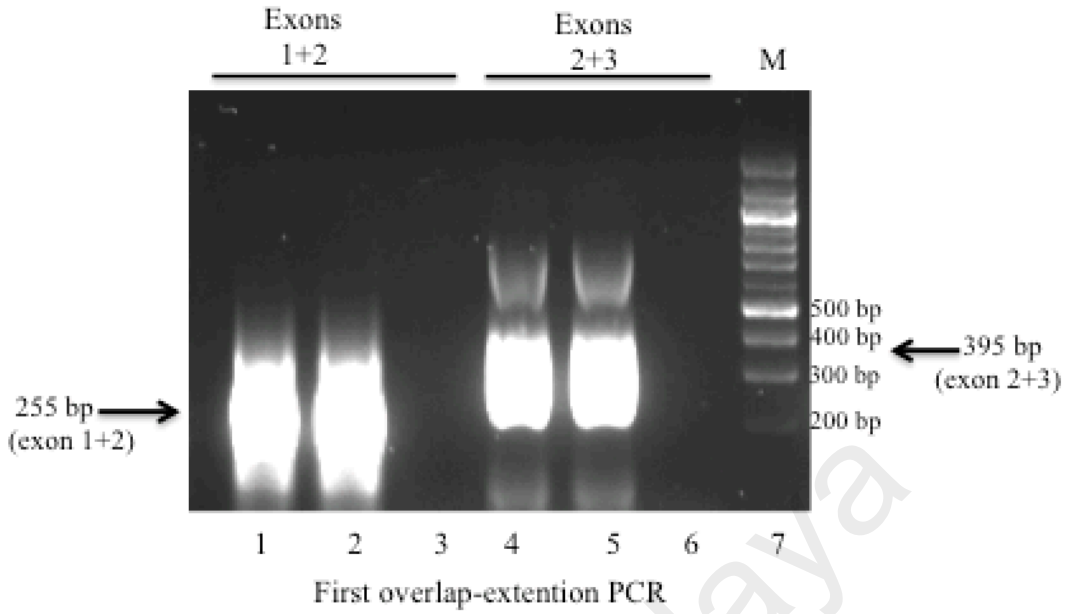


Figure 3.6: Construction of exon 1+2 and exon 2+3 r*fgf21* gene by overlap-extention PCR. The expected amplicon size for exon one and two is 255 bp, exon two and three is 395 bp.

Lane 1 & 2: PCR amplification of exon 1+2
Lane 4 & 5: PCR amplification of exon 2+3
Lane 3 & 6: Negative controls
Lane 7 :100bp DNA ladder (M)

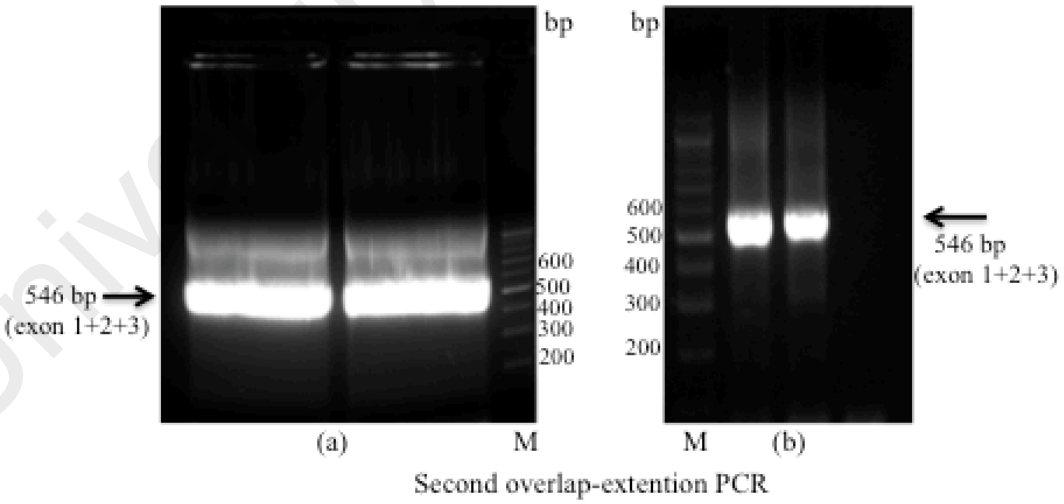


Figure 3.7: Construction of entire r*fgf21* gene by overlap-extention PCR. (a) Gel purification of *fgf21* fragment. (b) r*fgf21* after purification. The expected amplicon size for exon 1+2+3 is 546 bp.

M: 100bp DNA ladder.

3.2 Cloning

3.2.1 Cloning in *Escherichia coli* JM 109

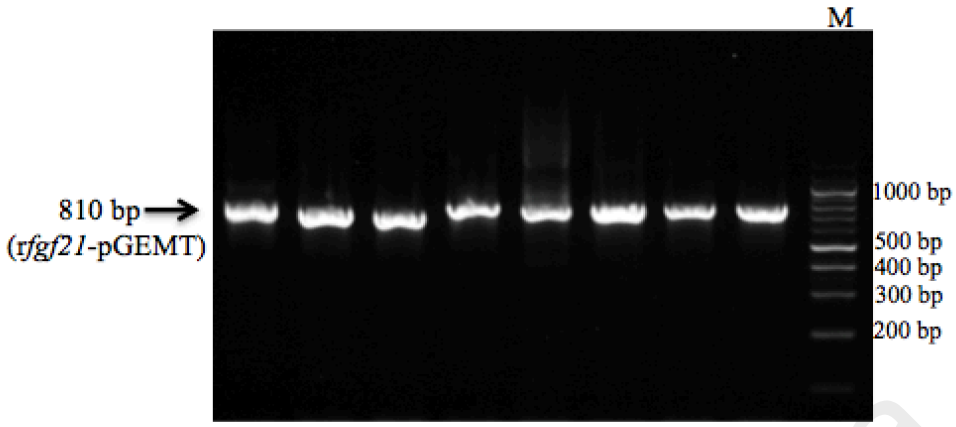
The construct ligated successfully to pGMT vector and cloned in *Escherichia coli JM 109*. Fragment with 810bp size and its correct DNA sequence confirmed the successful insertion of rFGF21 (Figure 3.8 and 3.9).

3.2.2 Cloning in *E. coli TOP 10*

The rFGF21 construct and vector digested successfully with restriction endonuclease *EcoR*I and *Not*I and produced overhangs at both ends, which provided the ligation of *fgf21* gene to the vector (Figure 3.10). The rFGF21 constructs successfully inserted into downstream of the AOXI promoter between the *EcoRI* and *Not*I sites of pPcIZα A vector, in a way that rFGF21 placed under the control of alcohol oxidase promoter on the vector and fused to a factor signal sequence and a C-terminl His6-Tag. The pPICZα-rFGF21 construct was successfully transformed into *E. coli*, TOP10 strain. PCR screening and sequence of positive pPICZα-rFGF21 construct with a fragment size of 750bp DNA confirmed the presence of the correct gene (Figure 3.11).

3.2.3 Cloning in *Pichia pastoris*

The positive confirmed pPICZα-*rfgf21* plasmids linearized successfully with *BstX*I as shown in Figure 3.12. Digestion of this DNA fragment that had homology to AOX1 at both of its ends, lead to replacement of genomic AOX1 by the fragment which generated a Mut^{s} recombinant strain. The vector integrated to the X33 competent cells successfully by easy comb method and its integration confirmed by PCR amplifications with AOXI universal primers which produced 1000bp DNA (Figure 3.13).



PCR colony amplification of r*fgf21*-pGEMT

Figure 3.8: PCR colony amplification of r*fgf21*-pGEMT transformant by using m13 forward and m13 reverse primers. Expected positive transformant amplicon size is 810 bp.

M: 100bp DNA ladder.

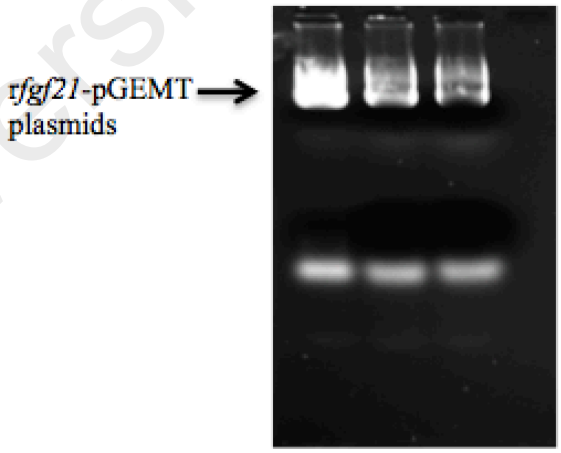


Figure 3.9: Plasmid extraction of the r*fgf21*-pGEMT transformants.

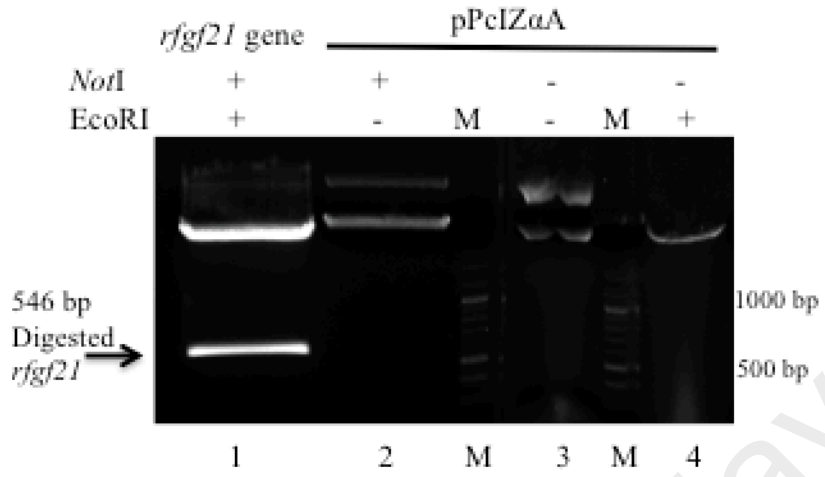
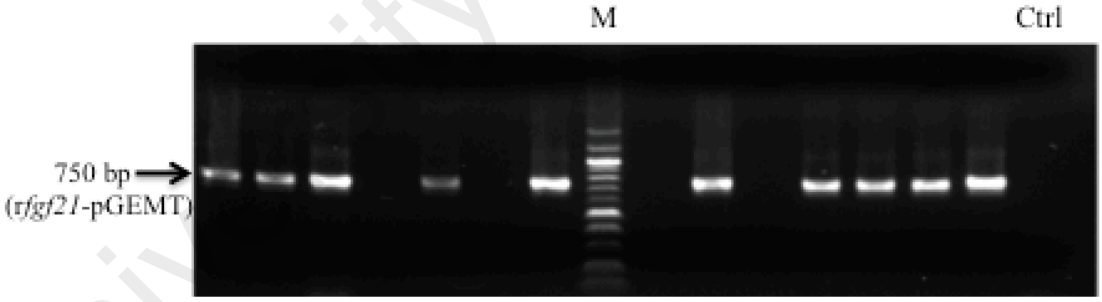


Figure 3.10: Agarose gel electrophoresis of recombinant *rfgf21*-pPICZαA vector after digestion with *Eco*RI and *Not*I.

Lane 1: Digested *rfgf21*-pPICZαA fragment with *Eco*RI and *Not*I
Lane 2: Digested *rfgf21*-pPICZαA fragment with *Not*I
Lane 3: Undigested *rfgf21*-pPICZαA fragment
Lane 4: Digested *rfgf21*-pPICZαA fragment with *Eco*RI
M: 100bp DNA ladder.



PCR colony amplification of pPICZαA-*rfgf21* transformant

Figure 3.11: PCR colony amplification of pPICZαA-*rfgf21* transformant by using primer 1Forward and AOX1 Reverse primer. Expected amplicon size positive transformant is 750 bp.

M: 100bp DNA ladder.
Ctrl: negative control.

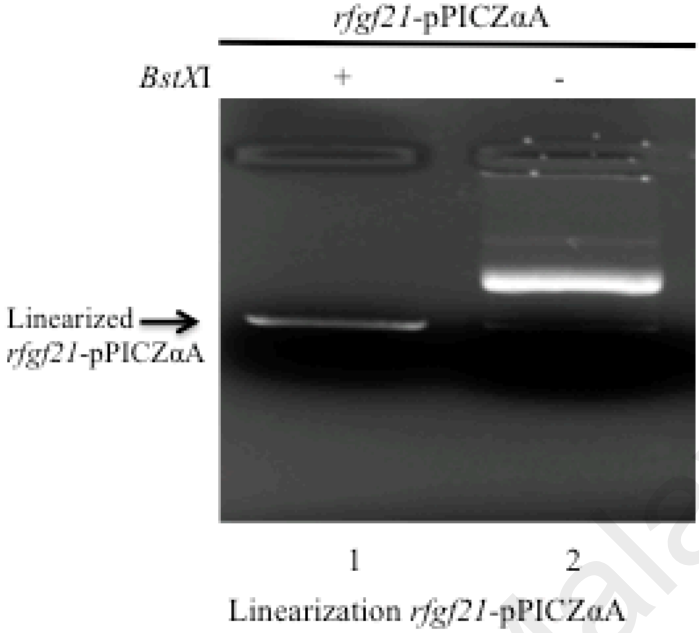


Figure 3.12: Digestion of *rfgf21*-pPICZαA with *BstX*I for *P. pastoris* expression. The circular recombinant plasmid was linearized with *BstX*I.

Lane 1: *rfgf21*-pPICZαA with *BstX*I
Lane 2: *rfgf21*-pPICZαA without *BstX*I (control)

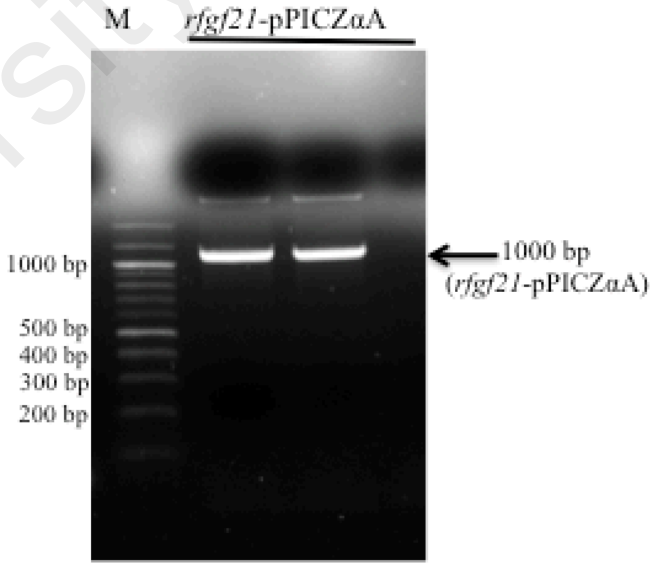


Figure 3.13: PCR amplification of integrated *rfgf21*-pPICZαA in X33 with AOXI universal primers. Expected positive transforman amplicon size is 1000 bp.

M: 100bp DNA ladder.

3.2.4 Cloning in *E. coli TB1*

The recombinant FGF21-pGEMT construct and vectors (c4X and p4X) digested with *Hind*III and *EcoR*I produced sticky ends (Figure 3.14). Both c4X-rFGF21 and p4X-rFGF21 constructs were successfully transformed into the *E. coli* competent cell (TB1 strain) into downstream of the maltose binding protein promoter between the *EcoRI* and *Hind*III sites of C4X(P4X) vector(s) which placed rFGF21 under the control of lactase promoter. The colonies screened by PCR using FGF21-*Hind*III-Ex3R and mal E Primers, showed a ±650~700bp fragments (Figure 3.15).

3.3 Protein expression

3.3.1 Protein expression in yeast

Transformant successfully induced and inoculated in shake flasks under normal conditions in BMGY for propagation and BMMY for induction purpose.

Different induction time (24 h, 48h, 72 h, 96 h), different growth temperature (20 °C and 30 °C) and different alcohol concentration (0.5%, 1%, 1.5%, 2%, 2.5%) investigated in order to obtain the highest yield of recombinant FGF21. More stable products was observed by dropping the induction time from 30 °C to 20 °C due to minimal extracellular proteolysis activity (data not shown)(Shi *et al.,* 2003; Li *et al.,* 2001). The optimal culture conditions were culture temperature at 20°C, 1% of methanol concentration and 72-hour of post-induction (Figures 3.16 and 3.17). Instead of a single band, a smear was detected in western blots analysis showing that *Pichia*-expressed recombinant FGF21 was highly glycosylated.

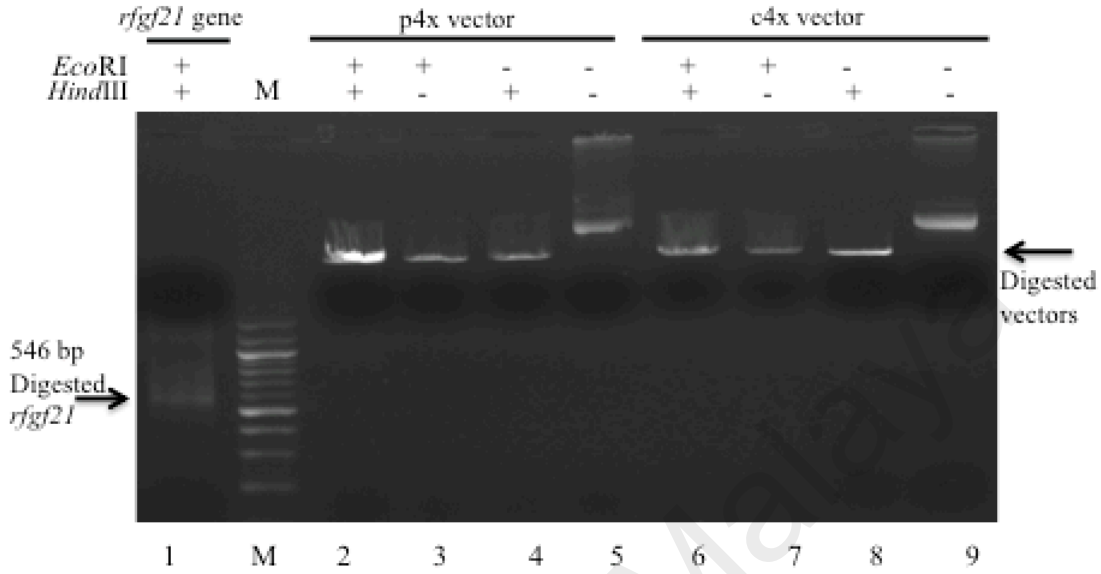
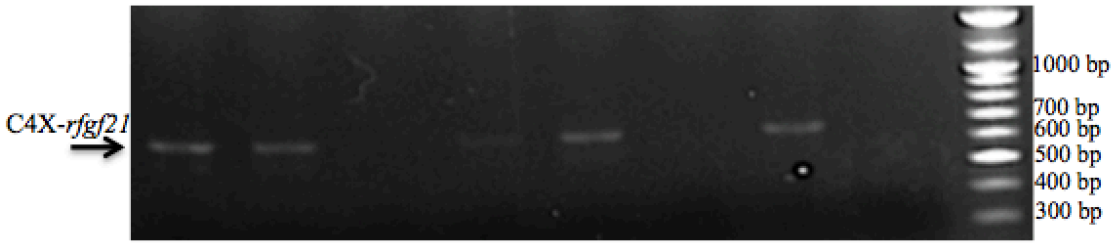


Figure 3.14: Digestion of *rfgf21* gene and C(P)4X vector with *Hind*III and *Eco*RI for *E. coli* expression.

Lane 1: *rfgf21* gene digested with *Hind*III and *Eco*RI
Lane 2: P4X vector digested with *Hind*III and *Eco*RI
Lane 3: P4X vector digested with *Eco*RI
Lane 4: P4X vector digested with *Hind*III
Lane 5: P4X vector without digestion (control).
Lane 6: C4X vector digested with *Hind*III and *Eco*RI
Lane 7: C4X vector digested with *Eco*RI
Lane 8: C4X vector digested with *Hind*III
Lane 9: C4X vector without digestion (control).
M: 100bp DNA ladder.



Colony selection of different transformant C4X-*rfgf21*

Figure 3.15: PCR colony amplification of C4X-*rfgf21* by using FGF21-Ex3R and malE Primers. Expected positive transformant amplicon size is 700 bp.

M: 100bp DNA ladder.

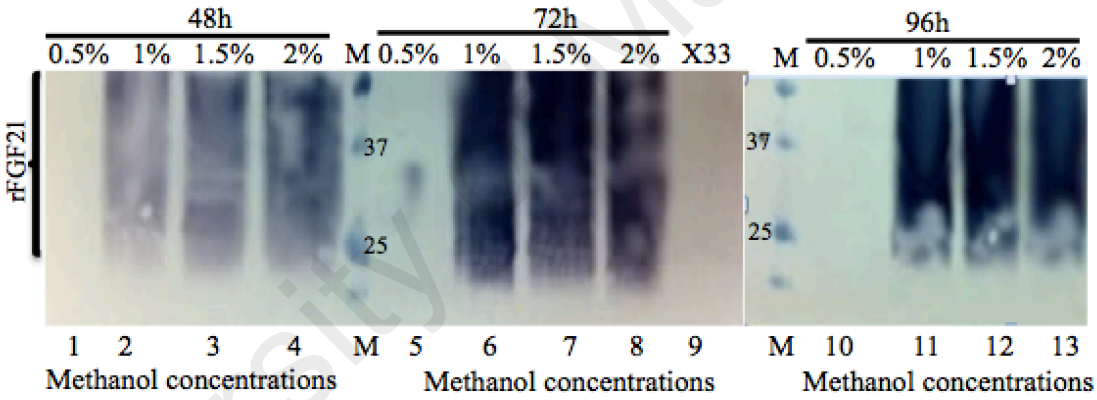


Figure 3.16: Westrn-blot analyses of rFGF21 expressed in *P. pastoris* after 0.5% -2% methanol induction at different time points.

Lane 1: 48h, 0.5% methanol
Lane 2: 48h, 1% methanol
Lane 3: 48h, 1.5% methanol
Lane 4: 48h, 2% methanol
Lane 5: 72h, 0.5% methanol
Lane 6: 72h, 1% methanol
Lane 7: 72h, 1.5% methanol
Lane 8: 72h, 2% methanol
Lane 10: 96h, 0.5% methanol
Lane 11: 96h, 1% methanol
Lane 12: 96h, 1.5% methanol
Lane 13: 96h, 2% methanol
Lane 9: wild type *P. pastoris* without *rfgf21* gene
M: Protein marker, precision plus protein standards from Bio Rad

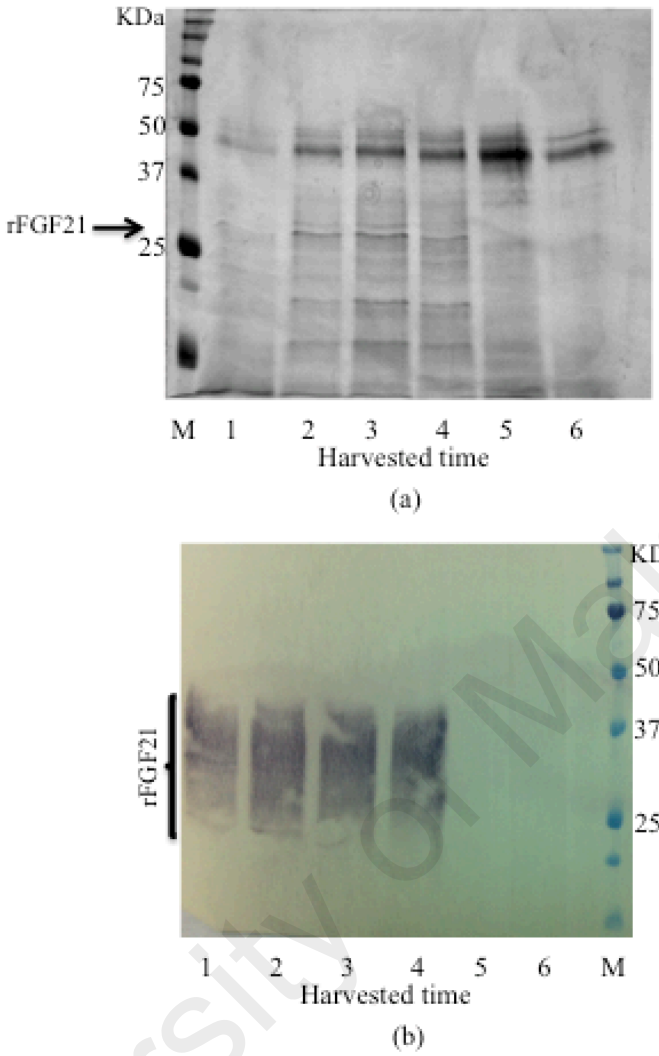


Figure 3.17: (a) Silver stained SDS-PAGE and (b) Western blot analyses of recombinant FGF21 expressed in *P. pastoris* after 1% methanol induction at different time points.

Lane 1: 24h Lane 3: 72h
Lane 2: 48h Lane 4: 96h
Lane 5: RWM, recombinant *P. pastoris* without methanol induction.
Lane 6: X33, wildtype *P. pastoris* without recombinant *fgf21* gene.
M: Protein marker, precision plus protein standards from Bio Rad.

3.3.2 Protein expression in *E. coli*

For *E. coli* samples, level of induction (IPTG) concentration from 0.3mM to 1mM (0.3mM, 0.5mM, 0.75mM, 1mM IPTG), different time course (one, two, three and four hour after induction) and different vectors (C4X, P4X) were tested to achieve and detect the higher yield. The highest yield of recombinant FGF21 was obtained after induction with 0.75mM of IPTG for 4 hours (Figure 3.18, 3.19 and 3.20). These proteins were detected and analyzed by SDS-PAGE followed by silver staining and western blot analysis.

3.4 Protein purification

3.4.1 Protein purification in Yeast

Purification successfully separated the expressed and secreted proteins in the culture media. The rFGF21P attached to HIS-6 tag protein in *P. pastoris* samples, facilitated purification of the expressed protein by affinity chromatography using Nickel charged column (figure 3.21). In yeast samples an addition of 2.5 kDa was added to recombinant protein weight (19.4 kDa) due to α factor signal weight. The smears in the western blot analysis showed that rFGF21P was highly glycosylated.

3.4.2 Protein purification in *E. coli*

In *E. coli* the fusion of maltose binding protein to recombinant FGF21E allowed the rFGF21 protein to be purified from rest of the proteins using amylose resin column chromatography. After purification, the concentration of recombinant FGF21E decreased dramatically due to loss, which is expected in purification process (Figure 3.22). The expected molecular weight of recombinant FGF21 was 19.4 kDa, however, rFGF21E was about 70 kDa. This difference was due to the fusion of MBP (~45 kDa) to the N-terminal of rFGF21E.

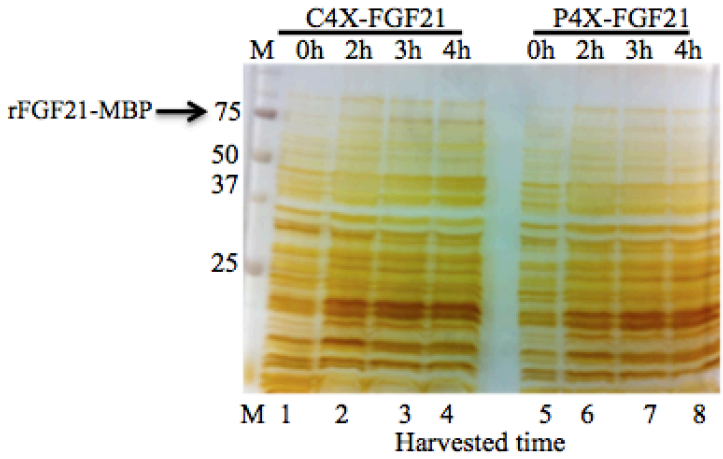


Figure 3.18: Silver stained SDS-PAGE analyses of recombinant FGF21 expressed in *E. coli* at different time points after induction with 0.75mM IPTG stained with specific anti-FGF21 from Abnova.

Lane 1: C4Xvector before induction
Lane 2: C4Xvector two hours
Lane 3: C4Xvector three hours
Lane 4: C4Xvector four hours
Lane 5: P4Xvector before induction
Lane 6: P4Xvector two hours
Lane 7: P4Xvector three hours
Lane 8: P4Xvector four hours
Lane M: Protein marker, precision plus protein standards from Bio Rad.

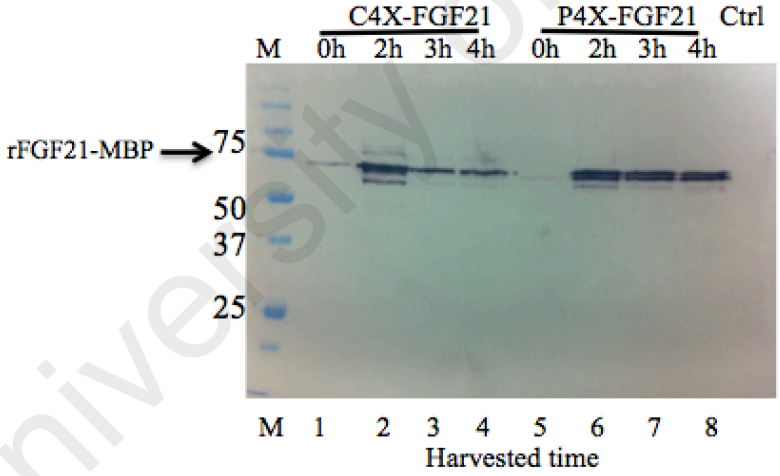


Figure 3.19: Western blot analyses of recombinant FGF21 expressed in *E. coli* at different time points after induction with 0.75mM IPTG stained with specific anti-FGF21 from Abnova.

Lane 1: C4Xvector before induction
Lane 2: C4Xvector two hours
Lane 3: C4Xvector three hours
Lane 4: C4Xvector four hours
Lane 5: P4Xvector before induction
Lane 6: P4Xvector two hours
Lane 7: P4Xvector three hours
Lane 8: P4Xvector four hours
Lane M: Protein marker, precision plus protein standards from Bio Rad.

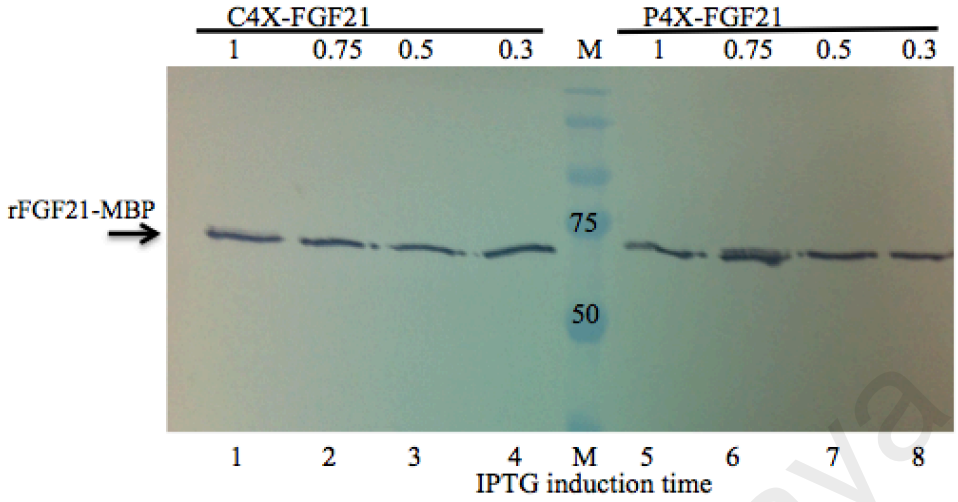


Figure 3.20: Western blot analyses of recombinant FGF21 expressed in *E. coli* at different IPTG concentrations stained with specific anti-FGF21 from Abnova after four hours induction.

Lane 1: C4X with 1mM IPTG
Lane 2: C4X with 0.75mM IPTG
Lane 3: C4X with 0.5mM IPTG
Lane 4: C4X with 0.3mM IPTG
Lane 5: P4X with 1mM IPTG
Lane 6: P4X with 0.75mM IPTG
Lane 7: P4X with 0.5mM IPTG
Lane 8: P4X with 0.3mM IPTG
Lane M: Protein marker, precision plus protein standards from Bio Rad.

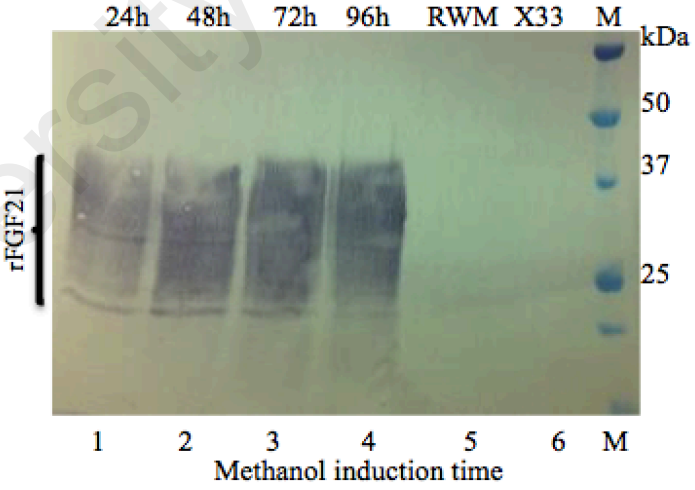


Figure 3.21: Western blot analysis of purified recombinant FGF21 produced in *P. pastoris* after 1% methanol induction at different time points. Smear and a clear band at 25kDa was appeared after purification with Ni column.

Lane 1: 24h after induction
Lane 2: 48h after induction
Lane 3: 72h after induction
Lane 4: 96h after induction
Lane 5: RWM, recombinant *P. pastoris* without methanol induction.
Lane 6: X33, wildtype *P. pastoris* without recombinant *FGF21* gene.
M: Protein marker, precision plus protein standards from Bio Rad.

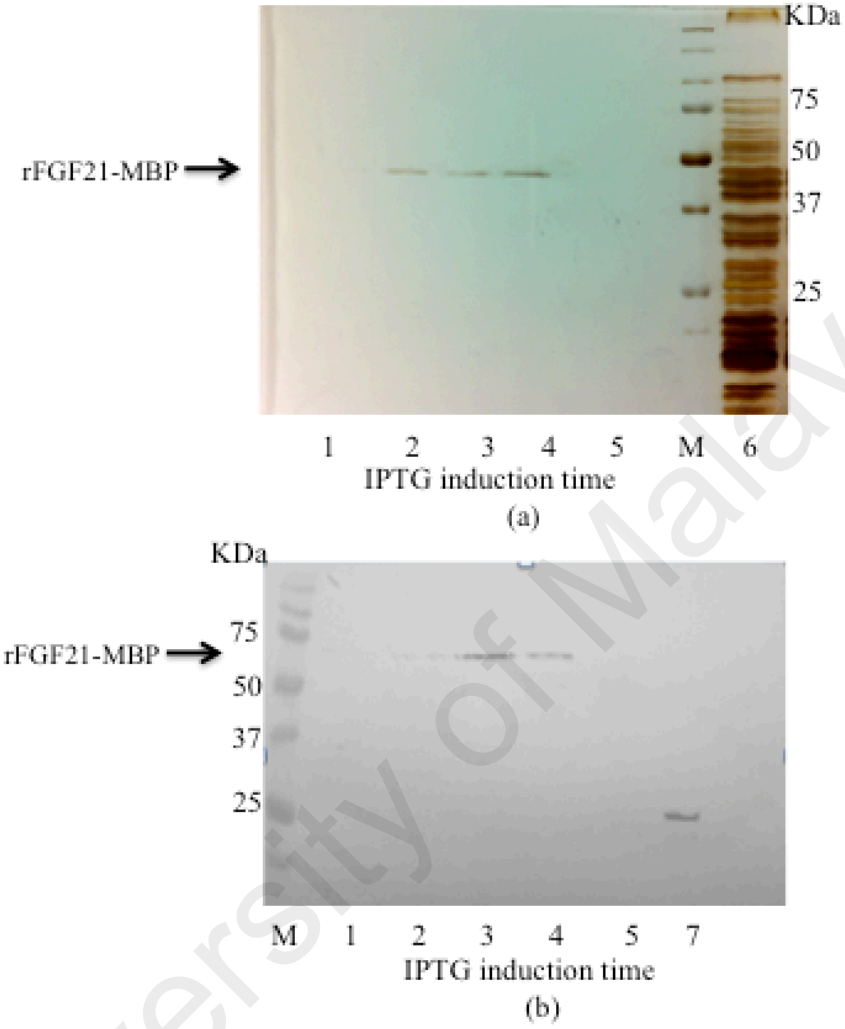


Figure 3.22: (a) Silver stained SDS-PAGE and (b) Western blot analyses of recombinant purified P4X-FGF21 expressed in *E. coli* at different IPTG concentrations after four hours with amylose column, stained with specific anti-FGF21 from Abnova. A clear band around 75 KDa was appeared after purification.

Lane 1: 0.3mM IPTG.
Lane 2: 0.5mM IPTG.
Lane 3: 0.75mM IPTG.
Lane 4: 1mM IPTG.
Lane 5: Competent cell (TB1) without recombinant insertion.
Lane 6: rFGF21 without purification.
Lane 7: Commercial rFGF21 from Sigma.
Lane M: Protein marker, precision plus protein standards from Bio Rad.

3.4.3 Deglycosylation of *Pichia*-expressed FGF21

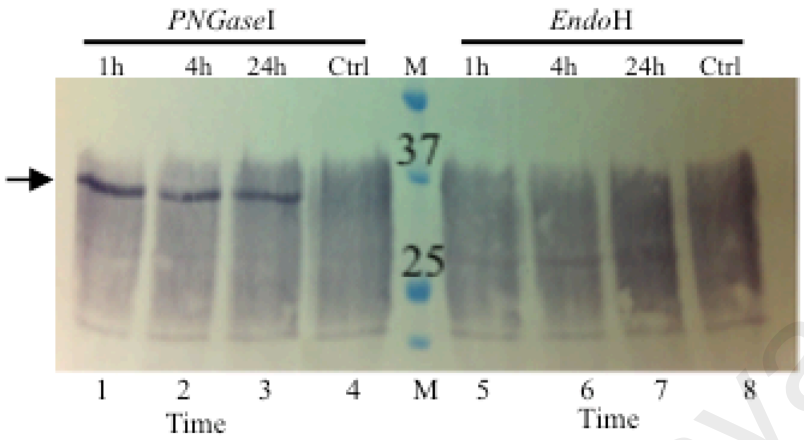
The smears in the western blot analysis showed that *Pichia*-expressed recombinant FGF21 was highly glycosylated. After deglycosylation by *PNGase F* and *Endo H* a smear was still remained but a clearer band with a molecular weight of about 30kDa was observed due to incomplete deglycosylation or unidentified glycoproteins present in the sample. In the result of deglycosylation by *Endo H*, a faint band with a molecular weight of about 22kDa was also observed (Figure 3.23).

3.4.4 Removal of maltose-binding protein from *E. coli*-expressed FGF21

MBP was cleaved by treating the product with Factor Xa in 4 °C for 2, 4, 8 and 24 hours (Figure 3.24, 3.25 and 3.26). A band with a molecular weight of 30kDa was detected by anti-FGF21 polyclonal antibody.

3.4.5 Bradford assay

The expression levels were measured by Bradford assay. It indicates that the concentration of rFGF21 in yeast was ~4.9 mg/L and in *E. coli* was ~2.3 mg/L respectively.



Fiqure 3.23: Western blot analysis of purified recombinant FGF21 expressed in *P. pastoris* after 1% methanol induction after deglycosylation with *PNGase F* and *Endo H*, for 1, 4 and 24 hours. Smear was still remained after deglycosylation but a clearer band with a molecular weight of about 30kDa appeared in *PNGase F* experiment.

Lane 1: one hour treating with *PNGase F*.
Lane 2: four hours treating with *PNGase F*.
Lane 3: twenty four hours treating with *PNGase F*.
Lane 4: without *PNGase F* treatment.
Lane 5: one hour treating with *Endo H*.
Lane 6: four hours treating with *Endo H*.
Lane 7: twenty four hours treating with *Endo H*.
Lane 8: without *Endo H* treatment.
M: Protein marker, precision plus protein standards from Bio Rad.

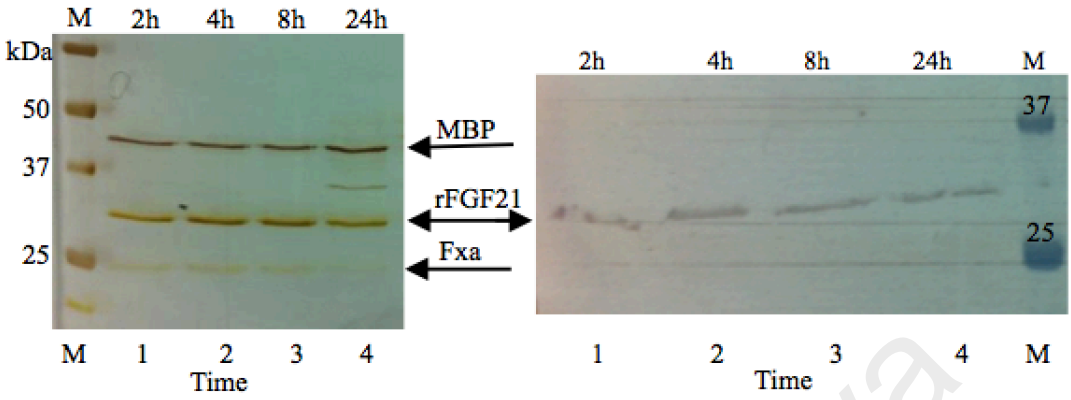


Figure 3.24: (a) Silver staining and (b) Western blot analysis of purified recombinant P4X-FGF21 expressed in *E. coli* with 0.75mM IPTG induction treated with 1µg Factor Xa at 4°C for 2, 4, 8 and 24 hours with amylose column, stained with specific anti-FGF21 from Abnova. Three different bands were appeared in silver staining gel pictures. the band around 40 kDa is MBP, around 30kDa is rFGF21 separated from MBP, and around 25kDa is Fxa.

Lane 1: two hours treatment
Lane 2: four hours treatment
Lane 3: eight hours treatment
Lane 4: twenty four hours treatment
Lane M: Protein marker, precision plus protein standards from Bio Rad.

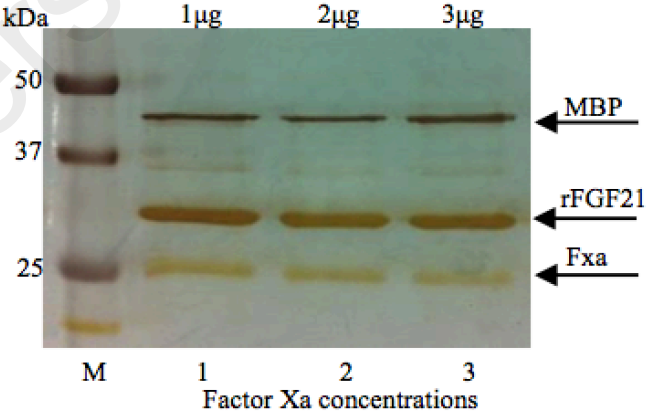


Figure 3.25: Silver staining of purified recombinant P4X-FGF21 expressed in *E. coli* after induction with 0.75mM IPTG treated with different Factor Xa concentration at 4°C for four hours with amylose column. Three different bands were appeared in silver staining gel pictures. the band around 40 kDa is MBP, around 30 kDa is rFGF21 separated from MBP, and around 25 kDa is Fxa.

Lane 1: 1µg Factor Xa
Lane 2: 2µg Factor Xa
Lane 3: 3µg Factor Xa
Lane M: Protein marker, precision plus protein standards from Bio Rad.

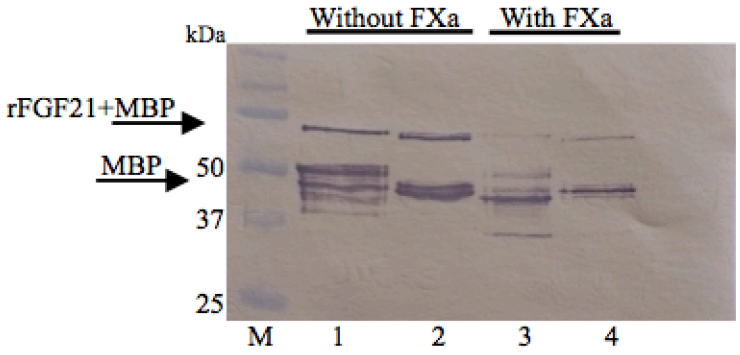


Figure 3.26: Western blot analysis of purified recombinant P4X-FGF21 and C4X-FGF21 expressed in *E. coli* induced with 0.75mM IPTG treated with Factor Xa at 4°C for four hours with amylose column, stained with specific anti-MBP from Abnova. After treatment with Fxa, maltose binding protein was separated from rFGF21+MBP and this rFGF21+MBP can not be detected by anti-MBP anti-body.

Lane 1: Purified rFGF21 expressed with C4Xvector
Lane 2: Purified rFGF21 expressed with P4Xvector
Lane 3: Purified rFGF21 expressed with C4Xvector treated with 1µg Factor Xa
Lane 4: Purified rFGF21 expressed with P4Xvector treated with 1µg Factor Xa
Lane M: Protein marker, precision plus protein standards from Bio Rad.

3.5 *In vivo* study

3.5.1 Acute and sub-chronic study

Acute (14 days) and sub-chronic (27 days) study was conducted to assess potential hazards and risks of the recombinant protein to the animal model. FGF21 was administered as a single daily dose 100 μg/kg body weight of FGF21 dissolved in dH_2O (Low-dose injection) and 300 μg/kg body weight of FGF21 dissolved in dH_2O (High-does injection) in different groups. 100 and 300 μg/kg body weight of FGF21 dissolved in dH_2O were chosen based on Kharitonenkov researches in 2007. At the end of the exprimensts no differences noted between treated and untreated group and data are available from all 60 rats, none of the rats died or shows any adverse signs related to treatment. Concentration of Serum alkalin phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), G- glutamyl transferase (GGT), sodium, potassium, chloride, CO2, urea and creatinine measured using standard enzymatic methods. These blood parameters all were on average tending toward untreatment group levels and shown no significant difference between them (Data are available in appendix D).

Histopathological examination on the liver and kidneys also revels no effect of toxicity or pathological effects beyond the treated and control samples (Figure 3.27 and 3.28). Since no interrelation between toxicity and dosage was found and survival rate is 100%, the doses were safe and used in our following studies.

3.5.2 Comparison of rFGF21 produced in *E. coli* and *P. pastoris*

Comparison of biological activity between rFGF21 produced in *E. coli* (rFGF21-E) and *P. pastoris* (rFGF21-P) was investigated in three different experiments. Effects of rFGF21 on blood glucose and lipids were investigated in male SD rats to find out whether the produced protein is effective in lowering the blood glucose and lipid profile in diabetic situation in our first experiment. Diets high in saturated fats and carbohydrates are linked with glucose intolerance; obesity and type 2 diabeties mellitus and male usually show (Motshakeri *et al*., 2015).

For this purpose, male SD rats were used since insulin resistance develops only in male rats (Kim *et al*., 1999). Rats fed with high sucrose high fat diet for one months to prouce insulin resistance rats after which a daily dosing paradigm of FGF21 were injected to the rats for 14 days and blood glucose were measured 24h after the last injection.

Before FGF21 administration, mean baseline fasting plasma glucose in animals fed with HSHF was 9mM/L, which is considered as a regular sign of diabetes comparing to 5mM/L plasma glucose in the first day of the experiment or negative group which was in agree with previous researches to convert the normal *Sprague-Dawley* rats to prediabetic status by feeding them HSHF diet, which reduced insulin sensitivity and exerted insulin resistance (Motshakeri *et al*., 2015).

After FGF21 injection, in treatment diabetic rodent group FGF21 notably lowered mean plasma glucose from the overtly diabetic level to near normal (6mM/L). This clearance of FGF21 was slower but not significantly in rFGF21 produced in bacteria.

The effects of FGF21 on lipids in SD rats were evaluated by clinical chemistry analysis (Figure 3.29). Concentration of Serum HDL, LDL, total cholesterol and tri-glycerids measured using standard enzymatic methods. The mean baseline TG level, as assessed by clinical chemistry analyzer, was 0.5 mmol/l, the mean baseline LDL level, was 0.7 mmol/l, the mean baseline HDL level 1.1 and TC was 1.4 which was similar to untreated group (TG: 0.68, LDL: 0.6, HDL: 1.3, TC: 1.6) showing that FGF21 was effective in improving lipid profile and they were on average tending toward untreatment group levels (table 3.1).

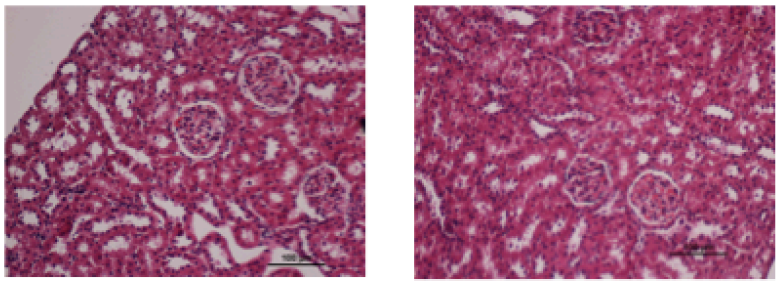
Table 3.1: Effect of rFGF21, on HSHF diet group

	rFGF21P	rFGF21E	Normal Ctrl	Negative Ctrl
TG	0.533333333±0.07	0.583333333±0.02	0.68±0.09	0.97±0.1
LDL	0.716666667±0.07	0.74±0.1	0.613333333±0.08	1.073333333±0.05
HDL	1.133333333±0.05	1.066666667±0.1	1.3±0.03	0.5±0.1
TC	1.433333333±0.1	1.466666667±0.05	1.616666667±0.07	1.866666667±0.1

During the experiment, all animals began to gain weight, and the average body weight level trended upward over time The administration of FGF21 led to a slight weight loss during each dosing phase compared with preadministration but this loss is not significant ($p<0.05$). The results showed in figure 3.30.

In second and third experiments OSTT (effects of high and low doses of FGF21 on blood glucose and lipid profile in rats fed with high fat high sucrose) and OGT (Oral Glucose Tolerance) (effects of rFGF21 in reducing oral glucose) test were investigated in normal SD female rats. In second experiment HSHF gavegaed to rats, after which different concentrations of rFGF21 was injected to rats to check for best dose that is efficient to show rFGF21 effects. OSTT test was examined to investigate the effect of rFGF21 on sucrose. Sucrose cleaved in body to glucose and fructose. In all treatment

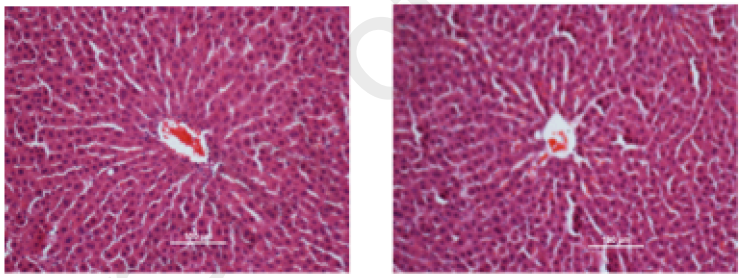
group insulin works together with FGF21 and in these group glucose plasma faster reduced comparing to untreated group. In OGTT group, animals was fed with glucose and rFGF21 was injected after that to check the efficancy of the rFGF21 in reducing plasma glucose. Based on previous works, the concentration of insluine in presence of FGF21 decreases slightly, and in this case glucose concentration sharply decrease in untreated group with insulin effects while in treated group this decrease is based on FGF21 effect, with slower gradiant reduction and so blood glucose levels at 60 and 120 minutes were higher than controls. In addition in FGF21 treated group the plasma glucose were less than untreated group (Pan *et al.*, 2014). In conclusion our results showed that both types of recombinant proteins were active and significantly lowered blood glucose in all the experiments (Figure 3.29, 3.30, 3.31 and Figure 3.32)($p<0.05$).



(a) (b)

Figure 3.27: Example of histological analysis of High dose (300 µg/kg body weight)/ Low dose (100 µg/kg body weight) rFGF21 on Sprague Dawley rats kidney in sub-chronic toxicity test. In all rats, female and male injected with rFGF21 from yeast or bacteria no differences between control and treatment Sprague Dawley rats were observed.(H&E staining, original magnification: 20X).

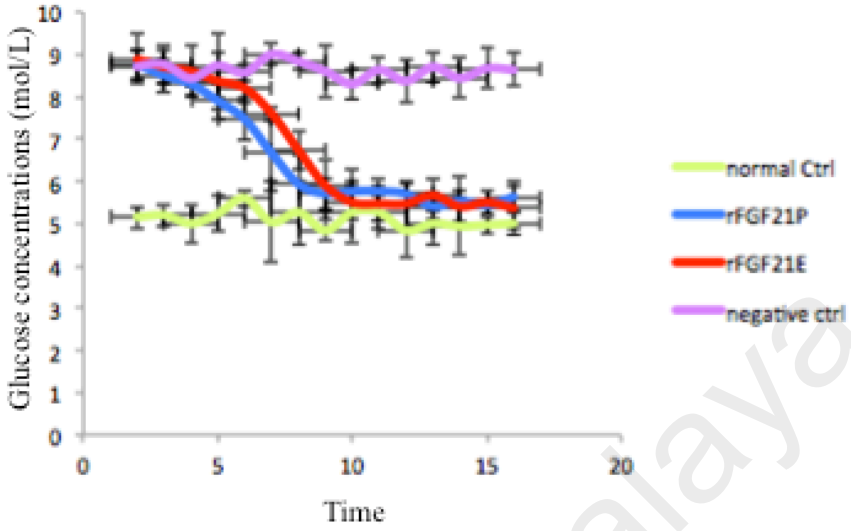
(a): rFGF21 treated Sprague Dawley.
(b): Sprague Dawley without FGF21 treatment.



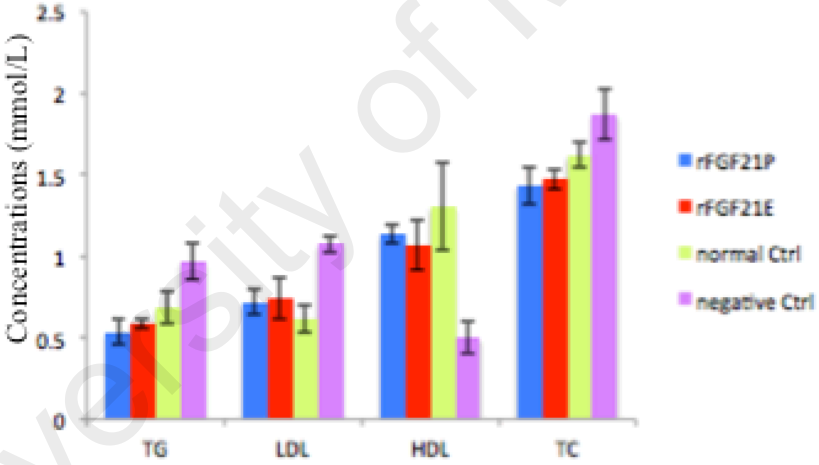
(a) (b)

Figure 3.28: Example of histological analysis of (300 µg/kg body weight)/Low dose (100 µg/kg body weight) rFGF21 on Sprague Dawley rats liver on sub-chronic toxicity test. In all rats, female and male injected with rFGF21 from yeast or bacteria no differences between control and treatment Sprague Dawley rats was observed. (H&E staining, original magnification: 20X).

(a): rFGF21 treated Sprague Dawley
(b): Sprague Dawley without treatment



(a)



(b)

Figure 3.29: Treatment of SD rats with rFGF21. Male SD rats were treated with fresh high sucrose-high fat daily for four weeks to induce insulin resistance rats. rFGF21 produced in *E. coli* and *P. pastoris* injected for 14 days to rats. (a) Blood glucose measured daily after rFGF21 administration. After daily administration of rFGF21 for 2 weeks in rats, rFGF21 improved the insulin resistancy significantly(P value<0.05), while the HSHF control group blood glucose (purple line) remained high. Besides rats in NC groups (blue line) with normal chow fed, did not show any changes in blood glucose levels over this period. (b) Lipid profiles measured at the end of the experiment. lipid profiles altered significantly to almost normal control levels comparing to HSHF control group. n=3 (P value<0.05)(error bars represent±SEM)

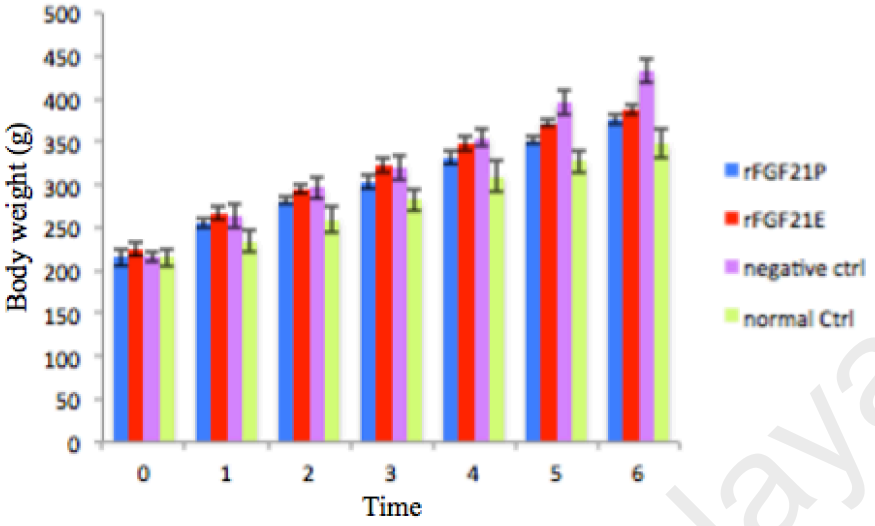


Figure 3.30: Body weights in Male SD rats treated with fresh high sucrose-high fat daily for four weeks following by FGF21 administration for 14 days. During this experiment, all animals began to gain weight, and the average body weight level increased over time. Treatment of semi-diabetic rats with rFGF-21 produced in *E. coli* and yeast led to a slight weight loss comparing to both control groups. n=3 (error bars represent ±SEM)

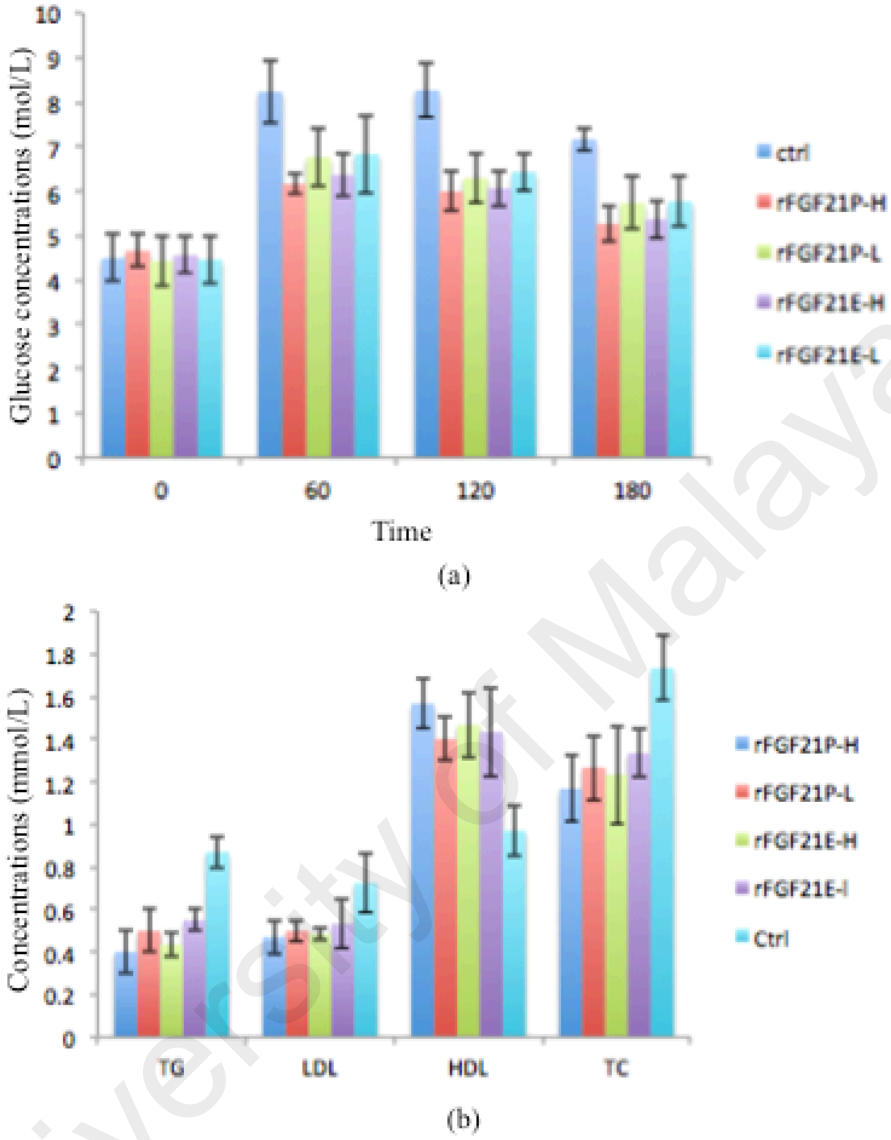


Figure 3.31: Treatment of SD rats with rFGF21 - OSTT test, female SD rats treated with fresh high sucrose-high fat, were injected with 100µg/kg as low dose and 300µg/kg as high dose rFGF21 produced in *E.coli* (rFGF21-E) and *P. pastoris* (rFGF21-P). (a) Blood glucose measured at 1,2,3 hour after rFGF21 administration. (b) Lipid profiles measured at the end of the experiment. All doses significantly lowered blood glucose and fasting plasma TGs while simultaneously raising HDL. n=3 (P value<0.05)(error bars represent ±SEM)

rFGF21P-H: high dose with 300µg/kg rFGF21 produced in *P. pastoris*
rFGF21P-L: low dose with 100µg/kg rFGF21 produced in *P. pastoris*
rFGF21E-H: high dose with 300µg/kg rFGF21 produced in *E.coli*
rFGF21E-L: low dose with 100µg/kg rFGF21 produced in *E.coli*
Ctrl: HSHFSD rats injected with normal saline.

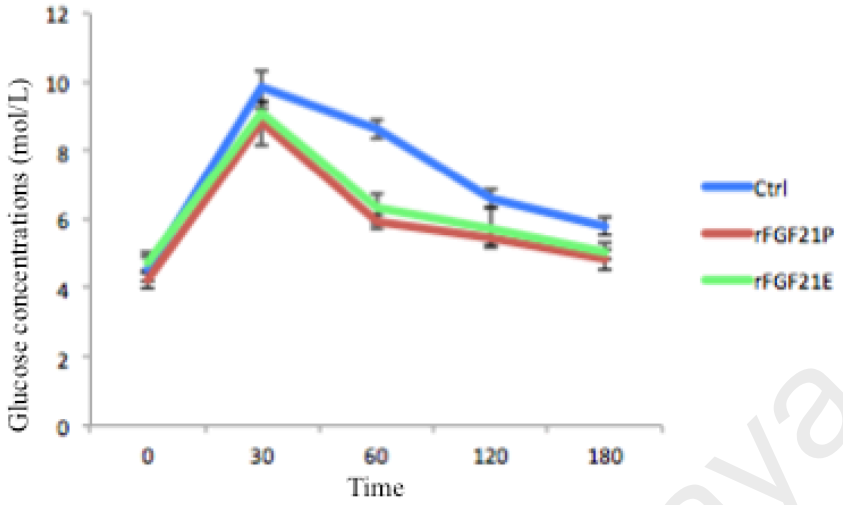


Figure 3.32: Treatment of SD rats with rFGF21 - OGTT test, female SD rats were treated with 2g/kg BW glucose, via gavage. Recombinant FGF21 produced in *E.coli* (rFGF21-E) and *P. pastoris* (rFGF21-P) was injected. Blood glucose measured by tail vein insicion at 0(before glucose intake),30,60,120,180 minutes after rFGF21 administration. The blood glucose concentrations during OGTT increased dramatically in the NC and treatment group after 30 minutes post glucose injection while in treatment group the peak is lower comparing to control group. At time 60 and 120 min the values were significantly lower than the NC rats. n=3 (P value<0.05) (error bars represent±SEM)
rFGF21P-H: SD rats treated with 200µg/kg rFGF21 produced in *P. pastoris*
rFGF21E-H: SD rats treated with 200µg/kg rFGF21 produced in *E.coli*
Ctrl: SD rats injected with normal saline.

3.5.3 Effects of rFGF21 protein on the reproduction system

3.5.3.1 Behavioral observations

No adverse signs or mortality related to treatment were observed in this study and all animals are in good health during the experiment. No difference in food consumption between groups was observed while male animal group consumes more food comparing to female animals.

3.5.3.2 Whole body weight

An increase in the whole body weight was observed in all experimental rats throughout the four weeks of this study (Figure 3.33). After FGF21 injection the treatment group gains weight slightly slower ($p>0.05$) compared to the controls due to rFGF21 functions which can reduce body weight in individuals.

3.5.3.3 *In situ* study of the reproductive tissues

The reproductive tissues of the treated and untreated animals were observed *in situ* carefully. The ovaries in all study groups were observed to be kidney-shaped buried in a mass of fat and tissue. In rFGF21 treated groups the uterine lumen width increased comparing to control group as shown in figure 3.34. Testes were observed to be light pink, bean shaped and slightly bigger in size in treated group comparing to control group as shown in figure 3.35.

3.5.3.4 Organ weights

An increase in the whole weight of the reproductive system in experimental groups was observed. This increase was significant compared to the controls as shown in table 3.1 and figure 3.36.

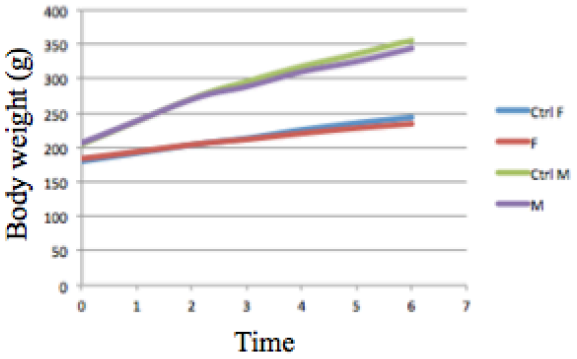


Figure 3.33: Body weights in Male and female SD rats treated with rFGF21 for one month. An increase in the whole body weight was observed in both experiments. The treatment group gains weight slightly slower compared to the controls. (P value>0.05)

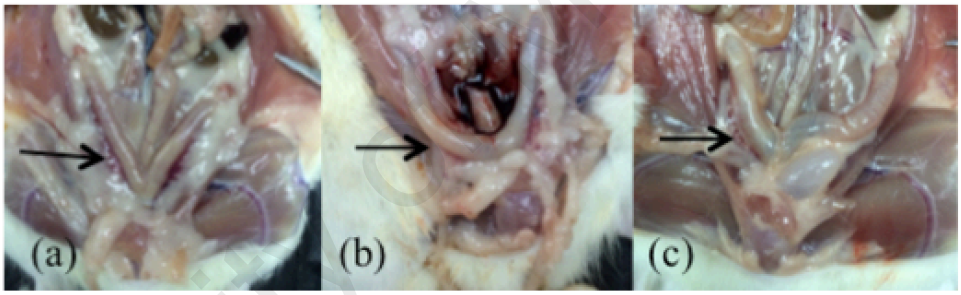


Figure 3.34: Photographs of the female SD rats reproductive system after one month injection with yeast rFGF21. The rFGF21 treated rats uterine width (b,c) increased in response to rFGF21 compared to control group (a).

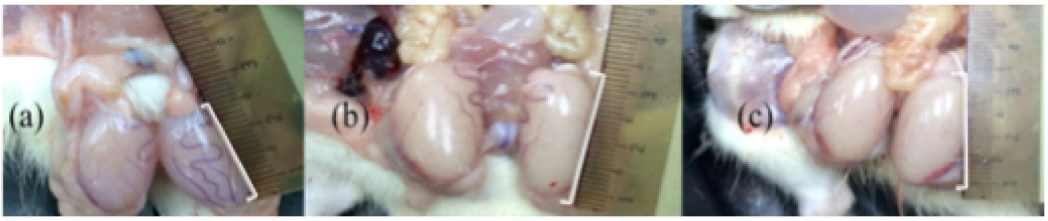


Figure 3.35: Photographs of the male SD rats reproductive system after one months injection with yeast rFGF21. The rFGF21 treated rats testis size (b, c) increased in response to rFGF21 compared to control group (a).

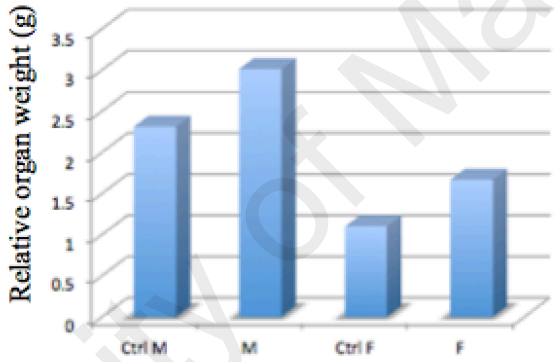


Figure 3.36: Relative (g/g body weight) organ weights. A significant increase in the whole weight of the reproductive system groups was observed comparing to treatment group. ($p < 0.05$)
Ctrl M: male organ weight control
M: treated male organ weight
Ctrl F: female organ weight control
F: treated female organ weight

Table 3.2: Summary of absolute and relative of organ weights.

Reproductive organ weight absolute	Body weight(gr) absolute	Relative weight (gr)	Mean	Reproductive organ weight absolute	Body weight (gr) absolute	Relative weight (gr)	Mean
Male control				Female control			
8.41	349	2.41	2.31	3.1	249	1.2	1.1
7.67	345	2.22		2.3	235	1	
8.6	374	2.3		2.63	247	1.1	
Male treated				Female treated			
9.6	338	2.84	3.01	4.2	238	1.8	1.7
10.32	346	2.98		3.4	230	1.5	
11.2	348	3.21		4	235	1.7	

Significant increase in the whole weight of the reproductive system in experimental groups was observed comparing to treatment group ($p<0.05$).

3.5.3.5 Histopathology

Stained tissues with H&E observed under a light microscope (Olympus CH-BI45- 2). In figure 3.37(a), the whole section of treated and untreated ovaries was shown. For female reproductive system, follicles and corpora lutea were identified and histopatological examination was done carefully on evaluation of follicular and corpora lutea developments. Presence and absence of follicles (primary follicles, atretic follicles and tertiary follicles) and corpora lutea and number of follicles and corpora lutea were determined under 20X magnification as shown in figure 3.37(b). The majority of the ovarian parenchyma was composed of corpora lutea in both groups but in different growth stage.

In male reproduction system, evaluation was done by carefully evaluating the spaces between cells and the cells itself separately to find out any abnormality. Ledgid cells, spermatogonia cells, spermatocyte, spermatids and somatic sertoli cells were defined. Testicular architecture is normal in treated and untreated groups as shown in figure 3.38.

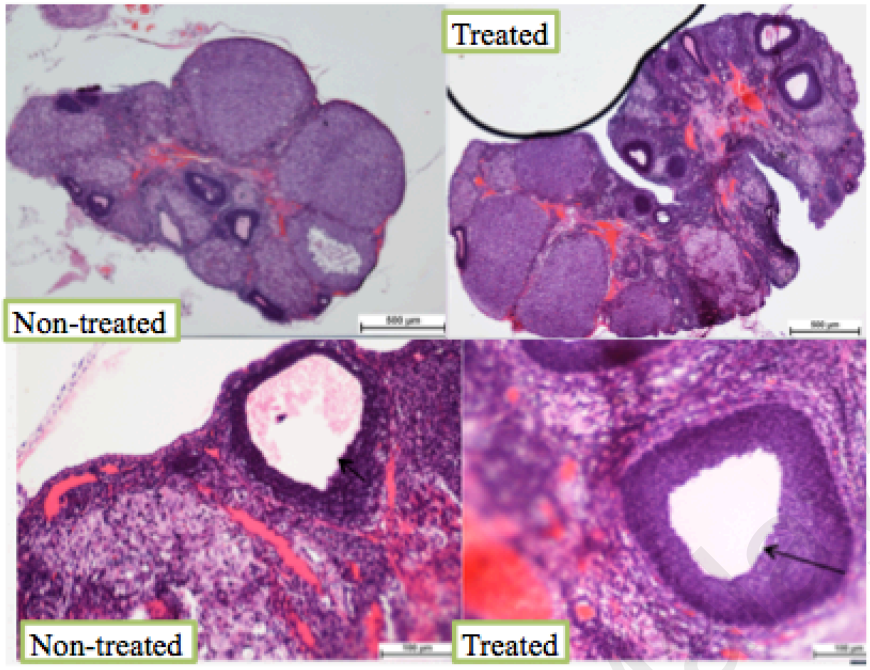


Figure 3.37: Histological analysis of rFGF21 on Sprague Dawley rats ovary (a) untreated and (b) treated groups. The majority of the ovarian parenchyma was composed of corpora lutea in both groups but in different growth stage in treated group. layers around the atretic and tertiary follicles in below pictures were more in treated group. H&E staining, original magnification: 4X top pictures and 20X for below pictures.

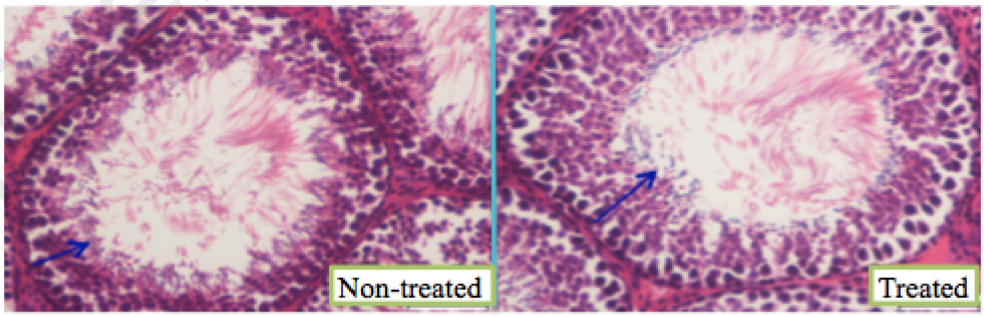


Figure 3.38: Histological analysis of rFGF21 on Sprague Dawley rats testis untreated and treated groups. H&E staining, original magnification: 40X. More layers of cells and hence more sperms was observed.

CHAPTER 4: DISCUSSION

Diabetes prevalence globally was 6.4% in 2010 and was predicted to increase to 7.7% by 2030 (Shaw *et al.*, 2010). Type 2 diabetes continues to be a growing global health concern, and as such there is a dire need for more effective, safer therapies other than conventional strategies.

FGF21 is a novel metabolic protein that plays a unique role in the regulation of carbohydrate and lipid metabolism, and had been demonstrated as a favorable metabolic protein that can be used to treat patients with Type 2 diabetics. FGF21 regulates glucose homeostasis through a combination of multiple mechanisms; it stimulates glucose uptake in adipose tissues, it suppresses glucose output in liver and it preserves B-cell mass and islets function in pancreases. FGF21 also lowers plasma TG through inhibiting hepatic fatty acid synthesis, while increasing fatty acids oxidation (Inagaki *et al.,* 2007, Kharitonenkov *et al.,* 2005, 2007; Xu *et al.,* 2009).

Kharitonenkov was the first to report the production of recombinant FGF21 in 2005. To produce bioactive recombinant protein as described by Kharitonenkov, multiple denaturation and purification steps were essential as the product accumulate and forms inclusion bodies. Inclusion bodies require several costly purification steps, which in turn, is expected to reduce yield (Hecht *et al.*, 2012, Fisher *et al.*, 2010, Kharitonenkov *et al.*, 2007; Kharitonenkov *et al.*, 2005). Although Kharitonenkov's method to produce recombinant FGF21 is still widely used by many researches, there is a need to focus efforts toward finding an alternative method which is not only cost effective in producing more soluble rFGF21 with high yield, but also establishing a method that offer other advantages such as increased *in vivo* activity and prolonged protein half-life (for increased drug efficiency).

In this study, two different recombinant protein expression systems (*E. coli* and *Pichia pastoris)* to produce rFGF21 were investigated. While the *E. coli* based expression system is widely used, the use of *Pichia pastoris* as an expression system was only known relatively recently. This yeast based has been reported to be easy to handle, cost effective and the cell culture is easy to maintain. Moreover, the *Pichia pastoris* system offers several advantages over the conventional bacteria based system in detailed later in this chapter. The efficiency of FGF21 expression in *E. coli* and *P. pastoris* was compared, and the bioactivity of recombinant FGF21 from both systems was investigated.

4.1 *In vitro* construction of *rfgf21* gene

FGF21, located on chromosome 19, has three exons, which are separated by two introns, and has 209 amino acids in sequence. Introns enhance mRNA stability in humans, and genes without introns will form structures that promote endonucleolytic cleavage (Wang *et al*., 2007). Cloning the entire *fgf21* gene with introns on bacteria or lower eukaryote is not ideal since they do not have splicing system. Cloning the entire gene on *pichia* expression system that has splicing also is not usually efficient as introns are often not correctly omitted due to differences in splicing systems (Rukin *et al*., 1986). In yeast, almost 70% of genes are intronless, or only one or two introns exist in intron-containing genes, illustrating the fact that the splicing mechanism is not a common event in yeast, so its better to remove the introns before expression.

Several methods are available for constructing or assembling a recombinant gene for biological and recombinant DNA technology research, among which ligation-based synthesis (Borodina *et al*., 2003); SfiI-based ligation method (Lu *et al.,* 2007) and primer extension (Wang *et al*., 2010) has been used to amplify *fgf21* gene. Most of these methods are time-consuming and require the use of a combination of enzymes, including restriction endonucleases, ligases, exonucleases and DNA polymerases. The

more popular methods of gene construction are PCR-based, as they are usually rapid, simple and cost effective approaches.

To produce recombinant FGF21, usually the cDNA of the gene was synthesized by reverse transcription from mRNA or restriction enzymes were employed to construct the gene. In this study, three sets of primers were designed to amplify the three exons. Every primer was designed to have extra overlapping bases at the 5' end complementary to adjacent exons that would function as the anchoring site for fragment overlapping extension reactions. We constructed the whole *fgf21* gene by joining all three exons of the *fgf21* gene together using overlap-extension PCR. Overlap-extension PCR is a bi-directional PCR amplification of specific alleles, which performs the amplification rapidly in a single reaction (Liu *et al*., 1997) and has three steps: (i) generation of the exonic fragments (with overlapping ends) by PCR, (ii) self-annealing at the overlap region of two adjacent fragments and (iii) extension and amplification of the overlapped fragment to obtain joined product. A series of overlap extension procedures performed. At the end of each stage, gel purification was carried out to obtain the original DNA template and to remove other unspecific products that might serve as template and interfere with amplification in the second PCR step. This approach is much more economical and the number of PCR reactions steps is also reduced (Urban *et al.*, 1997, Horton *et al.*, 1989; Horton *et al.*, 1997). Since it uses template-based DNA for synthesis, errors in the resulting product was also decreased. (Hoover & Lubkowski, 2002).

Throughout the amplification procedures *Pfu* DNA polymerase has been used to eliminate nucleotide misincorporation errors. It is estimated that *Pfu* DNA polymerase only makes 0.012 errors per kilobase (Cline *et al*., 1996), and this enzyme possesses 3' to 5' exonuclease activity and thus able to proofread. An additional advantage is that

unlike *Taq* DNA polymerase, extra adenine base is not attached to the 3‟ end of PCR products.

Upon successful splicing of all three exons and subsequent ligation into appropriate expression vector, the fidelity of DNA copying, synthesis and splicing was determined dan verified by DNA sequencing.

4.2 Bacterial expression

Bacterium is the first organism to be chosen as host strain in recombinant protein therapeutics. The most commonly used bacteria species is *Escherichia coli.*

E. coli is the preferred host and well documented to produce recombinant protein because it is rather easy to genetically manipulate and it produce high yields. Its expression is fast, typically producing protein in a single day with an acceptable cost (Braun & LaBaer, 2003, Jonasson *et al.,* 2002; Studier & Moffatt, 1986).

However, in spite of the extensive knowledge on its genetics and molecular biology of *E. coli*, not every gene can be expressed efficiently in this organism and it has some disadvantages as an expression host. *E. coli* is not capable of producing eukaryotic post-translational modifications and so the eukaryotic proteins expressed in *E. coli* may not be functional and work properly. Interrupted translation, frame shifting, mis-incorporation of amino acids and inhibition of protein synthesizes and cell growth are often occurred. In this case, they may need to be modified after translation in order to become active and/or adopt the proper structure. Typically, heterologous proteins synthesized in bacteria are not secreted into the culture medium and the solubility is a big issue. These proteins either form inclusion bodies in the cytoplasm or accumulate in the periplasm if they fused with a secretion signal. Inclusion body can be solubilized and refolded but this additional step hampers the purification process (Singh & Panda,

2005).

Expression level and solubility in bacteria can be affected by growth and culture temperatures, concentration of inducer (IPTG), host strain, protein size and structure and toxicity (Larentis *et al.,* 2014). Since the production of rFGF21 in bacteria was among the proteins that formed inclusion bodies, in this study we investigated and used tag affinity technology to express more soluble FGF21. The use of fusion tags is known to facilitate the purification process and to increase the solubility of recombinant proteins. In 2010 wang and his college used affinity tag modification technology and fused rFGF21 with SUMO tag that resulted in the expression of a more soluble rFGF21 in *E. coli*. In this study maltose binding protein (MBP) and His-tag were used in *E. coli* and *P. pastoris* expression systems respectively. MBP is one of the most effective solubility enhancer that also works as a natural affinity taq (Sun, 2011).

To optimize the condition and increase the expression levels in this study, level of induction concentration (IPTG), different time course and different vectors were tested to achieve and detect the optimal yield.

Different concentration of IPTG, from 0.3mM to 1mM (0.3mM, 0.5mM, 0.75mM, 1mM IPTG) was investigated. The highest yield of recombinant FGF21 was obtained after induction with 0.75mM of IPTG (Figure 3.20). In this study we observed that in using IPTG concentrations higher than 0.75mM, there was little or no effect in terms of further increasing the expression level of the desired protein. Another critical parameter that was investigated was the time of induction. For this purpose products were harvested at one, two, three and four hour after induction. It was then observed that the best time when the cells produced a higher yield of recombinant protein was four hours after adding IPTG (Figure 3.18 and 3.19).

Also, in the effort to obtain higher expression and yield, the influence and preformance of different expression vectors, namely C4X and the P4X, were also monitored, P4 vector release its product into peri plasmic space while C4 release its product intracellular space. The rFGF21 produced in P4X vector resulted in higher level of expression and based on this finding, the subsequent stages of experiments used the P4X-FGF21 as the expression construct of choice.

Purification was done after induction by using amylose column. Maltose binding protein as fusion tags attached FGF21 to amylose column and increased as well as simplified the solubility and purification of the desired expression product. Although after purification level of purified level decreased that is a normal fact because some part of the sample washed out from the column during the purification.

4.3 Expression in yeast

The advent of various technological advances has enabled the production of recombinant proteins of high quality and more similar to human proteins. Expression of proteins in yeast is a common alternative to prokaryotic and higher eukaryotic expression (wang *et al.,* 2007).

Its rapid growth, highly developed genetic system, ease of use, reduced time input and cost have made yeast eukaryotic cells like *Pichia pastoris* as an attractive organism for producing large scale eukaryotic proteins (Wang *et al.,* 2007).

Pichia pastoris has been developed and established as a protein expression system in the recent 30 years. Its advantages and disadvantages in the production of various recombinant proteins have been widely discussed (Higgins & Cregg, 1998, Cregg *et al.,* 2000, Cereghino *et al.,* 2002, Ilgen *et al*., 2005; Macauley-Patrick *et al*., 2005). As known for its rapid and ease of growth in simple medium, recombinant proteins could

be detected and harvested from *P. pastoris* culture in relatively short time and low cost.

Three different types of *Pichia pastoris* are available for expression; the wild-type *Pichia* pastoris strain X-33, GS115 and KM71H. The choice of host strain depends on the type of protein being expressed.

GS115 is a strain that has a mutation in the histidinol dehydrogenase gene (his4), and can only grow in media supplemented with histidine while KM71H is a methanol utilization slow (Mut^S) strain, which grows more slowly on methanol. Mut^S strain requires longer time to perform post-translational processes hence it may be better for expression of certain types of recombinant proteins.

However, compared to GS115 and KM71H, the wild-type strain X-33 offers several advantages and as such was chosen in this study. X-33 can grow on methanol and produce higher expression of recombinant protein while they need minimal media and does not need to add additional supplements to maintain optimal growth (Sirén *et al*., 2006). Initial observations in this study revealed that recombinant *P. pastoris* X-33 strains containing *rfgf21* gene grew as efficiently as the non-transformed wild-type control strain, X-33. This suggests that the integration of *rfgf21* gene construct into the host genome did not have any detectable negative effect on normal cell growth.

Multiple factors may play different roles in influencing the rate of cellular productivity including physiological, environmental and biochemical aspects. Molecular genetic aspects of the recombinant gene system (e.g. gene copy number, mRNA stability and/or unfolded protein response (UPR)) and cultivation, which includes temperature, pH and medium composition, monitoring the protein expression and secretion level influence the amount of the final recombinant proteins produced. In this study some factors such as growth temperature, methanol concentration and cultivation

period were monitored during flask cultivation to maximize the yield of rFGF21 in *P. pastoris*.

P. pastoris cells grow well at 28 to 30°C (*Pichia* Expression Instruction Manual). Growth was faster at 30°C, since cells entered stationary phase earlier (Li *et al.,* 2001) but rapid growth does not necessarily translate to maximal recombinant protein production, as several reports (as well as in this study) have shown low expression of heterologous proteins at the optimal growth temperature of 30°C. Conversely, a number of heterologous proteins have been successfully expressed at higher levels by shifting towards lower induction temperature. Lower temperature resulted in increased cell viability, and sustained growth much longer thus maximizing the production of recombinant proteins (Hong *et al.,* 2002).

In this study we observed that cultivation temperature was an important parameter for efficient expression of rFGF21 in *P. pastoris.* In the present study, the expression of rFGF21 could not be detected in the recombinant clones at 30°C. It was suspected that this was due to very low level of recombinant protein expression, which necessitated modifications to be made to the basic protocol to solve the problem, so cultivation temperature was reduced to 20 °C. This lower cultivation and induction temperatures significantly improved the expression of rFGF21 and minimized the extra cellular proteolysis and greatly enhanced the yield of the biologically active protein produced in *P. pastoris*. By lowering the induction temperature from 30°C to 20°C, the amount of accumulated rFGF21 in the culture medium was significantly increased from undetectable levels to 5 mg/L.

Other advantages of lower cultivation temperatures include increasing the efficiency of post-translational processing, such as protein folding in the ER, which may improve at lower temperatures and thus results in higher secretion rate of properly matured

protein (Hong *et al.*, 2002). Maintaining a suitable pH is also important as the change of pH may lead to cell death followed by the release of proteases.

Methanol is the carbon source and inducer for the AOX1 promoter and drives the expression of heterologous proteins. Methanol induction is essential in nutrient consumption and absorption and is essential for optimal cell growth (Liu *et al*., 2004). Recombinant proteins produced as long as the AOX1 promoter remains induced by methanol. However, its cytotoxicity to cell growth at high concentrations is also widely known. Therefore, optimization was necessary to determine the optimal concentration of methanol for maximum expression and yield of heterologous proteins. In this study, cultures were fed with different methanol concentrations (from 0.5% to 2.5%). The overall results indicated that the highest yield of rFGF21 was achieved at 20°C with 1.0% methanol feeding.

The inclusion of the α-factor signal peptide in pPICZαA recombinant vectors directed the secretion of recombinant proteins into the culture medium, facilitating the purification process. According to previous studies on *P. pastoris,* the use of α-factor containing signal sequences produce highly glycosylated products. Glycosylation is a common post-translational modification event in the secretory pathway in most eukaryotic expression systems; however, it is known to be species-, tissue- and cell-type-specific (Brooks, 2006). Various researches have shown that in general, glycosylation activities in *P. pastoris* generate heterologous recombinant proteins that are higher in molecular weight compared to those produced in mammalian expression systems (Braren *et al.,* 2007). However, there are also cases where the recombinant proteins produced in *P. pastoris* show lower molecular weights. Sadhukhan and Sen (1996) postulated that this could be due to the absence of other post-translational modification process in yeast, such as phosphorylation, sulphation and sialylation.

In the present study, Western blot results almost invariably showed broad smears rather than discrete bands of defined sizes. It was also noted that the size range of these smears suggested the molecular sizes of rFGF21 produced in *P. pastoris* were much larger than native FGF21.

To remove the glycosylated residues, deglycosylation with *PNGase F* and *Endo H* were done and after deglycosylation treatment, a distinct band appeared in the size of 40 kDa, revealed that these discrepancies in molecular size were due to variations in glycosylation of the rFGF21 (Figure 3.23). The two-fold increase in size could be due to dimerization of the protein, or perhaps the presence of different O-glycosylation residues. This phenomenon is not unheard of, as hyperglycosylation or hypermannosylation had been shown to frequently occur in yeast expression systems (Wu *et al*., 2002).

The highest yield achieved during this study in *P. pastoris*, was at culture growing temperature 20°C, methanol concentration 1%, and the best time to collect the products was at 72 hour after induction.

Nevertheless, despite the difficulties and problems outlined in this study, production of recombinant proteins in *Pichia* remains an attractive alternative expression system. The only remaining problem is whether proteins produce in yeast can be more human-like and if they are functional. Perhaps this negates the use of the more recently developed humanized glycosylation strain of *Pichia* (eg. *Pichia* GlycoSwitch® system) that claims that this system enables the production of controlled human-like glycosylation although this possibility has not been investigated in this study.

4.4 *In vivo* investigation

The present study used animal models to investigate the functionality of this recombinant FGF21 by conducting different experiments. For this purpose first acute and sub-chronic toxicity recombinant FGF21 were investigated and checked for the toxic nature and the potential of the product to raise any mortal effects. Besides the correct dose in which the protein was effective and does not cause any side effects or death in rats was also investigated. Rats were administered intraperitoneally (ip) with vehicle or rFGF21 variants at indicated doses. Blood parameters and body weights were measured at different time points before and after rFGF21 injection. The histopathological examination on the liver and kidneys and hematological changes induced by rFGF21 revels no effect of toxicity or pathological effects beyond the treated and control samples and so the recombinant product was safe and did not have any side effects on rats.

The first functionality experiment was conducted to investigate the effect of rFGF21's itself in a diabetic model that insulin is not working. The animals were placed on an obesogenic high fat-high sucrose diet for four weeks to become mimic to diabetic. Excessive ingestion of sucrose promotes development of type 2 diabetes mellitus, which is associated with obesity and insulin resistance in rodents. Reduced insulin sensitivity in body results in reduced glycogenesis and glucose disposal in target tissues that generates suitable model to study parameters related to type 2 diabetics. Since systematic administration of FGF21 pharmacologically have beneficial metabolic effects in obese and diabetic models as an insulin sensitizer, it should improve insulin sensitivity.

Fasting blood glucose above 6.1 mM/L considered as semi diabetic (Motshakeri *et al*., 2015). In our experiment blood glucose reached <9 mM/L, which can be considered

as mild diabetics. After daily administration of rFGF21 for 2 weeks in male rats fed with high fat high sucrose diet, no indication of hypoglycemia was observed and rFGF21 improved the insulin resistancy as expected, while the HSHF control group blood glucose remained high. Besides rats in NC groups with normal chow fed, did not show any changes in blood glucose levels over this period. The result achieved was consistent with previous observations that have been previously made in normal and diabetic rodents (Kharitonenkov *et al.,* 2005; Kharitonenkov *et al.,* 2007).

Lipid profile levels were tested at the end of this experiment. Lipid profiles reached to almost normal control levels compared to high fat high sucrose diet control group since the animals were still fed with high fat high sucrose diet until the end of the experiment period. During the high sucrose-high fat diet, all animals began to gain weight, and the average body weight level increased over time. Treatment of semi-diabetic rats with rFGF21 produced in *E. coli* and yeast led to a slight weight loss comparing to both control groups.

In the second experiment, different doses of rFGF21 were tested with high fat high sucrose diet. All doses lowered blood glucose and fasting plasma TGs significantly while simultaneously raising HDL. A dose of 300μg/kg body weight, lowered blood glucose faster comparing to 100μg/kg body weight doses. Since the sucrose compound will be cleaved to glucose and fructose in the body, the peak would appear slower than OGTT diagram. Both insulin and FGF21 simultaneously reduced blood glucose to normal levels in treatment groups although in the presence of FGF21, the insulin was secreted less than usual and in this case glucose concentration sharply decrease in untreated group with insulin effects while in treated group this decrease is based on FGF21 effect, with slower gradiant reduction and so blood glucose levels at 60 and 120 minutes were higher than controls. In addition in FGF21 treated group the plasma

glucose were less than untreated group (Pan *et al.*, 2014).

In our third experiment, effects of a single dose of rFGF21 were investigated by OGTT test. As demonstrated in Figure 3.32 the blood glucose concentrations during OGTT, increased dramatically in the NC and treatment group after 30 minutes post glucose injection while in treatment group the peak is lower comparing to control group. At time 60 and 120 min the values were significantly lower than the NC rats, since both insulin and FGF21 works together to reduce glucose level. Meanwhile FGF21 at least needs 4 hour to bring the blood glucose to normal rate, so the effects would be more stable and steadier in the body.

In general similar to previous studies, both rFGF21 produced in *E. coli* and *P. pastoris* were effective in lowering the blood glucose by improving the glucose uptakes compared to control groups and in lipid profiles respectively in all our experiments.

After confirming the functionality of the rFGF21 produced in *E. coli* and *P. pastoris,* we proceeded to investigate the potential effects of FGF21 on the histopathology (or perhaps development) of reproductive system in female and male rats. To date there is a lack of reports on the effects of FGF21 on the reproductive systems, and as such, its biological function in these organs still remains unclear. FGF21 was considered to be a novel target to treat metabolic diseases (Dostálová *et al.*, 2009; Kliewer *et al.,* 2010). Metabolic disease has been known to be associated to the development of polycystic ovary syndrome (PCOS) and deteriorated semen quality, which are the most common cause of infertility in women (Franks, 1995) and men (Kasturi *et al.,* 2008). These situations are normally associated with hyperinsulinemia, hyperandrogenism and insulin resistance (Gambineri *et al.*, 2002). In this study we tested Sprague Dawley female and male adult rats to determine histologic changes between treated and untreated groups administered with FGF21. For this purpose 300μg/kg body weight per day active

rFGF21 produced in yeast were administered for 28 days on these rats.

Whole body weight were increased in all experimental rats throughout the four weeks of this study but this increase was not significant ($p<0.05$) between treated and control groups although FGF21 treated group gained weight slower comparing to untreated group due to FGF21 function that reduce weight mildly.

***Females**:* No gross lesions were observed as a sign of necroses when female reproductive systems were evaluated *in situ*. However, microscopic and morphologic evaluations indicated treatment- related effects in the reproductive organs. The effects of rFGF21on uterine growth were shown in Figure 3.37. In rFGF21 treated groups the uterine lumen width were different compared to control group, where in rFGF21 treated group the lumen were wider. In addition to uterus size, following four weeks of treatment, the weights of uterus and ovaries in rFGF21 treated group increased significantly compared to the control group. And the relative organ/body weight ratio also improved significantly as shown in Table 3.2 due to FGF21 effects. Uterus swelling and increase in weight usually occurred due to the effect of estrogenic substances that increase imbibition and usually occured in the proestrus cycle (cook *et al*., 1997, O'connor *et al*., 1996; Reel *et al*., 1996). During proestrus in rats, the uterine lumen is distended with clear fluid (Yuan & Foley, 2002). In this situation cellular component of the uterus respond to steroid hormones such as estrogen, which stimulates DNA synthesis and cellular proliferation in the uterus of mammals (Mendoza-Rodriguez *et al*., 2003).

Rats reach full sexual maturity at approximately 7 weeks (Kohn & Clifford, 2002) and based on published reports, normal rats should have ovaries that have undergone 1 or more cycles of ovulation and the first regressing corpora lutea occurred at week 8 (Yuan & Foley, 2002). H&E staining photos of our study revealed no effects of

abnormality or ovarian autrophy in any of the groups of this study. An increase in the number of atretic follicles (AF) was observed in treated group while the number of primary follicles was more in control group. Atretic follicles in the same stage in treated group appeared to be more likely to occure and layers around the atretic and tertiary follicles were also more in treated group as shown in Figure 3.37. The layers around the atretic follicles reflects the progesteronal influence during proestrus, that may be stimulated by FGF21. In FGF21 treated animal's number of growing corpora lutea is more and fewer corpora lutea in regressing stage was observed. Corpora lutea were smaller and fewer comparing to controls. In fact, rFGF21 possess positive influences on histo-architecture of the ovaries of female rats suggesting positive influences on the reproductive health of the animals that can improve fertility. Future studies should be done to find out the exact function and pathway through which FGF21 effects on female reproductive organs and if FGF21 has estrogenic effects that can activate and keep ovary in proestrus stage or if most likely they activate other factors involved in the ovarian regulation and uterine growth.

Males: No gross abnormalities, including retained or small testis were detected during evaluation of male reproductive system. Similar to females, evaluations indicated treatment- related effects in the male reproductive organs. In the treated group, testes were slightly bigger in size and weight compared to animals in the control group as shown in Figure 3.35 due to effects of rFGF21. Absolute reproductive tissue weight and relative weight in males was significantly increased as shown in Table 3.2.

No sign of hyperplasia or hypertrophy was observed during histological evaluations of seminiferous tubule as shown in Figure 3.38. No effects of degeneration of ledgid cells, spermatogonia cells, spermatocyte, spermatids and somatic sertoli cells were observed and the presence of all the cellular elements without any distortion in

arrengment of spermatogenic cells were identified. No abnormal spaces in interstitial space were defined and testicular architecture is complete. Since the cells grow more, slight spaces between spermatogonia cells is observed in treated group comparing to the controls due to rFGF21 function and cells are not in a condense manner as we can see in control group. The histological sections showed abundant spermatogenic cells and because of that cellular secretions (sperms) increased in the lumen of the tubes, which indicated that spermatogenesis, was positively facilitated by rFGF21 while spermatogenic cell layers of control showed less dense packing of spermatogenic cells and the lumen of control was less densely filled with sperms. Future studies should be done to evaluate the mechanism of action FGF21 on male reproductive organ to evaluate its function clearly as physiologic modulators of spermatogenic cells. Previous studies indicated that Sertoli cells are major factors in regulation of spermatogenesis that can alter rates of sperm produced in testis (Griswold & Russell, 1993). Thus, it is possible that FGF21 could have interacted with Sertoli cells as well (Syazana *et al.,* 2011).

Xin Jiang demonstrated that FGF21 has anti oxidative function like FGF1 and FGF2, this feature can affects and increase testostron production, the key hormone involved in the production and maturation of spermatozoa in the seminiferous tubules of the testis which is in agree with our results (Ganong, 2003; Guyton & Hall, 1998). Based on Xin Jiang report that suggest *fgf21* gene may be involved in maintaining normal spermatogenesis and based on our result we conclude FGF21 could be an important factor for spermatogenesis and present a new paradigm to treat infertility, hence *fgf4* gene was also identified as a spermatogenesis enhancer in the testis (Yamamoto *et al.,* 2000; Jiang *et al.,* 2013).

CHAPTER 5: CONCULSION

In conclusion, the entire specific hypothesizes were achieved. The full length of FGF21 gene was constructed by overlapping exon 1, 2 and 3 by using overlapping – extension PCR method. FGF21 gene was cloned successfully in p AML vector and p PICZαA vector, which produced soluble FGF21 protein in *E.coli*. and *Pichia Pastoris.* Large scale expression and purification of FGF21 protein in bacteria and yeast were successfully achieved and finally the effect and bioactivity of these recombinant FGF21 on the blood glucose and lipid profile rats and on the tissue construction of the reproductive organs in female and male rats were investigated. In conclusion, the yeast production system seems to product rFGF21 with more yield based on Bradford assay results comparing to bacteria in order to produce the large scale production of rFGF21 and the purification is easier although its more time consuming. *P. pastoris* products also were more active in lowering blood glucose comparing to *E. coli* on all the experiments conducted in this project. We confirmed that FGF21 has positive effects in improvement of reproductive system of rats and therefore may be useful in treatment of infertility.

REFERENCES

Aaronson, S., Bottaro, D., Miki, T., Ron, D., Finch, P., Fleming, T., & Rubin, J. (1991). Keratinocyte growth factor: A fibroblast growth factor family member with unusual target cell specificity. *Annals of the New York Academy of Sciences, 638*(1), 62-77.

Akpantah, A., Oremosu, A., Ajala, M., Noronha, C., & Okanlawon, A. (2003). The effect of crude extract of Garcinia Kola seed on the histology and hormonal milieu of male sprague-dawley rats reproductive organs. *Nigerian Journal of Health and Biomedical Sciences*, *2*(1), 40-46.

Algül, H. (2009). A novel role for the fibroblast growth factor 21 in acute pancreatitis. *Gastroenterology, 137*(5), 1577-1579.

Andrews, M., Squire, T., Bowen, C., & Rollins, M. (1998). Low-temperature carbon utilization is regulated by novel gene activity in the heart of a hibernating mammal. *Proceedings of the National Academy of Sciences, 95*(14), 8392-8397.

Arner, P., Pettersson, A., Mitchell, P., Dunbar, J., Kharitonenkov, A., & Rydén, M. (2008). FGF21 attenuates lipolysis in human adipocytes–a possible link to improved insulin sensitivity. *FEBS Letters, 582*(12), 1725-1730.

Badman, M., Pissios, P., Kennedy, A., Koukos, G., Flier, J., & Maratos-Flier, E. (2007). Hepatic fibroblast growth factor 21 is regulated by PPARα and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metabolism, 5*(6), 426-437.

Banting, F., Best, C., Collip, J., Campbell, W., & Fletcher, A. (1991). Pancreatic extracts in the treatment of diabetes mellitus: preliminary report. 1922. *CMAJ: Canadian Medical Association Journal*, *145*(10), 1281–1286.

Basilico, C., & Moscatelli, D. (1992). The FGF family of growth factors and oncogenes. *Advances in Cancer Resarch, 59*(115), 65.

Bergman, R., & Afifi, A. (1974). *Atlas of microscopic anatomy: A companion to histology and neuroanatomy.* Philadelphia: Saunders.

Bernal-Mizrachi, E., Fatrai, S., Johnson, J., Ohsugi, M., Otani, K., Han, Z., & Permutt, M. (2004). Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet β cells. *The Journal of Clinical Investigation, 114*(7), 928-936.

Beyer, T., Werner, S., Dickson, C., & Grose, R. (2003). Fibroblast growth factor 22 and its potential role during skin development and repair. *Experimental Cell Research, 287*(2), 228-236.

Bhushan, A., Itoh, N., Kato, S., Thiery, J., Czernichow, P., Bellusci, S., & Scharfmann, R. (2001). FGF10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development, 128*(24), 5109-5117.

Bikfalvi, A., & Han, Z. (1994). Angiogenic factors are hematopoietic growth factors and vice versa. *Leukemia, 8*(3), 523-529.

Bland, J., & Altman, D. (2009). Analysis of continuous data from small samples. *British Medical Journal*, *338*, a3166.

Boden, G., & Zhang, M. (2006). Recent findings concerning thiazolidinediones in the treatment of diabetes. *Expert Opinion on Investigational Drugs*, *15*(3), 243-250.

Borodina, T., Lehrach, H., & Soldatov, A. (2003). Ligation-based synthesis of oligonucleotides with block structure. *Analytical Biochemistry, 318*, 309-313.

Bottcher, R., & Niehrs, C. (2005). Fibroblast growth factor signaling during early vertebrate development. *Endocrine Reviews, 26*(1), 63-77.

Braren, I., Greunke, K., Umland, O., Deckers, S., Bredehorst, R., & Spillner, E. (2007). Comparative expression of different antibody formats in mammalian cells and *Pichia pastoris*. *Biotechnology Applied and Biochemistry, 47*, 205-214.

Braun, P., & LaBaer, J. (2003). High throughput protein production for functional proteomics. *Trends in Biotechnology, 21*(9), 383-388.

Brogi, E., Winkles, J., Underwood, R., Clinton, S., Alberts, G., & Libby, P. (1993). Distinct patterns of expression of fibroblast growth factors and their receptors in human atheroma and nonatherosclerotic arteries. Association of acidic FGF with plaque microvessels and macrophages. *Journal of Clinical Investigation, 92*(5), 2408.

Brooks, S. (2006). Protein glycosylation in diverse cell systems: Implications for modification and analysis of recombinant proteins. *Expert Review Proteomics*, *3*, 345- 359.

Burgess, S., Leone, T. C., Wende, A. R., Croce, M. A., Chen, Z., Sherry, A. D., & Finck, B. N. (2006). Diminished hepatic gluconeogenesis via defects in tricarboxylic acid cycle flux in peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α)-deficient mice. *Journal of Biological Chemistry, 281*(28), 19000-19008.

Burgess, W. H., & Maciag, T. (1989). The heparin-binding (fibroblast) growth factor family of proteins. *Annual Review of Biochemistry, 58*(1), 575-602.

Cahill, G. F. (2006). Fuel metabolism in starvation. *Annual Review of Nutrition, 26*, 1-22.

Cancilla, B., Davies, A., Ford-Perriss, M., & Risbridger, G. P. (2000). Discrete cell-and stage-specific localisation of fibroblast growth factors and receptor expression during testis development. *Journal of Endocrinology, 164*(2), 149-159.

Carballada, R., Yasuo, H., & Lemaire, P. (2001). Phosphatidylinositol-3 kinase acts in parallel to the ERK MAP kinase in the FGF pathway during Xenopus mesoderm induction. *Genes & Development, 128*(1), 35-44.

Card, P. B., & Gardner, K. H. (2005). Identification and optimization of protein domains for NMR studies. *Methods in Enzymology, 394*, 3-16.

Cereghino, G. P., Cereghino, J. L., Ilgen, C., & Cregg, J. M. (2002). Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. *Current Opinion in Biotechnology*, *13*(4), 329-332.

Cereghino, G. P., Sunga, A. J., Cereghino, J. L., & Cregg, J. M. (2002). Expression of foreign genes in the yeast *Pichia pastoris*. *Genetic Engineering: Principles and Methods,* 157-169.

Cereghino G. P., & Cregg J. M. (1999) Applications of yeast in biotechnology: Protein production and genetic analysis. *Current Opinion in Biotechnology, 10*, 422–427.

Cereghino, J. L., & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews, 24*(1), 45-66.

Chen, W., Li, L., Yang, G., Li, K., Qi, X., Zhu, W., & Boden, G. (2008). Circulating FGF-21 levels in normal subjects and in newly diagnose patients with type 2 diabetes mellitus. *Experimental and Clinical Endocrinology and Diabetes, 116*(1), 65-68.

Chiu, I. M., Sandberg, P., Wang, W. P., & Hocks, L. A. (1990). Brain-derived heparin-binding growth factors and their oncogenic homologs. *Tropic Factors and the Nervous System*, *3*, 55-57.

Clark, J., Rager, D., & Calpin, J. (1997). Animal well-being I. General considerations. *Comparative Medicine*, *47*(6), 564-570.

Cline, J., Braman, J., & Hogrefe, H. (1996). PCR fidelity of Pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research, 24*, 3546-3551.

Colvin, J., Green, R., Schmahl, J., Capel, B., & Ornitz, D. (2001). Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell, 104*(6), 875-889.

Cook, J., Kaplan, A., Davis, L., & O'connor, J. (1997). Development of a Tier I screening battery for detecting endocrine-active compounds (EACs). *Regulatory Toxicology and Pharmacology*, *26*(1), 60-68.

Coskun, T., Bina, H., Schneider, M., Dunbar, J., Hu, C., Chen, Y., & Kharitonenkov, A. (2008). Fibroblast growth factor 21 corrects obesity in mice. *Endocrinology,* 149(12), 6018-6027.

Coulier, F., Ollendorff, V., Marks, I., Rosnet, O., Batoz, M., Planche, J., & Birnbaum, D. (1991). The *fgf6* gene within the FGF multigene familya. *Annals of the New York Academy of Sciences, 638*(1), 53-61.

Crabb, J., Armes, L., Carr, S. , Johnson, C., Roberts, G., Bordoli, R., & McKeehan, W. (1986). Complete primary structure of prostatropin, a prostate epithelial cell growth factor. *Biochemistry, 25*(18), 4988-4993.

Cregg, J., & Madden, K. (1988). Development of the methylotrophic yeast, *Pichia pastoris*, as a host system for the production of foreign proteins. *Developments in Industrial Microbiology, 29*, 33-41.

Cregg, J., Shen, S., Johnson, M., & Waterham, H. (1998). Classical genetic manipulation, *Pichia* protocols. *Humana Press*, 17–26.

Cregg, J., Cereghino, J., Shi, J., & Higgins, D. (2000). Recombinant protein expression in *Pichia pastoris*. *Molecular Biotechnology, 16*, 23-52.

Cuevas-Ramos, D., Almeda-Valdes, P., Aguilar-Salinas, C., Cuevas-Ramos, G., Cuevas-Sosa, A., & Gomez-Perez, F. (2009). The role of fibroblast growth factor 21 (FGF21) on energy balance, glucose and lipid metabolism. *Current Diabetes Reviews, 5*(4), 216-220.

Davidson, D., Blanc, A., Filion, D., Wang, H., Plut, P., Pfeffer, G., & Henderson, J. (2005). Fibroblast growth factor (FGF) 18 signals through FGF receptor 3 to promote chondrogenesis. *Journal of Biological Chemistry, 280*(21), 20509-20515.

Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A., & Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell*, *84*(6), 911-921.

Deo, D., Axelrad, T., Robert, E., Marcheselli, V., Bazan, N., & Hunt, J. (2002). Phosphorylation of STAT-3 in response to basic fibroblast growth factor occurs through a mechanism involving platelet-activating factor; JAK-2, and Src in human umbilical vein endothelial cells evidence for dual kinase mechanism. *Journal of Biological Chemistry, 277*(24), 21237-21245.

Dickson, C., Fuller, P., France, S., Kiefer, P., Acland, P., Macallan, D., & Peters, G. (1991). Expression, processing, and properties of int-2. *Annals of the New York Academy of Sciences*, *638*(1), 18-26.

Dostalova, I., Haluzikova, D., & Haluzik, M. (2009). Fibroblast growth factor 21: A novel metabolic regulator with potential therapeutic properties in obesity/type 2 diabetes mellitus. *Physiological Research, 58*(1), 1.

Drummond, A. E., Tellbach, M., Dyson, M., & Findlay, J. (2007). Fibroblast growth factor-9, a local regulator of ovarian function. *Endocrinology, 148*(8), 3711-3721.

Dümmler, A., Lawrence, A., & De Marco, A. (2005). Simplified screening for the detection of soluble fusion constructs expressed in E. coli using a modular set of vectors. *Microbial Cell Factories*, *4*(1), 34.

Eckenstien, F. (1994). Fibrolast growth factors in the nervous system. *Journal of Neurobiology*, *25*(11), 1467-1480.

Epstein, F., Shepherd, P., & Kahn, B. (1999). Glucose transporters and insulin action—implications for insulin resistance and diabetes mellitus. *New England Journal of Medicine, 341*(4), 248-257.

Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., & Guillemin, R. (1985). Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. *Proceedings of the National Academy of Sciences, 82*(19), 6507-6511.

Eswarakumar, V., Lax, I., & Schlessinger, J. (2005). Cellular signaling by fibroblast growth factor receptors. Cytokine & Growth Factor Reviews, *16*(2), 139-149.

Evans, S., Choudary, P., Neal, C., Li, J., Vawter, M., Tomita, H., & Stead, J. (2004). Dysregulation of the fibroblast growth factor system in major depression. *Proceedings of the National Academy of Sciences of the United States of America, 101*(43), 15506-15511.

Fantoni, A., Bill, R., Gustafsson, L., & Hedfalk, K. (2007). Improved yields of full-length functional human FGF1 can be achieved using the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification, 52*(1), 31-39.

Feldman, B., Poueymirou, W., Papaioannou, E., DeChiara, T., & Goldfarb, M. (1995). Requirement of FGF-4 for post implantation mouse development. *Science*, *267*(5195), 246-249.

Fisher, F., Chui, P., Antonellis, P., Bina, H., Kharitonenkov, A., & Flier, J. (2010). Obesity is a fibroblast growth factor21(FGF21)- resistance state. *Diabetes*, *59*, 2781-9.

Folkman, J., & Klagsbrun, M., (1987). Angiogenic factors. *Science 235*,442-448.

Franks, S. (1995). Polycystic ovary syndrome. *New England Journal of Medicine*, *333*(13), 853-861.

Fu, L., John, L., Adams, S., Yu, X., Tomlinson, E., Renz, M., & Moffat, B. (2004). Fibroblast growth factor 19 increases metabolic rate and reverses dietary and leptin-deficient diabetes. *Endocrinology, 145*(6), 2594-2603.

Fukao, T., Lopaschuk, G., & Mitchell, G. (2004). Pathways and control of ketone body metabolism: On the fringe of lipid biochemistry. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 70*(3), 243-251.

Gälman, C., Lundåsen, T., Kharitonenkov, A., Bina, H., Eriksson, M., Hafström, I., & Rudling, M. (2008). The circulating metabolic regulator FGF21 is induced by prolonged fasting and PPARα activation in man. *Cell Metabolism, 8*(2), 169-174.

Galzie, Z., Kinsella, A., & Smith, J. (1997). Fibroblast growth factors and their receptors. *Biochemistry and Cell Biology, 75*(6), 669-685.

Gambineri, A., Pelusi, C., Vicennati, V., Pagotto, U., & Pasquali, R. (2002). Obesity and the polycystic ovary syndrome. *International Journal of Obesity*, *26*(7), 883.

Geiser, F. (2004). Metabolic rate and body temperature reduction during hibernation and daily torpor. *Annual Review of Physiology, 66*, 239-274.

Georgiou, G. (1996). *Protein engineering: Principles and practice*. New York: Wiley-Liss.

Gimeno, R., & Moller, D. (2014). FGF21-based pharmacotherapy–potential utility for metabolic disorders. *Trends in Endocrinology & Metabolism*, *25*(6), 303-311.

Goeddel, D. (1990). Systems for heterologous gene expression. *Methods in Enzymology, 185*, 3-7.

Goetz, R., Beenken, A., Ibrahimi, O., Kalinina, J., Olsen, S., Eliseenkova, A., & Linhardt, R. (2007). Molecular insights into the klotho-dependent, endocrine mode of action of fibroblast growth factor 19 subfamily members. *Molecular and Cellular Biology, 27*(9), 3417-3428.

Goldfarb, M., Bates, B., Drucker, B., Hardin, J., & Haub, O. (1991). Expression and possible functions of the FGF-5 gene. *Annals of the New York Academy of Sciences, 638*(1), 38-52.

Goldfarb, M., Schoorlemmer, J., Williams, A., Diwakar, S., Wang, Q., Huang, X., & Vega, A. (2007). Fibroblast growth factor homologous factors control neuronal excitability through modulation of voltage-gated sodium channels. *Neuron, 55*(3), 449-463.

Goldman, J., Laws, S., Balchak, S., Cooper, R., & Kavlock, R. (2000). Endocrine-disrupting chemicals: Prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations. *Critical Reviews in Toxicology*, *30*(2), 135-196.

Gospodarowicz, D., Cheng, J., Lui, G., Baird, A., & Böhlent, P. (1984). Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: Identity with pituitary fibroblast growth factor. *Proceedings of the National Academy of Sciences, 81*(22), 6963-6967.

Gospodarowicz, D., Bialecki, H., & Greenburg, G. (1978). Purification of the fibroblast growth factor activity from bovine brain. *Journal of Biological Chemistry, 253*(10), 3736-3743.

Gospodarowicz, D. (1975). Purification of a fibroblast growth factor from bovine pituitary. *Journal of Biological Chemistry, 250*(7), 2515-2520.

Gräslund, S., Eklund, M., Falk, R., Uhlén, M., Nygren, P., & Ståhl, S. (2002). A novel affinity gene fusion system allowing protein A-based recovery of non-immunoglobulin gene products. *Journal of Biotechnology*, *99*(1), 41-50.

Griswold, M., & Russell, L. (1993). *The Sertoli cell biology* (2nd ed.). San Diego: Elsevier Academic Press.

Gronenborn, B. (1976). Molecular gene. *Genetics, 148*, 243–250.

Guyton, A., & Hall, J. (1998). *Medical physiology* (12th ed.), Philadelphia: Elsevier Academic Press.

Haluzik, M., Parizkova, J., & Haluzik, M. (2004). Adiponectin and its role in the obesity-induced insulin resistance and related complications. *Physiological Research, 53*(2), 123-130.

Harmer, J., Ilag, L., Mulloy, B., Pellegrini, L., Robinson, V., & Blundell, L. (2004). Towards a resolution of the stoichiometry of the fibroblast growth factor (FGF)–FGF receptor–heparin complex. *Journal of Molecular Biology, 339*(4), 821-834.

Havel, P. J. (2002). Control of energy homeostasis and insulin action by adipocyte hormones: Leptin, acylation stimulating protein, and adiponectin. *Current Opinion in Lipidology, 13*(1), 51-59.

Hecht, R., Li, Y., Sun, J., Belouski, E., Hall, M., & Hager, T. (2012). Rationale-based engineering of a potent long-acting *FGF21* analog for the treatment of type 2 diabetes. *Plos One, 7*(11) e49345.

Heimo, H., Palmu, K., & Suominen, I. (1997). Expression in *Pichia pastoris* and purification of Aspergillus awamori glucoamylase catalytic domain. *Protein Extract Purif*ication *10*, 70-79.

Hermansen, K., & Davies, M. (2007). Does insulin detemir have a role in reducing risk of insulin‐associated weight gain? *Diabetes, Obesity and Metabolism, 9*(3), 209-217.

Higgins, R., & Cregg, J. (1998). Introduction to *Pichia pastoris*. *Pichia Protocols*, 1-15.

Higgins, C., Petukhova, L., Harel, S., Ho, Y., Drill, E., Shapiro, L., & Christiano, A. (2014). FGF5 is a crucial regulator of hair length in humans. *Proceedings of the National Academy of Sciences*, *111*(29), 10648-10653.

Hodgson, J. (1993). Expression systems: A user's guide. Emphasis has shifted from the vector construct to the host organism. *Biotechnology, 11*(8), 887-893.

Holt, J., Luo, G., Billin, A., Bisi, J., McNeill, Y., Kozarsky, K., & Goodwin, B. (2003). Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes and Development, 17*(13), 1581-1591.

Hong, F., Meinander, N., & Jönsson, L. (2002). Fermentation strategies for improved heterologous expression of laccase in *Pichia pastoris*. *Biotechnology Bioeng. 79*, 438- 449.

Hoover, D., & Lubkowski, J. (2002). DNA works: An automated method for designing oligonucleotides for PCR-based gene synthesis. *Nucleic Acids Research*, *30*,e43.

Horton, M. (1997). Methods in molecular biology, PCR cloning protocols. *Humana Press*, *67*,141–149.

Horton, M., Hunt, S., Ho, J., & Pease, L. (1989). Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. *Gene, 77*,61-68.

Housa, D., Housova, J., Vernerova, Z., & Haluzik, M. (2006). Adipocytokines and cancer. *Physiological Research*, *55*(3), 233.

Hu, M., Qiu, W., Wang, Y., Hill, D., Ring, B., Scully, S., & Simonet, W. (1998). FGF-18, a novel member of the fibroblast growth factor family, stimulates hepatic and intestinal proliferation. *Molecular and Cellular Biology, 18*(10), 6063-6074.

Huang, X., Yu, C., Jin, C., Yang, C., Xie, R., Cao, D., & McKeehan, W. (2006). Forced expression of hepatocyte‐specific fibroblast growth factor 21 delays initiation of chemically induced hepatocarcinogenesis. *Molecular Carcinogenesis, 45*(12), 934-942.

Hutley, L., Shurety, W., Newell, F., McGeary, R., Pelton, N., Grant, J., & Prins, J. (2004). Fibroblast growth factor 1 a key regulator of human adipogenesis. *Diabetes, 53*(12), 3097-3106.

Ibrahimi, O., Zhang, F., Langhrstka, S., Mohammadi, M., & Linhardt, R. (2004). Kinetic model for FGF, FGFR, and proteoglycan signal transduction complex assembly. *Biochemistry, 43*(16), 4724-4730.

Ilgen, C., Lin-Cereghino, J., & Cregg, J. (2005). *Pichia pastoris*, production of recombinant proteins: Novel microbial and eukaryotic expression systems. *Yeast*, 143-162.

Inagaki, T., Choi, M., Moschetta, A., Peng, L., Cummins, C., McDonald, J., & Richardson, J. (2005). Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metabolism, 2*(4), 217-225.

Inagaki, T., Dutchak, P., Zhao, G., Ding, X., Gautron, L., Parameswara, V., & Esser, V. (2007). Endocrine regulation of the fasting response by PPARα-mediated induction of fibroblast growth factor 21. *Cell Metabolism, 5*(6), 415-425.

Inagaki, T., Lin, V., Goetz, R., Mohammadi, M., Mangelsdorf, D., & Kliewer, S. (2008). Inhibition of growth hormone signaling by the fasting-induced hormone FGF21. *Cell Metabolism, 8*(1), 77-83.

Ito, S., Kinoshita, S., Shiraishi, N., Nakagawa, S., Sekine, S., Fujimori, T., & Nabeshima, Y. (2000). Molecular cloning and expression analyses of mouse βklotho, which encodes a novel Klotho family protein. *Mechanisms of Development, 98*(1), 115-119.

Itoh, N., & Ornitz, D. (2004). Evolution of the *fgf* and *fgfr* gene families. *Trends in Genetics, 20*(11), 563-569.

Itoh, N., & Ornitz, D. (2008). Functional evolutionary history of the mouse *fgf* gene family. *Developmental Dynamics, 237*(1), 18-27.

Itoh, N. (2010). Hormone-like (endocrine) FGFs: Their evolutionary history and roles in development, metabolism, and disease. *Cell and Tissue Research, 342*(1), 1-11.

Izumiya, Y., Bina, H., Ouchi, N., Akasaki, Y., Kharitonenkov, A., & Walsh, K. (2008). FGF21 is an Akt-regulated myokine. *FEBS Letters, 582*(27), 3805-3810.

Jeon, W., Aceti, D., Bingman, C., Vojtik, F., Olson, A., Ellefson, J., & Seder, K. (2005). High-throughput purification and quality assurance of Arabidopsis thaliana proteins for eukaryotic structural genomics. *Journal of Structural and Functional Genomics, 6*(2-3), 143-147.

Jetton, T., Lausier, J., LaRock, K., Trotman, W., Larmie, B., Habibovic, A., & Leahy, J. (2005). Mechanisms of compensatory β-Cell growth in insulin-resistant rats roles of Akt kinase. *Diabetes*, *54*(8), 2294-2304.

Jiang, X., Skibba, M., Zhang, C., Tan, Y., Xin, Y., & Qu, Y. (2013). The roles of fibroblast growth factors in the testicular development and tumor. *Journal of Diabetes Research*, *2013*, 1-8.

Jonasson, P., Liljeqvist, S., Nygren, P., & Ståhl, S. (2002). Genetic design for facilitated production and recovery of recombinant proteins in Escherichia coli. *Biotechnology and Applied Biochemistry, 35*(2), 91-105.

Kasturi, S., Tannir, J., & Brannigan, R. (2008). The metabolic syndrome and male infertility. *Journal of Andrology*, *29*(3), 251-259.

Kelleher, F., O'Sullivan, H., Smyth, E., McDermott, R., & Viterbo, A. (2013). Fibroblast growth factor receptors, developmental corruption and malignant disease. *Carcinogenesis*, *34*(10), 2198-2205.

Kennedy, G., & Mitra, J. (1963). Hypothalamic control of energy balance and the reproductive cycle in the rat. *The Journal of Physiology*, *166*(2), 395.

Kersten, S., Desvergne, B., & Wahli, W. (2000). Roles of PPARs in health and disease. *Nature, 405*(6785), 421-424.

Kharitonenkov, A., Shiyanova, T., Koester, A., Ford, A., Micanovic, R., Galbreath, E., & Owens, R. (2005). FGF-21 as a novel metabolic regulator. *The Journal of Clinical Investigation, 115*(6), 1627-1635.

Kharitonenkov, A., Wroblewski, V., Koester, A., Chen, Y., Clutinger, C., Tigno, X., & Etgen, G. (2007). The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21. *Endocrinology, 148*(2), 774-781.

Kharitonenkov, A., & Shanafelt, A. (2008). Fibroblast growth factor-21 as a therapeutic agent for metabolic diseases. *BioDrugs*, *22*(1), 37-44.

Kharitonenkov, A., & Larsen, P. (2011). FGF21 reloaded: Challenges of a rapidly growing field. *Trends in Endocrinology & Metabolism*, *22*(3), 81-86.

Kharitonenkov, A., & Adams, A. (2014). Inventing new medicines: The FGF21 story. *Molecular Metabolism, 3*(3), 221-229.

Kim, H., Shin, J., Moon, H., Kim, T., Kang, I., Seok, J., & Han, S. (2002). Evaluation of the 20-day pubertal female assay in Sprague-Dawley rats treated with DES, tamoxifen, testosterone, and flutamide. *Toxicological Sciences*, *67*(1), 52-62.

Kim, J., Nolte, L., Hansen, P., Han, D., Kawanaka, K., & Holloszy, J. (1999). Insulin resistance of muscle glucose transport in male and female rats fed a high-sucrose diet. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *276*(3), R665-R672.

Kim, Y., Dementieva, I., Zhou, M., Wu, R., Lezondra, L., Quartey, P., & Joachimiak, A. (2004). Automation of protein purification for structural genomics. *Journal of Structural and Functional Genomics, 5*(1-2), 111-118.

Kjeldsen, T., Pettersson, A., & Hach, M. (1999). Secretory expression and characterization of insulin in *Pichia pastoris*. *Biotechnology Applied Biochem*istry *29*,79-86.

Kliewer, S., & Mangelsdorf, D. (2010). Fibroblast growth factor 21: From pharmacology to physiology. *The American Journal of Clinical Nutrition, 91*(1), 254S-257S.

Klock, H., White, A., Koesema, E., & Lesley, S. (2005). Methods and results for semi-automated cloning using integrated robotics. *Journal of Structural and Functional Genomics, 6*(2-3), 89-94.

Knaust, R., & Nordlund, P. (2001). Screening for soluble expression of recombinant proteins in a 96-well format. *Analytical Biochemistry*, *297*(1), 79-85.

Kohn, D., & Clifford, C. (2002). Biology and diseases of rats. *Laboratory Animal Medicine*, 121-165.

Konishi, M., Mikami, T., Yamasaki, M., Miyake, A., & Itoh, N. (2000). Fibroblast growth factor-16 is a growth factor for embryonic brown adipocytes. *Journal of Biological Chemistry, 275*(16), 12119-12122.

Kontaridis, M., Liu, X., Zhang, L., & Bennett, A. (2002). Role of SHP-2 in fibroblast growth factor receptor-mediated suppression of myogenesis in C2C12 myoblasts. *Molecular and Cellular Biology, 22*(11), 3875-3891.

Kubicky, R., Wu, S., Kharitonenkov, A., & De Luca, F. (2012). Role of fibroblast growth factor 21 (FGF21) in under nutrition-related attenuation of growth in mice. *Endocrinology*, *153*(5), 2287-2295.

Kurosu, H., Choi, M., Ogawa, Y., Dickson, A., Goetz, R., Eliseenkova, A., & Kuro, M. (2007). Tissue-specific expression of βKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *Journal of Biological Chemistry, 282*(37), 26687-26695.

Larentis, S., Tolsma, J., Fallahazad, B., Dillen, D., Kim, K., Macdonald, A., & Tutuc, E. (2014). Band offset and negative compressibility in graphene-MoS2 heterostructures. *Nano Letters*, *14*(4), 2039-2045.

Lesley, S. (2001). High-throughput proteomics: Protein expression and purification in the postgenomic world. *Protein Expression and Purification, 22*(2), 159-164.

Leung, K., Pippalla, V., Kreutter, A., & Chandler, M. (1998). Functional effects of FGF-13 on human lung fibroblasts, dermal microvascular endothelial cells, and aortic smooth muscle cells. *Biochemical and Biophysical Research Communications, 250*(1), 137-142.

Li, Z., Xiong, F., Lin, Q., d'Anjou, M., Daugulis, A., Yang, D., & Hew, C. (2001). Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. *Protein Expression and Purification*, *21*(3), 438-445.

Li, Z., Leung, W., Yon, A., Nguyen, J., Perez, V., Vu, J., & Gomez, S. (2010). Secretion and proteolysis of heterologous proteins fused to the Escherichia coli maltose binding protein in *Pichia pastoris*. *Protein Expression and Purification*, *72*(1), 113-124.

Lin-Cereghino, G., Leung, W., & Lin-Cereghino, J. (2007). Expression of protein in *Pichia pastoris*. *Expression Systems: Methods Express,*123–145.

Liu, Y., & Chiu, I. (1997). Assignment of FGF12, the human FGF homologous factor 1 gene, to chromosome 3q29→ 3qter by fluorescence *in situ* hybridization. *Cytogenetic and Genome Research, 78*(1), 48-49.

Liu, R., Yang, K., Lin, J., Lin, Y., Zhang, Z., Zhang, J., & Xia, N. (2004). High-yield expression of recombinant SARS coronavirus nucleocapsid protein in methylotrophic yeast *Pichia pastoris*. *World Journal Gastroenterol, 10*, 3602-3607.

Logan, A., Frautschy, S., & Baird, A. (1991). Basic fibroblast growth factor and central nervous system injury. *Annals of the New York Academy of Sciences*, 638, 474.

Lu, C., Mansoorabadi, K., & Jeffries, T. (2007). Comparison of multiple gene assembly methods for metabolic engineering. *Applied Biochemistry Biotechnology, 137*(1-12), 703-710.

Lundåsen, T., Gälman, C., Angelin, B., & Rudling, M. (2006). Circulating intestinal fibroblast growth factor 19 has a pronounced diurnal variation and modulates hepatic bile acid synthesis in man. *Journal of Internal Medicine*, *260*(6), 530-536.

Lundåsen, T., Hunt, M., Nilsson, L., Sanyal, S., Angelin, B., Alexson, S., & Rudling, M. (2007). PPARα is a key regulator of hepatic FGF21. *Biochemical and Biophysical Research Communications, 360*(2), 437-440.

Macauley-Patrick, S., Fazenda, M., McNeil, B., & Harvey, L. (2005). Heterologous protein production using the *Pichia pastoris* expression system. *Yeast,* 22,249-270.

Mai, K., Schwarz, F., Bobbert, T., Andres, J., Assmann, A., Pfeiffer, A., & Spranger, J. (2011). Relation between fibroblast growth factor–21, adiposity, metabolism, and weight reduction. *Metabolism, 60*(2), 306-311.

Makrides, S. (1996). Strategies for achieving high-level expression of genes in Escherichia coli. *Microbiological Reviews, 60*(3), 512-538.

Marie, P. (2003). Fibroblast growth factor signaling controlling osteoblast differentiation. *Gene*, *316*, 23-32.

Martin, G. (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes & Development, 12*(11), 1571-1586.

McAvoy, J., Chamberlain, C., De Iongh, R., Richardson, N., & Lovicu, F. (1991). The role of fibroblast growth factor in eye lens development. *Annals of the New York Academy of Sciences, 638*(1), 256-274.

McKeehan, W., Wang, F., & Kan, M. (1998).The heparan sulfate–fibroblast growth factor family: Diversity of structure and function. *Progress in Nucleic Acid Research and Molecular Biology, 59*, 135-176.

McWhirter, J., Goulding, M., Weiner, J., Chun, J., & Murre, C. (1997). A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. *Genes & Development, 124*(17), 3221-3232.

Mendoza‐Rodríguez, C., Merchant‐Larios, H., Segura‐Valdez, M., Moreno‐Mendoza, N., Cruz, M., Arteaga‐López, P., & Cerbón, M. (2003). C‐fos and estrogen receptor gene expression pattern in the rat uterine epithelium during the estrous cycle. *Molecular Reproduction and Development*, *64*(4), 379-388.

Micanovic, R., Raches, D., Dunbar, J., Driver, D., Bina, H., Dickinson, C., & Kharitonenkov, A. (2009). Different roles of N‐and C‐termini in the functional activity of FGF21. *Journal of Cellular Physiology, 219*(2), 227-234.

Miyamoto, M., Naruo, K., Seko, C., Matsumoto, S., Kondo, T., & Kurokawa, T. (1993). Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. *Molecular and Cellular Biology, 13*(7), 4251-4259.

Mohammadi, M., Olsen, S., & Goetz, R. (2005). A protein canyon in the FGF–FGF receptor dimer selects from an a la carte menu of heparan sulfate motifs. *Current Opinion in Structural Biology, 15*(5), 506-516.

Moore, E., Bendele, A., Thompson, D., Littau, A., Waggie, K., Reardon, B., & Ellsworth, J. (2005). Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. *Osteoarthritis and Cartilage, 13*(7), 623-631.

Moore, E., Bendele, A., Thompson, D., Littau, A., Waggie, K., Reardon, B., & Ellsworth, J. (2004). Fibroblast growth factor-18 stimulates chondrogenesis and promotes cartilage repair in a rat model of injury-induced osteoarthritis. *Transactions of the Orthopaedic Research Society*, *29*, 199.

Motshakeri, M., Goh, Y., & Ebrahimi, M. (2015). Metabolic effects of high sucrose and saturated oil feeding on insulin resistance in Sprague-Dawley rats. *Indian Journal of Experimental Biology, 53*, 264-272.

Moyers, J., Shiyanova, T., Mehrbod, F., Dunbar, J., Noblitt, T., Otto, K., & Kharitonenkov, A. (2007). Molecular determinants of FGF - 21 activity—synergy and cross - talk with PPARγ signaling. Journal of Cellular Physiology, *210*(1), 1-6.

Mraz, M., Bartlova, M., Lacinova, Z., Michalsky, D., Kasalicky, M., Haluzikova, D., & Haluzik, M. (2009). Serum concentrations and tissue expression of a novel endocrine regulator fibroblast growth factor - 21 in patients with type 2 diabetes and obesity. *Clinical Endocrinology, 71*(3), 369-375.

Mu, J., Pinkstaff, J., Li, Z., Skidmore, L., Li, N., & Myler, H. (2012). FGF21 analogs of sustained action enabled by orthogonal biosynthesis demonstrate enhanced antidiabetic pharmacology in rodents. *Diabetes 61*, 505– 512.

Mu, X., Kong, N., Chen, W., Zhang, T., Shen, M., & Yan, W. (2008). High-level expression, purification, and characterization of recombinant human basic fibroblast growth factor in *Pichia pastoris*. *Protein Expression and Purification, 59*(2), 282-288.

Muise, E., Azzolina, B., Kuo, D., El-Sherbeini, M., Tan, Y., Yuan, X., & Wong, K. (2008). Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor γ and altered metabolic states. *Molecular Pharmacology, 74*(2), 403-412.

Myers, R., Chedid, M., Tronick, S., & Chiu, I. (1995). Different fibroblast growth factor 1 (FGF-1) transcripts in neural tissues, glioblastomas and kidney carcinoma cell lines. *Oncogene, 11*(4), 785-789.

Naderali, E., Wong, C., & Hussain, M. (2009). Fibroblast growth factor-21: A link for glucose homeostasis. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews, 3*(4), 237-239.

Nicholes, K., Guillet, S., Tomlinson, E., Hillan, K., Wright, B., Frantz, G., & Stephan, J. (2002). A mouse model of hepatocellular carcinoma: Ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice. *The American Journal of Pathology, 160*(6), 2295-2307.

Nishimura, T., Nakatake, Y., Konishi, M., & Itoh, N. (2000). Identification of a novel FGF, FGF-21, preferentially expressed in the liver. *Gene Structure and Expression, 1492*(1), 203-206.

O'connor, J., Cook, J., Craven, S., Van Pelt, C., & Obourn, J. (1996). An *in vivo* battery for identifying endocrine modulators that are estrogenic or dopamine regulators. *Toxicological Sciences*, *33*(2), 182-195.

Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S., & Itoh, N. (2000). FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochemical and Biophysical Research Communications, 277*(3), 643-649.

Ogawa, Y., Kurosu, H., Yamamoto, M., Nandi, A., Rosenblatt, K., Goetz, R., & Kuro, M. (2007). β-Klotho is required for metabolic activity of fibroblast growth factor 21. *Proceedings of the National Academy of Sciences, 104*(18), 7432-7437.

Ornitz, D., & Itoh, N. (2001). Fibroblast growth factors. *Genome Biology*, *2*(3), 1-12.

Ornitz, D., & Itoh, N. (2015). The fibroblast growth factor signaling pathway. *Developmental Biology, 4*(3), 215-266.

Oron, G., Fisch, B., Zhang, X., Gabbay-Benziv, R., Kessler-Icekson, G., Krissi, H., & Abir, R. (2012). Fibroblast growth factor 10 in human ovaries. *Reproductive Biomedicine Online, 25*(4), 396-401.

Page, R., Moy, K., Sims, E., Velasquez, J., McManus, B., Grittini, C., & Stevens, R. (2004). Scalable high-throughput micro-expression device for recombinant proteins. *Biotechniques*, *37*, 364-369.

Pagliassotti, M., Prach, P., Koppenhafer, T., & Pan, D. (1996). Changes in insulin action, triglycerides, and lipid composition during sucrose feeding in rats. *American Journal of Physiology-Regulatory, 271*(5), R1319-R1326.

Pagliassotti, M., & Prach, P. (1995). Quantity of sucrose alters the tissue pattern and time course of insulin resistance in young rats. *American Journal of Physiology-Regulatory, 269*(3), R641-R646.

Pagliassotti, M., Shahrokhi, K., & Moscarello, M. (1994). Involvement of liver and skeletal muscle in sucrose-induced insulin resistance: Dose-response studies. *American Journal of Physiology-Regulatory, 266*(5), R1637-R1644.

Pan, Q., Ren, Y., Zhu, J., Hu, Y., Zheng, J., Fan, H., & Liu, W. (2014). Resveratrol increases nephrin and podocin expression and alleviates renal damage in rats fed a high-fat diet. *Nutrients*, *6*(7), 2619-2631.

Pelech, S., Olwin, B., & Krebs, E. (1986). Fibroblast growth factor treatment of Swiss 3T3 cells activates a subunit S6 kinase that phosphorylates a synthetic peptide substrate. *Proceedings of the National Academy of Sciences, 83*(16), 5968-5972.

Peti, W., & Page, R. (2007). Strategies to maximize heterologous protein expression in Escherichia coli with minimal cost. *Protein Expression and Purification*, *51*(1), 1-10.

Potthoff, M., Inagaki, T., Satapati, S., Ding, X., He, T., Goetz, R., & Kliewer, S. (2009). FGF21 induces PGC-1α and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. *Proceedings of the National Academy of Sciences, 106*(26), 10853-10858.

Powers, C., McLeskey, S., & Wellstein, A. (2000). Fibroblast growth factors, their receptors and signaling. *Endocrine-related Cancer, 7*(3), 165-197.

Primrose, S., Old, R., & Twyman, R. (2001). *Principles of gene manipulation: An introduction to genetic engineering*. New Jersey: Blackwell Science.

Puigserver, P., & Spiegelman, B. (2003). Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α): Transcriptional coactivator and metabolic regulator. *Endocrine Reviews*, *24*(1), 78-90.

Reel, J., Lamb, J., & Neal, B. (1996). Survey and assessment of mammalian estrogen biological assays for hazard characterization. *Toxicological Sciences*, *34*(2), 288-305.

Remmers, E., Sano, H., Lafyatis, R., Case, J., Kumkumian, G., Hla, T., & Wilder, R. (1991). Production of platelet derived growth factor B chain (PDGF-B/c-sis) mRNA and immunoreactive PDGF B-like polypeptide by rheumatoid synovium: Coexpression with heparin binding acidic fibroblast growth factor-1. *The Journal of Rheumatology*, *18*(1), 7-13.

Reuss, B., & Halbach, O. (2003). Fibroblast growth factors and their receptors in the central nervous system. *Cell and Tissue Research,313*(2),139-157.

Rhee, J., Inoue, Y., Yoon, J., Puigserver, P., Fan, M., Gonzalez, F., & Spiegelman, B. (2003). Regulation of hepatic fasting response by PPARγ coactivator-1α (PGC-1): Requirement for hepatocyte nuclear factor 4α in gluconeogenesis. *Proceedings of the National Academy of Sciences, 100*(7), 4012-4017.

Romanos, M. (1995). Advances in the use of *Pichia pastoris* for high-level gene expression. *Current Opinion Biotechnol*ogy *6*,527-533.

Rudolph, R. (1996) *Protein Engineering: Principles and practice*. New York: Wiley-Liss.

Rukin, B., Pikielny, C., Rosbash, M., & Green, M. (1986). Alternative branch points are selected during splicing of a yeast pre-mRNA in mammalian and yeast extracts. *Proceedings of the National Academy of Science, 83*,2022-2026.

Sadhukhan, R., & Sen, I. (1996). Different glycosylation requirements for the synthesis of enzymatically active angiotensin-converting enzyme in mammalian cells and yeast. *Journal of Biology Chemistry*, *271*,6429-6434.

Sakaue, H., Konishi, M., Ogawa, W., Asaki, T., Mori, T., Yamasaki, M., & Kasuga, M. (2002). Requirement of fibroblast growth factor 10 in development of white adipose tissue. *Genes & Development, 16*(8), 908-912.

Sambrook, J., & Russell, D. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). New York: Coldpring Harbor Laboratory Press.

Sano, H., Engleka, K., Mathern, P., Hla, T., Crofford, L., Remmers, E., & Wilder, R. (1993). Coexpression of phosphotyrosine-containing proteins, platelet-derived growth factor-B, and fibroblast growth factor-1 *in situ* in synovial tissues of patients with rheumatoid arthritis and Lewis rats with adjuvant or streptococcal cell wall arthritis. *Journal of Clinical Investigation*, *91*(2), 553.

Sano, H., Forough, R., Maier, J., Case, J., Jackson, A., Engleka, K., & Wilder, R. (1990). Detection of high levels of heparin binding growth factor-1 (acidic fibroblast growth factor) in inflammatory arthritic joints. *The Journal of Cell Biology, 110*(4), 1417-1426.

Santos, J., Menezes, V., Barberino, R., Macedo, T., Lins, T., Gouveia, B., & Matos, M. (2014). Immunohistochemical localization of fibroblast growth factor 2 in the sheep ovary and its effects on pre-antral follicle apoptosis and development *in Vitro*. *Reproduction in Domestic Animals, 49*(3), 522-528.

Sarruf, D., Thaler, J., Morton, G., German, J., Fischer, J., Ogimoto, K., & Schwartz, M. (2010). Fibroblast growth factor 21 action in the brain increases energy expenditure and insulin sensitivity in obese rats. *Diabetes*, *59*(7), 1817-1824.

Schwartz, S., & Liaw, L. (1993). Growth control and morphogenesis in the development and pathology of arteries. *Journal of Cardiovascular Pharmacology, 21*, S31-S49.

Scott, A., & Robert, S. (2006) Survival analysis. *Gastroenterology & Hepatology*, *2*(5), 380–383.

Shaw, J., Sicree, R., & Zimmet, P. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice, 87*(1), 4-14.

Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S., & Hegedus, D. (2003). Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*. *Protein Expression and Purification*, *28*(2), 321-330.

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., & Yamashita, T. (2004). FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. *Journal of Bone and Mineral Research, 19*(3), 429-435.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., & Klagsbrun, M. (1984). Heparin affinity: Purification of a tumor-derived capillary endothelial cell growth factor. *Science, 223*(4642), 1296-1299.

Singh, S., & Panda, A. (2005). Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering*, *99*(4), 303-310.

Sirén, N., Weegar, J., Dahibacka, J., Kalkkinen, N., Fagervik, K., Leisola, M., & von, N. (2006). Production of recombinant HIV-1 Nef (negative factor) protein using *Pichia pastoris* and a low-temperature fed-batch strategy. *Biotechnology Applied Biochemistry, 44*,151-158.

Smallwood, P., Munoz-Sanjuan, I., Tong, P., Macke, J., Hendry, S., Gilbert, D., & Nathans, J. (1996). Fibroblast growth factor (FGF) homologous factors: New members of the FGF family implicated in nervous system development. *Proceedings of the National Academy of Sciences, 93*(18), 9850-9857.

Soden, D., Ocallaghan, J., & Dobson, A. (2002). Molecular cloning of a laccase isozyme gene from Pleurotus sajor-caju and expression in the heterologous *Pichia pastoris* host. *Microbiology 148*, 4003-4014.

Storlien, L., Kraegen, E., Jenkins, A., & Chisholm, D. (1988). Effects of sucrose vs. starch diets on *in vivo* insulin action, thermogenesis, and obesity in rats. *The American Journal of Clinical Nutrition*, *47*(3), 420-427.

Studier, F., & Moffatt, B. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology*, *189*(1): 113-130.

Suckow, M., Weisbroth, S., & Franklin, C. (2005). *The laboratory rat* (2nd Ed.). Toronto: Academic Press.

Sun, P., & Waugh, D. (2011). Enhancing the solubility of recombinant proteins in Escher-ichia coli by using hexahistidine-tagged maltose-binding protein as a fusion partner. *Methods Molecular Biology, 705*,259-274.

Suzuki, M., Uehara, Y., Motomura-Matsuzaka, K., Oki, J., Koyama, Y., Kimura, M., & Imamura, T. (2008). βKlotho is required for fibroblast growth factor (FGF) 21 signaling through FGF receptor (FGFR) 1c and FGFR3c. *Molecular Endocrinology, 22*(4), 1006-1014.

Suzuki, N., Rubin, D., Lidman, C., Aldering, G., Amanullah, R., Barbary, K., & Dawson, K. (2012). The Hubble Space Telescope Cluster Supernova Survey. V. Improving the dark-energy constraints above z> 1 and building an early-type-hosted supernova sample. *The Astrophysical Journal*, *746*(1), 85.

Syazana, D., & kamaruddin, K. (2011). Effects of Gelam honey on sperm quality and testis of rat. *Sains Malaysia*, *40*(11), 1243-1246.

Szebenyi, G., & Fallon, J. (1998). Fibroblast growth factors as multifunctional signaling factors. *International Review of Cytology, 185*, 45-106.

Tagashira, S., Harada, H., Katsumata, T., Itoh, N., & Nakatsuka, M. (1997). Cloning of mouse FGF10 and up-regulation of its gene expression during wound healing. *Gene, 197*(1), 399-404.

Takahashi, J., Mori, H., Fukumoto, M., Igarashi, K., Jaye, M., Oda, Y., & Hatanaka, M. (1990). Gene expression of fibroblast growth factors in human gliomas and meningiomas: Demonstration of cellular source of basic fibroblast growth factor mRNA and peptide in tumor tissues. *Proceedings of the National Academy of Sciences, 87*(15), 5710-5714.

Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H., & Matsumoto, K. (1992). Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. *Proceedings of the National Academy of Sciences*, *89*(19), 8928-8932.

Terpe, K. (2006). Overview of bacterial expression systems for heterologous protein production: From molecular and biochemical fundamentals to commercial systems. *Applied Microbiology Biotechnology, 72*,211-222.

Thissen, J., Underwood, L., & Ketelslegers, J. (1999). Regulation of insulin-like growth factor–I in starvation and injury. *Nutrition Reviews, 57*(6), 167-176.

Tomlinson, E., Fu, L., John, L., Hultgren, B., Huang, X., Renz, M., & French, D. (2002). Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology, 143*(5), 1741-1747.

Unsicker, K., Engels, S., Hamm, C., Lüdecke, G., Meier, C., Renzing, J., & Flanders, K. (1992). Molecular control of neural plasticity by the multifunctional growth factor families of the FGFs and TGF-βs. *Annals of Anatomy-Anatomischer Anzeiger, 174*(5), 405-407.

Urban, A., Neukirchen, S., & Jaeger, K. (1997). A rapid and efficient method for site-directed mutagenesis using one-step overlap extension PCR. *Nucleic Acids Research*, *25*, 2227-8.

Vanderwalt, J., Noureddine, M., Kittappa, R., Hauser, M., Scott, W., Mckay, R., & Scott, B. (2004). Fibroblast growth factor 20 polymorphisms and haplotypes strongly influence risk of Parkinson disease. *The American Journal of Human Genetics, 74*(6), 1121-1127.

Vandisselemiliani, F., Deboerbrouwer, M., & Derooij, D. (1996). Effect of fibroblast growth factor-2 on Sertoli cells and gonocytes in coculture during the perinatal period. *Endocrinology, 137*(2), 647-654.

Vernet, E., Kotzsch, A., Voldborg, B., & Sundström, M. (2011). Screening of genetic parameters for soluble protein expression in Escherichia coli. *Protein Expression and Purification*, *77*(1), 104-111.

Wang, H., Xiao, Y., Fu, L., Zhao, H., Zhang, Y., & Wan, X. (2010) High-level expression and purification of soluble recombinant FGF21 protein by SUMO fusion in *Escherichia coli*. *BMC Biotechnology, 10*,14.

Wang, Y., Yuan, S., Wang, P., Liu, X., Zhan, D., & Zhang, Z. (2007). Expression, purification, and characterization of recombinant human keratinocyte growth factor-2 in *Pichia pastoris*. *Journal of Biotechnology*, *132*(1), 44-48.

Wente, W., Efanov, A., Brenner, M., Kharitonenkov, A., Köster, A., Sandusky, G., & Gromada, J. (2006). Fibroblast growth factor-21 improves pancreatic β-cell function and survival by activation of extracellular signal–regulated kinase 1/2 and Akt signaling pathways. *Diabetes, 55*(9), 2470-2478.

Wu, J., Lee, C., & Hsu, T. (2002). Asparagine-linked glycosylational modifications in yeast. *Cell Engineering*, *3*,215-232.

Xu, J., Lloyd, D., Hale, C., Stanislaus, S., Chen, M., Sivits, G., & Lindberg, R. (2009). Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. *Diabetes, 58*(1), 250-259.

Xu, J., Stanislaus, S., Chinookoswong, N., Lau, Y., Hager, T., Patel, J., & Graham, M. (2009). Acute glucose-lowering and insulin-sensitizing action of FGF21 in insulin-resistant mouse models—association with liver and adipose tissue effects.

American Journal of Physiology-Endocrinology and Metabolism, *297*(5), E1105-E1114.

Yamagata, H., Chen, Y., Akatsu, H., Kamino, K., Ito, J., Yokoyama, S., & Kondo, I. (2004). Promoter polymorphism in fibroblast growth factor 1 gene increases risk of definite Alzheimer's disease. *Biochemical and biophysical Research Communications, 321*(2), 320-323.

Yamamoto, S., Mikami, T., Konishi, M., & Itoh, N. (2000). Stage-specific expression of a novel isoform of mouse FGF-14 (FHF-4) in spermatocytes. *Gene Structure and Expression, 1490*(1), 121-124.

Yie, J., Hecht, R., Patel, J., Stevens, J., Wang, W., Hawkins, N., & Fisher, S. (2009). FGF21 N-and C-termini play different roles in receptor interaction and activation. *FEBS Letters, 583*(1), 19-24.

Yoon, J., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., & Granner, D. (2001). Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature*, *413*(6852), 131-138.

Yoshida, T., Sakamoto, H., Miyagawa, K., Sugimura, T., & Terada, M. (1991). Characterization of the hst-1 gene and its product. *Annals of the New York Academy of Sciences*, *638*(1), 27-37.

Yuan, Y., & Foley, G. (2002). Female reproductive system. *Handbook of Toxicologic Pathology*, *2*, 847-894.

Yuan, H., Corbi, N., Basilico, C., & Dailey, L. (1995). Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes & Development*, *9*(21), 2635-2645.

Zhang Y., Xie Y., Berglund, E., Coate, K., & He. T. (2012). The starvation hormone, fibroblast growth factor-21, extends lifespan in mice. *eLife*, *1*, e00065.

Zhang, X., Yeung, D., Karpisek, M., Stejskal, D., Zhou, Z., Liu, F., & Lam, K. (2008). Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. *Diabetes*, *57*(5), 1246-1253.

Zhang, X., Ibrahimi, O., Olsen, S., Umemori, H., Mohammadi, M., & Ornitz, D. (2006). Receptor specificity of the fibroblast growth factor family, the complete mammalian fgf family. *Journal of Biological Chemistry, 281*(23), 15694-15700.