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Purification and extraction of fibroblast growth factor 21 (FGF-21) protein by sumo fusion in *Escherichia coli.*

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Abstract: Fibroblast growth factor 21 has recently discovered its pivotal role in glucose, lipid metabolism and regulation of energy homeostasis. Further, it has helped in forming great strides for treatment of chronic diseases like diabetes and inflammation. FGF-21 was sub-cloned into the SUMO vector and was induced for expression in *Escherichia coli* Rosetta. The recombinant plasmid was transformed into *Escherichia coli* strain. FGF-21 was induced by IPTG and purified by Ni-NTA agarose (Nickel-nitrilotriacetic acid) column. The purified fusion protein was cleavaged by SUMO protease I to obtain recombinant FGF-21 with high purity. The purified protein was tested for its biological activity of FGF-21. HepG2 cell model was used to detect the regulation of glucose uptake activity of FGF-21 and were further treated with different concentrations of FGF-21.The residual glucose content in medium was measured using the glucose oxidase-peroxidase method. The results indicated that FGF-21 protein had a role in regulating the glucose uptake on HepG2 cells and the effect was significantly dose-dependent manner. In order to further verify whether purified FGF-21 protein obtained has biological activity in diabetic model. Studies have demonstrated that FGF-21 had a greater efficacy in dropping blood glucose in streptozotocin induced diabetic mice.

Keywords: FGF21, SUMO, HepG2, glucose oxidase-peroxidase (GOD-POD).

INTRODUCTION

Fibroblast growth factor has attracted substantial importance in these years specifically with regard to its growing role in the intermediary metabolism involving the utilization of glucose and lipids. The fibroblast growth factor family comprise of 23 members with broad series of biological functions which include angiogenesis, cell growth, wound healing and metabolism (Galzie *et al*., 1997, Itoh and Ornitz, 2004, Khoso *et al*., 2018). FGF-21 is a metabolic hormone primarily produced by the liver. It is also expressed in adipocytes and pancreas which play role in regulating endocrine, lipid metabolism, glucose metabolism, anti-oxidation and anti-inflammation (Kharitonenkov *et al*., 2005, Kharitonenkov *et al*., 2007, Kralisch and Fasshauer, 2011, Nishimura *et al*., 2000, Opoku *et al*., 2019). Moreover, it has predicted that it may become a candidate for treatment-related metabolic diseases drug (Amacher, 2014) (Kharitonenkov and Shanafelt, 2009).

Fibroblast growth factor 21 has exposed great perspective for clinical treatment due to the discovery of more biological functions. FGF21 has been newly measured as a metabolic hormone regulated by dietary eminence with various positive effects on lipid metabolism and glucose homeostasis reducing body weight, improving insulin sensitivity, and accelerating body insulin resistance. In fact, FGF-21 had enhanced insulin sensitivity, sugar and lipid metabolism and retained b-cell functions in diabetic mice models (Kharitonenkov *et al*., 2005, Kralisch and Fasshauer, 2011, Sarruf *et al*., 2010, Wente *et al*., 2006). Further, FGF-21 has the treatment of alcoholic and non-alcoholic fatty liver through anti-inflammatory treatment of rheumatoid arthritis, anti-oxidative stress to inhibit cell apoptosis after apoptosis, relieve senescence, cerebrovascular disease and fibrosis prevention and treatment of liver cancer and other biological functions (Tezze *et al*., 2019). Therefore, studies on FGF21 will help to develop new drugs for broad treatment of multiple diseases. Besides, unlike most members of the FGF cluster, Fibroblast growth factor 21 is free of tumorigenic and proliferating effects (Huang *et al*., 2006, Wente *et al*., 2006). Research illustrated that its biological activity does not depend on heparin as it is mostly involved in the regulation of lipid and glucose metabolism in the body which improve insulin resistance and insulin sensitivity, lower blood sugar without causing hypoglycemia. Further,

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it was discovered that the body in fibroblast growth factor-21 expression in the liver was significantly increased in starvation and ketogenic diets. Fibroblast growth factor-21 was significantly attenuated by exogenous injection of FGF-21 protein in T2 diabetic mice and their insulin resistance was improved. Therefore, FGF-21 is probably to become a novel drug for the treatment of type 2 diabetes. However, FG-F-21 shows potential as an innovator disease revising treatment for type 2 diabetes and linked metabolic disorders. Recently, Fibroblast growth factor-21 has been depicted as a potential novel drug candidate against metabolic syndromes. However, the production of FGF-21 by conventional methods, like plasmid recombination in Escherichia coli, produces unsatisfactory results. The experiments by Kharitonenkov showed that recombinant FGF21 formed and accumulates in inclusion bodies of transformed Escherichia coli.

Some researchers have also shown that the fibroblast growth factor-21 without fusion expression is more likely to form inclusion in bodies making it hard to produce bioactive protein. Meanwhile the inclusion bodies require denaturation, annealing and purification through several chromatography columns. SUMO is a small molecule ubiquitin-like modified protein, which is widely distributed in various eukaryotic cells (Johnson, 2004). SUMOylation has been shown to be involved in regulating various physiological processes such as apoptosis, stress response, protein stability, protein activation, cell cycle progression, signal transduction, RNA transcription and nuclear transport of proteins as a reversible post translational modification process (Zhou *et al*., 2005).

In recent years, it has been found that SUMO can be used as a fusion tag and a molecular chaperone for recombinant protein expression as it has anti-protease hydrolysis, a significant increase in recombinant protein expression, and promotion of correct folding of target proteins and improving solubility (Butt *et al*., 2005, Zuo *et al*., 2005a). The small molecule ubiquitin-like modified protein (SUMO) and ubiquitin (Ub) have only 18% homology in primary structure. However, the tertiary structure and biological functions are very parallel (Malakhov *et al*., 2004). Studies have shown that its use as a fusion tag of recombinant proteins can increase protein stability.

In this study, the pSUMO expression system was used to efficient, stable and soluble expression of a human FGF-21 fusion protein. Moreover, human FGF-21 with higher purity can be obtained by cleavage with SUMO protease I as no amino acid residue remains. The purified recombinant human FGF-21 was identified by the glucose oxidase-peroxidase (GOD-POD) method in the HepG2 cell model, The results showed that the purified recombinant human FGF-21 had a dose-dependent activity to promote glucose uptake in adipocytes (Xu *et al*., 2016b).

MATERIALS AND METHODS

Plasmids and bacterial strains

The SUMO-FGF21 expression vector was constructed in our Biopharmaceutical Lab. *Escherichia coli* Rosetta (DE3) plysS cells strains bought from TaKaRa Company. Primer synthesis were from Invitrogen Company; HepG2 cell line were grown at 37ºC under 5% CO2. HepG2 cells were preserved in Dulbecco modified Eagle medium (DMEM; GIBCO "Grand Island, New York, USA") accompanied with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Glucose test kit was purchased from Sichuan Mike Technology Co., Ltd. Restriction enzyme was purchased from NEB Company. IPTG (isopropylthio-β-D-galactoside), lysozyme, and ampicillin (Amp) were purchased from TaKaRa Company. Ni-NTA agarose granules were purchased from QIAGEN Company.

Experimental mice and cells

The 6 week's old db/db mice weighed about 30g were purchased from Changchun Yisi Laboratory Animal Co. Ltd. (Changchun, Jilin, PR China). HepG2 cells were provided by the Biopharmaceutical Research lab.

Expression and purification of human fibroblast growth factor-21

The recombinant hFGF-21 was expressed in *Escherichia coli* Rosetta (DE3) and transformed with the Small ubiquitin-related modifier (SUMO)-hFGF-21 plasmid. The expression strain Rosetta was used to inoculate *Escherichia coli* bacteria solution, inoculated on the surface of Luria-Bertani solid medium with ampicillin 100 g/mL (Sigma, St. Louis, MO, USA) and incubated in a shaking incubator at 37°C until the cell density OD-600 value was reached 0.4 to 0.6, then IPTG (Dingguo Bio Inc, Beijing, China) was added to a final concentration of 0.25 mmol/L, and finally incubated at 30ºC for 4 to 6 hours. The collected bacterial cells were suspended in lysis buffer, subsequently, the bacteria cell were interrupted by sonication methods and centrifuged at 12,000g, 4°C, for 10 to 15 minutes. The supernatant after sonication of the expressed bacteria was subjected to affinity chromatography on a Ni-NTA column, and the eluted first peak was collected to obtain a fusion protein. The expressed fusion protein was cleaved by SUMO protease I, and then subjected to Ni-NTA column affinity chromatography to collect the only elution peak of FGF-21 mature protein, which was detected by 15% SDS-PAGE electrophoresis.

Glucose uptake activity assay of FGF-21 in HepG2 cells

The activity of FGF-21 in cell line experiment, after

measuring the concentration of FGF-21, the FGF-21 protein was diluted in DMEM medium containing 10% NCS and cultured at 37°C, 5% CO2 and saturated humidity. HepG2 cells were seeded into 96-well plates and starving cells were cultured in serum-free DMEM medium for 12h and cells were stimulated with different concentrations (10, 100 and 1000nmol/L) of FGF21 protein for 24h respectively, by adding blank medium hole as Normal control. After 24 hours treatment, the residual glucose content in the medium was measured using the glucose oxidase-peroxidase method.

Two microliters of the culture medium supernatant were added to 200µl of the glucose detection solution. Each well was repeated at least 3 times and after 5 to 10 minutes at 37°C, the OD was measured at a wavelength of 500 nm. Calculate glucose consumption rate and use statistical analysis of experimental results. The residual glucose concentration and cell glucose consumption rate in the culture medium were calculated by the following formula:

Glucose concentration / (mmol / L) = (A sample / A standard) × 5.55

Cell glucose consumption rate % = [(C blank glucose-C administered glucose) / C blank glucose] × 100%

Detection of FGF-21 activity in diabetic model

The activity of purified FGF-21 protein obtained through mice model of type 1 diabetes was established by streptozotocin method. The 6 week's old mice weighed about 30g. After feeding for 3 weeks with high fat and high sugar fasted for 12 hours. Intraperitoneal injection of streptozotocin at a concentration of 30 mg/kg was injected once every 3 days. After injected for thrice i.e. 10 days the fasting blood glucose level in mice was measured.

When the blood glucose level was 11.1 mmol/L, the serum insulin levels were significantly decreased considering as diabetes models. The Diabetes mice were divided into two groups; Diabetic group and FGF-21 treatment group. Each group consists of 10 mice. The treatment group was injected with FGF-21 intraperitoneally at a dose of 1mg/kg for a week; Model group were injected with the same volume of 0.9% normal saline. Blood samples were taken from the tail vein, and the blood glucose was measured before and after injection. The blood glucose level of each mouse was measured every day.

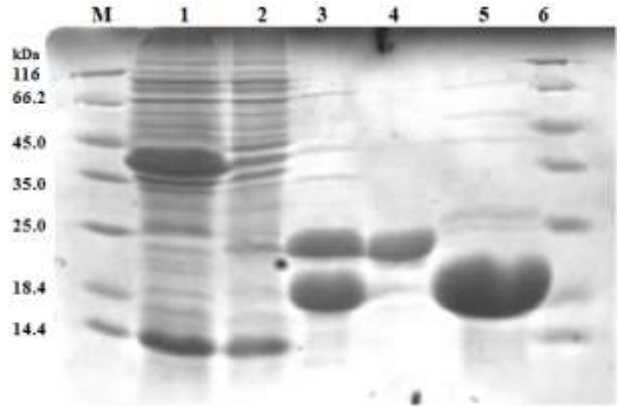
STATISTICAL ANALYSIS

All data analyzed as means ± S.E.M. Mean Value Comparisons between two groups were performed using the Student t test. Statistical Significant was considered at $P<0.05$.

RESULTS

Preparation and expression of fibroblast growth factor-21

The Psumo FGF-21 recombinant plasmid was transformed into Rosetta competent strain, and the plasmid was induced to the culture at 30°C, 80 rpm, add 0.25 mmol/L IPTG for 6 hours. After the expression, the bacterial liquid was collected, ultrasonically disrupted, clarified by hollow fiber column, and purified by Ni column. The protein was analyzed by 15% SDS-PAGE expression. The results showed that size of the fusion protein SUMO-FGF-21 was approximately 40 kDa. Protein is highly expressed in Escherichia coli and ratio of SUMO-FGF21 about 70%. And the target protein was mainly expressed in the supernatant as exposed in fig. 1.



M: Marker; 1: Total Protein; 2: Total Protein without IPTG induction; 3: Protein after purification and Enzyme digestion; 4: Mature FGF21; 5 SUMO; 6: Marker

Fig. 1: SDS-PAGE Electrophoresis analysis of expression of the SUMO-FGF21 protein in *Escherichia coli.*

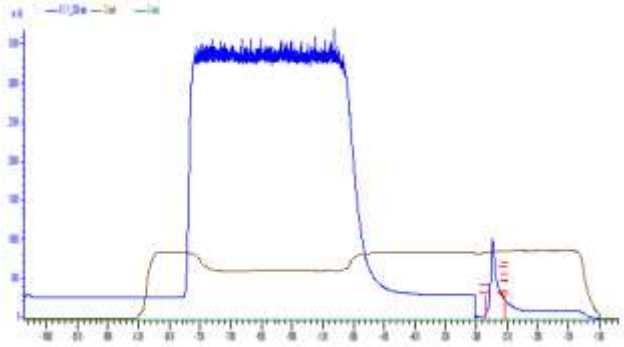
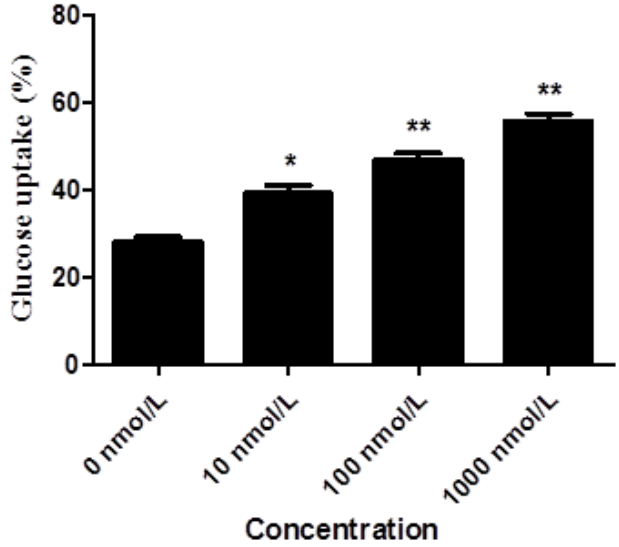


Fig. 2: Purification of FGF-21

Purification of FGF-21

SUMO-FGF-21 was first purified by affinity chromatography to obtain moderately pure SUMO-FGF21. Then SUMO-FGF-21 protein was digested with SUMO protease enzyme overnight at 4°C. After the cleavage was finished, it was exposed to second affinity chromatography. Finally, the FGF-21 mature protein (The molecular weight of mature FGF21 is about 22 kDa) with

high purity was gained. The purity of the FGF-21 protein was exposed in fig. 2.



The glucose uptake activity of HepG2 cells, and HepG2 cells treated with different concentrations of FGF-21 (10, 100 and 1000 nmol/L) for 24 h. The value (±SE) shows an average of three independent measurements. *P<0.05, ** P<0.01 compared with control group (0 nmol/L).

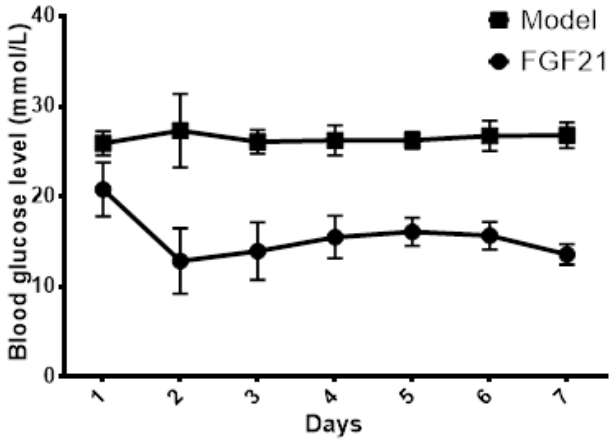
Fig. 3: Effect of FGF-21 on the glucose uptake of HepG2 cells. P<0.05, compared with "0" control group.

Biological activity assay of fibroblast growth factor -21 in HepG2 cells

Glucose absorption test is usually used to check the activity of FGF-21. In this study, HepG2 cells were seeded in 96 well plates incubated for overnight at 37°C with 5% CO2 under saturated moisture condition. In this study, HepG2 cell model was used to detect glucose uptake activity of FGF-21 protein and HepG2 cells treated with different concentrations of FGF-21, 0 nmol/L, 10nmol/L, 100nmol/L and 1000nmol/L respectively. The data compared with the blank control group. After 24 hours stimulation, results showed that the FGF-21 protein significantly stimulated the glucose uptake on HepG2 cells (P<0.05) and the effect was significantly dose-dependent manner. The results are shown in fig. 3.

FGF-21 activity of regulating blood glucose in diabetic mice (in-vivo)

In order to verify whether FGF21 has biological activity, the regulatory effect of FGF21 on blood glucose in streptozotocin (STZ) induced diabetic mice at animal level was studied. Results as shown in fig. 4, Blood glucose level in the treatment group continued to decrease for 7 days which was in sharp difference to the diabetic model group. The blood glucose level of diabetic mice treated with FGF21 was significantly decreased. The difference was significant compared with the model group (P<0.05). The result showed that the prepared Fibroblast growth factor-21 had the activity of regulating the blood glucose in diabetic mice. The results are exposed in fig. 4.



Effect of FGF-21 in diabetic mice model. Diabetic mice were treated with FGF21 at a dose of 1mg/kg. The values (±SE) shown are the average of at least 4 independent measurements.

Fig. 4: FGF-21 regulates blood glucose activity in diabetic mice model

DISCUSSION

Fibroblast growth factor -21 is a member of the FGF family. In recent years, with the in-depth study of the structure and function of fibroblast growth factor-21. FGF-21 is safe, reliable, independent of insulin and glucose regulation and lipid metabolism while improving insulin resistance with no side effects. It has become a potential drug for the treatment of metabolic diseases such as diabetes, obesity and fatty liver (Coskun *et al*., 2008).

Studies have shown that FGF-21 can regulate blood glucose level independently and compared with insulin, fibroblast growth factor-21 has a slow and lasting effect without insulin dependent. Moreover, studies have shown that FGF-21 has the effect of lowering blood triglyceride and cholesterol lipoprotein in regulating lipid metabolism (Xu *et al*., 2016a). It selectively reduces the content of low-density cholesterol lipoproteins and increases the content of high-density cholesterol lipoproteins.

Moreover, experiments have shown that FGF-21 is different from other FGF family members, and that FGF-21 does not have a mitogenic effect and Long-term injections in animals do not cause any side effects (Kharitonenkov and Shanafelt, 2008). In addition, FGF-21 does not cause side effects such as hyperinsulinemia, hypoglycemia, weight gain and edema compared with the main diabetes drugs in the market at present. Further it is acceptable that long-term injection of fibroblast growth factor-21 can also resist diet-induced obesity.

In this experiment, recombinant human FGF-21 was successfully expressed and purified by pSUMO and the naturally active target protein was obtained by cleavage with SUMO protease I. Subsequently SUMO protease I

and SUMO proteins incorporated poly histidine could be bound to Ni-NTA agarose particles so that active recombinant mature proteins may be isolated by Ni-NTA affinity chromatography. In recent years, it has been found that SUMO can be used in molecular chaperones to increase the stability and solubility of foreign proteins. Its mechanism may be that SUMO proteins, as a highly hydrophobic core, it provide nucleation sites (Su *et al*., 2006) for the folding of target proteins, and promote the interaction between proteins and make them correctly folded and ultimately enhance the solubility of fusion proteins (Zuo *et al*., 2005b).

In order to investigate the fusion protein, FGF-21 was successfully expressed by SUMO vector and the purified protein was tested for its biological activity of FGF-21, HepG2 cell model was used we used to detect the regulation of glucose uptake activity of FGF-21 protein, and HepG2 cells treated with different concentrations of FGF-21, 0nmol/L, 10nmol/L, 100nmol/L and 1000 nmol/L respectively. The residual glucose content in the medium was measured using the glucose oxidase-peroxidase method. The results indicated that FGF-21 protein has a role in regulating the glucose uptake on HepG2 cells and the effect was significantly dose-dependent manner. In order to further verify whether purified FGF-21 protein obtained has biological activity in diabetic model, the regulatory effect of FGF21 on blood glucose in streptozotocin (STZ) induced diabetic mice at animal level was also studied. These experimental results laid the foundation for the development of FGF-21 as a therapeutic agent for type 2 diabetes.

REFERENCES

Amacher DE (2014). Progress in the search for circulating biomarkers of nonalcoholic fatty liver disease. *J. Biomark.*, **19**(7): 541-552.

Butt TR, Edavettal SC, Hall JP and Mattern MR (2005). Sumo fusion technology for difficult-to-express proteins. *Protein Expres Purif*, **43**(1): 1-9.

Coskun T, Bina HA, Schneider MA, Dunbar JD, Hu CC, Chen Y, Moller DE and Kharitonenkov A (2008). Fibroblast growth factor 21 corrects obesity in mice. *Endocrin*, **149**(12): 6018-6027.

Galzie Z, Kinsella AR and Smith JA (1997). Fibroblast growth factors and their receptors. *Biochem Cell Biol*, **75**(6): 669-685.

Huang X, Yu C, Jin C, Yang C, Xie R, Cao D, Wang F and Mckeehan WL (2006). Forced expression of hepatocyte-specific fibroblast growth factor 21 delays initiation of chemically induced hepatocarcinogenesis. *Mol. Carcinogen*, **45**(12): 934-942.

Itoh N and Ornitz DM (2004). Evolution of the fgf and fgfr gene families. *Trends Genet*, **20**(11): 563-569.

Johnson ES (2004). Protein modification by sumo. *Annu. Rev. Biochem*, **73**(1): 355-382.

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

Kharitonenkov A and Shanafelt AB (2009). Fgf21: A novel prospect for the treatment of metabolic diseases. *Curr. Opin. Investig. Drugs*, **10**(4): 359-364.

Kharitonenkov A, Shiyanova TL, Koester A, Ford AM, Micanovic R, Galbreath EJ, Sandusky GE, Hammond LJ, Moyers JS and Owens RA (2005). Fgf-21 as a novel metabolic regulator. *J. Clin. Invest.*, **115**(6): 1627-1635.

Kharitonenkov A, Wroblewski VJ, Koester A, Chen YF, Clutinger CK, Tigno XT, Hansen BC, Shanafelt AB and Etgen GJ (2007). The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21. *Endocrin*, **148**(2): 774-781.

Khoso MH, Fanrui M, Rasoul LM, Wang WF, Khoso M A, Opoku YK, Abdelazez A, Hussain S and Li D (2018). The sterategic role of fibroblast growth factor-21in obesity. *Pak. J. Biotechnol.*, **15**(3): 855-864.

Kralisch S and Fasshauer M (2011). Fibroblast growth factor 21: effects on carbohydrate and lipid metabolism in health and disease. *Curr. Opin. Clin. Nutr. Metab. Care*, **14**(4): 354-359.

Malakhov MP, Mattern MR, Malakhova OA, Drinker M, Weeks SD and Butt TR (2004). Sumo fusions and sumo-specific protease for efficient expression and purification of proteins. *J. Struct. Funct. Genomics*, **5**(1-2): 75-86.

Nishimura T, Nakatake Y, Konishi M and Itoh N (2000). Identification of a novel fgf, fgf-21, preferentially expressed in the liver. *Biochim. Biophys. Acta Gene Struc. Expression*, **1492**: 203-206.

Opoku YK, Liu Z, Afrifa J, Khoso MH, Ren G and Li D (2020). Therapeutic role of fibroblast growth factor 21 (fgf21) in the amelioration of chronic diseases. *Int J Pept Res Ther.*, **26**: **1**07-119.

Sarruf DA, Thaler JP, Morton GJ, German J, Fischer JD, Ogimoto K and Schwartz MW (2010). Fibroblast growth factor 21 action in the brain increases energy expenditure and insulin sensitivity in obese rats. *Diabetes*, **59**(7): 1817-1824.

Su Z, Huang Y, Zhou Q, Wu Z, Wu X, Zheng Q, Ding C and Li X (2006). High-level expression and purification of human epidermal growth factor with sumo fusion in *Escherichia coli*. *Protein Peptide Lett*, **13**(8): 785-792.

Wente W, Efanov AM, Brenner M, Kharitonenkov A, Koster A, Sandusky GE, Sewing S, Treinies I, Zitzer H and 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.

Xu P, Zhang Y, Jiang X, Li J, Song L, Khoso MH, Liu Y, Wu Q, Ren G and Li D (2016a). Canine fibroblast growth factor 21 ameliorates hyperglycemia associated

with inhibiting hepatic gluconeogenesis and improving pancreatic beta-cell survival in diabetic mice and dogs. *Plos One*, **11**(5): E0155598.

Xu P, Zhang Y, Song L, Khoso MH, Li J, Jiang X, He J, Li J, Ma X and Ren G (2016b). Efficacy of a combination of high and low dosage of pegylated fgf-21 in treatment of diabetes in db/db mice. *Biomed. Pharmacother.*, **84**(1): 97-105.

Zhou F, Xue Y, Lu H, Chen G and Yao X (2005). A genome‐wide analysis of sumoylation - related biological processes and functions in human nucleus. *Febs Letters*, **579**(16): 3369-3375.

Zuo X, Li S, Hall J, Mattern MR, Tran H, Shoo J, Tan R, Weiss SR and Butt TR (2005a). Enhanced expression and purification of membrane proteins by sumo fusion in *Escherichia coli*. *J. Struct. Funct. Genomics*, 6**,** 103-111.

Zuo X, Mattern MR, Tan R, Li S, Hall J, Sterner DE, Shoo J, Tran H, Lim P and Sarafianos SG (2005b). Expression and purification of sars coronavirus proteins using sumo-fusions. *Protein Expres Purif*, **42**(2-3): 100-110.

Tezze C, Romanello V and Sandri M (2019). Fgf21 as modulator of metabolism in health and disease. *Front Physiol.*, **10**(1): 419.