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AMPLIFICATION, CLONING AND EXPRESSION OF THE REG3 Δ GENE FROM MOUSE PANCREAS

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ABSTRACT

Introduction: Reg proteins are a group of regenerating proteins which are implicated in the pancreas developmental biology. The aim of the study was to explore suitable conditions for cloning and expression of this recombinant protein that can ultimately be explored in further studies for testing its potential as a promising therapeutic for diabetes by inducing regeneration/neogenesis of pancreatic beta cells. *Methodology*: Total RNA was isolated from BALB/C mouse pancreas, cDNA was synthesized and Reg3 δ gene was amplified with gene specific primers. A recombinant plasmid (pET28a vector) was constructed with Reg3 δ gene. Recombinant protein was expressed in BL21 (DE3) strain in LB media. Expressed protein was isolated and separated on 12% SDS-PAGE. *Results:* Clones were confirmed with restriction and colony PCR and SDS-PAGE confirmed the 16 kDa band in IPTG induced samples. Further work for the purification of this protein will be pursued in future.

Keywords: Reg proteins, cloning, protein isolation, Reg3 δ, INGAP-rP, pancreas, mouse.

INTRODUCTION

Regenerating gene family is the member of C-type lectin superfamily of proteins, which is involved in the liver, pancreatic, gastric and intestinal cell proliferation and differentiation. All members of this conserved protein family share some structural and functional properties involved in injury, inflammation, diabetes and carcinogenesis. It includes small secretory proteins, which can function as acute phase reactants, lectins, anti-apoptotic factors or growth factors for pancreatic β-cells, neural cells and epithelial cells in the digestive system (Liu *et al.*, 2008). All family members share some common features like sequence homology, tissue expression profiles and exon-intron junction genomic organization (Zhang *et al.*, 2003).

Considerable research has been done on Reg family and its structurally related molecules as these proteins are involved in diseases etiology in mammals including human. Over the last one and half decade, 17 members of the Reg family have been cloned and sequenced.

Reg and related genes from human and rodents are classified into 1, 2, 3 sub-classes (Okamoto 1999). The mouse Reg gene family was mapped on chromosome 6, contiguous 75 kb region, including Reg1, Reg2, Reg3 α, Reg3 β, Reg3 γ, and Reg3 δ. Reg3 δ was expressed predominantly in the exocrine pancreas, whereas both Reg1 and Reg2 were expressed in hyperplastic islets and Reg3 α, Reg3 β and Reg3 γ were expressed strongly in the intestinal tract and weakly in the pancreas (Parikh *et al.*, 2012). In the context of the current study, Reg 3 related studies are further highlighted.

Reg3 δ protein is an important member of Reg3 family as its hamster counterpart known as "Islet Neogenesis Associated Protein, INGAP" is involved in pancreatic β-cell neogenesis and cellular growth. Protein product of INGAP is constituent of ilotropin (a protein lysate of regenerating pancreata) (Rafaeloff *et al.*, 1997). A 15 amino acid long bioactive peptide of INGAP is considered to hold the same function as of full length protein and reverses diabetes and improves glucose homeostasis (Rosenberg *et al.*, 2004). INGAP is a product of novel gene expressed in regenerating pancreas of hamster and reduces mortality by 50% in Streptozotocin (STZ) induced diabetic mouse (Rafaeloff *et al.*, 1997). There is an extensive research being done on the INGAP and its potential as therapeutic agent (Rafaeloff *et al.*, 1997; Barbosa *et al.* 2006; Dungan *et al.*, 2009), transgenic mice with over expression of INGAP (Taylor-Fishwick *et al.* 2006), its genomic expression (Taylor-Fishwick *et al.* 2003) and islet specific expression pattern (Taylor-Fishwick *et al.*, 2008). However, its mouse counterpart known as the Reg3 δ protein is a rather less explored member (Kulis and Shuker 2006). Reg3 δ protein bears 72% homology with amino acid sequence of hamster INGAP. The

potential of Reg3 protein as a therapeutic agent for the regeneration of β-cells for diabetes mellitus is needed to be explored.

As diabetes mellitus is a complex disease and in spite of its earlier discovery there is no stable therapy or cure developed for it so far. Hence, many therapeutic agents are being tested for their potential to treat diabetes in stable way. In this regard, plant extracts like *Cinnamonum zeylanicum*, A*llium sativum* and Z*ingiber Officinale* (Rind et al., 2010; Rind et al., 2013) have been recently reported with some good results. Some other herbal compounds like Stevioside have also been studied as potential therapeutic for diabetes (Jehan and Ashoush 2008). On the other hand, Reg3 δ is of animal origin but being the member of superfamily of Reg proteins holds an important place to be considered as a therapeutic agent for diabetes as it can enhance regeneration or neo-genesis of insulin producing cells.

Owing to the importance of this protein as a close relative of INGAP protein and to analyze its potential, we wanted to express this protein with recombinant technology. Here, in this paper, we summarize our experimental approach for the cloning and expression of this protein and discuss the issues involved for reliable expression of such protein in a developing country laboratory with interest in diabetes biology and recombinant DNA technology.

Materials and Methods

Animal Handling and Tissue Harvesting: A colony of Balb/C mice (8 weeks old) was procured from the National Institute for Health (NIH), Islamabad and maintained at the animal facility of NIBGE. These mice were maintained on commercial animal diet and free access to drinking water was provided. Pancreas tissues were harvested from 5 mice after euthanizing by neck dislocation and immediately removed pancreas tissue within one minute of neck dislocation. All animal related procedures were followed according to the ethics committee of our institute.

RNA Isolation and cDNA Synthesis: Tissues were immediately frozen in the liquid Nitrogen then crushed in sterilized mortar and pestle following the homogenization in the Trizol solution. Total RNA isolation was carried out from these homogenized pancreatic tissues as described earlier (Pittenger *et al.* 2007). cDNA was synthesized with Revert Aid first strand cDNA synthesis kit following the manufacturer's instructions.

PCR Amplification: Reg3 δ gene was amplified by Polymerase Chain Reaction (PCR) without putative signal peptide using the following primers resulting in the product of 474 bp. *Forward primer:* 5' CCGGATCCTTGGGTACA AGGGGAAC 3' R*everse primer:* 5' CTGAGC TCGAATCCTTAGGCCAGAGTC 3' PCR was performed on compact 48-well MJ Mini™ thermal cycler (Bio-Rad, USA). cDNA was denatured for 5 minutes at 94˚C followed by 30 cycles of further denaturation at 94˚C for 45 seconds, annealing at 55˚C for 45 seconds and extension at 72˚C for 45 seconds. A final extension of 72 ˚C was also carried out for 5 minutes after 30 cycles, Taq DNA polymerase (thermo scientific, USA) was used. Amplified DNA product was analyzed on 1% agarose gel in TAE buffer.

TA Cloning: Amplified Reg3δ gene was ligated in pTZ57R/T vector and cloned using the InsTA-clone PCR Cloning Kit by (Thermo scientific, USA). Blue/white selection method was used, white colonies were picked and inoculated in the LB media. Plasmids were isolated by phenol-chloroform method and sent for the sequencing.

Reg3 δ Expression Plasmid Construct: Expression construct was prepared by ligating the Reg3 δ gene into an expression vector, pET 28a. Following methodology was used for this purpose.

*Restriction Analysis***:** A double digest restriction was performed with *Nde* I and *Bam*H I restriction enzymes on pTZ57R/T-Reg3δ clones as primers for Reg3 δ gene have restriction sites for these enzymes and on pET 28a vector. Both reaction products were analyzed on 1% agarose gel and extracted with GeneJET Gel Extraction Kit by Thermo Scientific.

Ligation*:* Purified PCR product with sticky ends was ligated with the restricted and dephospho-rylated pET 28a vector with T4 DNA ligase (Thermo Scientific,USA) in the presence of ATP.

Expression of Reg3 δ protein: Ligated vectors were transformed into BL21 (DE3) strain and plated on LB media supplemented with Kana-mycin and chloramphenicol (30 and 35 g/mL, respectively). Three colonies were picked and grown in separate flasks containing 50 mL LB broth with Kanamycin and chloramphenicol. Bacterial growth was carried out at 37˚C to get the 0.6 absorbance at 600 nm in spectrophoto-meter and added the 1mM final concentration of the IPTG. Cells were incubated at 28˚C for 20 hours to produce Reg3 δ protein. Cells were centrifuged at 5000rpm at 4˚C and resuspended in the denaturing buffer [(100mM NaCl, 25mM Tris HCl (pH 8), 0.02% NaN_3 (azide), and 0.2mg/ml

Lysozyme)]. For this purpose, 50 mL cell culture pellets were resuspended in 5mL denaturing buffer. The resuspended cells were lysed by sonication and left on ice for 2-3 hours to allow the extraction of protein. To separate out the cellular debris from the protein the lysate was centrifuged at 14000 rpm. The supernatant was detected by SDS-PAGE.

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel was prepared with the 12% concentration of acrylamide (29:1 Acrylamide: Bisacrylamide) with 10% SDS and 10% APS. Samples were prepared by heating in the sample loading Laemmli buffer (Laemmli 1970) with 2% β-mercaptoethanol and bromophenol blue dye, Gel was run in the glycine buffer on 40 mA current.

RESULTS

Amplification of Reg3 δ gene: Mouse pancreatic tissues were used to isolate the total RNA, this total RNA was subjected to the synthesis of cDNA. Full length Reg3 δ gene was successfully amplified from the cDNA made out of total RNA isolated from the mouse pancreas. A full length gene of 626 bp length was amplified by using gene specific primers. Figure 1 is showing the amplification of Reg3 δ gene from mouse pancreas. The Reg3 δ protein has a 26 amino acids long signal peptide, according to literature this signal peptide is toxic for the bacteria.

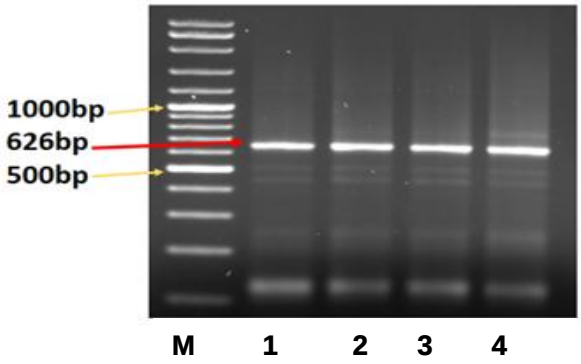

Figure 1: PCR amplification of full length Reg3δ gene from mouse pancreas.
Lane M is 100 bp ladder (Vivantis, USA), lanes 1-4 have PCR products (626 bp) for Reg3 δ gene.

Another PCR was performed for Reg3 δ gene with out putative signal peptide, to amplify the 474 bp long DNA fragment without the sequence of signal peptide (Figure 2). Specific primers were designed for this purpose with restriction sites of Nde I and BamH I restriction enzymes.

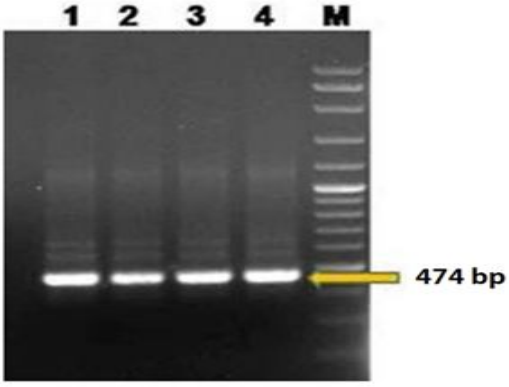

Figure 2: Amplification of Reg3δ gene without signal peptide for expression.
Lane M is 100 bp ladder (Vivantis, USA), lanes 1-4 have PCR products for Reg3 δ gene without signal peptide (474 bp).

Cloning in TA vector and restriction analysis: Full length amplified gene product was ligated in pTZ57 R/T vector and transformed into calcium chloride competent one Shot® TOP10 cells. The positive clones in blue/white colony selection method were further grown in LB media. Plasmids were isolated and sent for the sequencing. The sequence obtained was subjected to NCBI BLAST

alignment and obtained 0.0 E-value and 95% identity with the Reg3 δ mRNA sequence accession #: AB035204.1 showing that this is the right sequence, as previously it was amplified from duodenum of mouse (Figure 3).

>NF sequence of Reg3δ
GCCTGCACTCATTGGCTATATTGAGCATATTACTGCGAACACACAGAACCTGGGCCT
AATGCAGAGATCCACTGCCAGAAGCATTTCTCAGGACACCTGGCATTTCTGCTCACT
TATGGTGAAATTATCTTTGTGTCCTCTCTGGTGAAAAACAGTTTGACCACATTCCCAT
ACATCTGGATTGGACTCCATGATCTGTCACTTGGGAGTTAGCCCAATGAAAATGGAT
GGAAGTGGAGCAGCTCTGACCCTCTGACCTTCTATAACGGGAGATACCACCCTCCAT
GTCTGCACACCACGGCTACTGTGCAGCTTGTTCTCAGGCCTCAGGTCATCAGAAGT
GGAGAGATTATCATTGTGACACAATATTTCCCTATGTCTGCAAATTCAGGGGTAGGG
CAGTTCTGATTACACTGCCTGAAAGTATTCTGAAGATCCATGACAAAGAGCAGCATG
ATGCTACCAGAAGTCTCTCAACCCCAACGATCCTATTATTCGCGTCTCAAGGATCCGG
GCCGGCTAGATCACCGGAAGA

a: Sequence of Reg3 δ gene with forward primer sequence.

>NR sequence of Reg3 δ
TACTCAGCAGAATACGCTTATGGAAGAACAGCAGATATCGTATGATAGAGGACTTTCG
GTGTCGGGGTAGTGAGAAGGACTTACTCGGTGATGCCGTGCATGCTTGCTCCTTTGTC
TATGTGATCTTCAGGATACTTTCAGGCAGTTGAAATCAGAACTGGCCTAACCCTTGAAT
TTGCAGACATAGGGAAATATTGTGTCACAATAATAATCTCTCCACTTCTGATAACCTGA
GGCCTGAGACAAAGCTGCACAGTAGCCGTGGTGTGCAGACATGGAGGGTGGTATCT
CCAGTTATAGAAGGTCAGAGGGTCAGAGCTGCTCCACTTCCATCCATTTTCATTGGGC
AAACTCCCAAAGTGACAGATCATGGAGTCCAATCCAGATGTATGGGAATGTGCTCAAA
CTGTTTTTCACCAGAGAGGACACAAAGATAATTTCACCATAAGTGAGCAGAAATGCCA
GGTGTCCTGAGACATGCTTCTGNAGTGGATCTCTGCATTAGCCAGGTCTGTGGTCCAG
AATCAGTAAAAGCAATAGGAGCCATAAGCTTGGTCTCCTGGGGGACAGCTGATGCGT
GAAGAAGACAGTTTTTGCTAGGATTGTCCCCTGNACCCAGGTCTTGGGAA

b: Sequence of Reg3 δ gene with reverse primer sequence.

Figure 3: Sequence of Reg3 δ gene with forward and reverse primer sequence.

Expression vector construct: After confirmation of sequence the recombinant plasmid was prepared in pET28a vector, (Figure 4) which provided an IPTG inducible promoter and kanamycin antibiotic selection. For this purpose the Reg3 δ gene amplified without its signal peptide was ligated in the pET28a vector and transformed into the one Shot® TOP10 calcium chloride Competent *E. coli* cells for the cloning of recombinant vector. Positive cloned recombinant vectors were confirmed for the presence of Reg3 δ gene in it, with restriction analysis and colony PCR (Figure 5 & 6). Colony PCR was performed by using the diluted cultures as template DNA in PCR mix. Both of the confirmatory analysis (Colony PCR and Restriction analysis) were positive.

Protein expression: Confirmed plasmids were further transformed into chemically competent BL21 (DE3) strain for expression of Reg3 δ protein. Antibiotic selection was carried out with kanamycin, three positive colonies were subjected to further growth at 37 ^{o}C. After induction of protein expression with 1mM final concentration of IPTG at 28 ^{o}C for 20 hours the cultures were processed for isolation of protein by cell lysis. The lysates were centrifuged and the supernatant and pellets both were prepared for SDS-PAGE.

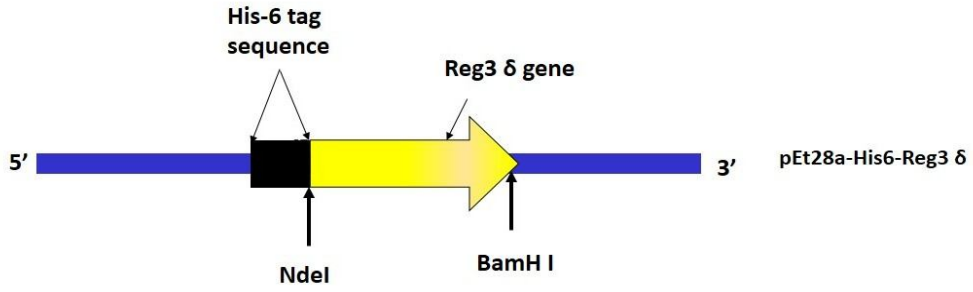

Figure 4: Graphical representation of pET28a based expression system encoding Reg3 δ gene.

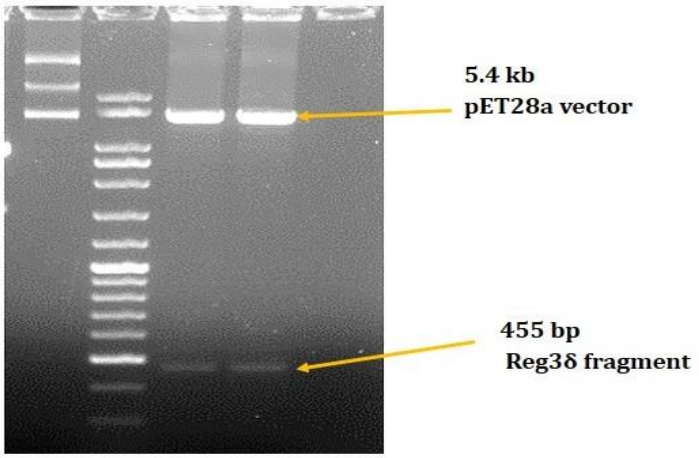

Figure 5: Restriction analysis of recombinant pET28a plasmid.

Figure showing the restricted Reg3 δ fragment of 455 bp and restricted plasmid of 5.4 kb size

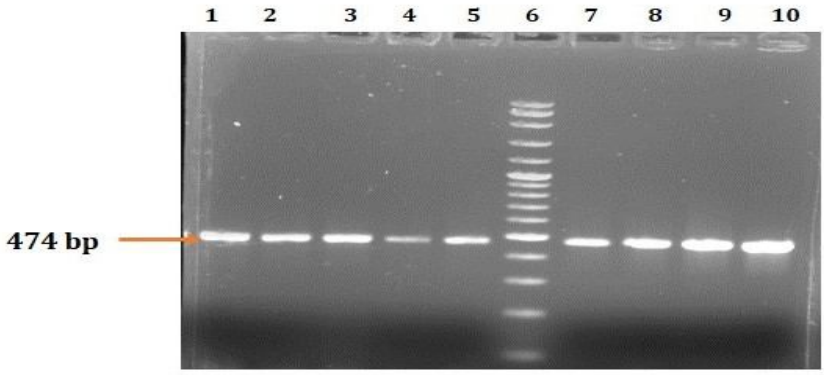

Figure 6: Colony PCR for Reg3 δ protein

Colony PCR from positive cultures showing the presence of Reg3 δ gene in the recombinant vectors Lane 6 has ladder and all other lanes have PCR products from the cultures.

SDS-PAGE: SDS-Polyacrylamide Gel Electrophoresis (12% acrylamide) were performed to analyze the proteins in different samples of induction and in supernatant and pellet. Denatured samples by heat and β-mercapto-ethanol were loaded on the 12% SDS-Polyacrylamide gel and separated on 40mA electric current. Separated proteins showed in Figure 7 indicating a band of the size of 16 kDa in the samples of IPTG induced pellet lane 5-7. These

results indicate the presence of recombinant protein in the inclusion bodies.

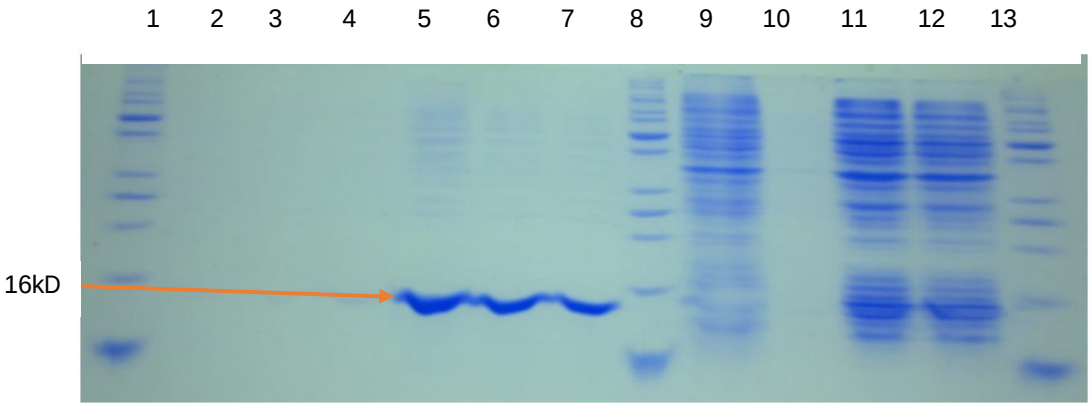

Figure 7: SDS PAGE for the IPTG induced and un-induced recombinant plasmids.
Lane 1 = Protein Ladder, Lane 2-4 = 1mM IPTG induction (supernatant) recombinant vector, Lane 5-7 = 1 mM IPTG induction (pellet) recombinant vector, Lane 8 = Protein Ladder, Lane 9 = IPTG induced (supernatant) non-recombinant vector pET28a, Lane 10=IPTG induced (pellet) non-recombinant vector pET28a Lane 11= Un-Induced recombinant plasmid pET28a, Lane 12 = Un-Induced recombinant plasmid pET28a, Lane 13 = Protein Ladder

DISCUSSION

The Reg proteins are very important group of pancreatic proteins as they play important roles in the pancreatic growth and different diseases. One member of this family, the INGAP (Islet Neogenesis Associated Protein) was found to be implicated in the differentiation and cell growth of endocrine pancreas in mammals (Tam *et al.*, 2004; Taylor-Fishwick *et al.*, 2006). The INGAP was first identified in cellophane wrapped pancreata in hamster (Rafaeloff *et al.*, 1997) and latter, it was reported that INGAP from hamster also express in the normal pancreata (Flores *et al.*, 2003). The mouse counterpart of hamster's INGAP is found 72% identical and termed as INGAP related Protein (INGAP-rP) and classified in the Reg family of proteins as Reg3 δ. This Reg3 δ protein express in normal pancreas of mice, which hindered exploitation of this protein as a potential therapeutic for diabetes. Hence, the aim of the current study was to optimize cloning and expression strategies for the recombinant production of mouse Reg3 δ in prokaryotic system for its ready availability to be used in subsequent evaluation as potential therapeutics in mouse models of diabetes.

During this study several challenging situations were encountered like isolation of good quality RNA from the pancreatic tissue. During extraction with Trizol solution (guanidinium thiocyanate and phenol), the grinding of fresh tissue led to the degradation of RNA. As a matter of fact, pancreas is a ribonuclease rich tissue that presents many challenges during RNA extraction. RNases work without cofactors, hence they are extremely stable and active enzymes under stringent conditions (Dastgheib *et al.*, 2014) and offer a great challenge during RNA isolation particularly from the tricky tissues like pancreas. Thus, we subjected the pancreas tissue to snap freezing in liquid Nitrogen and tissue was ground to powder, which was further used for the RNA isolation with Trizol.

Amplification of this gene from cDNA took some optimization with primer concentration and PCR profile. Finally the successful reaction of 25µL was settled with the 25 pmol primer concentration and the 1mMol $MgCl_2$ final concentration. Latter during the protein expression, several concentrations of IPTG were tried and 1mM final concentration was found optimal.

The recombinant clone has 6X-His tag on N-terminus of the protein for further purification and use for animal administration. The BL21 (DE3) cells though expressed the protein yet not giving a good yield, possibly due to the inclusion body problem, so that in future we are planning to use other strains for getting a good yield for appropriate use for animal testing. In this paper, we tried to summarize the research

methodology and challenges to express a Reg3 δ recombinant protein, which we encountered during the process and could be a guideline for the other researchers in this field.

REFERENCES

Barbosa, H., S. Bordin, L. Stoppiglia, K. Silva, M. Borelli, H. Del Zotto, J. Gagliardino and A. Boschero, Islet Neogenesis Associated Protein (INGAP) modulates gene expression in cultured neonatal rat islets. Regul. Pept. **136**: 78-84 (2006).

Dastgheib,S., C. Irajie, R. Assaei, F. Koohpeima and P. Mokarram, Optimization of RNA Extraction from Rat Pancreatic Tissue. Iranian Journal of Medical Sciences **39**: 282-288 (2014).

Dungan, K.M., J.B. Buse and R.E. Ratner, Effects of therapy in type 1 and type 2 diabetes mellitus with a peptide derived from islet neogenesis associated protein (INGAP). Diabetes Metab. Res. Rev. **25**: 558-565 (2009).

Flores,L.E., M.E.Garcia, M.I.Borelli, H.Del Zotto, M.E.Alzugaray, B. Maiztegui and J.J. Gagliardino, Expression of islet neogenesis-associated protein in islets of normal hamsters. J.Endocrinol. **177**:243-248 (2003).

Jehan, A.E.S. and I.S. Ashoush, Antihyper-glycemic effects of stevioside on diabetic rats. Pak. J. Biotechnol. **5**: 21-26 (2008).

Kulis, M.D., Jr. and S.B. Shuker, Expression, purification, and refolding of mouse islet neogenesis associated protein-related protein for NMR studies. Protein Expr. Purif. **48**: 224-231 (2006).

Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacterio-phage T4. Nature **227**: 680-685 (1970).

Liu, J.L., W. Cui, B. Li and Y. Lu, Possible roles of reg family proteins in pancreatic islet cell growth. Endocr Metab Immune Disord Drug Targets **8**: 1-10 (2008).

Okamoto, H., The Reg gene family and Reg proteins: with special attention to the regeneration of pancreatic beta-cells. J. Hepatobiliary Pancreat Surg. **6**:254-262 (1999).

Parikh, A., A.F. Stephan and E.S. Tzanakakis, Regenerating proteins and their expression, regulation and signaling. Biomol. Concepts. **3**: 57-70 (2012).

Pittenger,G.L., D.A.Taylor-Fishwick, R.H.Johns, N.Burcus, S.Kosuri and A.I.Vinik, Intra-muscular injection of islet neogenesis-associated protein peptide stimulates pancreatic islet neogenesis in healthy dogs. Pancreas **34**: 103-111 (2007).

Rafaeloff, R., G.L. Pittenger, S.W. Barlow, X.F. Qin, B. Yan, L. Rosenberg, W.P. Duguid and A.I. Vinik, Cloning and sequencing of the pancreatic islet neogenesis associated protein (INGAP) gene and its expression in islet neogenesis in hamsters. J. Clin. Invest. **99**: 2100-2109 (1997).

Rind,N.A., M.Umar Dahot, S.A.M.Malik and M.Kumar, Antidiabetic effect of Cinnamon (*Cinnamonum zeylanicum*) used to control diabetes mellitus in alloxan induced diabetic male rabbits.Pak.J.Biotechnol.**7:**81-90 (2010).

Rind, N.A., M. Umar Dahot, S.A Malik and M. Kumar, Comparative antihyperglycemic activity of aqueous extracts of garlic (*Allium sativum*) and ginger (*Zingiber Officinale*) in alloxan-induced male rabbits. Pak. J. Biotechnol. **10**: 53 – 62 (2013).

Rosenberg,L., M.Lipsett, J.W.Yoon, M. Prentki, R. Wang, H.S. Jun, G.L. Pittenger, D. Taylor-Fishwick and A.I. Vinik, A penta-decapeptide fragment of islet neogenesis-associated protein increases beta-cell mass and reverses diabetes in C57BL/6J mice. Ann. Surg. **240**:875-884 (2004).

Tam, J., L. Rosenberg and D. Maysinger, INGAP peptide improves nerve function and enhances regeneration in streptozotocin-induced diabetic C57BL/6 mice. The FASEB Journal (2004).

Taylor-Fishwick, D.A., A.Bowman, N. Hamblet, P. Bernard, D.M. Harlan and A.I. Vinik, Islet neogenesis associated protein transgenic mice are resistant to hyperglycemia induced by streptozotocin. J. Endocrinol. **190**: 729-737 (2006).

Taylor-Fishwick,D.A., A.Bowman, M. Korngie bel- Rosique and A.I.Vinik, Pancreatic islet immuno-reactivity to the Reg protein INGAP. J. Histochem. Cytochem. **56**: 183-191 (2008).

Taylor-Fishwick, D.A., S. Rittman, H. Kendall, L. Roy, W. Shi, Y. Cao, G.L. Pittenger and A.I. Vinik, Cloning genomic INGAP: a Reg-related family member with distinct transcrip -tional regulation sites. Biochim. Biophys. Acta **1638**: 83-89 (2003).

Zhang,Y.W., L.S. Ding and M.D.Lai, Reg gene family and human diseases. World J. Gastro-enterol. **9**: 2635-2641 (2003).