ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

miRNA ANALYSIS IN MOLECULAR PATHOGENESIS OF MODY

M.Sc. THESIS

Oğuzhan Fatih BALTACI

Department of Molecular Biology-Genetics and Biotechnology

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SEPTEMBER 2015

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MODY'NİN MOLEKÜLER PATOGENEZİNDE miRNA ANALİZİ

YÜKSEK LİSANS TEZİ

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EYLÜL 2015

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FOREWORD

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September 2015 Oğuzhan Fatih BALTACI

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ABBREVIATIONS

µg : Microgram
µl : Microliter
µM : Micromolar
Amp : Ampicillin
cDNA : Complementary DNA
Ct : Cycle threshold
dH_2O : Distilled water
DM : Diabetes mellitus
DMEM : Dulbecco's modified Eagle medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide
E. Coli : *Escherichia Coli*
EDTA : Ethylenediaminetetraacetic acid
FBS : Fetal Bovine Serum
g : Gram
GCK : Glucokinase
HNF1A : Hepatocelluar Nuclear Factor 1-A
LB : Lysogeny broth
M : Molar
ml : Mililiter
MIN6 : Mouse Insulinoma 6
mRNA : Messenger Ribonucleic Acid
miRNA : Micro Ribonucleic Acid
MODY : Maturity onset diabetes of the young
ng : Nanogram
nm : Nanometer
PBS : Phospate buffered saline
PCR : Polymerase Chain Reaction
pH : Power of Hydrogen
RNA : Ribonucleic Acid
qRT-PCR : Reverse Transcription Polymerase Chain Reaction
rpm : Recolutions per minute
siRNA : Short interfering Ribonucleic Acid
TBE : Tris-Boric Acid-EDTA
TE : Tris-EDTA
UTR : Untranslated region

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miRNA ANALYSIS IN MOLECULAR PATHOGENESIS OF MODY

SUMMARY

Maturity onset diabetes of the young (MODY) is a monogenic form of Diabetes mellitus (DM) characterized with abnormal beta cell function, autosomal dominant inheritance, hyperglycemia, lack of auto-immunity in non-obese young patients. The prevalence of MODY changes with the population, but MODY3 associated with *HNF1A* gene mutations is the most common form of MODY. MODY3 is a progressive form of hyperglycemia with diabetes usually diagnosed in adolescence/early adulthood. MODY3 has been associated with *HNF1A* gene mutations.

The *HNF1A* gene is expressed in pancreas, liver, and kidneys and encodes for the transcription factor HNF1A. HNF1A regulates expression of the genes involved in glucose metabolism, glucose transportation, insulin gene as well as liver specific genes. There are more than 300 different *HNF1A* gene mutations in the human genome mutation database associated with MODY3. MODY3 exhibits variable clinical phenotype. Clinical phenotype of MODY3 can vary within the same family even within the patients who have the same mutation. MODY3 phenotype depends on both mutation type and the location of mutation along the *HNF1A* gene.

MODY3 is frequently misdiagnosed as Type 1 DM due to the similar clinical features and early onset of diabetes. Correct diagnosis of MODY3 is important for the preventive treatment since MODY3 patients are under higher risk of diabetic complications such as heart attacks and strokes. Studies for the molecular diagnosis and determination of the *HNF1A* gene mutation profile in the Turkish MODY3 patients have been started in the Department of Molecular Biology and Genetics, Istanbul Arel University. It is important to determine the molecular mechanism of DM which has been considered as an epidemic disease due to increasing prevalence worldwide to decrease its morbidity and mortality rate. However, the exact pathogenesis of diabetes has not been resolved yet.

Recent studies have revealed that micro RNAs (miRNAs) have important roles in the development of diabetes, diabetic complications, insulin expression, insulin secretion and insulin action. For example, miR-375 in the pancreatic beta cells has been shown to be involved in insulin secretion and pancreas development, miR-133 in the cardiac cells has been shown to be involved in diabetic cardiac complications, miR-192 in the kidney has been shown to be involved in the diabetic nephropathy.

The main purpose of the study was to discover miRNAs that might be involved in the molecular pathogenesis of MODY by discovering the specific miRNAs that are regulated by HNF1A transcription factor. For this purpose, miRNAs that are differentially expressed in *HNF1A*-overexpressed and *HNF1A*-silenced MIN6 cells. MIN6 cells are mouse insulinoma cell lines that expresses *HNF1A* endogenously. MIN6 cells were transfected with the *HNF1A* expression vector carrying the human HNF1A cDNA sequence for the overexpression experiments. In order to silence *HNF1A* expression, MIN6 cells were treated with siRNA molecules which

specifically bind to HNF1A mRNA and inhibits its expression in post-transcriptional level. Overxpression and silencing of *HNF1A* gene was confirmed with Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Western Blot techniques.

miRNA sequencing results of *HNF1A*-overexpressed cells and *HNF1A*-silenced cells were compared to identify differentially expressed miRNA molecules. As a result, 238 known and 91 novel miRNAs were determined to have significant differential expression. Among 238 known miRNAs, five of them which are miR-129-1-3p, miR-129-2-3p, miR-200b-3p, miR-296-3p, and miR-378a-5p were found to have the most significant and the highest ratio of differential expression. In order to validate these candidate miRNA molecules regulated by HNF1A and involved in the molecular pathogenesis of MODY, further studies with cell lines and patients are required.

MODY'NİN MOLEKÜLER PATOGENEZİNDE miRNA ANALİZİ

ÖZET

Diabetes Mellitus (DM), tüm dünyada yüksek sıklıkla görülen, pankreas hücrelerinden insülinin salgılanamaması veya periferik hücrelerin insüline karşı duyarlı olmamaları ile karakterize edilen; karbonhidrat, yağ ve protein metabolizmasında bozuklukların olduğu bir metabolik hastalıklar grubudur. DM içinde yer alan metabolik hastalıkların ortak özelliği hiperglisemi olarak tanımlanan plazma glikoz seviyesinin yüksek olmasıdır.

Sağlıklı durumda periferik dokularda insüline hassasiyetin azalması, insülin salgılanmasının artmasına neden olarak normoglisemi sağlanır. İnsülin kandaki glikozun kas ve adiposit hücrelerine girişini ve bu hücrelerde glikoz metabolizmasını stimüle ederken, karaciğerde glikoneojenesisi engelleyerek glikoz hemostazını sağlar. İnsülinin salgılanması plazma glikoz seviyesi ile düzenlenir. Yemekten sonra insülin seviyesi 10 kat artış gösterir ve insülin glikoz taşıyıcısının (pankretik beta hücrelerinde GLUT2) glikozu hücre içine almasını sağlar. Hücre içinde glikoz sensörü olan glikokinaz (GCK) enzimi glikozu fosforilasyon ile glikoz-6-fosfat'a çevirir. Glikoz-6-fosfat daha sonra mitokondride glikolize uğrayıp ATP üretimi için kullanılır. ATP'den gelen enerji, hücre içi potasyumu, ATP'ye bağlı potasyum pompaları ile dışarı atmak için kullanılır. Potasyum iyonlarının hücre dışına çıkması hücre zarı potensiyel enerjisinde değişikliğe sebep olur, bu da hücre zarında bulunan voltaja bağlı kalsiyum kanallarının açılmasını ve hücre içine kalsiyum iyonlarının girmesini sağlar. Kalsiyum iyonları beta hücrelerinde depolanan insulinin hücre dışına salınmasına sebep olur.

Plazma glikoz seviyesinin normal sınırlar içinde olmaması hastalarda retinopati, nefropati, nöropati ve kardiyovasküler komplikasyonlara neden olurken, diyabetli hastalarda ölüm riski diyabetli olmayanlara göre iki kat daha fazla olduğu yapılan çalışmalarda gösterilmiştir. Türkiye Diyabet Önleme ve Kontrol Programı'nda verilen bilgiye göre Türkiye'de diyabetin artış hızı yüzde 220 ile dünya ve Avrupa genelinin üzerindedir. Dünya Sağlık Örgütünce, 2030 yılında Türkiye'de diyabetli sayısının 6.422.000 kişi olacağı tahmin edilmektedir.

Diyabet farklı tedavi yöntemlerinin uygulandığı Tip 1, Tip 2, spesifik nedenlere bağlı gelişen ve hamilelik diyabeti olmak üzere dört alt gruba ayrılmaktadır. Diyabetin en sık görülen tipi olan Tip 2 Diyabet pankreatik beta hücrelerinin insülin salgılamasında azalma olması ve/veya hedef dokuların insüline cevap oluşturmamasından dolayı gelişir ve tüm diyabet olgularının %90'ını oluşturur. Diyabette ikinci en sık görülen tip olan Tip 1 Diyabet otoimmune bir hastalık olup, pankreastaki beta hücrelerinin yıkımı nedeniyle insülinin üretilememesinden kaynaklanır ve tüm diyabet olgularında yüzde 5-10 sıklıkla görülür.

Maturity onset diabetes of the young (MODY) beta hücresi fonksiyon bozukluğunun olduğu diyabetin spesifik nedenlerle gelişen monogenik alt tiplerindendir İlk olarak 1974 yılında diyabetten farklı bir klinik olgu olarak tanımlanan MODY otozomal

dominant kalıtım gösteren, hasta aile bireylerinden en az bir tanesine 25 yaş öncesi tanı konulduğu, insüline bağlı olmayan diyabet tipidir.

MODY'nin farklı genler ile ilişkilendirilmiş, hastalığın ortaya çıkma yaşı, hiperglisemi seviyesi, tedaviye cevap gibi konularda farklı klinik özellikler sergileyen birçok alt grubu vardır. Glikokinaz gen mutasyonları (MODY2) ve transkripsiyon faktör HNF1A (MODY3), HNF4A (MODY1) mutasyonları en sık görülen MODY tipleridir. *HNF1A* gen mutasyonları insulin üretiminde ve salgılanmasında zamanla artan bir düşüşe sebep olur. Başlangıçta hastalar sülfonilüre ile tedavi edilebilse de ilerleyen zamanlarda insulin tedavisine ihtiyaç duyabilmektedirler. *GCK* gen mutasyonları ise daha hafif bir hastalık seyrine sebep olmakta, diyet ve egzersiz ile hastalığın kontrolü mümkün olmaktadır. Fare modelleri ile yapılan çalışmalar MODY ile ilişkilendirilen transkripsiyon faktörlerinin embriyonik pankreas gelişiminden, beta hücrelerinin farklılaşmasından, beta hücrelerinde gen ekspresyonun düzenlenmesinden, özellikle insülin ve GLUT2 genlerinin sorumlu olduğunu göstermiştir.

MODY tüm diyabet olgularının %1-5'ini oluşturmaktadır, ancak klinik olarak Tip 1 ve Tip 2 Diyabet ile benzer fenotip sergilemesinden dolayı, dünyada MODY'nin prevelansının daha yüksek olabileceği düşünülmektedir.

MODY'de yaşanan komplikasyonlar diyabetin sık görülen Tip 1 ve Tip 2 türlerinde karşılaşılan komplikasyonlardan farklı değildir. Örneğin MODY3 hastaları kalp krizi, felç gibi diyabetik komplikasyon riski altındadır.

HNF1A geninde meydana gelen mutasyonlar ile ilişkilendirilen MODY3, en sık görülen MODY tipidir. Homeodomain transkripsiyon faktör olan HNF1A, pankreas, karaciğer ve böbrek gibi farklı dokularda da ekspres edilir. HNF1A karaciğer spesifik genler dışında, glikoz metabolizmasında yer alan, glikoz transportunda yer alan genlerin ve insülin gen ekspresyonunda rol oynar. DNA'ya homodimer veya HNF1B proteini ile heterodimer olarak bağlanan HNF1A molekülü; N-terminal dimerizasyon, pseudo-POU ve Homeodomain motif içeren DNA bağlanma bölgesi, ve C-terminal transaktivasyon bölgesinden oluşur. HNF1A, 12. kromozomda yer alan ve 10 ekzondan oluşan *HNF1A* geni tarafından kodlanır. MODY3 hastalarında tespit edilmiş, insan genom mutasyon veritabanında listelenen, tüm gene yayılmış 300'den fazla farklı *HNF1A* mutasyonu vardır. Hamilelik sırasında bebeğin maternal diyabete maruz kalması, mutasyonun *HNF1A* geninin 1. ile 6. ekzonu arasında yer alması hastalığın açığa çıkma yaşını daha erkene alabilmektedir.

MikroRNAlar (miRNA), kodlama yapmayan yaklaşık 22 nükleotid uzunluğunda, post-transkripsiyon evresinde gen anlatımının düzenlenmesinde rol oynayan ribonukleotidlerdir. miRNAlar, hedef gen transkriptlerinin 3'UTR bölgesine bağlanarak hedef mRNAların yıkıma uğramasını veya protein sentezine girmesini engelleyerek gen ifadesinin regülasyonunu sağlar. İnsan genomunda yaklaşık 1000 miRNA olduğu ve transkriptlerin %30'nun miRNAlar tarafından düzenlendiği tahmin edilmektedir. Son yıllardaki çalışmalar kanser, kardiovasküler ve metabolik hastalıklarda miRNA seviyelerinin değiştiğini göstermektedir. Diyabet ile ilgili yapılan çalışmalarda miRNA'ların pankreas beta hücrelerinin fonksiyonunda, pankreas gelişiminde, insülin üretimi ve salgılanmasında rol oynadığı gösterilmiştir. Örneğin beta hücrelerinde anlatımı olan miR-375'in glikozla uyarılmış hücrelerde insülin salınımını negatif regüle ettiği ve bunu da Myotrophin (*Mtpn*) ve 3'phosphoinositidite-dependent protein kinase-1 (*PDK1*) genlerinin anlatımını kontrol ederek yaptığı çalışmalarda bulunmuştur. Pankreas beta hücrelerinde anlatımı

çok olan ve beta hücrelerinin gelişiminde rolü olduğu gösterilen miR-124a'nın ise hedef genlerinden birinin beta hücrelerinin farklılaşmasında, pankreas gelişimde, glikoz metabolizması ve insülin salınımında görevli FoxA2 (HNF3B) transkripsiyon faktörü olduğu bulunmuştur.

Tip 1 ve Tip 2 diyabet ile yapılan çalışmalarda miRNAların diyabet tanısında biyobelirteç olarak kullanılabileceğini göstermiştir. Örneğin, 2011 yılında yapılan bir çalışma Tip 2 diyabet patogenezinde miR-144'ün insülin sinyal yolağında etkili bir biyobelirteç olarak kullanılabileceği gösterilmiştir.

Bu çalışmanın amacı, HNF1A transkripsiyon faktörü tarafından kontrol edilen miRNAları saptayıp. bu doğrultuda MODY'nin moleküler patogenezinde rol alabilecek miRNAları keşfetmektir. Bu amaçla, *HNF1A* gen anlatımının susturulduğu ve overekspresе edildiği MIN6 hücrelerindeki miRNA anlatım seviyeleri karşılaştırılmıştır. Overekspresyon deneyi için yabanıl tip *HNF1A*-cDNA'sını içeren ekspresyon vektörü MIN6 hücrelerine transfekte edilmiştir. Susturma deneyleri için ise HNF1A mRNA'sına spesifik olarak bağlanan ve bu genin anlatımını baskılayan siRNA-HNF1A (short interfering RNA-HNF1A) kullanılmıştır. *HNF1A* geninin susturulduğu ve overekspresе edildiği, Gerçek Zamanlı Polimeraz Zincir Reaksiyonu ve Western Blot yöntemleriyle doğrulanmıştır.

HNF1A'nın overekspresе edildiği hücrelerdeki miRNA dizileme sonuçları HNF1A'nın susturulduğu hücrelerdeki miRNAlar ile karşılaştırıldığında, anlatım seviyesi istatistiksel olarak anlamlı bir şekilde değişiklik gösteren 238 adet bilinen miRNA saptanmıştır. Bununla birlikte anlatım seviyesi farklılık gösteren, miRNA olabilecek 91 adet yeni RNA molekülü keşfedilmiştir. 238 adet bilinen miRNA arasında, ifadesi en anlamlı olarak değişiklik gösteren ve en yüksek değişim katsayısına sahip miRNAlar; miR-129-1-3p, miR-129-2-3p, miR-200b-3p, miR-296-3p ve miR-378a-5p'dir. MODY'nin ortaya çıkmasında önemli bir yere sahip olan HNF1A transkripsiyon faktörünün anlatımını regüle ettiği muhtemel bu miRNAlar, aynı zamanda MODY'nin moleküler patogenezinde de rol alabilir. Hastalığın doğrudan saptanması ve tedavi edilmesi sürecinde, bulunan bu miRNAların etkisi araştırılmalıdır.

1. INTRODUCTION

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is group of metabolic disorders characterized with chronic hyperglycemia due to defects in insulin secretion and/or action. Hyperglycemia represents when excessive amount of glucose circulates in blood. In long-term, DM can cause dysfunction and failure of many organs such as nerves, eyes, kidneys, heart and blood vessels. The classic symptoms are polyuria, increased thirst, blurring of vision and weight loss (World Health Organization, WHO, 1999).

There are two main types of DM, Type 1 DM (insulin dependent diabetes mellitus, IDDM) and Type 2 DM (non insulin dependent diabetes mellitus, NIDDM). Type 1 DM is characterized by the destruction of pancreatic beta cells and insulin deficicency (Figure 1.1). The second form of DM is called Type 2 DM represents with insulin resistance.

The major mechanism underlying Type 1 DM is the autoimmune attack to the islets of Langerhans which contain pancreatic β-cells where insulin is produced in the body. Thus, autoimmune attack to the islets of Langerhans leads to decreased insulin production which is an essential hormone for the glucose metabolism in the body. Insulin regulates glucose homeostasis by transporting glucose from blood to muscles and adipocytes (Nyunt *et al*., 2009; Rother, 2007).

Type 1 DM can be diagnosed with the determination of certain antibodies such as anti-GAD (Glutamic Acid Decarboxylase). It is a complex disease associated with genetic and environmental factors. *IDDM1* gene (Insulin-Dependent Diabetes Mellitus 1), also known as *HLA-DQB1* located in major histocompatibility complex class II region on chromosome 6, is defined as the highest risk factor for Type 1 DM (Bluestone *et al*., 2010). In addition, virus infection might also be possible cause for Type 1 DM. For example, Coxsackie B viruses (CVBs) are associated with human diabetes by proposing that the immune system attacks the infected cells along with the pancreatic β-cells (Fairweather and Rose, 2002).

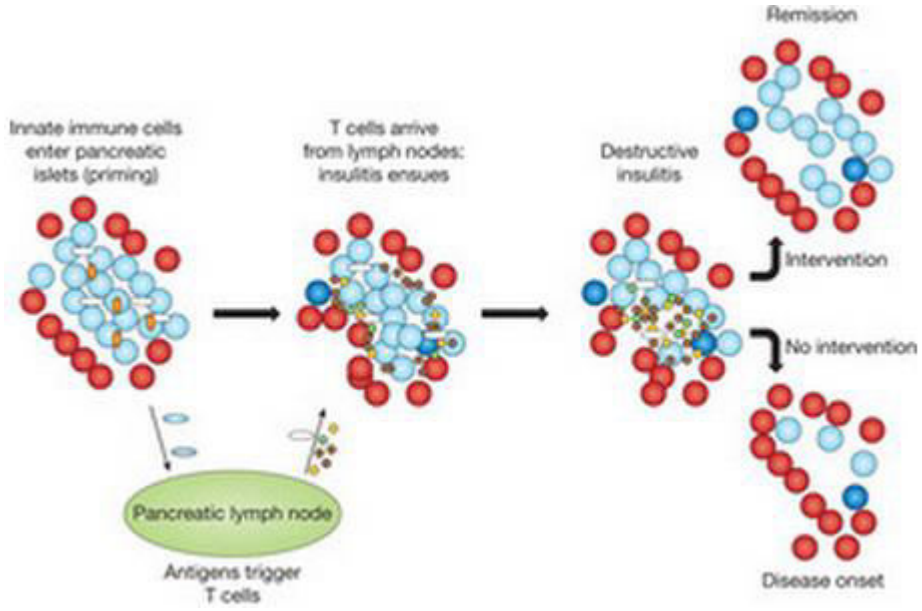

Figure 1.1 : Immunologic history of Type 1 DM (Bluestone *et al*., 2010)

Type 2 DM is charactarized by deficiencies in insulin secretion and insulin resistance. Insulin resistance is the inability to sense the secreted insulin hormone by target cells. It is the most common type of DM covering up to 90% of all cases. The pathogenesis involves when the required amount of insulin for compensating the resistance cannot be produced any longer by beta cells. Patients do not need to use insulin for the treatment; however, might require it for the control of blood glucose levels if this cannot be achieved with hypoglycemic agents or diet (Zimmet *et al*., 2001). The risk of developing Type 2 DM depends on genetic and environmental factors. Individuals whose both parents have Type 2 DM are under 70% risk of developing the disease (Rashmi *et al*., 2015).

Studies revelad genes associated with the risk of developing Type 2 DM. *Calpain-10*, a cysteine protease involved in glucose metabolism, is identified as the first susceptibility gene identified through linkage studies. However, variants of transcription factor 7-like 2 (*TCF7L2*) is known as the most successfully correlated gene so far (Horikawa *et al*., 2000).

1.2 Maturity Onset Diabetes of the Young (MODY)

Maturity onset of the young (MODY) is a monogenic form of Type 2 DM associated with loss of pancreatic β-cell function before the age of 25 years. It was first described in 1974 as a disorder different than diabetes and charactarized by

autosomal dominant inheritance, asymptomatic hyperglycemia, and absence of autoantibodies (Bonnefond *et al*.,2012; Fajans, 1990; Tattersall RB, 1974). MODY has at least 10 types associated with different genes and demonstrating different clinical features regarding the onset of the disease, the level of hyperglycemia, and the response to the treatment. MODY1, MODY2 and MODY3 are the most common forms of MODY (Table 1.1) characterized with *Hepatocyte Nuclear Factor 4-alpha* (*HNF4A*), *Glucokinase* (*GCK*), and *Hepatocyte Nuclear Factor 1-alpha* (*HNF1A*) mutations, respectively (Bonnefond *et al*., 2012; Fajans and Bell, 2011; Nyunt *et al*., 2009; Ellard and Colclough, 2006).

MODY constitutes 1 to 10% of all diabetes cases. In addition, it is thought that MODY prevalence might be higher than this percentage, since the disease is clinically misdiagnosed as Type 1 and 2 DM (Nyunt *et al*., 2009).

MODY is clinically and genetically a heterogenous disease. Even though MODY1, MODY2, and MODY3 are the most common forms, different gene mutations may cause the disease depending on population. Although 9 forms of MODY has been reported until 2009, there are currently more than 10 different types of MODY associated with different genes, with different prevalences in different populations. Patients suffering from MODY are under the risk of heart attack and paralysis (Thanabalasingham and Owen, 2011; Ellard *et al*., 2008; Gat-Yablonski *et al*., 2006).

Clinical features and treatments in MODY differ depending on the mutation type. For example, *HNF1A* mutations can cause a decrease in insulin production and secretion. Although patients are successfully treated with sulfonylurea at first, insulin might be necessary in late stages. However, *GCK* mutations cause less severe damage and the disease can be controlled with diet and exercise (Colom *et al*., 2010).

Table 1.1 : Clinical and genetic features of MODY1, MODY2 and MODY3 (Yamagata, 2003).

	MODY1	**MODY2**	**MODY3**
Gene	*HNF4A*	*GCK*	*HNF1A*
Chromosomal location	20q12-13.1	7p15-13	12q-24
Frequency	rare	relatively common	most common
Severity of hyperglycemia	severe	mild	severe

1.3 MODY3

MODY3 is the most common type of MODY and associated with the mutations in *HNF1A*. Prevalence of MODY3 differs depending on populations. According to Kropff and his colleagues, there are approximately 5000 MODY3 cases in the United Kingdom of whom almost 90% are misdiagnosed (Kropff *et al*., 2011). The onset and prognosis of the disease can vary depending on location of the *HNF1A* mutation. For example, in patients with mutations between exon 1 and 6, the onset of the disease can be earlier than expected (Colclough *et al*., 2013; Ellard and Colclough, 2006).

Approximately 70% of MODY3 patients develop this disease before age of 25; however, people who are diagnosed with MODY3 later than 25 years have been reported (Frayling *et al*., 2001). MODY3 is usually treated with sulfonylurea with good results; however, patients might need insulin in long-term because of the progression of insulin secretory capacity (Thanabalasingham *et al*., 2011).

1.4 HNF1A

Hepatocyte Nuclear Factor 1-alpha (HNF1A) is a transcription factor expressed in many tissues such as pancreas, liver, and kidney. It is a 631 amino acid long dimeric protein with the molecular weight of is 67,325 kDA. It has 10 exons and three functional domains which are N-terminal dimerization, DNA binding site that involves pseudo-POU and homeodomain motif, and C-terminal transactivation site (Figure 1.2).

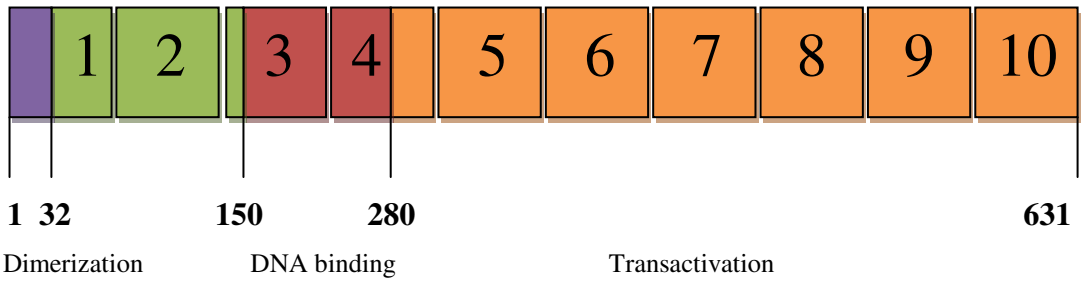

Figure 1.2 : Functional domains of HNF1A transcription factor (Yagamata, 2003).

HNF1A binds to DNA as a homodimer or a heterodimer via HNF1B protein by recognizing the inverted palindrome 5'-GTTAATNATTAAC-3'. It is associated with cellular processes and regulates the expression of hundreds of downstream genes

involved in glucose homeostasis, glucose import, and insulin secretion (Figure 1.3). *HNF4A*, for example, is regulated by HNF1A and it is required for the normal functioning of liver and pancreas. It is also a transcription factor that is in a positive feedback mechanism with HNF1A. (Odom *et al*., 2004).

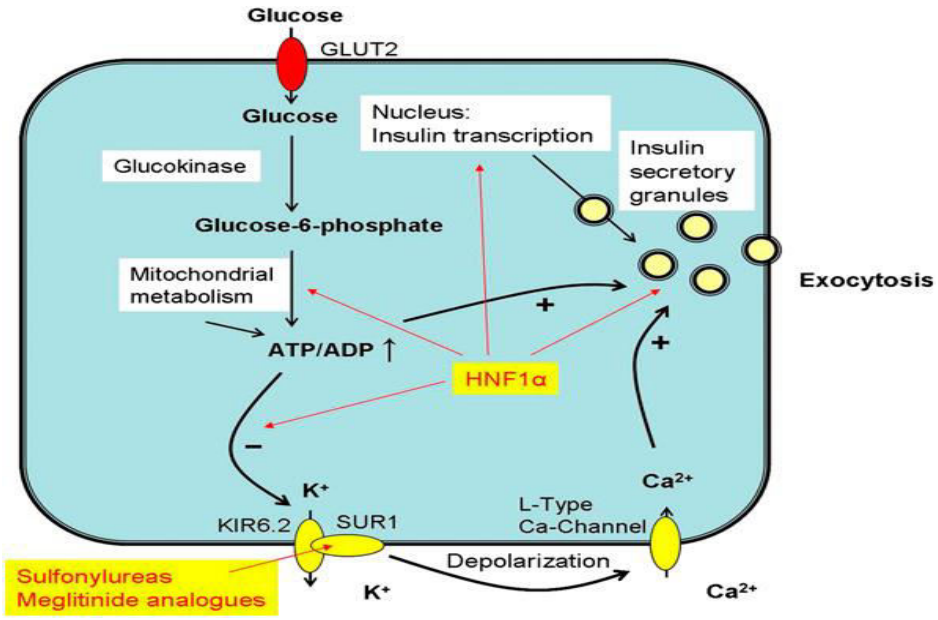

Figure 1.3 : Function of HNF1A in glucose metabolism (Becker *et al*., 2013).

HNF1A gene is located in the long arm of 12th chromosome and composed of 24726 base pairs. It is conserved in mouse, rat, zebrafish, chicken, Rhesus monkey, cow, dog, frog, and chimpanzee. More than 300 different mutations have been reported in the HNF1A gene in MODY3 patients (Human Genome Mutation Database, HGMD; Ellard and Conclough, 2006). Among these mutations, the most common one is Pro291fsinsC-HNF1A frameshift mutation which is in the 4th exon (Figure 1.4).

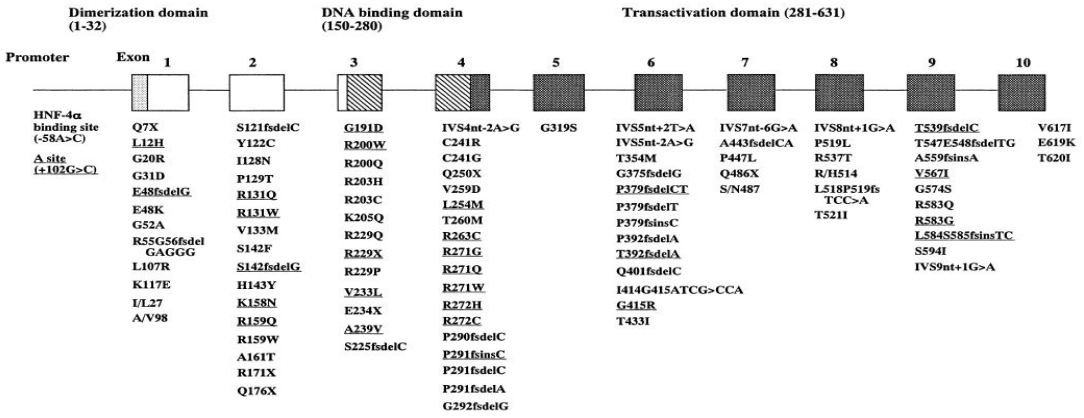

Figure 1.4: Mutations and polymorphisms in *HNF1A* gene (Yamagata, 2003).

1.5 Micro RNAs

Micro RNAs (miRNAs) are ~ 22 nucleotide noncoding single-stranded ribonucleic acids that can play important roles in regulation of gene expression in post-transcriptional level (Bartel, 2004). miRNAs are synthesized as large precursors called pri-miRNAs at first, then processed into stem-loop structured pre-miRNAs and transferred to cytoplasm by RAN-GTP and exportin-5 (Figure 1.5). In the cytoplasm, premiRNAs are excised from their stem-loop structure and unwinded to the single-stranded form called mature miRNAs by an RNase III enzyme, Dicer. miRNAs regulate gene expression by either imperfectly binding to 3' untranslated region (UTR) of a target mRNA or specific binding to a target mRNA (Esquela-Kerscher and Slack *et al*., 2006). It is estimated that there are nearly 1000 miRNAs in human genome and these miRNAs regulate 30% of the human transcripts (Bartel, 2004).

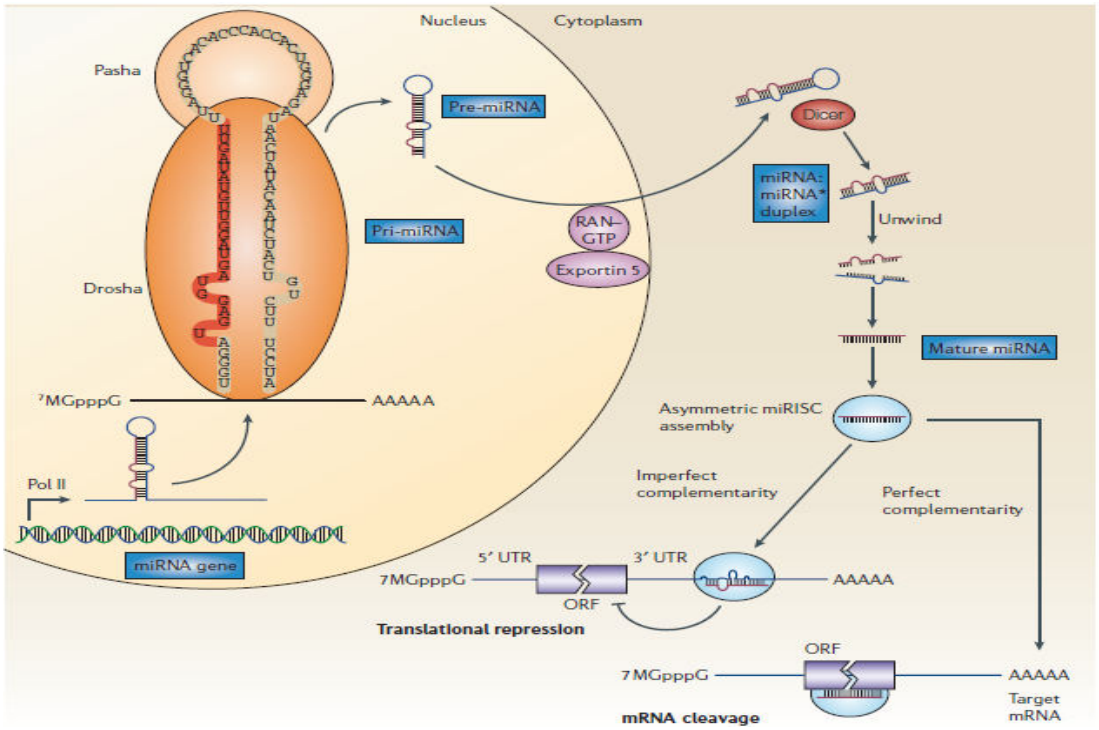

Figure 1.5 : Summmary of miRNA biogenesis (Esquela-Kerscher and Slack, 2006).

In the studies based on diabetes, miRNAs have been shown to play important roles on pancreatic beta cell function, pancreatic development, insulin production and secretion (Poy *et al*., 2004; Baroukh *et al*., 2007; Tang *et al*., 2008). For instance, it was found that miR-375 negatively regulates insulin secretion in glucose-stimulated cells by controlling the expression of myotrophin and 3'phosphoinositidite-

dependent protein kinase-1 (*PDPK1*) genes (El Quaamari *et al*., 2008; Poy *et al*., 2004).

miR-124 is highly expressed in pancreatic beta cells and has been shown to have a crucial role on the development of beta cells. It was found to be associated with HNF3B that is a transcription factor involved in glucose metabolism, pancreatic development, beta cell differentiation and insulin secretion (Baroukh *et al*., 2007).

There are studies that show miRNAs can directly be involved in insulin synthesis. Tang and his colleagues demonstrated that in mouse insulinoma (MIN6) cells which can synthesize insulin upon glucose stimulation, insulin is up-regulated when miR-30d level is increased, and it is down-regulated when miR-30d is decreased (Tang *et al*.; 2009). This indicates that miR-30d might be the regulator of a gene that can inhibit insulin expression. Also it has been reported that miR-30d regulates the expression of MafA transcription factor which is a protein that inhibits the expression of insulin (Zhao *et al*., 2012).

In the last decade, there have been studies showing that miRNAs are also involved in diabetic complications. miRNA-133 was found to have decreased expression in human and mouse models of diabetic cardiomyopathy which is a sign that it plays a role in cardiovascular complication of diabetes (Care *et al*., 2007).

Studies on Type 1 and 2 DM have shown that miRNAs might be used as biomarkers for the diagnosis of diabetes. miR-144 was shown to be an effective biomarker in insulin signal pathway of Type 2 DM pathogenesis (Karolina *et al*., 2011). In addition, miR-103 and miR-224 were shown to be upregulated in the serum of MODY carriers comparing them with healthy people (Bonner *et al*., 2013).

1.6 Aim of the Study

HNF1A is a protein that is responsible for regulation of many genes which are involved in molecular pathways such as glucose metabolism and insulin transport. Mutations in *HNF1A* gene were correlated with MODY3 disease which is the most common form of MODY. MODY is a form of diabetes that is often confused with Type 1 and 2 DM since the differential diagnosis of this disease has not been discovered yet.

Studies on Type 1 and 2 DM have shown that miRNAs might be used as biomarkers for the clinical diagnosis of DM. miR-144 was shown to be an effective biomarker in insulin signal pathway of Type 2 DM pathogenesis (Karolina *et al*., 2011). Also, recent studies provided ideas that miRNAs might play an important role in the diagnosis of MODY such as miR-103 and miR-224 (Bonner *et al*., 2013).

The aim of the present study is to identify the miRNAs regulated by HNF1A transcription factor. For this purpose, the *HNF1A* gene will be overexpressed and silenced in MIN6 cells. miRNA molecules that are differentially expressed in the *HNF1A*-overexpressed and silenced MIN6 cells will be determined by miRNA sequencing.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory equipments

The laboratory equipment that was used in this study is shown in Table 2.1.

Table 2.1 : Laboratory equipment.

Equipment	Supplier	Product Number
Freezer (-80°C)	New Brunswick	U410
Freezers (-20°C, +4°C)	Arçelik	7273220104
Ice machine	Scotsman	AF 80
Microwave	Arçelik	MD564
Vortex	Dragon Lab	MX-S
Water Bath	Wisd	1068
Incubator	Nüve	EN 400
Shaking Incubator	Heildoph	Unimax100
Incubator with CO_2	MMM Group	LSIK/ICV111
Inverted Light Microscope	Olympus	CKX41
Thermal Cycler	BioRad	T100

Table 2.1 (cont'd) : Laboratory equipment.

UV görüntüleme cihazı	UVITec Cambridge	11200152
Microcentrifuge	Sigma	1-15PK
Highspeed centrifuge	Beckman Coulter	Allegra X-30R and 64R
LightCycler ® Nano Instrument	Roche Life Science	06407773001
NanoDrop 2000 Spectrophotometer	Thermo Scientific	NanoDrop 2000
Spectrophotometer	Jenway	6300/05/20D
Hemacytometer	Sigma-Aldrich	Z359629
Biosafety Cabinet	TezSan	Class II
Autoclave	Nüve	OT 40L
Magnetic stirrer	Wisd	MSH-20A
Dry bath incubator	Major Science	MC-01N-01S
pH meter	Hanna Instruments	HI2211
Distilled water system	ELGA	Purelab Option-Q, DV25
LightCycler ® 8-Tube strips	Roche Life Science	06612601001
iblot™ Gel Transfer Device	Invitrogen	IB1001EU
Powerease® 90W	Life Technologies	PS0091
Bolt™ Mini Gel Tank	Life Technologies	B4477599

2.1.2 Commercial kits and reagents

In Table 2.2, commercial kits used in the study are shown.

Table 2.2 : Commercial kits and reagents.

Product Name	Supplier	Product Number
X-tremeGENE HP DNA Transfection Reagent	Roche Life Science	06366236001
Genopure Plasmid Midi Kit	Roche Life Science	03143414001
Lipofectamine® RNAiMAX Reagent	Life Technologies	13778150
mirVana miRNA Isolation Kit, with phenol	Life Technologies	AM1560
Transcriptor First Strand cDNA Synthesis Kit	Roche Life Science	04374012001
FastStart Essential DNA Probes Master	Roche Life Science	06402682001
RealTime ready Custom Single Assays	Roche Life Science	05583055001
RealTime ready Custom Single Assays	Roche Life Science	05532957001
Opti-4CN™ Substrate Kit	BioRad	170-8235

2.1.3 Chemicals and enzymes

Below chemicals and enzymes used in this study are listed (Table 2.3).

Table 2.3 : Chemicals and enzymes.

Product Name	Supplier	Product Number
Bolt 4-12% Bis-Tris Plus	Life Technologies	BG04120BOX
iblot® Gel Transfer Stacks nitrocellulose, Regular	Life Technologies	IB301001
Seeblue Plus Prestained Protein Standard	Life Technologies	1368755
Bolt™ Sample Reducing Agent (10X)	Novex	B0009
LDS Sample Buffer (4X)	Novex	B0007
Rabbit polyclonal HNF1A antibody	Santa Cruz	sc-10791
Rabbit monoclonal β-actin antibody	Cell Signaling	4967S
Ethanol	Sigma-Aldrich	32205
Isopropanol	Sigma-Aldrich	W292907
DMSO	Sigma-Aldrich	472301
HCl	Applichem	A3452,0250
NaOH	Applichem	A1432,1000
Ampicilin	Sigma-Aldrich	A0166-5G
PBS	Gibco	10010023
Trypsin-EDTA	Gibco	25300054

Table 2.3 (cont'd) : Chemicals and enzymes.

DMEM	Gibco	41966-029
Opti-MEM	Gibco	31985062
FBS	Gibco	16000044
L-glutamine	Gibco	25030-081
Penicilin/Streptomycin	Gibco	15070063
B-Mercaptoethanol	Gibco	21985-023
Tween-20	AppliChem	A4974,0100
BSA	AppliChem	A7252,0500
Skimmed milk powder	OXOID	LP0031
Agar	Merck	1.01614

2.1.4 Buffers and solutions

2.1.4.1 Tris-EDTA buffer

Tris-EDTA (TE) buffer was used in plasmid isolation as an elution buffer. HCl and NaOH were used for adjusting the pH values of Tris and EDTA to 8 using pH meter (Table 2.4).

Table 2.4 : TE buffer contents.

Ingredient	**Volume (ml)**
Tris-HCl, 1M pH 8.0	5
EDTA, 0.5M pH 8.0	1
dH_2O	496

2.1.5 Cell line

Pancreatic β-cell line MIN6 which is immortalized by SV40 T-antigen was used in this study. They are pancreatic β-cells derived from mouse pancreas. MIN6 cells are adherent and are capable of secreting insulin when they are exposed to high dosage of glucose.

2.1.6 Bacterial strain

E. coli, One Shot® OmniMAX™ 2 T1R strain was used in this study.

2.1.7 Expression vector

The expression vector expressing wild type human HNF1A gene was kindly provided by Dr. Eiji Kawasaki from University of Nagasaki, Japan.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Stock cell transfer to the culture flask

The frozen stock of MIN6 cells was taken out of -80°C and thawed in water bath that was adjusted to 37°C. After that, cells were taken into the 50 ml falcon tube, diluted with MIN6 medium, and centrifuged for 10 minute (min) at 900 x g. Pellet was resuspended in MIN6 medium and put into the T25 culture flask.

2.2.1.2 Cell culture media

Cells were grown in DMEM supplemented with FBS, L-glutamine, Penicilin/Streptomycin, and β-Mercaptoethanol as shown in Table 2.5.

Table 2.5 : Contents of MIN6 media.

Ingredient	Volume(ml)
DMEM	450
L-Glutamine	1
FBS (15%)	75
Penicilin/Streptomycin	5
B-Mercaptoethanol	0.45

2.2.1.3 Cell passage

After cells were reached 90% confluency, they were transferred to new culture flasks. First, culture medium was discarded and cells were rinsed with 3 ml PBS. After discarding PBS, cells were trypsinized with 2 ml trypsin-EDTA and incubated for 2 min at 37°C. Trypsinization was inhibited after adding 10 ml medium. Cells were taken into falcon tubes and pelleted for 10 min at 900 x g. Pellet was resuspended in 10 ml medium and poured into new culture flasks.

2.2.1.4 Cell counting

Cells were counted using hemacytometer. When the cell suspension was obtained during cell passaging, 10 µl of suspension was put onto the hemacytometer. Number of cells per squares was counted and cell number was calculated using the equation below.

Cell number per ml= Amount of counted cells x 10^4 (constant number) **(2.1)**

2.2.1.5 Cell freezing

After cell passage, some of the cell suspension was supplied with 5 % DMSO and transferred to cryogenic storage tubes. Tubes were stored at –80°C.

2.2.2 Bacterial culture media

Luria broth (LB) was used as the bacterial culture medium. Trypton, yeast extract, and NaCl were dissolved in dH_2O using the amounts shown in Table 2.6. Prepared LB medium was sterilized by autoclaving for 20 min at 121°C. After the solution cooled down, 1 ml of 100 mg/ml ampicilin was added.

In order to prepare LB agar plates, 15 g/L agar was added to the LB medium and the mixture was sterilized by autoclaving for 20 min at 121°C. After the solution cooled down, 1 ml of 100 mg/ml ampicilin was added. Then, the solution was poured into cell culture dishes and culture dishes were stored at 4°C after they solidified.

Table 2.6 : Contents of LB media.

Ingredient	Amount
Trypton	10 g
Yeast extract	5 g
NaCl	10 g
dH_2O	Up to 1 L

2.2.3 Determination of nucleic acid concentration

Nucleic acid concentrations and purities were assessed via Thermo Scientific Nanodrop 2000 spectrophotometer.

2.2.4 Transformation of competent cells

HNF1A-cDNA plasmid was transformed to and grown in One Shot OmniMAX2 T1 phage-resistant cells as described below:

a. Competent cells were thawed on ice.
b. 5 µl plasmid DNA was added to the cells, and the mixture was incubated on ice for 30 min.
c. Cells were heat-shocked for 30 seconds (sec) in water bath equilibrated to 42°C.
d. Cells were removed from water bath, put back on ince and incubated for 2 min.
e. 250 µl S.O.C. medium was added to the mixture.
f. Vial was capped and shaken horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
g. The transformation mix was diluted with LB medium as 1:50 and 50 µl of the mix was spreaded to the LB agar plate supplied with ampicillin.
h. Plates were incubated at 37°C overnight.

2.2.5 Plasmid preparation

2.2.5.1 Starter culture

5 ml LB medium supplied with 5 µl of 100 mg/ml ampicilin poured into a 15 ml falcon tube. A single bacterial colony was selected from the plate involving transformed bacteria and taken into LB medium. Tube was loosely capped and incubated for 16 hours at 37°C with vigorous shaking at 220 rpm.

2.2.5.2 Isolation culture

1 ml of starter culture and 40 ml LB medium were mixed in a 50 ml falcon tube. Mixture was incubated for 16 hours at 37°C with vigorous shaking at 220 rpm.

2.2.5.3 Plasmid isolation

Plasmid DNA was isolated with Genopure Plasmid Midi Kit, Roche Life Science using the manufacturer's protocol for low copy number plasmids as described below:

a. Before beginning, centrifuge temperature was adjusted to 4°C and all centrifuges were carried out in this temperature.

b. Isolation culture was put into Beckman centrifuge tube and sedimented for 10 min at 4000 x g.

c. Supernatant was discarded and pellet was resuspended in 4 ml suspension buffer+RNAse.

d. 4 ml lysis buffer was added to the cell suspension and mixed gently by inverting the tube 6 times. Mixture was incubated for 2 min at room temperature (RT).

e. 4 ml chilled neutralization buffer was added and the solution was mixed by inverting the tube 6 times. When a homogenous suspension was formed, the tube was put on ice and incubated for 5 min.

f. The solution was centrifuged for 45 min at 13000 x g.

g. During centrifugation, the column was placed into a collection tube and equilibrated with 2.5 ml equilibration buffer. Column was allowed to empty by gravity flow and flow through was discarded.

h. Supernatant obtained from centrifugation was removed from the tube and loaded onto the equilibrated column. Column was allowed to empty by gravity flow.

i. Flow through was collected and loaded a second time onto the column. Column was allowed to empty by gravity flow and flow through was discarded.

j. The column was washed with 4 ml washing buffer. Column was allowed to empty by gravity flow and flow through was discarded.

k. The column was washed with 4 ml washing buffer again. Column was allowed to empty by gravity flow and flow through was discarded.

l. The column was washed a third time with 4 ml washing buffer. Column was allowed to empty by gravity flow. Flow through and collection tube were discarded.

m. Column was re-inserted into a Beckman centrifuge tube and the plasmid was eluted with 2.5 ml prewarmed elution buffer. Column was allowed to empty by gravity flow and flow through was collected.

n. Plasmid was eluted a second time with 2.5 ml prewarmed elution buffer. Column was allowed to empty by gravity flow and eluates were combined.

o. Eluted plasmid DNA was precipitated with 3.6 ml isopropanol and the mixture was centrifuged for 105 min at 13682 x g.

p. Supernatant was discarded and pellet was washed with 3 ml chilled 70% ethanol. The mixture was centrifuged for 20 min at 13682 x g.

q. Supernatant was discarded and pellet was air-dried for an hour.

r. The plasmid DNA pellet was dissolved in 30 µl TE buffer.

s. Concentration of plasmid DNA was measured with Nanodrop 2000 spectrophotometer and DNA was stored at -20°C for further experiments.

2.2.5.4 DNA sequencing

Purified plasmid samples were sent to BGI company for sequencing to verify that the target DNA was obtained.

2.2.6 Transfection of MIN6 cells for *HNF1A* overexpression

$6x10^5$ MIN6 cells were seeded to 6-well plates and grown overnight. After that, cells were transfected with the expression vector to overexpress *HNF1A* gene using Xtregene gene HP transfection reagent, Roche Life Science as described below:

a. All materials were equilibrated to RT.

b. 200 µl DMEM, 2 µl of 1 µg plasmid DNA, and 6 µl transfection reagent were were mixed in a 1.5 ml eppendorf tube. They were incubated for 20 min at RT.

c. The mixture was dropwisely added to the cells.

d. Transfected cells were incubated for 48 hours.

2.2.7 Transfection of MIN6 cells for *HNF1A* silencing

$6x10^5$ MIN6 cells were seeded to 6-well plates and grown overnight. After that, cells were transfected with HNF1A-specific siRNA to silence the expression of *HNF1A* using Lipofectamine RNAimax reagent as described below:

a. Mixture-1: 4.5 µl siRNA and 150 µl Opti-MEM were mixed in a 1.5 ml microcentrifuge tube.

b. Mixture-2: 9 µl Lipofectamine reagent and 150 µl Opti-MEM were mixed in a 1.5 ml microcentrifuge tube.

c. Mixture 1 and 2 were mixed as 1:1 ratio (150 µl:150 µl).

d. siRNA-reagent complex were incubated for 5 min at RT and then, 250 µl of the mixture was dropwisely added to the cells.

e. Transfected cells were incubated for 24 hours.

2.2.8 RNA isolation

Total RNA was isolated from transfected cells using mirVana™ miRNA isolation kit as described below:

a. Cell culture medium was discarded from the plate and cells were rinsed with 2 ml PBS.

b. PBS was discarded from the plate and cells were lysed using 600 µl lysis buffer.

c. Lysed cells were collected with a cell scraper, pipetted into a 1.5 ml microcentrifuge tube, and vortexed until a homogenous lysate were obtained.

d. miRNA homogenate additive was added to the lysate and the mixture was vortexed.

e. The mixture was incubated on ice for 10 min.

f. Acid-Phenol:Chloroform was added to the mixture.

g. The mixture was vortexed for 30 sec.

h. The mixture was centrifuged for 5 min at 10000 x g to separate the aqueous and organic phases.

i. After centrifugation, aqueous phase were removed without disturbing the organic phase and transferred into a fresh 1.5 ml centrifuge tube.

j. Absolute ethanol was added to the aqueous phase.

k. A filter cartridge was placed into a collection tube supplied with the kit and lysate/ethanol mixture was pipetted onto this filter cartridge.

l. The mixture was centrifuged for 20 sec at 10000 x g and flow-through was discarded.

m. miRNA wash solution 1 was applied to the filter cartridge and centrifuged for 10 sec at 10000 x g. Flow-through was discarded.

n. Wash solution 2/3 was applied to the filter cartridge and centrifuged for 10 sec at 10000 x g. Flow-through was discarded.

o. The previous step was repeated.

p. After discarding the flow-through from the last wash, the filter cartridge was replaced in the same collection tube.

q. The assembly was spinned for 1 min at 12000 x g to remove residual fluid from the filter.

r. Elution buffer pre-heated to 95°C was applied to the center of the filter and centrifuged for 30 sec at 18000 x g.

s. Eluate was collected and stored at -80°C for further experiments.

2.2.9 cDNA synthesis

As a first step of qRT-PCR, cDNA was synthesized from total RNA that was extracted from transfected cells. Synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit, Roche and the protocol is as follows:

a. Components shown in Table 2.7 were mixed in a PCR tube and incubated for 10 min at 65°C in BioRad thermal cycler to ensure that possible RNA secondary structures were denatured.

Table 2.7 : Template-primer mix.

Component	Volume (µl)	Final Concentration
Total RNA	variable	1 µg
Anchored-oligo $(dT)_{18}$ primer	1	2.5 µM
Random hexamer primer	2	60 µM
Water, PCR grade	variable	to make total volume=13 µl
Total	**13**	

b. After denaturation, the mixture were immediately put on ice.

c. As a second step for the synthesis, components given in Table 2.8 were added to the mixture.

Table 2.8 : Reverse transcription mix.

Component	Volume (µl)	Final Concentration
Transcriptor Reverse Transcriptase Reaction Buffer, 5X	4	1X (8 mM $MgCl_2$)
Protector RNase Inhibitor, 40 U/µl	0.5	20 U
Deoxynucleotide Mix, 10 mM each	2	1 mM each
Transcriptor Reverse Transcriptase, 20 U/µl	0.5	10 U
Final	**20**	

d. cDNA was synthesized according to the program shown in Table 2.9.

Table 2.9 : Reverse transcription program.

Temperature (°C)	**Time (min)**
25	10
55	30
85	5

e. cDNA was stored at -20°C for further experiments.

2.2.10 Quantitative real-time PCR

In order to confirm that overexpression and silencing of HNF1A in MIN6 cells were accomplished, HNF1A expression was assessed using quantitative Realtime PCR (qRT-PCR) which is a technique that allows simultaneous amplification and quantification of a targeted DNA molecule. HNF1A expression in transfected MIN6 cells were analysed with qRT-PCR based on TaqMan probe system. Reaction was performed using FastStart Essential DNA Probes Master, Roche and RealTime ready Custom Single Assay, Roche. To determine the expression level of HNF1A, human HNF1A assay (ID:112176) for HNF1A plasmid and mouse HNF1A assay (ID:316865) for internal expression of HNF1A were used (Table2.10). Also mouse GAPDH assay (ID:307884) were utilized for normalization.

Table 2.10 : HNF1A assays.

Assay	**Amplicon Start**	**Amplicon End**	**Complementarity with Human HNF1A**	**Complementarity with Mouse HNF1A**
Human HNF1A	1659	1747	100%	91%
Mouse HNF1A	1968	2070	85%	100%

qRT-PCR was performed in LightCycler ® Nano Instrument using the program shown in Table 2.11.

Table 2.11 : qRT-PCR program.

Step	Temperature (°C)	Time (sec)	Cycle
Initial Denaturation	95	600	1
Denaturation	95	20	45
Amplification	60	40	

2.2.11 Western blot analysis

For validation of *HNF1A* overexpression and silencing in protein level, western blot analysis was carried out using Bolt® Welcome Pack + iBlot® 2 System, invitrogen. MIN6 cells were transfected with *HNF1A* expression vector, HNF1A-siRNA, or an empty plasmid (mock) as described in section 2.2.6 and 2.2.7.

2.2.11.1 Cell lysis

Cells were lysed using RIPA Lysis Buffer, Santa Cruz. Complete lysis buffer was prepared according to Table 2.11.

Table 2.12 : Complete RIPA buffer.

Content	Volume (µl)
PMSF solution	10
Sodium orthovanadate solution	10
Protease inhibitor	15
1X RIPA Lysis buffer	1000

a. Plates were placed on ice and cell media were discarded.

b. Cells were washed with 1 ml PBS twice.

c. 300 µl complete RIPA buffer was put on wells and cells were incubated on ice for 20 min in a shaking incubator at 15000 rpm.

d. After incubation, lysed cells were transferred to the 1.5 ml centrifuged tubes using cell scrapers and centrifuged at 4°C for 10 min at 10000 x g.

e. Supernatants were carefully taken to the fresh 1.5 ml centrifuge tubes without disturbing pellets and stored at -80°C.

2.2.11.2 Determination of protein concentration

Protein concentration of cell lysates were measured with Jenway spectrophotometer using Bradford Assay according to the manufacturer's instructions.

2.2.11.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is an electrophoresis technique that is used for separation of proteins according to electrophoretic mobility. SDS is used for disruption of tertiary structures of proteins to separate them according to their size.

a. Samples were prepared as shown in Table 2.13 and incubated for 5 min at 95°C in to prevent possible secondary structures.

Table 2.13 : Loading mixture.

Sample amount	LDS Buffer (4X)	Sample Reducing Agent (10X)	dH_2O
50 µg/well	15 µl	6 µl	Up to 60 µl

b. Samples were loaded on the 4-12 % bis-tris gradient gel,invitrogen and run in 1X MES running buffer, invitrogen for 30 min at 165 volt.

2.2.11.4 Western blot

a. After running was finished, polyacrylamide gel was taken out of the cassette and placed into the transfer sandwich composed of a nitrocellulose membrane, filter papers, anode, and cathode.

b. The sandwich was placed in the dry blotting device and proteins were transferred to the membrane for 7 min.

c. After transfer, membrane was put into PBST and washed for 5 min in a shaking incubator.

d. Membrane was put into the blocking solution composed of 5% milk powder in PBST at RT for 1 hour in a shaking incubator.

e. Membrane was washed with PBST twice for 5 min in a shaking incubator.

f. Membrane was incubated for 1 hour at RT with primary antibody solutions which are 1:50 polyclonal rabbit anti-HNF1A or monoclonal β-actin in PBST in a shaking incubator.

g. After primary antibody incubation, membrane was washed three times with PBST for 5 min and transferred to the secondary antibody solution which is a HRP-conjugated goat anti-rabbit antibody diluted as 1:5000 in PBST-1%BSA. Membrane was incubated gently in a shaking incubator for 1 hour at RT.

h. After secondary antibody incubation was finished, membrane was washed three times with PBST for 5 min in a shaking incubator.

i. For detection, Opti-4CN Detection Kit, goat anti-rabbit, BioRad was used.

j. 9 ml distilled water, 1 ml Opti-4CN diluent, and 200 µl Opti-4CN substrate were mixed and poured onto the membrane.

k. Membrane was incubated gently in a shaking incubator until proteins become visible.

l. After proteins are detected visually, detection mixture was discarded and membrane was washed with distilled water for 15 min.

m. Water was discarded and photographs were taken. Band densities were measured using ImageJ software.

2.2.12 Next generation sequencing of total RNA samples

After veryfing overexpression and silencing of HNF1A in MIN6 cells, total RNA samples isolated from transfected cells were sent to BGI, South Korea for miRNA sequencing. Illumina Truseq techology were used (Figure 2.1). In this process, small RNAs were extracted from total RNAs using PAGE separation method. Then, 5' and 3' adaptors were ligated to these small RNAs and RNAs were reverse transcribed into cDNAs. Those cDNAs were amplified using PCR and purified for sequencing experiment. Sequencing was made using HiSeq 2500.

In order to obtain high quality data, filtering steps were applied. In these steps, unattached tags, oversized insertions, poly A reads, and reads shorter than 18 nucleotides were eliminated.

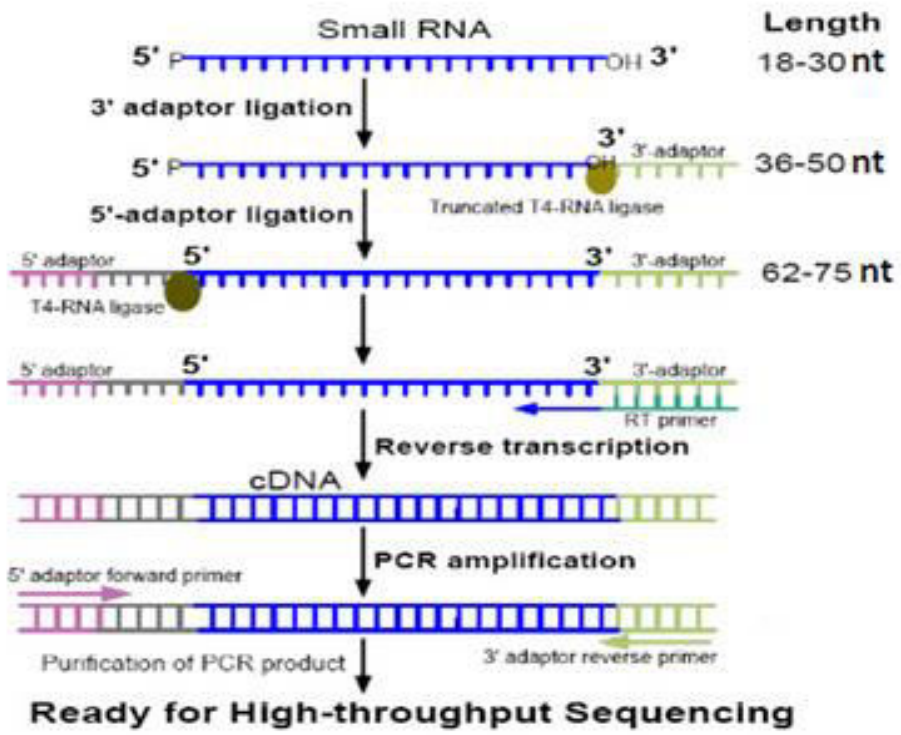

Figure 2.1 : Truseq experiment process.

3. RESULTS AND DISCUSSION

3.1 Verification of HNF1A Expression Vector

Plasmid DNA which will be used to overexpress *HNF1A* in MIN6 cells was isolated as described in Section 2.2.5 and sent to BGI, South Korea for DNA sequencing. Sequence analysis confirmed that the expression vector contains wild type human *HNF1A* cDNA.

3.2 RNA Isolation

Total RNAs isolated from *HNF1A*-overexpressed and *HNF1A*-silenced MIN6 cells were measured using Nano Drop 2000 spectrophotometer, Thermo Fisher Scientific and the results were shown in Table 3.1. According to the literature, the absorbance ratio of A_{260}/A_{280} of a pure RNA should be around 2,00, whereas the ratio of A_{260}/A_{230} should be higher than 1,80. Results showed that total RNAs have values in expected range and could be used for further analysis.

Table 3.1 : RNA isolation results (O:Overexpression, C:Control, S:Silencing).

Samples	**O1**	**O2**	**O3**	**S1**	**S2**	**S3**	**C**
Conc(ng/µl)	245	219	267	247	206	175	482,7
A_{260}/A_{280}	2,08	2,30	2,10	2,05	2,03	2,35	2,03
A_{260}/A_{230}	2,19	1,98	1,91	2,20	1,92	2,35	2,08

3.3 Quantitative Real-Time PCR

To verify overexpression and silencing of *HNF1A* gene in MIN6 cells, qRT-PCR was performed as described in Section 2.2.9.2. Ratios were calculated using the Ct values of qPCR results (Figure 3.1, 3.2, and 3.2) and equations below. *HNF1A* levels were normalized to *GAPDH*. Transfection was performed three times and qRT-PCR

experiments were run in duplicate. Statistical analyses were made by a paired *t* test using Graphpad software. $P < 0,05$ was considered statistically significant.

$$\Delta Ct = (Ct \text{ value of target gene} - Ct \text{ value of reference gene}) \quad \textbf{(3.1)}$$

$$\text{Relative Ratio} = 2^{-(\Delta Ct \text{ of target sample} - \Delta Ct \text{ of control sample})} \quad \textbf{(3.2)}$$

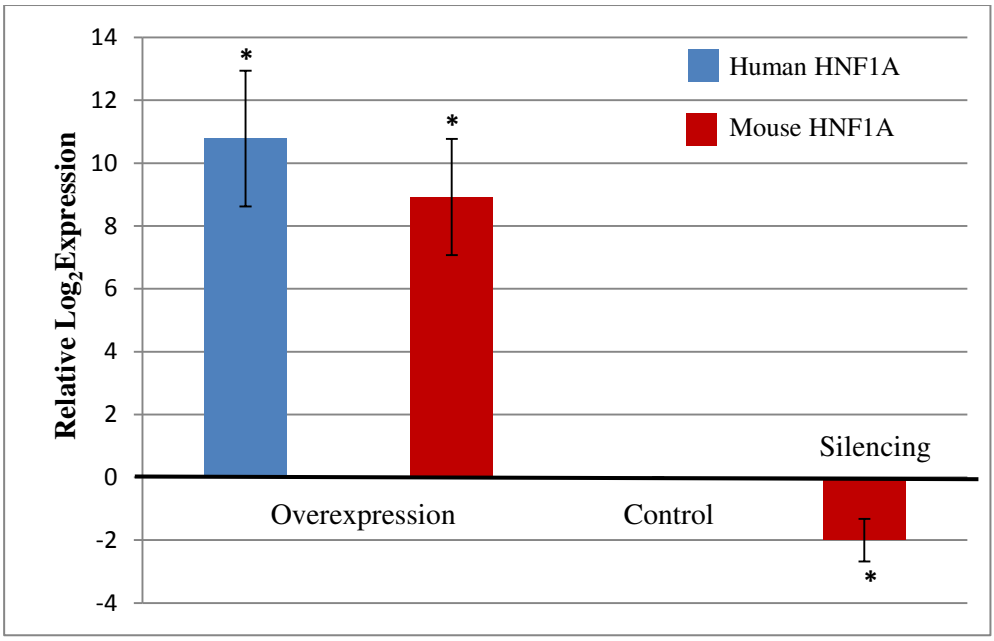

Figure 3.1 : Relative $\log_2$Expression of human and mouse HNF1A in MIN6 cells. n=3, * $P < 0,05$.

Figure 3.1 illustrates the expression of *HNF1A* in MIN6 cells. Both human and mouse *HNF1A* levels were significantly increased in overxpression group, but decreased in silencing group, comparing with control group. Also, there was no sign of human *HNF1A* expression in control group which demonstrates that the overexpression of HNF1A was due to the transfection of mouse-derived MIN6 cells with human *HNF1A* cDNA expression vector. On the other hand, increase of mouse *HNF1A* levels in overexpression group rose a question whether the homology between mouse and human *HNF1A* could be the reason. Also, the positive feedback mechanism between *HNF1A* and *HNF4A* might caused the significant increase in mouse *HNF1A* level.

As stated in Section 2.2.10, two different assays were used to determine *HNF1A* levels; one that contains mouse *HNF1A* specific primers and probes to measure endogenous expression in cells, and another that has human HNF1A specific system for observing plasmid expression. The mouse HNF1A assay enables the amplification of the 1968-2070 site in transcript and this site has 85% similarity with

the same site in human HNF1A transcript (see Table 2.10). So that primers and probes might also be interacting with also human HNF1A as they amplified mouse HNF1A, therefore, causing mouse HNF1A levels to increase significantly.

3.4 Western Blot

HNF1A overexpression and silencing in MIN6 cells were also validated with western blot in protein level as described in Section 2.2.11. Experiments were repeated twice. As a first step, transfected and untransfected cells were lysed and protein concentrations were measured with Bradford assay as described in section 2.2.11 (Table 3.2).

Tablo 3.2 : Protein concentrations. O1, O2: Overexpression, M1, M2: Mock, C1, C2: Control, S1, S2: Silencing.

Name	O1	M1	C1	S1	O2	M2	C2	S2
Concentration (µg/µl)	1,93	1,76	2,51	1,82	2,21	1,92	2,04	1,85

HNF1A protein levels (67,325 kDa) were measured by normalizing them to β-actin which is a housekeeping protein (Figure 3.2).

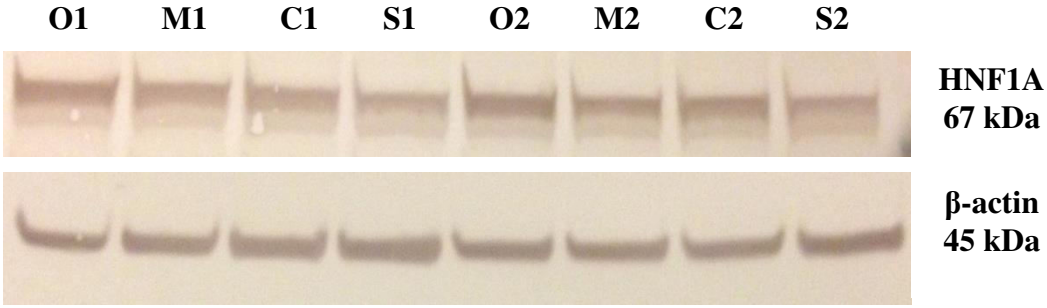

Figure 3.2 : HNF1A and β-actin levels. O1, O2: Overexpression, M1, M2: Mock, C1, C2: Control, S1, S2: Silencing.

As it can be seen in Figure 3.2, the bands of overexpression samples are much denser than mock, control, and silencing samples which suggest higher HNF1A protein expression, while β-actin densities look similar verifying that loading amounts of proteins are almost same.

Protein densities on the membrane were measured using ImageJ software to have quantitive information about the expression levels of HNF1A. β-actin levels were

used for normalization of the data (Figure 3.3). Results are shown in Figure 3.3 and each bar in the graph represents average of two measurements.

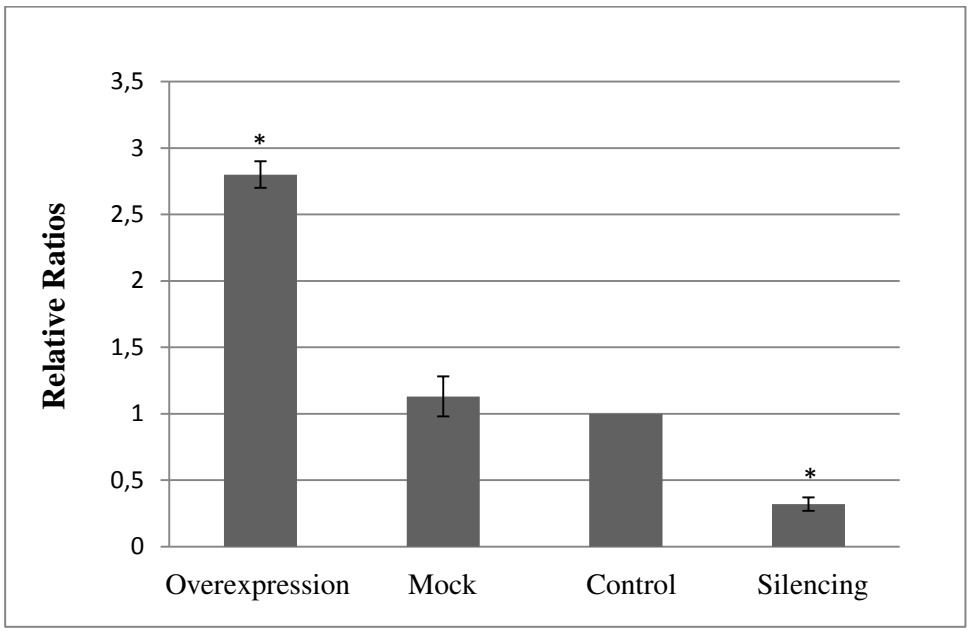

Figure 3.3 : Normalized HNF1A protein levels. n=2, * $P < 0,05$. Mock: transfection of cells with an empty vector.

Relative ratios that measured after normalization with β-actin indicate higher HNF1A expression in overexpression samples and less HNF1A expression in silencing comparing them with control group, although the expression difference between overexpression and silencing samples were not as much as the difference that was shown with qRT-PCR.

3.5 miRNA Sequencing Analysis

After verifying the overexpression and silencing of *HNF1A* gene in MIN6 cells, isolated total RNA samples were sent to BGI, South Korea for miRNA sequencing. Total RNAs isolated from three of overexpression and silencing group were sequenced. RNAs that are 18-30 nucleotide in length were considered as small RNAs. Also additional filtering was applied to have high quality reads (Table 3.3). After applying filtering steps, 97.29% of the data were described as clean reads.

Table 3.3 : Data quality.

Type	Count	Percent (%)
Total reads	11841971	100
Unattached 5' adaptor	40273	0.34
Unattached 3' adaptor	46179	0.39
Inserts without tags	12425	0.10
RNAs smaller than 18 nucleotide	222436	1.88
PolyA	30	0.00
Clean reads	11520628	97.29

Differential expression analysis was made by comparing silencing and overexpression sequencing data. 238 known miRNAs which were identified from miRBase database and 91 novel miRNAs that were predicted by MIREAP and Mirdeep softwares were found to have significant differential expression. According to Kyoto Encyclopedia of Genes and Genoms (KEGG) database, known miRNAs that were differentially expressed associated with molecular mechanisms such as Mitogen-Activated Protein Kinase (MAPK) signaling pathway, regulation of actin cytoskeleton, endocytosis, metabolic pathways and cancer (Figure 3.4).

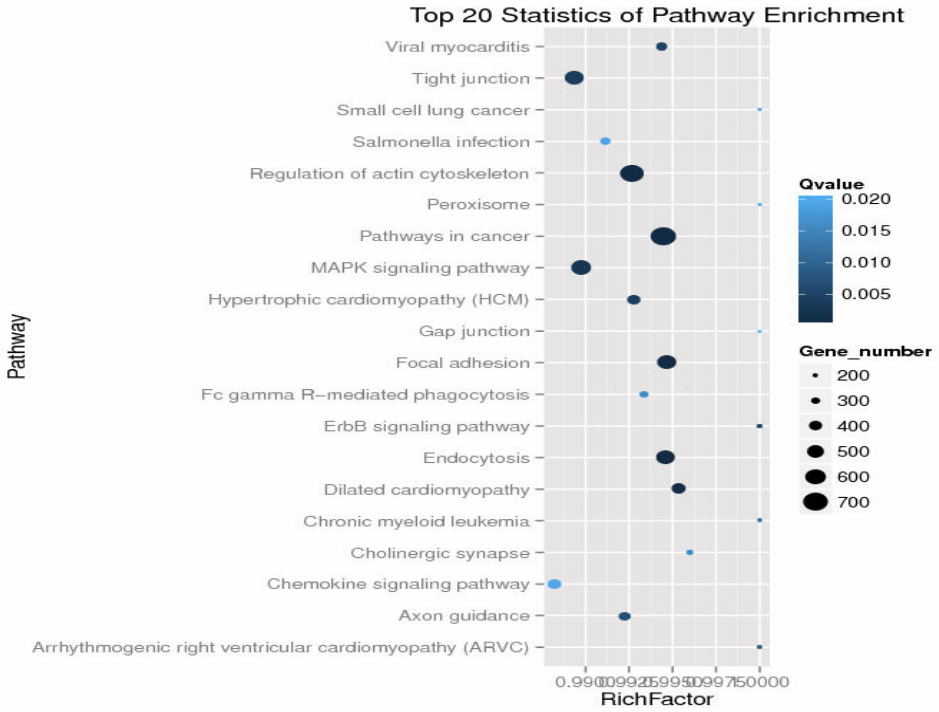

Figure 3.4: KEGG pathway analysis of known miRNAs having differential expression. Bigger rich factor means there is more obvious enrichment, whereas smaller Q value is an indication of more significant result.

In order to have more valid data, additional filtering steps were applied to the miRNA sequencing analysis. miRNAs having log_2fold change lower than 1 were eliminated. After that, miRNAs that have the most significant ($P < 0,05$) differential expression were selected. As mentioned before, three overexpression and three silencing samples were sequenced and each sample was compared to each other in the differential expression analysis. miRNAs that have significant differential expression in each comparison were determined. miRNAs that passed through these filtering steps were shown in Table 3.4.

Table 3.4 : Fold change and *p* values of known miRNAs having the most significant differential expression. *P* values smaller than 1e-350 are shown as 0.

Name	Log_2Fold Change (Overexpression/Silencing)	*P* value
miR-129-1-3p	2,63	0
miR-129-2-3p	2,87	0
miR-299a-5p	3,60	2,76e-27
let-7b-3p	2,46	7,6e-15
miR-378a-5p	2,24	6,7e-30
miR-1306-5p	1,88	1,32e-44
miR-3072-3p	2,41	7,74e-37
miR-8112	1,91	4,48e-35
miR-324-5p	2,26	2,12e-26
miR-1983	2,01	2,73e-3

A relative expression graph was created by normalizing overexpression results to silencing (Figure 3.5).

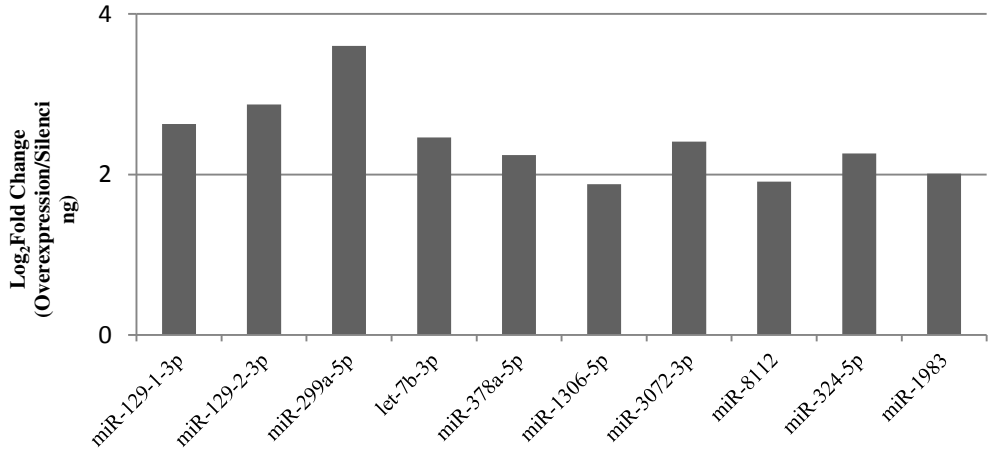

Figure 3.5 : Levels of known miRNAs having the most significant differential expression.

Among the miRNAs shown in Figure 3.5 miR-3072-3p, miR-8112, and miR-1983 are not present in humans. miR-1306-5p and miR-324-5p, on the other hand are conserved in both human and mouse. They are recently identified, therefore there are only a few study about their function. None of these studies implicates a relation with diabetes. The rest 5 miRNAs that were determined after filtering were shown to have strong association with diabetes and/or metabolic pathways related with diabetes (Table 3.5).

Table 3.5 : Differentially expressed miRNAs correlated with diabetes.

Name	Association with metabolic pathways
miR-129-1-3p	Glucose metabolism
miR-129-2-3p	Glucose metabolism
miR-200b-3p	Insulin signaling, diabetic neuropathy
miR-296-3p	Glucose-related miRNAs
miR-378a-5p	Insulin signaling

According to a study, overexpression of miR-378a-5p was shown to display hepatic insulin resistance by blocking insulin signaling through targeting *p110α* which is a critical component of insulin signaling (Liu *et al*., 2014). This indicates that it might be a key player in diabetes and also in MODY since its expression is controlled by HNF1A transcription factor according to the findings in this study. Another miRNA, miR-200b-3p, which was differentially expressed in *HNF1A*-overexpressed and silenced MIN6 cells, was associated with diabetic nephropathy by inhibiting the expression of FOG2 protein. FOG2 blocks the function of Akt which is a kinase that induces cell growth. Inhibiton of *FOG2* gene by miR-200b-3p prevent its blocking effect on Akt which can lead to increase in cell volume in nephrons, therefore causing diabetic nephropathy (Park *et al*., 2013). Also, miR-200b-3p was reported to facilitate the insuling signaling by blocking FOG2 (Hyun *et al*., 2009). This might be an indication of an indirect inhibiton of HNF1A on FOG2 by enhancing the expression of miR-200b-3p, thus facilitating insuling signaling since the expression of miR-200b-3p in HNF1A-overexpressed cells is higher than HNF1A-silenced cells.

These findings make miR-200b-3p a possible player in the molecular pathogenesis of MODY.

Among the differentially expressed miRNAs, miR-129 and miR-296 were shown to exhibit a role in glucose metabolism in earlier studies (Raitoharju *et* al., 2014; Tang *et al*., 2014). miR-296 was described as a glucose-related miRNA due to its downregulation when the cells are treated with high dosage of glucose. Insulin production and secretion are tightly controlled in cells by changes in glucose level so that miR-296 might be involved in the progression of diabetes as a glucose-related miRNA as well as it can take part in the molecular pathogenesis of MODY as an HNF1A-related miRNA.

91 new miRNAs predicted by MIREAP and Mirdeep softwares were identified as differentially expressed novel miRNAs in the presence of overexpression and silencing of HNF1A. Same filtering steps were applied to these miRNAs as they were applied to the known miRNAs. After that, remaining novel miRNAs were determined (Table 3.6).

Table 3.6 : Fold change and *p* values of novel miRNAs having the most significant differential expression. *P* values smaller than 1e-350 are shown as 0.

Name	**Log_2Fold Change (Overexpression/Silencing)**	***P* value**
novel_miR-3	-1,75	5,34e-25
novel_miR-43	-2,19	5,69e-8
novel_miR-19	-1,31	0,0001
novel_miR-69	-1,60	0,001
novel_miR-17	1,37	0,001

A relative expression graph was created by normalizing differential expression data of novel miRNAs of *HNF1A*-overxpressed cells to the data of novel miRNAs of *HNF1A*-silenced cells (Figure 3.6).

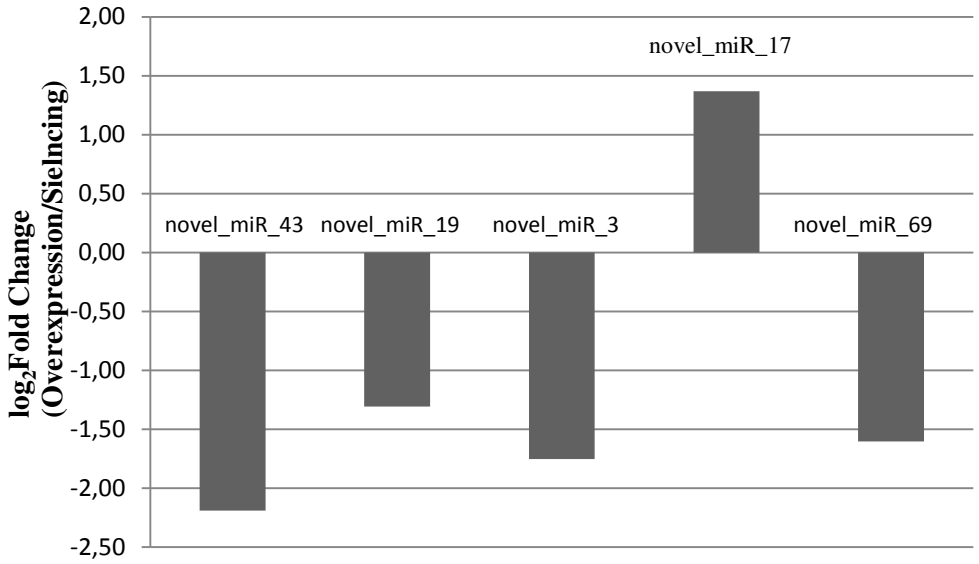

Figure 3.6: Levels of novel miRNAs having the most significant differential expression.

Predicted novel miRNAs shown in Figure 3.6 were expressed differentially, according to the changes in the expression of HNF1A transcription factor. They might be involved in metabolic pathways such as insulin production and glucose metabolism, therefore they can play key roles in diabetic pathways and pathogenesis of MODY. However, further experiments, for example qRT-PCR, should be carried out to confirm their existence as miRNAs before associating them with MODY or any other disease or pathway.

4. CONCLUSIONS AND FUTURE DIRECTIONS

MODY is a heterogenous form of diabetes that manifests loss of pancreatic β-cell function and is characterized by asymptomatic hyperglycemia and autosomal dominancy. It appears in a small part of diabetes cases; however, its pathogenesis and appearance in generations over a 20 year time period makes it an attractive model to investigate its progression. miRNA profiling in Type 1 and Type 2 DM has been investigated for a long time, but the differential expression of miRNAs in MODY and their potential to be diagnostic biomarkers are still unclear. In this study, miRNA expression profile was characterized by searching differentially expressed miRNAs in *HNF1A*-overexpressed and *HNF1A*-silenced MIN6 cells. After verifying overexpression and silencing of *HNF1A*, miRNA sequencing was carried out and comparative miRNA expression analysis was made in MIN6 cells.

According to the sequencing analysis, 238 known miRNAs were found to be differentially expressed by changes in HNF1A level. HNF1A is a transcription factor that targets many genes involved in many metabolic processes such as insulin expression, signaling and glucose transport. It is known that mutations in this gene cause MODY. miRNAs that were identified as HNF1A-related miRNAs in this study also might be involved in the progression of MODY. miR-129-1-3p, miR-129-2-3p, miR-200b-3p, miR-296-3p, miR-296-3p, and miR-378a-5p were the ones having the most significant differential expression and each of them were associated with diabetic pathways in the literature. To further support the findings of the study, differential expression levels of these miRNAs should be confirmed with further experiments. These miRNAs can also play important roles in the progression of MODY since they are regulator of many genes that are involved in diabetic pathways. Therefore, their potential to be diagnostic markers should be investigted by analyzing their levels in MODY carriers. Also, 91 new miRNAs that were predicted.to be novel should be validated with additional experiments to further define them defined as miRNAs and these miRNAs should be studied for their possible link with MODY.

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APPENDICES

APPENDIX A: Complete list of differentially expressed known miRNAs**.**

APPENDIX B: Complete list of differentially expressed novel miRNAs.

APPENDIX A

miR_name	Log2Ratio (S2,S3,S4/O1,O3,O4) normalized	*P*-value
miR-182-5p	-0,46	0
miR-375-3p	0,07	0
miR-741-3p	0,51	0
miR-26a-5p	-0,45	0
miR-30a-5p	-0,35	0
miR-92b-3p	1,2	0
miR-99b-5p	0,13	0
miR-871-3p	0,13	0
miR-434-3p	-0,44	0
let-7i-5p	-0,32	0
miR-486-5p	0,6	0
miR-3107-5p	0,6	0
miR-191-5p	-0,18	0
let-7a-5p	-0,3	0
miR-465a-3p	-0,31	0
miR-465b-3p	-0,31	0
miR-465c-3p	-0,31	0
miR-30c-5p	-0,68	0
miR-541-5p	-0,29	0
miR-25-3p	-0,36	0
miR-129-1-3p	-2,64	0
miR-129-2-3p	-2,88	0
miR-125a-5p	0,17	1,69e-307
let-7f-5p	-0,11	3,42e-266
miR-470-5p	0,08	1,21e-261
miR-186-5p	-0,34	1,26e-255
miR-378a-3p	-0,4	5,54e-251
miR-383-5p	-0,63	3,03e-241
miR-148a-3p	-0,91	2,34e-220
miR-30e-5p	-0,27	1,04e-213

miR-30d-5p	-0,2	2,36e-211
miR-22-3p	-0,18	5,99e-210
miR-183-5p	-0,42	1,29e-197
miR-127-3p	-0,08	1,38e-191
miR-301a-3p	-0,48	9,14e-188
miR-16-5p	-0,25	9,31e-184
miR-141-3p	-0,37	2,82e-182
miR-181a-5p	-0,12	1,01e-175
let-7d-3p	-1,06	3,51e-169
miR-340-5p	-0,2	2,52e-133
miR-879-5p	-1,78	1,76e-132
miR-93-5p	-0,39	4,67e-130
miR-30b-5p	-0,52	3,42e-125
miR-540-3p	0,22	8,06e-112
miR-423-5p	0,19	2,38e-111
miR-27b-3p	-0,09	7,55e-105
miR-181b-5p	0,2	2,30e-94
miR-128-3p	-0,72	2,66e-87
miR-381-3p	-0,23	1,47e-85
miR-431-5p	-0,43	5,54e-81
miR-151-3p	-0,21	8,22e-79
miR-143-3p	0,84	2,94e-78
miR-871-5p	-0,18	5,35e-76
miR-410-3p	-0,27	1,22e-74
miR-465c-5p	-0,15	1,09e-71
miR-872-3p	-0,67	3,01e-69
miR-98-5p	-0,29	4,17e-63
miR-881-3p	0,11	7,62e-63
miR-129-5p	-0,46	2,95e-61
let-7d-5p	-0,23	9,09e-61
miR-100-5p	-1,55	1,42e-60
miR-21a-5p	-0,19	2,17e-60
miR-103-3p	-0,26	1,98e-59

let-7b-5p	0,26	3,19e-57
miR-411-5p	-0,12	4,15e-57
miR-708-3p	-0,72	1,68e-51
miR-30a-3p	-0,29	1,47e-48
miR-30e-3p	-0,23	4,76e-48
miR-106b-3p	-0,35	3,19e-46
miR-1306-5p	-1,89	1,32e-44
miR-15b-5p	-0,45	1,02e-42
miR-409-5p	-0,18	1,20e-42
miR-298-5p	-0,73	6,07e-42
miR-149-5p	-0,64	4,36e-41
miR-192-5p	-0,08	4,84e-41
miR-342-3p	-0,43	1,73e-39
miR-10a-5p	-0,52	1,24e-38
let-7j	-0,35	4,73e-38
miR-3072-3p	-2,41	7,74e-37
miR-140-3p	-0,32	1,41e-35
miR-29a-3p	-0,23	3,17e-35
miR-8112	-1,92	4,48e-35
miR-484	-0,49	5,06e-35
miR-210-3p	-0,36	1,23e-33
miR-96-5p	-0,46	9,25e-33
miR-339-5p	-1,42	4,42e-31
miR-200b-3p	-1,03	1,88e-30
miR-378a-5p	-2,25	6,70e-30
miR-132-3p	-0,68	7,19e-30
miR-320-3p	-0,31	1,24e-29
miR-421-3p	-0,29	3,48e-29
miR-301b-3p	-0,61	1,31e-28
miR-7a-2-3p	-1,02	1,25e-27
miR-299a-5p	-3,6	2,76e-27
miR-324-5p	-2,26	2,12e-26
miR-465b-5p	-0,18	5,69e-26

miR-878-5p	-0,13	4,11e-25
miR-532-5p	-0,28	2,11e-24
miR-341-3p	-0,19	1,56e-23
miR-99a-5p	-0,39	2,92e-23
miR-463-5p	-0,18	3,19e-23
let-7g-5p	-0,14	3,64e-23
miR-126a-5p	-1,11	1,03e-22
miR-6240	-1,79	1,09e-22
miR-377-3p	-0,5	1,43e-22
miR-101a-3p	-0,16	2,56e-21
miR-1839-5p	-0,2	4,29e-21
miR-130a-3p	-0,17	1,01e-19
miR-667-3p	-0,6	1,39e-19
miR-666-5p	-0,36	2,03e-19
miR-136-3p	-0,16	3,73e-19
miR-483-3p	-0,91	5,50e-19
miR-107-3p	-0,26	6,63e-19
miR-344d-3p	-0,7	1,07e-18
miR-379-5p	-1,09	1,10e-18
miR-429-3p	-1,52	1,24e-18
miR-872-5p	-0,24	2,28e-18
miR-743b-3p	-0,1	2,76e-18
miR-1249-3p	-2,72	2,54e-17
miR-138-5p	-0,31	4,41e-17
miR-1224-5p	-0,25	5,66e-17
miR-325-3p	-0,53	7,21e-17
miR-26b-5p	-0,2	2,52e-16
let-7a-1-3p	-1,2	3,04e-16
let-7c-2-3p	-1,2	3,04e-16
miR-8114	-1,13	3,31e-16
miR-488-5p	-0,55	5,72e-16
miR-666-3p	-0,56	7,18e-16
miR-425-5p	-0,41	9,69e-16

miR-3473b	-2,15	1,21e-15
miR-532-3p	-1,35	2,87e-15
let-7b-3p	-2,46	7,60e-15
miR-184-3p	-0,1	1,57e-14
miR-200a-3p	-0,84	3,41e-14
miR-378c	-0,51	3,46e-14
miR-369-3p	-0,56	3,68e-14
let-7e-5p	-0,09	1,90e-13
miR-1983	-2,01	2,72e-13
miR-28a-3p	-0,52	6,75e-13
miR-488-3p	-0,43	6,82e-13
miR-376a-5p	-0,65	1,63e-12
miR-668-3p	-0,52	3,76e-12
miR-3473e	-1,93	4,80e-12
miR-152-3p	-1,14	5,93e-12
miR-574-3p	-1,35	1,37e-11
miR-671-3p	0,25	1,45e-11
miR-328-3p	-0,54	2,03e-11
miR-200c-3p	-0,61	3,91e-11
miR-3078-5p	-0,8	8,91e-11
miR-1981-3p	-1,18	1,07e-10
miR-195a-5p	-0,25	2,48e-10
miR-664-3p	-2,71	2,67e-10
miR-673-5p	-0,19	2,92e-10
miR-183-3p	-1,33	6,29e-10
miR-345-3p	-1,31	6,37e-10
miR-106b-5p	-0,33	6,88e-10
miR-7688-5p	-0,63	7,79e-10
miR-337-5p	-0,24	8,45e-10
miR-126a-3p	-1,61	1,16e-09
miR-199a-3p	-1,14	2,99e-09
miR-199b-3p	-1,14	2,99e-09
miR-329-5p	-0,54	3,92e-09

miR-880-3p	-0,32	4,71e-09
miR-877-5p	0,18	8,61e-09
miR-345-5p	-0,83	1,15e-08
miR-300-3p	-0,2	1,37e-08
miR-136-5p	-0,18	1,78e-08
miR-1199-5p	-0,74	1,96e-08
miR-98-3p	-1,06	2,10e-08
miR-1982-3p	-3,55	3,27e-08
miR-540-5p	-1,24	4,92e-08
miR-325-5p	-0,57	6,75e-08
miR-700-5p	-0,93	8,11e-08
miR-7082-3p	-5,75	8,92e-08
miR-743b-5p	-0,27	1,79e-07
miR-331-3p	-2,48	1,82e-07
miR-3068-3p	-0,63	2,59e-07
miR-199a-5p	-5,63	2,80e-07
miR-323-3p	-0,58	3,95e-07
miR-26b-3p	-0,52	4,16e-07
miR-361-3p	-0,39	6,22e-07
miR-153-5p	-0,61	7,94e-07
miR-431-3p	-0,33	1,23e-06
miR-674-3p	-0,5	1,24e-06
miR-487b-3p	-0,36	1,45e-06
miR-1843a-5p	-0,39	1,50e-06
miR-27a-3p	-0,39	1,65e-06
miR-362-3p	-0,79	2,31e-06
miR-574-5p	-0,94	2,48e-06
miR-370-3p	0,22	3,01e-06
miR-382-5p	-0,69	3,64e-06
miR-296-3p	-2,51	4,13e-06
miR-383-3p	-4,37	5,99e-06
miR-758-3p	-0,75	7,44e-06
miR-741-5p	-0,73	7,88e-06

miR-471-5p	-0,48	9,08e-06
miR-124-3p	-0,35	1,14e-05
miR-1981-5p	-0,92	1,26e-05
miR-148b-3p	-0,21	1,71e-05
let-7c-5p	0,03	2,32e-05
miR-29b-3p	-0,39	2,40e-05
miR-181a-1-3p	0,12	2,47e-05
miR-350-5p	-0,43	2,47e-05
miR-873a-5p	-1,12	2,59e-05
miR-205-5p	4,13	3,11e-05
miR-200b-5p	-5,05	3,20e-05
miR-883b-3p	-0,47	3,20e-05
miR-222-3p	-0,31	3,22e-05
miR-494-3p	-0,51	3,59e-05
miR-451a	0,7	3,86e-05
miR-1191	-0,99	3,89e-05
miR-29c-3p	-0,22	5,27e-05
miR-3057-3p	-2,63	5,46e-05
miR-1839-3p	-1,47	5,85e-05
miR-8103	0,92	6,14e-05
miR-6540-3p	-2,27	6,30e-05
miR-340-3p	-0,19	7,21e-05
miR-194-5p	-0,33	8,44e-05
miR-7679-5p	1,01	9,13e-05
miR-15a-3p	-1,13	0,00010
miR-384-5p	-0,44	0,00012
miR-142-5p	-0,67	0,00015
miR-30c-1-3p	-0,28	0,00017
miR-145a-3p	-3,14	0,00020
miR-742-5p	-1,17	0,00023
miR-7015-3p	0,67	0,00024
miR-146b-5p	-0,12	0,00025
miR-494-5p	-0,42	0,00026

miR-330-5p	-0,36	0,00033
miR-615-3p	-4,63	0,00038
miR-329-3p	-0,75	0,00038
miR-30c-2-3p	-0,14	0,00040
miR-455-5p	-0,36	0,00044
miR-350-3p	-0,79	0,00045
miR-6989-3p	-3,75	0,00049
miR-155-5p	4,55	0,00049
miR-676-5p	-0,58	0,00066
miR-301a-5p	-0,45	0,00068

APPENDIX B

miR_name	Log2Ratio (S2,S3,S4/O1,O3,O4) normalized	*P*-value
novel_mir_37	-9,06	6,47e-63
novel_mir_38	-8,94	5,64e-59
novel_mir_142	8,63	1,29e-34
novel_mir_2	-7,88	2,34e-32
novel_mir_91	8,22	1,65e-27
novel_mir_3	1,76	5,34e-25
novel_mir_63	-7,27	6,90e-23
novel_mir_121	7,83	3,63e-22
novel_mir_5	-6,71	1,60e-16
novel_mir_4	-5,79	1,41e-09
novel_mir_18	-5,75	2,26e-09
novel_mir_39	-5,60	1,51e-08
novel_mir_143	6,13	1,70e-08
novel_mir_43	2,20	5,69e-08
novel_mir_101	5,87	2,52e-07
novel_mir_106	5,79	5,00e-07
novel_mir_123	5,54	4,06e-06
novel_mir_92	5,45	8,27e-06
novel_mir_124	5,45	8,27e-06
novel_mir_11	-4,93	9,22e-06
novel_mir_93	5,35	1,696e-05
novel_mir_94	5,35	1,69e-05
novel_mir_64	-4,79	2,56e-05
novel_mir_95	5,24	3,50e-05
novel_mir_144	5,24	3,50e-05
novel_mir_145	5,24	3,50e-05
novel_mir_13	-4,71	4,29e-05
novel_mir_96	5,13	7,29e-05
novel_mir_97	5,13	7,29e-05
novel_mir_98	5,13	7,29e-05

novel_mir_19	1,31	0,00010
novel_mir_40	-4,47	0,00020
novel_mir_42	-4,47	0,00020
novel_mir_90	4,87	0,00032
novel_mir_99	4,87	0,00032
novel_mir_125	4,87	0,00032
novel_mir_146	4,87	0,00032
novel_mir_147	4,87	0,00032
novel_mir_148	4,87	0,00032
novel_mir_149	4,87	0,00032
novel_mir_100	4,71	0,00069
novel_mir_126	4,71	0,00069
novel_mir_127	4,71	0,00069
novel_mir_128	4,71	0,00069
novel_mir_150	4,71	0,00069
novel_mir_17	-1,37	0,00103
novel_mir_102	4,54	0,00150
novel_mir_103	4,54	0,00150
novel_mir_104	4,54	0,00150
novel_mir_105	4,54	0,00150
novel_mir_107	4,54	0,00150
novel_mir_108	4,54	0,00150
novel_mir_109	4,54	0,00150
novel_mir_129	4,54	0,00150
novel_mir_130	4,54	0,00150
novel_mir_131	4,54	0,00150
novel_mir_132	4,54	0,00150
novel_mir_151	4,54	0,00150
novel_mir_152	4,54	0,00150
novel_mir_153	4,54	0,00150
novel_mir_69	1,61	0,00151
novel_mir_65	-4,05	0,00172
novel_mir_67	-4,05	0,00172

novel_mir_68	-4,05	0,00172
novel_mir_32	-3,93	0,00297
novel_mir_110	4,35	0,00328
novel_mir_122	4,35	0,00328
novel_mir_133	4,35	0,00328
novel_mir_134	4,35	0,00328
novel_mir_154	4,35	0,00328
novel_mir_155	4,35	0,00328
novel_mir_156	4,35	0,00328
novel_mir_15	-3,79	0,00513
novel_mir_36	-3,79	0,00513
novel_mir_41	1,24	0,00543
novel_mir_8	0,77	0,00630
novel_mir_111	4,13	0,00727
novel_mir_112	4,13	0,00727
novel_mir_113	4,13	0,00727
novel_mir_114	4,13	0,00727
novel_mir_135	4,13	0,00727
novel_mir_136	4,13	0,00727
novel_mir_157	4,13	0,00727
novel_mir_158	4,13	0,00727
novel_mir_159	4,13	0,00727
novel_mir_44	-3,64	0,00888
novel_mir_45	-3,64	0,00888
novel_mir_46	-3,64	0,00888
novel_mir_72	-3,64	0,00888
novel_mir_73	-3,64	0,00888
novel_mir_74	-3,64	0,00888

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