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Proteomic and genomic analysis of PITX2 interacting and regulating networks

Yue Huang[a], **Kan Huang**[a], **Goran Boskovic**[d], **Yulia Dementieva**[e], **James Denvir**[f], **Donald A. Primerano**[a,d], and **Guo-Zhang Zhu**[a,b,c,*]

a *Biomedical Science Graduate Program, Marshall University, Huntington, WV 25755, USA*

b *Department of Biological Sciences, Marshall University, Huntington, WV 25755, USA*

c *Cell Differentiation and Development Center, Marshall University, Huntington, WV 25755, USA*

d *Department of Biochemistry and Microbiology, Marshall University, Huntington, WV 25755, USA*

e *Department of Mathematics, Marshall University, Huntington, WV 25755, USA*

f *Department of Statistics, West Virginia University, Morgantown, WV 26506, USA*

Abstract

PITX2 is a homeodomain transcription factor that has a substantial role in cell proliferation and differentiation in various tissues. In this report, we have conducted a systematic study, using proteomic and genomic approaches, to characterize PITX2-interacting proteins and PITX2-regulating genes. We identified four novel PITX2-associated protein partners YB-1, hnRNP K, nucleolin and hnRNP U in mass spectrometry analysis. We also found that overexpression of PITX2 upregulated 868 genes (two-fold to twenty five-fold) and downregulated 191 genes (twofold to fifteen fold) in DNA microarray analysis. These data provide an insightful perspective for further studying PITX2 function and mechanism of action.

Keywords

PITX2; YB-1; β-catenin; proliferation; differentiation

1. Introduction

Transcription factor PITX2 is a member of the homeobox gene family. A number of studies have demonstrated that PITX2 has a diverse role in cell proliferation, differentiation, hematopoiesis and organogenesis [1–4]. During early embryogenesis, PITX2 is a key regulator in the establishment of embryo left-right asymmetry[5]. In response to Wnt and other growth factors, PITX2 regulates cell-type specific cell proliferation during the development of cardiac outflow tract[3]. Mutations of PITX2 have been identified in several human disorders, such as Axenfeld-Rieger syndrome, iridogoniodysgenesis syndrome and sporadic Peter syndrome[6, 7]. *Pitx2*-deficient mice are embryonic lethal and show severe defects in heart, eye, pituitary

*Corresponding author. Department of Biological Sciences, Marshall University, One John Marshall Drive, Huntington, WV 25755, USA. Fax: +1 304 696 7136. Email address: zhu4@marshall.edu.

gland and tooth organogenesis[3]. Previous studies have shown that PITX2 cooperates with β-catenin and LEF/TCF and thus regulates cell proliferation by directly activating transcription of cyclin Ds and c-myc[3,8,9]. Besides β-catenin and LEF/TCF, other functional binding partners of PITX2, such as NF-1, HMG-17, MEF2A, Pit-1 and GcMa, have also been reported [1,10–13].

In this study, we analyzed the co-immunoprecipitated protein complex of PITX2 by mass spectrometry and successfully identified four proteins, YB-1, nucleolin, hnRNP K and hnRNP U, as novel PITX2-interacting partners. We also investigated the regulatory effects of PITX2 by examining gene expression profile of HEK293 cells with transient overexpression of PITX2. Our result indicated that 868 genes and 191 genes were upregulated and downregulated more than two-fold, respectively. Many of these regulated genes have previously been linked to cell proliferation, cell differentiation, and organogenesis of muscle and eye. Taken together, our findings provide an insightful perspective on PITX2 function and related molecular mechanism.

2. Materials and methods

2.1. Cell culture

HEK293 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, 10% fetal bovine serum, and penicillin-streptomycin (100 IU/ml) at 37°C in a humidified atmosphere with 5% CO_2.

2.2. Immunoprecipitation and immunoblotting

The open reading frame of human PITX2c, along with a FLAG epitope inserted between Met1 and Asn2, was amplified by PCR with the following primers: forward primer (5′-ACTGaagcttgccaccATGGATTACAAGGATGACGACGATAAGAACTGCATGAAAGGCCCGCTTCAC-3′, HindIII and Kozak site in lowercase) and reverse primer (5′-AGCTggtacctcaCACGGGCCGGTCCACTG-3′, KpnI site and stop codon in lowercase). The PCR product was cloned into the HindIII/KpnI sites of the vector pEGFP-N1 (Clontech). The resulting construct pEGFP-NFLAG-PITX2c, along with the control vector pEGFP-N1, was transfected into HEK293 cells using FugeneHD transfection reagent (Roche). The cells were observed 24 and 48 hours post-transfection, and no significant difference of cell growth and cell viability was noticed between the test and control groups. Cells were then lysed in immunoprecipitation buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) after 48 hours of initial transfection. For nuclear protein extraction, cells were incubated in hypotonic buffer (10 mM HEPES, 10 mM KCl, 1.5mM $MgCl_2$, 0.5 mM DTT) for 10 minutes before adding of 0.5% CA-630. Cell nuclei were then isolated by centrifugation. Nuclear proteins were extracted by the immunoprecipitation buffer. Cell lysates were pre-cleared by protein G beads (Pierce) for 2 h at 4°C, and then incubated with anti-FLAG M2 conjugated-agarose beads (Sigma) or rabbit anti-YB-1 (Epitomics) for 2 h or overnight at 4°C. The beads were washed five times using the immunoprecipitation buffer. To eliminate indirect protein-protein interactions, protein complexes were washed by consecutive addition of 2% and 4% Triton X-100 solutions. Washed beads were boiled in reducing SDS loading buffer for 10 min to elute proteins, which were then subjected to mass spectrometry and immunoblotting. Immunoblotting was performed as described previously [16]. Primary antibodies were mouse anti-PITX2 (Abnova), rabbit anti-β catenin and anti-YB-1 (Cell Signaling), rabbit anti-hnRNP K (Epitomics), rabbit anti-nucleolin and anti-hnRNP U (Abcam).

2.3. Peptide mass fingerprinting and LC-MALDI mass spectrometry

Proteins in the PITX2-immunoprecipitated complex were separated by SDS-PAGE and silver stained. Protein bands with high abundance were excised and analyzed by LC-MALDI (ABI Tempo LC MALDI) and MALDI MS/MS (ABI 4800 MALDI TOF/TOF) mass spectrometry analysis by Protea Bioscience (WV, USA). Peptides were identified by performing Mascot MS/MS Ion search (maximum missed cleavages: 2; peptide mass tolerance: ± 1–2 Da; fragment mass tolerance: ± 0.8–1 Da) on the non-redundant fasta database obtained from the National Center for Biotechnology Information (NCBI) and Swissprot database. The peptides and proteins with significant Mowse score ($p<0.05$), except trypsins, were reported.

2.4. RNA preparation

HEK293 cells were transiently transfected with pEGFP-NFLAG-PITX2c or pEGFP-N1. Forty eight hours after transfection, cells were harvested for total RNA extraction by using TRI Reagent (Ambion). RNA quality was assessed by electrophoretic analysis on an Agilent Model 2100 Bioanalyzer. All RNA samples used in this study had RNA Integrity Numbers greater than 8.0.

2.5. Microarray analysis

We employed a balanced block design with dye swap using four biological replicates from HEK293 cells transfected with either pEGFP-N1 or pEGFP-NFLAG-PITX2c. For microarray probes, total RNA (250 ng) was used as the template for synthesis of internally labeled cRNAs using Agilent QuickAmp Labeling kit and cyanine 3-CTP and cyanine 5-CTP (Perkin Elmer, Waltham, MA) and a modified QuickAmp protocol [14]. A total of 825 ng of cyanine 3- and cyanine5-labeled cRNAs was combined and hybridized onto Agilent Whole Human Genome 4 × 44 K microarrays at 65°C for 17 hours and then washed according to the manufacturer's protocol. Slides were scanned on Agilent DNA Microarray Scanner.

2.6. Statistical analysis of microarray data

Data were lowess-normalized and extracted from arrays using Agilent Feature Extraction v9.5. Features for which both channels had low signal were excluded. Log2 expression ratios of PITX2-overexpressed to control samples were imported to the Multiple Experiment Viewer (MeV) v4.0 to perform statistical analysis[15]. Values were compared for significant deviation from zero using one-class Significance Analysis of Microarrays (SAM)[16]. Only probes for which at least three replicates passed the low signal filter were included. SAM was performed with the maximum number of unique permutation, and delta was chosen to give 0% reported 90^{th} percentile False Discovery Rate (FDR). Data for significantly differentially expressed genes are shown in supplementary Table 1. Raw data from the microarray experiment are available at the NCBI Gene Expression Omnibus with accession number GSE13216.

2.7. Semi-quantitative RT-PCR analysis

To validate microarray data, we performed semi-quantitative RT-PCR. The cDNAs were reverse transcribed from total RNA in the presence of random primers (Clontech), and used as templates in the PCR under the following parameters: 94 C for 2 min, 1cycle; 94°C for 20 sec, 56°C for 20 sec (58°C for FOXJ1 and H19), 72°C for 2 min, 32 cycles; followed by a 6-min extension at 72 C. The primers used for this study are listed in supplementary Table 2.

3. Results

3.1. Identification of novel PITX2-associated proteins

Despite the substantial role of PITX2 in cell proliferation and differentiation, the molecular mechanism underlying PITX2 action is still limited. Identifying novel PITX2-interacting

protein partners would further our understanding of how PITX2 works. To do so, we first transiently overexpressed PITX2 in HEK293 cells (Figure 1A) and then co-immunoprecipitated PITX2 and its associated protein complex from the cell lysates. After the protein complex was separated by SDS-PAGE, five protein bands with high abundance, which did not appear in the pEGFP-N1 control sample (data not shown), were excised and subjected to mass spectrometry analysis. In addition to PITX2, four novel PITX2-associated proteins, YB-1, hnRNP K, nucleolin and hnRNP U, were identified from each protein band, respectively (Table 1). These four proteins achieved the highest significant scores among other candidates and their molecular weights matched the PAGE-determined molecular weights. To further confirm the association of these four proteins with PITX2, we performed immunoblotting on PITX2-immunoprecipitated proteins. As shown in Figure 1B, we indeed detected YB-1, hnRNP K, nucleolin and hnRNP U in the PITX2-immunoprecipitated protein complex. As a positive control for the PITX2 complex, we also demonstrated the presence of β-catenin, a previously reported binding partner of PITX2 (Figure 1B). Using an YB-1 antibody, we also detected the interaction of PITX2 and YB-1 at endogenous levels (Figure 1C). We were not able to test for interactions between PITX2 and other novel binding partners since immunoprecipitation-suitable antibodies for these proteins are not currently available.

3.2. Identification of PITX2-regulated genes

Since PITX2 has been shown to participate in a number of physiological processes, we hypothesized that PITX2 may have numerous target genes. To explore this idea, we performed DNA microarray to examine the gene expression profile of HEK293 cells transiently overexpressing PITX2 compared to cells with normal PITX2 levels. Remarkably, we found that 868 genes and 191 genes were upregulated and downregulated, respectively, by more than two-fold. Many of these regulated genes can be clustered into different biological processes, such as cell proliferation, cell differentiation, and organogenesis of muscle and eye, as exemplified in Table 2. A more comprehensive Gene Ontology (GO) analysis is provided in supplementary Table 3. To verify microarray data, we used semi-quantitative RT-PCR to measure relative changes in the mRNA abundance of twelve genes. The RT-PCR results were very consistent with the microarray results (Figure 2).

4. Discussion

In this study, we have identified YB-1, nucleolin, hnRNP K and hnRNP U as the novel PITX2-interacting partners. The four proteins show consistency in terms of their functions in that all of them play roles in regulating cell proliferation and RNA processing [17–20]. YB-1 is a multifunctional protein that functions in the regulation of cell proliferation and drug resistance [21]. Nuclear YB-1 acts as a transcription factor which controls transcription of genes involved in cell proliferation, such as cyclin A [17,22]. In this regard, our two observations, upregulation of cyclin A upon overexpression of PITX2 and the association of YB-1 and PITX2, may well echo each other. YB-1 also can bind to mRNA and become part of messenger ribonucleoprotein particels (mRNPs), thus controls gene translation[21]. Nucleolin is a ubiquitously expressed protein and a major component of the nucleolus[18]. Nucleolin functions include chromatin-remodeling, regulation of mRNA processing, ribosome assembly and nucleo-cytoplasmic transport[18]. Nucleolin, hnRNP K and YB-1 have been previously reported to be assembled into a macromolecular complex regulating mRNA stability[23,24]. In this respect, it is tempting to postulate that PITX2, through association with YB-1, may regulate mRNA stability as well. Indeed, such function has been previously assigned to PITX2 [25]. The identification of novel PITX2-interacting partners provides a new look at the mechanistic aspect of PITX2 function. It also suggests that PITX2 may regulate its downstream targets at both transcription and translation levels. Hence, the functional implication of this novel PITX2-interacting network will be a valuable topic in future studies.

Previously, only a few PITX2 transcription targets have been identified, such as cyclin Ds, LEF and PLOD[3,9,26,27]. However, these target genes may not be capable of mediating all functions of PITX2. In this study, we have found a remarkable set of potential target genes of PITX2. Of these candidates, FOXJ1 and DKK2 have been reported as direct targets of PITX2 transcriptional regulation in two recently published studies[28,29], consistent with our findings. To this end, we cannot rule out the possibility that some of the genes detected in our expression profile study are not directly regulated by PITX2. Nonetheless, our map of the PITX2-regulated gene network opens new avenues for studying biological processes involving PITX2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PITX2
: pituitary homeobox 2

YB-1
: Y box binding factor-1

hnRNP U
: heterogeneous nuclear ribonucleoprotein U

hnRNP K
: heterogeneous ribonucleoprotein K

NF-1
: nuclear factor-1

HMG-17
: high mobility group-17

GcMa
: glial cells missing a

PLOD
: Procollagen Lysyl Hydroxylase

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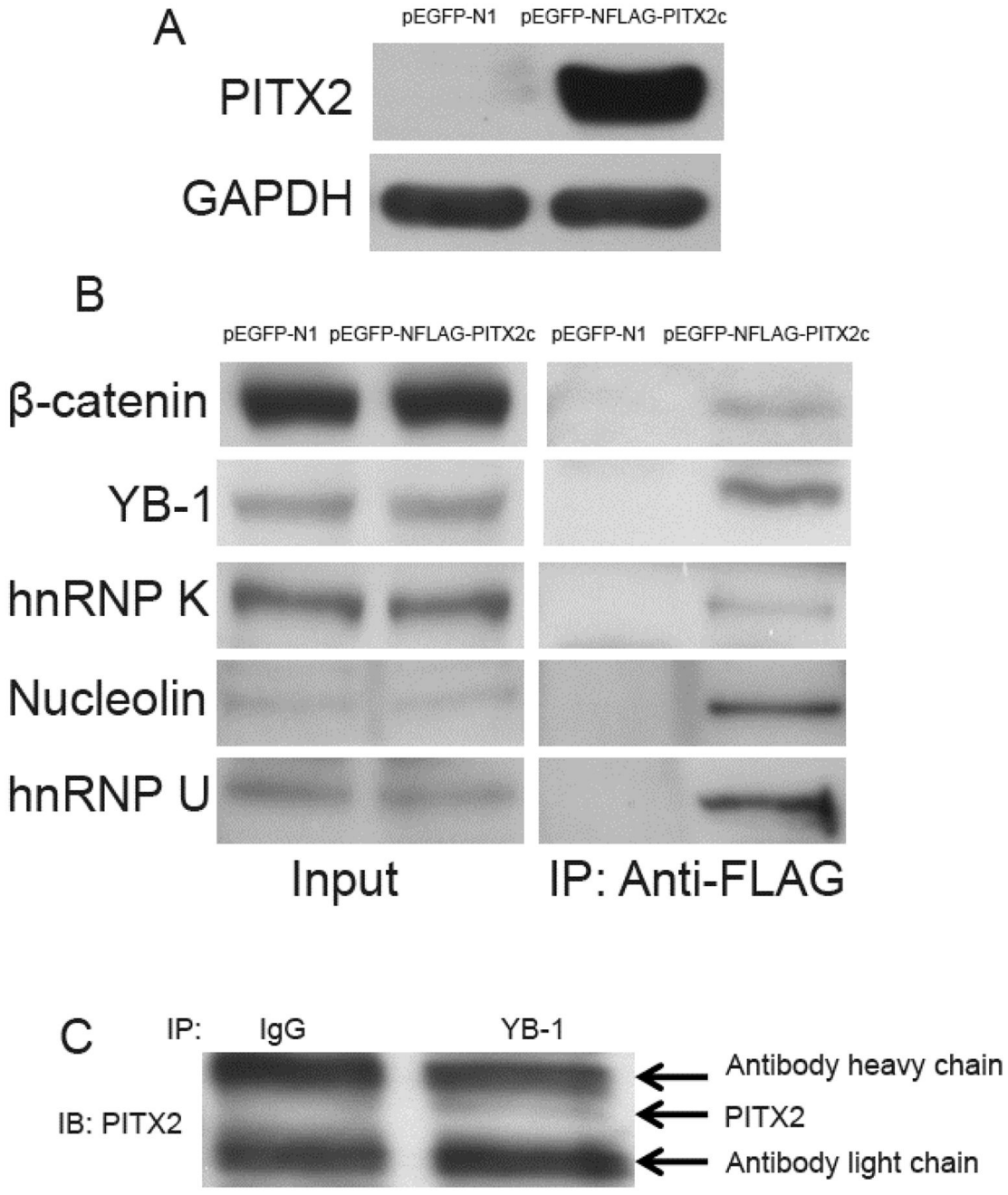

Figure 1.

(A) PITX2 was transiently overexpressed in HEK293 cells. The cell lysates from pEGFP-N1 and pEGFP-NFLAG-PITX2c were analyzed by immunoblotting. (B) YB-1, hnRNP K, nucleolin, hnRNP U and β-catenin are PITX2-interacting proteins. The PITX2-immunoprecipitated complex, along with the protein samples equivalent to 10% input, was probed with respective antibodies. (C) The endogenous protein complex of YB-1 and PITX2 was immunoprecipitated from HEK293 nuclear lysates by an YB-1 antibody and then detected by a PITX2 antibody.

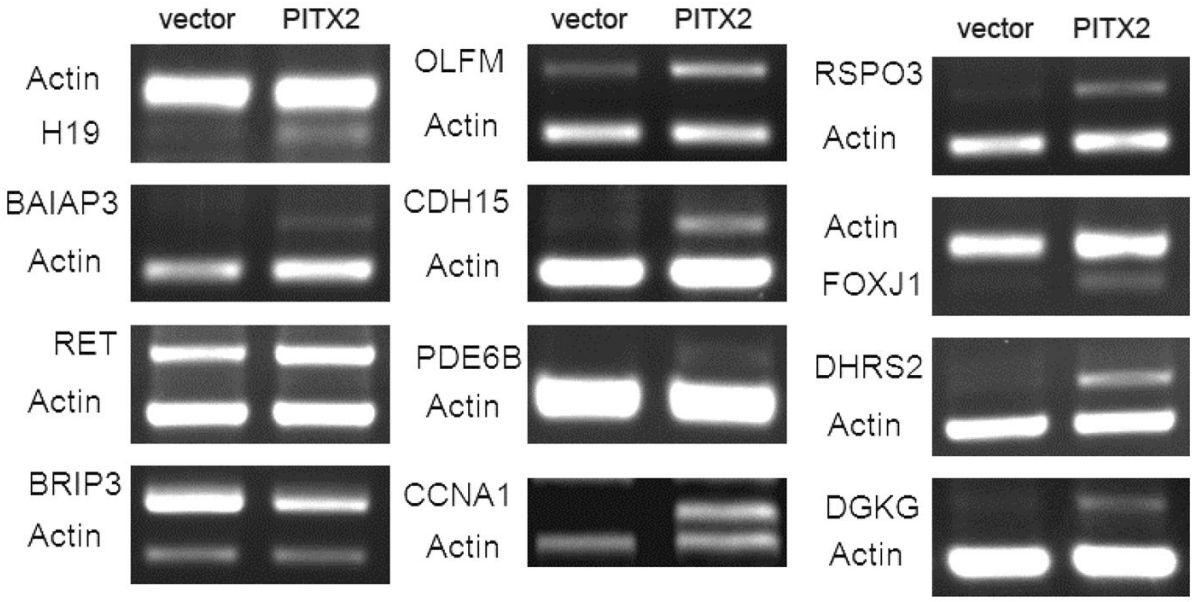

Figure 2.
Semi-quantitative RT-PCR analysis of PITX2-regulating genes. Actin was used as an internal control. Vector=pEGFP-N1; PITX2=pEGFP-NFLAG-PITX2c.

Table 1

List of proteins identified from the PITX2-immunoprecipitated complex, as described in Materials and methods. Proteins with ions scores > 32 indicate identity or extensive homology ($p<0.05$).

Protein	Peptides (ions score)	RefSeq No.	Gel region
Heterogeneous nuclear ribonucleoprotein U (hnRNP U)	(R) NFILDQTNVSAAAQR (123) (R) GYFEYIEENKYSR (16) (K) SSGPTSLFAVTVAPPGAR (50)	NP_114 032.2	100–120 kDa
Nucleolin	(K) EVFEDAAEIR (27) (K) GFGFVDFNSEEDAK (16) (K) GLSEDTTEETLKESFDGS (17)	NP_005 372.2	80–100 kDa
Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	(R) NLPLPPPPPPR (43)	NP_002 131.2	60–70 kDa
Y box binding protein-1 (YB-1)	(R) NEGSESAPEGQAQQR (49) (R) NGYGFINR (59)	NP_004 550.2	40–50 kDa
Pituitary homeobox 2 (PITX2)	(R) EEIAVWTNLTEAR (58) (R) THFTSQQLQELEATFQR (53)	NP_700 476.1	30–40 kDa

Table 2

Exemplified genes regulated by PITX2.

Accession No.	Gene	Fold	Description
Proliferation			
NR_002196	H19	12.0	imprinted maternally expressed untranslated
NM_003914	CCNA1	11.6	cyclin A1, cell cycle regulator
NM_003933	BAIAP	7.7	BAI1-associated protein 3
NM_001007139	IGF2	5.5	insulin-like growth factor 2 (somatomedin A)
NM_020975	RET	5.4	ret proto-oncogene
NM_032043	BRIP1	0.17	BRCA1 interacting protein C-terminal helicase 1
NM_004407	DMP1	0.16	dentin matrix acidic phosphoprotein
Muscle			
NM_004933	CDH15	9.5	cadherin 15, M-cadherin (myotubule)
NM_000257	MYH7	6.0	myosin, heavy chain 7, cardiac muscle, beta
AB002384	C6orf32	4.6	mRNA for KIAA0386 gene
Eye			
NM_014279	OLFM	9.1	olfactomedin 1
NM_000283	PDE6B	8.9	phosphodiesterase 6B, cGMP-specific, rod, beta (congenital stationary night blindness 3, autosomal dominant
NM_014421	DKK2	6.1	dickkopf homolog 2
NM_000327	ROM1	3.9	retinal outer segment membrane protein 1
Development/differentiation			
NM_032784	RSPO3	20.5	R-spondin 3 homolog
NM_000582	SPP1	9.5	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
NM_001454	FOXJ1	7.5	forkhead box J1
NM_030761	WNT4	6.8	wingless-type MMTV integration site family, member 4
Others			
NM_001346	DGKG	9.0	diacylglycerol kinase, gamma 90kDa
NM_182908	DHRS2	25.2	dehydrogenase/reductase (SDR family) member