Expression of *CXCL6* and *BBS5* that may be glaucoma relevant genes is regulated by PITX2

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Conflict of interest

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Abstract

The transcription factor PITX2 is implicated in glaucoma pathology. In an earlier study we had used microarray analysis to identify genes in the trabecular meshwork (TM) that are affected by knock down of *PITX2*. Here, those studies were pursued to identify genes that are direct targets of PITX2 and that may be relevant to glaucoma. Initially, bioinformatics tools were used to select among the genes that had been affected by PITX2 knock down those that have PITX2 binding sites and that may be involved in glaucoma related functions. Subsequently, the effect of PITX2 was tested using the dual luciferase assay in four cell cultures including two primary TM cultures co-transfected with vectors containing promoter fragments of six candidate genes upstream of a luciferase gene and a vector that expressed PITX2. Finally, the effect of PITX2 on endogenous expression of two genes was assessed by over expression and knock down of PITX2 in TM cells.

Thirty four genes were found to contain PITX2 binding sites in their putative promoter regions, and 16 were found to be associated with TM-specific and/or glaucoma associated functions. Results of dual luciferase assays confirmed that two of six genes tested were directly targeted by PITX2. The two genes were *CXCL6* (chemokine (C-X-C motif) ligand 6) and *BBS5* (Bardet-Biedl syndrome 5). Over expression and knock down of PITX2 showed that this transcription factor affects endogenous expression of these two genes in TM cells. *CXCL6* encodes a pro-inflammatory cytokine, and many studies have suggested that cytokines and other immune system functions are involved in glaucoma pathogenesis. *BBS5* is a member of the BBS family of genes that affect ciliary functions, and ciliary bodies in the anterior chamber of the eye produce the aqueous fluid that affects intraocular pressure. Immune related functions and intraocular pressure are both important components of glaucoma pathology. The role of PITX2 in glaucoma may be

mediated partly by regulating the expression of *CXCL6* and *BBS5* and thus affecting immune functions and intraocular pressure.

Keywords

Glaucoma; Intraocular pressure; trabecular meshwork; PITX2.

Abbreviations

IOP, Intraocular pressure; TM, trabecular meshwork.

1. Introduction

Glaucoma is the leading cause of irreversible blindness. It constitutes a group of neuropathies that manifest by optic nerve head cupping and degeneration of the optic nerve, resulting in a specific pattern of visual field loss (Foster et al., 2002). The pathogenic pathways of glaucoma are not well understood, although significant advances have been made. Increased intraocular pressure (IOP) is a major risk factor and the target of treatment protocols. Increased IOP is thought to be due mainly to defects in drainage of aqueous humor from the anterior chamber of the eye (Hollows and Graham, 1966; Wallace et al., 2014). The trabecular meshwork (TM), which is composed of an irregular network of connective tissue beams at the iridocorneal angle, is a major component of the drainage system. The fluid traverses this tissue, enters Schlemm's canal, and ultimately drains into the venous system.

Four genes that can independently cause primary open angle glaucoma (POAG), *MYOC*, *OPTN*, *WDR36*, and *NTF4*, and two that can independently cause primary congenital glaucoma (PCG), *CYP11B1* and *LTBP2*, have been identified (Stoilov et al., 1997; Ali et al., 2009; Narooie-Nejad et al., 2009; Fingert, 2011). However, glaucoma is usually a complex disorder caused by multiple genetic and environmental risk factors (Fingert, 2011; Ahram et al., 2015). The four known POAG genes together are estimated to account for disease status in less than 10% of POAG patients (Sarfarazi et al., 2003; Chitsazian et al., 2007). Genome-wide association studies and genetic analysis on quantitative traits relevant to glaucoma such as central corneal thickness (CCT) are approaches for identification of genes with less prominent effects, and *CAV1*, *CAV2*, *ELOVL5*, and *ZNF469* are among candidate genes thus identified (Fingert, 2011; Mabuchi et al., 2011; Loomis et al., 2014).

We implemented another approach for identification of genes that may have subtle and accumulative effects relating to glaucoma. Pituitary homeobox 2 (*PITX2*) and forkhead box C1 (*FOXC1*) are transcription factors that are expressed in the periocular mesenchyme and that affect the development of various mesoderm derived tissues, including ocular tissues (Kidson et al., 1999; Cox et al., 2002; Gage et al., 2005; Berry et al., 2006). Mice with targeted deletions in either of the encoding genes exhibit various eye development defects that include abnormalities of the optic nerve and absence of anterior chamber formation (Gage et al., 1999; Kidson et al., 1999; Kitamura et al., 1999; Lu et al., 1999). Additionally, mutations in either of the genes are cause of Axenfeld-Rieger syndrome (ARS) in a subset of patients (Semina et al., 1996; Alward, 2000; Strungaru et al., 2007). ARS is characterized by anterior eye segment defects and systemic anomalies (Semina et al., 1996; Alward, 2000; Strungaru et al., 2007). Notably, approximately 50% of ARS patients develop glaucoma, usually in adolescence or early adulthood, and patients with *PITX2* or *FOXC1* mutations are prone to developing glaucoma (Strungaru et al., 2007). Finally, both genes are expressed in the TM (Kidson et al., 1999; Kozlowski and Walter, 2000; Priston et al., 2001). Given their potential relevance to eye development and glaucoma, we earlier performed whole genome microarray analysis to identify genes whose expressions in human TM cultured cells were altered by knock down of these two transcription factors (Paylakhi et al., 2011; Paylakhi et al., 2013). In the present study, we pursued our findings on some of the *PITX2* affected genes. Specifically, we first used bioinformatics tools to select among the 41 genes that had been affected by *PITX2* knock down those that have *PITX2* binding sites and, therefore, are more likely to be direct targets of the protein. Subsequently, we identified candidate genes that may be involved in glaucoma and TM related functions. The bioinformatics findings relating to effects of *PITX2* on expression of *CXCL6* (chemokine (C-X-C motif) ligand 6) and *BBS5*

(Bardet-Biedl syndrome 5) were empirically confirmed. The significance of these results are discussed.

2. Methods

This research was performed in accordance with the Helsinki Declaration and with approval of the ethics boards of the University of Tehran and Tarbiat Modares University. Eye globes used for isolation of TM to be used for preparation of TM cell cultures were obtained from the Central Eye Bank of Iran.

2.1. Bioinformatics protocols

DECODE (<http://www.sabiosciences.com/chipqpcrsearch.php?app=TFBS>) was used to select among the 41 PITX2 target genes identified by knock down/microarray analysis, those with PITX2 binding sites within their promoter regions. This bioinformatics tool combines text mining applications and data from the UCSC Genome Browser to compile lists of human genes with predicted binding sites for over 200 human transcription factors. Additionally, the 3000 base pair region upstream of the A of the AUG translation initiation codon in both DNA strands of each gene was visually screened for presence of predicted PITX2 bicoid sequence elements 5'-TAATCC- 3' and 5'-CTAAGCC - 3' (Hanes and Brent, 1989; Treisman et al., 1989; Saadi et al., 2003). PipMaker (<http://pipmaker.bx.psu.edu/cgi-bin/pipmaker?basic>) was used for checking evolutionary conservation of the potential binding sites in orthologous sequences of chimpanzee and mouse genomes. For the purpose of performing empirical studies, we aimed to identify genes with TM-specific and/or glaucoma associated functions. The genes with conserved PITX2 binding sites were submitted to STRING (Search

Tool for the Retrieval of Interacting Genes/Proteins; string-db.org/), KEGG (Kyoto Encyclopedia of Genes and Genomes; www.genome.jp/kegg/), and GeneCards (www.genecards.org/). These bioinformatics tools provide a wealth of information on physical and functional properties of biological entities and interactions between these entities based on high- throughput genomic, transcriptomic, proteomic, epigenetic, and metabolomics studies, data available in the literature, and computational analyses. Results were evaluated for each of the genes, and those that have relevance to TM functions and glaucoma were chosen. Additionally, genes with conserved PITX2 binding sites were submitted to DisGeNET (www.disgenet.org/) to identify glaucoma associated genes; DisGeNET integrates available data on gene-disease associations from several public data sources and the literature (Pinero et al., 2015).

2.2. Empirical verification of direct targeting of genes by PITX2

Empirical testing was first performed by cloning promoter regions of candidate genes upstream of a firefly luciferase gene and comparison of expression of the luciferase in the presence and absence of PITX2 expression vectors in several cell lines. Promoter fragments of six genes that contained all predicted PITX2 binding sites of the respective genes and the promoter region of a negative control gene without PITX2 binding site, *LDLRAD2* (low density lipoprotein receptor class A domain containing 2), were amplified by PCR (Fig. 1). The fragments ranged in size from 1810 to 3054 base pairs. The primer pairs used for their amplification created *ACC651* and *SacI*, *XhoI* and *EcoRV*, *SacI* and *XhoI*, *ACC651* and *EcoRV*, or *ACC651* and *XhoI* restriction enzyme recognition sites at, respectively, the 5' and 3' termini (Table S1). The fragments were cloned adjacent to the firefly luciferase reporter gene in the pGL4.14 vector (Promega Corporation, Madison, WI, USA).

Cloned fragments were sequenced for verification of accuracy. Cultured cells were co-transfected with pGL4.14 recombinant plasmids, pRL-TK (Promega Corporation, Madison, WI, USA), and empty pcDNA4 or pcDNA4-PITX2 (kindly provided by Dr. Michael A. Walter). PRL-TK contains the renilla luciferase gene adjacent to the thymidine kinase promoter, and pcDNA4-PITX2 expresses PITX2 under the constitutively expressed cytomegalovirus promoter. Transfections were performed in 24-well plates. Additional control transfections included recombinant pGL4.14 + pRL-TK, non-recombinant pGL4.14 + pRL-TK + pcDNA4-PITX2, and mock transfections without vector. All transfections were done in triplicate in each of four types of cells. The four host cells were Hek-293 (National Institute of Genetic Engineering, Tehran, Iran), HeLa (National Institute of Genetic Engineering, Tehran, Iran), and human trabecular meshwork primary cultures TM5 and TM6. The TM cultures were prepared exactly as described before (Paylakhi et al., 2011). Transfection reactions were performed using 400 ng of plasmid DNAs, Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA, USA) and 2×10^5 cells. Forty eight hours after transfection, firefly and renilla luciferase activities were measured using dual luciferase assays (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. For genes whose promoters were directly targeted by PITX2 as assessed by the luciferase assays, effects of over expression and knock down of PITX2 on endogenous expression of respective genes cells were tested in TM5 and TM6. In the over expression studies, cells were transfected with pcDNA4-PITX2. In the knock down experiments, cells were treated with PITX2 siRNA (Dharmacon Research, Lafayette, CO; J-017315-05) as described before (Paylakhi et al., 2011). All experiments were performed in triplicate. Total RNA was isolated 48 hours after transfections, and cDNAs were synthesized by standard procedures. Real time PCR for *PITX2* and the relevant genes was performed on a on a Corbett 65H0 instrument (Corbett Research, Sidney,

Australia) using the QuantiFast SYBR Green PCR Kit (QIAGEN, Germantown, MD). *ACTB* (actin, beta) was used as control gene. The real time PCR primers used are shown in Table S2. Statistical analysis was done using the Relative Expression Software Tool (REST) software (Pfaffl et al., 2002).

3. Results

Among the 41 genes that were assessed to be affected by PITX2 knock down in the microarray experiments, DECODE and visual screening identified the same 34 genes to contain PITX2 binding sites in their putative promoter regions (Table 1). The number of binding sites per gene ranged from one to seven, and the maximum number was present in the promoter regions of *PLP2* (proteolipid protein 2) (seven sites), *BBS5* (six sites), and *CBF β* (core-binding factor, beta subunit) (six sites). Based on information derived from STRING, KEGG, and GeneCards, we assessed that at least 16 genes were associated with TM-specific and/or glaucoma associated functions (Table 1). The terms aqueous humor and apoptosis were included or implicated in the annotations of, respectively, three and six of the genes. The terms eye development, oxidative stress response, extracellular matrix functions, ciliary functions, cytoskeleton functions, RHO signaling, and TGF β signaling were each associated with one of the 16 genes. DisGeNET designated that four of the 34 genes with PITX2 binding sites were associated with glaucoma, and, as expected, these were included in the set of 16 genes identified using the other bioinformatics tools. These genes were *ALDH1A1* (aldehyde dehydrogenase 1 family, member A1), *ADAMTS5* (ADAM metalloproteinase with thrombospondin type 1 motif, 5), *CXCL6*, and *MEIS2* (Meis homeobox 2).

Six TM/ glaucoma relevant genes identified by using the bioinformatics approach were selected for empirical studies: *PLP2*, *BBS5*, *CBF β* , *ALDH1A1*,

ADAMTS5, and *CXCL6*. The first three had the largest number of PITX2 binding sites in their promoter regions, and the last three were among the four genes designated by DisGeNET to be associated with glaucoma. Results of the dual luciferase assay evidenced that the presence of pcDNA4-PITX2 caused increased expression of firefly luciferase when the enzyme's encoding sequence was placed under the regulation of promoter fragments of *BBS5* or *CXCL6* (Fig. 2). Increase expression of firefly luciferase was not observed with the use of *PLP2*, *CBF β* , *ALDH1A1*, and *ADAMTS5* promoter fragments. The results were consistent in Hek-293, HeLa, and the two TM cell cultures used. The effect of PITX2 on *BBS5* or *CXCL6* endogenous transcripts in two TM cell cultures were consistent with the luciferase results, as PITX2 over expression evidenced increased levels of the respective transcripts in real time PCR experiments, and PITX2 knock down evidenced decreased levels of the transcripts (Fig. 3).

4. Discussion

Earlier we had confirmed the reliability of our PITX2 siRNA knock down microarray data by performing real time and Western blot analysis on several selected genes (Paylakhi et al., 2011). To make best use of the array data, we here aimed to identify genes that may be direct targets of the transcription factor. We found that a large per cent (83%, 34/41) of the genes identified by microarray analysis contained in their putative promoter regions the predicted PITX2 bicoid sequence binding elements. Unfortunately, highly informative data on PITX2 binding elements is not available. For example, the binding sequence of PITX2 is not available in TRANSFAC (<http://www.gene-regulation.com/pub/databases.html>) which is a database that reports transcription factor binding sequences based on experimental data. Although the bicoid sequence binding elements are in all

likelihood part of most functional PITX2 binding elements, their presence may not be sufficient to ensure PITX2 binding. The presence of the bicoid binding elements in a large percent of genes affected by PITX2 knock down may in some cases be fortuitous and expected simply because of the short length of the sequences. The results of the luciferase data discussed below support the conjecture that presence of the bicoid binding sequences does not ensure direct targeting by PITX2.

Considering that presence of the bicoid binding sequence in promoter regions may be a necessary feature of genes directly regulated by PITX2, we sought glaucoma relevant genes among the 34 genes that contained the bicoid sequences. Annotations in various data bases evinced that the sixteen selected genes had relevance to glaucoma. For example, the aqueous fluid whose volume affects intraocular pressure is produced by ciliary bodies in the anterior chamber (Hollands and Graham, 1966; Hara et al., 1977; Wallace et al., 2014). Features of the extracellular matrix of the trabecular meshwork affect the facility with which this fluid traverses the TM before drainage into the venous system (Gong et al., 1996; Borrás, 2003; Acott and Kelley, 2008; Keller et al., 2009). Oxidative stress is now widely recognized as an important component in the etiology of glaucoma (Tezel, 2006; Liton and Gonzalez, 2008; Sacca and Izzotti, 2008; Sacca et al., 2015). TGF β levels are elevated in the aqueous humor of glaucoma patients, and TGF β is implicated in both oxidative response and extracellular matrix related functions (Wordinger et al., 2007; Fatma et al., 2009). TM dysfunctions can cause apoptosis in the cells of this tissue (Grierson, 1987; Johnson et al., 1989). Apoptosis of TM cells is a common histological finding in POAG, and apoptosis of retinal ganglion cells is a major glaucoma risk factor (Alvarado et al., 1984; Sommer, 1989; Nickells, 1999). Actin filaments in the cytoskeleton are responsible for contractile properties and for modulation of aqueous humor outflow resistance in the TM (Tian et al., 2000; Rao and Epstein, 2007; Tian et al., 2009; Last et al., 2011). Members of

the RHO family of proteins including RhoA and ROCKs are expressed in human TM cells, and are involved in modulation of TM contraction and aqueous humor outflow (Thieme et al., 2000; Honjo et al., 2001a; Honjo et al., 2001b; Rao et al., 2001; Nakajima et al., 2005; Rao and Epstein, 2007; Tokushige et al., 2007; Pattabiraman and Rao, 2010;).

It is emphasized that the identification of potentially glaucoma relevant genes whose expression is somehow affected by PITX2 is important, irrelevant of whether the regulation is direct or mediated by other molecules. For example, further studies on PITX2 interactions with ADAMSTS5 may be valuable as ADAMSTS5 is an extracellular matrix protein and the relevance of the *ADAMSTS5* gene to glaucoma has been signaled in various global gene expression studies (Rozsa et al., 2006; Huang et al., 2009; Luna et al., 2009;). Identification of the directly affected genes is simply one step in deciphering a complicated regulatory network that includes PITX2. In fact, among six candidate genes tested, the luciferase assay and subsequent over expression and knock down studies confirmed direct regulation by PITX2 only for *CXCL6* and *BBS5*. Of course, it is quite possible that the chosen promoter regions of some genes used for the luciferase assay were not sufficiently long to contain all regulatory regions required to faithfully replicate endogenous expression regulation of the genes.

Expression of *CXCL6* in the human eye and secretion of the encoded protein in significant quantities by TM cells was first reported prior to our microarray knock down studies (Shifera et al., 2010). Our microarray data showed that PITX2 knock down resulted in a threefold decrease of *CXCL6* expression which was notable (Paylakhi et al., 2011). We have shown that FOXC1 also affects the expression of this gene (Paylakhi et al., 2013). Furthermore, *CXCL6* was among genes identified to be relevant to glaucoma pathogenesis in three other glaucoma related global gene expression studies (Liton et al., 2006; Lukas et al., 2008; Comes and Borrás, 2009).

CXCL6 encodes a pro-inflammatory cytokine that induces migration of monocytes and neutrophils (Yoshie et al., 2001). Multiple clinical and experimental studies have suggested that cytokines and other immune system functions are involved in glaucoma pathogenesis (Tezel and Wax, 2004). In this regard, monocytes facilitate conductivity of human Schlemm canal endothelial cells and play an important role in aqueous humor homeostasis (Alvarado et al., 2005; Alvarado et al., 2010). Notably, immune related activities were prominent in the bioinformatics analysis of functions attributed to the 41 *PITX2* affected genes identified by our microarray experiments (Paylakhi et al., 2011). The results presented here suggest that some of the immune related functions of *PITX2* may be mediated by directly affecting *CXCL6* expression that in turn can affect aqueous fluid homeostasis. This may at least partially explain manifestation of secondary glaucoma in ARS patients with *PITX2* mutations and may implicate immune related roles for *PITX2* in the etiology of primary glaucoma.

BBS5 is a member of the BBS family of genes that consists of at least 19 members (Beales et al., 1997; Yen et al., 2006). The functions of the encoded proteins are not well understood. It has been suggested that some BBS proteins are involved in intracellular and intraflagellar transport (Blacque et al., 2004; Kim et al., 2004; Yen et al., 2006). A protein complex named the BBSome consisting of 7 BBS proteins including *BBS5* has been identified (Nachury et al., 2007). This complex localizes in primary cilia, and results of various studies suggest that disruptions of the complex cause basal body and/or cilium dysfunction (Nachury et al., 2007). Such dysfunctions may be the cause of Bardet-Biedl syndrome (BBS), a pleiotropic primary cilium associated genetic disease (Blacque and Leroux, 2006; Nachury et al., 2007; Su et al., 2014). Previously, *BBS5* was reported as a protein that interacts with *PITX2* (Huang et al., 2009). Now, identification of *BBS5* as a

direct target of PITX2 regulation is intriguing because the ciliary bodies in the anterior chamber are the source of aqueous humor (Ethier et al., 1986; Heimark et al., 2002), and PITX2 may regulate aqueous humor outflow through its effects on *BBS5*.

5. Conclusion

PITX2 affects the transcription of at least 16 genes that may have relevance to glaucoma etiology. *CXCL6* and *BBS5* are two of these genes that are directly targeted by PITX2. *CXCL6* encodes a pro-inflammatory cytokine, and many studies have suggested that cytokines and other immune system functions are involved in glaucoma pathogenesis. *BBS5* is a member of the BBS family of genes that affect ciliary functions, and ciliary bodies in the anterior chamber of the eye produce the aqueous fluid that affects intraocular pressure. Immune related functions and intraocular pressure are both important components of glaucoma pathology. The role of PITX2 in glaucoma may be mediated partly by regulating the expression of *CXCL6* and *BBS5* and thus affecting immune functions and intraocular pressure.

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Figure Legends

Fig. 1: Distribution of PITX2 binding sites in promoter regions of target genes. The putative binding sites were identified using DECODE and visual inspection of the promoter regions of target genes for presence of PITX2 bicoid sequence elements. Evolutionary conservation was assessed by PipMaker in orthologous sequences of chimpanzee and mouse genomes: ‡, conserved in human, chimpanzee and mouse; +, conserved in human and chimpanzee. On the line that represents the promoter region of each gene, the nucleotide positions shown are with respect to A of ATG initiation codon. Bars above and below the gene lines, respectively, show sites on the coding and non-coding strands of the DNA. The bolded portions of the gene lines represent fragments that were cloned into the vector pGL4.14.

Fig. 2: Dual luciferase assays. Dual luciferase assay results in Hek-293, HeLa, TM5, and TM6 cells in presence of various combinations of vectors. For each cell type, experiments were done under five conditions (conditions 1-5). The conditions are described in upper left panel. For each gene, results from left to right are for experimental conditions 1 to 5. Standard deviations based on three replicate transfection experiments are shown. *** indicates $p < 0.001$ for comparisons of firefly/luciferase ratios of conditions 3 and 4.

Fig. 3: Effects of PITX2 knock down and over expression on endogenous expression of *CXCL6* and *BBS5* in TM cells. In the knock down studies, expression levels in the presence of PITX2 specific siRNA and scrambled siRNA are compared. In the over expression studies, expression levels in the presence of pcDNA4-PITX2 and empty vector are compared. Standard deviations for real time PCR data are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 1. PITX2 target genes based on siRNA knock down/ microarra

Table 1. PITX2 target genes based on siRNA knock down/ microarray analysis in TM cells			
Genes	Number of PITX2 binding sites	Relevance to TM functions and glaucoma ^a	Association with glaucoma ^b
<i>ADAMTS5</i>	1	Maintenance of extracellular matrix integrity (Dudhia, 2005; Stanton et al., 2005; Durigova et al., 2011)	+
<i>ALDH1A1</i>	1	Response to the oxidative stress damages (Piatigorsky, 2000)	+
<i>AUH</i>	3	Regulation of the apoptosis through its interaction with ACAA2 (Cao et al., 2008)	-
<i>BBS5</i>	6	Regulation of the cilia formation and ciliary trafficking (Ethier et al., 1986; Heimark et al., 2002; Li et al., 2004; Nachury et al., 2007; Su et al., 2014)	-
<i>BHLHB3</i>	1		-
<i>C7orf47</i>	5		-
<i>C7orf63</i>	3		-
<i>CBFB</i>	6	Regulation of blood homeostasis and its derivatives including aqueous humor (Wang et al., 1996)	-
<i>CD4</i>	3		-
<i>CEBPG</i>	5		-
<i>CFL2</i>	1	Regulation of the actin-filament dynamics (Gillett et al., 1996; Bamburg et al., 1999; Maciver and Hussey, 2002; Abbhi and Piplani, 2013)	-
<i>CTXN1</i>	2		-
<i>CXCL6</i>	3	Regulation of the aqueous humor outflow (Alvarado et al., 2005; Alvarado et al., 2010)	+
<i>DIRAS3</i>	4	Regulation of the trabecular meshwork cells contraction and aqueous humor outflow (Yu et al., 1999; Abbhi and Piplani, 2013)	-
<i>ELK3</i>	4		-
<i>FAM70A</i>	3	Interaction with RHO family components (Rao and Epstein, 2007)	-
<i>FLG</i>	1		-
<i>GALNT1</i>	0		-
<i>GBP2</i>	1		-
<i>GBP5</i>	4		-
<i>GRP</i>	2		-
<i>HAPLN1</i>	2		-
<i>IRS2</i>	1		-
<i>KHDRBS3</i>	0		-
<i>LIN7A</i>	2		-
<i>LMNB1</i>	4	Regulation of cell cycle and apoptosis through its interaction with CDK1 (Kokkinakis et al., 2005)	-
<i>LOC653602</i>	0		-
<i>MEIS2</i>	1	Eye development (Conte et al., 2010)	+
<i>MICALL2</i>	0		-
<i>NOMO2</i>	4	Regulation of TGFβ signaling (Schier, 2003; Haffner et al., 2004; Strizzi et al., 2008)	-

Fig. 1: Distribution of PITX2 binding sites in promoter regions

Fig. 2: Dual luciferase assays

	1	2	3	4	5
pGL4.14 (without promoter)	-	-	-	-	+
recombinant pGL4.14 (with promoter fragment)	-	+	+	+	-
pRL-TK	-	+	+	+	+
Empty pcDNA4	-	-	+	-	-
pcDNA4-PITX2	-	-	-	+	+

ALDH1A1

PLP2

CXCL6

CBF β

ADAMTSS5

BBS5

LDLRAD2

Fig. 3: Effects of PITX2 knock down and over expression on endog