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Prediction of Regulatory Networks in Mouse Abdominal Wall

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Abstract

Sequence specific transcription factors are essential for pattern formation and cell differentiation processes in mammals. The formation of the abdominal wall depends on a flawless merge of several developmental fields in time and space. Absence of *Pitx2* leads to open abdominal wall in mice, while mutations in humans result in umbilical defects, suggesting that a single homeobox transcription factor coordinates the formation and patterning of this anatomical structure. Gene expression analysis from abdominal tissue including the abdominal wall after removal of the major organs, of wild type, *Pitx2* heterozygote and mutant mice, at embryonic day 10.5, identified 275 genes with altered expression levels. *Pitx2* target genes were clustered using the “David Bioinformatics Functional Annotation Tool” web application, which bins genes according to gene ontology (GO) key word enrichment. This provided a way to both narrow the target gene list and to start identifying potential gene families regulated by *Pitx2*. Target genes in the most enriched bins were further analyzed for the presence and the evolutionary conservation of *Pitx2* consensus binding sequence, TAATCY, on the –20kb, intronic and coding gene sequences. Twenty *Pitx2* target genes that passed all the above criteria were classified as genes involved in cell transport and growth. Data from these studies suggest that *Pitx2* acts as an inhibitor of protein transport and cell apoptosis contributing to the open body wall phenotype. This work provides the framework to which the developmental network leading to abdominal wall syndromes can be built.

Keywords

Pitx2; Homeobox; Development; Gene Expression Analysis; Gene Regulation; Bioinformatics

1. Introduction

The abdominal wall holds in the intestinal organs and provides stability to the trunk. Classic ventral body wall defects are characterized by a thin body wall, muscular dysplasia and/or absence of midline fusion (Brewer and Williams, 2004a). Congenital abdominal wall defects such as gastroschisis or omphalocele, and abdominal injuries can be a challenge to be repaired due to the musculofascial continuity and the complex vascular supply (Chang et al., 1992).

The molecular mechanisms involved in the developmental processes that occur during the formation of the abdominal wall are not well understood. Cells from the definitive endoderm

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and lateral plate mesoderm migrate to form the abdominal wall. Abdominal level somites extend ventrally and form the abdominal muscles. In the mouse, ventral abdominal wall formation is initiated at embryonic day 9, (E9.0) when the primary ventral body forms, consisting of a thin epithelial peridermal membrane. The secondary body wall begins to form at E12.0 in both the thoracic and abdominal areas, with the primary contributor being cells from the somites (Christ et al., 1983; Ogi et al., 2005). The developing abdomen is composed of mesenchymal connective tissue and somatic-derived muscles which converge to enclose the internal organs (Brewer and Williams, 2004a, b; Suzuki et al., 2009).

Signaling molecules assist communication between cells and initiate the activation of certain sequence specific transcription factors (SSTFs) in the cells that receive the signal. The combination of active SSTFs at a given time and location defines the regulatory state of the cell. The complementary part of the regulatory apparatus is the regulatory genome that is common to all cells. Expression of each gene is controlled by regulatory sequences, the *cis*-regulatory modules (CRM) that contain clusters of different transcription factor binding sites. A regulatory gene (node) contains numerous CRMs that control its expression in a spatiotemporal manner during development. Signaling molecules and SSTFs form a network that is essential for pattern formation and cell specification. The identification of gene regulatory networks during abdominal wall development will provide a better understanding of how cells from the mesendoderm will differentiate to form the lining of the abdominal wall and the intestinal tissue.

Several SSTFs are involved in the pathogenesis of congenital body wall defects. The homeobox transcription factor *Pitx2* regulates body wall formation and muscle specification (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999). Mutations in human *Pitx2* contribute to Axenfeld-Rieger syndrome and Omphalocele and VATRE-Like syndrome (Katz et al., 2004), which are characterized by umbilical abnormalities. *Pitx2* functions upstream of several growth factor signaling pathways, that regulate cell-type specific proliferation.

In this study we performed a systems biology approach to identify *Pitx2* target genes during mouse abdominal wall development. Gene expression data from microarray experiments were integrated with online gene ontology databases and in house CRM prediction scripts. Abdominal wall tissue from *Pitx2* mutant, heterozygote and wild type E10.5 mice were prepared for analysis on Mouse Genome 430 2.0 microarrays, with RMA normalized data (Koussis and Gross, 2008) and analyzed with SAMExcel (Tusher et al., 2001). This resulted in 324 probe sets, representing 275 unique genes that were differentially expressed. Genes were placed into functional bins such as “differentiation”, “migration”, “adhesion”, and “signaling” based on their gene ontology annotations using DAVID Bioinformatics Functional Annotation Tool (Dennis et al., 2003; Huang et al., 2009). This tool bins genes based on the frequency of occurrence of ontology key words between our genes of interest versus the entire database of known genes. The most enriched bin (Bin1), containing 87 genes, was further analyzed for predicted *Pitx2* binding sites in the -20kb region. Genes with a minimum of two *Pitx2* binding sites conserved in at least 3 and 4 species respectively narrowed the list to 25 genes. Target genes containing a second gene with *Pitx2* binding sites in both intronic and/or exonic sequences in this -20kb region were eliminated. This resulted in 20 genes that were further analyzed for conserved *Pitx2* sites within the gene itself, which included the coding and intronic sequences. These CRM prediction scripts were used to place *Pitx2* and its targets in contact to specific tissues of interest. A predicted network model was constructed by using BioTapestry Version 4 to visually link the *Pitx2* with its target genes.

2. Materials and methods

2.1. Mice

The *Pitx2*^{+/LacZ} mice (Lin et al., 1999) were used. Female mice were checked for the presence of vaginal plug (E0.5). Embryos were isolated at E10.5 and yolk sacs were used for genotyping. Tissue from the abdominal wall from each genotype was isolated by cutting across embryos behind the forelimb and in front of the hindlimb, with removal of the neural tube and obvious internal organs.

2.2. RNA Preparation and Microarray Analysis

Abdominal wall tissue was dissected from E10.5 *Pitx2*^{+/+} (Wild Type, WT), *Pitx2*^{+/LacZ} (Heterozygote, HET) and *Pitx2*^{LacZ/LacZ} (Homozygote, MUT) mice. Total RNA was prepared using Qiagen RNeasy Mini kit, labeled using Affymetrix one-step labeling, and used to probe the Affymetrix Mouse Genome 430 2.0 array. The raw *.cel files were normalized by RMA using RMAExpress (Bolstad et al., 2003; Kioussi and Gross, 2008). The data sets were further processed with SAMExcel with a delta value of 4 and a minimum fold change of 1.5. The significantly differentially expressed genes were analyzed with DAVID Bioinformatics Functional Annotation Tool (Dennis et al., 2003; Huang et al., 2009). The DAVID Functional Annotation Clustering application provided functional clusters of genes that were individually searched for the *Pitx2* binding sites.

2.3. Pitx2 Binding Site Analysis and Biotapestry

For initial screening, genomic sequences with -20kb flanking each gene were downloaded from MGI's link to the mouse reference genome (NCBI v37, mm9). These were processed with a script, *binding_site_search.pl* that was generated by our lab to determine the location of *Pitx2* binding sites, TAATCY, relative to the start of the gene (Amendt et al., 1998; Wilson et al., 1996). For identifying the evolutionary conservation of these *Pitx2* binding sites, the -20kb region of selected genes as defined by the gene entry locations in the UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly available at <http://genome.ucsc.edu/>, was downloaded and formatted for *binding_site_compare.pl*. The script concatenated the alignments from the UCSC Genome Browser, identified the absolute *Pitx2* binding site locations for each gene based on the mouse sequence, and reported the species for which each binding site was conserved within. Cytoscape 2.6.3 was utilized for composing visualizations of gene expression data (Shannon et al., 2003). BioTapestry Version 4 was utilized for composing the predicted gene network in the abdominal wall (Longabaugh et al., 2005).

3. Results and Discussion

3.1. Pitx2 Target Gene Clusters in Abdominal Wall

Gene expression analysis was used to identify *Pitx2* target genes during abdominal wall development. Total RNA was prepared from tissue of three biological replicates for WT, HET and MUT embryos. Probed RNAs were applied to nine Mouse Genome 430 2.0 microarrays. Scatter plot comparisons (data not shown) within each of the biological triplicates indicated that one of the three array sets was inconsistent with the other two. Thus, results from the six comparable arrays were normalized by RMA. Array results have been deposited for public access at ArrayExpress under the accession number E-MEXP-2332, (Hilton et al., 2010).

Data represented by 45,101 probes and >39,000 transcripts were processed with SAMExcel (Tusher et al., 2001). *Pitx2* target genes were selected based on a Δ of 4 and a minimum fold change of 1.5. These thresholds identified with a 1.5% false discovery rate, 324 statistically significant altered probe sets based on t-tests, representing a total of 275 known unique genes.

The DAVID Functional Annotation Clustering tool was applied to the above genes and generated 31 bins that covered 265 of the above unique genes. The first 10 bins, with enrichment scores greater than 2.0, can be seen as an example of DAVID output in Table 1. Selected key terms (GOTERMs) such as signal, cell differentiation, organ development etc, were ranked according to their relative frequency of occurrence in the *Pitx2* target genes gene-ontology, as compared to their occurrence in all genes identified in the mouse genome. These classifications helped categorize the types of *Pitx2* target genes in the developing abdominal wall. The most enriched cluster contained the GOTERM “signal” and “secreted” (Table 1), which include regulatory genes, signaling molecules and sequence specific transcription factors (SSTF) that represent the regulatory apparatus of the cell.

3.2. Evolutionarily conserved *Pitx2* Binding Sites

To evaluate the *Pitx2* target genes, comparative genome analysis was performed, which included the identification of *Pitx2* binding sites in promoter sequences, coding and intronic regions. Initially, the 20kb region upstream of the transcription codon in the genomic sequences of all 265 *Pitx2* target genes from Bin1 to Bin10 were searched for the presence of the TAATCY motif to further confirm regulation by *Pitx2*. The number of *Pitx2* binding sites varied from 6 to 33 per gene (Table 2). If genes were directly regulated, one would expect that bins with the highest enrichment score would have the highest number of potential *Pitx2* binding sites. Indeed, a decrease of the average number of sites was observed as the enrichment score was decreased. Indirect regulation through the recruitment of co-factor binding complexes however cannot be dismissed, and would likely account for the non linear correlation of average binding sites and enrichment scores. However, we chose to focus on the direct regulation of genes by *Pitx2* interaction with predicted binding sites. Because Bin1 was the most significantly enriched as determined by significant P-values from David bioinformatics tools and contained a greater than average number of *Pitx2* binding sites, it was chosen for further analysis of *Pitx2* binding site conservation. A Perl script was developed that could take in the alignment output for each gene from the UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly, and identify the absolute location of each binding site, along with the species that the site was conserved (Kent et al., 2002).

All *Pitx2* target genes with at least 2 *Pitx2* binding sites were originally selected based on having one site conserved in 3 species (rat, human, orangutan) and an additional second site conserved in 4 species (rat, human, orangutan, dog), resulting in a total of 25 genes. Within these genes, 5 genes were eliminated because of the existence of another gene within the –20kb sequences. All *Pitx2* target genes that passed all the above criteria were summarized in Table 2. The top three genes, Hepatocyte growth factor (Hgf), Sulfatase1 (Sulf1) and Thansthyrenin (Ttr) were characterized by up to eight *Pitx2* binding sites conserved in up to eight species, while the majority of the target genes had an average of 2–4 conserved *Pitx2* binding sites. Almost all of the *Pitx2* target genes had multiple highly conserved *Pitx2* binding sites at proximal promoter sequences (Fig 1).

Comparative analysis of the predicted *Pitx2* genes based on the evolutionary conserved binding sites was further analyzed in the intronic and coding sequences (Fig 1). When *Pitx2* target genes were placed in order based on the number of *Pitx2* binding sites per gene length, the order did not change significantly, when compared to the order based on the number of *Pitx2* binding sites within the promoter sequences. The top and bottom genes remained at their respective locations, with some rearrangements occurring in the middle.

3.3. Function of *Pitx2* Target Genes

The order of *Pitx2* target genes based on the absolute fold change of the Affymetrix gene expression array data was different than the one based on the number of conserved *Pitx2*

binding sites (Table 3). However, genes at the low end of Table 2 were also positioned at the end of Table 3 suggesting that genes with <2 fold change harbored less Pitx2 binding sites. This group included the chemokine ligand 12 (Cxcl12), cysteine rich transmembrane BMP regulator 1 (Crim1), follistatin-like 1 (Fstl1), phospholipase A2 receptor 1 (Pla2r1), laminin gamma1 (Lamy1) and tissue inhibitor of metalloprotease 2 (Timp2). Laminins, a family of adhesive glycoproteins, are involved in cell attachment, differentiation and migration (Timpl et al., 1979). Fstl1, an extracellular glycoprotein with antiproliferative effects, acts as a muscle-derived secreted factor with favorable actions on the heart and vasculature (Oshima et al., 2008). Pla2r1, a transmembrane glycoprotein related protein, is involved in cell proliferation, cell migration, lipid mediator production and smooth muscle contraction (Hanasaki et al., 1997). The majority of the *Pitx2* target genes with 1.5–2.0 fold change are glycoprotein type proteins involved in vascular development and maintenance (Hanasaki et al., 1997; Jaworski et al., 2006; Lieberam et al., 2005; Wilkinson et al., 2007; Yang et al., 2009).

Pitx2 target genes with a fold change between 2.0 and 2.5 were more in order compared the number of Pitx2 binding sites. The genes of this group glypican (Gpc4), Insulin growth factor binding protein 1 (Igfbp2), podocalyxin-like 1 (Podxl1) and gelsolin (Gsn) are involved in cell growth and apoptosis (Kleffens et al., 1999; Maltby et al., 2009; Mejillano et al., 2004; Ybot-Gonzalez et al., 2005). Deletions in the Gpc4 gene, a heparan sulfate proteoglycan, are associated with the gigantism syndrome Simpson-Gobaldi-Beckwith (SGBS, OMIM: 312870), (Veugelers et al., 2000). Gpc4 is part of the Wnt/planar cell polarity pathway for convergence and extension during zebrafish gastrulation (LeClair et al., 2009). Gsn promotes apoptotic resistance and epithelia mesenchymal transformation via actin filament remodeling (Silacci et al., 2004). Mutation in Gsn is associated with the Familial Amyloid, Finnish type polyneuropathy (FAF, OMIM: 105120), (Levy et al., 1990). These genes are also cell cycle related genes, which is significant as Pitx2 regulates organ development upon binding and activation of cell cycle control genes, such as cyclins (Kioussi et al., 2002). Collectively, the gene expression array data and the bioinformatics analysis of the evolutionary conserved Pitx2 binding sites suggest that Pitx2 binds to CRMs of these genes and inhibits tissue specific cell cycle (Fig. 3).

Pitx2 target genes with a fold change >3 were the ones with the highest number of conserved Pitx2 conserved binding sites in coding and non-coding sequences. Hgf, commonly known as scatter factor (SF), is a secreted protein that functions as a mitogen with morphogenetic activities in a variety of epithelial cells (Soriano et al., 1995). Hgf is mediated by the Met tyrosine kinase receptor for paracrine signaling during placenta, liver, muscle and neuron morphogenesis (Ebens et al., 1996; Maina et al., 1997; Schmidt et al., 1995; Uehara et al., 1995). Hgf mutant mice exhibit impaired migration of the myotome towards the forelimbs (Schmidt et al., 1995). Pitx2 mutant mice exhibit severe muscular malformations in head, abdomen and limbs (Hilton et al., 2010; Shih et al., 2007). Met induces Wnt signaling that activates Pitx2 in several tissues. The strong presence of Pitx2 binding sites suggests that Pitx2 promotes Hgf expression in the migrating myotome to the abdominal wall and limbs. Sulfatases (Sulf1, 2) are essential regulators of cellular heparan sulfate-6-O-sulfation for matrix transmission and reception of GDNF signal from muscle to innervating neurons (Ai et al., 2007). Pitx2 can act as a repressor of Sulf1 expression in the abdominal wall tissue (Table 3), and inhibit the GDNF signaling cascade from muscles to neurons. Sulf1 regulates Wnt signaling by modulating sulfation state of cell surface heparin surface proteoglycans (Lin and Perrimon, 2002). A possible mechanism is that Pitx2 inhibits Sulf1 expression that activates Wnt to stimulate Pitx2 expression (Fig. 3). Transthyrein (Ttr), a transporter of hormones and proteins in the plasma is expressed in the visceral endoderm and degraded in the adult liver, kidney and muscle (Kwon and Hadjantonakis, 2009; Makover et al., 1988). The clinical syndromes associated with TTR aggregation are familial amyloid polyneuropathy (FAP) and cardiomyopathy (FAP), in which mutant TTR protein aggregates in peripheral and autonomic

nerves and heart (Saraiva, 2001). *Pitx2* is highly expressed in the endoderm derived intestinal tissue, and *Pitx2* mutants exhibit hypocellular and severely malformed intestines. *Pitx2* might act as a repressor to *Ttr* and further inhibit the transportation of critical molecules for the intestinal growth and development. The Dkkopf homolog 1 (*Dkk1*), a secreted extracellular protein acts as inhibitor of the Wnt signaling pathway, with high levels in the abdominal organs. A proposed mechanism is that the Wnt/ β -catenin pathway activates *Pitx2* (Kioussi et al., 2002), which inhibits *Dkk1*, which in turn negatively regulates *Wnt1*. Fibulin (*fbln-5*) is an elastin-binding protein required for assembly and organization of elastic fibers. *Fbln-5* is an inhibitor of vascular smooth muscle cell proliferation and migration (Spencer et al., 2005). *Pitx2* can inhibit *Fbln-5* and promote indirectly the smooth muscle cell proliferation. Interaction of *Pitx2* with its target genes is demonstrated in Fig 2. The strength of *Pitx2* interactions with its target genes is demonstrated in Fig 2. Stronger interactions as defined by a high number binding sites or a large fold change are represented as genes positioned closer to the core, while weaker interactions are displayed further away. Targets such as *ttr*, *sulf*, and *hgf* remain close to the core, while targets such as *Pla2r1*, *Tmp2*, *Cxcl12* and *Fstl* remain far from the core, when comparing their strength of interaction as defined by binding sites or fold change. Genes in the middle rankings of either method of ordering tend to shift within in the middle regions, with a notable exception being Albumin (*Alb*).

The Albumin (*Alb*) gene exhibited a lower number of conserved binding sites with a higher fold change when compared to the other genes that were selected for analysis. One might explain this discrepancy to the different developmental fields involved in the abdominal wall development. Gene expression analysis was performed in a mixed cell population rather than a specific cell lineage. Another factor might also be the fact the bioinformatics approach is based on direct protein DNA interactions of *Pitx2* to its target genes. This is not always the case, as co-regulatory complexes are usually present for the SSTF activation in specific cell lineages. Biological validations should verify the expression profiles of all identified *Pitx2* target genes, from which predicted network models as developed in programs such as BioTapestry can be revised. A proposed regulatory network model has been generated based on the network data from these studies and the current literature (Fig 3). This model indicated *Pitx2* as a key node for abdominal wall development.

Furthermore, we suggest that *Pitx2* regulates cell growth and migration by regulating the expression of genes involved in Wnt trafficking. Twenty percent of the target genes discussed previously in this study have been shown to be involved in the Wnt pathway including *Gpc4*, *HGF*, *Sulf1*, and *Dkk1*. *Pitx2* binding sites might represent CRMs that control gene expression in abdominal wall developmental fields, including skeletal and smooth muscle and endoderm-derived cells. The *Pitx2*-dependent gene expression can be regulated by modification of the local chromatin due to the presence of coactivator and/or corepressor complexes that have been initiated by *Pitx2*. *Pitx2* occupies cell cycle specific genes, upon Wnt/ β -catenin activation, releases the histone deacetylase 1 (HDAC1) and promotes proliferation in muscles (Kioussi et al., 2002). *Pitx2* can also occupy and suppress the transcriptional activity of several members of the T-box family in the abdominal wall, by changing the acetylation state of their chromatin (Hilton et al., 2010). *Pitx2* may link the Wnt signaling to changes in developmental fields involved in abdominal wall development through the regulation of genes involved in enzymatic activities for transporting information to the cell membrane that can influence cell specification and migration.

4. Conclusion

These studies introduced a systems biology approach to predict the *Pitx2* target genes during abdominal wall development. *Pitx2* is required for abdominal wall closure and organ formation. We used SAMExcel to analyze microarray data from *Pitx2* mutant, heterozygote and wild type

mouse embryos to extract significantly altered transcripts. DAVID Functional Annotation Clustering Tool identified genes grouped according to enrichment of reported gene ontology keywords. This provided a lead to investigate the Pitx2 dependent cellular/biological processes in the developing abdominal wall. Our studies suggest that Pitx2 regulates abdominal wall development by regulating the expression of genes encoding for cell surface glycoproteins involved in transporting molecules into the cell, genes involved in enzymatic activities that determine cell specification, and genes involved in cell migration; all which are essential in being able to coordinate the closure of the abdominal wall. This study helps form the transcriptional network for abdominal wall development, placing Pitx2 as an essential node in the developmental circuit. Further biological validations should follow to confirm the predicted network at E10.5, with future studies at later developmental stages to establish the network in a temporal manner.

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Figure 1. Evolutionary Conserved Pitx2 binding Sites

Comparative analysis of Pitx2 consensus binding sites in the *Pitx2* target genes. Genomic consensus sequences 20kb upstream of the transcriptional start site were mapped against the aligned sequences in the top twenty Pitx2 target genes. Red dots indicate a predicted Pitx2 binding site. Vertical red dots indicate the number of species the motif was found.

Figure 2. *Pitx2* Target Genes Visualized with Cytoscape

Each circle represents an individual gene with *Pitx2* as the core regulator. Red and green lines represent repressed and activated *Pitx2* target genes respectively. The length of the lines represents the strength of their relationships. **(A)** Representation of target gene relations to *Pitx2* based on the sum of binding sites and number of species conserved from Table 2. Genes with a greater net number of sites and species are mapped closer to the *Pitx2* core. **(B)** Representation of target gene relations to *Pitx2* based on fold change. The greater the fold change the closer the mapping of the gene to *Pitx2*. It appears that *Pitx2* is a general repressor for the majority of our target genes.

Figure 3. Predicted Pitx2 Network Model in Abdominal Wall

BioTapestry was used to create a model of the Pitx2 regulatory network in the abdominal wall. Genes within the nested regions are identified as different gene regulatory components. Red and green links represent repressed and activated interactions from pitx2 as found in this study, while blue links represent interactions identified from literature.

Table 1

Functional Annotation Clusters for Ptx2 Target Genes in the Abdominal Wall

Annotation Bin	Enrichment Score	GOTERM	# of Genes	P-Value	Benjamini corrected P-Value	Average # Ptx2 Binding Sites
1	7.7	Signal	86	8.89E-14	7.60E-11	17.7
		Secreted	33	2.00E-12	8.70E-10	
2	6.5	Developmental Process	86	1.49E-10	2.90E-05	16.6
		Organ Development	49	2.20E-08	2.70E-01	
3	5.3	Extracellular Matrix	19	7.03E-07	9.50E-05	16.0
		Basement Membrane	7	4.90E-04	4.70E-02	
4	4.8	Biological Adhesion	28	2.30E-06	1.70E-03	17.7
		Cell Adhesion	28	2.26E-06	5.50E-02	
5	2.4	Enzyme Regulatory Activity	23	7.00E-04	3.20E-01	17.1
		Protease Inhibitor	10	9.60E-04	2.50E-01	
6	2.3	Cell Proliferation	19	9.60E-04	2.50E-01	15.3
		Regulator of Cell Proliferation	15	1.20E-03	2.90E-01	
7	2.2	Embryonic Development	14	1.50E-05	8.30E-01	14
		Appendage Morphogenesis	6	4.8E-03	5.40E-01	
8	2.18	Regulation of Biological Quality	20	9.70E-03	6.90E-01	15.6
		Growth	11	8.90E-03	6.70E-01	
9	2.1	Vitamin Binding	11	2.80E-06	2.60E-03	19.8
		Cofactor Binding	6	1.40E-01	1.00E00	
10	2.04	Actin Binding	6	9.30E-06	1.60E-3	12.7
		Cytoskeleton	4	1.30E-02	6.30E-1	

Significantly differentially regulated probe sets as identified by SAMExcel were entered into DAVID Functional Annotation Clustering tool. Selected GO terms from the first 10 bins with at minimum of a 2.0 enrichment score. Annotation Bin1 exhibited the most significant P values.

Table 2

Pitx2 Signaling Target Genes With Evolutionary Conserved Binding Sites

Gene	Gene Location	Site Location	Species	Function	Bibliography
Hgf hepatocyte growth factor	Chr5: 16059368– 16125257	16044545	M, R, H, O, D, Ho	Organogenesis placenta, liver, muscles, neurons	Dietrich et al., 1999; Ebens et al., 1996; Maina et al., 1997; Schmidt et al., 1995; Uehara et al., 1995
		16047737	M, R, O, D, Ho		
		16050952	M, R, H, O, D, Ho		
		16051906	M, R, H, O, D, Ho		
		16052042	M, R, Ho		
		16052047	M, R, H, O, D, Ho		
		16052876	M, R, H, O, D		
Sulf1 sulfatase 1	Chr1: 12708626– 12850453	12689935	M, D, Ho	Adult Muscle Development	Langsdorf et al., 2007
		12690965	M, R, H, O, D, Ho		
		12694348	M, H, O		
		12700950	M, R, H, O, D		
		12703579	M, H, O		
		12703612	M, R, H, D, Ho		
		12704923	M, R, H, O, D, Ho		
		12705011	M, R, H, O, D, Ho		
Ttr transferrin	Chr18: 20823914– 20832383	20805080	M, R, H	Visceral Endoderm Development Nerve Regeneration	Fleming et al., 2007; Kwon and Hadjantonakis, 2009
		20816233	M, R, H, O, Ho		
		20816262	M, H, O		
		20816278	M, R, H, O, Ho		
		20818368	M, H, O, D, Ho		
		20819472	M, R, Ho		
		20819896	M, R, H, O		
		20823753	M, R, H, O, D, Ho, C, Op		
			M, D, Ho		
			M, R, H, O, Ho		
Dkk1 dickkopf homolog 1	Chr19: 30620353– 30624155	30625758	M, D, Ho	Limb Development and Patterning	Verani et al., 2007
		30629096	M, R, H, O, Ho		
		30639102	M, R, H, O, D, Ho		
		30643643	M, H, O		
Sema3d semaphorin 3D	Chr5: 12383556– 12587058	12367488	M, R, H, O	Spinal Motor Neuron Development	Cohen et al., 2005
		12369334	M, R, D, Ho		
		12370226	M, H, O, Ho		
		12383276	M, R, H, O, Op		
Tnc tenascin C	Chr4: 63620819– 63681759	63683738	M, H, O, D	Neuron Axon Guidance Myoblast Fusion, Tendon Formation, Sarcomere Assembly	El-Karef et al., 2007
		63689818	M, H, O, D, Ho		
		63690696	M, H, O, Ho		
		63696163	M, H, O, D		
Apcdd1 adenomatosis polyposis coli down- regulated 1	Chr18: 63081981– 63112849	63062451	M, H, O, D, Ho	Bone Development	(Hecht et al., 2007)
		63062984	M, H, D		
		63068603	M, H, O, D, Op		
		63075658	M, H, O		

Gene	Gene Location	Site Location	Species	Function	Bibliography
Fbln5 Fibulin5	Chr12: 102988153– 103057298	103058272 103068145 103071053 103075085	M, R, H, O, Ho M, R, H M, R, H, O, Ho M, H, O	Smooth Muscle and Vascular Development	(Spencer et al., 2005)
Gpc4 glypican 4	ChrX: 49406198– 49518429	49522591 49530364 49535530 49537545	M, D, Ho M, R, H, Ho M, R, Ho M, R, H, O, D	Cell Growth	Ybot-Gonzalez et al., 2005
Igfbp2 insulin growth factor binding protein 2	Chr1: 72871077– 72899048	72852648 72860887 72867615 72870401	M, Ho, Op M, O, D, Ho, Op M, H, D, Ho M, H, O	Cell Growth	Kleffens et al., 1999
Lamy1 laminin, gamma 1	Chr1: 155066052– 155179916	155181670 155184013 155193997	M, R, H, O, Ho M, R, H, D M, R, H, O, D, Ho	Muscle and Vascular Development	Bader et al., 1998; Tiger and Gullberg, 1997
Podxl podocalyxin-like	Chr6: 31469488– 31513981	31515298 31521648 31525429 31526653	M, H, O, Ho M, R, H, O, Ho M, R, O M, R, Ho	glycosylation	Maltby et al., 2009
Alb1 albumin	Chr5: 90889929– 90905627	90873620 90878108 90879959	M, R, H, O, H M, D, Ho M, R, H, O, D, Ho	Serum Transport Carrier Protein	Jin et al., 2009
Gsn gelsolin	Chr2: 35135183– 35163412	35124567 35125503 35128519 35132821	M, H, O M, R, Ho M, H, O, Ho M, H, O, Ho	Cell Growth and Apoptosis	Mejillano et al., 2004
Crim1 cysteine rich transmembrane BMP regulator 1	Chr17: 78596408– 78773654	78580487 78582857	M, R, H, O, D, Ho M, R, H, O	Vascular Endothelial Development	Wilkinson et al., 2007
Cxcl12 chemokine ligand 12	Chr6: 117118553– 117131385	117101734 117102255	M, R, H, O, D, Ho, Op M, R, D	Angiogenesis in Early Development Motor Axon Patterning	Lieberam et al., 2005
F3 coagulation factor III	Chr3: 121426527– 121437960	121423171 121424657	M, R, H, O, Ho M, H, O	Vascular Development	Ong et al., 2000
Fstl1 follistatin-like 1	Chr16: 37776959– 37836600	37757499 37762158	M, R, H, O M, H, O	Nervous System Mesenchyme Development	Yang et al., 2009
Pla2r1 phospholipase A2 receptor 1	Chr2: 60255600– 60391365	60392795 60400516	M, H, O, H M, R, D	Lipid Metabolism	Hanasaki et al., 1997
Timp2 tissue inhibitor of	Chr11: 118162383– 118217054	118224740 118233948	M, R, B, H8 M, R, B	Extracellular Matrix Maintenance	Jaworski et al., 2006

Gene	Gene Location	Site Location	Species	Function	Bibliography
metalloproteinase 2					

M, mouse; R, rat; O, orangutan; D, dog; H, human; Ho, horse; Op, opossum, C, chicken

Table 3Gene Expression Profile of *Pitx2* Target Genes

Gene	WT	HET	MUT	Δ Fold
Alb	2533 $\pm$ 202	2146 $\pm$ 48	497 $\pm$ 58	-5.1$\pm$0.2
F3	122 $\pm$ 31	220 $\pm$ 3	435 $\pm$ 25	3.6$\pm$0.7
Tnc	836 $\pm$ 76	923 $\pm$ 48	3029 $\pm$ 113	3.6$\pm$0.5
Fbln5	247 $\pm$ 15	395 $\pm$ 47	785 $\pm$ 57	3.2$\pm$0.0
Ttr	884 $\pm$ 62	520 $\pm$ 40	2750 $\pm$ 212	3.1$\pm$0.0
Hgf	329 $\pm$ 39	181 $\pm$ 14	126 $\pm$ 1	-2.6$\pm$0.3
Dkk1	87 $\pm$ 1	85 $\pm$ 0	224 $\pm$ 3	2.6$\pm$0.0
Gsn	307 $\pm$ 11	431 $\pm$ 44	761 $\pm$ 39	2.5$\pm$0.0
Igfbp2	2443 $\pm$ 152	4593 $\pm$ 645	5580 $\pm$ 180	2.3$\pm$0.2
Sulf1	470 $\pm$ 28	732 $\pm$ 80	1034 $\pm$ 21	2.2$\pm$0.1
Podxl	202 $\pm$ 2	529 $\pm$ 155	446 $\pm$ 236	2.2$\pm$1.2
Gpc4	741 $\pm$ 6	1588 $\pm$ 11	1472 $\pm$ 9	2.0$\pm$0.0
Cxcl12	1478 $\pm$ 1	2509 $\pm$ 167	811 $\pm$ 75	-1.8$\pm$0.2
Apcdd1	461 $\pm$ 12	800 $\pm$ 9	832 $\pm$ 37	1.8$\pm$0.0
Crim1	233 $\pm$ 1	322 $\pm$ 37	428 $\pm$ 5	1.8$\pm$0.0
Lamc1	691 $\pm$ 49	819 $\pm$ 42	1173 $\pm$ 28	1.7$\pm$0.1
Fstl1	4861 $\pm$ 177	5925 $\pm$ 34	7884 $\pm$ 572	1.6$\pm$0.1
Pla2r1	157 $\pm$ 5	220 $\pm$ 8	252 $\pm$ 1	1.6$\pm$0.1
Timp2	1016 $\pm$ 44	1321 $\pm$ 123	1656 $\pm$ 65	1.6$\pm$0.1
Sema3d	219 $\pm$ 7	210 $\pm$ 19	330 $\pm$ 6	1.5$\pm$0.1