Gene 469 (2010) 1–8

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Prediction of regulatory networks in mouse abdominal wall

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ARTICLE INFO

Article history:
Accepted 16 August 2010
Available online 24 August 2010

Received by I.B. Rogozin

Keywords:
Pitx2
Homeobox
Development
Gene expression analysis
Gene regulation
Bioinformatics

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. The absence of Pitx2 leads to an 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 −20 kb, 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.

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 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 VATER-like syndrome (Katz et al., 2004), which are characterized by

Abbreviations: GO, gene ontology; SSTF, sequence specific transcription factor; CRM, *cis*-regulatory module; RMA, robust multichip average; WT, wild type; HET, heterozygote; MUT, mutant; MGI, mouse genome informatics.

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doi:10.1016/j.gene.2010.08.008

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 (Kioussi 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 −20 kb 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 biding sites in both intronic and/or exonic sequences in this −20 kb 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 −20 kb 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 −20 kb 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://www.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 20 kb 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