Gene 513 (2013) 287–291

Contents lists available at SciVerse ScienceDirect

Gene

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

Short Communication

Akt1 as a putative regulator of *Hox* genes

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ARTICLE INFO

Article history:
Accepted 20 October 2012
Available online 12 November 2012

Keywords:
Hox gene
Akt1
In silico analysis
DNA methylation

ABSTRACT

In mammals, precise spatiotemporal expressions of *Hox* genes control the main body axis during embryogenesis. However, the mechanism by which *Hox* genes are regulated is poorly understood. To discover the putative regulator of *Hox* genes, *in silico* analyses were performed using GEO profiles, and *Akt1* emerged as a candidate regulator of *Hox* genes in E13.5 MEFs. The results of the RT-PCR showed that 5′ *Hoxc* genes, including ncRNA were upregulated in *Akt1* null MEF. Combined bisulfite restriction analysis (COBRA) and bisulfite sequencing showed that the CpG island of a 5′ *Hoxc* gene was hypomethylated in *Akt1* null cells. These results indicate that *Hox* expression could be controlled by the function of *Akt1* through epigenetic modification such as DNA methylation.

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1. Introduction

Hox genes, which encode transcription factors containing a homeodomain, play an important role in establishing the anterior–posterior (A–P) body axis. The homeodomain is a highly conserved protein motif found in a wide range of animal species, including vertebrates. In mammals, 39 *Hox* genes are located on four different chromosomal loci as clusters and these are classified into 13 paralogues in the cluster. The chromosomal order of *Hox* genes is collinear, as to the relative position and time of their expression along the A-P axis in the embryo; the most 3′ gene is expressed early in the anterior, and the most 5′ gene is expressed late in the posterior structures during development (Lemons and McGinnis, 2006; Pearson et al., 2005). For proper pattern formation during embryogenesis, the regulation of *Hox* gene expression has to be accurate. However, the transcriptional mechanisms involved in *Hox* expression have not been clearly understood to date (Wellik, 2009).

Thus far, several upstream regulatory factors controlling *Hox* genes have been identified. Members of the nuclear receptor superfamily and their cognate ligands have been reported to regulate *Hox* gene expression. One of them, a retinoic acid (RA) plays a key role in the regulation of *Hox* expression. Retinoic acid response elements (RAREs) were identified in several *Hox* genes, and the aberrant RA activity has led to the distinct homeotic transformations through alterations of *Hox* expression (Alexander et al., 2009; Deschamps and van Nes, 2005). Other upstream transcription factors involved in *Hox* gene regulation include CDXs (Bansal et al., 2006; Tabaries et al., 2005), Jpk (Lee and Kim, 2006), Krox-20, SOX/OCT family members, and the HOX proteins themselves (Kobrossy et al., 2006).

Recently, epigenetic regulation of *Hox* gene expression has been suggested, along with the trans-acting factors: chromatin conformations (Ferraiuolo et al., 2010; Lee et al., 2010), chromatin status (Simon, 2010; Woo et al., 2010), boundary elements (Kim et al., 2011), non-coding RNAs (Rinn et al., 2007; Wang et al., 2011), etc. Among these, polycomb-mediated gene repression and DNA methylation have been well-understood and known to play an important role in *Hox* gene regulation. Binding of the polycomb repressive complex 1 (PRC1) component, Bmi1, and the polycomb repressive complex 2 (PRC2) component, embryonic ectoderm development (EED)-enhancer of zeste homolog 2 (EZH2) complex, to the *Hox* promoter led to the enrichment of H2A ubiquitination and H3K27 trimethylation, respectively (Cao et al., 2005, 2008; Wu et al., 2008). Moreover, the PRC components recruit DNA methyltransferase (DNMT) leading to inhibit *Hox* gene transcription (Velasco et al., 2010; Wu et al., 2008; Xi et al., 2007).

In mammals, there are three closely related isoforms of protein kinase B (PKB)/Akt family members: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3. As one of the downstream targets of phosphatidylinositol 3-kinase (PI3K), a serine–threonine kinase PKB/Akt is reported to play a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration. Among these three Akt isoforms, Akt1 is the most predominantly expressed isoform in most tissues (Hanada et al., 2004), and shown to be involved in cellular survival pathways, by inhibiting the apoptotic processes. *Akt1*$^{-/-}$ mice have growth retardation and attenuation of spermatogenesis in males (Chen et al., 2001). Akt2 is expressed predominantly in insulin target tissues. Mice

Abbreviations: Hox, homeobox gene; Akt, v-akt murine thymoma viral oncogene homolog; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; MEF, mouse embryonic fibroblast; GEO, gene expression omnibus; COBRA, combined bisulfate restriction analysis; DNMT, DNA methyltransferase; H3K27, histone H3 lysine 27; ROS, reactive oxygen species; HDAC, histone deacetylase; PRC, polycomb repressive complex; CDX, caudal type homeobox; SOX/OCT, SRY (sex determining region Y)-box/octamer-binding transcription factor; Jpk, Jopock.

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http://dx.doi.org/10.1016/j.gene.2012.10.034

Table 1
Primers for real-time PCR of *Hoxc* genes.

Hox	Accession no.	Sequence (5′→3′)
C4	NM_013553	F-GACTCCCCAACACCAAAGTG R-GACAAGGGAGGAGGCAAGAG
C5	NM_175730	F-CATGCCTGTTTGCTGTCATC R-CATTGTGGAAGGCTGGAGAG
C6	NM_010465	F-CCTTGCACCCCTCTCTTCTC R-CAGACAAGCCAGGAAGAAGC
C8	NM_010466	F-CACGTCCAAGACTTCTTCCACCACGGC R-CACTTCATCCTTCGATTCTGGAACC
C9	NM_008272	F-CGACCTGGACCCTAGCAAC R-TCATCTTCATCCTCCGGTTC
C10	NM_010462	F-CAAGGGACCTATGGTTCGTG R-GAAATGCAGCAAGGAAGGAG
C11	NM_001024842	F-CGAGGAGGAGAACACGAATC R-TGTCGGTCTGTCAGGTTCAG
C12	NM_010463	F-GAATCCGACTCCAGTTCGTC R-CAGAAACTCGCCCTCCAG
C13	NM_010464	F-GTCCCATTCCACCCCTAATC R-GCACAGGAAGAGGCAAGAAC

lacking *Akt2* display a profound diabetic phenotype; in addition to beta-cell dysfunction, these mice are impaired in glucose homeostasis, indicating that Akt2 plays an important role in diabetes (Chen et al., 2009). Mice lacking Akt3 have small brains, but the role of Akt3 remains less clear (Easton et al., 2005). These Akt isoforms are similarly activated and phosphorylate downstream substrates of more than 50 proteins.

In an attempt to search for upstream regulators of *Hox* genes, we applied *in silico* analysis and found one candidate, protein kinase B (PKB) alpha/Akt1. Here we report PKBα/Akt1 as a putative upstream regulator of *Hox* genes.

2. Materials and methods

2.1. Mouse embryonic fibroblast (MEF) cells and culture

Immortalized wild-type (*Akt1*$^{+/+}$) and *Akt1*$^{-/-}$ MEFs (E13.5) were generated as previously described (Bae et al., 2003; Cho et al., 2001). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM: WelGENE Inc.) supplemented with 10% fetal bovine serum (WelGENE Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (WelGENE Inc.) at 37 °C in 5% CO_2.

Table 2
GEO datasets showing different expression profiles of *Hoxc8*.

Dataset	Experiment	*Hoxc8* expression
GDS1784	Protein kinase B alpha knockout effect on adipogenesis	Up-regulation in *PKB alpha* null
GDS2149	PGC-1alpha null brown adipocyte response to cAMP	Down-regulation in *PGC-1 alpha* null
GDS2843	Mammary gland development	Down-regulation depending on pregnancy time
GDS2123	Brown fat cell response to PGC-1alpha and PGC-1beta deficiency	Down-regulation in *PGC-1 alpha* knockout
GDS1500	Mechanical stress effect on fibroblasts from various fetal tissues	Up-regulation in skin fibroblast
GDS2743	Brown and white adipocyte differentiation	Up-regulation in white pre-adipocyte
GDS2209	Spinal cord and dorsal root ganglion	Down-regulation in dorsal root ganglion
GDS2699	Mesenchymal and epithelial compartments of the developing intestine	Up-regulation in mesenchyme
GDS1272	Luteinizing hormone overexpression effect on the mammary gland	Down-regulation in LH over-expression

2.2. RT- and real-time PCR analysis

Total RNAs were extracted from MEFs using the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Prior to RT-PCR, genomic DNA was eliminated using the TURBO DNA-free Kit (Ambion). Two micrograms of total RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega) and cDNA was amplified with the specific *Hox*. Amplification conditions were as follows: 5 min at 94 °C;27–35 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C; followed by one cycle of 7 min at 72 °C. For real-time PCR analysis, Power SYBR Green PCR Master Mix (Applied Biosystems) was used. The specific *Hoxc* primers for real-time PCR are listed in Table 1.

2.3. DNA extraction and bisulfite modification

Genomic DNA was extracted from the MEFs using a standard protocol. For bisulfite modification, 10 μg of genomic DNA was sheared with a 1 ml syringe and 500 ng DNA samples were subjected to sodium bisulfite conversion using EZ DNA Methylation-Gold Kit (Zymo Research). Genomic DNA from *Akt1*$^{+/+}$ MEFs was treated with CpG

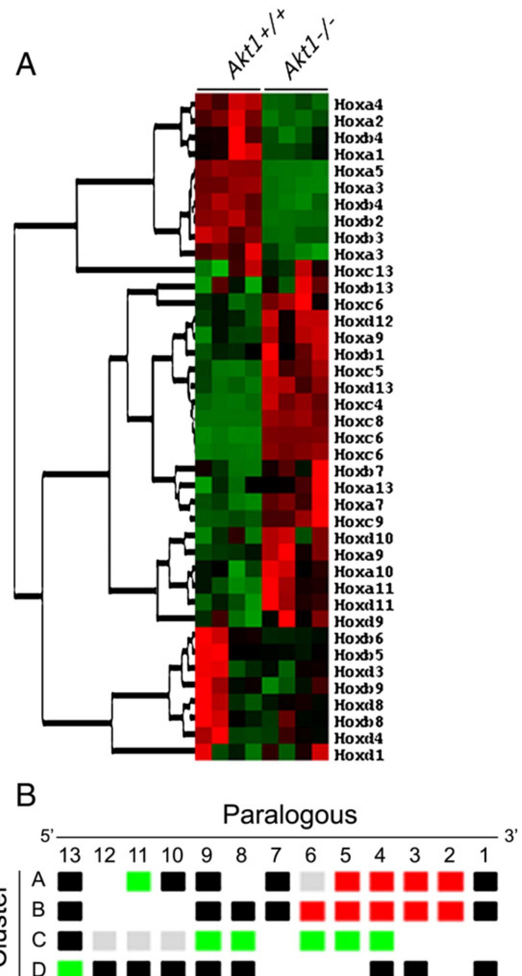

Fig. 1. Hierarchical clustering analysis of the GDS1784 dataset. (A) Hierarchical cluster heat map. The level of gene expression is color-coded. Red, higher expression; green, lower mRNA expression; black, no difference. (B) Summary of differentially expressed *Hox* genes. Each column represents a *Hox* paralogue (1 through 13), and each row a *Hox* cluster. Colors represent the expression level of *Akt1*$^{-/-}$/*Akt1*$^{+/+}$ (Red, <0.67; Black, between 0.67 and 1.5; Green, >1.5; Gray, missed *Hox* genes in this dataset).

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