RESEARCH NOTE

Title: *Sp1* was an important transcriptional regulation factor of *Forkhead box N1* in pig

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Running title: *Sp1* was a transcriptional regulation factor of *Foxn1*

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Sp1 was an important transcriptional regulation factor of *Forkhead box N1* in pig

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Introduction

The transcription factor *Foxn1* plays an important role in the development and function of thymic epithelial cells in vertebrates. However, the transcriptional regulation of *Foxn1* is still unknown. A series of dual luciferase report vectors were constructed and the relative activities of report vectors were detected. The 5'-untranslated regions contains two *cis*-acting elements, *Sp1* and *GATA-1*, as well as trans-acting elements between positions −332 to −438. Nevertheless, the relative activities of *Foxn1* promoter was significantly increased in PK15 cells only while the *Sp1* was overexpressed, suggesting that the *Sp1* was the most important sequence for *Foxn1* transcription activation in the pig. Regrettably, the exact trans-acting elements were not found.

The thymus, which is the main site of T lymphocyte development, is one of the most important organs of the immune system. Transcription factor forkhead box N1 (*Foxn1*) is an epithelial cell-autonomous gene that predominantly regulates the development of thymic epithelial cells (TECs) and skin keratinocytes (Palamaro *et al*., 2014). Mutations in the *Foxn1* gene result in failure in thymus development, hairless nude skin, and short life (Abitbol *et al*., 2015; Bryson *et al*., 2013). On the other hand, *Foxn1* upregulation in the thymus of aged mice could result in the regeneration in thymus function (Bredenkamp *et al*., 2014). *Foxn1* maintains TECs to support T-cell development via mcm2 (Ma *et al*., 2012). miR-18b and miR-518b could up-regulate *Foxn1*transcription (Kushwaha *et al*., 2014). The expression pattern of *Foxn1* had obviously temporally and spatially specific. Until now, it was only detected in thymus and skin. Its expression reached highest level after birth, and then declined (Rode *et al*., 2015).

Materials and methods

Three fragments of the 5' UTR of the pig *Foxn1* gene were obtained by PCR amplification. Primer details are presented in Table 1 (A, B, C, and R). The restriction enzyme *Nhe*I was introduced to the

sense primer, whereas the endonuclease *Hind*III was introduced to the reverse primer. The fragments were ligated upstream of the firefly luciferase gene in the plasmid pGL3-Basic. The accuracy of the insert fragment was detected by sequencing. PK-15 cells were transfected with Lipofectamine^{TM} 2000 Reagent. Cells were cotransfected with the *Foxn1* promoter constructs and a plasmid containing a *Renilla* luciferase (pRL-TK) to assess for transfection efficiency (pRL-TK: *Foxn1* promoter constructs = 1:399). Twenty-four hours after transfection, the cells were harvested and firefly and *Renilla* luciferases were assessed by using the Stop & Glo kit (Promega). Firefly luciferase activity was normalized to that of the Renilla luciferase activity, and the results were expressed relative to that of the promoterless vector. Cotransfections were performed four times, and the luciferase assays were conducted three times on individual transfection experiments. *Cis*- and *trans*-acting elements among 5'-UTR region were all verified using this method.

The complete CDS of *GATA-1* (NC_010461) and *Sp1* (NC_010447) were cloned by RT-PCR. The restriction enzyme *EcoR I* was introduced to the sense primer, whereas the endonuclease *Xba I* was introduced to the reverse primer. The primer sequences were list in the Table 1. The fragments were ligated in the plasmid pcDNA3.1(+). The accuracy of the insert fragment was detected by sequencing. pcDNA-*GATA-1* and pcDNA-*Sp1* were cotransfected with the pGL3-Basic-*Foxn1*, respectively. Firefly luciferase activity was measured as above.

All data were expressed as the mean ± SEM. Statistical analysis was performed using the student's *t*-test using the SAS 9.2 statistical software. The threshold of significance was defined as $P < 0.05$ or $P < 0.01$.

Results and discussion

The 5'-UTR sequence of the pig *Foxn1* of 1,001 bp was cloned, the indicated fragment length is the length of the total fragment and does not refer to the nts before the initiation codon. A series of 5'-UTRs of the *Foxn1* promoter at nucleotide (nt) positions −973, −620, and −224 bp and with a common 3'-terminus at position +28 bp were cloned into luciferase reporter vector pGL3-basic. In the present study, the first nucleotide of the initiation codon was designated as +1. The accuracy of the constructs was confirmed by sequencing.

The *Foxn1* promoter activities of the 5'-deletions mutants were assessed by using a dual-luciferase reporter assay after transient transfection into PK15 cells. Fig.1(a) shows that the most remarkable changes in promoter activity were observed between pGL3-basic (control) and (−224/+28 bp; $P < 0.01$), (−224/+28 bp) and (−620/+28 bp) ($P < 0.01$), (−620/+28 bp) and (−973/+28 bp) ($P < 0.01$). It is possible that *cis*-acting elements were present within the −224–+28 bp region and −620–−973 bp region, as well as *trans*-acting element within the −224–−620 bp region, *cis*-acting should be promoter elements and *trans*-acting should be repressor elements.

The online database (http://www.gene-regulation.com/cgi-bin/pub/ programs/patch/bin/patch.cgi) was used to analyze the potential transcription factor binding sites between −224 to +28 bp. Several transcription factors were detected such as *GATA-1*, *GATA-2*, *GATA-3*, *SRY*, *E2F*, *v-Myb*, *Sp1*, and

MZF1, and these all can bind to this region. To identify the most important *cis*-acting elements within the *Foxn1* promoter, overlap-extension PCR was performed for site-specific mutagenesis within the −224 to +28 bp regions. Three mutants were constructed. Mutant 1 showed a deletion of the sequence GGGTGATGGTGTCACTACTCCCGCCGCAGTCTG, which included the transcription factor binding site, *GATA-1*. Mutant 2 showed a deletion of the sequence TGAGGT and were replaced by the sequence CAACAC, which included the transcription factor binding site, *Sp1*. Mutant 3 showed a deletion of the sequence ATCTGTTT and was replaced by CCACACCC, which included the transcription factor binding site, *GATA-3*. Except for the deleted nucleotides, the rest of the other sequences were in complete agreement. Five primer pairs were designed to amplify the mutants (Table 2). Three mutants were produced and their activities were analyzed as earlier described. The activities of every mutant was compared to that of the −224/+28 bp fragment. Fig. 1(b) shows that the relative transcriptional activity of mutant 1 and mutant 2 was statistically significant ($P < 0.01$).

The eukaryotic expression vector of pcDNA3.1-*GATA-1* and pcDNA3.1-*SP1* were constructed. They were cotransfected with pGL3-basic-*Foxn1*, respectively. The relative luciferase activity of pcDNA3.1-*SP1* adds pGL3-basic-*Foxn1* is much higher than others (Fig. 1(c)). It means *Sp1* is one of the most important *cis*-acting elements of *Foxn1*. While the relative luciferase activity of pcDNA-*GATA-1* add pGL3-basic-*Foxn1* is as same as pGL3-basic-*Foxn1*. It means *GATA-1* is not the *cis*-acting element of *Foxn1*. There are some other factors among this region. *Sp1* is also a well-known transcription factor that has been implicated in a wide variety of essential biological processes such as cell growth, differentiation, apoptosis, and carcinogenesis. *Sp1* activates the transcription of various genes that contain putative CG-rich Sp-binding sites within their promoters (Vizcaino *et al*., 2015; Yan *et al*., 2015; Zhao *et al*., 2014).

To identify the most important *trans*-acting elements within the *Foxn1* promoter region, a series of fragments of different lengths between −224 bp and −620 bp were PCR amplified. Nt positions −332, −438, and −530 bp with a common 3'-terminus at position +28 bp were cloned into the luciferase reporter vector pGL3-basic (D, E, F, and R). The activities of every fragment were compared to that of the −620/+28 bp fragment, respectively. Fig.1(d) shows that the relative transcriptional activity between −332/+28 bp and −438/+28 bp were statistically significant different ($P < 0.01$). This finding suggested the existence of *trans*-acting elements within the −332 to −438 bp region. Afterwards, when the similar method was used to detect the exact transcription factors, no factors were found. It means there maybe some new transcription factors, while not be found.

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Table 1 Primer pairs used to amplify the *Foxn1* 5'-UTR sequence

Name	Sequence (5'-3')	Length (bp)	Location (bp)
A	CTAGCTAGCCACGCCACGGTGGTAACCCAAAC	1001	−973~+28
B	CTAGCTAGCGTTGGGAGTTCTCGTCGTGGCTCA	648	−620~+28
C	CTAGCTAGCAGGCTATTTAACTGCTTCCTTCTGT	252	−224~+28
D	CTAGCTAGCCAGACATTCCACAGTGGGCCAGTGT	360	−332~+28
E	CTAGCTAGCTCCGACTAGACCCCTAGCCTGGGAA	466	−438~+28
F	CTAGCTAGCAAGGATCCGGCGATGCCATGA	558	−530~+28
R	CCCAAGCTTCAGACTGCGGCGGGAGTAGTGACA		

GATA-1F	GAATTCATGGAGTTCCCTGGCCTC	1239	-
GATA-1R	TCTAGATCACGAGCTGAGCGG		
Sp1F	GAATTCATGAGCGACCAAGATCACT	2361	-
Sp1R	TCTAGATCAGAAGCCATTGCCACT		

Note: The enzyme cut sites are highlighted in box; protect base are highlighted in line.

Table 2 Primer pairs used to amplify the mutants

Primer	Location(bp)	Sequence (5'-3')	Size (bp)
mutant 1	-224~-6	CTAGCTAGCAGGCTATTTAACTGCTTCCTTCTGT CCCAAGCTTGGTCCTGGCCTCGAAGAAAGC	219
mutant 2-1	-224~-140	CTAGCTAGCAGGCTATTTAACTGCTTCCTTCTGT AGAGAGAACCCACACCAGAGACAGACAGA	85
mutant 2-2	-166~+28	GTCTGTCTCTCCACACCCTTCTCTCTCTC CCCAAGCTTCAGACTGCGGCGGGAGTAGTGACA	194
mutant 3-1	-224~--107	CTAGCTAGCAGGCTATTTAACTGCTTCCTTCTGT CTTCCCTTCCCCAACACTCCCTGGGCT	118
mutant 3-2	-106~+28	CCAGGGACACAACGGGAAGGGAAGCGG CCCAAGCTTCAGACTGCGGCGGGAGTAGTGACA	134

Note: The enzyme cut sites are highlighted in box; protect bases are highlighted in line; the mutation sites are highlighted in gray color.

Figure legends

Figure 1. The relative luciferase activity of Foxn1 promoter in PK15 cells. (a)First analysis of luciferase activity of Foxn1 reporter constructs in PK15 cell lines. The relative luciferase activities of each fragment are all significant difference to pGL3-basic. (b) Second analysis of luciferase activity of Foxn1 reporter constructs in PK15 cell lines. The relative luciferase activity of mutant 1 and mutant 2 are all significant difference to (−224/+28 bp) fragment, but mutant 3 is not. It is mainly for detecting the cis-acting element of Foxn1 promoter. (c) The relative luciferase activity of Foxn1 after cotransfected with *GATA-1* and *Sp1*. The first bar means only pGL3-basic plasmid. The second bar means only pGL3-basic-*Foxn1* plasmid. The third bar means pGL3-basic-*Foxn1* and pcDNA3.1-*GATA-1* cotransfection. The forth bar means pGL3-basic-*Foxn1* and pcDNA3.1-*Sp1* cotransfection. The relative luciferase activity of pGL3-basic-*Foxn1*+pcDNA-*Sp1* is significantly higher than others. (d) The relative luciferase activity of (−438/+28 bp) fragment is significant difference to (−332/+28 bp) fragment. It is mainly for detecting the trans-acting element of *Foxn1* promoter.

** means a significant difference at $P < 0.01$.