Application of the Dual-Luciferase Reporter Assay to the Analysis of Mouse Kit and Yy1 Promoter Activation Regulation by Sohlh1

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Abstract: Kit is significantly downregulated in Sohlh1 deletion ovaries and Sohlh1 was over-expressed in Yy1 cKO ovaries and whether Sohlh1 regulates kit and Yy1 transcription is unknown. Here, researchers examine the promoter transcription activation of Kit and Yy1 regulated by Sohlh1 using dual-luciferase reporter assays. The Kit responsiveness of the *Sohlh1* gene has been validated and provided evidence of Sohlh1 transcriptional activation of the *Kit* gene promoter in the mouse ovary. In contrast, Sohlh1 has little effect on the essential activity of Yy1 promoter. Researchers identifid -1769 to -297 as the transcriptional activitation region of Yy1 promoter and revealed strong inhibitor in -297 to +135 region where maybe G-quadruplexes motifs exist. Researchers found two Yy1 binding Sites in Sohlh1 promoter and surmise that Yy1 negatively regulates Sohlh1 in mouse ovary.

Key words: Mouse, Sohlh1, Kit, Yy1, dual-luciferase reporter assay, China

INTRODUCTION

Mouse are endowed with a large complement of primordial follicles within their ovaries at birth. Some primordial follicles are recruited to grow into primary and larger follicles but most oocytes die by apoptosis during primordial follicle assembly (Brown *et al.*, 2010; Xu *et al.*, 2011). Several transcription factors and signaling molecules such as Sohlh1 (spermatogenesis and oogenesis-specific bHLH transcription factor 1), Sohlh2 (spermatogenesis and oogenesis-specific bHLH transcription factor 2, Figla (factor in the germ line, alpha), *Nobox* (newborn ovary homeobox gene), *Lhx8* (Lim homeobox gene 8), Yy1 (Yin-Yang1), Kit and its Ligand (KitL) have been implicated in primordial to primary and growing follicle transition (De Felici, 2000; Rajkovic *et al.*, 2004; Hutt *et al.*, 2006; Pangas *et al.*, 2006; Joshi *et al.*, 2007; Choi *et al.*, 2008a, b; Griffith *et al.*, 2011).

Sohlh1 is preferentially expressed in germ cells of the embryonic ovary and in oocytes of primordial and primary follicles. Sohlh1 homozygous null females were infertile with atrophic ovaries that lacked oocytes at 10 weeks and occupied ≈1/10 of the WT volume. There appears to be a defect in follicle development during the primordial to primary follicle transition in Sohlh1 deletion ovaries (Pangas *et al.*, 2006).

During early oocyte and follicular development, paracrine communications between oocytes and surrounding granulosa cells are critical for normal cell development and function (Gilchrist *et al.*, 2004). KitL localized to granulosa cells is a growth factor that exerts an influence on oocytes through binding its tyrosine kinase receptor, Kit (localized to oocytes) (Hutt *et al.*, 2006). Kit-KitL interactions play an anti-apoptotic effect in fetal oogonia and oocytes and improve oocytes of primordial to progress beyond the primary follicle in postnatal ovaries (Driancourt *et al.*, 2000).

Yy1 is a multifaceted polycomb group gene that activates, represses or initiates transcription of a diverse assortment of genes (Hinkins *et al.*, 2005). Recent study had presented that YY1 function in the oocyte is critically required for oocyte growth and granulosa cell expansion (Griffith *et al.*, 2011). Deletion of Yy1 from oocytes of primary follicles and growing follicles results in a blockage of folliculogenesis. Yy1 cKO mice are infertile and their

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ovaries lack mature follicles due to defects in proliferation and expansion of granulose cells (Griffith *et al.*, 2011). Several germ cell specific genes are down regulated in Yy1 cKO ovaries such as Growth differentiation factor 9 (Gdf9) and Lhx8 which are also down regulated in Sohlh1 deletion ovaries (Jagarlamudi and Rajkovic, 2011).

Kit is significantly downregulated in Sohlh1 deletion ovaries and Sohlh1 was over-expressed in Yy1 cKO ovaries (Choi *et al.*, 2008a, b; Griffith *et al.*, 2011). Interestingly when Kit, Yy1 or Sohllh1 was absent there was much similarity in oogenesis and ovary clinical phenotype such as sterile, atrophic ovaries, postnatal oocyte loss and the blockage of early folliculogenesis (Hutt *et al.*, 2006; Pangas *et al.*, 2006; Griffith *et al.*, 2011). Researchers suppose the relationship of gene regulation may exist among the three genes. In early mouse folliculogenesis whether Sohlh1 regulates Kit and Yy1 transcription is unknown. Here, researchers examine the promoter transcription activation of Kit and Yy1 regulated by Sohlh1 using dual-luciferase reporter assays.

MATERIALS AND METHODS

All mouse experiments were carried out on a C57BL/6 background.

Mouse Kit and Yy1 promoter-luciferase reporter constructs: Plasmid pGL3-basic consisting of a multiple cloning site in front of the luciferase reporter gene was used to create Kit and Yy1 promoter-luciferase reporter constructs. PCR was performed using mouse tail DNA as template and Platinum® Pfx DNA Polymerase (Invitrogen). Primers were designed that would amplify the region from -2298 to +124 of the *Kit* gene (GenBank Accession Number: NC_000071.5): KitF 5' CGG<u>GGTACC</u>CAACC CAAGAGGAAAGTGTAGC 3' and KitR 5' CCC**AAGCTT**

CCACGGAGCAGGACCAA 3' (KpnI site is underlined and HindIII site is in bold), thermal cycling conditions consisted of an initial denaturation step at 94°C for 4 min followed by 30 cycles of 94°C for 20 sec, 60°C for 30 sec and 68°C for 2 min 30 sec final extension was 68°C for 10 min and -2202 to +135 of the *Yy1* gene (GenBank Accession Number: NC_000078.5): Yy1F 5' CGG<u>GGTACC</u> AGCCTTCCCACATTCATAC 3' and Yy1R 5' TCCCCCGG GCGTGGCGATGTA GAGGGTGT 3' (KpnI site is underlined and SmaI site is italicised), thermal cycling conditions consisted of an initial denaturation step at 94°C for 4 min followed by 30 cycles of 94 for 20 sec, 58°C for 30 sec and 68°C for 2 min 30 sec, final extension was 68°C for 10 min. KpnI and HindIII double digestions were carried out on the Kit promoter and pGL3-basic vector. Subcloning into pGL3-Basic placed the Kit (-2298 to +124) promoter fragments upstream of the promoter-less luciferase gene for promoter activity analysis. The successfully constructed plasmid were named pGL3-Kit-A. The method of the construction of pGL3-Yy1-A was identical to that of pGL3-Kit-A, only used KpnI and SmaI instead. AxyPrep™ Plasmid Miniprep Kit was used to purify the plasmid from Escherichia coli DH5α.

To generate of Kit and Yy1 promoter deletion constructs, the design of primers used for PCR amplifying different lengths of promoters was based on rightly sequenced pGL3-Kit-A and pGL3-Yy1-A as the templates (Table 1). There were difficulties with the SmaI digest hence HindIII was used instead to generate Yy1 promoter deletion constructs. The identities of all Kit and Yy1 promoter constructs were verified by restriction mapping and nucleotide sequencing.

Construction of expression vector for mouse Sohlh1: Total RNA was isolated from new born WT mouse ovaries by using TRIzol (Invitrogen). RNA

Table 1: Oligonucleotides used for PCR to generate Kit and Yy1 promoters deletion constructs

Primer name	Sequence	Products size/bp	Reaction condition
pGL3-Kit-B	F:5'-CGGGGTACCATGATACTCAATGAGGCCATG-3' R:5'-CCCAAGCTT CCACGGAGCAGGACCAA-3'	2054 (-1930 to +124)	94°C 20 sec, 60°C 30 sec, 68°C 2 min 20 sec 30 cycles
pGL3-Kit-C	F:5'-CGGGGTACCGGACTTAAAGAACCCCCAAAA-3' R:5'-CCCAAGCTT CCACGGAGCAGGACCAA-3'	1543 (-1419 to +124)	94°C 20 sec, 60°C 30 sec, 68°C 1 min 40 sec 30 cycles
pGL3-Kit-D	F:5'-CGGGGTACCATCTTGCTCCTAAGGCAGACC-3' R:5'-CCCAAGCTT CCACGGAGCAGGACCAA-3'	1002 (-878 to +124)	94°C 20 sec, 60°C 30 sec, 68°C 1 min 10 sec 30 cycles
pGL3-Kit-E	F:5'-CGGGGTACCCCGTCCTCCTCTACCAAC-3' R:5'-CCCAAGCTT CCACGGAGCAGGACCAA-3'	640 (-516 to +124)	94°C 20 sec, 60°C 30 sec, 68°C 50 sec 30 cycles
pGL3-Kit-F	F:5'-CGGGGTACCTGGTCTTAGAGGGCACAGCG-3' R:5'-CCCAAGCTT CCACGGAGCAGGACCAA-3'	308 (-184 to +124)	94°C 20 sec, 60°C 30 sec, 68°C 25 sec 30 cycles
pGL3-Yy1-B	F:5'-CGGGGTACC GTAGTTAACCTAGAAACGAGG-3' R: 5'-CCCAAGCTTCGTGGCGATGTAGAGGGTGT-3'	1904 (-1769 to +135)	94°C 20 sec, 58°C 30 sec, 68°C 2 min 30 cycles
pGL3-Yy1-C	F:5'- GGGGTACCCTGTTACTTATAATAGGGAAATC-3' R: 5'-CCCAAGCTTCGTGGCGATGTAGAGGGTGT-3'	1462 (-1327 to +135)	94°C 20 sec, 58°C 30 sec, 68°C 1 min 30 sec 30 cycles
pGL3-Yy1-D	F:5'-CGGGGTACCTCGTCAGAGCAGACTTTATTGCG-3' R: 5'-CCCAAGCTTCGTGGCGATGTAGAGGGTGT-3'	979 (-844 to +135)	94°C 20 sec, 58°C 30 sec, 68°C 1 min 30 cycles
pGL3-Yy1-E	F:5'-CGGGGTACCTCGGGGTTGAGGAGTTGGT-3' R: 5'-CCCAAGCTTCGTGGCGATGTAGAGGGTGT-3'	432 (-297 to +135)	94°C 20 sec, 58°C 30 sec, 68°C 30 sec 30 cycles

sample (2 µg) were reverse transcribed with Reverse Transcriptase System (Promega). The resulting cDNA samples were subjected to amplification using gene *Sohlh1* specific primers, Sohlh1F: 5' CGG<u>GGTACC</u>GCT TCCCTCCAACTT CGTGC 3', Sohlh1R: 5' CCGGAATTC TCAGGGGAAAAAG TCAGGGAAGATG 3' (KpnI site is underlined and EcoRIsite is italicised) and Platinum® Pfx DNA Polymerase (Invitrogen). PCR production contained full-length CDS sequence of sohlh1 was ligated into eukaryotic expression vector pcDNA3.0 to obtain recombinant plasmid pcDNA3.0-Sohlh1 which was identified by KpnI/EcoR I double digestion and nucleotide sequencing.

Cell culture, transfection and luciferase assays: Human Embryonic Kidney cells (HEK293) were grown in Dulbecco's modified Eagle's medium with 10% Fetal Bovine Serum (FBS). Cells (in 96 well plates) were cotransfected with Sohlh1 expression plasmid DNA (100 ng) or pcDNA3.0 (100 ng) and each promoter construct (100 ng) using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. To normalize for transfection efficiency the cells were cotransfected with 20 ng of pRL-TK plasmid which encodes renilla luciferase. Firefly luciferase values were divided by renill luciferase values to normalize for transfection efficiency.

About 44-48 h post-transfection, the cells were lysed with 20 µL 1 x passive lysis buffer, cultured for 15 min on a rocking platform at room temperature and then subsequently assayed for luciferase activity in cell lysate using the Dual Luciferase Reporter Assay System (Promega). Each 20 µL lysate was mixed with 80 µL of Luciferase Assay Reagent (LARII) in the 96 well multiwell plate which was then placed in the counter. Firefly luciferase activity was measured first followed by the addition of 80 µL stop/Glo® substrate to measure renilla lusiferase. Statistical significance was determined using the paired t-test and the GraphPad Prism 5 Software package (GraphPad Software). The p<0.05 was deemed to indicate statistical significance.

RESULTS AND DISCUSSION

Kit is important for multiple aspects of oocyte and follicle development including the establishment of primordial germ cells within the ovary, primordial follicle activation, oocyte survival and growth (Hutt *et al.*, 2006). In previous study, the activation of the Kit promoter appears cell-specific in hematopoietic stem cells and mast cells (Maeda *et al.*, 2010). Because the composition of transcription factors is cell-specific, the Kit promoter in
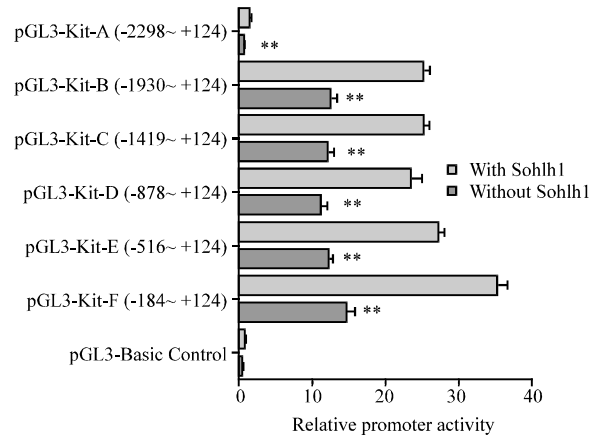

Fig. 1: The expression of Sohlh1 causes activation of mouse Kit promoter. The Kit promoter-firefly luciferase vectors (pGL3-Kit-A-F) was co-transfected with either pcDNA 3.0-Sohlh1 or pcDNA 3.0. All transfections included the control vector pRL-TK that expresses renilla luciferase to correct for differences in transfection efficiency between samples. The activity of all promoter fragments stimulated by Sohlh1 were significantly higher than those of Sohlh1 absence. Data was analyzed with the paired t-test using GraphPad Prism Software, *p<0.05, **p<0.01

oocytes possibly interacts with oocyte-specific transcription factors to appear different activity from hematopoietic stem cells and mast cells. We generated the reporter plasmid pGL3-Kit-A (-2298 to +124) and to screen cis-acting elements within the Kit promoter region, five deletion constructs carrying various lengths of 5'-flanking region were generated from -2298 bp.The programs of TFSEACH (http://mbs.cbrc.jp/research/db/TFSEARCH. html) and WWW Promoter Scan (http://www-bimas.cit. nih.gov/molbio/proscan/) were used to searching transcription factor binding Sites in Kit promoter. The program www promoter scan predicted promoter region in -1577 to -1327 in which a TATA-box (-1349) was found.

Sohlh1 is one of the oocyte-specific gene expressed mainly in early stage of follicles play important and unique roles in primordial follicle formation, activation and survival (Jagarlamudi and Rajkovic, 2011). Figure 1 shows luciferase values of various lengths of Kit promoter in relative activity, cotransfected with or without Sohlh1. In the absence of Sohlh1, the relative luciferase activity of all Kit promoter fragments were over 20 fold higher than that of pGL3- Basic, except pGL3-Kit-A (2 fold). The promoter activity significantly increased by the deletion of -2298 to -1930, suggested that the -2298 to -1930 region contains powerful negative cis-regulatory element. In the -2298 to

-1930 region. There is one E2F binding motif. E2F transcription factors can interact with pocket proteins and possibly function as transcription repressors (Biyashev and Qin, 2011). The activity of pGL3-Kit-F (-184 to +124) activated by Sohlh1 was highest >30 fold higher than that of pGL3-Basic. The program TFSEACH revealed the presence of GATA and Sp1 binding motifs in the -184 to +124 region. GATA-1 and Sp1 are transcription factors with zinc finger domains for DNA binding. The interaction between the GATA-1 and Sp1 zinc finger domains is important for synergistic transcriptional effects (Imanishi *et al.*, 2010). In mast cells, the -232 to +22 region is essential for Kit promoter activity and GATA2 interacts with this promoter region via Sp1 and maintains expression of the *Kit* gene (Maeda *et al.*, 2010). In the presence of Sohlh1, the activities of all promoter fragments were significantly higher than that of Sohlh1 absence and the maximal stimulation was also in the -184 to +124 region. The result suggested Sohlh1 could positively regulate the Kit promoter. Sohlh1 encodes basic Helix-Loop-Helix (bHLH) transcriptional regulator which binding to a consensus sequence CANNTG called the E-box. There are six E-box in the -1419 to +124 region. In mouse ovary which E-box was bound by Sohlh1 to activate Kit promoter requires further research.

Although, Yy1 has been reported to modulate a mounting list of genes such as c-myc, c-fos and p53, limited research has been carried out to investigate the mechanisms underlying how Yy1 is regulated even there is no study on the regulation of Yy1 promoter in oogenesis (Deng *et al.*, 2010). Figure 2 shows, regardless of the presence or absence of Sohlh1, there was no difference between the activites of the same Yy1 promoter fragments except the pGL3-Yy1-E (-297 to +135). Although, significantly activated by Sohlh1, the activity of pGL3-Yy1-E was drastically decreased which was <1/10 of those of pGL3-Yy1-B and pGL3-Yy1-C. The result suggested Sohlh1 was without the function to affect the essential activation of Yy1 promoter. Figure 3 shown in the -297 to +135 region, the G/C content is 78%, markedly higher than that of -2202 to +135(57.6%). G/C-rich strands of DNA can adopt four-stranded structures known as G-quadruplexes (G4) motif (Hurley, 2001). In human YY1 promoter (-250 to 0) and 5'-UTR, the G/C content is 78.4 and 80%, respectively which is similar to that of mouse Yy1. Recent reseach revealed the presence of G4 structures in the promoter and the 5'-UTR of human *YY1* gene and suggested an inhibitory role of the G4 motif in the YY1 promoter (Huang *et al.*, 2012). Researchers suspect that there should be G4 motifs near the transcription start site to inhibit the transcription
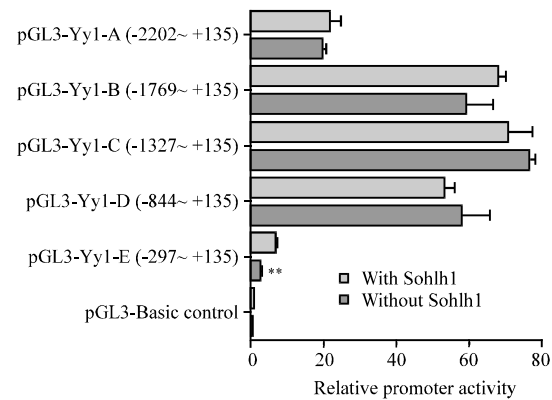

Fig. 2: Sohlh1 has little capability to affect Yy1 transcription. Regardless of the presence or absence of Sohlh1, there was no difference between the activites of the same Yy1 promoter fragments, except the pGL3-Yy1-E (-297 to +135). Although, significantly activated by Sohlh1, the activity of pGL3-Yy1-E was drastically decreased which was much lower than those of the other promoters. Data was analyzed with the paired t-test using GraphPad Prism Software,$^*p<0.05$, $^{**}p<0.01$

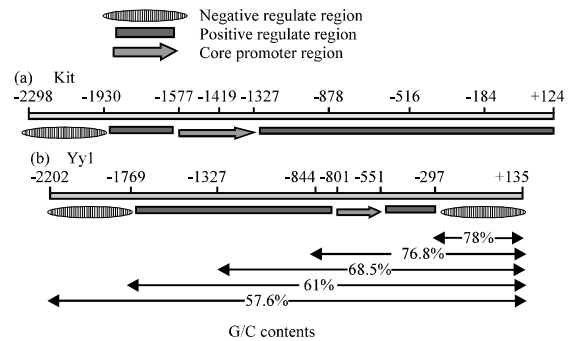Fig. 3: Schematic map of mouse Kit and Yy1 upstream regions; a) The program predicted *Kit* gene promoter region in -1577 to -1327 in which a TATA-box (-1349) was found. The promoter activity significantly increased by the deletion of -2298 to -1930, suggested that the -2298 to -1930 region contains powerful negative cis-regulatory element. The activity of pGL3-Kit-F (-184 to +124) activated by Sohlh1 was highest; b) The G/C content is 78% in the -297 to +135 region of *Yy1* gene where possibly G4 motifs exist to inhibit the transcription activity, markedly higher than that of -2202 to +135(57.6%). The program predicted *Yy1* gene promoter region in -801 to -551. Deletion of the -1769 to -297 region resulted in the promoter activity drastically decreased

activity of mouse Yy1. The -1327 to +135 region (pGL3-Yy1-C) got the maximal activity and the program www promoter scan predicted promoter region in -801 to -551. Since, Sohlh1 has little capability to affect Yy1 transcription why Sohlh1 was over-expressed in Yy1 cKO ovaries? By using the program TESS (http://www. cbil. upenn.edu/tess/), researchers found two Yy1 binding site(CCATNT) at -553 and -885 of Sohlh1 promoter. Yy1 can act as transcriptional activator and repressor whether Yy1 negatively regulates Sohlh1 in mouse ovary (Zhu *et al.*, 2011). However, this hypothesis requires further testing.

CONCLUSION

In this study, Kit has been validated as Sohlh1 responsive through promoter cloning and reporter gene analysis. Transient cotransfections of Kit-luciferase reporter vectors and Sohlh1 expression vector provided evidence of Sohlh1 transcriptional activation of the *Kit* gene promoter in the mouse ovary. Although, Sohlh1 has little capability to affect Yy1 transcription, we identifid -1769 to -297 as the transcriptional activitation region of Yy1 promoter and revealed strong inhibitor in -297 to +135 region where maybe G4 motifs exist.

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