Downstream Genes Regulated by Bcl2l10 RNAi in the Mouse Oocytes

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ABSTRACT : Previously, we have shown that Bcl2l10 as a member of Bcl-2 family, key regulators of the apoptotic process, is dominantly expressed in oocytes of ovary but several member of the Bcl-2 family are not expressed in oocytes. Recent our studies had been processed about roles and regulatory mechanisms of Bcl2l10 in oocytes. Microinjection of Bcl2l10 RNAi into the cytoplasm of germinal vesicle oocytes resulted in metaphase I (MI) arrest and exhibited abnormalities in their spindles and chromosome configurations (Yoon et al., 2009). The present study was conducted to elucidate the downstream genes regulated by Bcl2l10 and signaling networks in Bcl2l10 RNAi microinjected oocytes by using microarray analysis. Surprisingly, we found that a large proportion of genes regulated by Bcl2l10 RNAi were involved in the cell cycle and actin skeletal system regulation as important upstream genes of Bcl2l10. Among the transcripts with highly significant fold changes more than 2-fold, Tpx2 and Cep192 are 16.1- and 8.2-fold down regulated respectively by Bcl2l10 RNAi. Tpx2 and Cep192 are known as cofactors that control Aurora A kinase activity and localization. Therefore, we concluded that Bcl2l10 may have important roles during oocyte meiosis as functional upstream regulator of Tpx2 and Cep192.

Key words : Bcl2l10, RNAi, Microarray, Spindle, Tpx2.

INTRODUCTION

Bcl2l10, also called Diva, has known as a member of Bcl-2 family and has a critical role in apoptosis regulation in adult cells (Adams & Cory 1998; Song et al., 1999). Bcl-2-related proteins constitute two contradictory classes of the family: anti-apoptotic members (Bcl-2, Bcl-xL, Bcl-w, NR-13, A1 and Mcl-1) inhibit cell death (Lu et al., 1995), and pro-apoptotic members (Bax, Bak, Bad, Bik, Bid, Hrk, Bim and Bok/Mtd) promote apoptosis (Song et al., 1999; Naumann et al., 2001). Structurally, Bcl2l10 is a member of pro-apoptotic on sequence analysis but unlike to other Bcl2-related pro-apoptotic protein by lacking critical residues in the BH-3 domain which have correlation between BH-3 containing pro-apoptotic proteins and their binding partners (Inohara et al., 1998).

Bcl2l10 is also considered as involved in regulating the mouse oocyte maturation. We previously have shown that Bcl2l10 is highly expressed in mouse oocytes and Bcl2l10 RNAi caused abnormalities in spindle formation and chromosome segregation (Yoon et al., 2009). Using Mass Spectrometry, we also identified actin and troptomyosin as binding partners of Bcl2l10 which may play an important role in adjusting the cytoskeletal system during oocyte maturation (Yoon et al., 2009). This is consistent with HIP1R (Huntington-interacting protein 1-related) identified as interacting protein partners of Bcl2l10, which is characterized as involving clathrin-mediated endocytic machinery and actin assembly (Kim et al., 2009).

Therefore, we conducted the present study to investigate the downstream genes and important signaling networks for function of Bcl2l10 in the oocytes by using microarray analysis.

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MATERIALS AND METHODS

1. Animals

C57Bl6 mice were obtained from Koatech (Pyeoungtack, Korea) and mated to male mice of the same strain to produce embryos in the breeding facility at the CHA Research Institute of CHA University. All procedures described were reviewed and approved by the University of Science Institutional Animal Care and Use Committee, and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

2. Reagents

Chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless noted otherwise.

3. Isolation of Oocytes

For isolation of GV oocytes from preovulatory follicles, 4-week-old female C57Bl6 mice were injected with 5 IU eCG and sacrificed 46 hours later. Cumulus-enclosed oocyte complexes (COCs) were recovered from ovaries by puncturing the preovulatory follicles with 27-gauge needles. M2 medium containing 0.2 mM 3-isobutyl-1-methyl- xanthine (IBMX) was used to inhibit germinal vesicle breakdown (GVBD) while handling oocytes. Isolated oocytes were snap-frozen and stored at −70℃ prior to RNA isolation.

4. Messenger RNA Isolation

Messenger RNA was isolated from oocytes using the Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS, Oslo, Norway), according to the manufacturer's instructions. Briefly, oocytes were resuspended in 300 ㎕ lysis/binding buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT) for 5 minutes at room temperature. After vortexing, 20 ㎕ prewashed Dynabeads oligo dT_{25} was mixed with lysate and annealed by rotating for 5 minutes at room temperature. The beads were separated with a Dynal MPC-S magnetic particle concentrator and poly $(A)^+$ RNAs were eluted by incubation in 10 ㎕ Tris-HCl (10 mM Tris-HCl, pH 7.5) at 65℃ for 2 minutes.

5. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Complementary DNA (cDNA) was synthesized from mRNA using 0.5 ㎍ oligo dT primer, according to the Super Script Preamplification System protocol (Gibco-BRL, Grand Island, NY). PCR reactions (20 ㎕) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 25 pM each primer, and 2.5 U Taq DNA polymerase (Promega, Madison, WI). Single oocyte-equivalent cDNAs were used as templates for PCR analysis. PCR reaction conditions and primer sequences for the encoding genes are listed in Table 1.

6. Real-time RT-PCR

Quantitative real-time RT-PCR analysis was performed using the iCycler (Bio-Rad, Hercules, CA). The iQ™ SYBR Green Supermix PCR reagents (Bio-Rad) were used for monitoring amplification and results were evaluated with the iCycler iQ real-time detection system software. The reaction mixture contained cDNA, 20 pmol forward and reverse primers and SYBR Green Supermix 2 (100 mM KCl, 40 mM Tris-HCl [pH 8.4], 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein and stabilizers). Template was amplified with 40 cycles of denaturation at 95℃ for 40 sec, annealing at 60℃ for 40 sec, and extension at 72℃ for 40 sec. Upon completion of PCR, fluorescence was monitored continuously while slowly heating the samples from 60℃ to 95℃ at 0.5℃ intervals. The melting curves were used to identify any nonspecific amplification products. Quantitation of gene amplification was performed by determining the cycle threshold (C_T), based on the fluorescence detected within the geometric region of the semi-log amplification plot. Expression of each mRNA species was normalized to that of Gapdh mRNA. Relative quantitation of target gene expression was evaluated using the

comparative C_T method and experiments were repeated at least three times.

7. RNAi for Bcl2l10

To determine the possible role of Bcl2l10 in oocyte maturation, production of Bcl2l10 double-stranded RNA (dsRNA) and RNAi by microinjection was performed as described previously (Yoon et al., 2009). We prepared dsRNA for Bcl2l10 (551 bp) using the MEGAscript RNAi Kit (Ambion, Austin, TX). GV oocytes were microinjected with Bcl2l10 dsRNA in M2 medium containing 0.2 mM IBMX. Bcl2l10 dsRNA-injected oocytes were cultured in M16 medium containing 0.2 mM IBMX for 8 hours, followed by culture in the plain M16 for 16 hours in 5% CO_2 at 37℃. Control oocytes cultured in M16 medium containing 0.2 mM IBMX for 8 hours, followed by culture in the plain M16 for 8 hours in 5% CO_2 at 37℃.

8. Microarray Analysis

Due to the small amounts of initial total RNA from 350 oocytes, the process required an amplifying two-cycle target labeling assay step to obtain sufficient amounts of labeled cRNA target for analysis with microarrays. Total RNA was used to synthesize double-stranded cDNA with the MEGAscript kit (Ambion) with an oligo (dT) primer containing a T7 RNA polymerase promoter. The labeled cRNA was hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA), which covers transcripts and variants from 34,000 well-characterized mouse genes. Probe sets on this array are derived from sequences from GenBank and dbEST. The chips were analysed by using a Genechip Array scanner 3000 7G (Affymetrix) and GenPlex 3.0 (ISTECH) software.

RESULTS AND DISCUSSION

To identify molecular basis of Bcl2l10 function, we compared gene expression of MI oocytes arrested after Bcl2l10 RNAi to those of control MI oocytes. Top 10 functional pathways in which many genes were changed by Bcl2l10 RNAi are summarized in Table 2. Eighty six genes in the metabolic pathways were affected by Bcl2l10 RNAi. Over thirty genes were changed in pathways in cancer, cell cycle, spliceosome, regulation of actin cytoskeleton, and ubiquitin mediated proteolysis. Among these top 10 pathways, we focused in 2 pathways such as cell cycle and regulation of actin cytoskeleton based on our previous results demonstrated that Bcl2l10 is involved in the regulation of meiosis (Yoon et al., 2006; 2009). Previously, we found that the Bcl2l10 expression is oocyte-specific and the Bcl2l10-associated proteins were related to the actin and cytoskeletal system using Mass Spectrometry (Yoon et al., 2006). In addition, Bcl2l10 RNAi resulted in MI arrest with chromosomal and spindle abnormalities (Yoon et al., 2009).

Figure 1 and 2 depict the GO and KEGG pathway of transcripts encoding genes related to the cell cycle and regulation of actin cytoskeleton, respectively. Among changed genes (red box), we selected genes with interesting functions (blue box) to confirm their expression by real-time RT-PCR. Primer sequences for selected genes and their isomers for some genes are listed in Table 1. Up or down arrow beside the blue box indicates result of its expression in the microarray data and its real-time RT-PCR data displayed in Figure 3 and 4, respectively. Names of isomers of some genes are listed with original gene name in parenthesis in the Table 1.

In Figure 1, we selected 2 up-regulated (Bub3 and APCC) and 5 down-regulated (Smc1b, Smc3, Stag2, Rad2l, and CycB) genes but expressions of all these genes were down-regulated (Fig. 3). In Figure 2, 10 genes in the middle of the KEGG pathway were selected and confirmed by real-time RT-PCR. Among 10 genes, there were 3 isomers for PI3K and 2 isomers for MLCP (Table 1). Five genes (Nras, Ppp1cc, Ppp1r12a, Rac3, and 290073G15Rik) were up-regulated and 8 genes (Pik3ca, Pik3ca, Pik3r1, Tiam2, Rhoa, Rock1, Pak1, and Myh9) were down-regulated in the microarray data. Except 2 genes (Nras and 2900073G15Rik)

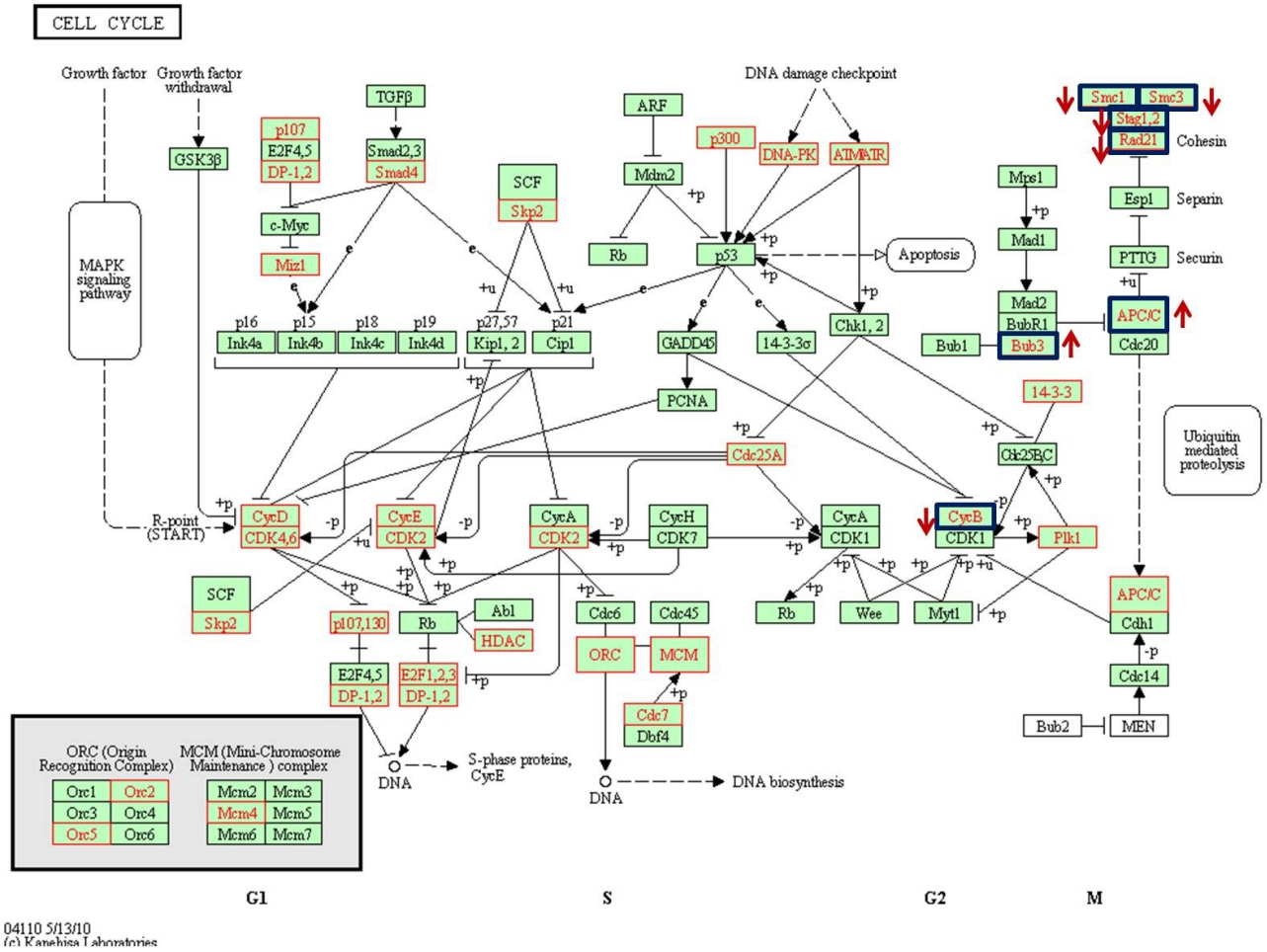

Fig. 1. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway display of transcripts participating in cell cycle regulation. The significantly up- and down-regulated transcripts by Bcl2l10 RNAi were defined by more than 2-fold change, and were shown in boxes that were marked in red. The genes confirmed by real-time RT-PCR are indicated in blue box. Red arrows showed up-(↑) and down-regulated genes (↓) by Bcl2l10 RNAi in microarray analysis.

of which expression was not changed after Bcl2l10 RNAi, expressions of the other 11 genes were all down-regulated (Fig. 4). This somewhat high false- positive rate may result from the fact that we did one round of microarray experiment due to the difficulty to get enough number of oocytes after RNAi.

However, list of genes changed after RNAi is extremely beneficial to search out downstream of the specific gene, such as Bcl2l10 in oocytes in this case. There were 644 and 1,166 genes up- and down-regulated more than 2 folds after Bcl2l10 RNAi. Top twenty up- regulated and down-regulated genes are summarized in Table 3 and Table 4, respectively. Mostly apparent attribute of the Table 3 is that there are 5 enzymes (pyrophosphate synthase, N-methyltransferase, and 3 kinases). Whereas most noticeable attribute of the Table 4 is that there are many genes of which function is related to the cytoskeletal organization, such as Tpx2, Cep192, Kir20b, Myo6, and Cd2ap. According to the gene ontology data, function of Tpx2, Cep192, Kir20b, Myo6, and Cd2ap is related to the spindle assembly, microtubule nucleation, microtubule-based movement, physical interaction, and actin polarization, respectively.

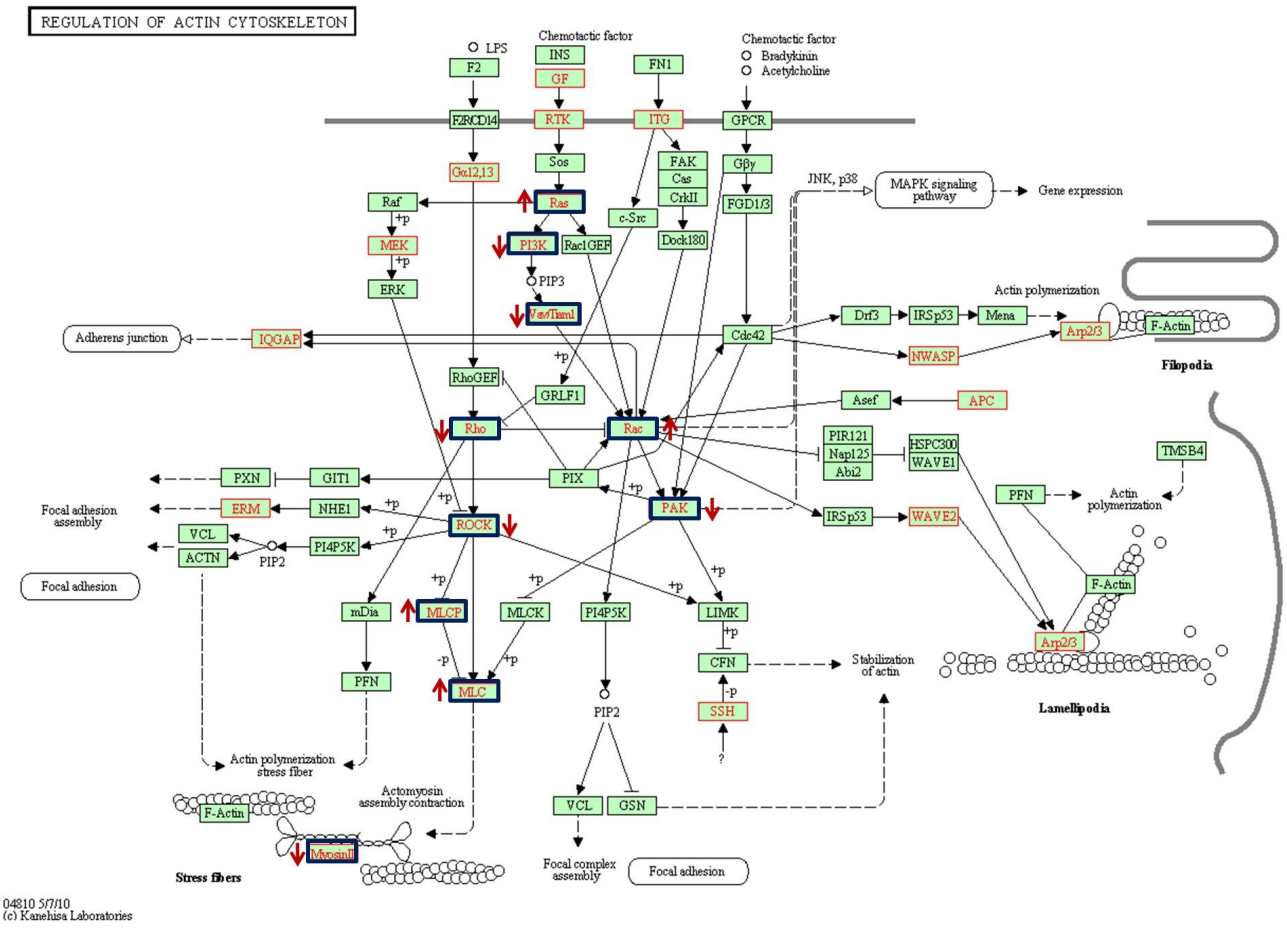

Fig. 2. GO and KEGG pathway display of transcripts encoding regulation of actin cytoskeleton. The significantly up- and down-regulated transcripts by Bcl2l10 RNAi were defined by more than 2-fold change, and were shown in boxes that were marked in red. The genes confirmed by real-time RT-PCR are indicated in blue box. Red arrows showed up-(↑) and down-regulated genes (↓) by Bcl2l10 RNAi in microarray analysis.

In Table 4, Tpx2 was the most down-regulated gene by Bcl2l10 RNAi and it was confirmed by real-time RT-PCR (data not shown). Microtubule binding protein, Tpx2, and centromere protein, Cep192, is well-known cofactors of Aurora A kinase (Joukov, 2011). Eukaryotes have one to three members of the Aurora family of serine-threonine kinases. Two members of the family, Aurora A and B, are present in *C. elegans, D. melanogaster* and in all vertebrates. The third member, Aurora C has only been found in mammals (Barr & Gergely, 2007). During mitosis, a fraction of Aurora A binds TPX2, which both activates the kinase and targets it to spindle microtubules (Tsai et al., 2003; Ozlu et al., 2005; Kufer et al., 2002; Eyers et al., 2003). Tpx2 controls localization of Aurora A at centrosomes, while Cep192 controls its activity in microtubules. Aurora A is an important oncogenic kinase that has well established roles in spindle assembly (Xu et al., 2011).

In addition to this down-regulation found in the cytoskeletal system, expression of two genes associated to the chromatin assembly or disassembly, Arid4a and Cenpm, was also down-regulated (Table 4). It is reported that Arid4a and Arid4b are members of epigenetic complexes that regulate genomic imprinting at the PWS/AS domain (Wu et al., 2006). Mechanism of Bcl2l10 work also involved with

Table 1. Sequence of oligonucleotide primers used in this study, their annealing temperature (AT), and expected PCR product sizes

Gene	Accession no.	Primer sequence	AT (℃)	Size (bp)
Anapc11 (APC/C)	NM_025389.4	F-TGGCGTTTAATGGCTGCT R-GCATTCAGCCACTTGAGGAT	60	115
Bcl2l10	AF067660	F-CTCTGTGACTAGGCAGATCC R-GTCTCTAGGCTGGAGGACTT	60	551
Bub3	NM_009774.3	F-AATGACGGAACTACGCTTGC R-TTTCTGCGTCTGTCACTTGG	60	106
Ccnb3 (CycB)	NM_183015.3	F-TGTGAGATGACCCTGCAAGA R-TATGGGATCGGAAGTTCAGC	60	199
Myh9 (MyosinII)	NM_022410.2	F-AGCAGCTTAAACGGCAGCTA R-CCACGCCTCAGTTTGTTCTT	60	154
Pak1 (PAK)	NM_011035.2	F-TGGACCCAAGGTTGACATCT R-CATCTCAAGACAGCGGTTCA	60	190
Pik3ca (PI3K)	NM_008839.2	F-AGCCTTGGACAAAACTGAGC R-CTGCTTGATGGTGTGGAAGA	60	115
Pik3cb (PI3K)	NM_029094.3	F-CTGAGCTCACATCGGTCAAA R-GCCTCGTCAAACTTCTGCTT	60	106
Pik3r1 (PI3K)	NM_001024955.1	F-CAAAGCGGAGAACCTATTGC R-CCGGTGGCAGTCTTGTTAAT	60	138
Ppp1cc (MLCP)	NM_013636.3	F-TAGGCTGGGGTGAAAATGAC R-TGGGTGCAGAAAACAGAGTG	60	171
Ppp1r12a (MLCP)	NM_027892.2	F-AGCTCAACAGGCCAAACAGT R-TCCAGTAGACCGTCGCTTTT	60	176
Rac3 (Rac)	NM_133223.4	F-AAGCTGGCACCCATAACCTA R-CTGGCTTTTTCACTGGAGGT	60	160
Rad21	NM_009009.4	F-CAGCAGGTAGAGCAAATGGA R-CATCTGCTGAGTGCGTTTGT	60	216
Nras (Ras)	NM_010937.2	F-TGACTTGCCAACAAGGACAG R-TAAAAGGCATCCTCCACACC	60	117
Rhoa (Rho)	NM_016802.4	F-CGCTTTTGGGTACATGGAGT R-GAGGCACCCAGACTTTTTC	60	119
Rock1 (Rock)	NM_009071.2	F-ATTGGAAGCTGAGCGAAGAG R-CTTGTCAGTTAGGCGTGCTTT	60	277
Smc1b (Smc1)	NM_080470.1	F-ATGCAGCCCTGGACAATACT R-TCAGTGCATCAGCTTTGGAG	60	126
Smc3	NM_007790.3	F-CACAGAAAAGCCGTGTCAGA R-ATCTTTGGCCATCTCTGCTG	60	168
Stag2	NM_021465.3	F-GAACACCATCAAACGCCTCT R-TCGGGCTTCAGTTCTGTTCT	60	200
Tiam2 (Tiam1)	NM_011878.2	F-ACTTCCACCACCCTCTGAAA R-CCTCTTGGTGCTGTTTGCTT	60	191
2900073G15Rik (MLC)	NM_026064.2	F-GCTTTGATGAGGAAGCCATC R-GGATGCGTGTGAACTCAATG	60	162

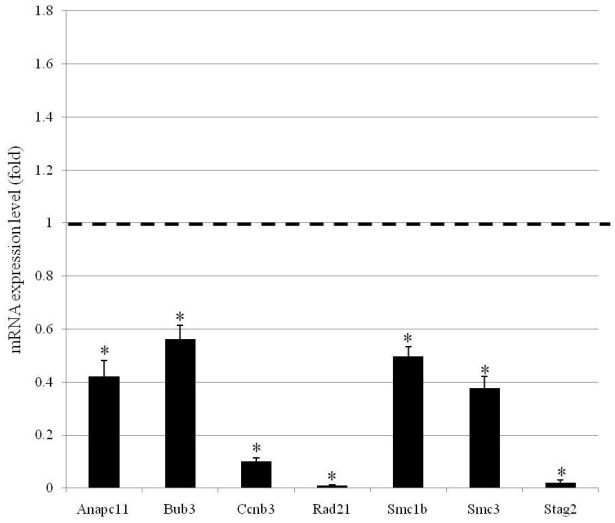

Fig. 3. Relative amount of seven cell cycle-related genes in MI-arrest oocytes after Bcl2l10 RNAi. Expression of mRNA was confirmed by quantitative real-time RT-PCR analysis. The x-axis represents the genes and the y-axis shows the relative expression levels. The expression levels were calculated C_T values, and then mRNA expression ratio was determined relative to that of control MI oocytes. Experiments were repeated at least three times, and data was expressed as mean±SEM. Asterisks represent statistical significance at $p<0.05$.

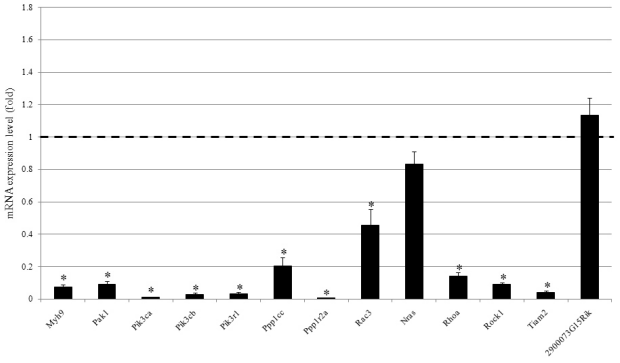

Fig. 4. Relative amount of thirteen actin cytoskeleton-related genes in MI-arrested oocytes after Bcl2l10 RNAi. Expression of transcript was confirmed by quantitative real-time RT-PCR analysis. The x-axis represents the genes and the y-axis shows the relative expression. The expression levels were calculated C_T values, and then mRNA expression ratio was determined relative to that of control MI oocytes. The number of biological replicates is three, and data was expressed as the mean±SEM. Asterisks represent statistical significance at $p<0.05$.

Table 2. Number of genes up- and down-regulated by Diva RNAi

KEGG pathway	Gene counts
Metabolic pathways	86
Pathways in cancer	51
Cell cycle	36
Spliceosome	33
Regulation of actin cytoskeleton	33
Ubiquitin mediated proteolysis	30
Focal adhesion	26
Endocytosis	26
MAPK signaling pathway	24
Progesterone-mediated oocyte maturation	23

centromere protein (Cenpm) and myosin (Myo6), too.

Based on our previous work, finding of binding or working partners of Bcl2l10 using Mass Spectrometry, we proposed that Bcl2l10 is working in the regulation of cytoskeletal system (Yoon et al., 2006). In the present study, we confirmed the similar results that Bcl2l10 intimately involved in the regulation of spindle formation working with Tpx2 and Cep192. In conclusion, we propose that Bcl2l10 is a regulator of meiotic spindle formation and its work is closely engage regulation of Tpx2 and Cep192. Further meticulous studies on the molecular mechanisms involved in regulation of meiosis by Bcl2l10 and its downstream Tpx2 and Cep192 are under investigation.

ACKNOWLEDGMENTS

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-314-E00120).

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Table 3. Top 20 genes up-regulated more than 2 folds by Diva RNAi

Genes	Gene titles	Fold changes
Nup54	nucleoporin 54	5.6
Gmfb	glia maturation factor, beta	4.7
Slc35a3	solute carrier family 35 member 3	4.4
Nus1	nuclear undecaprenyl pyrophosphate synthase 1 homolog (*S. cerevisiae*)	4.4
Nr2e1	nuclear receptor subfamily 2, group E, member 1	4.3
Slmap	sarcolemma associated protein	4.3
Prmt3	protein arginine N-methyltransferase 3	4.2
Csnk2a1	casein kinase 2, alpha 1 polypeptide /// predicted gene 10031	4.1
Clasp1	CLIP associating protein 1	4.1
Cnot4	CCR4-NOT transcription complex, subunit 4	4.0
C030046I01Rik	RIKEN cDNA C030046I01 gene	4.0
Dtna	dystrobrevin alpha	4.0
Slco4c1	solute carrier organic anion transporter family, member 4C1	3.9
C030046I01Rik	RIKEN cDNA C030046I01 gene	3.8
Snhg1	small nucleolar RNA host gene (non-protein coding) 1	3.8
Tmem188	transmembrane protein 188	3.8
Srpk2	serine/arginine-rich protein specific kinase 2	3.7
Cklf	chemokine-like factor	3.7
Luc7l	Luc7 homolog (S. cerevisiae)-like	3.6
Srpk2	serine/arginine-rich protein specific kinase 2	3.5

Table 4. Top 20 genes down-regulated more than 2 folds by Diva RNAi

Genes	Gene titles	Fold changes
Tpx2	TPX2, microtubule-associated protein homolog (*Xenopus laevis*)	−16.1
Rbm12b	RNA binding motif protein 12B	−15.3
Ptp4a1	protein tyrosine phosphatase 4a1-like	−14.6
Ranbp2	RAN binding protein 2	−10.1
Eea1	early endosome antigen 1	−9.3
Arid4a	AT rich interactive domain 4A (RBP1-like)	−9.1
Cep192	centrosomal protein 192	−8.2
Kif20b	kinesin family member 20B	−7.9
Psip1	PC4 and SFRS1 interacting protein 1	−7.8
Atad2b	ATPase family, AAA domain containing 2B	−7.5
Mki67	antigen identified by monoclonal antibody Ki 67	−7.4
Nexn	nexilin	−7.2
Eif4g3	eukaryotic translation initiation factor 4 gamma, 3	−6.9
Ccnb3	cyclin B3	−6.9
C430048L16Rik	RIKEN cDNA C430048L16 gene	−6.8
Cenpm	centromere protein M	−6.8
Leo1	Leo1, Paf1/RNA polymerase II complex component, homolog (*S. cerevisiae*)	−6.7
Myo6	myosin VI	−6.6
Tnfaip8	tumor necrosis factor, alpha-induced protein 8	−6.6
Cd2ap	CD2-associated protein	−6.5

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(Received 3 February 2011, Received in revised form 13 March 2011, Accepted 14 March 2011)