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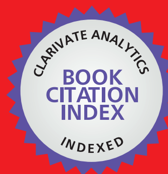

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Role of Bcl2l10 in Regulation of Meiotic Cell Cycle in the Mouse Oocyte

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1. Introduction

Bcl2l10, also known as Diva or Boo, is a member of the Bcl2 family. This factor is known to have opposing apoptotic functions in various tissues. That is, it can act as either a pro-apoptotic or an anti-apoptotic factor depending on the cellular milieu (Ke et al., 2001; Lee et al., 2001).

The pro-apoptotic factor Bad is expressed in various tissues including rat ovaries and testes (Kaipia et al., 1997). Bok, anti-apoptotic factor, has been detected in granulosa cells as well as in several reproductive tissues such as the ovaries, testes, and uterus (Hsu et al., 1997). Several anti-apoptotic Bcl2 homologs are expressed in the ovary. Mcl1, Bcl2, Bcl2l1 (Bcl-x), and other Bcl2 family members have also been detected in ovarian tissues (Hsu et al., 1997; Hsu and Hsueh, 2000; Kim and Tilly, 2004; Tilly et al., 1995). However, the expression patterns of these Bcl2 family members are quite different from those of Bcl2l10, which exhibits ovary- and oocyte-specific expression. Murine Bcl2l10 was first identified in expressed sequence tag clones from unfertilized, fertilized, and two-cell-stage mouse eggs (Inohara et al., 1998). Expression of this factor is restricted to the ovary and testis in adult mice (Inohara et al., 1998).

2. Expression of Bcl2l10 in the ovary

In a previous study, we used the annealing control primer-PCR method to investigate differentially expressed genes in the mouse oocytes. Using this approach, we found that Bcl2l10 was highly expressed in oocytes (Yoon et al., 2005). We confirmed that Bcl2l10 is constantly expressed in oocytes during oocyte maturation and found that its expression disappeared after the four-cell stage (Figure 1).

Since Bcl2l10 is a member of Bcl2 family, we first evaluated the relationship between Bcl2l10 expression and apoptosis in the ovary. Surprisingly, Bcl2l10 expression did not appear to be

related to granulosa cell apoptosis in ovarian follicles (Figure 2). Analysis of serial ovarian sections using immunohistochemical labeling for Bcl2l10 and TUNEL assay revealed that BCL2L10 expression is oocyte-specific but that this expression is mismatched with the apoptotic death of follicular granulosa cells. Therefore, Bcl2l10 may have a function that is not related to the regulation of apoptosis in oocytes.

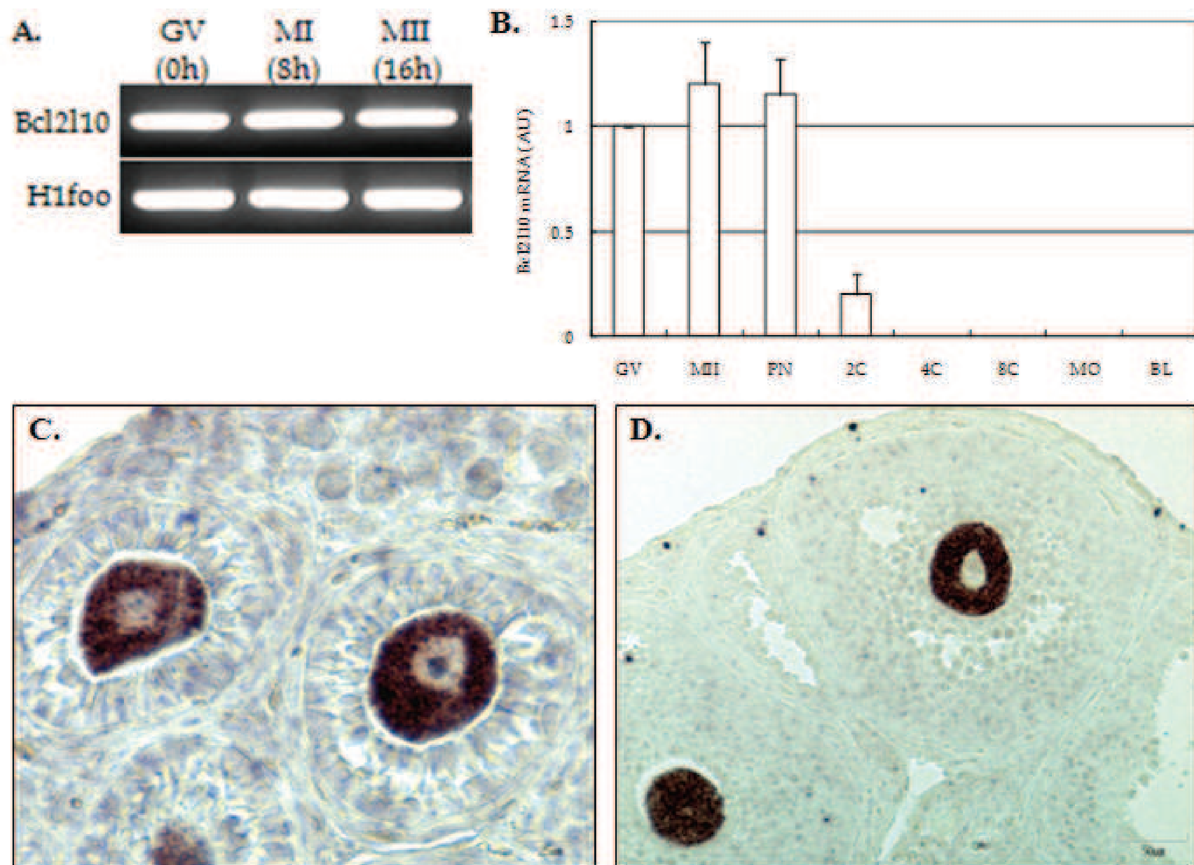

Fig. 1. Expression of Bcl2l10 mRNA in oocytes and preimplantational embryos. A) Semiquantitative RT-PCR analysis of Bcl2l10 expression during spontaneous oocyte maturation *in vitro*. GV, germinal vesicle-stage oocytes; MI, meiosis I oocytes collected after 8 h of culture; MII, meiosis II-stage oocytes collected after 16 h of in culture. B) Quantitative real-time RT-PCR of Bcl2l10 mRNA in oocytes and embryos. Messenger RNA isolated from oocytes and at various embryonic stages was reverse transcribed. For PCR, the cDNA from a single oocyte or an embryo equivalent served as a template for amplification. The expression level was calculated from the cycle threshold values (CT) based on the fluorescence detected within the geometric region of the semi-log plot, and the mRNA ratio (arbitrary units) was calculated with respect to that of GV oocytes. Experiments were repeated at least three times, and data are expressed as the mean ± SEM. PN, pronucleus one-cell zygote; 2C, two-cell; 4C, four-cell; 8C, eight-cell; MO, morula; BL, blastocyst-stage embryo. C, D) *In situ* hybridization of Bcl2l10 mRNA. Mouse ovaries of 2-week-old (C) and 4-week-old (D) were used, and we observed the oocyte-specific Bcl2l10 mRNA expression. Scale bars represent 25 μm and 50μm for C and D, respectively (C, D cited in Yoon et al., 2006, *Korean J Fertil Steril*).

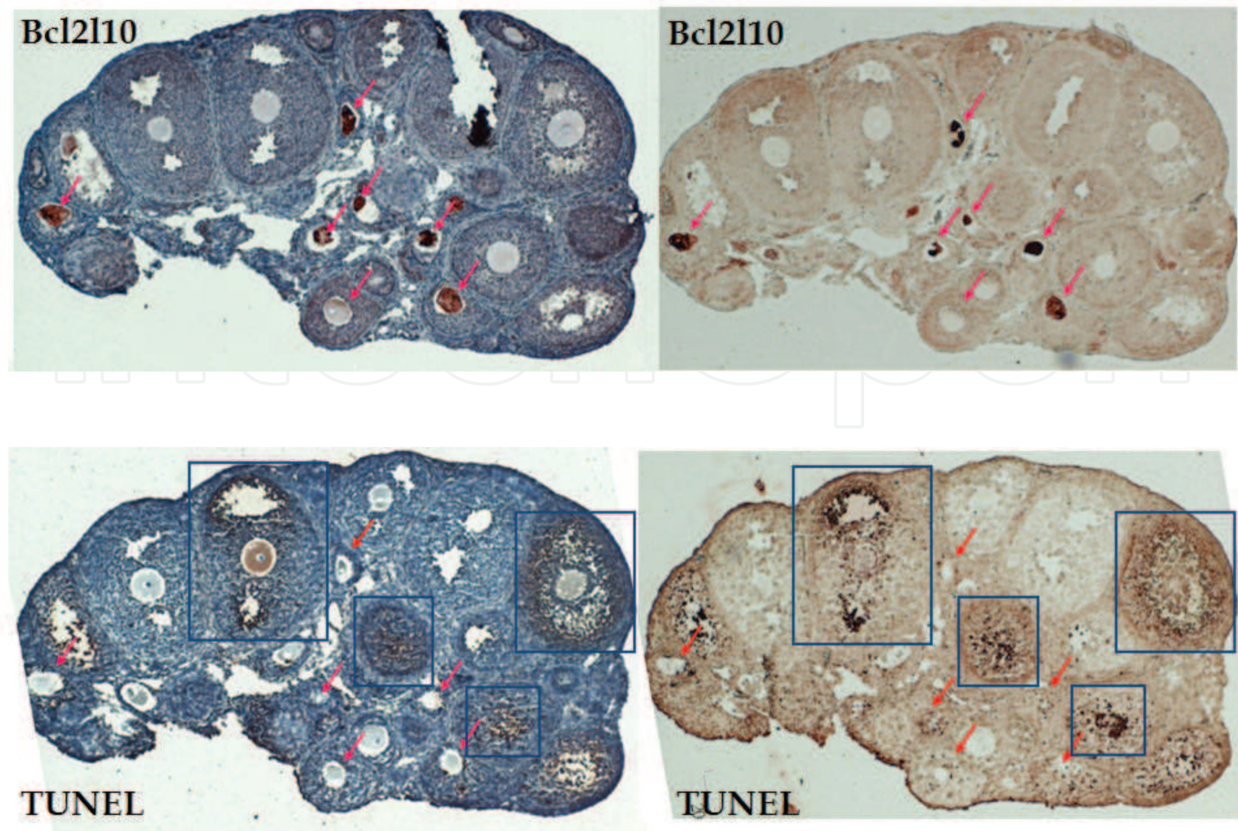

Fig. 2. BCL2L10 immunohistochemical labeling and TUNEL assay in serial sections. Tissue sections were obtained from 4-week mouse ovaries. Arrows indicate peculiar BCL2L10 expression in oocytes of preantral follicles, while boxes indicate apoptotic follicles with TUNEL-positive granulosa cells.

3. Bcl2l10 binding proteins

Next, we tried to identify the working partner(s) of Bcl2l10 using immunoprecipitation followed by mass spectrometry (Yoon et al., 2006). We transiently expressed FLAG-Bcl2l10 in NIH/3T3 cells and purified Bcl2l10-associated proteins (Figure 3). Specifically detected 14 bands were excised in the Coomassie Blue-stained 12% SDS-PAGE gels and identified using LC/MS/MS analysis. The protein band specific for Diva was identified as Bcl2-like 10 in the size of 21 kDa (Fig. 3, gel slice number 2) suggesting a successful IP analysis using monoclonal anti-FLAG antibody. List of potential Bcl2l10-binding partners is summarized in Table 1. Interestingly, many of the identified Bcl2l10-associated proteins are known to be associated with the cytoskeletal system. The identification of actin-related proteins such as actin, α-actinin, gelsolin, myosin, tropomyosin, and tropomodulin 3 as Diva-binding proteins suggests that Diva protein associates with the components of actin filaments. Actin was the most abundant Diva-associated protein as shown in Figure 3 (gel slice number 6). All these proteins, with the exception of actin, are known to bind actin to form, stabilize, and cross-linking the microfilaments.

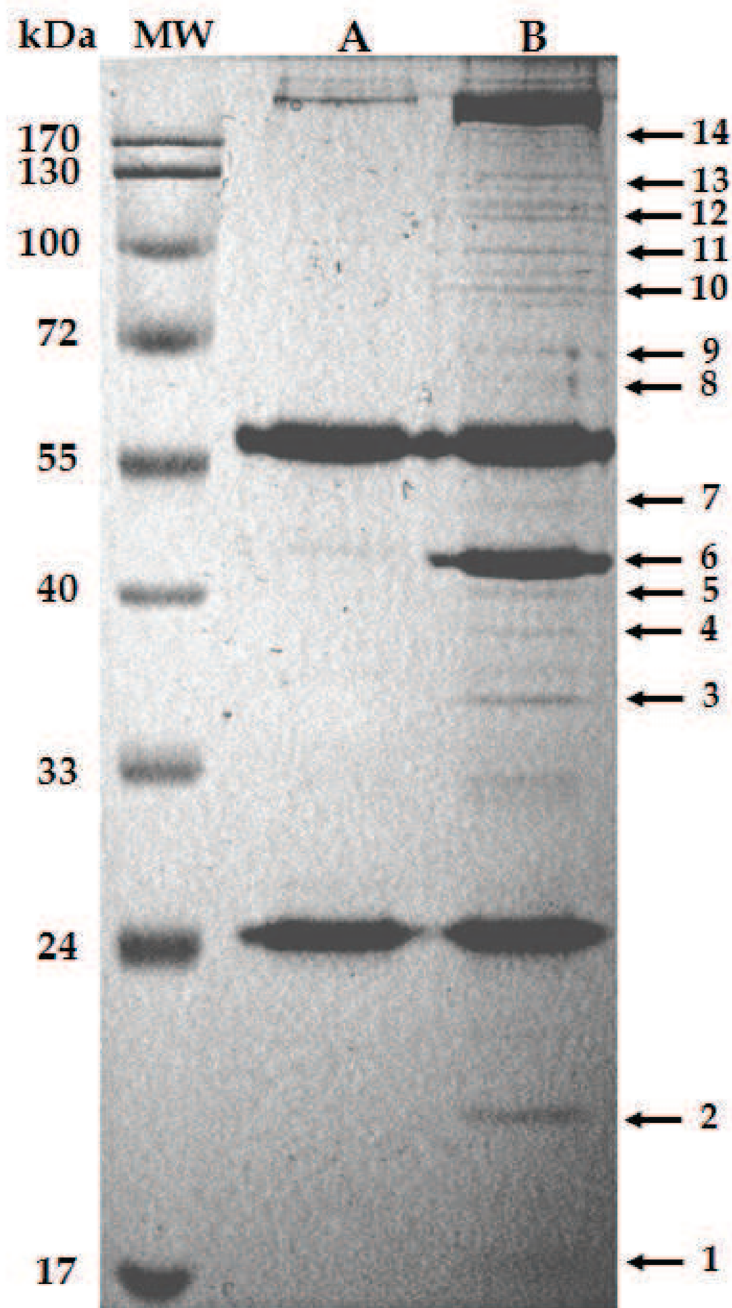

Fig. 3. Identification of BCL2L10-associated proteins. NIH/3T3 cells were transiently transfected for 24 h with empty vector or FLAG-BCL2L10-expressing vector. Total protein extracts were incubated with anti-FLAG-agarose beads. Anti-FLAG-agarose elutes from an equivalent amount of transfected cells (500 µg) were analyzed by 12% SDS polyacrylamide gel electrophoresis, and proteins were visualized by Coomassie Blue staining. The positions and relative molecular masses in kilodaltons (kDa) of protein size markers are indicated on the left. Protein-containing bands, as indicated by numbers on the right, were excised, and proteins present in gel slices were identified by mass spectrometry. MW, protein size markers; A) NIH/3T3, anti-FLAG-agarose elutes from NIH/3T3 cells transfected with pCMV-FLAG empty vector; B) FLAG-BCL2L10, NIH/3T3 cells transfected with pCMV-FLAG-BCL2L10 vector (Yoon et al., 2006).

Gel slice number	Accession No.	Score	Description	Unigene
1	gi_16307437	95	RIKEN cDNA 2900073G15	2900073G15Rik
2	gi_30851239	69	Bcl2l10	Bcl2l10
3	gi_1351289	69	Tropomyosin 1 alpha chain (alpha-tropomyosin)	Tpm1
4	gi_19353393	79	Tpm2 protein; Tropomyosin 2, beta	Tpm2
5	gi_52139168	90	Tropomodulin 3	Tmod3
6	gi_49868	109	Beta-actin (aa 27-375); Actin, beta, cytoplasmic	Actb
7	gi_80478706	45	ARP3 actin-related protein 3 homolog (yeast)	Actr3
8	gi_194362	123	Igh-4 protein	Igh-4
9	gi_27369615	29	PIF1 homolo: Expressed sequence AI449441	AI449441
10	gi_34871482	34	PREDICTED: similar to Transcription initiation factor	Taf6
11	gi_90508	39	Gelsolin, cytosolic	Gsn
12	gi_61097906	35	Actinin, alpha 1	Actn1
13	gi_47847434	42	mFLJ00150 protein: Centrosomal protein 110	Cep110
14	gi_17978023	80	Myosin, heavy polypeptide 9, non-muscle	Myh9

Table 1. Bcl2l10-associated proteins identified by immunoprecipitation and mass spectrometry.

Immunoprecipitation/Western blot analysis of ovarian tissue homogenates confirmed the association of actin and tropomyosin with Bcl2110 (Figure 4). These findings suggest that, in the ovary, Bcl2l10 plays roles unrelated to apoptosis and instead participates in the regulation of cytoskeletal systems. During meiosis, actin filaments have roles in migration of chromosomes, segregation of homologous chromosomes, development and maintenance of the cortex, formation of polarity, movement of peripheral cortical granules, and extrusion of the first polar body (Sun and Schatten, 2006).

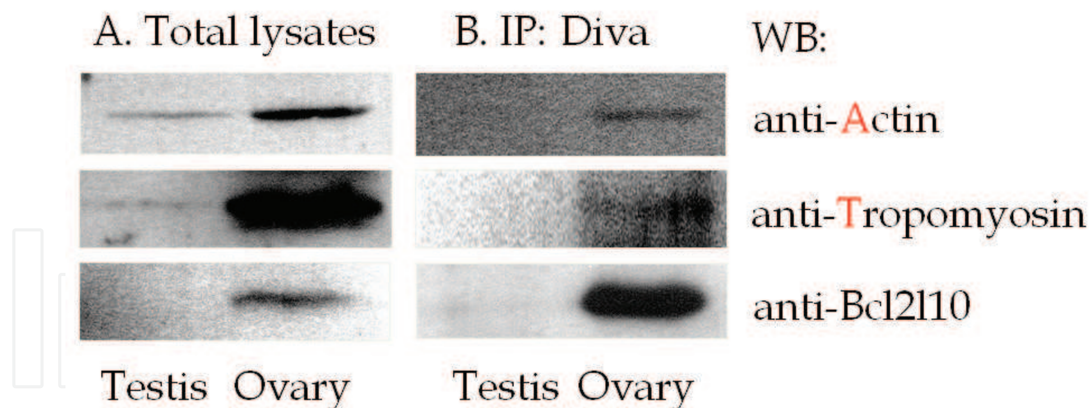

Fig. 4. Identification of BCL2L10-associated proteins using immunoprecipitation (IP)/Western blot analysis (WB). A) WB analysis. Total protein lysates (20 μg) from mouse testes and ovaries were electrophoresed and probed with anti-actin, anti-tropomyosin, or anti-BCL2L10 antibody. Testes lysates served as a negative control for BCL2L10 protein expression. B) IP/WB analysis. Total protein lysates from testes and ovaries (500 μg) were subjected to IP using anti-Bcl2l10 antibody. Anti-BCL2L10-agarose elutes were then electrophoresed and probed with anti-actin, anti-tropomyosin, or anti-BCL2L10 antibody to confirm of the association of actin and tropomyosin with BCL2L10 (as cited in Yoon et al., 2006, *Korean J Fertil Steril*).

A non-apoptotic function of Bcl2l10 has also been demonstrated. Specifically, Bcl2l10 plays a role in Huntington-interacting protein 1-related (HIP1R) protein-mediated endocytosis as well as in the regulation of actin machinery in 293T cells. HIP1R regulates clathrin-mediated endocytotic apparatus and actin assembly. An interaction between endogenous BCL2L10 and HIP1R has been shown by immunoprecipitation and Far-Western analysis (Kim et al., 2009).

4. Function of Bcl2l10 in oocytes

We have investigated the role of Bcl2l10 not only in the ovary, but also in oocytes. RNA interference (RNAi) was used for this purpose. To determine the role of Bcl2l10 during oocyte maturation, we microinjected *in vitro* transcribed dsRNA for Bcl2l10 into the cytoplasm of germinal vesicle-stage (GV) oocytes and monitored *in vitro* oocyte maturation. Bcl2l10 RNAi selectively reduced levels of endogenous Bcl2l10 and resulted in incomplete meiosis that was arrested in meiosis I (MI) (Yoon et al., 2009). MI-arrested oocytes had abnormalities in the spindles and chromosomes. The most prominent changes were the disappearance of spindles and aggregation of chromosomes (Figure 5).

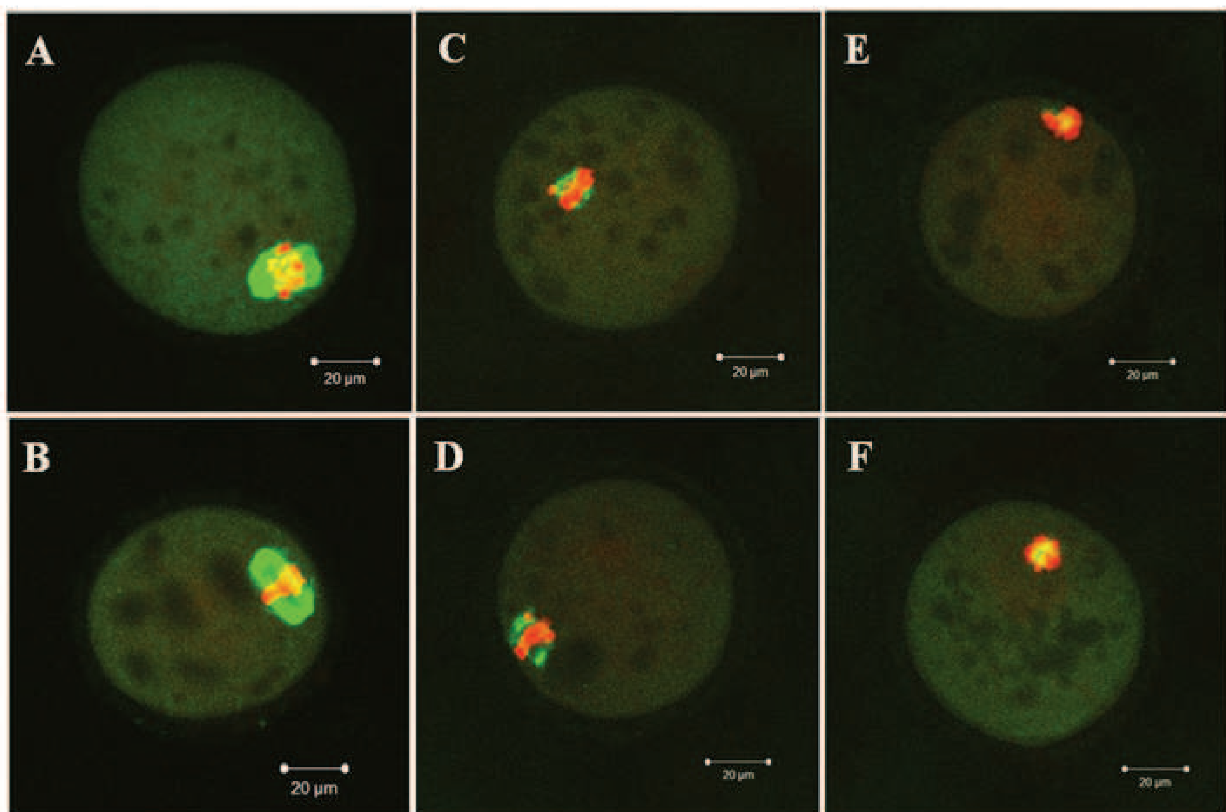

Fig. 5. Bcl2l10 RNAi-induced abnormalities in oocytes, as seen by α-tubulin immunofluorescent staining and chromosomal staining. Germinal vesicle oocytes were injected with Bcl2l10 dsRNA and cultured for 16 h. Oocytes were then fixed in 4% paraformaldehyde and stained with an α-tubulin antibody (green). Chromosome material was counterstained with propidium iodide (red). A) Control, uninjected oocyte. B) Buffer-injected control oocyte. C-F) Bcl2l10 dsRNA-injected oocytes arrested in meiosis I (as cited in Yoon et al., 2010, From Biol Reprod). Bars represent 20 μm.

5. Genes downstream of Bcl2l10

After discovering that Bcl2l10 RNAi induced changes in spindles and chromosomes, we set out to identify the network of factors acting downstream of Bcl2l10 in oocytes so that we could better understand the regulatory mechanisms underlying meiosis in oocytes. To identify downstream genes, we conducted microarray analysis of Bcl2l10 RNAi-induced changes in gene expression in mouse oocytes (Kim et al., 2011).

Due to the small amounts of initial total RNA obtained from the 350 oocytes, we performed an amplifying two-cycle target labeling assay so that we could obtain sufficient amounts of labeled cRNA target for microarray analysis. The labeled cRNA was hybridized to the Affymetrix GeneChip Mouse Genome 430 2.0 Array, which covers transcripts and variants from 34,000 well-characterized mouse genes.

Bcl2l10 RNAi induced a more than 2-fold up-regulation of 644 genes and down-regulation of 1,166 genes. Notably, the top 20 up-regulated genes included five enzymes (*i.e.*, pyrophosphate synthase, N-methyltransferase, and three kinases). The top 20 down-regulated genes were related to cytoskeletal organization (Table 2). These genes encoded proteins such as Tpx2, Cep192, Kir20b, Myo6, and Cd2ap. Tpx2 functions in spindle assembly, Cep192 in microtubule nucleation, Kir20b in microtubule-based movement, Myo6 in physical interactions, and Cd2ap in actin polymerization.

Genes	Gene title	Fold change
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	- 6.7
Myo6	myosin VI	- 6.6
Tnfaip8	tumor necrosis factor, alpha-induced protein 8	- 6.6
Cd2ap	CD2-associated protein	- 6.5

Table 2. Top 20 genes down-regulated more than 2-fold by Bcl2l10 RNAi.

Tpx2 was found to be down-regulated by 16.1-fold and Cep192 by 8.2-fold. The functions of these proteins are intimately related. Interfering with TPX2 function in HeLa cells causes

defects in microtubule organization during mitosis, and Tpx2 RNAi produces abnormalities in spindle formation (Gruss et al., 2002). Tpx2, a microtubule-binding protein, and Cep192, a centromere protein, are well-known cofactors of Aurora A kinase. Both proteins act to control the activity and localization of this kinase (Joukov et al., 2011). Eukaryotes have one to three members of the Aurora family of serine-threonine kinases. Aurora A is an important oncogenic kinase that has well-established roles in spindle assembly (Xu et al., 2011). During mitosis, a fraction of Aurora A binds Tpx2, activates the kinase, and targets it to spindle microtubules (Eyers et al., 2003, Kufer et al., 2002, Ozlu et al., 2005, Tsai et al., 2003). Tpx2 controls localization of Aurora A at centrosomes, whereas Cep192 controls its activity in microtubules (Joukov et al., 2010).

Our finding that Bcl2l10 RNAi induced concurrent down-regulation of Tpx2 and Cep192 leads us to conclude that Bcl2l10 may have important roles in regulating oocyte meiosis through its ability to act as an upstream regulator of Tpx2 and Cep192. Association between Bcl2l10 and Aurora kinase A is an interesting new area that warrants further investigation.

6. Conclusion

We have identified Bcl2l10 expression in oocytes and uncovered a role for this factor in regulating meiosis. Our findings point to new non-apoptotic function for this Bcl2 family member and open a challenging new area of research on Bcl2l10 regulation of meiosis through Bcl2l10 involvement in spindle assembly. We propose that Bcl2l10 is an important regulator of meiotic spindle formation and works closely with Tpx2, Cep192, and Aurora A kinase. The molecular mechanisms underlying meiotic regulation by Bcl2l10 and its downstream genes (Tpx2 and Cep192) as well as the relationship between Bcl2l10 and Aurora A kinase are currently under careful investigation.

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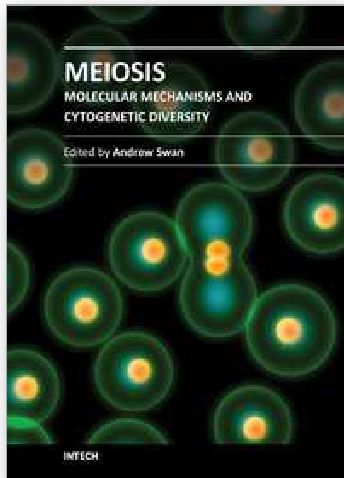

Meiosis - Molecular Mechanisms and Cytogenetic Diversity
Edited by Dr. Andrew Swan

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Meiosis, the process of forming gametes in preparation for sexual reproduction, has long been a focus of intense study. Meiosis has been studied at the cytological, genetic, molecular and cellular levels. Studies in model systems have revealed common underlying mechanisms while in parallel, studies in diverse organisms have revealed the incredible variation in meiotic mechanisms. This book brings together many of the diverse strands of investigation into this fascinating and challenging field of biology.

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