Impaired Spindle Checkpoint Response of Brca1-deficient Mouse Embryonic Fibroblasts (MEFs) to Nocodazole Treatment

Myoung-Ae Kim[1], Hyunju Kim[1] and Jeanho Yun[1]*

Medical Research Center for Cancer Molecular Therapy, College of Medicine, Dong-A University, Busan 602-714, Korea
[1]Department of Biochemistry, College of Medicine, Dong-A University, Busan 602-714, Korea

Received September 15, 2005 / Accepted December 6, 2005

Genetic alternation of Brca1 predispose of breast and ovarian cancer. Brca1 plays critical role in cell cycle regulation following DNA damage. Previous studies revealed that Brca1 plays an important role in S phase and G2/M checkpoint regulation. However, whether Brca1 involves in spindle checkpoint is unclear. In this study, the role of Brca1 in cell cycle response following nocodazole, which is a reagent that depolymerizes microtubules and activates the spindle checkpoint, has been examined using wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ mouse embryonic fibroblasts (MEFs). While wild type and Brca1-proficient MEFs showed an acute mitotic arrest, Brca1-deficient MEFs failed to arrest at mitotic phase in response to nocodazole treatment. In double-thymidine block and nocodazole treatment experiment, a portion of $p53^{-/-}Brca1^{-/-}$ MEFs were clearly by-passed nocodazole induced mitotic arrest. Consistent with this, in morphologic analysis, $p53^{-/-}Brca1^{-/-}$ MEFs showed growing cell morphology after nocodazole treatment. Taken together, these results suggest that Brca1 protein is an important component for normal induction of spindlecheckpoint and impairment of Brca1 function could induce dysregulation of mitotic cell cycle that ultimately results in genomic instability.

Key words – Brca1, Spindle checkpoint, nocodazole, cell cycle

Genetic stability is achieved by the coordinated regulation of DNA replication and repair, chromosomal segregation, and cell cycle checkpoints[7,11]. There are several checkpoints that act to ensure the orderly progression of critical events in the cell cycle. The mitotic spindle checkpoint functions to delay the metaphase to anaphase transition until all pairs of sister chromatids are attached to spindle microtubes, thereby ensuring the correct segregation of duplicated chromosomes into the two daughter cells[8,12]. A number of proteins have been identified that sense the kinetochore microtube attachment and regulate the separation of sister chromatids. Accumulating evidence indicates that dysregulated expression as well as functional inactivation of these checkpoint proteins can cause abnormal mitosis, leading to chromosomal mis-segregation, apoptosis, polyploidy, or multinucleated cells. In addition to the proteins that directly monitor the microtube attachment and regulate chromosomal segregation, many tumor suppressors are also involved in the spindle checkpoint response. For example, tumor suppressor p53 has been shown to prevent polyploidy by keeping cells with mitotic spindle damage from reinitiating DNA synthesis[3,5,9,10].

Previous studies demonstrated that Brca1 plays an important role to maintain genomic stability in response to genotoxic stress (reviewed in [15,24]). Brca1 is involved in DNA damage repair, transcription regulation, cell death and cell cycle checkpoint control. Brca1 has been shown to associate with many cell-cycle regulatory proteins[4]. Brca1 protein level increases and phosphorylated during late G1 and S phase[13]. In addition, it has been also shown that Brca1 is phosphorylated during mitosis[2]. Upon exit from M phase, Brca1 is dephosphorylated and its expression decreases[13,16]. This expression pattern implicated that Brca1 has a role in cell cycle checkpoint and indeed, previous studies revealed that Brca1 is essential in S and G2/M checkpoint[20,21]. Moreover, Wang RH et al., recently reported that MEFs expressing exon 11-deleted isoform of Brca1 ($Brca1^{\Delta 11/\Delta 11}$) failed to arrest metaphase in the presence of nocodazole[19]. However, whether the absence of the full-length Brca1 also induces same abnormality and the exact role of Brca1 in the spindle checkpoint is undetermined. In this communication, we examined the role of Brca1 in response to nocodazole treatment by analyzing cell cycle progression of isogenic wild type, $p53^{-/-}Brca1^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ mouse embryonic fibroblasts (MEFs).

***Corresponding author**
Tel : +82-51-240-2919, Fax : +82-51-241-6940
E-mail : yunj@dau.ac.kr

Materials and Methods

Cell culture

Wild type, $p53^{-/-}$ $Brca1^{-/-}$ and $p53^{-/-}$ $Brca1^{-/-}$ MEFs were generated as previously described[25]. All MEF cell lines used were cultured in DMEM high-glucose media containing 5% fetal calf serum.

Nocodazole treatment and cell cycle analysis

Nocodazole (Sigma, St. Louis MO, USA) was dissolved in distilled waster and freshly diluted with cell culture medium to 100 ng/ml concentration. After incubate for indicated time, to analyze DNA content by fluorescence -activated cell sorting (FACS), cells were harvested and fixed with 70% ethanol. The cells were stained with propidium iodide solution (50 μg/ml of RNase, 0.1% sodium citrate, 0.03% NP-40 and 50 μg/ml of propidium iodide in PBS). Flow cytometric analysis was performed using a FACSCalibur flow cytometer and CellQuest software (Beckton Dickinson, San Jose CA, USA). A total of 10,000 events were analyzed for each sample, and the experiment was repeated at least twice.

Double-thymidine block and nocodazole treament

For double-thymidine block synchronization, cells were treated with 2 mM thymidine (Sigma, St. Louis MO, USA) for 12 hrs, released into regular medium for 12 hrs, and then treated with 2 mM thymidine again for 12 hrs. Then cells released into either regular medium or 100 ng/ml nocodazole containing medium. After release from thymidine block, cells harvested at indicated time point and analyzed cell cycle distribution as described above.

Morphological analysis

5×10^5 MEFs were seeded into 10 cm^2 tissue culture dish. Cells in the presence or absence of nocodazole (100 ng/ml) were observed under phase-contrast microscopy (Olympus, Melville, N.Y., USA). Cell morphology images were captured with CCD camera.

Results and Discussion

$p53^{-/-}Brca1^{-/-}$ MEFs failed to arrest after nocodazole treatment

To address the role of Brca1 in mitotic cell cycle regulation, we first examined cell cycle response to nocodazole, which is a reagent that depolymerizes microtubules and activates the spindle checkpoint. Since Brca1 homozygous deletions are embryonic lethal[24], we used previously generated isogenic wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs [25]. It has been successfully demonstrated that our isogenic MEFs provide an ideal system for examining the roles of Brca1 despite $p53^{-/-}Brca1^{-/-}$ MEFs have p53-deficient background[22,23,25].

In cell cycle analysis by using FACS, we found that 4N peak of wild type MEFs were sharply increased after nocodazole treatment (Fig. 1A). After 24 hrs, almost all cells were arrested in G2/M peak indicating that nocodazole activated spindle checkpoint so that cells were arrested in mitotic phase. The quantitative analysis revealed that G1 cell cycle portion was decreased below 10% at 12 hrs time point and below 5% at 24 hrs later (Fig. 1B). $p53^{-/-}$ MEFs

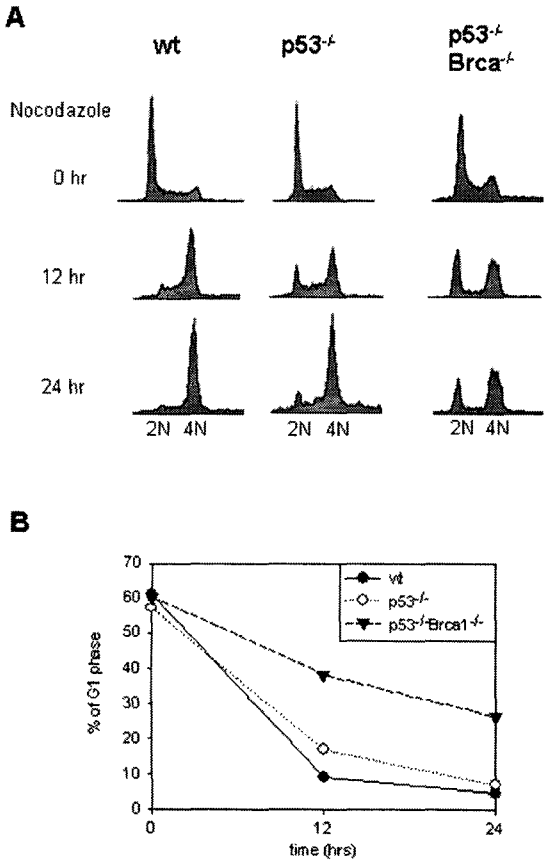

Fig. 1. $p53^{-/-}Brca1^{-/-}$ MEFs were not arrested in mitosis after nocodazole treatment. (A) Wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs were treated nocodazole (100 ng/ml) and harvested 12 hrs or 24 hrs later. Cells were fixed and cell cycle distribution was analyzed by using FACS as described in Materials and Methods. (B) Percentage of G1 cell cycle phase after nocodazole treatment was determined with CellQuest software and plotted.

also showed similar mitotic arrest in response to nocodazole although arrest kinetics was slightly slow compare to wild type MEFs suggesting spindle checkpoint function of $p53^{-/-}$ MEFs is not critically impaired. However, interestingly, $p53^{-/-}Brca1^{-/-}$ MEFs didn't show such acute mitotic arrest (Fig. 1A). A significant portion of G1 cells were observed after nocodazole treatment and the quantitative analysis revealed that almost 30% of cells showed in G1 phase at 24 hrs time point. These results suggest that $p53^{-/-}Brca1^{-/-}$ MEFs failed to arrest effectively at mitosis and grew continually.

However, above experiment didn't rule out the possibility that the nocodazole response of $p53^{-/-}Brca1^{-/-}$ MEFs is slower than wild type and $p53^{-/-}$MEFs. To address this question, we examined nocodazole response at longer time scale. As shown in Fig. 2, $p53^{-/-}Brca1^{-/-}$ MEFs still showed same G1 peak until 72 hrs after nocodazole treatment, indicating that it's not because of delayed response kinetics. Consistent with this, we found that the growth rate of wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs was not significantly different (data not shown).

In addition, we found that 8N cell started to appear from 36 hrs ($p53^{-/-}$ MEFs) or 48 hrs (wild type) time point,

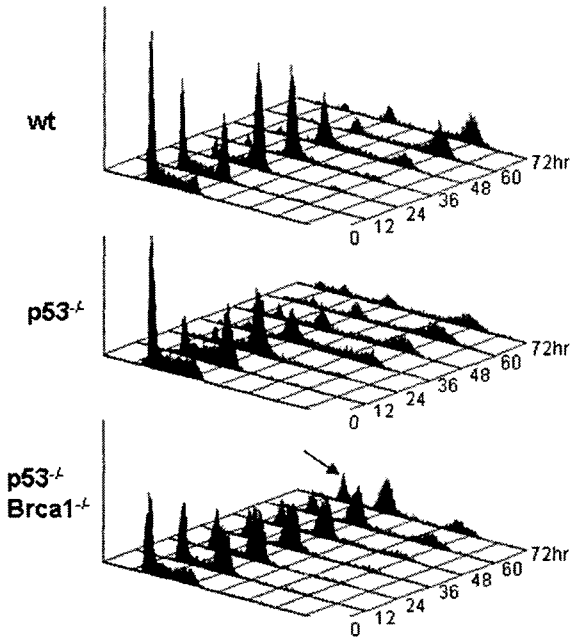

Fig. 2. $p53^{-/-}Brca1^{-/-}$ MEFs were defective to nocodazole response in long-term scale. Wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs were treated with 100 ng/ml of nocodazoleand harvested at indicated time point. Cell cycle distribution was analyzed by using FACS as described in Materials and Methods.

which is a typical signal for endoreduplication when cells arrested at mitotic spindle checkpoint for long time. This result is consistent with previous studies that p53 plays a role to prevent re-entering cell cycle rather than direct involvement to spindle checkpoint activation[10,14,17]. However, appearance of 8N cells in $p53^{-/-}Brca1^{-/-}$ MEFs was much delayed and less extent compare to wild type and $p53^{-/-}$ MEFs, suggesting spindle checkpoint activation in Brca1-deficient cell was not complete and efficient.

Spindle checkpoint defect in $p53^{-/-}Brca1^{-/-}$ MEFs

To further determine whether nocodazole-induced spindle checkpoint is defect in Brca1-deficient MEFs, we employed the double-thymidine block synchronization method. After double thymidine block, cells were arrested in G1/S phase border (Fig. 3, 0 hr). By releasing from thymidine block, cells synchronously progressed through S, G2/M phase and recovered normal cell cycle distribution about 24 hrs later (Fig. 3, upper panels). To address whether MEF cells exhibit normal spindle checkpoint, we treated nocodazole when cells were released from double thymidine block. As shown in Fig. 3 lower panel, wild type and $p53^{-/-}$ MEFs were arrested in mitotic phase after nocodazole treatmentand this arrest maintained until 24 hr time point. However, $p53^{-/-}Brca1^{-/-}$ MEFs never arrested in mitotic phase completely and G1 phase cells observed over all time point. This data suggest that a portion of $p53^{-/-}Brca1^{-/-}$ MEFs were clearly escaped from nocodazole-induced spindle checkpoint and continuously grew.

$p53^{-/-}Brca1^{-/-}$ MEFs bypass nocodazole-induced spindle checkpoint

To verify whether Brca1-deficient MEFs are actually growing after nocodazole treatment, we monitored cell morphology continuously by using a microscope. We found that the most of wild type and $p53^{-/-}$ MEFs became rounded 24 hrs after nocodazole treatment indicating cells were arrested in mitotic cell cycle phase (Fig. 4). However, a significant portion of $p53^{-/-}Brca1^{-/-}$ MEFs were not rounded, but still maintained normal cell morphology suggesting that a portion of $p53^{-/-}Brca1^{-/-}$ MEFs were actually growing in the nocodazole-containing medium.

This incomplete mitotic arrest may due to appearance of resistance cell to spindle checkpoint regulation. A large number of previous studies well demonstrated that absence of functional Brca1 induce defect in DNA repair and

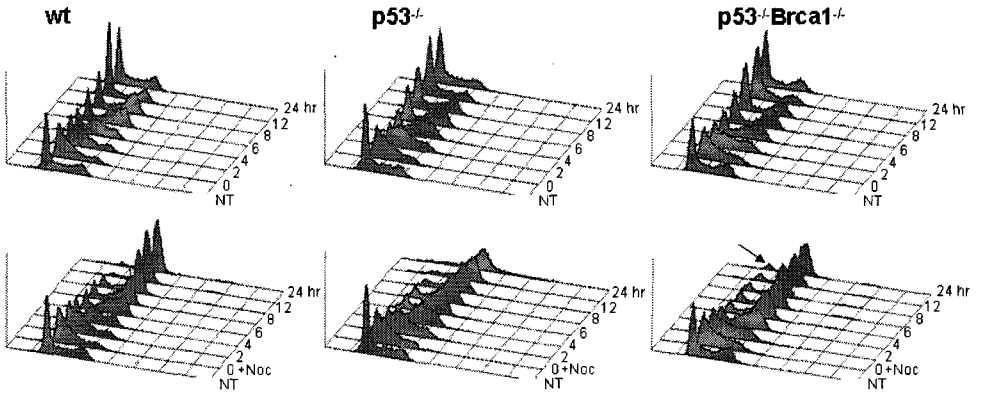

Fig. 3. Defect of spindle checkpoint in $p53^{-/-}Brca1^{-/-}$ MEFs. Wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFswere synchronized in G1/S border by double-thymidine block as described in Materials and Methods (0 hr). Cells released into either regular medium (upper panel) or nocodazole (Noc; 100 ng/ml) containing medium and harvested at indicated time points. Cell cycle distribution was analyzed by using FACS as described in Material and Methods. NT : no treatment.

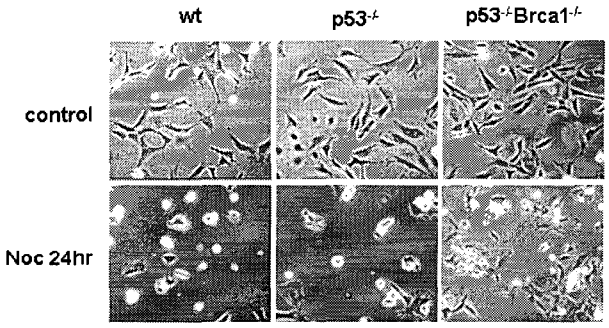

Fig. 4. Morphological analysis of Brca1-deficient MEFs. Wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFswere cultured in the presence or absence (control) of nocodazole (Noc; 100 ng/ml)for 24 hrs and the cell images were captured with CCD camera.

proper checkpoint regulation (reviewed in [15,22]). Brca1-associated breast cancer shows significantly higher levels of chromosomal abnormalities than sporadic breast cancers[1,6,18]. Because of the defect of DNA damage checkpoint, Brca1-deficient cells would accumulate DNA damage. Therefore, it is possible that some portion of $p53^{-/-}Brca1^{-/-}$ MEFs may exhibit altered spindle checkpoint regulation already. Another possibility is Brca1 exhibits a direct regulatory function to spindle checkpoint. Consistent with this notion, it has been recently revealed that Brca1 requires expression of Mad2, which is one of key component of spindle checkpoint[19]. It remains to be explored what is the precise biochemical function of Brca1 required for spindle checkpoint.

Taken together, our results show that Brca1-deficient cells defect in proper activation of nocodazole-induced spindle checkpoint, suggesting Brca1 plays an essential role in spindle checkpoint. Further study about the exact role of Brca1 in spindle checkpoint will provide valuable information whether Brca1 serves as a better target for a more effective cancer treatment.

Acknowledgements

This paper was supported by the Dong-A University Research Fund in 2004 (to J. Yun) and Korea Science and Engineering Research Center for Cancer Molecular Therapy at Dong-A University (to J. Yun).

References

1. Brodie, S. G., Xu, X., Qiao, W., Li, W. M., Cao, L., and Deng, C. X. 2001. Multiple genetic changes are associated with mammary tumorigenesis in Brca1 conditional knockout mice. *Oncogene* **20**, 7514-7523.
2. Chen, Y., Farmer, A. A., Chen, C. F., Jones, D. C., Chen, P. L., and Lee, W. H. 1996. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. *Cancer Res.* **56**, 3168-3172.
3. Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H., and Reid, B. J. 1995. A p53-dependent mouse spindle checkpoint. *Science* **267**, 1353-1356.
4. Deng, C. X., and Brodie, S. G. 2000. Roles of BRCA1 and its interacting proteins. *Bioassays* **22**, 728-737.
5. Di Leonardo, A., Khan, S. H., Linke, S. P., Greco, V., Seidita, G., and Wahl, G. M. 1997. DNA rereplication in the presence of mitotic spindle inhibitors in human and mouse fibroblasts lacking either p53 or pRb function. *Cancer Res.* **57**, 1013-1019.
6. Gasco, M., Yulug, I. G., and Crook, T. 2003. TP53 muta-

tions in familial breast cancer: functional aspects. *Hum. Mutat.* **21**, 301-306.
7. Hartwell, L. 1992. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell* **71**, 543-546.
8. Jallepalli, P. V., and Lengauer, C. 2001. Chromosome segregation and cancer: cutting through the mystery. *Nat. Rev. Cancer* **1**, 109-117.
9. Khan, S. H., and Wahl, G. M. 1998. p53 and pRb prevent rereplication in response to microtubule inhibitors by mediating a reversible G1 arrest. *Cancer Res.* **58**, 396-401.
10. Lanni, J. S., and Jacks, T. 1998. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol. Cell. Biol.* **18**, 1055-1064.
11. Lengauer, C., Kinzler, K. W., and Vogelstein, B. 1998. Genetic instabilities in human cancers. *Nature* **396**, 643-649.
12. Nigg, E. A. 2001. Mitotic kinases as regulators of cell division and its checkpoints. *Nat. Rev. Mol. Cell. Biol.* **2**, 21-32.
13. Ruffner, H., and Verma, I. M. 1997. BRCA1 is a cell cycle-regulated nuclear phosphoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7138-7143.
14. Sablina AA, Agapova LS, ChumakovPM, and Kopnin BP. 1999. p53 does not control the spindle assembly cell cycle checkpoint but mediates G1 arrest in response to disruption of microtubule system. *Cell. Biol. Int.* **23**, 323-334.
15. Ting, N. S., and Lee, W. H. 2004. The DNA double-strand break response pathway: becoming more BRCAish than ever. *DNA Repair (Amst)*, **3**, 935-944.
16. Vaughn, J. P., Davis, P. L., Jarboe, M. D., Huper, G., Evans, A. C., Wiseman, R. W., Berchuck, A., Iglehart, J. D., Futreal, P. A., and Marks, J. R. 1996. BRCA1 expression isinduced before DNA synthesis in both normal and tumor-derived breast cells. *Cell Growth Differ.* **7**, 711-715.
17. Vogel C, Kienitz A, Hofmann I, Muller R, and Bastians H. 2004. Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy. *Oncogene* **23**, 6845-6853.
18. Weaver, Z., Montagna, C., Xu, X., Howard, T., Gadina, M., Brodie, S. G., Deng, C. X., and Ried, T. 2002. Mammary tumors in mice conditionally mutant for Brca1 exhibit gross genomic instability and centrosome amplification yet display a recurring distribution of genomic imbalances that is similar to human breast cancer. *Oncogene* **21**, 5097-5107.
19. Wang, R. H., Yu, H., and Deng, C. X. 2004. A requirement for breast-cancer-associated gene 1 (BRCA1) in the spindle checkpoint. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 17108-17113.
20. Xu, X., Qiao, W., Linke, S. P., Cao, L., Li, W. M., Furth, P. A., Harris, C. C., and Deng, C. X. 2001. Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. *Nat. Genet.* **28**, 266-271.
21. Xu, X., Weaver, Z., Linke, S. P., Li, C., Gotay, J., Wang, X. W., Harris, C. C., Ried, T., and Deng, C. X. 1999. Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. *Mol. Cell.* **3**, 389-395.
22. Yun, J. Involvement of Brca1 in DNA interstrand cross-link repair through homologous recombination-independent process. *J. Life Sci.* **15**, 542-547.
23. Yun, J., Zhong, Q., Kwak, J. Y., and Lee, W. H. 2005. Hypersensitivity of Brca1-deficient MEF to the DNA interstrand crosslinking agent mitomycin C is associated with defect in homologous recombination repair and aberrant S-phase arrest. *Oncogene* **24**, 4009-4016.
24. Zheng, L., Li, S., Boyer, T. G., and Lee, W. H. 2000. Lessons learned from BRCA1 and BRCA2. *Oncogene* **19**, 6159-6175.
25. Zheng, L., Pan, H., Li, S., Flesken-Nikitin, A., Chen, P. L., Boyer, T. G., and Lee, W. H. 2000. Sequence-specific transcriptional corepressor function for BRCA1 through a novel zinc finger protein, ZBRK1. *Mol. Cell.* **6**, 757-768.

초록 : Brca1 결손 세포주에서 nocodazole 처리에 의한 spindle checkpoint 활성화 연구

김명애[1] · 김현주[1] · 윤진호[1]*
(동아대학교 의과대학 생화학교실, [1]동아대학교 암분자치료연구센터)

항암유전자 Brca1의 변이는 유방암 및 난소암에 대한 감수성을 증가시키며, Brca1은 DNA 손상신호후 세포주기 조절에 필수적인 역할을 한다. 연구결과, Brca1이 세포주기 S기와 G2/M 조절점에서 중요한 역할을 담당함이 밝혀졌다. 그러나, Brca1의 spindle checkpoint 관여여부는 알려져 있지 않다. 본 연구에서는 spindle checkpoint를 활성화시키는 nocodazole를 처리하여 야생형, $p53^{-/-}$ 그리고 $p53^{-/-}Brca1^{-/-}$ 세포주의 세포주기 변화를 조사하였다. 야생형과 $p53^{-/-}$ 세포주는 신속한 mitosis기 정지가 나타난 반면, $p53^{-/-}Brca1^{-/-}$ 세포주의 경우 모든 세포가 M기에서 정지하지 않았다. Double-thymidine block 기법에 의한 세포주기 동조화후 nocodazole 처리시에도 $p53^{-/-}Brca1^{-/-}$ 세포주에서는 일부세포가 M기 조절점을 통과하여 계속 G1기로 진행하였다. 형태학적 분석에서도 nocodazole 함유배지에서 계속 증식하는 세포형태가 관찰되었다. 이와 같은 결과들은 Brca1이 spindle checkpoint가 정상적으로 작동하는데 중요한 역할을 담당한다는 것을 의미하고 있다.