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Inhibition of Polo-like Kinase 1 Prevents the Male Pronuclear Formation Via Alpha-tubulin Recruiting in *In vivo*-fertilized Murine Embryos

Jeonghyeon Moon and Sangho Roh†

Cellular Reprogramming and Embryo Biotechnology Laboratory, Dental Research Institute, BK21, Seoul National University School of Dentistry, Seoul, Republic of Korea

Abstract

Polo-like kinase 1 (Plk1) has been known to be a critical element in cell division including centrosome maturation, cytokinesis and spindle formation in somatic, cancer, and mammalian embryonic cells. In particular, Plk1 is highly expressed in cancer cells. Plk1 inhibitors, such as BI2536, have been widely used to prevent cell division as an anticancer drug. In this study, the fertilized murine oocytes were treated with BI2536 for 30 min after recovery from the oviduct to investigate the effect of down-regulation of Plk1 in the *in vivo*-fertilized murine embryos. Then, the localization and expression of Plk1 was observed by immunofluorescence staining. The sperm which had entered into the oocyte cytoplasm did not form male pronuclei in BI2536-treated oocytes. The BI2536-treated oocytes showed significantly lower expression of Plk1 than non-treated control group. In addition, alpha-tubulin and Plk1 gathered around sperm head in non-treated oocytes, while BI2536-treated oocytes did not show this phenomenon. The present study demonstrates that the Plk1 inhibitor, BI2536, hinders fertilization by inhibiting the formation of murine male pronucleus.

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† Correspondence: Sangho Roh, D.V.M., Ph.D. (ORCID: 0000-0001-8082-6459)
Tel: +82-2-880-2333, Fax: +82-02-882-0228
E-mail address: sangho@snu.ac.kr

INTRODUCTION

Polo-like kinase 1 (Plk1) is a family of serine/threonine-protein kinase 13 (STPK13) (Wolf *et al*., 1997). Plk1 was first founded in Drosophila in 1988 (Sunkel and Glover, 1988). Plk1 plays a variety of roles in cells during cell division (Archambault and Glover, 2009). Plk1 regulates the centrosome maturation (Lane and Nigg, 1996), chromosome adhesion (Hauf *et al*., 2005), kinetochore-microtubule attachment (Brennan *et al*., 2007), cytokinesis and mitotic entry (Sumara I *et al*., 2004).

Plk1 is highly expressed in cancer cells, therefore it is considered a proto-oncogene (Li *et al*., 2017). Hence, Plk1 inhibitors are used to inhibit the proliferation of cancer cells (Yan *et al*., 2018; Wang *et al*., 2018). The Plk1 inhibitor, BI2536, is a highly effective molecule that inhibits the growth and proliferation of cancer cells (Merrick *et al*., 2018; Noor *et al*., 2018; Jeong *et al*., 2018). BI2536 was first developed by the Boehringer Ingelheim Company in 2007. Steegmaier and colleagues showed that BI2536 is a potent inhibitor of Plk1 that inhibits tumor growth both of *in vitro* and *in vivo* (Steegmaier *et al*., 2007). BI2536 has been approved for acceptable safety in some clinical trials. (Frost *et al*., 2012). However, through extensive preclinical testing is less than the rate of side effects, it is no longer used in monotherapy (Mross *et al*., 2012).

In a previous study, Plk1 is an essential factor in the first mitotic division in mouse zygote (Baran *et al*., 2013). It regulates the cleavage of one-cell (Zhao *et al*., 2010), spindle formation kinetics and APC/C activation in mouse zygote (Baran *et al*., 2016). Plk1 plays an important role not only in the mitotic division but also in the mouse oocyte maturation (Kheilová *et al*., 2015).

Recently, many research groups have demonstrated the role and function of Plk1 in the embryo of the pre-implantation stage (Zhang Z *et al*., 2017; Wang H. *et al*., 2017). However, the accurate mechanism of Plk1 in mouse pre-implantation stage embryos still remains unclear. This study investigated that the downregulation of Plk1 using BI2536 triggers the failure of fertilization through the formation of male pronucleus.

MATERIALS AND METHODS

1. Chemicals

All organic and inorganic compounds were purchased from Sigma-Aldrich Korea.

2. Collection the *in vivo*-fertilized embryos and animals

The 7.5 IU equine chorionic gonadotropin (eCG; Daesung Microbiology Lab, Korea) injected 6-8 weeks old C57BL6 X DBA2 F1-hybrid (B6D2F1) female mice (Orient Bio, Korea) by intraperitoneal for superovulation and 7.5 IU human chorionic gonadotropin (hCG; Daesung Microbiology Lab) after 48 h later. Immediately after hCG injection, female B6D2F1 mice were mated with 8-10 weeks old male B6D2F1 mice (Orient Bio, Korea). The *in vivo*-fertilized embryos were collected 15 h after hCG injection, and the oviducts were removed and transferred to 2 ml TCM-199 with Hepes-CZB medium (HCZB). After tearing the ampullae of the oviducts, the *in vivo*-fertilized embryos were released without cumulus cells. The *in vivo*-fertilized embryos were washed five times in HCZB. All animal experiments were approved under the agreement guidelines of the Institutional Animal Care and Use Committee of Seoul National University (approval number: SNU-130123-5-7 and SNU-130123-4-7)

3. Cell culture

Washed *in vivo*-fertilized embryos were cultured in KSOM medium for 3.5 to 4 days. Mouse embryonic stem cells (mESCs) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Welgene, Korea) with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific Korea, Korea), 1% penicillin/streptomycin solution (Life Technologies), 1% non-essential amino acids (Gibco, USA), 0.1 mM and 1000 U/ml leukemia inhibitory factor (LIF; Chemicon, USA). mESCs were cultured in 0.1% gelatin coated-dishes. Mouse skin fibroblasts (mSFs) were obtained from female B6D2F1 mice. To obtain murine skin fibroblast cells, skin tissue of B6D2F1 was washed twice with phosphate-buffered saline (PBS) including 3% penicillin/streptomycin solution and cultured in DMEM with 10% FBS and 1% penicillin/streptomycin solution. All of the cells were cultured in 37°C 5% CO2 humidified incubator.

4. Treatment of a Plk1 inhibitor, BI2536

The *in vivo*-fertilized embryos were treated with 4 nM of BI2536 (Axon medchem BV, Netherlands) to inhibit Plk1 which is essential to the mitotic division. The oocytes used as the experimental groups were placed in KSOM medium with 4 nM of BI2536 and 1% DMSO for 30 min after recovery from the oviduct in a 37°C humidified incubator. The oocytes used as the control group were cultured in KSOM medium with 1% of DMSO.

5. Immunofluorescence

The oocytes, mSFs and mESCs were fixed with 4% paraformaldehyde in PBS for 30 min, then the oocytes were washed three times in PBS containing 0.5% PVP and 0.1% Triton X-100. The fixed cells were set in PBS containing 0.25% Triton X-100 for 4 h to make the membrane permeable and then incubated in PBS with 0.1% Triton X-100 and 1% BSA for blocking for 2 h in a 37°C incubator. Plk1 was detected by rabbit polyclonal IgG antibodies (1:100; Santa Cruz Biotechnology, USA) and goat-anti rabbit polyclonal IgG antibodies (1:500; Millipore, USA). Alpha-tubulin (α-tubulin) was detected by mouse monoclonal IgM antibodies (1:100; Santa Cruz Biotechnology, USA) and goat-anti mouse monoclonal IgM secondary antibodies (1:500; Novus, USA). DAPI staining was used to assess nuclear morphology. All of stained cells were observed by confocal microscope. The oocytes were fixed and monitored at 0 h or 5 h after treatment of BI2536 respectively. The confocal microscopy images were acquired by LSM 700 (Zeiss, Germany). The confocal microscopy images were analyzed by Zen2 BLUE edition software (Zeiss, Germany).

6. Statistical analysis

All experiments were iterated three times. All percentage data obtained in this study are presented as the mean ± standard deviation (S.D.). To determine the significance of differences among groups, comparisons were made using Student's *t*-test as implemented in GraphPad Prism V5.0 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered significant.

RESULTS

To confirm the expression and location of Plk1 and α-tubulin in differentiated cells, they were subjected to immunofluorescence analysis. Plk1 was not detected in mSFs which were not in the mitotic phase (Figure 1A). However, in metaphase mESCs, Plk1 was highly expressed in the centrosome parts (Figure 1B, arrows).

The developmental rate of BI2536-treated zygotes (0.0 ± 0.0) was significantly lower than that of non-treated *in vivo*-fertilized embryos (75.3 ± 3.3; $p<0.05$; Table 1). Even BI2536-treated zygotes failed to develop in the first mitotic division. Therefore, the focus was placed on the localization and expression of Plk1 of the mouse *in vivo*-fertilized embryos in the following experiments.

The immunofluorescence images showed that the oocytes were fixed immediately after the treatment of BI2536 (Figure 2A and B). α-tubulin and Plk1 were highly expressed with sperm which entered oocyte cytoplasm in non-treated *in vivo*-fertilized embryos (Figure 2A, arrow). However, the expression of Plk1 and α-tubulin were low at location of sperm in BI2536-treated embryos (Figure 2B, arrow). Female nuclei have been arrested in metaphase Ⅱ without significant changes in both treated and non-treated groups (Figure 2A and B, arrowheads). The intensity of Plk1 around sperm in non-treated *in vivo*-fertilized embryos (Non-sperm) is significantly higher than the intensity of cytosol in non-treated *in vivo*-fertilized embryos (Non-cytosol) and BI2536-treated *in vivo*-fertilized embryos (BI-cytosol) (Figure 2C).

After 5 h later of treatment, the mouse zygotes formed the intact pronuclei (Figure 3A, arrows). Plk1 was not concentrated in specific region in non-treated oocytes. Instead, Plk1 spread widely in the cytosol of no treatment groups (Figure 3A). However, the expression of Plk1 was low in BI2536-treated oocytes (Figure

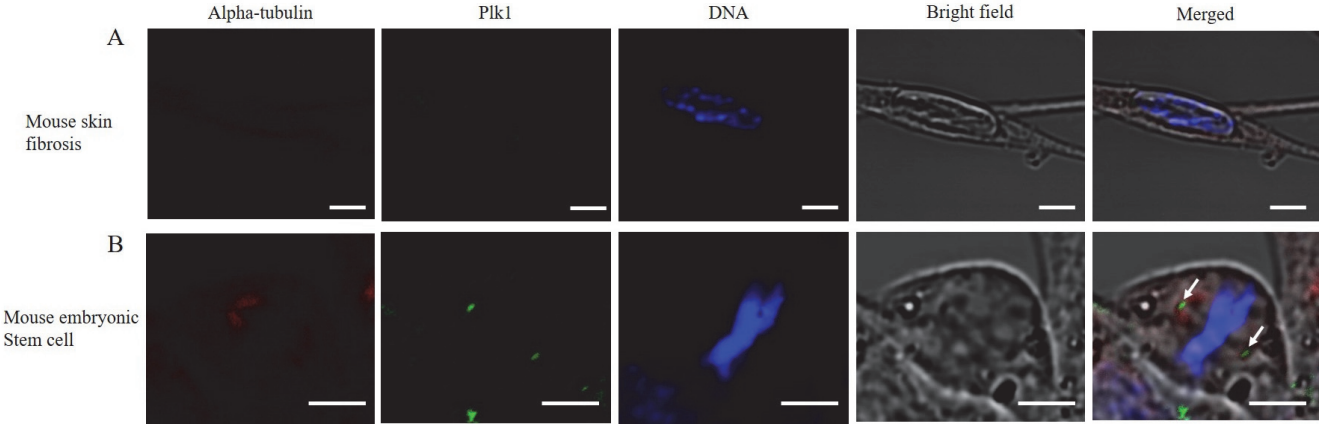

Figure 1. The immunofluorescence images of (A) mouse skin fibrosis and (B) mouse embryonic stem cell. Polo-like kinase 1 (Plk1) localized at the regions of centrosomes (arrows). Scale bar = 5 μm.

Table 1. Developmental rates of non-treated *in vivo*-fertilized embryos and BI2536-treated *in vivo*-fertilized embryos

Group	No. of embryos developed to (%)				
	1-cell	2-cell	4-cell	Morula	Blastocyst
Non-treated	73	62 (84.9 ± 3.8)	61 (83.6 ± 2.5)	58 (79.5 ± 0.7)	55 (75.3 ± 3.3)
BI2536 treated	74	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)*

*Different superscripts denote a significant difference compared with other groups (p<0.05).

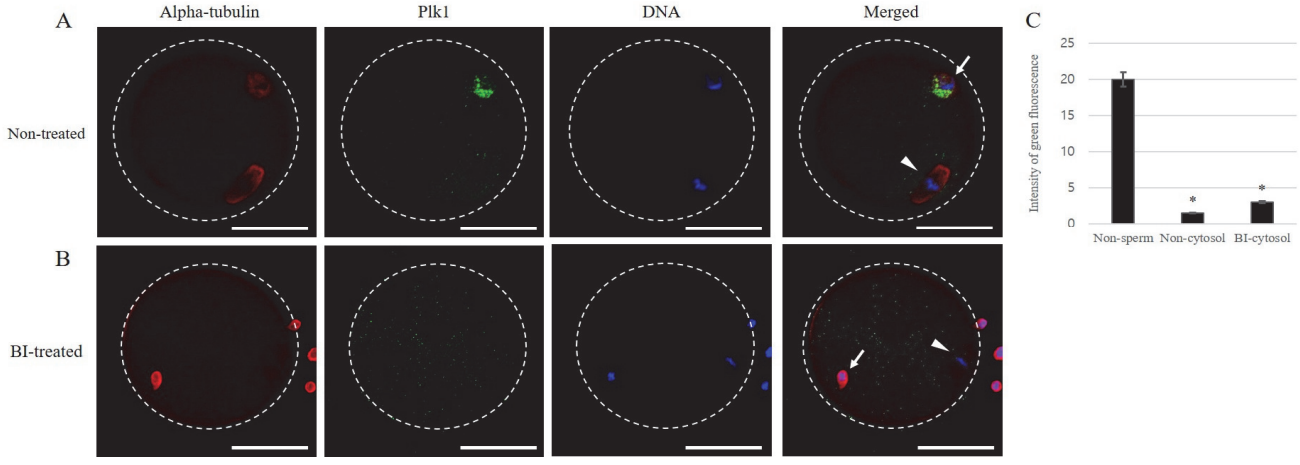

Figure 2. The immunofluorescence expression of Plk1, alpha-tubulin (α-tubulin) and DNA in (A) non-treated *in vivo*-fertilized embryos (B) BI2536-treated *in vivo*-fertilized embryos shortly after fertilization. Male pronuclei (arrows) and female pronuclei (arrowheads) were presented respectively. (C) The quantitative analysis data of Plk1 intensity. *p<0.05. Scale bar = 20 μm.

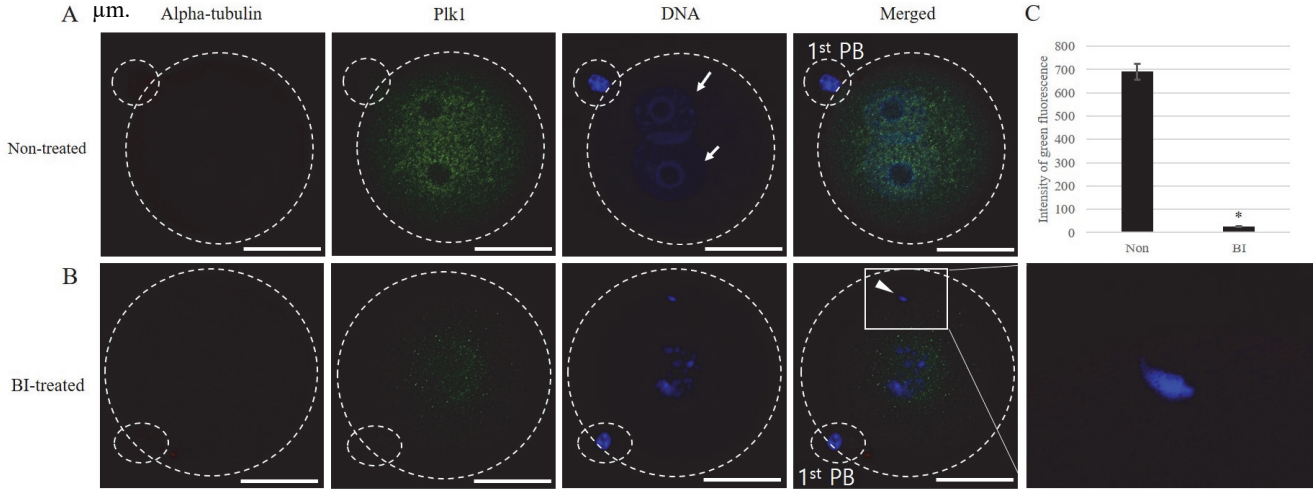

Figure 3. The immunofluorescence expression of Plk1, α-tubulin, and DNA in (A) non-treated *in vivo*-fertilized embryos. The pronuclei were presented in arrows. (B) BI2536-treated *in vivo*-fertilized embryos 5 h after fertilization. The intact sperm which had entered oocyte cytoplasm were observed (arrowhead). (C) The quantitative analysis data of expression of Plk1. PB = polar body, Non = no treatment group, BI = BI2536 treatment group *p<0.05. Scale bar = 20 μm.

3B). The sperm did not form the pronuclei in BI2536-treated groups. Mouse sperm which entered oocyte cytosol remained intact (Figure 3B, arrowhead). The intensity of Plk1 in no treatment groups (Non) was significantly higher than BI2536-treated oocytes (BI) (Figure 3C).

DISCUSSION

In previous studies, Plk1 is known as the essential element in mitotic cell division (Kang *et al*., 2006). In addition to the much-studied cyclin-dependent protein kinases, it is now appreciated that Plk1 functions as a crucial component for various mitotic

events. Plk1 is tightly adjusted both temporally and spatially throughout the cell cycle. Timely recruitment of Plk1 to specific subcellular structures is likely important for proper function of Plk1 at these locations.

In somatic cells, the localization of Plk1 to the interphase and mitotic centromeres as cells proceed through the mitotic and meiotic cell cycle. Plk1 regulates the ultimately leads to chromosome segregation and aneuploidy during mitotic cleavage stage (Lee *et al*., 2008). In particular, inhibition of Plk1 induces mitotic catastrophe following of the spindle assembly checkpoint in lung cancer cells (Choi *et al*., 2015). Plk1 inhibition elevates the antineoplastic activity in prostate cancer (Shao *et al*., 2015), breast cancer (Ha *et al*., 2018), pancreatic cancer (Mao *et al*., 2016).

When Plk1 and α-tubulin were observed by immunofluorescence, fully differentiated mSFs with few mitotic cleavages had the little expression of Plk1. However, actively dividing mESCs showed higher expression of Plk1 when compared with mSFs. In particular, Plk1 was located at the regions of centrosomes in metaphase of mESCs. This result has the same consequence with previous reports from others (Schmucker and Sumara, 2014; Colicino *et al*., 2018).

Plk1 inhibitors are even progressing to clinical trials (Gutteridge *et al*., 2016). In particular, BI2536 is widely used because it has a high effect of inhibiting plk1 and its low price (Gohda *et al*., 2018; Cheng *et al*., 2018). However, this study showed that BI2536 could have a negative effect on the male pronuclear formation. We treated BI2536 at a concentration of 100 nM or more. Consequently, the BI2536-treated embryos were proceeded apoptosis pathway (data not shown). This data was used BI2536 at a lower concentration (4 nM vs. 100 nM) and time (30 min vs. 18 h) than the results of other embryo research groups (Baran, 2013; Baran *et al*., 2016). Nonetheless, the male pronuclei of the BI2536-treated oocytes did not form intact morphology in this study. In previous studies, inhibition of Plk1 causes aberrant spindle (Liao *et al*., 2018), chromosome segregation via assembly inhibition of α-tubulin (Zhang *et al*., 2017). In this study, Plk1 and α-tubulin gather around the sperm which entered oocyte cytoplasm in non-treated oocytes. However, it did not show concentration of Plk1 and α-tubulin in BI2536-treated oocytes. Taken together, this data suggested that the BI2536, a Plk1 inhibitor, has the potential to cause infertility via inhibiting fertilization.

In conclusion, this study revealed that the Plk1 inhibitor BI2536 hinder fertilization by inhibiting the formation of male pronucleus via preventing α-tubulin from being normally recruited.

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REFERENCES

Archambault V, Glover DM. 2009. Polo-like kinases: conservation and divergence in their functions and regulation. Nat. Rev. Mol. Cell. Biol. 10:364.

Baran V, Solc P, Kovarikova V, Rehak P, Sutovsky P. 2013. Polo-like kinase 1 is essential for the first mitotic division in the mouse embryo. Mol Reprod Dev. 80:522-534.

Baran V, Brzakova A, Rehak P, Kovarikova V, Solc P. 2016. PLK1 regulates spindle formation kinetics and APC/C activation in mouse zygote. Zygote. 24:338-345.

Brennan IM, Peters U, Kapoor TM, Straight AF. 2007. Polo-like kinase controls vertebrate spindle elongation and cytokinesis. PLoS One. 2:e409.

Cheng CY, Liu CJ, Huang YC, Wu SH, Fang HW, Chen YJ. 2018. BI2536 induces mitotic catastrophe and radiosensitization in human oral cancer cells. Oncotarget. 9:21231-21243.

Choi M, Kim W, Cheon MG, Lee CW, Kim JE. 2015. Polo-like kinase 1 inhibitor BI2536 causes mitotic catastrophe following activation of the spindle assembly checkpoint in non-small cell lung cancer cells. Cancer Lett. 357:591-601.

Colicino EG, Garrastegui AM, Freshour J, Santra P, Post DE, Kotula L, Hehnly H. 2018. Gravin regulates centrosome function through PLK1. Mol Biol Cell. 29:532-541.

Frost A, Mross K, Steinbild S, Hedbom S, Unger C, Kaiser R, Trommeshauser D, Munzert G. 2012. Phase i study of the Plk1 inhibitor BI2536 administered intravenously on three consecutive days in advanced solid tumours. Curr Oncol. 19:e28-35.

Gohda J, Suzuki K, Liu K, Xie X, Takeuchi H, Inoue JI, Kawaguchi Y, Ishida T. 2018. BI2536 and BI-6727, dual Polo-like kinase/bromodomain inhibitors, effectively reactivate latent HIV-1. Sci Rep. 8:3521.

Gutteridge RE, Ndiaye MA, Liu X, Ahmad N. 2016. Plk1 Inhibitors in Cancer Therapy: From Laboratory to Clinics. Mol Cancer Ther. 15:1427-1435.

Ha GH, Kim DY, Breuer EK, Kim CK. 2018. Combination

treatment of Polo-like kinase 1 and tankyrase-1 inhibitors enhances anticancer effect in triple-negative breast cancer cells. Anticancer Res. 38:1303-1310.

Hauf S, Roitinger E, Koch B, Dittrich CM, Mechtler K, Peters JM. 2005. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol. 3:e69.

Jeong SB, Im JH, Yoon JH, Bui QT, Lim SC, Song JM, Shim Y, Yun J, Hong J, Kang KW. 2018. Essential role of Polo-like kinase 1 (Plk1) oncogene in tumor growth and metastasis of tamoxifen-resistant breast cancer. Mol Cancer Ther. 17:825-837.

Kang YH, Park JE, Yu LR, Soung NK, Yun SM, Bang JK, Seong YS, Yu H, Garfield S, Veenstra TD, Lee KS. 2006. Self-regulated Plk1 recruitment to kinetochores by the Plk1-PBIP1 interaction is critical for proper chromosome segregation. Mol Cell. 24:409-422.

Kheilová K, Petr J, Žalmanová T, Kučerová-Chrpová V, Řehák D. 2015. Src family kinases are involved in the meiotic maturation of porcine oocytes. Reprod Fertil Dev. 27:1097-1105.

Lane HA, Nigg EA. 1996. Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. J Cell Biol. 135:1701-1713.

Lee KS, Oh DY, Kang YH, Park JE. 2008. Self-regulated mechanism of Plk1 localization to kinetochores: lessons from the Plk1-PBIP1 interaction. Cell Div. 3:4.

Li Z, Liu J, Li J, Kong Y, Sandusky G, Rao X, Liu Y, Wan J, Liu X. 2017. Polo-like kinase 1 (Plk1) overexpression enhances ionizing radiation-induced cancer formation in mice. J Biol Chem. 42:17461-17472.

Liao Y, Lin D, Cui P, Abbasi B, Chen C, Zhang Z, Zhang Y, Dong Y, Rui R, Ju S. 2018. Polo-like kinase 1 inhibition results in misaligned chromosomes and aberrant spindles in porcine oocytes during the first meiotic division. Reprod Domest Anim. 53:256-265.

Mao Y, Xi L, Li Q, Cai Z, Lai Y, Zhang X, Yu C. 2016. Regulation of cell apoptosis and proliferation in pancreatic cancer through PI3K/Akt pathway via Polo-like kinase 1. Oncol Rep. 36:49-56.

Merrick DT, Edwards MG, Franklin WA, Sugita M, Keith RL, Miller YE, Friedman MB, Dwyer-Nield LD, Tennis MA, O'Keefe MC, Donald EJ, Malloy JM, van Bokhoven A, Wilson S, Koch PJ, O'Shea C, Coldren C, Orlicky DJ, Lu X, Baron AE, Hickey G, Kennedy TC, Powell R, Heasley L, Bunn PA, Geraci M, Nemenoff RA. 2018. Altered cell-cycle control, inflammation, and adhesion in high-risk persistent bronchial dysplasia. Cancer Res. 78:4971-4983.

Mross K, Dittrich C, Aulitzky WE, Strumberg D, Schutte J, Schmid RM, Hollerbach S, Merger M, Munzert G, Fleischer F, Scheulen ME. 2012. A randomised phase II trial of the Polo-like kinase inhibitor BI2536 in chemo-naïve patients with unresectable exocrine adenocarcinoma of the pancreas - a study within the Central European Society Anticancer Drug Research (CESAR) collaborative network. Br J Cancer. 107:280-286.

Noor A, Umelo IA, Kronenberger P, Giron P, De Vlieghere E, De Wever O, Teugels E, De Grève J. 2018. Targeting Polo-like kinase 1 and TRAIL enhances apoptosis in non-small cell lung cancer. Oncotarget. 9:28731-28744.

Schmucker and Sumara. 2014. Molecular dynamics of PLK1 during mitosis. Mol Cell Oncol. 1:e954507.

Shao C, Ahmad N, Hodges K, Kuang S, Ratliff T, Liu X. 2015. Inhibition of polo-like kinase 1 (Plk1) enhances the antineoplastic activity of metformin in prostate cancer. J Biol Chem. 290:2024-2033.

Sunkel CE, Glover DM. 1988. polo, a mitotic mutant of Drosophila displaying abnormal spindle poles. J Cell Sci. 1:25-38.

Steegmaier M, Hoffmann M, Baum A, Lénárt P, Petronczki M, Krssák M, Gürtler U, Garin-Chesa P, Lieb S, Quant J, Grauert M, Adolf GR, Kraut N, Peters JM, Rettig WJ. 2007. BI2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth *in vivo*. Curr Biol. 17:316-322.

Sumara I, Giménez-Abián JF, Gerlich D, Hirota T, Kraft C, de la Torre C, Ellenberg J, Peters JM. 2004. Roles of polo-like kinase 1 in the assembly of functional mitotic spindles. Curr Biol. 14:1712-1722.

Wang H, Choe MH, Lee IW, Namgoong S, Kim JS, Kim NH, Oh JS. 2017. CIP2A acts as a scaffold for CEP192-mediated microtubule organizing center assembly by recruiting Plk1 and aurora A during meiotic maturation. Development. 144:3829-3839.

Wolf G, Elez R, Doermer A, Holtrich U, Ackermann H, Stutte HJ, Altmannsberger HM, Rübsamen-Waigmann H, Strebhardt K. 1997. Prognostic significance of polo-like kinase (PLK) expression in non-small cell lung cancer. Oncogene. 14:543-549.

Yan W, Yu H, Li W, Li F, Wang S, Yu N, Jiang Q. 2018. Plk1 promotes the migration of human lung adenocarcinoma epithelial cells via STAT3 signaling. Oncol Lett. 5:6801-6807.

Yao D, Gu P, Wang Y, Luo W, Chi H, Ge J, Qian Y. 2018. Inhibiting polo-like kinase 1 enhances radiosensitization via

modulating DNA repair proteins in non-small-cell lung cancer. Biochem Cell Biol. 3:317-325.

Zhang Z, Chen C, Cui P, Liao Y, Yao L, Zhang Y, Rui R, Ju S. 2017. Plk1 inhibition leads to a failure of mitotic division during the first mitotic division in pig embryos. J Assist Reprod Genet. 34:399-407.

Zhang Z, Chen C, Ma L, Yu Q, Li S, Abbasi B, Yang J, Rui R, Ju S. 2017. Plk1 is essential for proper chromosome segregation during meiosis I/meiosis II transition in pig oocytes. Reprod Biol Endocrinol. 15:69.

Zhao Y, Ai J, Zhang H, Zhu G. 2010. Polo-like kinase-1 regulates first cleavage of one-cell embryos in culture during assisted reproduction. Saudi Med J. 31:247-252.