이학석사학위논문

유방암 세포주에서 중심립 숫자 조절에 관한 연구

Studies on the supernumerary centrioles in the breast cancer cell lines

2021 년 08 월

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ABSTRACT

Studies on the supernumerary centrioles in the breast cancer cell lines

Yejoo Lee

Centrosomes are major microtubule organizer in animal cells. It is critical to maintain two centrosomes in a single cell since they function as spindle poles to pull a set of chromosomes into two daughter cells. However, deregulations in centrosome numbers are frequently observed in cancer cells. Here, I investigated generation of the supernumerary centrioles in selected breast cancer cells. The number of centrioles at specific stages of the cell cycle was determined with the coimmunostaining analyses using the BrdU and centrin-2 antibodies. The results showed that supernumerary centrioles are maintained at specific subset of the breast cancer cell populations. The proportions of the cells with supernumerary centrioles were not identical during

the cell cycle. In case of MDA-MB-231 cells, proportion of the cells with supernumerary centrioles were maintained to 20% throughout the cell cycle. However, in case of Hs578t cells, supernumerary centrioles were detected in 5% of the S phase cells, but in 50% of the M phase cells. I also detected premature separation of centrioles in Hs578t cells at mitosis. These results suggest that supernumerary centrioles may be generated at specific cell cycle stages, possibly following distinct amplifying mechanisms. The breast cancer cell lines may adopt their own mechanisms for amplifying their centrioles.

Key Words: Supernumerary centrioles, Breast cancer cell line, PLK4, PCNT, CEP215, Interphase, Mitosis

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CONTENTS

LIST OF FIGURES

INTRODUCTION

The centrosome is a hub of microtubule organizing center (MTOC) consists of centrioles surrounded by pericentriolar material (PCM). Each centrosome organizes the microtubules to form the mitotic spindle in dividing cells, contributing to an accurate cell division (Nigg and Holland, 2018). Therefore, maintaining accurate number of centrosomes is crucial for normal division of cells, else it leads to aneuploidy and uncontrollable cell division. Furthermore, it has been observed that there is a strong relationship between abnormal centrosome number, morphology and aneuploid forming cancer cells. This indicates that a precise regulation of centrosome number and formation might be important for normal division of cell. (Nigg and Stearns, 2011)

Canonically, centrosome exist as two numbers. At G1 phase of the cell cycle, Mother and daughter centrioles are connected by proteinaceous linker1, 2. Around G1/S transition, each centriole assembles procentriole, mediated by conserved components serine/ threonine kinase Polo-like kinase 4 (PLK4), coiled-coil proteins centrosomal protein of 192 kDa (CEP192), (CEP152), spindle assembly abnormal protein 6 homologue (HsSAS-6), SCL-Interrupting locus protein (STIL) and centrosomal P4. 1-associated protein (CPAP) 3-5.

Especially PLK4, HsSAS-6 and STIL are significant in centriole duplication, since loss of any of these proteins prevents procentriole assembly, whereas their overexpression creates multiple daughter supernumerary centrioles. Throughout S/G2 phase, each centriole-procentriole pair are proximal to each other. When cell enters mitosis, PCM is accumulated in each centrosome. A centrosome and PCM complex emit myriad of microtubules and allow them to act as spindle poles. At the later stage of mitosis, centrioles disengage from each other as PCM disseminates. The separated daughter centrioles from mother centriole becomes a young mother centriole, and procure the ability to form new daughter centriole in another round of cell cycle (Wang *et al*., 2011).

Defects in numerical and structural centrosomes are frequently discovered in Human cancers (Gonczy., 2015; Prosser and Pelletier, 2017). Loss of centrosome likely arrests the cell, whereas tumor cells with abnormal centrosomes survive by inhibiting p53 function (Lambrus and Holland, 2017). In solid human cancer, centrosome amplification is commonly discovered, and it is known to be relevant to the multipole spindle formation, chromosome segregation errors and aneuploidy. (Zyss and Gergely, 2009; Chan., 2011; Yamamoto *et al*., 2011; Nolte *et al*., 2013; Denu *et al*., 2016; Marteil *et al*., 2018) Moreover, centrosomal microtubule nucleation derived by centrosome amplification provides a primary role in tumor initiation and progression.

(Levine *et al*., 2017) In healthy cells, these abnormalities can be rebounded by mitotic spindle assembly checkpoint (SAC), and a following cell death. However, cancer cells can detour this survival mechanism by clustering extra centrosomes into two spindle poles (Kramer *et al*., 2011; Ring *et al*., 1982). Until now, many studies targeting the supernumerary centrioles and the survival mechanisms was applied to cancer therapy. But still, the origins and consequences of such abnormalities are understudied. Although, it is suggested that cancer cells contain significant number of supernumerary centrosomes, the underlying mechanism and its origin is not clear yet. (Zyss and Gergely, 2009; Chan., 2011; Yamamoto *et al*., 2011; Nolte *et al*., 2013; Denu *et al*., 2016; Marteil *et al*., 2018) Identifying which phase extra centrosome form might provide better understanding in contributing factors leading to supernumerary centrioles. Mechanistic studies revealing the origin of amplified centrosomes are prevalent. Among all, over expression and deregulation of PLK4 was known to be the driving force leading to over duplicated centrioles at the S phase of the cell cycle. (Habedanck *et al*., 2005; Kleylein-Sohn *et al*., 2007; McCoy *et al.*, 2015) This is because PLK4 overexpression can generate multiple daughter centrioles. In addition, it is reported PLK4 is overexpressed in variety of cancer cells. (Liao *et al*., 2019) However, the other factors such as centriole fragmentation, de novo centrosome formation and reduplicated

centrioles after premature centriole disengagement is also suggested. (Sabat-Pospiech *et al*., 2019) Previously, our lab revealed lack of Pericentrosomal materials lead to premature centriole separation and centriole amplification during mitosis and provided new perspective in initiation of formation of supernumerary centrosome. (Kim *et al*., 2019) One of the possible explanations to this phenomenon is that the physical disconnection between mother and daughter centrioles no longer block the further round of centriole assembly, which induce centriole reduplication. (Loncarek *et al*., 2008; Loncarek *et al*., 2010; Tsou *et al*., 2009) However, the need of corroborating this phenomenon should be further studied by observing cells in each phase of the cell cycle. Moreover, we do not know whether this phenomenon is commonly discovered in cancer cell lines.

Here, I analyzed the supernumerary centrioles in breast cancer cell lines. I selected the breast cancer cells as my experimental model because it is reported that centriole amplification is highly prevalent in breast cancer carcinomas. We discovered the subset of cancer cell lines contain different proportion of supernumerary centrioles. I also discovered that amplified centrioles are discovered in overall stage of the cell cycle. However, for Hs578t cell line the frequency was higher especially in M phase compared to other stages of cell cycle. When observed in the change in number of centrioles in

time course, I found out that the number of centrioles increased in Mitotic phase of the cell cycle. Finally, I observed that in hs578t cell line, premature centriole disengagement was significantly discovered in M-phase-arrested cells.

MATERIALS AND METHODS

Cell culture, Cell synchronization and Drug treatments

MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-468 were kindly provided by Professors Won shik Han from Seoul National University, BT549, Hs578t from Sujae Lee. HCC1428 cell line was obtained from Korean Cell Line Bank. MDA-MB-231, MDA-MB-468, MDA-MB-157 and BT-549 are TNBC Breast cancer cell lines, whereas MCF7 and MDA-MB-157 are luminal breast cancer cell lines. The P53/PCNT/CEP215 Knockout HeLa cells expressing DD-PCNT were made in the Flp-INT-Rex Hela cells (Kim et al., 2019). All cell lines were cultured in their respective media supplemented with fetal bovine serum (FBS, Gibco), Penicillin (100 IU mL^{-1}, Gibco) and Streptomycin (100 µg mL^{-1}, Gibco). HeLa, TERT-RPE1, MDA-MB-231, MDA-MB-468, MDA-MB-157, Hs578t, MCF7 cells were cultured in DMEM, Bt549 and HCC1428 were cultured in RPMI, supplemented with 10% fetal bovine serum and antibiotics. For prometaphase arrest, cells were treated with 2mM thymidine and 2.5 µM STLC. For time course experiment, cells were arrested at G1/S boundary by the double thymidine block. 2mM thymidine was added to the culture medium for 16 hr,

cells were incubated in fresh medium for 8-15 hr, and were incubated again in 2mM thymidine for 16 hr and released into the fresh medium. To induce PLK4 ectopic expression, cells were treated with doxycycline for 24 hours, washed out and cultured for another 24 hours. Natural dividing mitotic cells were obtained from asynchronous cell plates. For drug treatments, the following compounds and concentrations were used: STLC (2.5μM), Palbociclib (1μM), Thymidine (2mM), Aphidicolin (5μM), RO3306 (5μM)

Antibodies

Antibodies for CEP135 (Kim *et al*., 2008), CP110 (Kim *et al*., 2008) were mentioned previously. Centrin (Millipore, 20H5), Cyclinb (Santacruz, Sc-245), BrdU (Sigma, 59-14-3) were purchased. Anti-mouse IgG-HRP (Sigma, A9044), anti-rabbit IgG-HRP (Calbiochem, DL03L) and Alexa 488, 555 (Invitrogen) were used as secondary antibodies for Immunocytochemistry analyses.

Immunostaining analysis

In order to detect centrosomes in BrdU labeled cells, the BrdU incorporated cells are seated in the coverglass and fixed with cold methanol for 10 min at 4℃, washed with

cold PBS, and blocked with blocking solution (3% bovine serum albumin, and 0.3% Triton X-100 in PBS) for 30 min. The samples were incubated with primary antibodies excluding BrdU for 1 h, washed with 0.1% PBST, incubated with secondary antibodies for 30 minutes, washed, post-fixed with 4% paraformaldehyde for two days in 4 °C. The cells are denatured by preheated 2N HCL, stabilized by Sodium Borate buffer (1M, pH 8.5) and washed with PBS. The samples are reblocked with blocking solution (3% bovine serum albumin, and 0.3% Triton X-100 in PBS) for 30 min. The samples were incubated with BrdU antibody for 1 h, washed with 0.1% PBST, incubated with BrdU targeting secondary antibody for 30 minutes. The samples were treated with 4,6-diamidino-2-phenylindole (DAPI) solution for up to 2 minutes. The cover glasses were mounted on a slide glass with ProLong Gold antifade reagent (P36930; Life Technologies). Images were observed with fluorescence microscopes with a digital camera (Olympus IX51) equipped with QImaging 336 QICAM Fast 1394 and processed in ImagePro 5.0 (Media Cybernetics). ImagePro 5.0 (Media 337 Cybernetics), Photoshop CC (Adobe) and ImageJ 1.51k (National Institutes of Health) were used for image processing.

Fluorescence-activated cell sorting

The cells were harvested and washed in PBS. Cold 70% ethanol were dropped to the sample while vortexing for fixation and stored at 4℃ for minimal 10 minutes. Cells were washed three times in PBS. Propidium iodide (PI) with RNase and EDTA (7.5pH) was used for DNA staining. 10000 cells were counted for each sample to draw the propidium iodide DNA histogram plot. The cells at each cell cycle phase were measured by using markers set within the plot representing the percentage for each cell cycle phase.

RESULTS

Supernumerary centrioles in the breast cancer cells

In the beginning, I determined the presence of the supernumerary centrioles in selected breast cancer cell lines. The breast cancer cell lines were kindly provided by Professors Won shik Han (MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-468) and Sujae Lee (BT549, Hs578t). HCC1428 cell line was obtained from Korean Cell Line Bank. The RPE1 cell line was used as a control which maintains a normal chromosome ploidy. RPE1 and the breast cancer cells were cultured at optimal conditions for high proliferation activities and subjected to the FACS analysis to determine the chromosome ploidies of the breast cancer cell lines. Most of the breast cancer cells used in this study had two peaks, 2n and 4n peaks, suggesting that they follow normal chromosome duplication and segregation during the cell cycle (Figure 1a). However, MDA-MB-157 revealed a broad FACS distribution with extra cell populations smaller than 2n and larger than 4n (Figure 1a). This result indicates that the chromosome ploidy of MDA-MB-157 is not tightly maintained during the cell cycle.

I performed immunostaining analysis to determine the number of

centrioles in the breast cancer cell lines. The centrioles were visualized with the centrin-2 antibody. In RPE1 cells, 55% of them had 1 or 2 centrioles and rest of them had 3 or 4 centrioles, suggesting that about a half of the cell population is G1 phase, and the rest were S, G2 and M phase (Figure 1b). This result is consistent with the cell cycle distribution predicted by the FACS analysis (Figure 1a). In fact. The number of centrioles in all the breast cancer cells examined in this study are consistent with the predicted proportion of the cell cycle stages in the FACS analysis (Figure 1a). At the same time, I found that a significant proportion of the breast cancer cells contained supernumerary centrioles. The proportion of cells with supernumerary centrioles may vary depending on the cell lines, so that supernumerary centrioles were detected at 18% of MDA-MB-157 cells and at 6% of Hs578t (Figure 1b). As a control, supernumerary centrioles were detected at 3% of RPE1 cells (Figure 1b).

These results suggest that supernumerary centrioles are common among breast cell cancer cells. Only a subset of breast cancer cells includes supernumerary centrioles while the others include expected numbers of centrioles.

The previous results revealed that a fraction of the breast cancer cells includes supernumerary centrioles. Next, I intended to determine at which stages of the cell cycle supernumerary centrioles are present. The cell cycle

stages of the individual cells were determined by coimmunostaining with the BrdU and cyclin B1 antibodies. The G1 phase cells were not immunostained at all, but the S and G2 phase cells were immunostained with the BrdU and cyclin B1 antibodies, respectively (Figure 2). I occasionally detected cells which were coimmunostained with both the BrdU and cyclin B1 antibodies and considered them as G2 phase cells (data not shown). The M phase cells were distinguished with the presence of condensed chromosomes in the DAPI staining (Figure 2). We were able to use the same secondary antibodies for detection of BrdU and cyclin B1 which are located at the nucleus and cytoplasm, respectively. In this way, I was able to save a secondary antibody for detection of centrioles in the same cell.

I determined the centriole numbers at specific cell cycle stages of the breast cancer cells. Most of the G1 phase RPE1 cells included 1 or 2 centrioles at G1 phase and 3 or 4 at S, G2 and M phases (Figure 3). Supernumerary centrioles were detected at 2-5% of the RPE1 cells of all cell cycle stages (Figure 3). Similarly, the breast cancer cells include 1 or 2 centrioles at G1 phase and 3 or 4 centrioles at S, G2 and M phases. However, supernumerary centrioles were detected in significant proportions of the breast cancer cells. For example, 22-42% of MDA-MB-157 cells included supernumerary centrioles at S, G2 and M phase (Figure 3). Even supernumerary centrioles were detected in

17% of the G1 phase MDA-MB-157 cells (Figure 3). These results confirm that a subset of the breast cancer cells include supernumerary centrioles throughout the cell cycle. The proportion of the cells with supernumerary centrioles are, however, not the same among the breast cancer cells. For example, the proportion of supernumerary centrioles in the Hs578t cells are higher at M phase than the other cell cycle stages (Figure 3).

Generation of supernumerary centrioles at M phase

To have a clue at which stages of the cell cycle supernumerary centrioles are generated, I treated the breast cancer cells with the cell cycle drugs. We used five different cell cycle drugs to arrest the cell cycle at different stages of the cell cycle. Palbociclib which is a CDK4/6 inhibiting agent is known to synchronize the cells at the G1 phase of the cell cycle. Thymidine and aphidicolin are widely used to arrest the cells at the S phase of the cell cycle. RO3306 which is a CDK1 inhibitor mostly arrest the cells at the G2 phase of the cell cycle. STLC, an Eg5 inhibitor, arrests the cells at the prometaphase phase of the cell cycle. The breast cancer cells were treated with the cycle drugs for 24 h and subjected to coimmunostaining analyses to determine centriole numbers at specific stages of the cell cycle.

The results showed that the progression of cell cycle was blocked at

expected points in general (Figure 4). However, we observed a few exceptions. For example, the FACS analysis revealed that palbociclib effectively arrested the cell cycle at G1 phase of all cells except MDA-MB-157 (data not shown). Both aphidicolin and thymidine block cell cycle progression at S phase but the coimmunostaining analyses revealed that many of them are still at G1 phase (Figure 4). RO3306 arrest the cell cycle at G2 phase but it also arrested it at G1 phase (data not shown). Even with such exception, I still managed to determine centriole numbers in the cells arrested at specific stages of the cell cycle. The experiments were repeated three times and the data were summarized in Table 1.

I observed that a significant proportion of MDA-MB-231 (23%), MDA-MB-157 (26%), Bt549 (8%), MCF7 (12%) and HCC1428(15%) already contained >2 centrioles at G1 phase after the palbociclib treatment (Figure 4). In contrast, RPE-1 (4%), MDA-MB-468 (3%), and Hs578t (2%) maintained normal number of centrioles in G1 phase of the cell cycle (Figure 4). These results indicate that some breast cancer cells contain supernumerary centrioles at the G1 phase. Treatment of thymidine and aphidicolin arrested the cell cycle of many breast cancer cells at the S phase. I also observed supernumerary centrioles in the S phase of MDA-MB-231 (15%), MDA-MB-468 (22%), MDA-MB-157 (19%), Bt549 (17%), MCF7 (16%) and HCC1428 (18%), but not of RPE-1 (5%) and hs578t (0%) cells (Figure 4). Supernumerary centrioles

were detected in significant proportions of the breast cancer cells at G2 phase with the RO3306 treatment, such that MDA-MB-231 (18%), MDA-MB-468 (19%), MDA-MB-157 (41%), Hs578t (25%), Bt549 (15%), MCF7 (21%) and HCC1428 (30%) but not much in RPE1 (5%) cells. Finally, I observed the amplified centrioles in the mitotic phase of the cell cycle, which is arrested by STLC. Significant proportion of the breast cancer cells had supernumerary centrioles. The proportion of the breast cancer cells at specific cell cycle stages after the cell cycle drug treatment are comparable to those in the naturally diving cells (Figures 3 and 4). These results revealed that the centriole numbers were not significantly affected by the cell cycle drug treatment.

The previous studies displayed three distinct pattern of abnormal centriole formation in the breast cancer cells: (1) Those in which supernumerary centriole already exist in G1 (MDA-MB-231(23%), MDA-MB-157(26%), Bt549(8%), MCF7(12%) and HCC1428(15%) and newly form centrioles in S phase, (2) those form novel amplified centrioles in S phase (MDA-MB-468), and that initially produce supernumerary centrioles in G2 and further amplify centrioles in Mitotic stage of the cell cycle (Hs579t). These observations suggest that supernumerary centrioles are generated in multiple pathological mechanism.

To have a clue how supernumerary centrioles are generated during the

cell cycle, I determined the centriole numbers in synchronized populations of selected breast cancer cells. The double thymidine block and release method was used to synchronize the cell cycle. The FACS analyses revealed that the cells were properly synchronized, so that most of the cells reached to G2 phase at 9^{th} h and entered to mitosis near 12^{th} h after the release.

I used HeLa cells as a control. Most of the HeLa cells had 1 or 2 centrioles in the beginning, but contained 3, 4 centrioles as soon as they entered S phase (Figure 5). In case of the PLK4-overexpressing HeLa cells, multiple centrioles were detected at S, G2 and M phases, suggesting that centrioles are overamplified at S phase (Figure 5). In case of the $TP53^{del};PCNT^{del};CEP215^{del}$ triple knockout cells, supernumerary centrioles were detected at G2 and M phases, suggesting that centriole amplification occurred at later stages of the cell cycle (Figure 5). Next, I examined the centriole numbers in the synchronized population of Hs578t cells. The results showed that most of the Hs578t cells has 3 or 4 centrioles at S phase, indicating that they normally duplicated the centrioles (Figure 5). However, the proportion of cells with supernumerary centriole increased to 60% of the M phase cells, suggesting that centriole amplification occurs at M phase in the Hs578t cells. In contrast, proportion of the MDA-MB-231 cells with supernumerary centrioles were maintained to 20% throughout the cell cycle, which is similar to the PLK4

overexpressing cells (Figure 5).

Precocious centriole separation of Hs578t cells at M phase

In *PCNT*-deleted Hela cells, centrioles prematurely separated and amplified in early mitosis (Kim et al., 2018). I wondered whether Hs578t also contains prematurely separated centrioles, since they form amplified centrioles at the late phase of the cell cycle. I coimmunostained the cells with Centrin2 and Cep135. CEP135 exist at both mother and newly formed centrioles. One Cep135 is seen as two dots before passage through telophase or early G1: a probable consequence of centriole disengagement during M phase exit (Arquint et al., 2012). Therefore, I used CEP135 as a centriole disengagement marker along with centrin2.

Similar to P53/PCNT/CEP215 KO Hela cell, centrin2 was prematurely separated in over 78.7% of the Hs578t mitotic cell. However, the centrin2 was slightly separated in the Hela cell and Plk4 overexpressing cell line. These results suggest that Hs578t might form supernumerary centrioles due to lack of some Pericentrosomal materials.

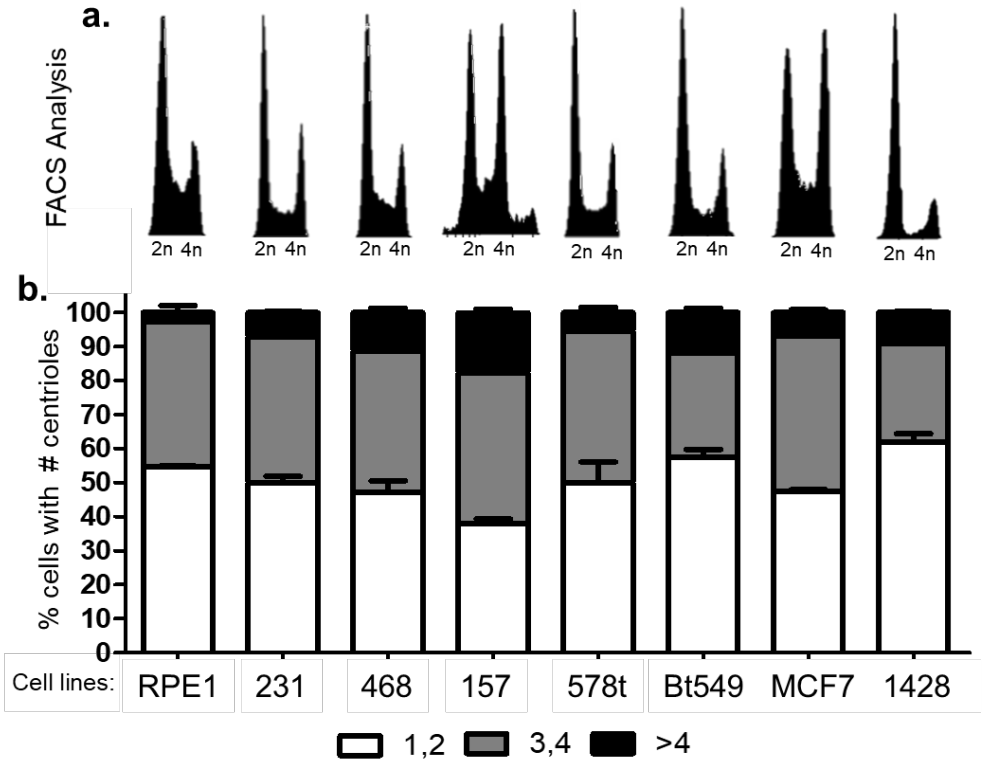

Figure 1. Supernumerary centrioles in the breast cancer cells. (a) RPE1 and the selected breast cancer cells, such as MDA-MB-231, MDA-MB-468, MDA-MB-157, Hs578T, Bt549, MCF7 and HCC1428, were cultured at optimal proliferation conditions and subjected to FACS analysis. (b) The number of centrioles were determined with the centrin-2 signals in the immunostaining analysis. The graphs show the proportion of the cells with indicated numbers of centrioles. Greater than 90 cells per group were analyzed in three independent experiments. Each value is represented as mean and standard deviation.

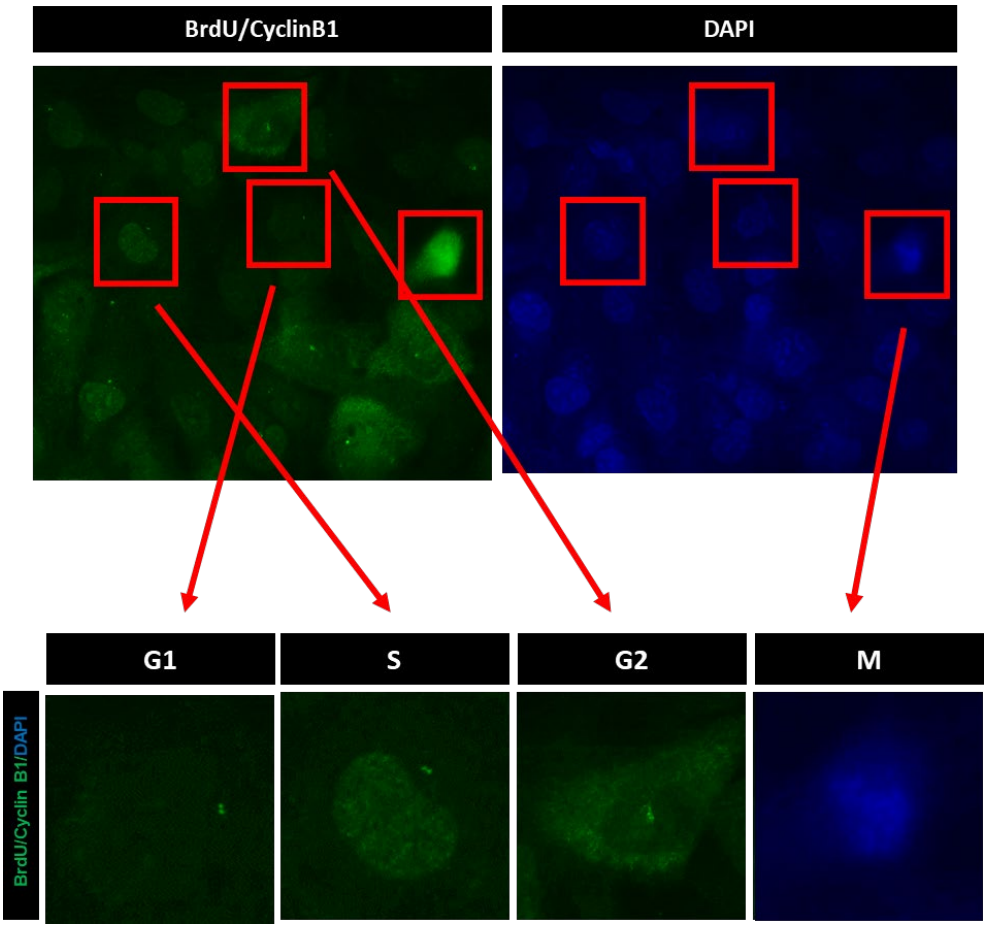

Figure 2. Determination of the cell cycle stages of individual cells. The cells were coimmunostained with antibodies specific to BrdU (green) and cyclin B1 (green). DNA was stained with DAPI (blue). Scale bar, 10 μm. The BrdU and cyclin B1 antibodies immunostained the S and G2 phase cells, respectively. The G1 phase cells were not immunostained with either antibody at all. The M phase cells were determined by condensed chromosomes in the DAPI staining.

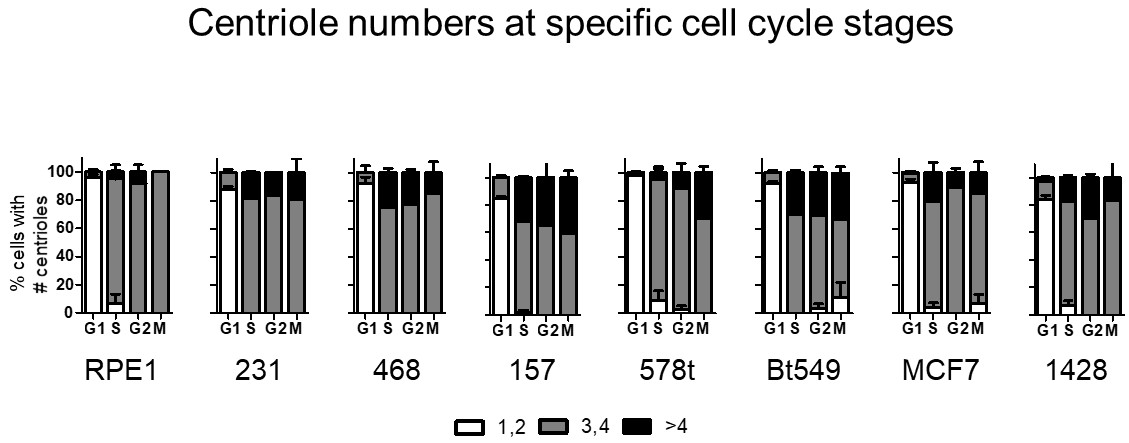

Figure 3. Determination of the number of centrioles at cell cycle stages of the breast cancer cells. To determine the centriole numbers at specific stages of cell cycle, the actively dividing RPE1 and the breast cancer cells were coimmunostained with the antibodies specific to BrdU, Cyclin B1, and centrin-2. DNA was stained with DAPI. The graphs show the proportion of the cells with indicated numbers of centrioles at specific cell cycle stages. Greater than 90 cells per group were analyzed in three independent experiments. Each value is represented as mean and standard deviation

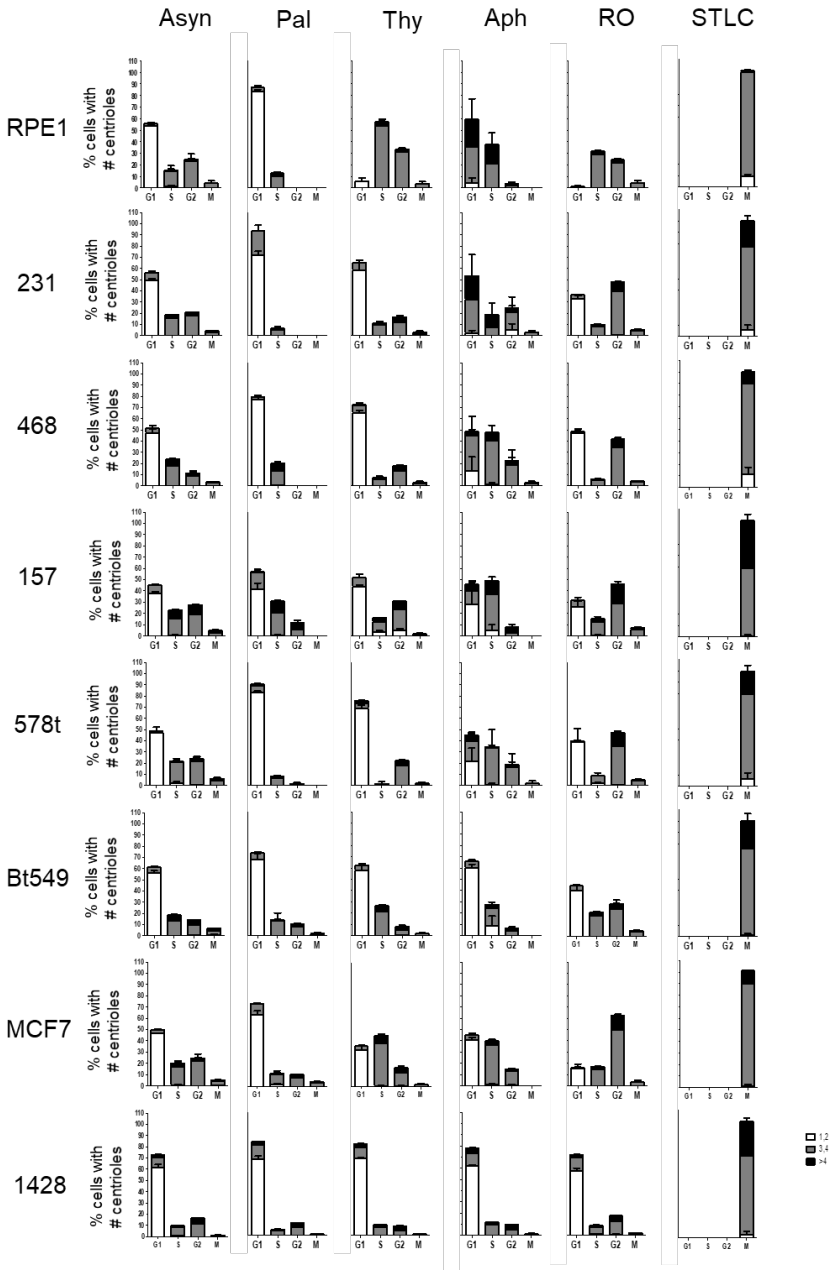

Figure 4. Determination of the number of centrioles in the breast cancer cells after the treatment of the cell cycle drugs The RPE1 and the breast cancer cells were treated with the cell cycle drugs (palbociclib, thymidine, aphidicolin, RO3306, and STLC) for 24 hours and subjected to immunostaining analyses to determine the centriole numbers at specific cell cycle stages. Proportion of the cells with indicated number of centrioles were presented at each cell cycle stage. Greater than 90 cells per group were analyzed in three independent experiments. Each value is represented as mean and standard deviation.

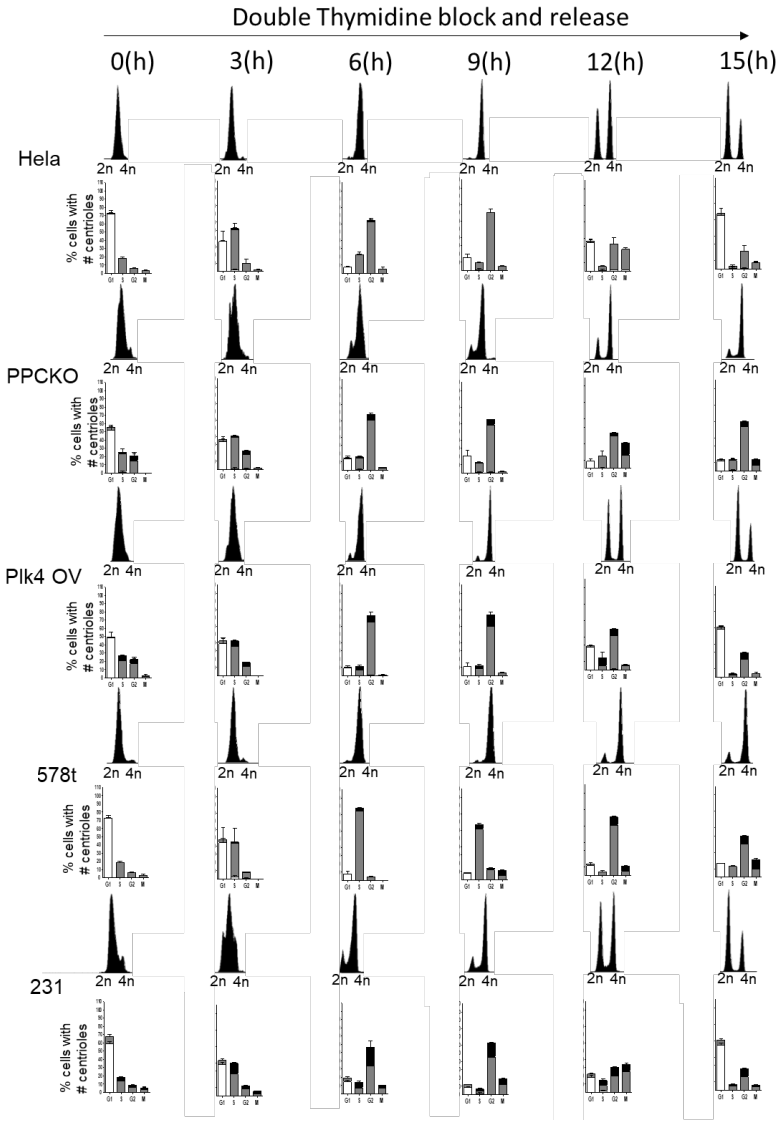

Figure 5. Determination of the number of centrioles in synchronized populations of the breast cancer cells Hela cells (*TP53*^{del}*;PCNT*^{del}*;CEP215*^{del} triple knockout and PLK4-overexpressing cells) and the breast cancer cells (Hs578t and MDA-MB-231) were arrested at G1/S phase with the double thymidine blocks and synchronously released in a fresh medium. The cells were harvested at every 3 h and subjected to FACS analysis to determine cell cycle stages and coimmunostaining analysis to determine the number of centrioles at specific cell cycle stages. Proportion of the cells with indicated number of centrioles were presented at each cell cycle stage. Greater than 90 cells per group were analyzed in three independent experiments. Each value is represented as mean and standard deviation.

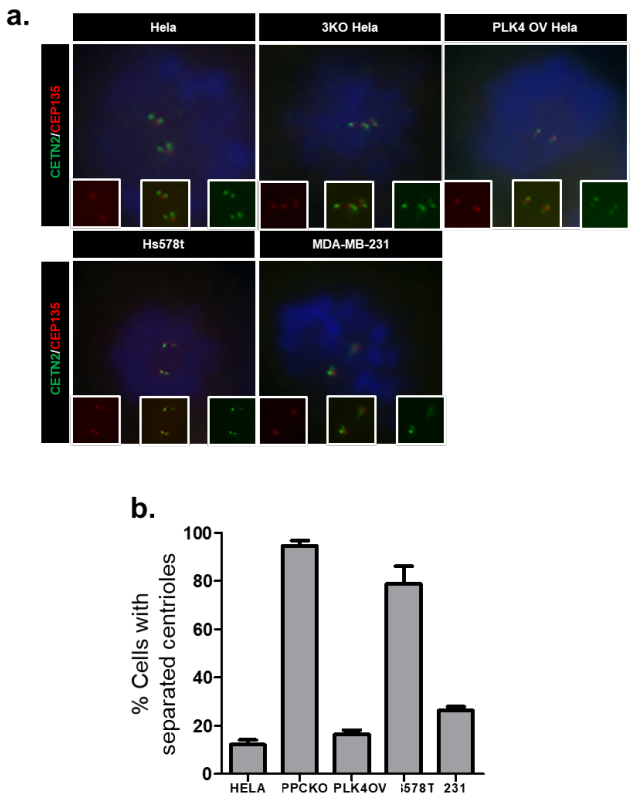

Figure 6. Premature centriole separation at M phase of the breast cancer cells (a) Hela cells (*TP53*^{del}*;PCNT*^{del}*;CEP215*^{del} triple knockout and PLK4-overexpressing cells) and the breast cancer cells (Hs578t and MDA-MB-231) were treated with STLC for 10 h and subject to immunostaining analysis with antibodies specific to centrin-2 (green) and CEP135 (red). DNA was stained with DAPI (blue). Scale bar, 10μm. (b) The number of M phase cells with separated centrioles were counted. Greater than 90 cells per group were analyzed in three independent experiments. Each value is represented as mean and standard deviation.

Table1. The statistical number and percentage of CETN2 dots discovered in cell cycle stages in asynchronous and drug treated breast cancer cell lines.

		Asynchronous (#(%))					Palbociclib					Thymidine					Aphidicolin					RO3306					STLC			
Cells	Stages	1,2	3,4	>4	Total	Total(%)	1,2	3,4	>4	Total	Total(%)	1,2	3,4	>4	Total	Total(%)	1,2	3,4	>4	Total	Total(%)	1,2	3,4	>4	Total	Total(%)	1,2	3,4	>4	Total
RPE1	G1	133(96)	6(4)	0	139(100)	57%	111(96)	5(4)	0(0)	116(100)	87%	8(100)	0	0	8(100)	6%	5(7)	43(62)	21(30)	69(100)	53%	79(99)	1(1)	0	80(100)	41%				
	S	2(4)	40(85)	5(11)	47(100)	19%	0	14(82)	3(18)	17(100)	13%	0	75(95)	4(5)	79(100)	59%	0	28(50)	28(50)	56(100)	43%	0	57(90)	5(10)	62(100)	32%				
	G2	0	53(96)	2(4)	55(100)	22%	0	0	0	0	0%	0	39(95)	2(5)	41(100)	31%	0	4(67)	2(33)	6(100)	5%	0	36(86)	6(14)	42(100)	22%	8(9)	80(90)	1(1)	89(100)
	M	0	4(100)	0	4(100)	2%						0	5(100)	0	5(100)	4%						0	9(100)	0	9(100)	5%				
231	G1	116(87)	17(13)	0	133(100)	56%	76(77)	23(23)	0	99(100)	93%	68(90)	8(11)	0	76(100)	68%	2(3)	46(68)	20(29)	68(100)	44%	62(77)	7(23)	0	69(100)	36%				
	S	0	37(82)	8(18)	45(100)	19%	0	6(86)	1(14)	7(100)	7%	0	11(85)	2(15)	13(100)	12%	0	17(59)	12(41)	29(100)	19%	0	16(75)	3(25)	19(100)	11%				
	G2	0	46(81)	11(19)	57(100)	23%	0	0	0	0	0%	0	16(80)	4(20)	20(100)	18%	5(10)	40(77)	7(13)	52(100)	34%	1(1)	63(81)	14(18)	78(100)	47%	5(5)	68(72)	21(22)	94(100)
	M	0	7(78)	2(22)	9(100)	4%						0	1(50)	1(50)	2(100)	2%	0	4(80)	1(20)	5(100)	3%	0	8(80)	2(20)	10(100)	6%				
468	G1	114(93)	8(7)	0	122(100)	49%	115(97)	3(3)	0	118(100)	69%	77(91)	7(8)	1(1)	85(100)	74%	11(25)	30(68)	3(7)	44(100)	40%	84(97)	3(3)	0	87(100)	50%				
	S	0	44(76)	14(24)	58(100)	23%	1(2)	22(62)	11(36)	34(100)	20%	0	7(78)	2(22)	9(100)	8%	1(2)	37(84)	6(14)	44(100)	40%	0	10(91)	1(9)	11(100)	6%				
	G2	0	45(79)	12(21)	57(100)	23%	0	10(56)	8(44)	18(100)	11%	0	14(78)	4(22)	18(100)	16%	0	15(83)	3(17)	18(100)	17%	0	55(81)	13(19)	68(100)	39%	10(11)	69(78)	9(10)	88(100)
	M	0	10(83)	2(17)	12(100)	5%						0	2(67)	1(33)	3(100)	3%	0	2(67)	1(33)	3(100)	3%	0	6(86)	1(14)	7(100)	4%				
157	G1	96(84)	18(16)	0	114(100)	45%	63(73)	22(26)	1(1)	86(100)	57%	84(87)	13(13)	0	97(100)	52%	32(60)	14(26)	7(13)	53(100)	45%	40(81)	11(19)	0	51(100)	31%				
	S	1(2)	39(66)	19(22)	59(100)	23%	1(2)	30(60)	16(38)	47(100)	31%	7(23)	18(58)	6(19)	31(100)	17%	4(7)	37(67)	14(25)	55(100)	47%	1(1)	21(67)	5(32)	27(100)	17%				
	G2	0	55(66)	28(34)	83(100)	28%	0	9(50)	9(50)	18(100)	12%	9(17)	33(61)	12(22)	54(100)	29%	0	3(33)	6(67)	9(100)	8%	0	42(59)	29(41)	71(100)	44%	1(1)	58(57)	42(42)	101(100)
	M	0	7(58)	5(42)	12(100)	5%						0	3(60)	2(40)	5(100)	3%						0	11(85)	2(15)	13(100)	8%				
578T	G1	101(97)	2(2)	1(1)	104(100)	48%	94(98)	2(2)	0	96(100)	91%	87(92)	5(5)	3(3)	95(100)	76%	20(42)	23(48)	5(10)	48(100)	43%	58(97)	2(3)	0	60(100)	38%				
	S	5(15)	40(82)	1(3)	46(100)	21%	0	7(90)	1(10)	8(100)	8%	0	2(100)	0	2(100)	2%	1(2)	43(93)	2(4)	46(100)	41%	3(12)	12(88)	0	15(100)	10%				
	G2	1(2)	47(87)	6(11)	54(100)	25%	0	2(100)	0	2(100)	2%	0	22(85)	4(15)	26(100)	21%	0	12(86)	2(14)	14(100)	13%	1(1)	54(74)	18(25)	73(100)	46%	6(7)	66(73)	18(20)	90(100)
	M	0	8(67)	4(33)	12(100)	6%						0	1(50)	1(50)	2(100)	2%	0	3(100)	0	3(100)	3%	0	7(78)	2(22)	9(100)	6%				
BT549	G1	105(91)	9(9)	0	114(100)	61%	98(92)	9(8)	0	107(100)	70%	151(92)	12(7)	1(1)	164(100)	63%	122(92)	11(8)	0	133(100)	64%	124(91)	13(10)	0	137(100)	49%				
	S	0	24(67)	10(33)	34(100)	18%	0	20(95)	1(5)	21(100)	14%	0	59(83)	12(17)	71(100)	27%	13(23)	35(63)	8(14)	56(100)	27%	0	56(85)	10(15)	66(100)	24%				
	G2	1(4)	17(65)	8(31)	26(100)	14%	0	11(79)	3(21)	14(100)	11%	1(5)	13(59)	8(4)	22(100)	8%	0	12(75)	4(25)	16(100)	8%	0	53(85)	9(15)	62(100)	22%	1(1)	56(76)	17(23)	74(100)
	M	1(8)	7(58)	4(33)	12(100)	6%	0	5(71)	2(29)	7(100)	5%	0	4(80)	1(20)	5(100)	2%	0	1(50)	1(50)	2(100)	1%	1(7)	10(71)	3(21)	14(100)	5%				
MCF7	G1	107(92)	8(7)	1(1)	116(100)	48%	94(87)	13(12)	1(1)	108(100)	74%	72(90)	8(10)	0	80(100)	36%	83(90)	9(10)	0	92(100)	45%	23(96)	1(4)	0	24(100)	15%				
	S	2(4)	41(80)	8(16)	51(100)	21%	2(12)	13(77)	2(12)	17(100)	12%	1(1)	86(84)	16(16)	103(100)	47%	2(3)	72(89)	7(9)	81(100)	40%	0	39(91)	4(9)	43(100)	26%				
	G2	0	57(90)	7(11)	64(100)	26%	0	11(85)	2(15)	13(100)	10%	1(3)	23(68)	10(29)	34(100)	15%	1(4)	22(85)	3(12)	26(100)	13%	1(1)	72(78)	19(21)	92(100)	56%	0	102(89)	13(11)	115(100)
	M	1(8)	9(75)	2(17)	12(100)	5%	0	5(83)	1(17)	6(100)	4%	0	3(100)	0	3(100)	1%	0	3(75)	1(25)	4(100)	2%	1(20)	4(80)	0	5(100)	3%				
HCC1428	G1	248(84)	37(13)	9(3)	294(100)	72%	241(82)	45(15)	9(3)	295(100)	82%	249(84)	36(12)	12(4)	297(100)	77%	243(80)	47(15)	15(5)	305(100)	79%	227(80)	45(16)	11(4)	283(100)	73%				
	S	3(8)	30(75)	7(18)	40(100)	10%	0	15(75)	5(25)	20(100)	6%	0	27(82)	6(18)	33(100)	12%	0	36(80)	9(20)	45(100)	12%	3(9)	27(77)	5(14)	35(100)	9%				
	G2	0	47(70)	20(30)	67(100)	17%	0	28(80)	7(20)	35(100)	11%	0	13(52)	12(48)	25(100)	7%	0	18(56)	14(44)	32(100)	8%	2(3)	41(67)	18(30)	61(100)	16%	2(3)	49(70)	21(30)	72(100)
	M	0	4(80)	1(20)	5(100)	1%	0	2(50)	2(50)	4(100)	1%	0	2(50)	2(50)	4(100)	1%	0	3(50)	3(50)	6(100)	2%	0	4(57)	3(43)	7(100)	2%				

Table 2. The statistical number and percentage of CETN2 dots discovered in cell cycle stages after the double thymidine block and release.

		0				3				6				9				12				15			
Cells	Stages	1,2	3,4	>4	Total	1,2	3,4	>4	Total	1,2	3,4	>4	Total	1,2	3,4	>4	Total	1,2	3,4	>4	Total	1,2	3,4	>4	Total
Hela	G1	31±0	0.3±1.2	0	31.3±0.3	22±13	0	0.3±0.3	22.7±13	3.3±0.2	0	0	3.3±0.2	7±2	0	0	7±2	16±4	1±1	0	21±5	30±2	1±0	0	31±2
	S	0	8±0.3	0	8±0.3	0.3±1.2	27.3±0.8	0.7±0.6	28.3±2.4	0	10±2.7	0.3±1.2	10±2.8	0	4±1	0	4±1	0	3±1	0	3±1	0	1±0	0	1±1
	G2	0	2.7±1.2	0	2.7±1.2	0	5.3±1.9	0	2.3±1.5	0	28±5.3	0	29±5.5	0	32±1	0	32±1	1±0	19±4	0	19±5	0	10±4	0	10±4
	M	0	1.3±0.6	0	1.3±0.6	0	1.3±1.5	0	1.3±0.6	0	1.3±0	0	2.3±0.3	0	2±0	0	2±0	1±0	15±2	0	16±2	0	3±1	0	4±1
ppcko	G1	32±3	2±1	0	34±3	23±	2±0	0	24±2	8±2	1±0	0	8±2	14±6	0	0	14±6	5±3	0	0	5±3	6±2	1±0	0	7±2
	S	0	13±2	1±0	14±2	0	27±4	1±0	27±4	0	8±0	0	8±0	0	7±1	0	8±1	0	9±5	0	9±5	0	6±1	0	6±2
	G2	0	12±8	5±4	17±12	0.3±	13±6	3±1	16±6	0	34±1	5±2	39±2	0	31±4	7±4	38±1	0	18±2	4±0	23±2	0	24±2	4±0	28±2
	M	0	0	0	0	0	1±1	0	2±1	0	1±1	0	2±1	0	1±1	0	1±1	0	13±1	5±1	17±2	0	5±1	5±1	7±1
plk4	G1	26±2	1±0	0	27±2	24±5	2±2	0	26±3	5±1	0	0	5±1	7±4	0	0	7±4	20±2	1±0	0	22±2	29±2	1±1	0	31±2
	S	0	11±0	3±0	15±0	0	22±1	4±1	26±1	0	4±1	1±0	6±1	0	6±4	2±2	8±5	0	3±2	1±1	4±0	0	1±1	1±0	2±1
	G2	0	10±6	3±1	13±7	0	7±2	3±0	10±2	0	40±7	9±2	49±9	0	35±5	5±4	27±12	0	30±1	6±1	36±0	0	11±1	4±1	15±1
	M	0	1±0	0	1±0	0	0	0	0	0	1±1	0	1±1	0	2±1	0	2±1	0	2±0	1±0	3±0	0	2±1	0	2±1
578T	G1	27±1	8±4	0	31±1	24±10	2±	0	26±11	3±1	0	0	3±1	4	0	0	4±1	6±1	1±1	0	7±1	9±1	0	0	9±1
	S	0	0	0	0	1±1	21±1	1	23±10	0	30±1	1	32±1	0	31±2	3±1	34±3	0	2±1	0	2±	0	7±1	0	7±1
	G2	0	0	0	0	0	4±10	0	3	0	2	0	2	0	6±0	1±0	7±1	0	32±2	5±1	37±2	0	22±0	5±1	28
	M	0	0	0	0	0	0	0	0	0	0	0	0	0	2±1	2±1	4±2	0	3±1	3±0	6±1	0	5±0	6±1	12±1

DISCUSSION

In this report, I analyzed numerical variation of breast cancer cell lines. Centriole abnormalities were commonly discovered in breast cancer cells. Centrosome amplification was widespread in cancer, and each cell line displayed different proportion of subpopulation of supernumerary centrioles. My follow up studies showed that in all cell cycle stages, supernumerary centrioles existed. Surprisingly, each breast cancer cell lines differed in the way supernumerary centrioles are structured. Largely, the breast cancer cells that I studied showed three possible scenarios for centrosome formation. (1) Those that already contains supernumerary centrioles at the G1 phase of the cell cycle; The possible explanation to this phenomenon is, it is likely that amplified centrioles that are present in previous cycle remains in next cell cycle. (Holland and Cleveland, 2009) This might be because it is reported that cancer cell lines are able to cope with centrosomal abnormalities through spindle pole clustering, each pole with more than two centrioles or centrosomes. The centrioles in each pole might divide and undergo further round of cell cycle. (Kramer *et al*., 2011) (2) The other increased its centriole at S phase of the cell cycle. This might be because it is unveiled that overexpressed PLK4 is prevalent in cancer. The

hyperactivity and overexpression of PLK4 might facilitate the formation of multiple procentrioles. (Habedanck *et al*., 2005; Kleylein-Sohn *et al*., 2007; McCoy *et al.*, 2015). And of the 7 groups of breast cancer cell line, (3) Hs578t initially formed amplified centrioles at the G2 phase of the cell cycle and further amplify centrioles at the mitotic stage of the cell cycle. This provided the novel possibility that centrosome amplification not happens only in S phase but in other stages of the cell cycle. My experiment revealed that various pathways can be the derivative of supernumerary centriole formation.

In the previous finding, they revealed that lack of PCNT induces precocious disengagement of centrioles and contributes to formation of multiple centrioles (Kim *et al*., 2019). They generated PCNT KO Hela cell lines as the experimental model to prove this hypothesis. They explained that the deficiency of Pericentrosomal materials might induce early separation of mother and daughter centrioles leading to reduplication of centrioles. Furthermore, the depletion of another pericentrosomal material CEP215, aggravated the formation of supernumerary centrioles, which put on weight to the hypothesis. (Jung., 2020) Therefore, a possible explanation of late supernumerary centriole formation present in Hs578t cell line might be the lack of some pericentrosomal materials. In my study, Hs578t had significant number of prematurely separated centrioles corresponding to P53/PCNT/CEP215 KO Hela cell line.

However, it remains to be unveiled whether any pericentrosomal materials related proteins are depleted in Hs578t cell lines. It would be interesting to test what abnormalities and deficiency in Pericentrosomal materials are discovered, and how this contributes to premature centriole disengagement in Hs578t cell line. In addition, I also need to validate whether the number (3) phenotype is prevalent in other cancer cell line. Further studies might provide better understanding in correlation of supernumerary centriole formation and early centriole disengagement in cancer cell model.

Currently, I do yet know why all cancer cell line show different patterns of supernumerary centriole production. To uncover the reason, I need to measure the proteinous level of supernumerary centriole generating factors. Many approaches have been done in the former studies to discover supernumerary centrioles forming candidate. The studies reported the level of PLK4 mRNA expression in breast cancer cells. (Mason *et al*., 2014; Lei *et al*., 2019) Surprising in these data, the level of PLK4 mRNA level of Hs578t cell line was relatively low compared to other cancer cell lines. If this case is true, I can exclude the possibility that Hs578t gains amplified centrioles due to overexpression PLK4 mRNA level. Moreover, I consider it might be interesting to deduce the origin of supernumerary centrioles by thoroughly observing centrosomal structures. In one study, the de novo centriole did not contain any

proximal and procentriole markers, whereas overduplicated centrioles contained multiple SAS-6, possibly because of presence of multiple daughter centrioles. (Balestra *et al*., 2021; Marteli *et al*., 2017) In addition, CDK1 inhibitor RO3306 induced PLK1-dependent centriole reduplication (Loncarek *et al*., 2010). Last but not least, multiple centrioles originated by fragmentation of elongated mother centrioles maintained multiple CP110 as well as small number of elongated centrioles. (Marteil *et al*., 2018)

I believe that studying the change in number of supernumerary centrioles according to different cell cycle described in this study has several benefits compared to drug induced cell cycle arrests. Discriminating cell cycle by antibody markers did not hinder the normal proliferation of cancer cell line. Since it is important that any chemical do not perturb the phenotype of cancer cell line, minimizing the drug effects was imperative. Altogether, my work demonstrates that centrosome amplification is widespread in cancer. My survey with publicly available data is invaluable tools for identifying molecular mechanisms of incontrollable homeostasis of centriole number. I want to expand the characterization of cancer cell lines to explore the question of origin of cancer cells with abnormal centrosome number. In the future, I look forward for the scientific community to pursuit novel clinical and diagnostic tools in cancer research.

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국문초록

중심체는 동물세포에만 존재하는 세포내 소기관으로, 미세소관의 구심점 역할을 한다. 중심체는 세포당 2개가 존재하여 세포분열시 염색체를 두 딸세포로 끌고 오는 역할을 담당한다. 하지만 일부의 암세포에서는 다수의 중심체가 발견되며, 그 결과 세포분열이 정지되거나, 염색체 일부를 유실 혹은 복제되는 결함을 야기한다. 본 연구에서 저자는 유방암 세포주들을 대상으로 중심체 수의 이상을 분석하였다. 개별 세포의 세포주기를 BrdU와 CyclinB1 항체를 이용한 면역화학염색기법으로 확인하고, 중심립의 수는 centrin-2로 세었다. 그 결과, 분석한 7종류의 유방암 세포주 모두에서 과다한 중심립의 수를 보유한 세포들을 관찰하였다. 과다한 중심립의 수를 보유한 세포의 비율은 세포주마다 차이가 있었다. 그리고 이는 모든 세포주기에서 비슷한 비율로 관찰되었다. 하지만 Hs578t 세포주의 경우, S 기의 5%, M기의 50% 세포에서 중심립이 과다하게 형성됨이 관찰되었다. 이는 Hs578t 세포에서는 중심립의 복제가 M기에서 일어날 수 있음을 암시한다. 본 실험실에서는 이전 연구에서 중심구 단백질이 결핍된 세포에서는 M 기에서 중심립 쌍이 조기에 이격되고, 중심립이 합성될 수 있을 가능성을 제시하였다.

실제로 Hs578t 세포주의 경우 중심립 이격이 M기에 관찰되었다. 결론적으로 본 연구는 일반 암세포에서도 중심립이 M기에 합성될 수 있음을 시사한다.