이학박사학위논문

TP53, PCNT 그리고 *CEP215* 결핍 세포주에서 과복제 중심립의 연구

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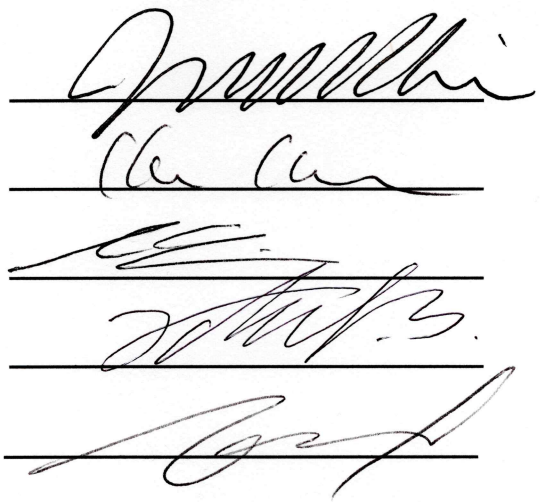

ABSTRACT

Studies on supernumerary centrioles in *TP53*, *PCNT* and *CEP215* triple knockout cells

Gee In Jung

The centrosome is a subcellular organelle that functions as a major microtubule organizing center in most animal cells. It is composed of centrioles and surrounding pericentriolar material (PCM). During mitosis, centrosomes function as spindle poles to pull a set of chromosomes into daughter cells, and abnormality in centrosome numbers leads to spindle pole disorder. Therefore, the centriole number has to be tightly regulated during the cell cycle for successful cell division. In fact, centrosome amplification is often observed in many cancer cells. In my dissertation, I generated HeLa cell lines in which the *TP53*, *PCNT* and *CEP215* genes are deleted and observed the phenotypes related to centriole behavior during the cell cycle.

In chapter I, I observed centriolar phenotypes in the *CEP215* deleted cells. CEP215 is a major PCM protein that recruits the γ-tubulin ring complex for

microtubule organization. In my dissertation research, I observed that daughter centrioles were prematurely separated from the mother centrioles in *CEP215* knockout cells. I also generated *TP53*, *PCNT* and *CEP215* triple knockout cells and observed centriole amplification as well as precocious centriole separation. Based on the observations, I propose that CEP215 is involved in maintaining the mother and daughter centriole association during mitosis.

In chapter II, I studied centriolar phenotypes in the *TP53*, *PCNT* and *CEP215* triple knockout cells. I observed the unscheduled amplification of centrioles in the triple knockout cells during mitosis. The amplified centrioles lack the ability to function as the template for centriole assembly during the subsequent S phase. They also lack the ability to organize microtubules. Nonetheless, I do not rule out the possibility that the amplified centrioles may occasionally disturb bipolar spindle pole formation during mitosis. My works propose a novel mechanism by which supernumerary centrioles are generated in the cells depleted of PCM in the mitotic centrosomes.

Key Words: Centrosome, Centriole, CEP215, PCNT, PCM

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PURPOSE

Background

1. Centrosome

Edouard Van Beneden found the centrosome in 1883, and Theodor Boveri ensured its existence in 1888 (Scheer, 2014). It does not exist in plant cells but exists only in animal cells (Scheer, 2014). The structure of the centrosome was revealed with the advance of microscopy (Fig. 1A). The centrosome is usually located between the nucleus and Golgi and comprises centrioles and pericentriolar material (PCM). The centrosome is one of the significant subcellular organelles like mitochondria and Golgi, but unlike them, PCM is not surrounded by the membrane.

1.1 Centriole

Centriole is composed of nine microtubule triplets in a cylindrical structure (Gonczy, 2012) (Fig. 1). The centriole is typed with three; mother centriole, daughter centriole and procentriole. Mother centriole is a mature centriole with distal appendages, and it can produce cilia (Fig. 1). The daughter centriole is a semi-mature centriole, which can make procentrioles but does not

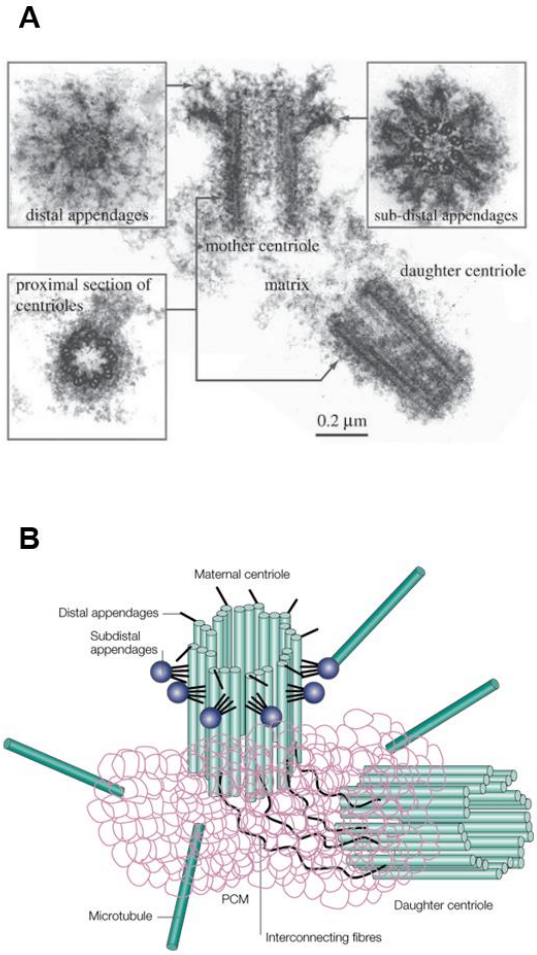


Figure 1. Structure of centrosome

(A) Electron micrographs of centrioles (Winey and O'Toole, 2014). The mother centriole has distal and sub-distal appendages. Centriole has a cartwheel structure, which is composed with nine triplets of microtubules. (B) Structure of centrosome (Stearns, 2004). The centrosome is composed of centrioles and pericentriolar material (PCM). Centrosomes are linked with interconnecting fibres.

have distal appendages during interphase (Fig. 1). Mother and daughter centrioles usually grow up to 500 nm (Gonczy, 2012). When the mother and daughter centrioles enter the S phase, procentrioles start to grow next at a perpendicular angle. They grow up to 250 nm and continue to grow until the next cell cycle (Kong et al., 2020).

1.2 Pericentriolar material (PCM)

The pericentriolar material (PCM) is a protein complex that surrounds centrioles (Lawo et al., 2012) (Fig. 1). It is composed of various proteins such as pericentrin (PCNT), CDK5RAP2 (CEP215), CEP192, CEP152 and γ-tubulin (Fig. 2). The primary function of PCM is to recruit γ-tubulin, and it allows the centrosome to act as a major microtubule organizing center (MTOC). PCM changes its size throughout the cell cycle. During the interphase, PCM forms a toroidal structure around the centrioles and holds two centrioles tightly (Lawo et al., 2012; Mennella et al., 2012) (Fig. 2). During the mitosis, PCM expands its size in a cloud shape and the interaction between PCNT and CEP215 is essential in this phenomenon (Kim and Rhee, 2014) (Fig. 3).

1.3 Function of centrosome

The centrosome's primary function is to act as a microtubule organizing

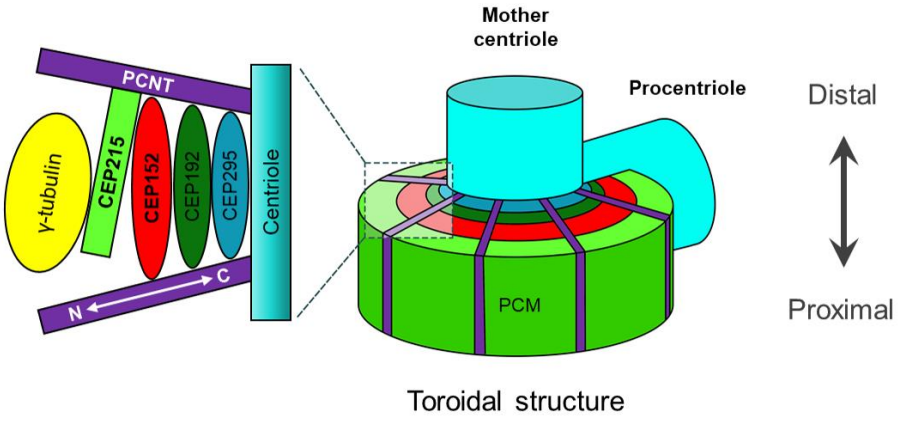


Figure 2. Toroidal structure of PCM during interphase

PCM forms a toroidal structure during interphase. PCNT works as a cornerstone of interphase PCM with its C-term targeting the wall of the centriole and N-term extending out. PCM components necessary in centriole assemblies such as CEP290, CEP192, and CEP152 are found close to the wall of the mother centriole. In contrast, PCM proteins involved in microtubule nucleation such as CEP215 and γ-tubulin are located in the outer layers of PCM. Interphase PCM follows a highly organized toroidal pattern, surrounding mother and procentrioles with different diameters (modified from Lawo et al., and Luders, 2012).

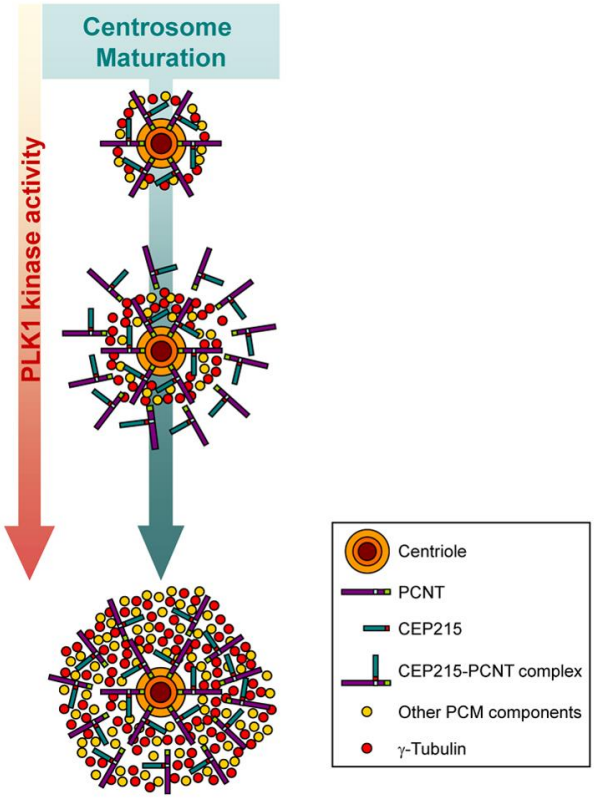


Figure 3. Centrosome maturation during mitosis

PCM structure is different from interphase to mitosis. Massive amount of proteins cluster to the centrosome when the cell enters mitosis and it is called centrosome maturation (also known as PCM expansion). It is important in proper cell division. PLK1 kinase activity and the interaction of PCNT and CEP215 are important in this phenomenon. The extended mitotic matrix is formed with PCNT, CEP215, γ-TuRC and various other PCM components. Mitosis PCM is no longer in an ordered structure, but disorganized and cloudlike shape (Kim and Rhee, 2014).

center and to make primary cilia. During the interphase, centrosome forms microtubule network and control the transportation of intercellular material, maintain the cell shape and manage cell to cell migration (Azimzadeh and Bornens, 2007) (Fig. 4A). During mitosis, it acts as a spindle pole body, forms a massive amount of microtubules, and divides the cell into two with an equal amount of chromosomes (Wang et al., 2014) (Fig. 4B). When the cell enters the G0 phase, it forms primary cilia (Avidor-Reiss and Gopalakrishnan, 2013) and acts as a sensory cellular antenna that coordinates several cellular signaling pathways (Nachury, 2014) (Fig. 4C). A massive amount of γ-tubulin ring complex (γ-TuRC) exists around the PCM and this γ-TuRC is the template for the microtubule (Kollman et al., 2011). By this, the centrosome can act as a major microtubule organizing center.

2. Centrosome cycle

The centrosome is duplicated once and only once per cell cycle (Stearns, 2004). It is crucial to maintain the number of centrosomes because the abnormality in centrosome numbers can cause genomic instability (Luders, 2012; Winey and O'Toole, 2014). Similar to DNA, the centrosome duplicates during the S phase and segregates at the mitotic exit. The centrosome duplication cycle

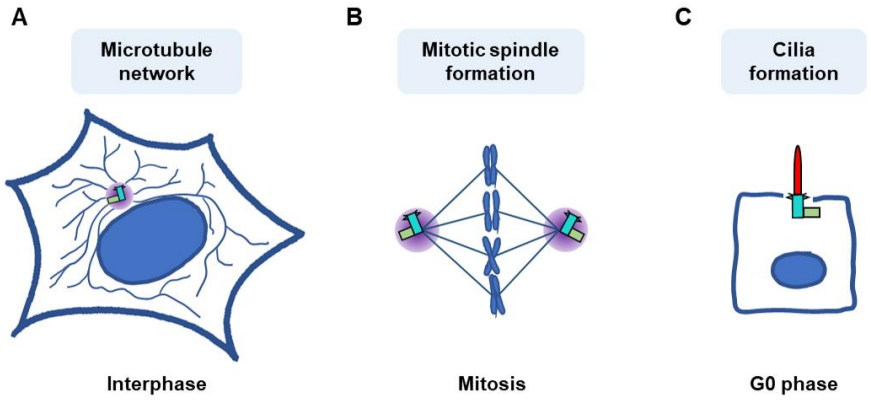


Figure 4. Function of centrosome

(A) Centrosome is important in organizing microtubule network during interphase. (B) The centrosome is important in mitotic spindle formation during mitosis. (C) The centrosome is important in cilia formation when the cell enters the G0 phase (modified from Fukasawa, 2007; Nigg and Raff, 2007).

is closely linked with the cell cycle, and it can be divided into six stages: centriole-to-centrosome conversion, centriole duplication initiation, procentriole elongation, centrosome maturation and separation, bipolar spindle assembly and lastly, centriole disengagement and separation (Fig. 5).

2.1 Centriole-to-centrosome conversion

During the G1 phase, the centriole that has been assembled previous cell cycle must convert into the centrosome, to produce a new procentriole. This phenomenon is called a centriole-to-centrosome conversion. Centriole-to-centrosome conversion requires sequential loading of CEP135, CEP295, CEP192 and CEP152 onto the procentriole (Wang et al., 2011). Then it becomes daughter centriole.

2.2 Centriole duplication initiation

When the cell enters the S phase, PLK4 concentrates on a single spot on the wall of the mother and daughter centrioles (Fukasawa, 2007). If it does not focus on a single spot, multiple centrioles are formed (Coelho et al., 2015). SAS6 is recruited on the concentrated spot of PLK4 and the formation of procentrioles starts (Dzhindzhev et al., 2014; Wang et al., 2014). Duplication of another centriole is prevented when the procentriole is closely attached next to the mother

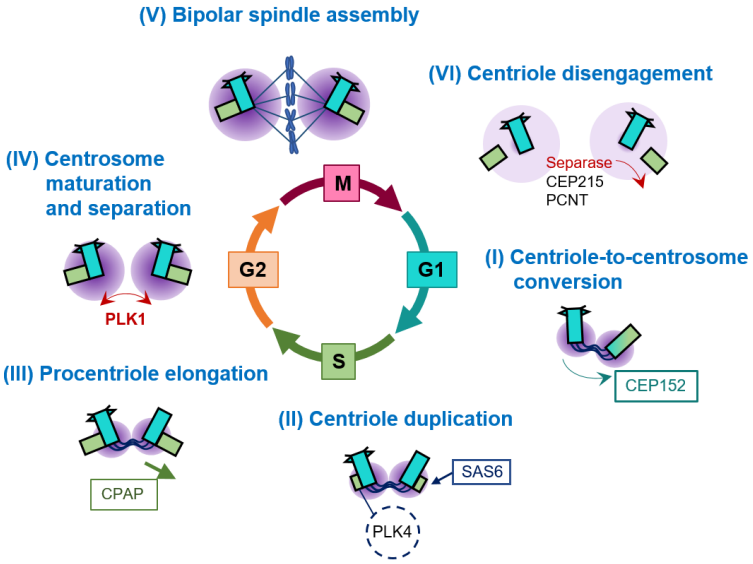


Figure 5. Centrosome duplication cycle

Centrosome duplication is similar to DNA synthesizes. The duplication cycle can be divided into six stages: (I) Centriole-to-centrosome conversion must happen during G1 phase to initiate the duplication for new procentrioles, and CEP152 is commonly refered as the indicator. (II) Centriole duplication start during S phase, and PLK4 is known as the master regulator for centriole duplication and it recruits SAS6, which is cartwheel protein. (III) Procentriole elongation happens during S to G2 phase, and CPAP is important in this phenomenon. (IV) Centrosome maturation and separation takes place at the end of G2 phase and early mitosis. PLK1 is responsible in centrosome separation. (V) Bipolar spindle assembly is the main event during mitosis. It is important to segregate chromosomes equally to each daughter cells. (VI) Centriole disengagement happens at the mitotis exit and the removal of CEP215 and PCNT is important. PCNT is cleaved by separase. (modified from Wang et al.) 2014).

centriole (Nigg and Raff, 2009; Saunders, 2005).

2.3 Procentriole elongation

The procentriole starts to elongate after the initiation. CPAP and CP110 are the two major proteins in this step (Cuomo et al., 2008). CPAP is essential in the accumulation of centriolar tubulins, and CP110 does the opposite (Cuomo et al., 2008). The procentriole elongates to about 250 nm and continues until the next two consecutive cell cycles (Kong et al., 2020). Centriole over elongation can lead to fragmentation, and it can lead to centriole amplification (Marteil et al., 2018; Sieben et al., 2018).

2.4 Centrosome maturation and separation

Centrosome maturation is the accumulation of γ-tubulin ring complexes and other PCM proteins at G2/M transition. At the onset of mitosis, PLK1 phosphorylates PCNT to initiate the centrosome maturation (Lee and Rhee, 2011), and the daughter centriole gains distal appendages and then becomes a young mother centriole. The interaction between PCNT and CEP215 is important in the enlargement of PCM size (Kim and Rhee, 2014). Centrosome maturation allows the centrosome to nucleate a massive amount of microtubules, enabling the cell to pass mitosis properly.

While the centrosomes enlarge their sizes, they also have to separate from each other to migrate to the opposite sides of the cell to form a bipolar spindle pole. It is called centrosome separation. During the interphase, two centrosomes are connected with linker proteins such as C-Nap1 and rootletin, and they are removed by Nek2 when the cell enters mitosis (Vitre et al., 2015). At the end of the G2 phase and during early mitosis, the degradation of CEP68 is initiated by PLK1 phosphorylation (Pagan et al., 2015) (Fig. 6). This degradation of CEP68 allows the removal of CEP215 and initiates centrosome separation (Pagan et al., 2015) (Fig. 6).

2.5 Bipolar spindle formation

After the maturation and separation, the centrosomes have to form microtubule asters (Denu et al., 2020). The asters nucleate spindle fibers, they bind to chromosomes and chromosome segregation starts. It is essential to form a proper bipolar spindle pole because multipole can cause chromosome segregation error and monopole can cause cytokinesis failure (Luders, 2012).

2.6 Centriole disengagement and separation

Centriole disengagement and separation is a different phenomenon from centrosome separation. At the mitotic exit, PLK1 mediates PCNT cleavage and

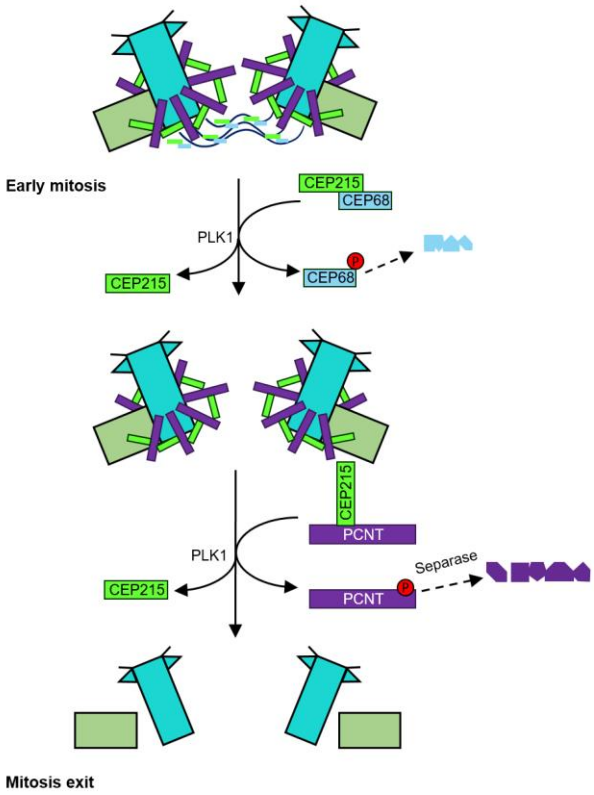


Figure 6. Importance of the disperse of CEP215 during mitosis

In early mitosis, PLK1 phosphorylation of CEP68 allows degradation of CEP68 with CEP215, leading to centrosome separation. In the mitotic exit, degradation of PCNT with separase along with CEP215 allows centriole separation. Centrosome separation and centriole separation are different phenomenon. Centrosome separation allows centrosomes to migrate to each end of the cell, and it forms monopolar spindle pole if it fails. Centriole separation allows centriole to convert into centrosome, and without this, centriole duplication is prevented. (Modified from Fry, 2015).

its cleavage allows removing CEP215 from the PCM (Pagan et al., 2015) (Fig. 6). Then the procentriole disengages from mother centriole and can become daughter centriole. Centriole disengagement has to take place for the conversion to happen. Centriole disengagement allows mother centriole to regain its ability to produce new centriole, so it's called a licensing mechanism for centriole duplication (Wong and Stearns, 2003). It was recently revealed that the deletion of *PCNT* could cause precocious centriole disengagement in early mitosis (Kim et al., 2019) (Fig. 7).

There are two models for centriole disengagement that have been proposed previously. One is that there is "glue protein" such as cohesin holding two centrioles together (Schockel et al., 2011). The other is that there is no specific protein that is holding the two centrioles together, but the PCM (Cabral et al., 2013; Lee and Rhee, 2012; Matsuo et al., 2012).

3. PCM proteins

PCM is consist of various proteins, such as γ-tubulin, PCNT, CEP215, and CEP192. The improvement in the super resolution microscopies revealed that PCM is a highly organized toroidal structure during interphase (Castellanos et al.,

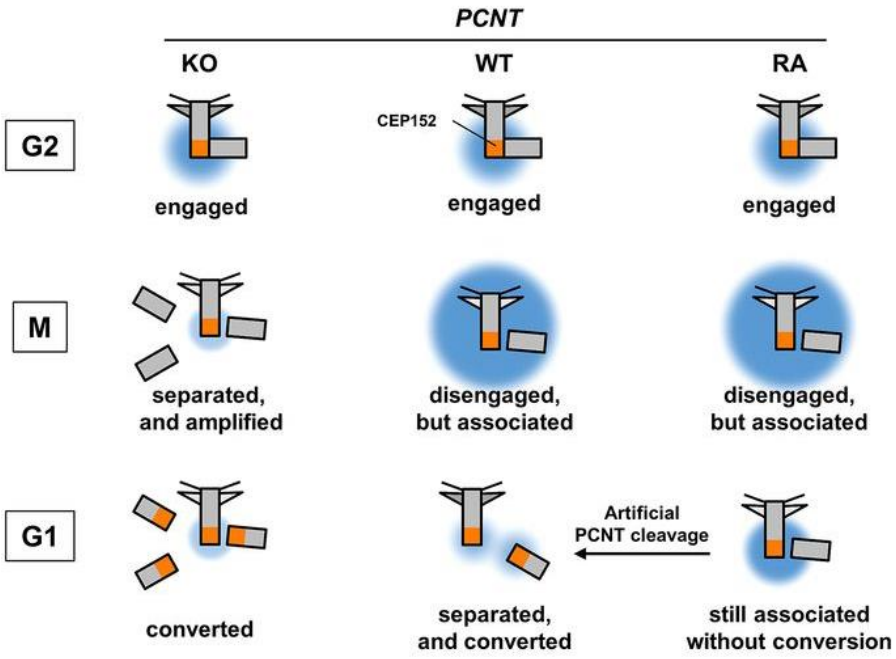


Figure 7. Importance of PCNT in centriole engagement

Deletion of *PCNT* leads to precocious centriole disengagement and over duplication in early mitosis. However, it does not cause centriole disengagement during interphase. The WT cells are disengaged during mitosis but remain associated with PCNT. The centrioles are separated and converted at the end of mitosis. The RA mutant is a non-degradable form of PCNT so the centriole separation is prevented at the mitotic exit. These associated procentrioles fail to convert into mother centrioles during the G1 phase. When this mutant is artificially cleaved, centriole-to-centrosome conversion then takes place (Kim et al., 2019).

2008; Fu and Glover, 2012; Lawo et al., 2012). Then it expands its size during mitosis as an amorphous mass of proteins.

3.1 Pericentrin (PCNT)

Pericentrin (PCNT) is an evolutionary conserved PCM protein, and is crucial in microtubule organization (Dictenberg et al., 1998). PCNT acts as the cornerstone of the toroidal structure during interphase (Lawo et al., 2012), and it is the key protein in centrosome maturation during mitosis (Lee and Rhee, 2011) (Fig. 3). PCNT controls spindle organization and mitotic entry (Martinez-Campos et al., 2004). It is also important in bipolar spindle formation and orientation (Chen et al., 2014; Richens et al., 2015). At the end of the mitosis, separase-mediated PCNT cleavage is controlled by PLK1, and it is the critical step for the centriole separation (Lee and Rhee, 2011) (Fig. 7). PCNT is also important in proper ciliogenesis (Li et al., 1998; Megraw et al., 1999; Vaizel-Ohayon and Schejter, 1999).

3.2 CDK5RAP2 (CEP215)

CDK5RAP2 (CEP215) also is a conserved protein throughout yeast to human. Mutation in CEP215 can cause microcephaly, and its depletion can cause a decrease in recruitment in centrosomal γ-tubulin and leads to a problem in bipolar spindle formation (Fong et al., 2008; Hertwig, 1942; Lee and Rhee, 2011). CEP215

is the key factor in recruiting γ-tubulin to the centrosome, and interaction with PCNT is significant in centrosome maturation during mitosis (Fong et al., 2008; Kim and Rhee, 2014). In neuronal progenitor cells, CEP215 controls the centrosome activity and segregation of chromosomes (Lizzaraga et al., 2010). During mitosis, CEP215 forms complex with HSET, the minus end directed microtubule motor protein, and holds the minus end of microtubule and keep the centrosome attached to the spindle pole (Chavali et al., 2016). It was recently discovered that CEP215 and ASPM work together to concentrate spindle poles on a single site (Boveri, 2008). The removal of CEP215 is important in centrosome separation and centriole separation at the end of mitosis (Boveri, 1902; Pagan et al., 2015) (Fig. 6). Altogether, CEP215 is an essential protein in the organization of the centrosomal microtubule.

4. Centrosome and disease

Dysfunction of the centrosome can cause various types of diseases. Mutation in PCNT and CEP215 can cause neurodegenerative diseases and centrosome amplification is often related to cancer.

4.1 Centrosome and microcephaly

The abnormalities in the *CEP215* cause microcephaly and Alzheimer (Levine

et al., 2017), and the patients missed C-term of CEP215 which can interact with PCNT (Adams et al., 2020; Bond et al., 2005; Hassan et al., 2007). Abnormalities in *PCNT* cause microcephalic osteodysplastic primordial dwarfism type II and seckel syndrome (Griffith et al., 2008). Mutation in other important PCM proteins such as ASPM, MCPH1, and CENPJ can also cause microcephaly (Levine et al., 2017). Dysfunction in cilia can cause retinal degeneration, cognitive impairment, obesity, and bardet-Biedel syndrome (Chavali et al., 2014). Interestingly, centrosome amplification in the mouse brain can also lead to microcephaly (Marthiens et al., 2013).

4.2 Centrosome and cancer

Centrosome amplification frequently occurs in many types of cancer, and is considered the major contributing factor for chromosome instability in cancer cells (Chan, 2011; Fukasawa, 2005; Marteil et al., 2018). There are many reasons for centrosome amplification. Procentrioles can be either amplified from mother centriole or de novo (Fig. 8). Fragmentation of overly long centriole or cytokinesis failure can also cause centriole amplification (Luders, 2012; Sabat-Pospiech et al., 2019) (Fig. 8). Overexpression of PLK4, commonly referred to as a master regulator of centriole duplication, can induce centrosome amplification (Habedanck et al., 2005; Kleylein-Sohn et al., 2007) (Fig. 8). PLK1 overexpression or expression in a

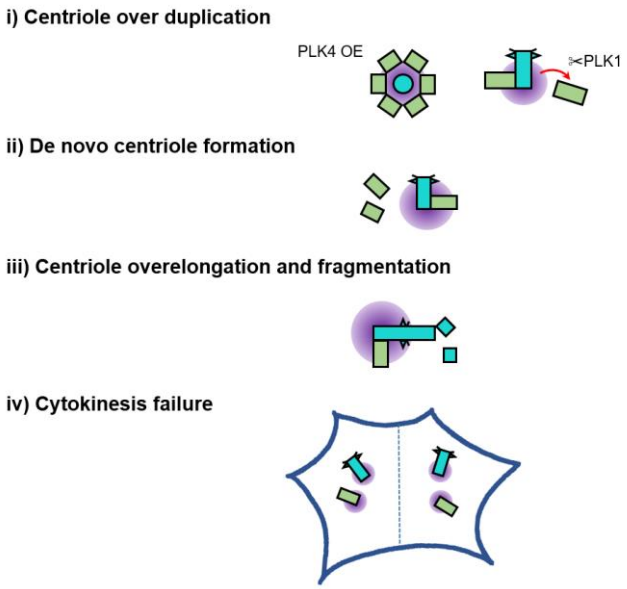


Figure 8. Various reasons for centriole amplification

There are various reasons to cause centriole amplification. (i) Centrioles can amplify from mother or daughter centrioles. PLK4 overexpression or precocious centriole disengagement during interphase induced by PLK1 overexpression can cause centriole amplification. (ii) Over duplicated centrioles can be assembled de novo. (iii) Centrioles can be over elongated by the over expression of PLK1 or prolonged mitosis. Over elongated centriole can be fragmented. (iv) Cytokinesis failure can also lead to centriole amplification (modified from Saunders, 2005).

wrong cell cycle phase, which is an important protein in centrosome maturation and separation, can also cause centrosome amplification (Barr et al., 2010; Lu and Yu, 2009; Yamamoto et al., 2006) (Fig. 8). Premature disengagement in centrioles during the G2 phase can cause centriole amplification (Kong et al., 2014) (Fig. 8). Deleting PCNT causes premature centriole disengagement during the M phase and leads to centriole amplification (Kim et al., 2019) (Fig. 7). Although several reasons can cause centrosome amplification, the question always remained whether centrosome amplification was sufficient to cause cancer (Raff and Basto, 2017).

Purpose

The centrosomes duplication is tightly linked with the cell cycle and closely regulated to ensure centrioles' assembly to once and only once per cell cycle. Despite this tight regulation, there are several reasons to cause centriole amplification and this phenomenon is common in many types of cancer. The debates about whether centriole amplification is the cause or consequence of cancer are still ongoing.

The purpose of my dissertation research is to investigate the amplified centrioles created by the disruption of PCM. First, I investigated the role of CEP215 in centriole engagement and duplication. Next, I studied the amplified centrioles created from the CEP215 and PCNT knockout, the two major PCM proteins, and examined when they are created and whether they can act as a centrosome.

CHAPTER 1.

Importance of CEP215 in centriole engagement and licensing during mitosis

Abstract

CEP215 is one of the major proteins in organizing PCM. Mutations in the *CEP215* gene lead to microcephaly. CEP215 interacts specifically with the γ-tubulin ring complex to create microtubules and allows the centrosome to act as a major microtubule organizing center. Since mitotic spindle poles robustly organize microtubules to pull a set of chromosomes into daughter cells, CEP215 is abundantly present in the centrosomes during mitosis and dissipates afterward. Here, I investigated the role of CEP215 in centriole engagement during mitosis. I observed that deletion of *CEP215* leads to precocious centriole disengagement during mitosis. The rescue experiments suggest that the interaction of CEP215 with PCNT is critical for keeping the two centrioles engaged. After, I investigated the role of CEP215 in centriole duplication. I observed that deletion of *CEP215* along with *PCNT* leads to centriole amplification. My works revealed the importance of CEP215 in centriole engagement and duplication during mitosis.

Introduction

The centrosome is a main microtubule organizing center, and is composed of two parts, the centrioles and pericentriolar material (PCM) (Bettencourt-Dias and Glover, 2007). PCM is where all the vital biochemical reaction happens. PCM changes its size from interphase to mitosis (Fig. 3). During interphase, PCM forms a toroidal structure around the mother centriole and organizes interphase microtubule dynamicity for the intercellular organization (Lawo et al., 2012) (Fig. 2). During mitosis, PCM expands its size in an amorphous structure and organizes the microtubule for proper cell division (Woodruff et al., 2014) (Fig. 3). The interaction between PCNT and CEP215 is important in this mitotic PCM expansion and mitotic PCM expansion is important in making proper bipolar spindle pole (Kim et al., 2015) (Fig. 3).

CEP215, also known as CDK5RAP2, is one of the major PCM proteins which is important in recruiting the γ-tubulin to the centrosome (Fong et al., 2008). Since γ-tubulin is the critical protein in microtubule nucleation and maintenance, CEP215 is also an important microtubule nucleation protein. Additionally, CEP215 is important in centrosome cohesion and cell division (Choi et al., 2010; Fong et al., 2008; Graser et al., 2007; Kim et al., 2015; Lee and Rhee, 2010). CEP215 has two conserved domains throughout the species,

CM1 and CM2. CM1 is important in the γ-tubulin attachment to the centrosome (Fong et al., 2008; Samejima et al., 2008; Zhang and Megraw, 2007) and this site is also fundamental in the attachment of the centrosome to mitotic spindle poles (Barr et al., 2010). CM2 is located at the C-term of CEP215 and interacts with PCNT and AKAP450 (Buchman et al., 2010; Wang et al., 2010). The mutant that phenylalanine 75th residue is substituted to alanine, also known as $CEP215^{F75A}$ mutant, cannot interact with γ-tubulin. CM2-truncated mutant, known as $CEP215^{\Delta C}$, cannot interact with PCNT (Kim and Rhee, 2014).

CEP215 was identified as the novel protein required for centrosome cohesion along with CEP68 in 2007 (Graser et al., 2007). In 2010, Barrera et al. reported that CEP215 regulates centriole engagement and cohesion in mice (Barrera et al., 2010). Pagan et al. reported that the removal of CEP215 is important in centriole separation in the mitotic exit. They argued that CEP68 and PCNT bind to different pools of CEP215 and their removal is to remove CEP215 from the PCM (Pagan et al., 2015) (Fig. 6). Also, they argued that CEP215 is inhibiting centriole licensing (Pagan et al., 2015). In this chapter, I generated *CEP215* deleted cells and investigated their precise role in centriole engagement and duplication.

Materials and Method

Cell culture, generation of deleted cell lines and synchronization

The deleted cell lines were made in the Flp-In T-Rex Hela cells (Kim et al., 2019). *CEP215* was deleted using the CRISPR/Cas9 technique in the *TP53* deleted cells. The gRNA sequences for *CEP215* deletion are (5'-ctgcagccgctgagcgtcccagg-3'). The triple KO cell was generated the *PCNT*; *TP53* double deleted cells expressing DD-PCNT (Kim et al., 2019). The gRNA sequences for *CEP215* deletion are (5'-ccagggacggtgacgtcctcttc-3') and (5'-ctgcagccgctgagcgtcccagg-3'). For mitotic synchronization, cells were sequentially treated with 2mM thymidine (T9250; Sigma-Aldrich, St. Louis, MO) and 5μM STLC (2191; Tocris, Bristol, United Kingdom).

Antibodies

The antibodies specific to centrin-2 [immunocytochemistry (ICC) 1:1,000; 04-1624; Merck Millipore, Billerica, MA], CEP152 (ICC 1:500, IB 1:100; 183911; Abcam), GAPDH (IB 1: 20,000; AM4300; Life Technologies, Carlsbad, CA), α-tubulin (ICC 1:2,000, IB 1: 20,000; ab18251; Abcam), γ-tubulin (ICC 1:1,000, IB 1:2,000; 11316; Abcam) were purchased. The antibodies specific to CEP215 (Lee and Rhee, 2010) (ICC 1:2,000, IB 1:500), and CEP135

(Kim et al., 2008) (ICC 1:2,000) were previously described. Secondary antibodies conjugated with fluorescent dyes (ICC 1:1,000; Alexa Fluor 488, 594 and 647; Life Technologies) and with horseradish peroxidase (IB 1:10,000; Sigma-Aldrich or Millipore) were purchased.

Immunostaining analysis

Cells on cover glass (0117520; Paul Marienfeld, Lauda-Königshofen, Germany) were fixed with cold methanol for 10 minutes 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 minutes. The samples were incubated with primary antibodies for 1 h, washed with 0.1% PBST, incubated with secondary antibodies for 30 minutes, washed, and 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 QICAM Fast 1394 and processed in ImagePro 5.0 (Media Cybernetics). ImagePro 5.0 (Media Cybernetics), Photoshop CC (Adobe) and ImageJ 1.51k (National Institutes of Health) were used for image processing. All images were obtained in an identical setting with the same exposure time for measuring fluorescence intensities at the centrosome. ImageJ was used for

measuring and the background signals were subtracted from the centrosomal signals.

Immunoblot analysis

Cells were washed with PBS, lysed on ice for 10 minutes with RIPA buffer (1% Triton X-100, 150 mM NaCl, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM Tris-HCl at pH 8.0, 1 mM Na3VO4, 10 mM NaF, 1 mM EDTA and 1 mM EGTA) containing a protease inhibitor cocktail (Sigma-Aldrich, P8340) and centrifuged for 10 minutes at 4°C. A fraction of the supernatants were used for the Bradford assays, and the rest were mixed with 4×SDS sample buffer (250 mM Tris-HCl at pH 6.8, 8% SDS, 40% glycerol and 0.04% bromophenol blue) and 10 mM DTT (0281-25G; Amresco). The mixtures were boiled for 5 minutes. For PCNT, CEP215, and CEP152, 3% stacking gel and 4% separating gel was used with 20mg of protein samples. For SAS-6 and CPAP, 5% stacking gel and 8% separating gel were used with 20mg of protein samples. The rest are loaded in 5% stacking gel and 10% separating gel with 10mg of protein samples. Proteins at gels were transferred to Protran BA85 nitrocellulose membranes (10401196; GE Healthcare Life Sciences). The membranes were blocked with a blocking solution (5% nonfat milk in 0.1% Tween 20 in TBS or 5% bovine serum albumin in 0.1% Tween 20 in TBS) for 2 h, incubated with primary antibodies

diluted in blocking solution for overnight at 4°C, washed with TBST (0.1% Tween 20 in TBS), incubated with secondary antibodies in blocking solution for 30 minutes and washed again. ECL reagent (LF-QC0101; ABfrontier, Seoul, Korea) and X-ray films (CP-BU NEW; Agfa, Mortsel, Belgium) were used to detect the signals.

Results

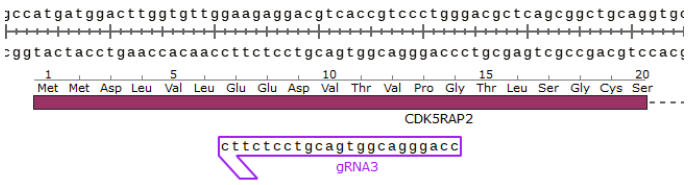
Generation of *TP53; CEP215* deleted cell line

To investigate the precise role of CEP215 when it is completely deleted from the cells, I generated *CEP215* knockout cell line using the CRISPR/Cas9 method. I expected knockout to reveal important phenotypes of CEP215 in centriole engagement which was not shown in previous knockdown experiments since it eliminates the target gene, like it did in the PCNT knockout experiment (Kim et al., 2019). The deletion of centrosomal proteins frequently goes under TP53-induced cell death (Lambrus et al., 2015; Srsen et al., 2006). To eliminate the possibility of cell death caused by p53 activation, I generated *TP53* knockout cells first. Four types of insertion were found from more than 20 DNA sequences I checked from the monoclonal *TP53* knockout cell line (Fig. 9). Next, I deleted the *CEP215* gene in this *TP53* knockout cell line. Four types of insertion and deletion were found from more than 20 DNA sequences I checked from the monoclonal *TP53; CEP215* double knockout cell line (Fig. 10). I studied the expression of endogenous CEP215 by immunoblotting and immunostaining. The complete disappearance of CEP215 was confirmed by both experiments, in whole cell lysate level and centrosomal level (Fig. 11A-C).

Figure 9. The in-del assay result of *TP53* knock out cell line

The *TP53* genes were deleted in HeLa cells using the CRISPR/CAS9 method. The sequence of gRNA was written inside the purple box. 4 types of insertion found in the in-del assay of the *TP53* gene were listed. Insertions were written in blue. More than 20 DNA sequences were analyzed.

***CDK5RAP2* exon1**



tactacctgaaccacaaccttctcctgcagtggc~~a~~gggaccctgcgagtcgccgacgtc (9/21)

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tactacctgaaccacaaccttctcctgcagtggc~~a~~agggaccctgcgagtcgccgacgtc (1/21)

Figure 10. The in-del assay result of *TP53; CEP215* knock out cell line

The *CEP215* genes were deleted in *TP53* knockout cells using the CRISPR/CAS9 method. The sequence of gRNA was written inside the purple box. 4 types of insertion and deletion found in the in-del assay of the *CEP215* gene were listed. Insertions were written in blue and deletions were written in red, marked as strikethrough. More than 20 DNA sequences were analyzed.

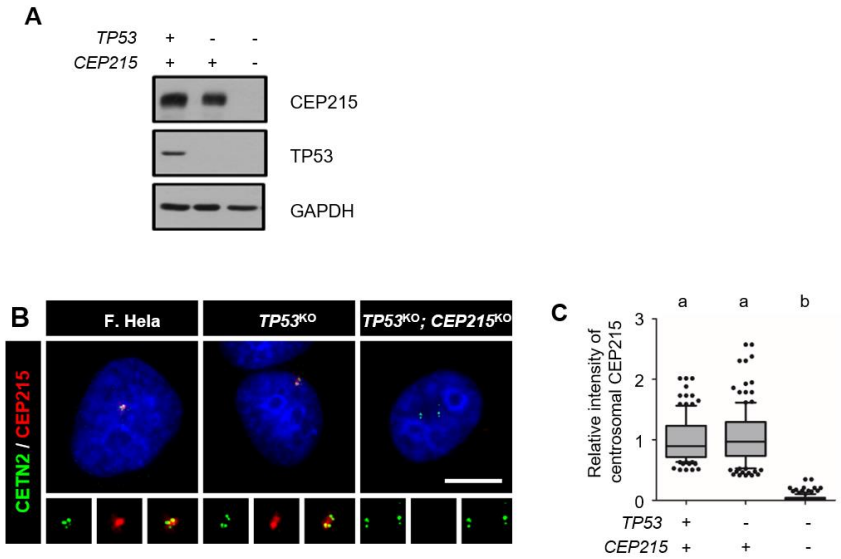


Figure 11. Generation of *TP53; CEP215* knock out cell line.

(A)The deletions were confirmed with the immunoblot analysis with antibodies specific to CEP215, TP53 and GAPDH. CEP215 blot was cropped from the 4% gel and TP53 and GAPDH blots were 10% gel. (B) The KO cells were coimmunostained with antibodies specific to centrin-2 (CETN2, green) and CEP215 (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (C) The relative intensity of centrosomal CEP215. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM. The statistical significance was analyzed using two-way ANOVA and indicated with lower cases ($P<0.05$).

Deletion of *CEP215* leads to premature centriole separation in early mitosis

Next, I investigated the role of CEP215 in maintaining centriole engagement during mitosis. CEP215 was an essential factor in centrosome cohesion in human (Graser et al., 2007) and centriole cohesion in mice (Barrera et al., 2010). It was recently revealed that the cleavage of PCNT mediates the removal of CEP215 from PCM and is important in centriole separation (Pagan et al., 2015), and the deletion of PCNT leads to precocious centriole separation (Kim et al., 2019). So I hypothesized that CEP215 also must be important in human centriole cohesion.

I checked whether the deletion of *CEP215* leads to precocious centriole separation in early mitosis. First, I arrested cells in prometaphase with STLC (S-trityl-L-cysteine), which is an Eg5 inhibitor. Then I immunostained the cells with centrin2 (CETN2), which detects the middle part of the centriole wall and CEP135, which detects the centriole's proximal end to determine centriole separation (Kim et al., 2019; Lee and Rhee, 2012; Saunders, 2005). Wild type control cells will show 2 to 1 ratio of CETN2 and CEP135 since most of the mother and daughter centrioles are remain associated in early mitosis, and they were grouped as type I (Fig. 12A). PCNT knockout cell line showed 1 to 1 ratio of CETN2 and CEP135 because the centrioles were prematurely separated in early mitosis (Kim et al., 2019), and they were grouped as type III (Fig. 12A).

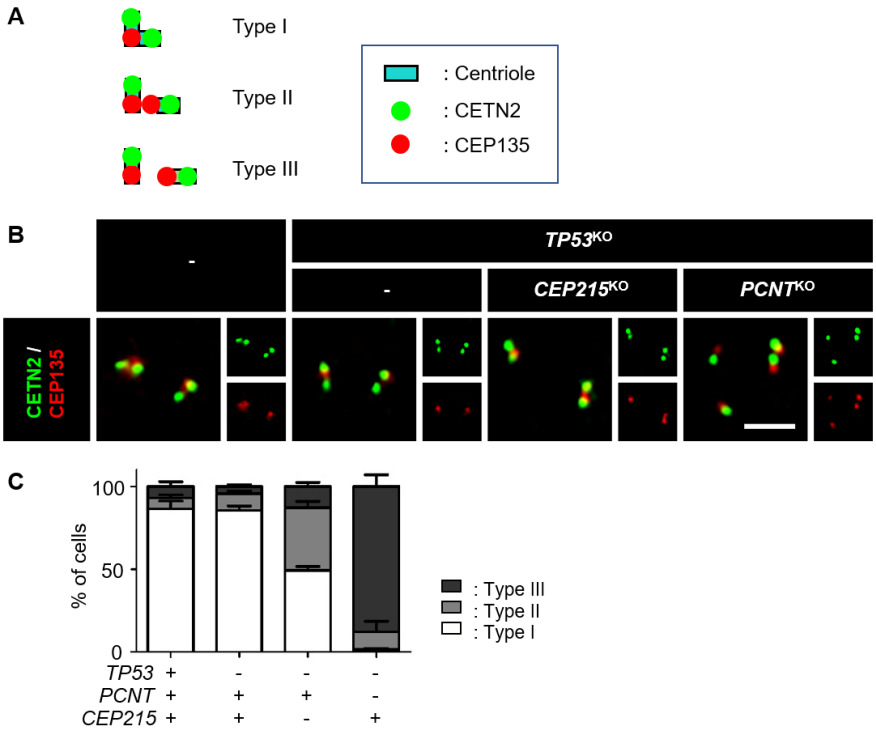


Figure 12. Precocious centriole separation in *CEP215* knockout cells in early mitosis

(A) Centriole disengagement types were categorized into three. (B) The prometaphase arrested cells were subjected to coimmunostaing analysis with antibodies specific to CETN2 (green) and CEP135 (red). Scale bar, 2 μm. (C) The number of cells with separated centrioles was counted, based on the 1:1 ratio of the centriolar CEP135 and CETN2 signals, and Fig. 12A categorization. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

What I observed from the *CEP215* knockout group was quite different from those two types. The ratio of CETN2 and CEP135 were 1 to 1 which indicated centrioles were separated and distanced far enough for our eyes to observe them as two dots. However, the two centrioles were still closely remained, not like PCNT knockout centrioles. So the two centrioles observed as 1 to 1 ratio but closely remained were grouped as type II (Fig. 12A).

The percentage of centriole separation type II increased to 40 percent in the *CEP215* knockout group compared to control groups (Fig. 12B). The majority of *PCNT* knockout cells had type III centrioles (Fig. 12B,C). This indicated that the removal of CEP215 leads to premature centriole separation. Since physical interaction between PCNT and CEP215 is important in forming mitotic PCM of the centrosome (Kim and Rhee, 2014), I hypothesized that this interaction also might be significant in protecting mitosis centrioles from separation. That without CEP215, PCNT fails to form the tight barrier and it leads to premature centriole separation.

The interaction between CEP215 and PCNT is important in maintaining centriole integrity.

To investigate whether the interaction between CEP215 and PCNT was essential in maintaining centriole integrity, I did a rescue experiment with several

mutant types of CEP215. I used CEP215^{F75A} mutant, which cannot interact with γ-tubulin, and CEP215$^{\Delta C}$ mutant, which cannot interact with PCNT (Kim and Rhee, 2014). I transfected vector only, CEP215^{WT}, CEP215^{F75A} and CEP215$^{\Delta C}$ mutants. CEP215^{WT} and CEP215^{F75A} significantly reduced the percentage of centriole separation type II compared to the vector only transfected group (Fig. 13A,B). However, CEP215$^{\Delta C}$ failed to reduce the percentage of disengagement type II (Fig. 13A,B). This result suggested that the interaction of PCNT and CEP215 might be important in protecting centrioles from separation.

However, the result was insufficient to conclude the theory because the localization of CEP215 to the centrosome is reduced to 70 percent in the CEP215$^{\Delta C}$ mutant (Kim and Rhee, 2014). To confirm the theory, I did a rescue experiment by adding the PACT domain to the mutants. PACT is a conserved domain in PCNT that targets the wall of the centrosome (Gillingham and Munro, 2000). Adding the PACT domain in CEP215$^{\Delta C}$ mutant can force the recruitment of the CEP215, which still lacks the interaction with PCNT (Kim and Rhee, 2014). I transfected vector only, CEP215^{WT}, CEP215$^{\Delta C}$, CEP215$^{WT+PACT}$ and CEP215$^{\Delta C+PACT}$ mutants into the *CEP215* knockout cell line. The rescued cell lines were immunostained with CETN2 (green) and CEP135 (red) (Fig. 14A). The CEP215^{WT} and CEP215$^{WT+PACT}$ rescued the centriole disengagement type II significantly as expected (Fig. 14B). However, CEP215$^{\Delta C}$ and CEP215$^{\Delta C+PACT}$

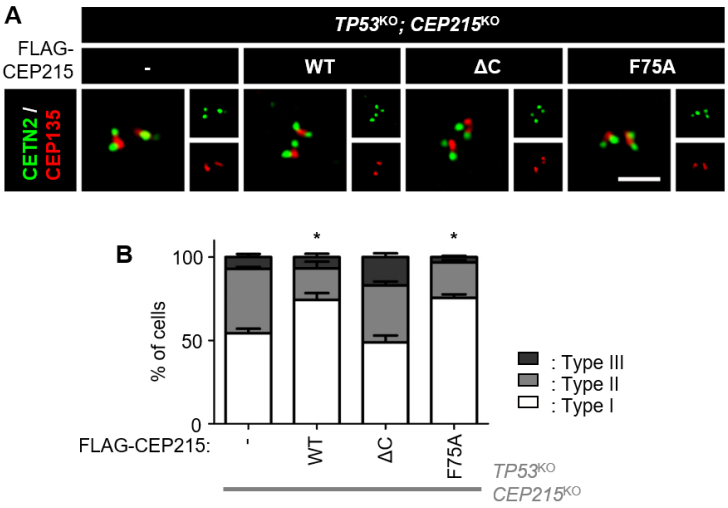


Figure 13. *CEP215* knockout cell lines are rescued with several mutants

(A) *CEP215* knockout cell line was rescued with vector only, $CEP215^{WT}$, $CEP215^{\Delta C}$ and $CEP215^{F75A}$ mutants. The prometaphase arrested rescued cells were immunostained with CETN2 (green) and CEP135 (red). Scale bar, 2 μm. (B) The number of cells with separated centrioles were counted, based on Fig. 12A categorization. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM. The statistical significance was analyzed using one-way ANOVA. *, $P<0.05$.

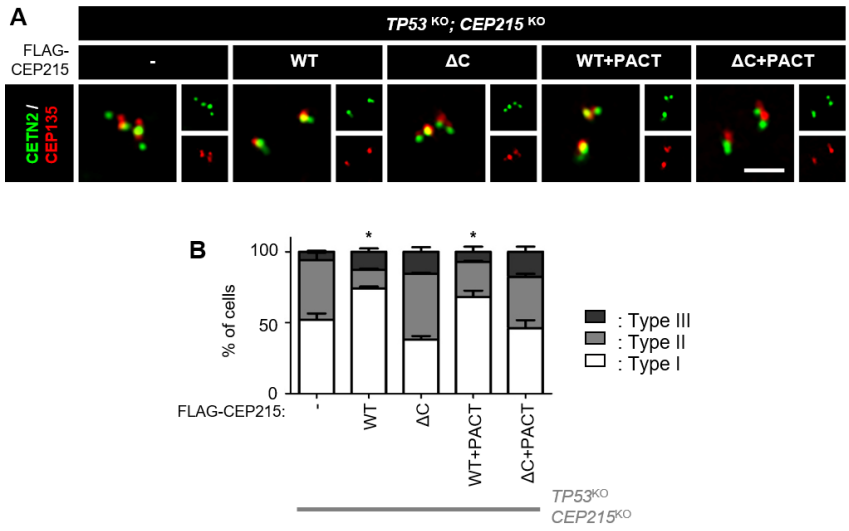


Figure 14. Interaction of CEP215 and PCNT is important in centriole engagement

(A) *CEP215* knockout cell line was rescued with vector only, $CEP215^{WT}$, $CEP215^{\Delta C}$, $CEP215^{WT+PACT}$ and $CEP215^{\Delta C+PACT}$ mutants. The prometaphase arrested rescued cell lines were immunostained with CETN2 (green) and CEP135 (red). Scale bar, 2 μm. (B) The number of rescued cells with separated centrioles were counted, based on Fig. 12A categorization. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM. The statistical significance was analyzed using one-way ANOVA. *, $P<0.05$.

failed to rescue the phenotype (Fig. 14B). This result indicated that rather than just the aggregation of CEP215, but the interaction of CEP215 and PCNT was important in centriole engagement during mitosis.

Generation of *TP53; PCNT; CEP215* deleted cell line

Centriole disengagement is an essential step to allow centrioles for duplication. When it is prevented with non-cleavable PCNT (R2231A), centrioles cannot duplicate (Kim et al., 2019; Pagan et al., 2015). However, CEP215 depletion by siRNA can reverse this phenotype, which indicates the removal of CEP215 is important in centriole duplication (Pagan et al., 2015). Although the deletion of PCNT causes precocious centriole separation in early mitosis in almost every cell, the over duplication happens in less than 20 percent of cells (Kim et al., 2019). I hypothesized that this might be because of CEP215, and to prove the hypothesis, I generated *CEP215* knockout in *TP53*; *PCNT* deleted cell line (Kim et al., 2019) using the CRISPR/Cas9 method. During the selection step, I realized that the triple KO cells failed to form a stable cell line, due to a low proliferation activity and cell apoptosis. Therefore, the triple KO cells were generated in the presence of the ectopic *PCNT* gene with a destabilization domain (*DD-PCNT*), whose expression can be induced by doxycycline and shield1 (Kim et al., 2019). 5 types of DNA sequences were found in the in-del assay and they

all made stop codon in CEP215 exon number 1. I analyzed more than 20 DNA sequences from monoclonal *TP53; PCNT; CEP215* triple knockout cell line (Fig. 15). The ectopic *DD-PCNT* gene was hardly expressed as far as doxycycline and shield1 were absent. Immunoblot analysis revealed that PCNT and CEP215 were below detection levels in the triple KO cells (Fig. 16A). Immunostaining analysis also revealed that the PCNT and CEP215 signals were absent at the centrosomes of the triple KO cells (Fig. 16B-D). These results indicated that the *TP53*, *PCNT* and *CEP215* triple KO cell line was properly generated.

Precocious centriole separation and amplification in the triple KO cells during M phase

Next, I examined precocious separation and amplification of centrioles at the M phase in the *TP53*, *PCNT* and *CEP215* triple KO cells. The cells were arrested at prometaphase using S-trityl-L-cysteine (STLC), an EG5 inhibitor, and determined centriole behaviors (Fig. 17A). I confirmed that PCNT and CEP215 were not detected in the deletion cells arrested at prometaphase (Fig. 17B). Precocious centriole separation was observed in most *TP53; PCNT* KO cells (Fig. 12B,C and 17C,D), and over duplicated centrioles were detected in about 30% of them (Fig. 17C,E). In the *TP53*; *CEP215* KO cells, precocious centriole separation was observed in about half of the cells (Fig. 12B,C and 17C,D) and

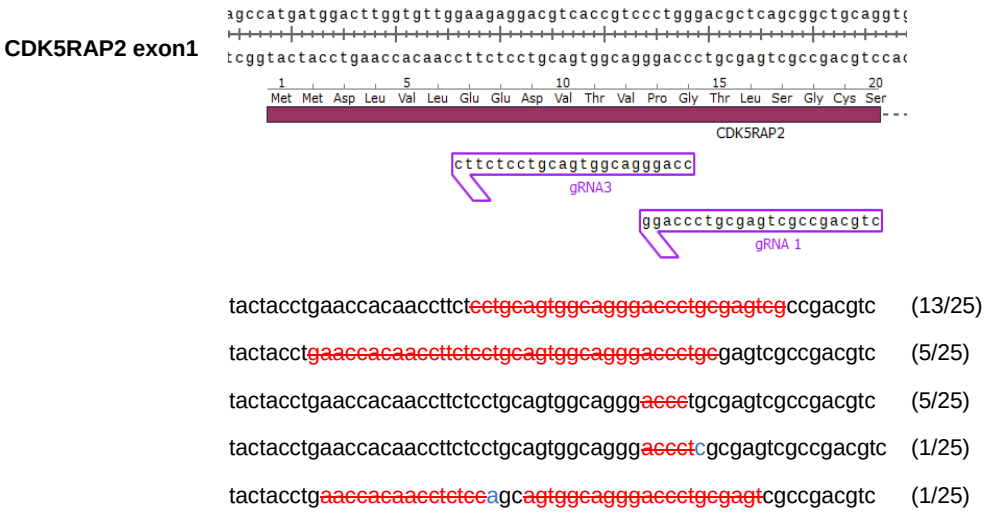


Figure 15. The in-del assay result of *TP53; PCNT; CEP215* knock out cell line

The *CEP215* genes were deleted in *TP53; PCNT* knockout cells using the CRISPR/CAS9 method. The sequences of gRNA were written inside the purple boxes. 5 types of insertion and deletion found in the in-del assay of the *CEP215* gene of *TP53; PCNT* knockout cell line are listed. Insertions were written in blue and deletions were written in red, marked as strikethrough. More than 20 DNA sequences were analyzed.

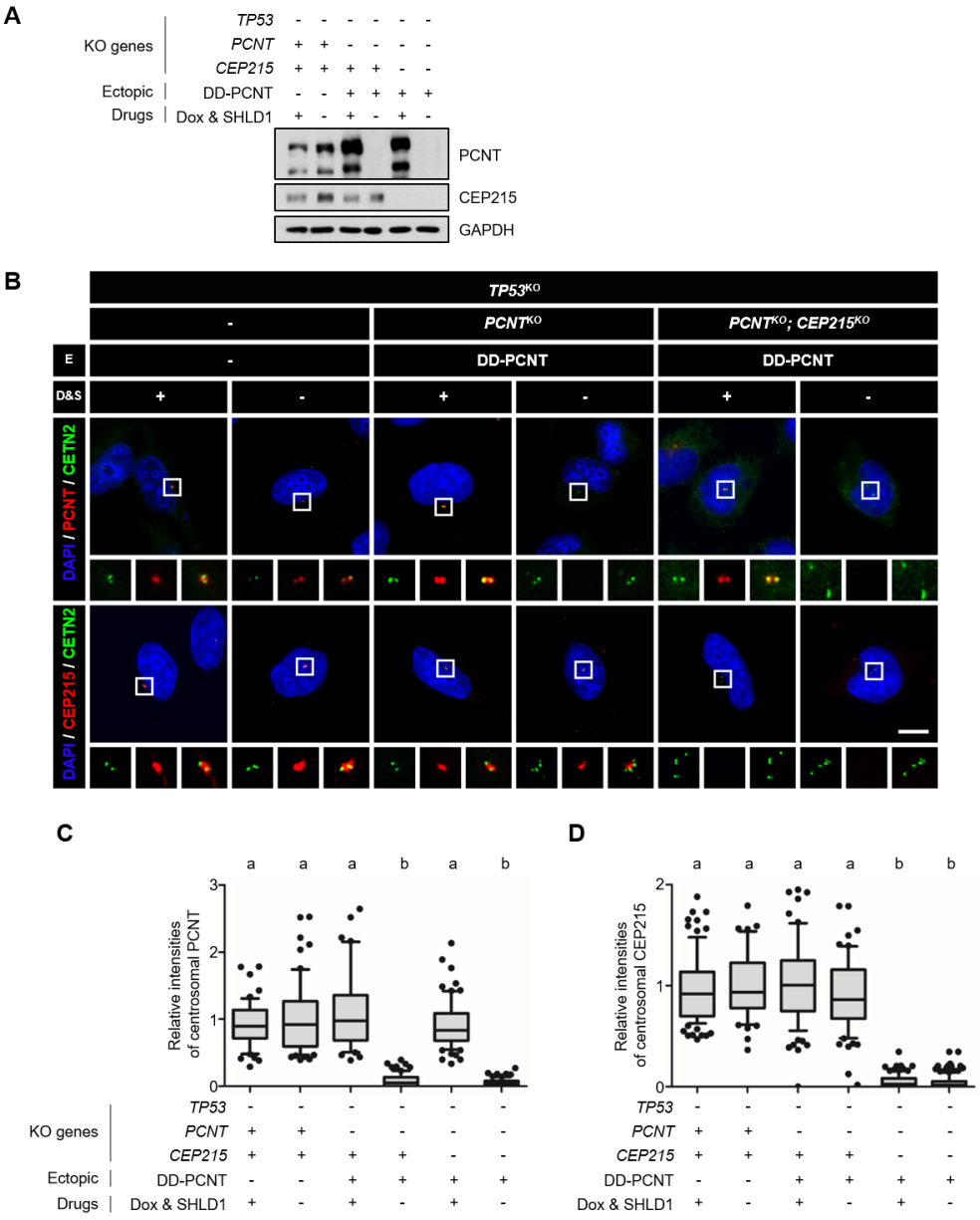
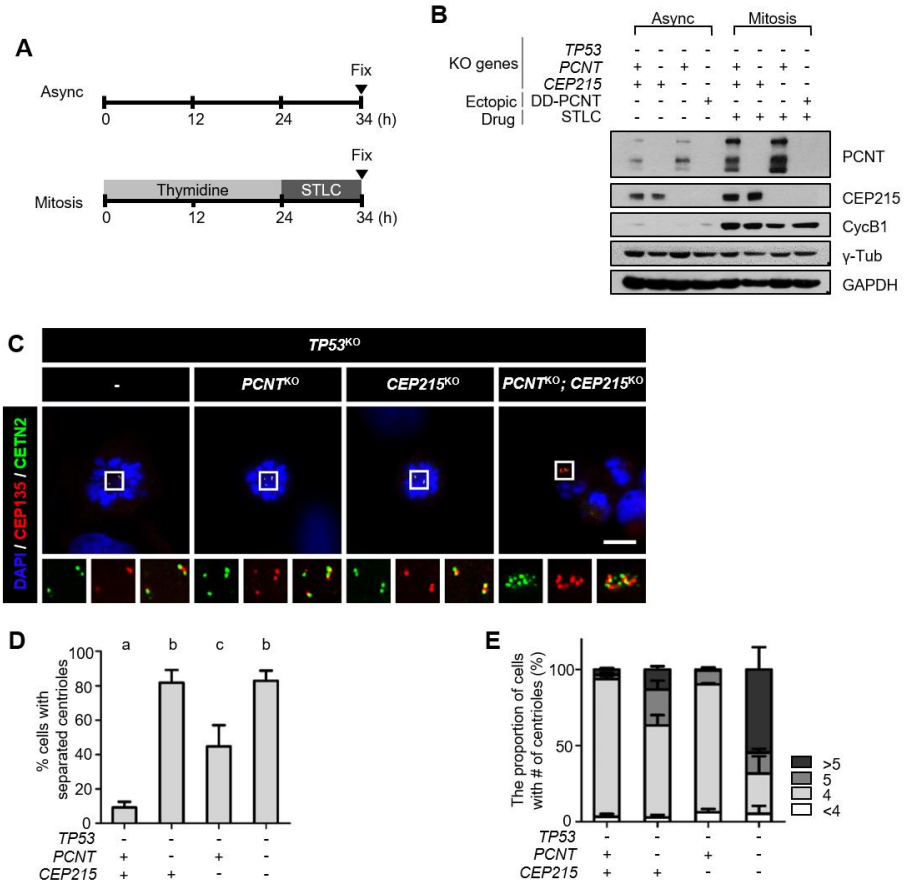

A
KO genes
Ectopic
Drugs
TP53
PCNT
CEP215
DD-PCNT
Dox & SHLD1
PCNT
CEP215
GAPDH
B
TP53KO
PCNTKO
PCNTKO; CEP215KO
E
D&S
DD-PCNT
DAPI / PCNT / CETN2
DAPI / CEP215 / CETN2
C
D
Relative intensities of centrosomal PCNT
Relative intensities of centrosomal CEP215
a a a b a b
a a a a b b

Figure 16. Generation of *TP53; PCNT; CEP215* deleted cells

(A) Endogenous *PCNT* was deleted in the presence of ectopic *DD-PCNT* gene whose expression was induced by doxycycline (Dox) and shield1 (SHLD1). The deletions were confirmed with the immunoblot analysis with antibodies specific to PCNT, CEP215 and GAPDH. PCNT and CEP215 blots were cropped from the same 4% gel and the GAPDH blot was from 10% gel. (B) The KO cells were coimmunostained with antibodies specific to CETN2 (green), PCNT (red) and CEP215 (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (C, D) Relative intensities of the centrosomal PCNT (C) and CEP215 (D) signals were determined. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM. The statistical significance was


A
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DD-PCNT
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STLC
PCNT
CEP215
CycB1
γ-Tub
GAPDH
C
TP53KO
-
PCNTKO
CEP215KO
PCNTKO; CEP215KO
DAPI / CEP135 / CETN2
D
% cells with separated centrioles
a
b
c
b
E
The proportion of cells with # of centrioles (%)
>5
5
4
<4

Figure 17. Precocious centriole separation and amplification at the M phase in the triple KO cells

(A) Timeline for preparation of prometaphase cells. The KO cells were treated with thymidine for 24 h followed by STLC for 10 h. (B) Immunoblot analyses were performed with antibodies specific to PCNT, CEP215, cyclin B1, γ-tubulin and GAPDH. PCNT and CEP215 blots were cropped from the same 4% gel, cyclin B1 blot was from the 8% gel, γ-tubulin and GAPDH blots were from the same 10% gel. (C) The prometaphase arrested cells were subjected to coimmunostaining analysis with antibodies specific to CETN2 (green) and CEP135 (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (D) The number of cells with separated centrioles was counted, based on the 1:1 ratio of the centriolar CEP135 and CETN2 signals. (E) The number of centrioles per cell was counted. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM. The statistical significance was analyzed using two-way ANOVA and indicated with lower cases ($P<0.05$).

no centriole amplification occurred (Fig. 17C,E). Precocious centriole separation was evident in the *TP53*; *PCNT; CEP215* triple KO cells and centriole amplification was fortified (Fig. 17C-E). Indeed, some of the triple KO cells included an exceeding number of extra centrioles up to 30 (Fig. 17C,E). These results indicated that the removal of CEP215 might be critical in centriole duplication and a cooperative function of PCNT and CEP215 in preventing precocious centriole separation and amplification during mitosis.

Discussion

Here, I generated *CEP215* knockouts in *TP53* and *TP53; PCNT* knockout cells and investigated the importance of CEP215 in centriole cohesion and duplication. I observed that the deletion of CEP215 causes precocious centriole separation (Fig. 18). Interaction of CEP215 and PCNT was essential for the centriole engagement. I also investigated that the triple KO cells had an exceeding number of centrioles.

It was reported that centriole distancing occurs right after mitotic entry (Shukla et al., 2015). However, the two centrioles remain associated until the mitotic exit (Kim et al., 2019). There are two hypotheses for centriole association in the spindle poles of mitotic cells. One is an unidentified “glue protein” that attaches the daughter centriole to the mother centriole (Schockel et al., 2011). The glue protein may be degraded during mitotic exit, resulting in the liberation of the daughter centriole from the mother centriole (Schockel et al., 2011). The other hypothesis states that the mother and daughter are closely associated during mitosis, surrounded by mitotic PCM (Cabral et al., 2013; Seo et al., 2014). At the end of mitosis, PCM is disintegrated, resulting in the mother and daughter centrioles' separation. Cleavage of PCNT initiates disintegration of PCM, leading to centriole separation (Lee and Rhee, 2012; Matsuo et al., 2012). The phenotype

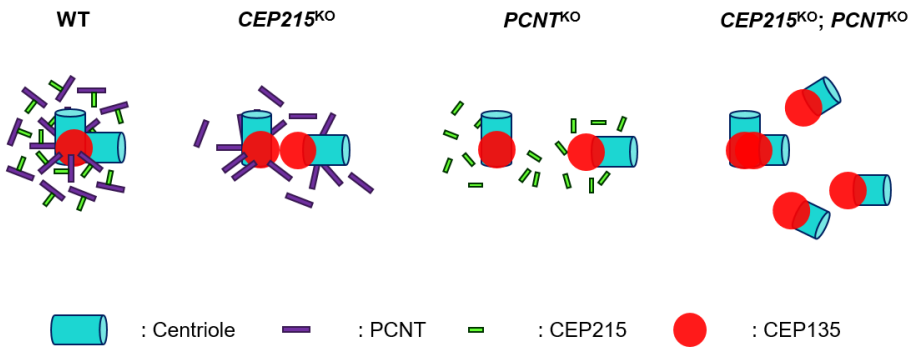

Figure 18. Hypothesized model for mitosis PCM of knockout cell lines

At the early M phase, mitotic PCM forms the tight barrier around the centrioles. Deletion of *CEP215* caused disruption in mitotic PCM and caused precocious disengagement. CEP215 and PCNT interaction was essential in protecting the centrioles, and just the forced recruitment of CEP215 cannot rescue this phenotype. Deletion of *PCNT* caused precocious disengagement, and overduplication of centrioles were observed as previously reported. The additional deletion of *CEP215* in *PCNT* knockout cells also showed precocious disengagement, and provoked centriole amplification. In conclusion, mitotic PCM is important in maintaining the integrity of the centriole.

of PCNT deleted cells suggested that PCM is important in centriole engagement during mitosis. CEP215 knockout cells also caused precocious centriole separation (Fig. 18), and the interaction between CEP215 and PCNT was important. Therefore, the results support that there is no specific glue protein, but PCM is the key player in centriole association during mitosis.

The importance of CEP215 for centriole duplication has been suggested previously (Barrera et al., 2010; Pagan et al., 2015). However, centriole amplification was not observed from previous knockdown experiments (Chavali et al., 2016; Fong et al., 2008; Kim and Rhee, 2014; Lee and Rhee, 2010). Here, I observed centriole amplification in the additional removal of *CEP215* in *PCNT* knockout cells. Double deletion of *CEP215* and *PCNT* have provoked centriole amplification during mitosis than *PCNT* alone. Further investigation is necessary to define the statement, however, I want to argue that the removal of CEP215 is important in centriole duplication. It must have increased the accessibility of the centriole assembly factor. Therefore, I insist that the mitotic PCM is important in maintaining the integrity of the centriole, preventing centriole disengagement and amplification.

In this chapter, I investigated the importance of CEP215 in centriole cohesion and duplication in *TP53* and *TP53; PCNT* knockout cells. Although I observed the phenotypes that have not been observed from previous knockdown

experiments by changing to knockout experiments, the disadvantages of using knockout cells cannot be ruled out. Because the knockout cells have to proliferate long periods without the target proteins, they might gain the new ability and exhibit different phenotypes from knockdown cells. Also, they might have side effects because they are forced to live. For instance, I had to knockout TP53 to force the cells to live, and even rescue the triple knockout cells with *DD-PCNT*. They might show different and unexpected phenotypes since they are destined to die in normal conditions.

Dysfunction of PCNT leads to microcephalic osteodysplastic primordial dwarfism type 2 disease (Kantaputra et al., 2011; Rauch et al., 2008). Dysfunction of CEP215 leads to primary microcephaly (Bond et al., 2005; Hassan et al., 2007; Pagnamenta et al., 2012). It has been suggested that the reason for this is due to the defects in centrosome maturation and mitotic spindle orientation (Lizarraga et al., 2010). However, centrosome amplification caused microcephaly in the mouse brain without causing spindle misorientation (Marthiens et al., 2013). My results showed the deletion of *CEP215* causes precocious centriole separation and the double deletion of *CEP215* and *PCNT* causes centriole amplification. Thus, my research might provide another explanation for the mechanisms of microcephaly caused by PCNT and CEP215 mutation.

CHAPTER 2.

Identification of the origin and fate of supernumerary centrioles in *TP53; PCNT; CEP215* triple knockout cells

Abstract

Cancer cells frequently include supernumerary centrioles. Here, I observed amplification of the centrioles in *TP53; PCNT; CEP215* triple knockout cell line. I determined when these supernumerary centrioles were created by comparing them with the supernumerary centrioles from the PLK4 overexpressed cell line. I observed the M phase centriole assembly from triple KO cells. Many of the triple KO cells maintained supernumerary centrioles throughout the cell cycle. The M phase assembled centrioles lack the ability to function as templates for centriole assembly during the S phase. They also lack the ability to organize microtubules in interphase and mitosis. My works revealed the origin and fate of supernumerary centrioles created in triple KO cells.

Introduction

The centrosome is a very small organelle organized with the centrioles and PCM (Bettencourt-Dias and Glover, 2007). During the G1 phase, the cell contains two centrosomes and one centriole each. The duplication starts during the S phase and centriole elongation happens during the G2 phase. So the cell has two centrosomes and two centrioles each. At the end of the G2 phase, the linker proteins holding two centrosomes together degrades and centrosome separation occurs. The centrosomes move to each end of the cell and form a bipolar spindle pole. At the end of the mitosis, centriole separation occurs, and centriole-to-centrosome conversion occurs during the G1 phase (Nigg and Stearns, 2011).

Centriole separation is an essential process in centriole-to-centrosome conversion (Kim et al., 2019). Centriole-to-centrosome conversion is an essential process for recruiting PCM and centriole duplication initiation (Wang et al., 2011). CEP295 is found to be the first step in conversion, and CEP152 is the last protein to be recruited to newly converted centriole (Fu and Glover, 2016; Tsuchiya et al., 2016). The conversion process is very complicated and the process underlying it still needs many investigations.

Centrosome amplification is a phenomenon that is commonly found in various cancer types (Raff and Basto, 2017). Premature centriole separation

during the S phase can initiate the duplication of new centriole (Agircan et al., 2014) and prolonged S phase can lead to over duplication of centrioles (Borel et al., 2002). The overexpression of PLK4 can also lead to centriole over duplication, and centrosome fragmentation or loss of centriole cohesion can also lead to centriole over duplication (Luders, 2012). Cell to cell fusion or cytokinesis failure can also cause centriole over duplication (Luders, 2012). It was recently revealed that prolonged mitosis could also cause centriole amplification (Kong et al., 2020; Marteil et al., 2018). The previous study showed that the deletion of *PCNT* could lead to premature centriole separation in early mitosis, leading to centriole over duplication during the M phase (Kim et al., 2019). I generated *TP53, PCNT* and *CEP215* triple knockout cell lines and observed centriole amplification in the previous chapter. In this chapter, I investigated the origin of supernumerary centrioles found in triple KO cells, and studied their fate whether they function as centrioles.

Materials and Methods

Cell culture and synchronization

HeLa cells were cultured in DMEM (LM001-05; Welgene, Gyeongsangbuk-do, Korea) with 10% FBS (S101-01; Welgene). For mitotic synchronization, cells were sequentially treated with 2mM thymidine (T9250; Sigma-Aldrich, St. Louis, MO) and 5μM STLC (2191; Tocris, Bristol, United Kingdom). For the time course experiment, cells were treated with doxycycline 24 hours after seeding to induce ectopic PLK4 expression, washed out and cultured for another 24 hours. Mitotic cells were obtained with a gentle shake off from asynchronous cell plates and collected with a warm medium at indicated time points.

Microtubule regrowth assay

Mitotic cells were obtained with mitotic shake off and cultured for 2 h to reach the early G1 phase. The cells were treated with 5μM of nocodazole (M1404; Sigma-Aldrich) for 2 hours at 37℃, placed on ice for 1 hour, and then transferred to a warm medium for microtubule growth. The cells were fixed with PEM buffer (80mM PIPES pH6.9, 1mM $MgCl_2$, 5mM EGTA, 0.5% Triton X-100) for 10

minutes at room temperature, incubated in phosphate-balanced buffer with 0.5% Triton-X (PBST) for 5 minutes to increase permeability, and subjected to immunostaining with antibodies specific to α-tubulin and CETN2.

Live cell observation

LAXS benchtop high-content analysis system was used for live observation of *CETN2-Dendra2* cells. The *CETN2-Dendra2* plasmid kindly provided by Alwin Krämer (Loffler et al., 2013) was stably transfected into the cells. The cells were synchronized at M phase with sequential treatments of thymidine and STLC, activated with 405nm wavelength for 10 seconds and captured with 488nm and 561nm wavelengths for up to 2 h.

Antibodies

The antibodies specific to CETN2 (ICC 1:1,000; 04-1624; Merck Millipore, Billerica, MA), CEP295 (ICC 1:500; 122490; Abcam, Cambridge, MA), CEP192 (ICC 1:1,000, IB 1:500; A302-324A; Bethyl Laboratories, Montgomery, TX), CEP152 (ICC 1:500, IB 1:100; 183911; Abcam), GAPDH (IB 1: 20,000; AM4300; Life Technologies, Carlsbad, CA), SAS-6 (ICC 1:200, IB 1:100; sc-376836; Santa Cruz Biotechnology, Dallas, TX), α-tubulin (ICC 1:2,000, IB 1: 20,000; ab18251; Abcam), γ-tubulin (ICC 1:1,000, IB 1:2,000;

11316; Abcam) were purchased. The antibodies specific to CEP215(Lee and Rhee, 2010) (ICC 1:2,000, IB 1:500), PCNT (Lee and Rhee, 2011) (ICC 1:2,000, IB 1:2,000), CEP135 (Kim et al., 2008) (ICC 1:2,000) and CPAP(Chang et al., 2010) (ICC 1:100; IB 1:500) were previously described. Secondary antibodies conjugated with fluorescent dyes (ICC 1:1,000; Alexa Fluor 488, 594 and 647; Life Technologies) and with horseradish peroxidase (IB 1:10,000; Sigma-Aldrich or Millipore) were purchased.

Immunostaining analysis

Cells on cover glass (0117520; Paul Marienfeld, Lauda-Königshofen, Germany) were fixed with cold methanol for 10 minutes 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 minutes. The samples were incubated with primary antibodies for 1 h, washed with 0.1% PBST, incubated with secondary antibodies for 30 minutes, washed, and 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 QICAM Fast 1394 and processed in ImagePro 5.0 (Media Cybernetics). ImagePro 5.0 (Media Cybernetics), Photoshop CC (Adobe)

and ImageJ 1.51k (National Institutes of Health) were used for image processing. All images were obtained in an identical setting with the same exposure time for measuring fluorescence intensities at the centrosome. ImageJ was used for measuring and the background signals were subtracted from the centrosomal signals.

Immunoblot analysis

Cells were washed with PBS, lysed on ice for 10 minutes with RIPA buffer (1% Triton X-100, 150 mM NaCl, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM Tris-HCl at pH 8.0, 1 mM Na3VO4, 10 mM NaF, 1 mM EDTA and 1 mM EGTA) containing a protease inhibitor cocktail (Sigma-Aldrich, P8340) and centrifuged for 10 minutes at 4°C. A fraction of the supernatants were used for the Bradford assays, and the rest were mixed with 4×SDS sample buffer (250 mM Tris-HCl at pH 6.8, 8% SDS, 40% glycerol and 0.04% bromophenol blue) and 10 mM DTT (0281-25G; Amresco). The mixtures were boiled for 5 minutes. For PCNT, CEP215, and CEP152, 3% stacking gel and 4% separating gel was used with 20mg of protein samples. For SAS-6 and CPAP, 5% stacking gel and 8% separating gel were used with 20mg of protein samples. The rest are loaded in 5% stacking gel and 10% separating gel with 10mg of protein samples. Proteins at gels were transferred to Protran BA85 nitrocellulose membranes

(10401196; GE Healthcare Life Sciences). The membranes were blocked with a blocking solution (5% nonfat milk in 0.1% Tween 20 in TBS or 5% bovine serum albumin in 0.1% Tween 20 in TBS) for 2 h, incubated with primary antibodies diluted in blocking solution for overnight at 4°C, washed with TBST (0.1% Tween 20 in TBS), incubated with secondary antibodies in blocking solution for 30 minutes and washed again. ECL reagent (LF-QC0101; ABfrontier, Seoul, Korea) and X-ray films (CP-BU NEW; Agfa, Mortsel, Belgium) were used to detect the signals.

Results

Limited centriole assembly at S phase in the triple knockout cells

First, I traced the fate of the amplified centrioles in the triple KO cells throughout the cell cycle to investigate when these supernumerary centrioles were assembled. I hypothesized that the amplified centrioles of triple KO cells would be created during mitosis since centriole disengagement happened during mitosis in *PCNT* deleted cells. In comparison, I used the PLK4 overexpressing cell line as a comparison, because PLK4 overexpressing cells generate extra centrioles during the S phase (Coelho et al., 2015; Habedanck et al., 2005). The extra centrioles were observed when the ectopic *PLK4* gene expression was induced by doxycycline for 24h (Fig. 19A,B).

Using the mitotic shake off method, I collected the M phase population of the PLK4 overexpressing cells and the triple KO cells, forced them to enter into the G1 phase synchronously and cultured them for up to indicated time points (Fig. 19A). In control cells, the number of centrioles was two in 2 hours after the mitotic shake off, and increased to four in the 17 hours when the cells entered the S phase (Fig. 19B). The centriole number eventually down to two in the 24 hours after the next mitosis (Fig. 19B,C). The PLK4 overexpressing cells included extra centrioles at the beginning of the culture and 60% of them had five or more

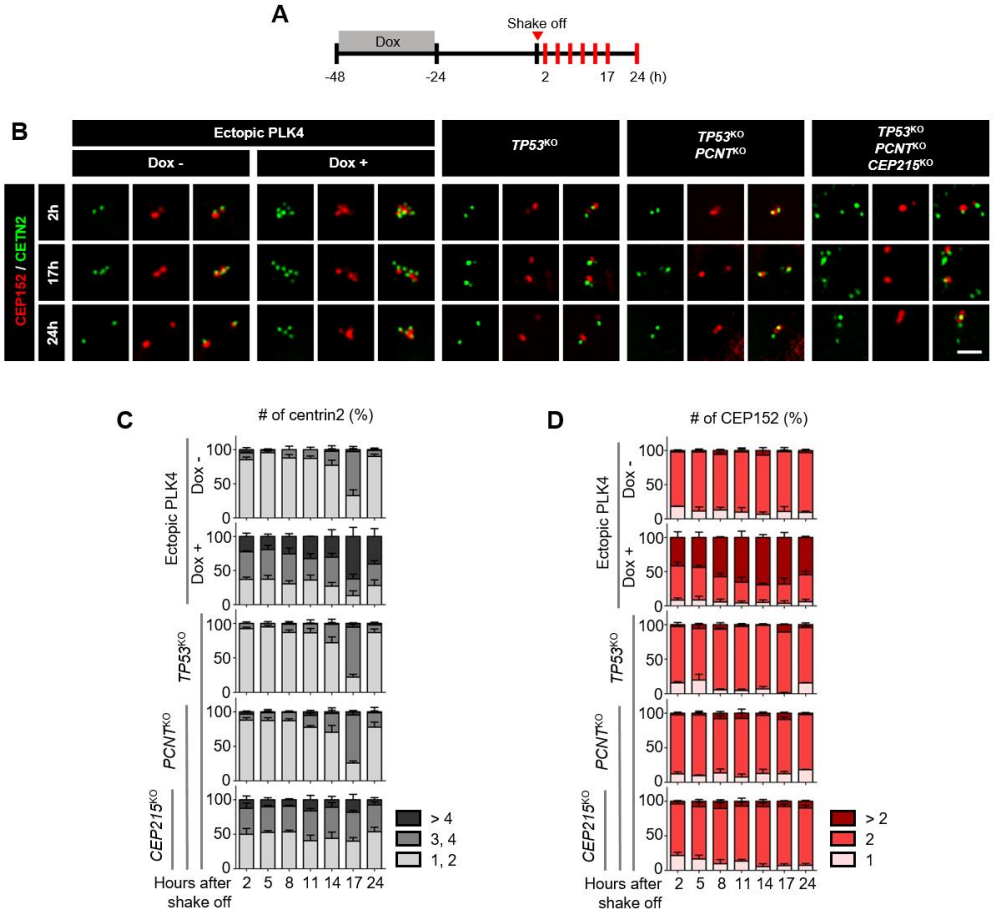

Figure 19. Limited S phase centriole assembly in the triple KO cells

(A) Timeline for preparation of synchronous interphase cells. Doxycycline was treated for 24 h to induce ectopic PLK4 expression, washed out and cultured for another 24 h. Mitotic cells were collected and cultured for up to 24 h. At indicated time points, the cells were subjected to coimmunostaining analyses. (B) The PLK4 overexpressing and KO cells were subjected to coimmunostaining analysis with antibodies specific to CETN2 (green) and CEP152 (red). Scale bar, 2 μm. (C, D) The number of CETN2 (C) and CEP152 (D) dots were counted in the PLK4 overexpressing and KO cells at indicated time points. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

centrioles at the 17 hours, indicating that most of the centrioles assembled new procentrioles during the S phase (Fig. 19B,C). The triple KO cells also included multiple centrioles at the G1 phase. However, the number of centrioles in the triple KO cells showed only a slight increase during the S phase (Fig. 19B,C) unlike PLK4 overexpressing group. These results indicated that not every amplified centriole from triple KO cells duplicated during interphase. These results suggested that the M phase assembled centrioles survive but do not duplicate during interphase.

CEP152 is a mother centriole protein that functions as an adaptor for PLK4 (Hatch et al., 2010). In control cells, the number of CEP152 positive centrioles were two throughout the cell cycle (Fig. 19B,D). About 40% of the PLK4 overexpressing cells included three or more CEP152 positive centrioles at the beginning of the culture and this proportion was maintained throughout the cell cycle (Fig. 19B,D). This observation was consistent with the previous reports in which multiple centrioles in the PLK4 overexpressing cells could duplicate during the S phase (Coelho et al., 2015). It is interesting that the triple KO cells included only two CEP152 positive centrioles out of multiple centrioles and this number was maintained throughout the cell cycle (Fig. 19B,D). These results indicated that only a single out of numerous daughter centrioles converted into a mother centriole in the triple KO cells during mitotic exit.

M phase centriole assembly in triple knockout cells

I used centrinone, a PLK4 inhibitor, to determine centriole assembly in the S phase in the PLK4 overexpressing cells and the triple KO cells (Wong et al., 2015). As expected, the number of centrioles in control cells remained two at the S phase (Fig. 20). The average number of centrioles in PLK4 overexpressing cells started with seven and increased to ten during the S phase (Fig. 20). However, the S phase assembly of the centrioles was inhibited with the centrinone treatment (Fig. 20). The average number of centrioles in the triple KO cells was 3.5, which hardly increased even at the S phase (Fig. 20). Centrinone had a little effect on the triple deletion cells, confirming that the triple KO cells barely assembled procentrioles in the S phase (Fig. 20).

To examine whether these amplified centrioles are assembled during mitosis since there was no significant increase in the number of centrioles during interphase, I used a photo-convertible fluorescent protein called Dendra2 (Loffler et al., 2013). Dendra2 is a photoconvertible fluorescent protein that can be activated by UV light. Before the activation, it only expresses green signals and when it is activated with light, it expresses both green and red signals. So the CETN2 coupled to the Dendra2 allows differentiation between pre-existing and newly formed centrioles after the photoconversion (Loffler et al., 2013). The

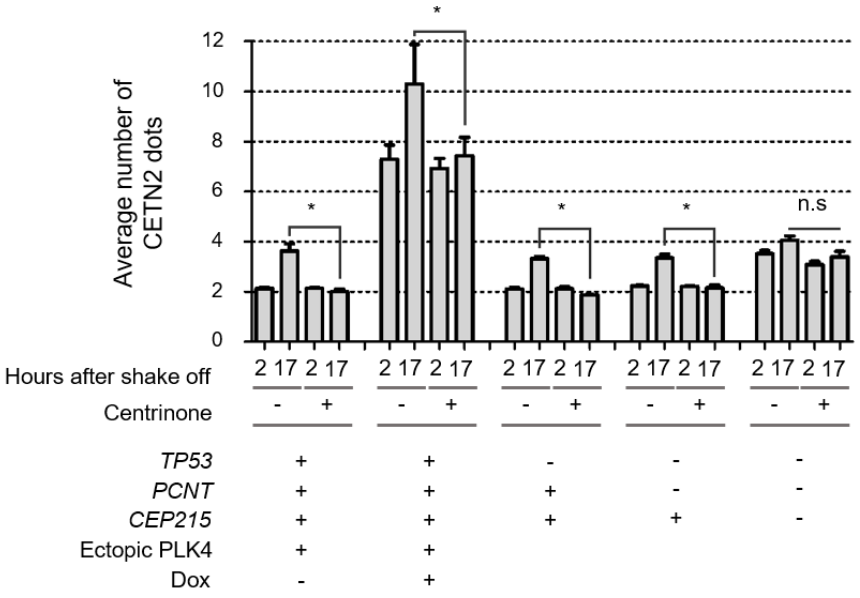

Figure 20. Triple KO cells hardly assemble procentrioles during the S phase
(A) After mitotic shake off, the PLK4 overexpressing and KO cells were cultured in the presence of centrinone for 2 h and 17 h, and immunostained with the CETN2 antibody. The number of CETN2 dots per cell was counted and the average value is derived. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM. The statistical significance was analyzed using T-test in indicated groups. *, $P<0.05$.

CETN2 dots that existed during interphase will show green and red signals and newly formed centrioles after the light activation will show only green signals by activating the cells before the mitosis. The *CETN2-Dendra2* expressing cells synchronously entered mitosis and the cells were activated with the light right before the mitosis. Centrioles with only green signals started to appear in prometaphase arrested triple KO cells (Fig. 21A,B). However, no centriole was generated in the *TP53* deleted control cells (Fig. 21B). This result indicated mitosis centriole assembly in the triple KO cells.

Next, I studied whether these amplified centrioles were made with cartwheel structure like during interphase (Nigg and Stearns, 2011). I immunostained the cells with SAS6 (Fig. 22A) because SAS6 was a cartwheel protein (Nakazawa et al., 2007). During mitosis, the number of SAS6 was usually 2 in the control cells since one cartwheel structure made one procentriole. Amplified centrioles from PLK4 overexpressing cell line showed more than 2 SAS6 dots, and the number of SAS6 was half of the number of CETN2 dots during mitosis. Since their amplified centrioles are made during the S phase, the number of SAS6 dots did not exceed the number of CETN2 dots during mitosis. However, if the amplified centrioles of triple knockout cells were made with cartwheel structure during mitosis, they would be stained with SAS6 and the number of CETN2 dots co-stained with SAS6 dots would exceed the number of

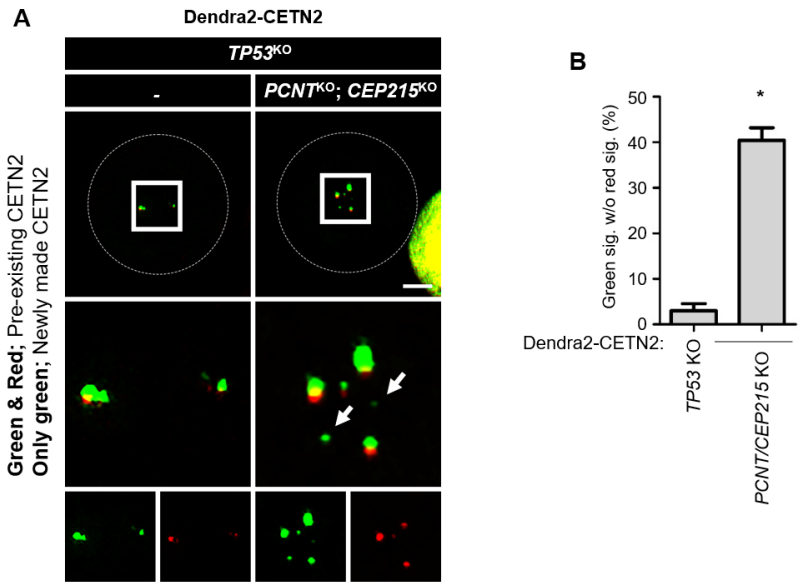

Figure 21. Centriole assembly during M phase in the triple KO cells

(A) The *CETN2-Dendra2* expressing *TP53* KO and triple KO cells were treated with thymidine 24 h followed by STLC for 8 h. After the light activation, the CETN2-Dendra2 signals were observed for up to 2 h. Light activation makes CETN2-Dendra2 detected with 594 nm fluorescence (red). Nascent centrioles were detected only with 488 nm fluorescence (green, white arrow) in triple KO. Scale bar, 4.42 μm. (B) The number of cells containing the centrioles with only green signals was counted. Greater than 20 cells per group were analyzed in three independent experiments. Values were means ± SEM. The statistical significance was analyzed using T-test. *, $P<0.05$.

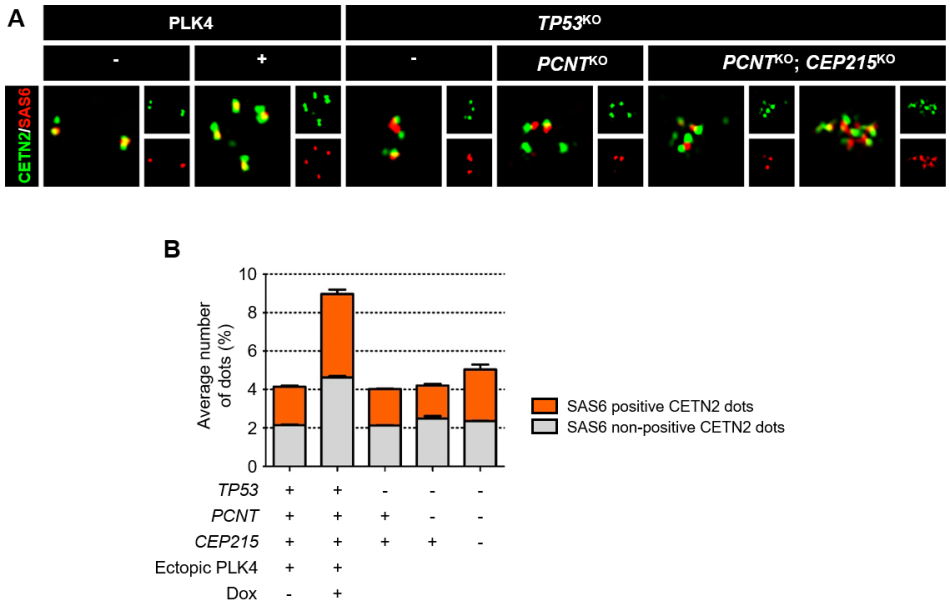

Figure 22. SAS6 signals found in newly made centrioles during M phase

(A) The PLK4 overexpressing and KO cells were treated with STLC for 10 h and coimmunostained with CETN2 (green) and SAS6 (red). Scale bar, 2 μm. (B) The average number of dots of SAS6 positive CETN2 dots (orange) or SAS6 non-positive dots (gray) were counted. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

CETN2 dots those do not co-stained with SAS6 dots. In triple KO cells, about 2 CETN2 dots were SAS6 non-positive dots. The rest were SAS6 positive dots, which positively indicated that the two SAS6 non-positive dots were mother centriole and newly made amplified centrioles were SAS6 positive.

Centriole-to-centrosome conversion in the precociously separated centrioles

I investigated centriole-to-centrosome conversion after precocious centriole separation at the M phase. When *PCNT* deleted cells were arrested at prometaphase with STLC, their centrioles readily separated (Kim et al., 2019) (Fig. 19D). I determined the localization of CEP295 and CEP152 in the precociously separated centrioles at the M phase. The results showed that about halves of the *TP53; PCNT* deleted and *TP53; PCNT; CEP215* deleted cells included three and more CEP295 and CEP152 signals in their centrioles (Fig. 23A,B). On the other hand, CEP295 and CEP152 signals were detected at a centriole pair in most of the *TP53* and *TP53; CEP215* deleted cells (Fig. 23A,B). These results suggested that daughter centrioles readily convert to centrosomes even at the M phase as soon as they were separated from the mother centrioles

Defective centriole-to-centrosome conversion in the triple KO cells

The presence of only two CEP152 positive centrioles out of multiple

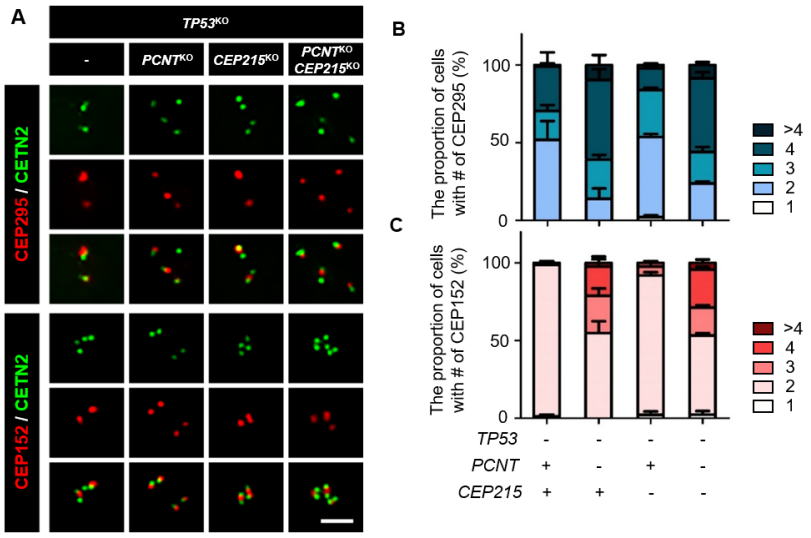

Figure 23. Centriole-to-centrosome conversion in precociously separated centrioles during M phase

(A) The PLK4 overexpressing and KO cells were treated with STLC for 10 h and coimmunostained with CETN2 (green) and CEP295 or CEP152 (red). Scale bar, 2 μm. (B) The numbers of CEP295 and CEP152 signals were counted in the cells. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

ones in the triple KO cells suggested that only a pair of centrioles could recruit PLK4 to generate new procentrioles during the S phase. To examine the intactness of the centrioles in the triple KO cells, I performed coimmunostaining analysis with CEP152 and selected centrosome proteins (Fig. 24A). As expected, most of the centrioles in the control cells were CEP152 positive and also coimmunostained with antibodies specific to CEP295, CEP192, CEP135 and γ-tubulin (Fig. 24B). Most of the multiple centrioles in the PLK4 overexpressing cells were immunostained with all the antibodies I used (Fig. 24B). However, only two centrioles in the triple KO cells were positive for CEP152 (Fig. 24B). Furthermore, CEP295, CEP192, CEP135 and γ-tubulin were detected almost exclusively at the CEP152 positive centrioles (Fig. 24B). These results strongly suggested that only a pair of the CEP152 positive centrioles were intact mother centrioles whereas the rest were defective centrioles in the triple KO cells.

I performed a similar coimmunostaining analysis with the KO cells at mitosis (Fig. 25A). Consistent with the previous results, the control and the KO cells at interphase, had two centrosomes positive to CEP192 and γ-tubulin (Fig. 25A,B). In the mitotic control cells, both CEP192 and γ-tubulin signals were detected at two pairs of centrioles (Fig. 25A,B). The *PCNT* and triple KO cells had centrioles separated and frequently amplified at mitosis. Both the CEP192 and γ-tubulin signals were detected in many of the separated and amplified

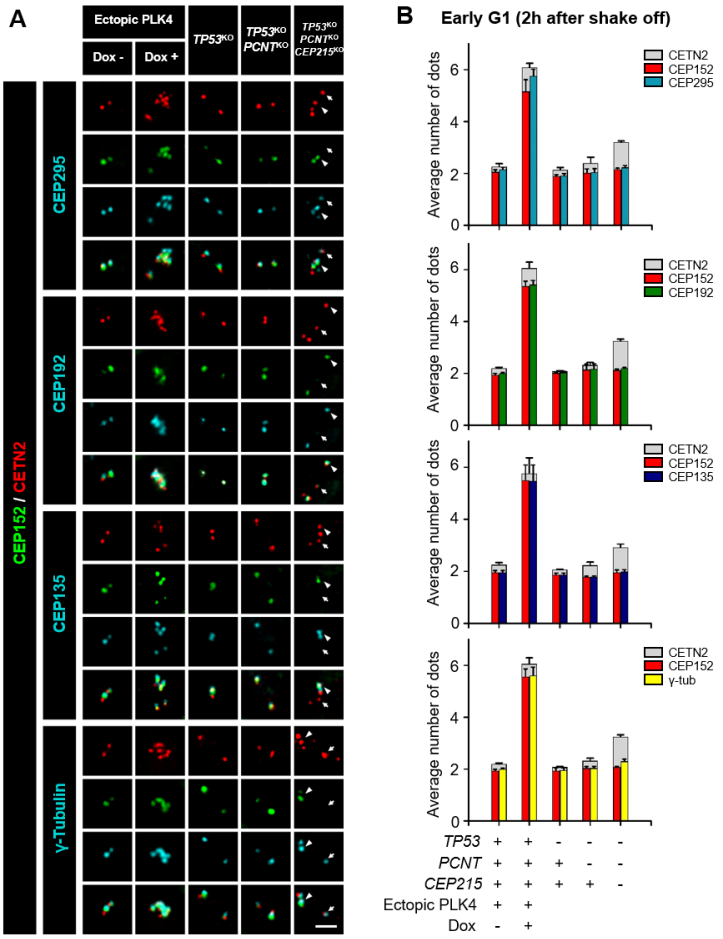
A
Ectopic PLK4
Dox -
Dox +
TP53KO
TP53KO PCNTKO
TP53KO PCNTKO CEP215KO
CEP152 / CETN2
CEP295
CEP192
CEP135
γ-Tubulin
B
Early G1 (2h after shake off)
Average number of dots
CETN2
CEP152
CEP295
CEP192
CEP135
γ-tub
TP53
PCNT
CEP215
Ectopic PLK4
Dox

Figure 24. Determination of intact centrioles in the triple KO cells

(A) The PLK4 overexpressing and KO cells at the G1 phase were triple-stained with antibodies specific to CEP295, CEP192, CEP135 and γ-tubulin (cyan), along with CETN2 (green) and CEP152 (red). Scale bar, 2 μm. Arrows and arrowheads mark the CEP152 positive and negative centrioles, respectively. (B) The number of centrioles with CEP152 and the indicated antibodies were counted. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

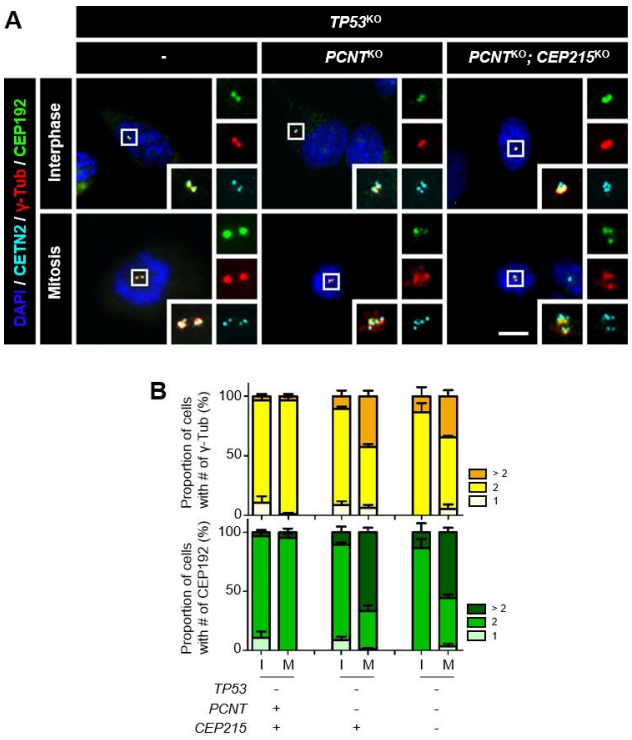

Figure 25. Difference between interphase and mitosis PCM

(A) The KO cells at interphase and mitosis were co-immunostained with antibodies specific to CEP192 (green), γ-tubulin (red) and CETN2 (cyan). Scale bar, 10 μm. (B) The number of CEP192 and γ-tubulin signals were counted in cells at interphase (I) and mitosis (M). Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

centrioles of the deletion cells at mitosis (Fig. 25A,B). This result suggested that precociously separated centrioles might disturb mitotic progression.

Defective microtubule organization in the triple KO cells

I performed microtubule regrowth assays to determine the biological activities of the centrosomes in the PLK4 overexpressing cells and the triple KO cells. In control cells, microtubules started to be organized from both centrosomes present at the G1 phase (Fig. 26A,B). About 6 centrosomes were present in the PLK4 overexpressing cells and 91% of them were able to organize microtubules (Fig. 26A,B). However, in the tripled KO cells, only 73% of the centrosomes organized microtubules, leaving 27% without the activity (Fig. 26A,B). These results suggested that a significant fraction of the centrosomes in the triple KO cells have functional defects in microtubule organization during interphase.

I determined spindle configurations in mitotic cells of the PLK4 overexpressing and triple KO cells. Spindle pole phenotypes were categorized into five groups following Watanabe et al (Watanabe et al., 2019); lagging chromosome, chromosome misalignment, monopole, bipole and multipole (Fig. 27A,B). As expected, most of the mitotic control cells formed bipolar spindle poles (Fig. 27B). In PLK4 overexpressing cells, about 60% of mitotic cells

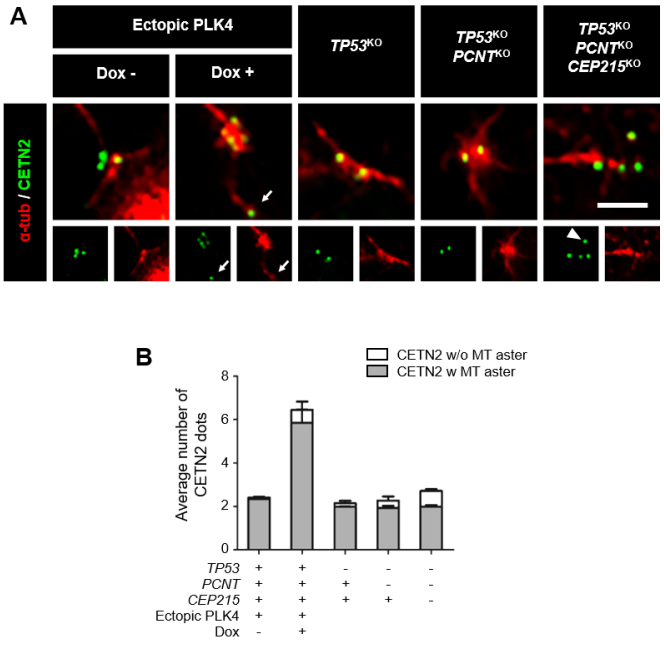

Figure 26. MTOC activity in the supernumerary centrioles during interphase

(A) The PLK4 overexpressing and KO cells at the G1 phase were subjected to microtubule regrowth assays. The cells were co-immunostained with antibodies specific to CETN2 (green) and α-tubulin (red). Scale bar, 2 μm. (B) The number of CETN2 dots with and without microtubule asters were counted. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

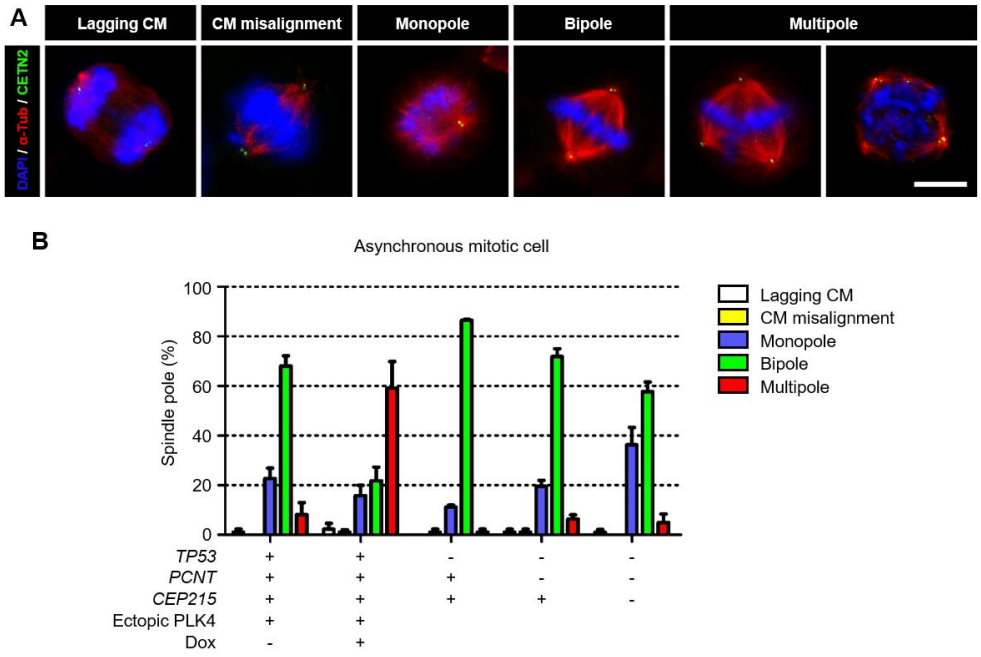

Figure 27. MTOC activity in the supernumerary centrioles during mitosis

(A) The PLK4 overexpressing and KO cells at mitosis were subjected to co-immunostaining analysis with antibodies specific to CETN2 (green), α-tubulin (red) and DAPI (blue). Representative abnormalities of the spindle poles were shown. Scale bar, 10 μm. (B) Mitotic cells with abnormal spindle poles were counted. Greater than 30 cells per group were analyzed in three independent experiments. Values were means ± SEM.

formed multipoles (Fig. 27B). On the other hand, the proportion of monopoles were significantly increased up to 40% in the triple KO cells but the mitotic cells with multipoles were insignificant (Fig. 27B). These results indicate that many of the separated centrioles in the triple KO cells have limited ability to function as spindle poles during mitosis.

Discussion

Here, I generated *TP53, PCNT* and *CEP215* triple KO cell lines and determined their phenotypes at the centrosome. I observed that centrioles in the triple KO cells precociously separated and amplified at the M phase. Many of the triple KO cells maintained supernumerary centrioles throughout the cell cycle. However, the number of centrioles did not double during the S phase. It is likely that, in the triple KO cells, supernumerary centrioles, many of which were assembled during the M phase, could not function as templates for centriole assembly during the S phase (Fig. 28). On the other hand, supernumerary centrioles in PLK4 overexpressing cells served as the template for centriole assembly at the subsequent S phase (Fig. 28).

The M phase assembled centrioles in the triple KO cells failed to duplicate during the S phase. In contrast, the S phase assembled centrioles in PLK4 overexpressing cells doubled in the next S phase. These results strongly suggested that the majority of the M phase assembled centrioles lack the ability to function as the template for nascent centriole assembly during the S phase. Only two out of multiple centrioles in the triple KO cells were positive to CEP152, a scaffold for PLK4 in mother centrioles. Known preceding components for the centriole-to-centrosome conversion, such as CEP135, CEP295 and CEP192,

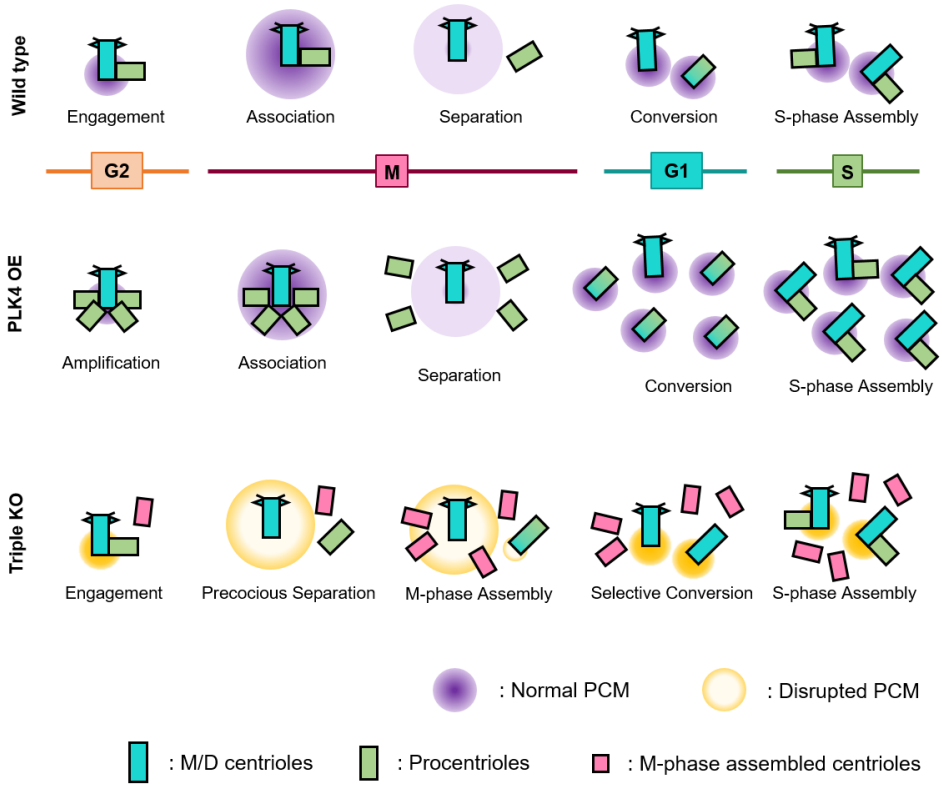

Figure 28. Summarized model for the duplication amplified centrioles.

At the early M phase, daughter centrioles readily disengaged from mother centrioles, but remain associated with mitotic PCM. Daughter centrioles eventually separated from the mother centrioles after PCM was disintegrated at the end of mitosis. PLK4 overexpression generated supernumerary centrioles most of which could function as templates for centriole assembly in the subsequent S phase. Deletion of *PCNT* and *CEP215* makes mitotic PCM disorganized. As a result, daughter centrioles precociously separated from mother centrioles at the M phase and centriole amplification occurred. The M phase assembled centrioles, however, could not convert to mother centrioles at the mitotic exit. They did not organize microtubules, nor function as templates for centriole assembly in the subsequent S phase, and were detected throughout the cell cycle.

were also detected almost exclusively at the CEP152 positive centrioles, indicating that only a single out of many centrioles in a triple KO cell could convert from centriole-to-centrosome during mitotic exit. It could be suspected that two CEP152 positive centrioles might be generated at the previous S phase, while the other centrioles were assembled at the M phase. It remained to be investigated why the M phase assembled centrioles could not convert to the centrosome during mitotic exit. A possibility is that centriole assembly processes cannot compressively proceed within a short M phase. As a result, the M phase assembled centrioles might not recruit a series of centrosomal proteins necessary for the conversion until the end of mitosis. It remains to be identified what factors are critically absent for the M phase assembled centrioles to undergo conversion.

Once a daughter centriole converts to a mother centriole during mitotic exit, it acquires an ability to recruit PCM (Fu and Glover, 2016; Wang et al., 2011). I observed that almost all amplified centrioles in the PLK4 overexpressing cells could organize microtubules. As a result, the majority of the PLK4 overexpressing cells formed multipoles in the M phase. On the other hand, a significant proportion of centrioles in the triple KO cells failed to organize microtubules in interphase. These results also support the notion that the M phase assembled centrioles could not convert to centrosomes during mitotic exit. Consequently, they hardly function as microtubule organizing centers during the

cell cycle. Nonetheless, I do not rule out the possibility that a fraction of the M phase assembled centrioles may acquire an ability to organize microtubules, especially during mitosis.

Supernumerary centrioles are common in cancer cells (Chan, 2011; Marteil et al., 2018). Many cells with supernumerary centrioles can complete mitosis by forming a bipolar spindle with clustered centrosomes (Ganem et al., 2009). Nonetheless, bipolar spindle formation through centrosomal clustering is associated with an increased frequency of lagging chromosomes during anaphase, thereby explaining the link between supernumerary centrosomes and chromosomal instability (Ganem et al., 2009).

In this work, I argue that supernumerary centrioles in the *TP53; PCNT; CEP215* deleted cells are not sufficient enough to generate tumors. These supernumerary centrioles could not act as a microtubule organizing center, nor seem to be capable of duplication during interphase so they could not create normal procentriole. Although supernumerary centrioles are found in triple KO cells, only two centrosomes act properly during the cell cycle. This might be the reason why the growth of tumors was not observed in Plp (Drosophila PCNT) and cnn (Drosophila CEP215) mutant injected Drosophila larval brain tissue (Castellanos et al., 2008). The M phase assembled centrioles might not be as harmful as the S phase assembled centrioles. Nonetheless, they still have a chance

to organize microtubules in the M phase and disturb the progress. My works have been done on a cellular basis, eliminated *TP53* to avoid the apoptotic pathway. So the limitation remains whether this phenomenon I observed can be applied to the actual model. Future works are required to determine the heterogeneity of centrioles in diverse cancer cells and knockout mice.

CONCLUSION

PCM surrounds centrioles and regulates diverse centrosomal functions, including microtubule organization. In the dissertation, I removed *CEP215*, an important PCM protein, in *TP53* and *TP53; PCNT* knockout cells and studied the phenotypes. The centrioles in the knockout cells precociously separated and duplicated during mitosis. The centrosome amplification phenomenon was intensified in the *PCNT* and *CEP215* double knockout cells than the *PCNT* single knockout cells. Based on the results, I propose another function of PCM, which protects centrioles from precocious separation and amplification during mitosis.

Centriole amplification is frequently observed in many cancer cells. Several mechanisms have been proposed to elucidate centriole amplification, such as cytokinesis failure and PLK4 overexpression. My research proposes another pathway for centriole amplification, but did not examine it yet. Therefore, it is important to determine that centriole amplification occurs with the PCM failure in other cancer cells.

CEP215 is one of genes whose mutations are detected in the microcephaly patients. However, it remains to be clearly elucidated how *CEP215* mutations causes microcephaly. My results revealed that centrioles are

precociously separated in *CEP215* deleted cells and sometimes amplified with additional mutations of other genes. This mutant phenotype may be linked to microcephaly. Therefore, it is worth to determine abnormalities in mitotic PCM in the microcephalic brains of the *CEP215* deleted mice.

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국문 초록

중심체는 동물 세포에서 미세 소관을 형성하는 주요 기관으로, 중심립과 중심구로 이루어져있다. 중심구는 미세 소관의 말단에 집적하여 세포내 미세소관 망을 구축하는 역할을 하고, 중심립은 이러한 중심구를 모으는 중추의 역할을 한다. 중심체의 복제는 세포 주기와 밀접하게 연관되어있다. DNA 복제 주기와 비슷하게 S기 동안에는 중심립의 개수가 2개에서 4개로 늘어나고 M기에는 두개의 중심체가 세포의 양끝으로 이동해 양극성 방추사를 형성한다. 중심체는 간기 동안에는 세포의 모양, 세포 내 운 수송을 용이하게 하고 중기 동안에는 세포 분열에 중추적인 역할을 한다. 미세 소관을 만드는 역할은 중심구에서 이루어지지만 이러한 중심구를 모으는게 중심립이기 때문에 중심립 개수의 이상은 세포의 중기에 특히 문제를 야기하고 이는 염색체 수 이상, invapodopia를 유발하여 암과 밀접하게 연관되어 있다고 알려져 있다. 중심체의 주요 단백질인 CEP215와 PCNT가 동시에 결실된 상황에서 이의 표현형을 관찰함으로써 이들 단백질들의 기능을 총체적으로 이해하고자 하였다.

제 1장에서는 중심체 주요 단백질인 CEP215의 세포분열기에 중심립 결합과 새로운 중심립 복제에 있어서의 중요성에 대하여 연구하고자 하였다. CEP215는 기존 knockdown실험으로 밝혀진

바에 의하면 γ-tubulin의 중심체로의 밀집에 중요한 역할을 한다고 밝혀져 있고, 중기에 중심체의 결합에 중요하다고 알려져 있다. CEP215의 유전자가 아예 발현하지 않는 세포 주를 제작해 이른 중기를 관찰한 결과, 중심립의 이격 현상을 관찰할 수 있었다. CEP215의 다양한 발현 억제 표현형을 관찰한 결과 이 이격 현상에는 CEP215와 PCNT의 결합이 중요하다는 것을 확인할 수 있었다. 또한 CEP215와 PCNT의 동시 결핍 세포 주를 제작, 관찰한 결과에서는 CEP215가 새로운 중심립을 형성시킬 수 있게 하는 것에 있어 중요함을 알 수 있었다.

제 2장에서는 CEP215와 PCNT의 동시 결핍 세포 주에서 보이는 과 복제 중심립들이 언제 만들어지는지, 이 중심립들이 어떻게 되는지 연구하고자 하였다. PLK4를 과 발현 시켜 만들어지는 과 복제 중심립들과 세포 주기 동안 비교 분석하였다. 중심체 단백질들이 없어지면서 만들어지는 이 과 복제 된 중심립들은 M기에 형성되는 것으로 보였으며, PLK4가 과 발현되면서 만들어지는 중심립들은 S기 동안 증폭되는 것을 관찰 할 수 있었다. 또한 knockout 세포 주의 과 복제 중심립들은 PLK4 과 발현을 통해 만들어진 과 복제 중심립들과 다르게 새로운 중심립을 만들지 못하며 미세소관도 만들지 못하는 것을 관찰 할 수 있었다. 따라서 이 연구를 통해 중심구의 파괴도 중심립의 과 복제 현상을 야기시킬수

있으며, 이로 인해 M기에 만들어진 중심립들은 세포주기동안 중심체로서 역할을 하지 못하는 것을 발견할 수 있었다.

주요어: 중심체, 중심체 접합, 중심립, 중심립 복제, 중심립 과복제

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