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Centriole Duplication in PE (SPEV) Cells Starts before the Beginning of the DNA Replication

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Abstract—Centrosome includes two centrioles and is a structural basis of mitotic spindle pole. Duplication of this organelle and doubling of chromosomes quantity during DNA replication are two principal events of cell cycle in the course of preparation for cell division. In this work, cells of pig kidney embryonic cell line PE (SPEV) were individually monitored after mitosis and procentriole appearance was detected by electron microscopy as soon as 5–6 h after mitosis. This period was 1–2 h shorter than minimal duration of G_1-phase in PE cell line. Ultrastructural analysis of centrosomes in the cells with known "cell cycle age" in combination with autoradiography study of the same cells using ^{3}H-thimidine directly confirmed that duplication of centrioles started earlier than cells entered in S-phase of cell cycle, i.e., preceded the DNA replication.

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Process of centriole duplication by analogy to the process of DNA doubling is often called "replication", because the new copy of this organelle is usually formed close to already existing centriole. As it has been shown earlier for DNA [1, 2], "replication" of centrioles is semi-conservative; experiments with biotinilated tubulin injection in cells have shown that each daughter cell gets one "old" centriole and one newly formed centriole [3]. However, the mechanisms of DNA replication and "replication" of centrioles are essentially different.

Centriolar cylinders can appear in the cell in two ways. First, new centriolar cylinders can arise de novo without direct connection with already existing centriole [4–7]. Alternatively, they appear near the surface of pre-existing centrioles or basal bodies, that is characteristic for the majority of cell types. Process of centriole "replication" has no analogous mechanisms; the new copy is created not along with the old one, but perpendicular to it. Moreover, a small distance filled by fine fibrillar material is always present between the mother and daughter centriolar cylinders [8].

Near each parent centrioles only one new centriolar cylinder usually appears; Gall proposed to name it the "procentriole" [8]. In the majority of cells, the number of centriolar cylinders corresponds to the number of genome copies (ploidity): in diploid cell in G_1 phase of cell cycle there are two centrioles, and in tetraploid cell in G_2 phase, four centrioles [9]. This accordance maintains to a certain extent for polyploid cells, too [10].

In contrast to DNA, whose doubling can be revealed by the inclusion of labelled precursors, the beginning of centrioles doubling can be shown reliably only by electron microscopy. The data about centriole ultrastructure in cells of different stages of a cell cycle allowed correlating events of the cell cycle and of the centriolar cycle [9, 11]. Stubblefield proposed to subdivide the centriolar cycle into the phases of maturation, reproduction and separation of centrioles [11]. Robbins with co-authors [9] for the first time described the basic events of centriolar cycle of somatic cells and found that the first structural reorganization of centrosome structure occurred in early G_1-phase, when centrioles loose the typical for mitotic cells mutual-perpendicular orientation. He added the phase of "disorientation" in description of centrosome cycle. Unlike the phases of a nuclear cycle replacing each other, the phases of centriolar cycle are usually overlapped. In particular, in significant part of cell types the centrioles separate simultaneously or just after the disorientation phase in early G_1-phase [12]; in other cell types, the separation occurs after the beginning of duplication centrioles, i.e. during the phase of maturation [13].

The principal question of nuclear and centriolar cycles is what starts earlier, the duplication of centrioles or DNA replication? Different authors do not have the common opinion. Robbins with co-authors showed that in synchronized HeLa cell fraction with 20% cells in S-phase, centriole replication at the early stages was detected in 65–70% of cells [9]. It means that at least in part of the cells, centriole replication started in G_1. On the other hand, in one of the classical works on the ultrastructural analysis of centriolar cycle in PE (SPEV) cells, procentrioles were found only during S-phase of cell cycle [14].

Independence of the beginning of centrioles duplication from DNA synthesis was shown experimentally [15, 16]. The principal mechanism of cellular regulation of centrioles doubling was described in [17–19]. However, no detailed studies of the succession of nuclear and centriolar events in cells with known "age" in a cell cycle have been performed yet.

In the present study, the ultrastructural analysis of centrosomes on serial sections of cells that were previously observed after mitosis was used in combination with autoradiography study of the same cells; the data show that centriole duplication in PE (SPEV) cells can start before the beginning of DNA replication.

EXPERIMENTAL

Vital observation of cells. Cell line PE (SPEV; pig kidney of embryonic age) was obtained from the Russian collection of cell cultures (RCCC, St. Petersburg). Cells were cultured at 37°C in 5% of CO_2 atmosphere in cell culture medium "199" supplemented with 10% embryonic calf serum (Vector, Russia) and antibiotics. For experiments, the cell were plated on coverslips and cultured in Petri dishes. For vital observation a special camera was constructed allowing medium change during vital observations. The camera volume was 1.25 ml; 25-mm coverslips forming top and bottom of the camera were strictly parallel, to allow for high-quality phase contrast (objective Plan-Neofluar 40/0.9, Opton, Germany). During the observations and recordings, orange filter was used, and the temperature of the microscope stage was kept constant at 37°C. Cells were photographed using MFN-12 photo equipment 2–3 times during mitosis, and subsequently once an hour until fixation.

Autoradiography and electron microscopy. For autoradiography, cells were cultured in the full medium containing [^{3}H] thymidine (400 kBq/ml) during one hour before fixation. The cells were then fixed in 2.5% glutaraldehyde (Fluka, Germany) in phosphate buffer (pH 7.4), postfixed with osmium tetroxyde (Agar Scientific LTD, UK), dehydrated in ethanol and acetone, and embedded in EPON-812 resin according to a standard technique. After polymerization of the resin, coverslips were removed from the blocks in liquid nitrogen and the surfaces the blocks were covered (under a weak red light) with photo emulsion of type "M" (NIIKhIM-FOTOPROEKT, Russia). Several control samples of the same block containing individually monitored cells were covered by photo emulsion simultaneously with the sample, so that to determine an optimal time of exposition, which was found to be 10 days. In the developed autographs, the experimental cells were then located and photographed by means of the light microscope equipped by phase-contrast system. Serial ultra thin sections (70 nm) of these cells were obtained using ultramicrotomes LKB-III and LKB-V (LKB, Sweden). Sections were mounted on single slot grids with Formwar film and were additionally contrasted by incubation (25 min) in aqueous solution of 4% uranyl acetate and in lead citrate by Reynolds. Samples were investigated and photographed using electron microscope HU-11, HU-12 (Hitachi, Japan) or Philips CM-12 (Netherlands).

RESULTS

The detailed ultrastructural analysis of the cells monitored after mitosis has shown that centriole replication starts much earlier than it could be expected on the basis of results of the previous study of SPEV cells [14], even if the duration of a G_1-phase in these cells was minimal. The present work was initiated by the previous study, which showed an unusually early beginning of the centriole replication (6 h after mitosis) in control sister cells with intact centrosomes [20]. A detailed monitoring of cells after the end of mitosis performed in the present study has confirmed the results obtained in [20].

In the first series of experiments, 19 cells were monitored individually and used for the ultrastructural analysis. In cells fixed at 3–4 h (5 cells) and 4 h–4 h 40 min (5 cells) after the beginning of anaphase, the replicated centrioles were never found.

For the first time the beginning of procentriole growth near the proximal ends of mother centrioles was detected in the cell fixed 5 h 6 min after the beginning of anaphase, i.e., less than 5 h after the end of mitosis. In all investigated cells fixed 5–6 h (4 cells) and 6 h–7 h 10 min after mitosis (5 cells) the short procentrioles were found. Thus, according to these experiments, replication of centrioles starts at least 1–2 h prior to the beginning of DNA replication, which for SPEV cells begins not earlier than 7 h (10 h in the average) after mitosis [21].

However, all these results, as well as the data of previous works, are indirect. Theoretically, it cannot be excluded that the investigated cells had an abnormally short G_1-phase. For the direct verification of the obtained data, the experiments with parallel evaluation of the "age in cell cycle" by means of vital monitoring, the assessment of the phase of a cell cycle by autoradiography, and the ultrastructural analysis of centrosome in the same cells were carried out. Live cells were monitored and fixed at 5 h 48 min to 8 h 50 min (9 cells) after mitosis. Due to the high quality of the photo emulsion and the optimal time of exposition, the labelling of cell nucleus in synthetic phase of a cell cycle differed from background by a factor of 100 or more, which considerably facilitated the subsequent analysis of the results.

Some nuclei of the investigated cells in G_1-phase did not reveal any ^{3}H-thymidine incorporation; an example of a centrosome region of one such cell is shown in Fig. 1. None of the nine individually monitored cells incorporated ^{3}H-thymidine in their nucleus, i.e., all of them were in G_1-phase at the moment of fixation. Three

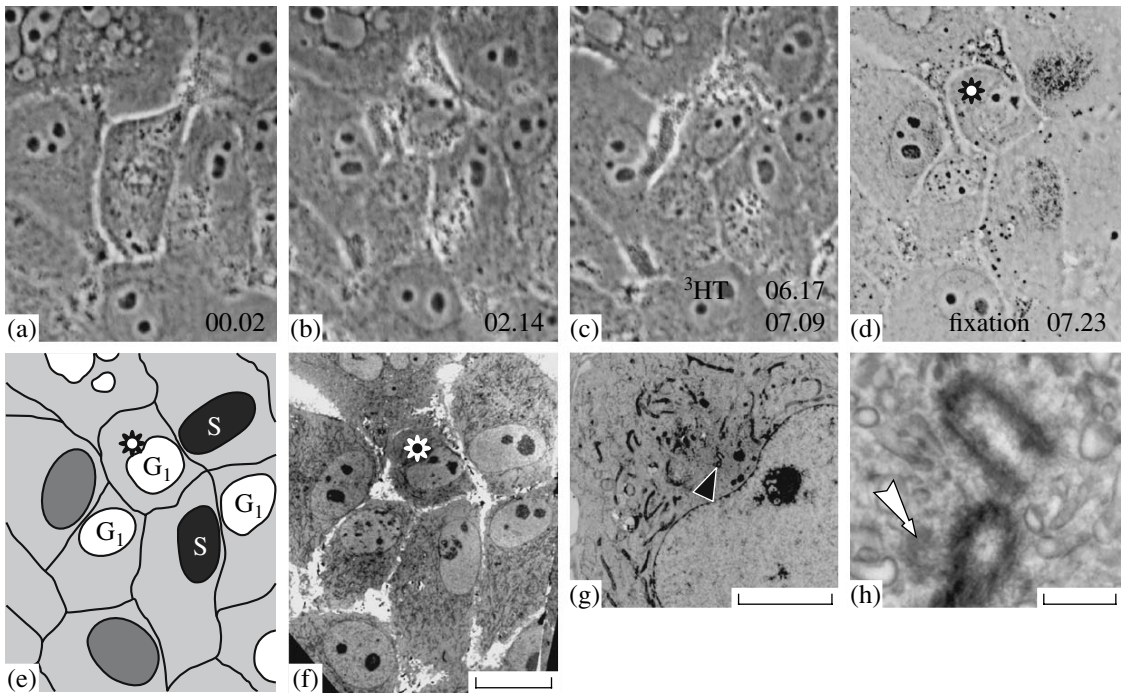

Fig. 1. Autoradiography study of cells and centrosome ultrastructure 7 h 23 min after the beginning of anaphase. (a–c) Vital observation of the cells; time after anaphase of mitosis is shown on the photos. In (c) the time of [^{3}H] thymidine addition to the culture medium is also indicated. (d) Cells after fixation and autoradiography development, time period from anaphase of mitosis to fixation is shown. (e) Scheme of cells contours corresponding to phase contrast (d) and low magnification EM (f); letters on cell nuclei designate a phase of a cell cycle; dark grey hatched cells may be either in G_2 or in G_1 phase. (g, h) Centrosome region at intermediate and high magnification. Asterisk in (d–f) designates the cell whose centrosome was further studied; the arrowhead in (g) shows a centrosome region, and the arrow in (h) points to a young procentriole. Scale bar, 20 (a–f), 4 (g), and 0.2 μm (h).

out of these cells were used for the ultrastructural analysis. In all of them procentrioles were found near the parent centrioles (Figs. 1g and 1h). There are two cells with intensively labelled nucleus (cells in S-phase), and also some cells with small unlabelled nuclei (cells in G_1-phase), including a cell which was monitored from the beginning of anaphase and in which centrosome was further investigated by means of electron microscopy of serial sections (Figs. 1d and 1e).

Besides, there were two cells with large nuclei, one of them had an unlabelled nucleus, and in the other, the nucleus was faintly labelled (Fig. 1d). These cells could be either in G_2- or in G_1-phase (Fig. 1e), since in SPEV cell culture there is always a certain proportion of polyploid cells with nuclei of greater size than in normal diploid cells. Thus, the data of direct experiment have confirmed a hypothesis that replication centrioles in cells SPEV can precede DNA replication.

Interestingly, the length of procentrioles in all investigated cells did not exceed 100 nm, i.e., noticeable lengthening of procentrioles did not occur during G_1-phase. We did not found any clear correlation between the cell age and the length of procentrioles; procentrioles in more "advanced" (within a cell cycle) cells often were shorter than in "younger" cells. At the same time, in spite of the fact that the majority of procentrioles were 75–95 nm in length, in three cells the procentrioles were shorter than 50 nm. This possibly corresponded to the initial stages of centriole duplication (Fig. 2). "Age" of these cells in a cell cycle was either less (Figs. 2a and 2b) or bigger (Fig. 2c) than the age of the cells with ~80-nm procentrioles (Fig. 1).

DISCUSSION

For a better understanding of centrosomal cycle and other intracellular processes, a combination of morphological and biochemical approaches is necessary. Biochemical mechanisms of the regulation of centrioles replication were discovered independently in three laboratories [17–19]. The basic stages of this process are the following. In the second half of G_1 restriction point (R-point) a cell "decides" whether to enter the next mitosis or not. At that moment, a complex of the biochemical reactions starts. These reactions depend on a number of factors, such as cell size, the presence of external growth factors and other conditions of growth of a cell, and on the cell interactions with surrounding cells. The complex of Cyclin D/CDK 4/6 phosphorylates the protein pRB. This protein loses the ability to connect with factors of activation of transcription EF2. Released factor EF2 activates the synthesis of cyclins E and A that leads to the beginning of centriole duplication [17–19].

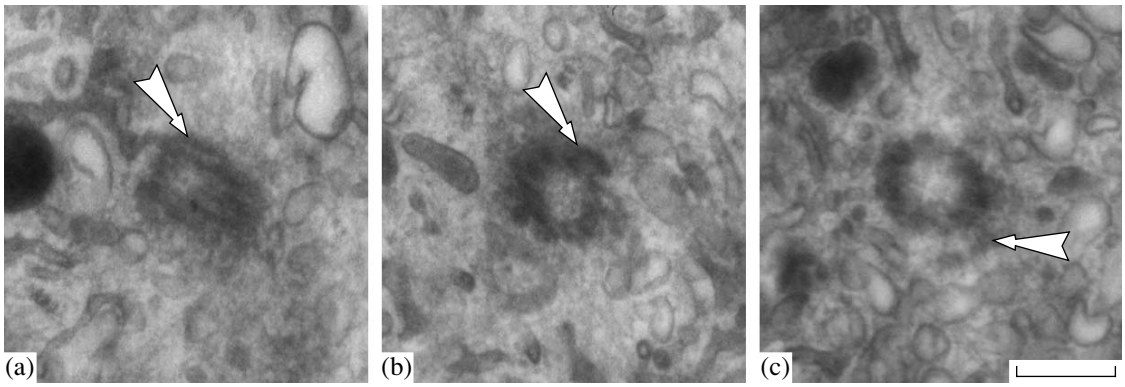

Fig. 2. Ultrastructure of centrosome during the second half of G_1-phase of a cell cycle. Early stages of centriole duplication. (a, b) The appearance of procentrioles (arrow) near the surface of two parent centrioles in the cell fixed 6 h 14 min after the beginning of anaphase. (c) Procentriole at the stage of annular structure formation at a 50 nm distance from parent centriole in a cell fixed 8 h 23 min after the beginning of anaphase. Scale bar, 0.2 μm.

The processes of DNA replication and duplication of centrioles are regulated by the cytoplasmatic mechanism involving cyclins. Centrioles doubling cannot begin before the achievement of the restriction point, or, in other words, prior to the beginning of the activation of synthesis of cyclins E and A. Location of key regulators in centrosome gives the reason to believe that centrosome is the site of the cell "decision" to pass restriction point and to continue cell cycle. In the present work, a comparative study of nuclear and centrosomal cycles in SPEV cells has been carried out. The obtained results are in a good agreement with the data obtained on HeLa cells [9] and L929 cells [15], but differ from the data obtained on SPEV cells earlier [14].

In reports [9, 15], cells were synchronized in mitosis, and the analysis of cell cycle stages combined with ultrastructural analysis of centrioles was further performed during a long-term cultivation of the cells. In contrast to the present work, the study of a transition time to S-phase relative to the time of the procentriole appearance was exclusively statistical, without simultaneous analysis of individual cells by both methods. In these works it was shown that the percentage of cells with procentrioles in the same fractions was always higher than the percentage of cells in S-phase [9, 15]. Furthermore, procentriole occurrence took place after the inhibition of the beginning of DNA replication by arabinosyl cytosine [15]. Thus, SPEV cell line was an exception from the general rule and characterized by "late" procentriole occurrence in cell cycle [14].

It is well known that parameters of a cell cycle essentially depend on cell culture conditions and in particular, on cell density, time after cell replating, and even on the position of a cell relative to the edge of cell islet [22]. Although the composition of cell medium is standardized, the composition of serum, which is a source of growth factors, may vary and thus influence the parameters of a cell cycle of the same cell culture. This can be one of the explanations of the difference of the data presented here from the results reported in [14]. According to these results, centrioles in SPEV cells start to duplicate only during S-phase of a cell cycle.

However, cell cycle stages of analysed cells were determined exclusively on the basis of autoradiography without monitoring live cells after mitosis. Therefore, cells in late G_1-phase could be excluded from the analyzed group in this work [14]. Furthermore, authors did not find the duplicated centrioles in the part of S-phase cells [14]. Several explanations of the contradiction of these results to our observations are plausible. First, in the present work the quantity of analysed cells was probably insufficient to find the cells without procentrioles in a late G_1-phase. The detection of the earliest stages of procentriole formation more than 8 h after mitosis could signify that probably not all of the cells started centriole duplication prior to the beginning of an S-phase. Since in the present work S-phase cells were not studied, it is not possible to prove that centriole duplication could not begin later than what was shown here. Second, the variability of growth factors in serum used for cell culture could lead to the displacement of centriolar cycle relative to the nuclear cycle. Third, at the earliest stages the procentrioles are clearly visible only on sections that have a "good" orientation; thus, a part of procentrioles at the early stages might not be found or were not identified as procentrioles by the authors of [14]. The last supposition is highly probable, as the length of the "earliest" procentrioles in that study was ~0.1 μm [14]. On the other hand, in the present work all procentrioles found in G_1-stage cells were shorter than 100 nm and even shorter that 50 nm in three cells (Fig. 2), which at other orientation of the section could be invisible. The first description of the earliest stages of procentriole formation was made by Sorokin in chick duodenal epithelial cells [5]. The morphology of very young procentrioles in SPEV cells is practically identical to this description. At the first stage, an electron-dense extension was formed on a surface of the parent centrioles (Fig. 3 in [5] and Fig. 2a, b in the present paper).

Further, the newborn procentriole formed at the distance about 50 nm from surface of parent centriole with annular structure accompanied with visible "antrum" appearance in the central part of centriole (Fig. 4 in [5] and Fig. 2c in the present paper).

In summary, there are grounds to assume that once a cell reaches a restriction point, the centriole replication process can be activated at a certain period of cell cycle, starting from the second half of G_1-phase. SPEV cell line is not an exception in this regard, and the dynamics of the procentriole occurrence generally coincides with that in HeLa [9] and L929 cells [15].

It should be noted that centrosome intactness plays a key role in the cell cycle progression. A damage or removal of centrioles at various phases of cell cycle may have various consequences. If centrioles were damaged by a microirradiation in anaphase of mitosis, cells could finish division [23] but never reached S-phase [24]. However, if centrioles were destroyed by a laser microirradiation after transition of a restriction point (synchronization by hydroxyurea between G_1- and S-phase during the time period equivalent to 1.5 cell cycles), new centrioles formed de novo similarly to their formation in mouse blastomers [25]. Such cells were capable to continue cell cycle and to enter mitosis. The results of the present work can explain, why cells synchronized by hydroxyurea (or aphidicolin) were capable to continue centriolar cycle [16] and even to re-establish the destroyed centriolar cylinders [25]. As these cells have already passed a restriction point, cytoplasm of these cells already possessed the factors necessary for centriole replication, irrespective of whether replication DNA proceeds or not. Restoration of centrioles after microirradiation [25] is one of the proofs that reproductive ability of centrioles is not directly correlated with their triplet structure. Transfer of the information about triplet structure can occur not only through the deuterosomes (ciliogenesis), but also through other cytoplasm precursors that have no centriolar structure, as it takes place during oogenesis and embryogenesis.

We reported here that lengthening of procentrioles over 100 nm did not occur during G_1-phase. This corresponds to the results of Gorgidze and Vorobjev [26] who found that in cytoplasts, i.e., in cells with removed nuclei, new procentrioles could appear, but their lengthening was not detected. On the contrary, if cell cycle was experimentally blocked in mitosis, the lengthening of centrioles did occur so that their length exceeded their normal size [27].

Thus, it may be concluded that restriction point is the point of coordination of cell (nuclear) and centrosome cycles. The complex of external and internal signals triggers centriole replication that serve a morphological criterion of cell transition through the restriction point from the G_1-post-mitotical (G_1-pm) to the G_1-presynthetical (G_1-ps) phases [28].

However, the procentriole growth stops at a certain stage, since the cell enters S-phase. If DNA replication is normal, cell "allows" the continuation of centriole elongation process. Further on, after the end of DNA replication, centrosomes receive a signal for the separation and formation of the poles of mitotic spindle.

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