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The importance of a single primary cilium

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The centrosome is the main microtubule-organizing center in animal cells, and helps to influence the morphology of the microtubule cytoskeleton in interphase and mitosis. The centrosome also templates the assembly of the primary cilium, and together they serve as a nexus of cell signaling that provide cells with diverse organization, motility, and sensory functions. The majority of cells in the human body contain a solitary centrosome and cilium, and cells have evolved regulatory mechanisms to precisely control the numbers of these essential organelles. Defects in the structure and function of cilia lead to a variety of complex disease phenotypes termed ciliopathies, while dysregulation of centrosome number has long been proposed to induce genome instability and tumor formation. Here, we review recent findings that link centrosome amplification to changes in cilium number and signaling capacity, and discuss how supernumerary centrosomes may be an important aspect of a set of cilia-related disease phenotypes.

Introduction

The centrosome is a complex organelle that functions as the major microtubule-organizing center (MTOC) in animal cells. It plays an important structural role in a plethora of cellular processes including intracellular transport, cell polarity, migration and division. The centrosome also functions as a signaling hub that mediates important aspects of cell physiology, such as orchestrating G1/S transition, entry into mitosis, cytokinesis, and monitoring of DNA damage. At the core of the centrosome is a pair of pinwheel-shaped microtubule structures called centrioles, which are surrounded by pericentriolar material (PCM; Fig. 1). In animal cells, centrioles contribute to centrosomal functions through recruitment and organization of the PCM, thus facilitating the organization of both the interphase microtubule array as well as the mitotic spindle. However, it is becoming increasingly evident that centrioles have evolved for the primary purpose of templating the assembly of cilia, essential sensory and motile organelles.

Except for a few specialized cell types, almost all cells in the human body contain a single centrosome and a single (primary) cilium. The primary cilium is a non-motile microtubule-based organelle that protrudes from the apical surface of most mammalian cells (Fig. 1). Its assembly is templated by the older of the two centrioles in quiescent cells, and it is disassembled as cells re-enter the cell cycle. Primary cilia are a nexus of cell signaling, associated with regulation of the Notch, Hedgehog, PDGF, TOR and Wnt signaling pathways, among others. Components of these signaling pathways are concentrated within the ciliary compartment, and this is critical for efficient pathway activation. It is now well established that mutations in proteins that localize to, and function through, cilia cause human disease conditions termed ciliopathies, a group of clinically and genetically overlapping disorders whose etiologies lie in defective ciliary structure and function (for recent reviews see refs. 15–17). Since the assembly of the cilium is dependent on centrioles, mutations that affect the structure and function of centrioles, and the centrosome in general, can also impact normal ciliary function.

Keywords: centrosome, cilium, centriole, ciliopathy, cancer

Abbreviations: MTOC, microtubule-organizing center; PCM, pericentriolar material; PKD, polycystic kidney disease; TSC, tuberous sclerosis; MKS, Meckel-Gruber syndrome; Shh, Sonic hedgehog; Smo, smoothened

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Cells control centrosome number using a duplication and segregation mechanism linked to the cell cycle. Centrosome number is predominantly regulated through the control of centriole duplication. Like DNA, centrioles duplicate in a semi-conservative fashion once and only once per cell cycle, ensuring that each daughter cell inherits one centrosome (with two centrioles) at the end of cell division. The two centrioles present in a quiescent or G₁ animal cell differ in age: the older of the two centrioles (which was assembled at least two cell cycles ago) is termed the mother centriole, and the younger centriole (which was assembled in the previous cell cycle) is called the daughter centriole. Centriole duplication begins at the G₁ to S phase transition with the formation of one procentriole adjacent to each pre-existing parental centriole. The procentrioles continue to grow during the G₂ phase of the cell cycle, and the newly formed centriole pairs move to opposite sides of the cell and help establish the two mitotic spindle poles. During mitosis, the two pairs of centrioles are segregated by the mitotic spindle such that each daughter cell inherits a centriole pair, and thus ends up with one centrosome and the ability to form one primary cilium. To ensure the fidelity of this process, cells have evolved regulatory mechanisms to tightly control the centrosome duplication process throughout the cell cycle. So, what happens when the regulation of centrosome number goes awry?

**Centrosome Amplification and Cancer**

An important connection between centrosome abnormalities and cancer was first proposed by Theodore Boveri over a century ago. Since then, researchers have been trying to determine how centrosome defects contribute to cell transformation and tumorigenesis. It has been widely observed that an increase in centrosome number per cell, called centrosome amplification, is a common characteristic of most solid and hematological cancers. Overall, centrosome amplification is a rarity in healthy adult tissues, the presence of supernumerary centrosomes has been noted in early pre-malignant lesions and correlates with increased tumor grade, size and metastasis in various types of cancers of the brain, breast, colorectum, prostate, kidney, liver and bone. These extra centrosomes can arise through several mechanisms: deregulation of the centrosome cycle (resulting in successive rounds of duplication in the same S phase), a failure of cell division (thereby generating a tetraploid cell with two centrosomes), through fusion of two neighboring cells (due to infection by fusogenic viruses), or through unregulated de novo synthesis of centrioles.

The presence of supernumerary centrosomes in tumors raises the question of whether they are simply innocent bystanders or whether they play a causative role in tumor evolution. Extensive work over the past 30 years has attempted to answer this simple yet challenging question, and the findings are summarized in some excellent reviews. Overall, centrosome amplification was proposed to result in the formation of multipolar spindles during mitosis, leading to an increased frequency of aberrant chromosome segregation, causing genetic instability and
ultimately driving tumorigenesis. This model persisted, unchallenged, for many years even though it presented a paradox: when multipolar mitosis does occur as a consequence of cells having too many centrosomes, the resulting daughter cells are usually not viable and thus unlikely to contribute to tumor formation.\textsuperscript{39-40} Indeed, several mechanisms exist that limit the detrimental consequences of supernumerary centrosomes and encourage the formation of a normal bipolar mitosis. These include centrosome removal (extrusion of extra centrosomes from cells), centrosome inactivation (a bipolar mitosis is achieved by silencing MTOC activity of all but 2 centrosomes), and perhaps most importantly centrosome clustering, where cells coalesce their extra centrosomes into two groups to form a bipolar spindle.\textsuperscript{52} So how do supernumerary centrosomes contribute to cell transformation?

Recently, two landmark studies revealed a mechanism by which centrosome amplification may drive tumorigenesis. The authors showed that, even in cells with supernumerary centrosomes that cluster at each pole of the mitotic spindle, the extra centrosomes increase the frequency of merotelic kinetochore attachments, causing chromosome mis-segregation and increased aneuploidy.\textsuperscript{39,40} Further evidence comes from the demonstration that induction of centrosome amplification can directly initiate tumor formation and metastasis in flies.\textsuperscript{52} Finally, centrosome amplification has been shown to disrupt cell polarity, migration and cell cycle progression, all hallmarks of transformed cells.\textsuperscript{32} Thus, it is becoming increasingly evident that rather than serving as a mere beacon of malignancy, supernumerary centrosomes actually drive malignant transformation. However, the recent discovery that centrosome amplification is prevalent in non-cancer related human disease conditions suggests other possible mechanisms by which these supernumerary centrosomes may perturb normal cell function.

**Centrosome Amplification in Ciliopathies**

Mutations that disrupt the structure and function of cilia are associated with a broad spectrum of human disease phenotypes that include polydactyly, brain malformation, situs inversus (defects of left-right patterning), obesity and polycystic kidney disease.\textsuperscript{15,17} Due to the critical role of the centrosome as the structural support for primary cilium formation and a coordinator of ciliary protein trafficking, dysregulation of the centrosome often leads to defective ciliary assembly and signaling. Thus, abnormal centrosome function can contribute to cilia-related disease phenotypes. Interestingly, centrosome amplification has recently been reported in tissues from patients and animal models harboring mutations in ciliopathy genes.

Polycystic kidney disease (PKD) is the most common disorder caused by defects in the chemo- and mechano-sensory functions of the primary cilium.\textsuperscript{43,44} Polycystin-1 (Pkd1) and Polycystin-2 (Pkd2) are membrane-spanning proteins that localize to the centrosome and cilium in renal epithelial cells, and work together in a complex to sense ciliary bending induced by urine flow.\textsuperscript{45} Mutations in Pkd1 and Pkd2 cause defects in ciliary signaling, cell polarity, cell division, hyperproliferation of tubular epithelial cells and formation of cysts that profoundly alter the organ architecture and impair renal function.\textsuperscript{43-45} Recent experiments have uncovered a potentially important role for both Pkd1 and Pkd2 in the maintenance of centrosome integrity. Depletion of Pkd1 was shown to induce centrosome amplification in cells in vitro, while supernumerary centrosomes were observed in kidneys of a Pkd1 knockout animal model and in human renal tissue from Pkd1 patients in vivo.\textsuperscript{56} The presence of supernumerary centrosomes was noted in seemingly normal tubular cells, suggesting that centrosome amplification is an early event that precedes cyst formation. Similarly, centrosome amplification was reported in fibroblast cells isolated from Pkd2 transgenic mice and in mesenchymal cells of Pkd2 knockout embryos, indicating that Pkd2 dysregulation also alters centrosome number.\textsuperscript{57}

These centrosome number aberrations extend to other cilia-related disease syndromes. Loss of the tumor suppressor genes TSC1 and TSC2, mutations in which cause tuberous sclerosis, also lead to centrosome amplification.\textsuperscript{48,49} Depletion of the centrosomal Mks1 or Mks3 proteins, mutated in Meckel-Gruber syndrome, similarly results in centrosome amplification.\textsuperscript{50} Although a causative role for centrosome amplification in the pathogenesis of these disease phenotypes has not been well established, the fact that PKD, TSC and MKS are all attributed to defects in ciliary signaling raises the question of whether changes in centrosome number might affect the structure and/or function of the primary cilia in those cells.

**Super-Ciliated Cells**

Since it is unclear what phenotypes are conferred upon cells having supernumerary centrosomes that might lead to cilia-related diseases, we considered the possibility that extra centrosomes might result in aberrant cilium number and thus affect normal ciliary signaling. To test this hypothesis, we induced centrosome amplification by briefly expressing the kinase Plk4, which is known to cause formation of multiple centrioles in a diversity of cells and organisms.\textsuperscript{51-53} As expected, this led to the formation of supernumerary daughter centrioles, which matured into mother centrioles by passage through the cell cycle.\textsuperscript{54} Remarkably, we discovered that the presence of extra centrosomes invariably led to the formation of more than one primary cilium per cell (Fig. 2). These super-ciliated cells contained up to six primary cilia per cell.\textsuperscript{54}

Next, we wondered whether an increase in the number of primary cilia might perturb signaling pathways that rely on ciliary function. We quantified the concentration of signaling molecules that reside within the primary cilium, focusing first on the transmembrane protein Smoothened (Smo). In response to Sonic Hedgehog (Shh) ligand, Smo translocates into the cilia,\textsuperscript{55} and this is essential in activating downstream signaling in mammalian cells.\textsuperscript{56} Using quantitative single-cell immunofluorescence microscopy, we determined that the amount of Smo per unit length cilium decreased proportionally as the number of cilia per cell increased.\textsuperscript{54} We refer to this decrease in ciliary protein concentration as a “ciliary
processes that depend on cilium function. Ciliary signaling is essential in organizing the architecture and polarity of epithelial cells through regulation of both the canonical and non-canonical (planar cell polarity) Wnt signaling pathways. We found that the presence of supernumerary centrosomes and cilia disrupted epithelial cell polarity and organization in vitro. Furthermore, super-ciliated cells displayed slow progression through the cell cycle as well as defective directional cell migration (our unpublished results), both processes that rely on proper ciliary function.

It is important to note that the observed deficiencies in cell polarity, migration and division in super-ciliated cells are similar to cellular defects caused by mutations in canonical ciliopathy genes. It is tempting to speculate that the reduction in ciliary protein concentration, due to supernumerary centrosomes and cilia, might lead to the same phenotypic outcome in patients.

The observation that supernumerary centrosomes resulted in the formation of extra cilia and caused defective recruitment of ciliary Smo protein was intriguing, and raised the question of whether other signaling pathways are similarly affected. To address the generality and nature of the ciliary dilution phenotype, we examined the ciliary concentrations of additional proteins that localize to cilia in super-ciliated cells. We found that the serotonin 6 (Htr6) receptor, the fibro-cystin (PKHD) protein, and the GTPase Arl13b all exhibited a ciliary dilution phenotype similar to that of Smo in super-ciliated cells. The ciliary dilution phenotype was observed even under conditions in which the total concentration of the transported protein was not limiting. Because super-ciliated cells assembled cilia of similar length to those in mono-ciliated cells, we reasoned that components of the ciliary machinery might not display the ciliary dilution phenotype. Consistent with this hypothesis, the ciliary concentration of IFT88, a component of the intraflagellar transport machinery required for cilium assembly, was equal in mono- and super-ciliated cells. Lastly, we found that the ciliary dilution phenotype was dependent on number of cilia rather than number of centrosomes, suggesting that the mechanism determining ciliary protein levels assesses the number of centrosomes per cell.

Our experiments demonstrate that centrosome amplification can cause the formation of ectopic cilia, and leads to a reduction in ciliary signaling capacity. To determine the functional consequences of having too many cilia in a cell, we tested the effect of extra cilia on cellular processes that depend on cilium function. Ciliary signaling is essential in organizing the architecture and polarity of epithelial cells through regulation of both the canonical and non-canonical (planar cell polarity) Wnt signaling pathways. We found that the presence of supernumerary centrosomes and cilia disrupted epithelial cell polarity and organization in vitro. Furthermore, super-ciliated cells displayed slow progression through the cell cycle as well as defective directional cell migration (our unpublished results), both processes that rely on proper ciliary function.

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and tissues, both in vitro and in vivo. It raises the interesting possibility that aberrant primary cilium-associated signaling, due to the presence of ectopic centrosomes and cilia, might be an important aspect of a set of cilia-related disease phenotypes. However, a causal role for centrosome amplification in cystogenesis, or other ciliopathy phenotypes, has not been established. Similarly, it is not known whether the presence of excess cilia is in fact contributing to cell transformation, cyst formation or tumorigenesis in vivo. Future work, particularly at the organism level, will be essential to ascertain the impact of centrosome amplification on ciliary function during development, and how it may contribute to disease.

Questions and Answers

Dr Helen Liapis, Professor of Pathology and Immunology, Washington University School of Medicine: Moe this was a wonderful talk. Beautiful pictures, I am using the last one you showed in the new Heptinstall’s Pathology of the Kidney textbook; it is probably going to be on the cover. I still don’t understand...
why there are no data in human disease, particularly polycystic kidney disease, to show structural abnormalities of the cilia. As you know, this has cast doubts of the relevance of ciliary biology (which is beautiful) to human disease. Why do you think that is?

Dr Mahjoub: Actually, structural defects in cilia have been observed in a number of cilia-related diseases. If you were to completely abrogate cilia structure, you get embryonic lethality, so there is a reason why there aren’t humans walking around completely lacking cilia. But there are specific sub domains within the cilium, like the transition zone, the periciliary necklace, and the ciliary pocket that control trafficking of proteins in and out of the cilium. These are structural components that you may not see by EM per se, but there are proteins that reside in, and help define, these unique regions. These proteins are commonly mutated in ciliopathies, and we can count those as structural defects. So even though the cilium in itself is fully assembled, the defects in those little structures change which proteins get in and change which proteins go out, and that alters the activity of the signaling pathways downstream of those proteins. So you might not think of them as a structural defect in the gross term but they still impact the signaling capacity of the cilium.

Dr Ying Chen, Assistant Professor Medicine, Washington University School of Medicine: So Moe, I’m thinking that in autosomal dominant polycystic kidney disease, research has proven that there is a phenotypic switch in the second mesenchyme. Research has proven that there is a disease, research has proven that there is a switch in the second mesenchyme. But what we found is that more cilia in a cell actually led to less signaling, and less cell division. But I only showed you data for pro-proliferative pathways. If we consider inhibitory pathways that function through cilia, then the presence of supernumerary cilia would presumably dilute those proteins in the same way, so you could have a release of inhibition on cell proliferation. But we have not tested that yet.

Dr Jeffrey Miner, Professor of Medicine, Washington University School of Medicine: Can you bypass the dampened sonic hedgehog signaling when you have the abnormal multiple cilia by overexpressing Smoothened?

Dr Mahjoub: Although we did not test it specifically for the Shh pathway, overexpression of ciliary membrane proteins does not lead to more of those proteins localizing in cilia. We did overexpress a number of ciliary signaling molecules, and although you can have lots of them in the cytoplasm, they do not get imported into the cilium. At first we thought that one of the trafficking components might become saturated, such that you can have all the cargo you want in the cell body, but only so much of it could be loaded onto the trafficking machinery. That doesn’t seem to be the case. The experiments in which we unclustered the centrioles, so that the multiple cilia in the cell were separated and residing in their own ciliary pocket proved that point. In those cells, proteins were targeted normally, so the limiting factor was the structure of the ciliary pocket itself. Think of it as a little train station: normally, cargo only has one train (cilium) to get loaded onto, but in super-ciliated cells there are three or more trains for the cargo to get loaded on, so it gets distributed differentially among the multiple trains (cilia).

Dr Keith Hruska, Professor of Pediatrics and Medicine, Washington University School of Medicine: One clinical insight into ADPKD comes from the skeleton, where the osteocyte cilium is responsible for transmitting mechanical load. What the clinical part is, we find that there is a tremendous decrease in remodeling rate in the ADPKD osteocytes, both in vivo and in vitro, and related to that there is an increase in FGF23 secretion. So when you are talking about Smoothened in the signaling pathways, here the signaling pathway is Wnt since it is the main anabolic factor in the skeleton. So have you had an opportunity to look at Wnt pathways?

Dr Mahjoub: We did not look directly at Wnt pathway activity in the super-ciliated cells, although we did the functional assay where we showed that cell polarity (canonical and planar cell polarity) was disrupted in those cells. We picked Sonic Hedgehog because it is very well characterized with regards to its role in the cilium. We used that as a tool to test the activity of that pathway and then we looked at a number of other ciliary signaling molecules just to show that it is likely happening to other pathways too. I should mention that a recent paper from the Gleson lab demonstrated a reduction in canonical Wnt pathway activation in super-ciliated cells, which nicely supports our findings.

Dr Feng Chen, Associate Professor of Medicine, Washington University School of Medicine: A couple of questions. The first one is really simple but I am curious. You mentioned that during asymmetric divisions in mono-ciliated cells, the older mother cilium is always inherited by one of the daughter cells, the stem cell for example. I wonder how the centrioles are distributed within the super-ciliated cells. Would all the older centrioles and all of the cilia within a cell go to one daughter cell when the cell divides?

Dr Mahjoub: The asymmetric inheritance of ciliums based on their age has been reported only in certain cell types so far, such as neural progenitor cells. However, this process is not occurring in our cultured cells in vitro, so I cannot address that directly. What happens in our experiments is that we induce the formation of multiple daughter centrioles at once, which are template by the original mother and original daughter centrioles. Instead of the normal duplication cycle, where you get 1-to-1 ratios of new daughter centrioles to parental centrioles, these now make 5 or 6 new daughter centrioles each so you got a bunch of young centrioles at once. The cells then go through mitosis at least twice, and a fraction of them become mother centrioles because they have undergone maturation.
An interesting experiment would be to induce centriole amplification in certain cell types (like neural progenitors), and see whether the very oldest mother centriole and the original daughter centriole are segregated differentially, even if extra centrioles are around. We would have to do it in a cell type that has asymmetric cell division as part of its program, and see if that disrupts cell fate. The cells we are looking at do not divide this way, they are just epithelial or fibroblast cells that divide symmetrically.

Dr Marc Hammerman, Chromalloy Professor, Washington University School of Medicine: How many generations of centrioles are there? Is there such a thing as a great grandmother centriole?

Dr Mahjoub: In theory, we can trace the lineage of centrioles in dividing cells through time indefinitely, although it is technically challenging. A cell starts out with a mother and a daughter centriole, builds a pair of new daughter centrioles in S-phase then segregates the two pairs to its sister cells following division. This means that the original mother centriole will always be the oldest centriole in the entire generation of cells. In theory, one should be able to follow the location of that centriole forever because every time that cell divides, one of the two cells will inherit that oldest centriole, because these organelles do not disassemble and reassemble. We should be able to trace their lineage. What that means to the biology of that cell, we do not know yet.

Hani Suleiman, Staff Scientist, Department of Pathology and Immunology Washington University School of Medicine: Regarding the directional migration, could you elaborate on why you would have directional migration defects if you still have the 3 or 2 cilia in the same spot at the leading edge of a polarized cell?

Dr Mahjoub: Dr Soren Christensen’s lab has done a lot of good work to show that the cilium responds to PDGF ligand through the PDGF-α receptor, which is localized to the cilium. So the primary cilium appears to be important for receiving extracellular signals that help orient cells during migration, for example during wound healing. I showed you that having extra cilia causes defects in the signaling capacity of those cilia, because there is less of each signaling protein, and thus less interaction among the components. Therefore, the cell migration experiments were another way to test the functionality of the cilia in super-ciliated cells. Presumably, these cilia have less PDGF receptor per unit length just like the other proteins we looked at, and are not able to receive that extracellular signal very well. The result is that cells do not migrate properly; although they fill in the wound over time in our scratch assay, the directionality of migration is perturbed. One experiment we would like to do is to examine this at the single cell level, instead of the tissue level. We would feed the cells a specific ligand (e.g., PDGF) and track its movement toward or away from the stimulus. We would do this with cells that have one or more cilia and see how each cell responds.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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