Centriole Age Underlies Asynchronous Primary Cilium Growth in Mammalian Cells

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Summary

Primary cilia are microtubule-based sensory organelles that play important roles in development and disease [1]. They are required for Sonic hedgehog (Shh) [2–4] and platelet-derived growth factor (PDGF) [5] signaling. Primary cilia grow from the older of the two centrioles of the centrosome, referred to as the mother centriole. In cycling cells, the cilium typically grows in G1 and is lost before mitosis, but the regulation of its growth is poorly understood. Centriole duplication at G1/S results in two centrosomes, one with an older mother centriole and one with a new mother centriole, that are segregated in mitosis. Here we report that primary cilium grow asynchronously in sister cells resulting from a mitotic division and that the sister cell receiving the older mother centriole usually grows a primary cilium first. We also show that the signaling proteins inversin [6] and PDGFRA localize asynchronously to sister cell primary cilia and that sister cells respond asymmetrically to Shh. These results suggest that the segregation of differently aged mother centrioles, an asymmetry inherent to every animal cell division, can influence the ability of sister cells to respond to environmental signals, potentially altering the behavior or fate of one or both sister cells.

Results and Discussion

We investigated the kinetics of primary cilium biogenesis by using NIH 3T3 mouse fibroblasts that stably express GFP-centrin 2 to label centrioles [7]. Dividing cells were collected by mitotic shake-off and replated at low density so that sister cells could be identified by proximity and then fixed and stained for acetylated α-tubulin to label primary cilia [3]. In many sister cell pairs, only one sister cell had a primary cilium (Figure 1A). Such asymmetric pairs were the predominant class 4–8 hr after mitotic shake-off (Figure 1B).

To determine whether both cells in a given sister pair were capable of generating a primary cilium, we collected mitotic cells and plated them in medium with reduced serum to arrest the cells in G0, the cell-cycle state maximally permissive for cilium formation. Only one sister cell had a primary cilium in most pairs 2 hr after mitotic shake-off, but both sister cells had primary cilia in most pairs at later time points (Figure 1C). To ensure that this asymmetry was not caused by perturbation of the cells during mitotic shake-off, primary cilia were counted in NIH 3T3 cells pairs identifiable as sisters by the presence of a midbody remnant in unperturbed cultures of cells. Only one sister cell had a primary cilium in many of these pairs as well (21.7%, n = 300 cells). Asynchronous primary cilium growth in sister cells was also observed in untransfected NIH 3T3 cells (see below), hTert-RPE1 human epithelial cells (see Figure S2 available online), and primary mouse embryonic fibroblasts (data not shown), both after mitotic shake-off and in unsynchronized cultures.

Asynchronous primary cilium growth might result from differing rates of cell attachment or cell-cycle progression between recently divided sister cells. This was tested by manipulating cells so that both centrosomes remained in the same cytoplasm after mitosis. Mitotic NIH 3T3 cells expressing GFP-centrin 2 were collected and treated with cytochalasin B to prevent cytokinesis, producing binucleate cells with four centrioles (Figure 1D). Cytochalasin B was removed [8], and primary cilium in binucleate cells were counted at 2 hr intervals after shake-off. All four centrioles were often clustered together (Figure 1D), but most ciliated cells still had only a single primary cilium from 2 to 8 hr after shake-off (Figure 1E). This suggests that the asynchrony is a result of an intrinsic difference between the two mother centrioles rather than other physiological differences.

Centriole age is a fundamental intrinsic difference between mother centrioles in sister cells. The age difference derives from the centriole replication cycle: cells in G1 have two centrioles, a mother and daughter from the previous cell cycle; in S phase, both centrosomes become mothers as new daughter centrioles grow from their sides. Thus, one of the centrosomes in a G2 cell has the older mother centriole, and the other has the new mother centriole [9]. Between S phase and mitosis, the new mother centriole acquires a set of mother-centriole-specific proteins [10–12] associated with centriolar appendages [9]. After mitosis, one sister cell has the centrosome containing the older mother centriole, and the other sister cell has the centrosome containing the new mother centriole (see schematic in Figure S1).

To test the relationship between mother centriole age and the timing of primary cilium growth, we used pulsed expression of an epitope-tagged tubulin protein to distinguish centrioles by age. The microtubules making up the centriole are stable [13]; therefore, centrioles formed after transfection incorporate the tagged tubulin, whereas preexisting centrioles do not (see schematic in Figure 2A). NIH 3T3 cells were transfected with a plasmid expressing myc-tagged α-tubulin [14] and cultured for 68 hr. In sister cell pairs that expressed myc-tagged α-tubulin for two rounds of centriole duplication, three of the centrioles contained myc-tagged tubulin and one centriole, the oldest, lacked it (Figure 2A). In 33 of 35 such pairs in which one primary cilium was present, the cell with the unlabelled (oldest) mother centriole had the primary cilium (p < 0.001; Figure 2B). Thus, the cell receiving the older mother cilium usually grows a primary cilium first after mitosis.

We next sought to identify molecular differences between old and new mother centrioles that might explain the asynchronous primary cilium growth. Previously described mother-centriole-specific proteins have been shown to first localize to new mother centrioles in S, G2, or M phases, but their relative

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levels at the centrosomes of sister cells have not been reported. Proteins were localized in sister pairs from unsynchronized cultures that had not yet generated primary cilia, and in sister pairs 2 hr after mitotic shake-off. Ninein, centriolin, pericentrin, γ-tubulin, and polaris localized symmetrically to the mother centrioles of both sister cells (difference in signal between centrosomes ≤ 15%). In contrast, cenexin/ODF2 localized asymmetrically to centrosomes during late mitosis and early G1 (Figure 3A). Cenexin/ODF2 has been reported to localize asymmetrically to centrosomes early in mitosis [10, 15] and is required for primary cilium growth [16, 17]. We found that the cenexin/ODF2 signal was greater on the centrosome of one cell in a sister pair after mitotic shake-off (64.4% brighter, n = 100 centrosome pairs at 30 min). At 2 hr after mitotic shake-off, the centrosome with greater cenexin/ODF2 staining had the primary cilium in 84% of sister pairs with a single primary cilium (n = 58 pairs, p < 0.001). Thus, accumulation of a sufficient amount of cenexin/ODF2 might be a rate-limiting step in ciliogenesis.

The correlation between greater cenexin/ODF2 staining and primary cilium growth suggested that the new mother centriole might not become fully competent for ciliogenesis until the G1 phase of the cell cycle following its formation. To test whether this reflects a specific cell-cycle requirement, such as progression through mitosis, or simply time dependence, we uncoupled cell-cycle progression from centriole maturation. NIH 3T3 cells were synchronized in S phase by double thymidine treatment, followed by a 6 hr release to allow progression into G2 phase. Only a small fraction of cells had two primary cilia after this synchronization (Figure 3C). Cells were then arrested in G2/M with 20 μM etoposide [18]. After 18 hr of treatment, there was a 7-fold increase in the number of cells with four centrioles and two primary cilia (Figures 3B and 3C), demonstrating that the new mother centriole had become competent to generate a primary cilium during the arrest. The mother-centriole-specific proteins cenexin/ODF2 (Figure 3D) and centriolin (data not shown) were present at both mother centrioles in these cells. Similarly, a 3-fold increase in cells with two primary cilia was observed after arresting cells in G2/M for 18 hr by an independent mechanism with the CDK1 inhibitor RO-3306 [19]. The above results show both that primary cilia in NIH 3T3 cells can grow in G2 as well as in G1 and that the new mother centriole can, with sufficient time in G2, acquire competence for ciliogenesis within a single cell cycle.

Given the importance of the primary cilium in signal transduction, we reasoned that asynchronous ciliogenesis might have functional consequences for sister cells after division. We first tested whether ciliary signaling proteins are delivered asynchronously to the primary cilium following mitosis. Inversin [6] is required for the establishment of left-right axis asymmetry [20] and has been implicated in cell-cycle control [21]. Sister NIH 3T3 cells stably expressing tdTomato-inversin were imaged at 3 min intervals. The average time from anaphase to the appearance of a focus of inversin was 179 ± 80 (standard deviation) min (n = 187 cells), similar to reported kinetics of primary cilium growth [22]. In all cases, an inversin focus appeared and intensified in one cell before the other (Figure 4A; Movie S1), with an average lag of 66.7 min (n = 37 pairs) between appearance of the foci.

We next tested whether platelet-derived growth factor receptor α (PDGFRα), a growth factor receptor that requires localization to the primary cilium for activity [5], localizes asynchronously in sister cells. Mitotic cells were collected, plated in low-serum medium, and stained for acetylated α-tubulin and PDGFRα. In 39 of 48 sister pairs in which both cells had primary cilia, only one cell had ciliary PDGFRα staining (Figure 4B), suggesting that asynchronous cilia formation can result in asynchronous acquisition of functional components by the cilium. Indeed, PDGFRα has been reported to localize at the base of the ciliary axoneme after 12 hr of serum starvation but becomes enriched along the length of the axoneme after 24 hr of serum starvation [5], consistent with the accumulation of functional components in the cilium over time.

To test whether asynchronous primary cilium formation might allow sister cells to respond differentially to an environmental signal, we examined Sonic hedgehog (Shh) pathway activity in sister cells. NIH 3T3 cells were exposed to conditioned medium containing ShhN, an active fragment of Shh,
A sister pair had a primary cilium, one cilium was Smo+ in 34% to Shh while the other sister cell is not. Even when both cells in that the sister cell with a primary cilium can become responsive Smo+ (73% of pairs, n = 63; Figure 4C). This demonstrates. In most pairs with one primary cilium, that cilium was and primary cilia in these pairs were scored for Smo localiza-

for 24 hr and stained for acetylated α-tubulin and Smoothened (Smo), a Shh pathway component that localizes to the primary cilium only after Shh stimulation [3, 4]. Recently divided sister pairs were identified by the presence of a midbody remnant, and primary cilia in these pairs were scored for Smo localization. In most pairs with one primary cilium, that cilium was Smo+ (73% of pairs, n = 63; Figure 4C). This demonstrates that the sister cell with a primary cilium can become responsive to Shh while the other sister cell is not. Even when both cells in a sister pair had a primary cilium, one cilium was Smo+ in 34% of cases, and both cilia were Smo+ in 61% of cases (n = 96 pairs; Figure 4D). Inferring from the results for inversin and PDGFRα, a differential Shh response in pairs with two cilia might reflect the asynchronous acquisition of Shh receptors or signaling components by the cilia in the sister cells. Ciliary Smo was not observed in sister pairs exposed to control medium lacking ShhN. These results demonstrate that asynchronous primary cilium growth can lead to differential responses of sister cells to a uniform environmental signal.

We have shown that primary cilium formation in recently divided cells is asynchronous, that this is a result of the inherent asymmetry in mother cilentrode age between sister cells, and that asynchronous primary cilium formation imparts the ability to respond to an environmental stimulus to the sister cell that first forms a cilium. In biflagellate unicellular algae, cilentrode age difference has been shown to influence cilium formation and structure. In Pleurochrysis carterae, flagellum length is dependent on cilentrode age: new ciliotyles make a short flagellum in the first cell cycle and a long flagellum in subsequent cycles [23]. In Chlamydomonas, the unit8 mutation revealed a difference in the ability of older and newer ciliotyles to grow a flagellum, with the newer cilentrode requiring an extra generation to do so [24]. These results reinforce the conclusion that cilentrode age influences cilium formation, structure, and function.

We speculate that asynchronous primary cilium growth might have consequences for cell fate determination by allowing sister cells to differentially detect environmental signals in the context of tissue development. In the case of a signal that is transient or limited in abundance, the cell that generates a primary cilium first would be able to respond to that signal, whereas its sister cell would not (Figure S3A). Both PDGF and Shh can induce cell proliferation [25, 26], and if either of these signals were limiting, the cell that sensed the signal first might progress through the cell cycle more quickly than its sister. In the case of a persistent or abundant signal, the cell that generates a primary cilium first might respond to the signal and then modify the responsiveness of its sister cell (Figure S3B). Alternatively, the duration of signaling might be critical [27], and the cell that generates a primary cilium first would experience the signal for a longer time. In both scenarios, differential sensing of the environmental signal could lead to a different fate for each sister cell. Additionally, in cases in which stem cell identity depends on cell positioning, primary cilium might serve as positional sensors [28], and asynchronous cilium growth might bias the orientation of sister cells with respect to the stem cell niche. Interestingly, during asymmetric stem cell divisions in the Drosophila male germline, the older centrosome remains in the stem cell, whereas the younger centrosome is segregated to the differentiating cell [29]. Testing the physiological significance of the possibilities described above will require the development
of models to examine centriole age with respect to cell division, primary cilium formation, and cell differentiation in vivo.

Experimental Procedures

Cells, Media, and Drugs
NIH 3T3 cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle medium (DMEM) + 10% normal calf serum (Invitrogen). hTert-RPE1 cells (Clontech) were grown in DMEM/F12 + 10% fetal bovine serum (Invitrogen). NIH 3T3 lines stably expressing GFP-centrin 2 or tdTomato-inversin were generated by transfection of pTS1175 or pTS1641 with FuGENE 6 transfection reagent (Roche) followed by selection with 450 \( \mu \)g/ml Geneticin (Invitrogen) and clonal isolation. Cytochalasin B (Sigma) was dissolved in dimethyl sulfoxide at 2 \( \mu \)g/ml for 1 hr to prevent cytokinesis, producing binucleate cells with four centrioles. Cytochalasin B was removed by washing five times with fresh medium over 20 min.

Plasmids
pCDNA1 expressing myc-tagged Mus musculus \( \alpha \)-tubulin isoform 2 (pTS488) has been described previously [14]. A pEGFP-C1 (Clontech) derivative expressing GFP-tagged Homo sapiens centrin 2 (pTS1175) was a gift from J. Salisbury. The Mus musculus inversin coding region was amplified by polymerase chain reaction from pEF BOS EX + invGFP (a gift from H. Hamada) with SalI and SacII sites added and cloned into pCMV-tdTomato (a gift from R. Tsien) to generate pTS1641.
Antibodies
Antibodies were diluted in PBS-BT (phosphate-buffered saline [PBS] + 3% bovine serum albumin [BSA] + 0.1% Triton X-100); 1% normal goat serum was used instead of BSA for Shh experiments. Primary antibodies used were mouse anti-acetylated α-tubulin clone 6-11B-1 (1:2000, Sigma), rabbit anti-PDGFRa and rabbit anti-my c (both 1:200, Santa Cruz Biotechnology), rabbit anti-hCenexin [15] (1:1000, a gift from K. Lee), and rabbit anti-Smo [4] (1:1000; gift from R. Rohatgi). Secondary antibodies used were Alexa 488- or 594-conjugated goat anti-mouse and anti-rabbit (1:200, Invitrogen). DNA was stained with 50 ng/ml DAPI.

Mitotic Shake-off
Cells from four 10 cm plates grown to 50% confluence were collected [30], replated in one 3.5 cm plate, fixed in −20°C methanol (or 4% paraformaldehyde in PBS for PDGFRα and Smo staining) at the indicated time points, and processed for immunofluorescence as described previously [31].

Pulse Labeling of Centrioles
NIH 3T3 cells were transfected with pTS488 via calcium phosphate precipitation. After 68 hr, cells were extracted for 20 min on ice in cold extraction buffer (100 mM PIPES + 1% Triton X-100; 50 mM HCl) and processed for immunofluorescence as described previously [31].

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures, three figures, and one movie can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01458-4.

Acknowledgments
We thank J. Salisbury, H. Hamada, R. Tsien, K. Lee, R. Rohatgi, and P. Beachy for reagents; L. Schneider for staining advice; and A. Vasey and members of the Stearns laboratory for comments on the manuscript. This work was supported by NIH grant GM52022 (T.S.) and National Science Foundation Graduate Research and Stanford Graduate fellowships (C.T.A.).

Received: March 7, 2009
Revised: July 9, 2009
Accepted: July 9, 2009
Published online: August 13, 2009

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