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SHORT COMMUNICATION

Primary cilia found on HeLa and other cancer cells

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Abstract

For many years now, researchers have known of a sensory appendage on the surface of most differentiated cell types called primary cilium. Primary cilia are both chemo- and mechano-sensory in function and have an obvious role in cell cycle control. Because of this, it has been thought that primary cilia are not found on rapidly proliferating cells, for example, cancer cells. Here we report using immunofluorescent staining for the ciliary protein Arl13b that primary cilia are frequently found on HeLa (human epithelial adenocarcinoma) and other cancer cell lines such as MG63 (human osteosarcoma) commonly used for cell culture studies and that the ciliated population is significantly higher (ave. 28.6% and 46.5%, respectively in starved and 15.7–18.6% in un-starved cells) than previously anticipated. Our finding impacts the current perception of primary cilia formed in highly proliferative cells.

Keywords: Arl13b; cilia; ciliopathies; cancer cells; HeLa cells; primary cilia

Introduction

Primary cilia are single non-motile cilia found on the surface of non-dividing or quiescent cells (Pan and Snell, 2007). First discovered by Zimmerman (1898), the existence of primary cilia was known for some time, however without knowledge of their function. Most early descriptions of this cellular projection were accomplished through electron microscopy, but recent advances in immunofluorescent microscopic techniques including antibodies directed against proteins found primarily in motile and primary cilia have allowed for further analysis of the primary ciliary role.

Primary cilia are thought to be both mechano- and chemo-sensory and to function in coordinating several signaling pathways, including sonic hedgehog, Wnt, and RTK (Satir et al., 2010; Barral et al., 2012; Christensen et al., 2012; Goto et al., 2013; Oh and Katsanis, 2013; Mukhopadhyay and Rohatgi, 2014). Ciliopathies, the dysfunction or lack of cilia, have been implicated in obesity, diabetes, situs inversus, polydactyly, Joubert, orofaciodigital, and Bardet-Biedl syndromes, and other developmental complications (Pan et al., 2005; Satir et al., 2010). Like other cilia, the axoneme is structurally formed by stable microtubules composed primarily of acetylated- and glutamylated-tubulin, but unlike other cilia, which have a $9+2$ configuration, primary cilia have a $9+0$ arrangement (Satir and Christensen, 2007; Satir et al., 2010). The cilium projects from the basal body, which originates from the active mother centriole. Because of these structural roots, the primary cilium is expected to be involved in the regulation of progression into the cell cycle (Tucker et al., 1979; Pan and Snell, 2007; Pugacheva et al., 2007; Plotnikova et al., 2008; Plotnikova et al., 2009; Jackson, 2011; Goto et al., 2013). Additionally, evidence for the primary cilium's role in regulating the cell cycle has been published describing the localization of several critical cell cycle proteins to the cilium, including Aurora A, which functions in deciliation and prevention of cilium regeneration (Pugacheva et al., 2007; Inoko et al., 2012; Goto et al., 2013).

In general, cancer cells are thought to have lost their ability to form primary cilia, since by definition cancer is the loss of the cells' ability to control growth and results in cells entering the cell cycle aberrantly (Seeley et al., 2009; Yuan et al., 2010; Hassounah et al., 2012). However, implementing antibodies directed against a protein called Arl13b (ADP-ribosylation factor-like 13b), a small GTPase found in the axoneme of cilia, here we describe the frequent presence of primary cilia on HeLa and MG63 (human epithelial adenocarcinoma and osteosarcoma, respectively) cancer

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Abbreviations: Arl13b, ADP-ribosylation factor-like 13b; ATCC, American type culture collection; DIC, differential interference contrast; RTK, receptor tyrosine kinase; Wnt, wingless-related integration site

cells. Arl13b is a small GTPase whose cellular localization is restricted to the axoneme of cilia and to some extent to some actin rich structures of migrating cells (Sun et al., 2004; Caspary et al., 2007; Duldulao et al., 2009; Casalou et al., 2014). Arl13b is known to function in the maintenance of ciliary structure, however in a not yet completely understood fashion. Mutations in the Arl13b gene, which lead to Joubert syndrome, a disease that manifests itself in brain malformations, ocularmotor apraxia, kidney cysts, and polydactyly, are also poorly understood and can sometimes lead to the loss of cilia (Juric-Sekhar et al., 2012; Delling et al., 2013; Higginbotham et al., 2013; Miertzschke et al., 2014).

Materials and methods

Cell culture

HeLa, human epithelial adenocarcinoma cells (CCL-2, two different lots, 59681574 and 60143948 purchased on 10/12/2012 and 3/24/2014, respectively), mouse embryo NIH3T3 fibroblasts (CRL-1658), MG63 human osteosarcoma cells (CRL-1427), and MC3T3-E1 subclone 4 mouse pre-osteoblasts (CRL-2593) were purchased from American Type Culture Collection (ATCC, Manassas,VA). All cell types were maintained at 37°C in a 5% CO_2 atmosphere and 100% humidity in Dulbecco's modified Eagles medium (Sigma, St. Louis, MO) (HeLa and NIH3T3), Eagles Minimum Essential Medium (MG63), or alpha-modified Eagles medium (Gibco/Invitrogen, Grand Island, NY) (MC3T3-E1) completed with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 1% L-glutamine (HyClone, Logan, UT) and 1% penicillin/streptomycin (Corning, Corning, NY) according to vendor's instructions.

Immunofluorescence staining

Cells were seeded onto poly-L-lysine (Sigma, St. Louis, MO) coated cover slips placed into 3.5 cm diameter tissue culture plastic dishes and cultured for 2–3 days in complete medium to approximately 60–70% confluence or in a series of dilutions of suspended cells resulting in a range in confluency of approximately 20–80% and were then fixed or serum starved for 24 h (Li et al., 2011) before fixation. Cells were fixed in either 3.7% formaldehyde (in 1xPBS) for 15 min at room temperature (RT) followed by permeabilization in ice-cold methanol (5 min) or were fixed and permeabilized directly in ice-cold methanol (5 min). Then, cells were blocked in 5% FBS/1xPBS at RT for 30 min. Primary antibodies directed against anti-Arl13b (mouse monoclonal–NeuroMab, Davis, CA Cat. # 73–287; at 1:200), anti-acetylated-tubulin (rabbit polyclonal, Sigma, St. Louis, MO cat # 6-11B-1; at 1:250), anti-PCNA (rabbit polyclonal, Santa Cruz, Dallas, TX Cat. #Sc-7907; at 1:200) and anti-γ-tubulin (rabbit polyclonal, Sigma, St. Louis, MO Cat. # T3559; at 1:500) were diluted in blocking solution and incubated with cells for 1 h at RT. Cells were then incubated in secondary antibodies Alexa488-conjugated goat-anti-mouse and Alexa568-conjugated goat-anti-rabbit (Molecular Probes/Invitrogen, Grand Island, NY, Cat. # A11029 and A11036, respectively) diluted at 1:200 for 1 h at RT. Nuclei were stained using DAPI (Molecular Probes, Eugene, OR, Cat. # D1306). Slides were imaged using a Nikon Eclipse TE2000-E inverted fluorescence microscope equipped with 40x and 60x Plan Apo, NA 1.4 oil immersion objectives and a forced-air-cooled Photometrics CoolSnap HQ CCD camera (Roper Scientific, Duluth, GA). Images were captured using MetaVue (Molecular Devices, Sunnyvale, CA) software version 6.1r5.

Quantitative image analyses

Images of seven randomly chosen areas on each coverslip ($n = 5$) were acquired by scanning the coverslips in the UV excitation channel (DAPI, showing blue emitting nuclei). Next, the filter cubes were rotated to the blue excitation channel (showing green Arl13b emission), focused and imaged. Both authors evaluated and analyzed the images independently. HeLa cells were counted from two different lots from ATCC (lot # 59681574 and 60143948) totaling 1845 counted cells and 551 counted cilia. Variation in counts between evaluators was below 15%. MetaVue software was used to count cell nuclei to determine the total number of cells present in the field of view. Similarly, the cilia in the same field of view were counted to determine the number of ciliated cells in the population. The same analysis protocol was used for NIH3T3 cells ($n = 3$), MG63 cells ($n = 2$), and MC3T3-E1 cells ($n = 3$); 175, 56, and 185 cells were counted, respectively.

Results and discussion

Since primary cilia contribute to cell cycle control and are usually present during the G_0 and G_1 phase of the cell cycle (Pan and Snell, 2007; Li et al., 2011; Inoko et al., 2012; Goto et al., 2013), we arrested HeLa cells growing on cover glasses at the G_1/S phase by serum starvation to enrich for cells containing cilia as described by Li et al. (2011). Unexpectedly and contrary to earlier reports (Alieva et al., 1999; Alieva and Vorobjev, 2004), we frequently detected primary cilia on Hela cancer cells, as shown by DIC imaging and indicated by staining positive for Arl13b, a specific protein marker for ciliary axonemes (Figures 1A, 1C and 1D). Additionally, the base of each primary cilium stained positive for γ-tubulin (Figures 1A, 1C and 1D), indicating that the cellular projection was rooted at a centriole, another characteristic of primary cilia (Wheatley et al., 1996). Although unlikely, to exclude the possibility that our HeLa cell cultures (lot 59681574) were unintentionally contaminated with another

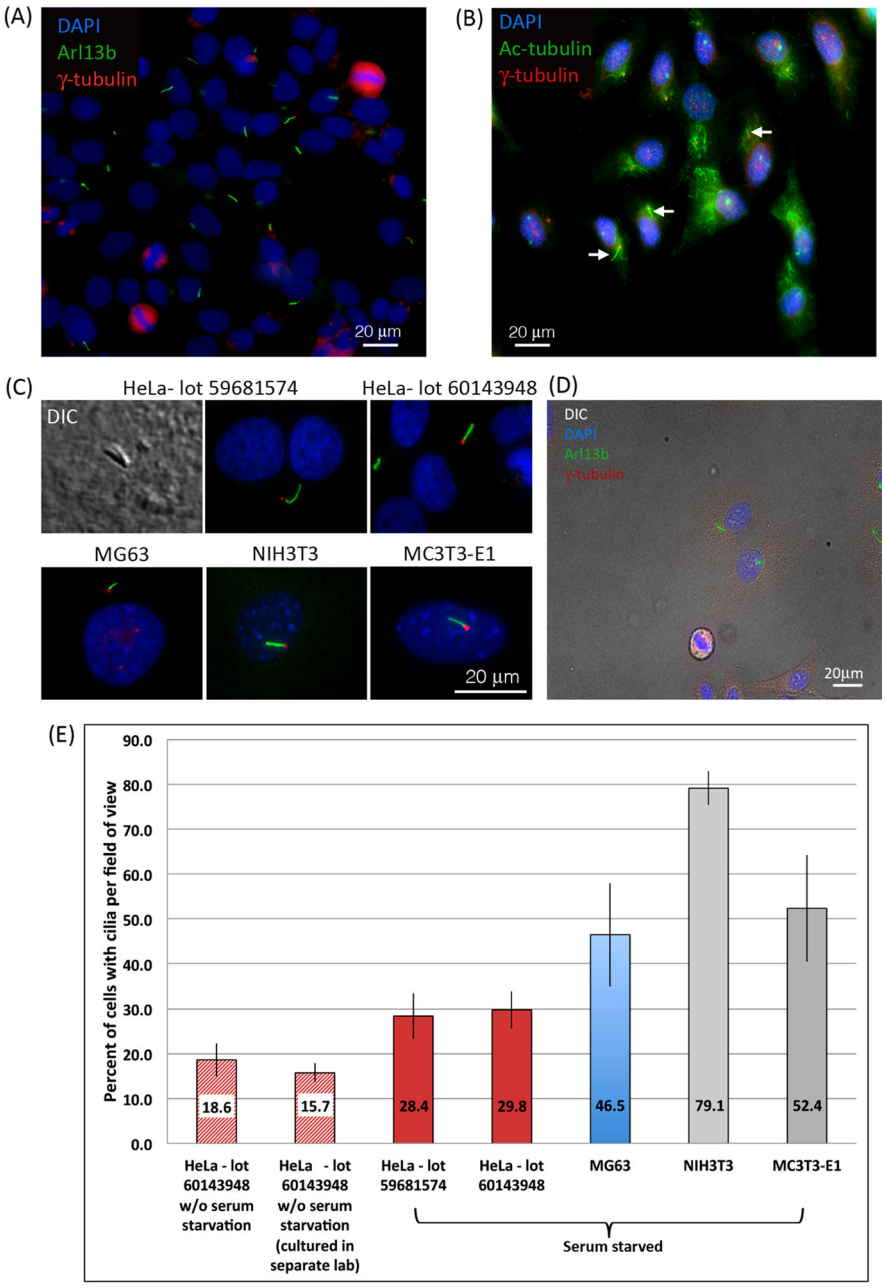

Figure 1 **Primary cilia are frequent on HeLa cancer cells** (A, C) Two different lots of HeLa cells purchased from ATCC stained positive for Arl13b (green), a protein localized with high specificity to the axonemes of cilia. At the base of the cilia, centrioles visualized using an antibody targeting γ-tubulin (red) are clearly detectable as well. Cell nuclei are stained with DAPI (blue). (B) A more traditional stain for primary cilia, acetylated-tubulin (green) and γ-tubulin (red), also stains primary cilia (arrows) but not as distinctly as with antibodies directed against Arl13b. (C) Primary cilia were also detected using DIC. NIH3T3 cells, reported as "the gold standard" for primary ciliated cells, and MC3T3-E1 cells both exhibited a similar staining pattern to HeLa cells. (D) Fluorescent Arl13b (green), γ−tubulin (red), and DAPI (blue) stains merged with a DIC image demonstrate that even at very low density HeLa cells form primary cilia. (E) Quantitative analysis of primary cilia detected in different lots of starved and un-starved HeLa cells, as well as in starved MG63 osteosarcoma, NIH3T3 fibroblasts, and MC3T3-E1 pre-osteoblast cells. The number of HeLa cells that stain positive for primary cilia that we detected is significantly higher (28.6% after starvation, 18.6% in un-starved cells) than had been reported previously (<2%). In NIH3T3 and MC3T3-E1 comparable values to previously published reports were detected (Alieva et al., 1999; Schneider et al., 2005; Malone et al., 2007).

cell type (Lorsch et al., 2015; Yu et al., 2015), we purchased a second vial of a later batch of HeLa cells from ATCC, the main repository of the U.S. (lot 60143948) and investigated cilia formation after the cells had been sub-cultured only once. No significant differences in the overall number of ciliated cells from the two different lots of HeLa cells were detected (Figures 1C and 1E). To quantify the number of HeLa cells that were ciliated under our standard starvation cell culture conditions, we counted cell nuclei and cilia and then compared the number of ciliated cells to the total number of cells. Of the 1845 HeLa cells counted, 28.6% +/− 5% (Figure 1E) stained positive for both Arl13b and γ-tubulin indicating the presence of a primary cilium (Figures 1A, 1C and 1D).

To investigate whether other cancer cells also form primary cilia, we cultured and stained human MG63 osteosarcoma cells. Also on these cells, we frequently detected primary cilia with 46.5% +/− 11.5% of cells containing primary cilia (Figures 1C and 1E). Mouse NIH3T3 fibroblasts and mouse MC3T3-E1 pre-osteoblasts reported previously to exhibit primary cilia in a large number of cells were grown and stained in parallel as a positive control (Figure 1C). We determined that 79.5% +/− 3.7% and 52.4% +/− 11.9% of NIH3T3 fibroblasts and MC3T3-E1 pre-osteoblasts, respectively, were ciliated (Figure 1E), similar to published numbers (Alieva et al., 1999; Schneider et al., 2005; Malone et al., 2007). As another control, we used a more traditional stain for primary cilia, acetylated-tubulin and γ-tubulin (Figure 1B). This stain, while commonly used in our hands, was less cilium specific since, in addition to staining ciliary acetylated-tubulin, it also stained acetylated-tubulin in the cytoplasm. Thus, the additional stain of acetylated cytoskeletal microtubules may have obscured cilia present on some cells and this may have contributed to our enhanced efficiency in detecting primary cilia in HeLa and MG63 cancer cells using Arl13b staining.

To exclude the possibility that we were inducing a quiescent G_0–like state causing the HeLa cells to aberrantly form cilia, we grew HeLa cells without serum starvation. These HeLa cells formed less cilia than serum starved cells, 18.6% +/− 3%, and 15.7% +/− 2.1% compared to 28.6% +/− 5% (Figure 1E); however they formed significantly more primary cilia than has been reported previously (none or <2%) (Alieva et al., 1999; Alieva and Vorobjev, 2004; Inoko et al., 2012). This result is in agreement with our current understanding of primary cilia formation in that serum starvation increases the number of ciliated cells. Additionally, as culture conditions including culture facilities, growth media, passage numbers, etc. may affect cell culture behavior, we analyzed HeLa cells for Arl13b expression that were independently purchased by another laboratory from ATCC (lot # 60143948) and were cultured without serum starvation in their facility using their own consumables (medium, culture dishes, coverglasses, etc.). Also in these cells, a significant amount of primary cilia were detected (15.7% +/− 2.1%), similar to what we detected previously in the HeLa cell batches that we had cultured (Figure 1E).

As described above, we provide evidence that HeLa as well as other cancer cell lines form primary cilia under starved and un-starved culture conditions. To exclude the possibility that we induced primary cilia formation by growing HeLa cells at a too high confluency unintentionally inducing an arrested stage (although an unlikely assumption as cancer cells such as HeLa are not contact inhibited and continue to grow in three dimensions when reaching total confluency), we performed a series of serial dilutions ranging from approximately 20–80% confluency. We did not observe a difference in the number of cells containing primary cilia as depicted by the representative merged DIC and fluorescence image of the lowest dilution shown in Figure 1D. Since the presence of duplicated centrosomes indicates that the cells are in late S- or G_2-phase of the cell cycle, we counted the number of cells containing primary cilia that had two centrosomes. Approximately 13% of serum starved HeLa cells containing a primary cilium also had two centrosomes (a representative image is shown in Figure 2A, centrosomes depicted with arrows), suggesting that actively dividing HeLa cells were also ciliated. To further support this conclusion, we stained HeLa cells for Arl13b as well as PCNA (proliferating cell nuclear antigen). PCNA is a DNA clamp that promotes polymerase δ interaction with DNA during the S-phase of the cell cycle to increase processivity (Herce et al., 2014). While PCNA is found in the nucleus at all stages of the cell cycle, it becomes concentrated and localized in foci of active DNA synthesis during S-phase forming a specific, punctate staining pattern within the nucleus (Herce et al., 2014). As shown in Figure 2B, HeLa cells that exhibited the distinctive PCNA S-phase staining pattern (Figure 2B, depicted with arrow) in addition stained positive for Arl13b (Figure 2B, depicted with arrowhead), further suggesting the presence of primary cilia in cells that are in the S-phase of the cell cycle. Taken together, these results suggest that in addition to quiescent cells (G_0), actively proliferating HeLa cells exhibit primary cilia.

Recently, Emoto et al. (2014) reported that both primary pancreatic cells isolated from tumor biopsies as well as PANC-1 and CFPAC-1 cells, pancreatic cancer cell lines, stained positive for primary cilia. The authors reported that 25 of 100 tissue biopsies (25%) not only stained positive for primary cilia but that these samples also correlated with increased lymph node metastasis and decreased patient survival (Emoto et al., 2014). Additionally, Yuan et al. (2010) described that cells of healthy breast epithelial tissue typically had primary cilia while cells of cancerous epithelial tissue lacked primary cilia, except in the case of a very

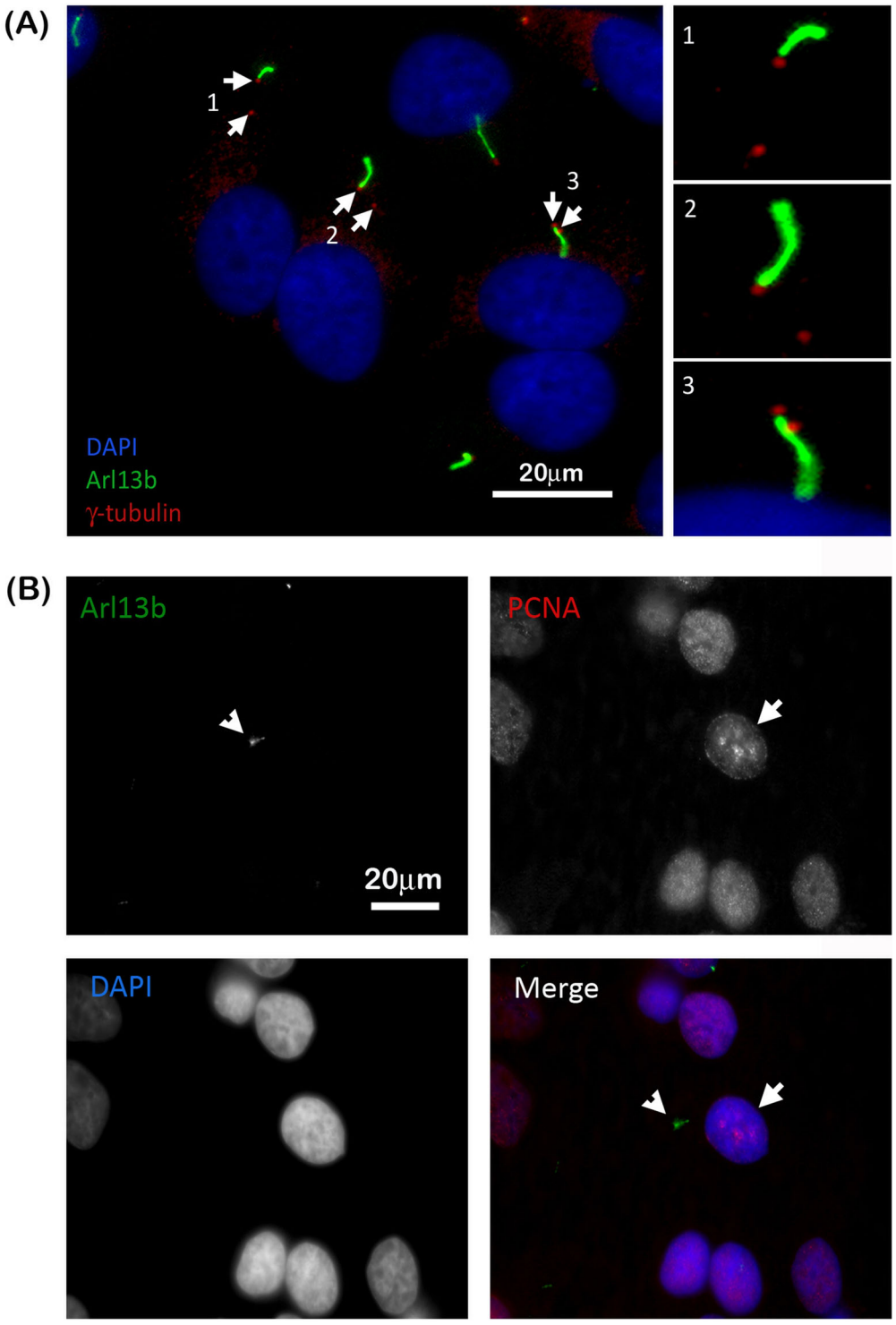

Figure 2 **Actively proliferating HeLa cells also have primary cilia** (A) Arl13b (green) and γ-tubulin (red) staining reveals several HeLa cells with two centrioles (depicted with arrows) indicating that these cells are in either the S- or G2-phase of the cell cycle. Higher magnifications of the regions containing the cilium and centrioles are to the right. (B) Arl13b staining (depicted with arrowhead) together with PCNA staining further indicates that proliferating cells have primary cilia as indicated by the distinctive punctate PCNA S-phase staining pattern in the nuclei of cells (depicted with arrow) containing primary cilia. Cell nuclei are stained with DAPI (merged panel: Arl13b, green; PCNA, red; nuclei, blue).

invasive and metastatic cancer type called basal B subtype (Yuan et al., 2010). These two examples suggest that cancer cells with primary cilia may be more aggressive in nature than un-ciliated cancer cells. Knowing the history of HeLa cancer cells and their highly aggressive behavior, detecting primary cilia on these cells may be less of a surprise.

Conclusions

Because of the particular cellular localization of Arl13b to the cilium shaft and the high specificity of the commercially available mouse monoclonal antibodies, detection of primary cilia, as shown here in HeLa and MG63 cancer cells, as well as in other cell lines known to exhibit high numbers of primary cilia (NIH3T3, MC3T3-E1), became more feasible. Taken together, our results indicate that proliferating malignant cancer cells, such as human epithelial HeLa adenocarcinoma cells or MG63 osteosarcoma cells robustly exhibit primary cilia, similar to other non-cancer cell lines and that these cells do not need to reach a state of quiescence (G_0) but also exhibit primary cilia during G_1-, S-, and G_2-phases of the cell cycle. Thus, our finding sheds new light on the expression of primary cilia in highly proliferative cells, such as cancer (reported here) and embryonic stem (ES) cells (Kiprilov et al., 2008; Bangs et al., 2015).

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