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Detection of primary cilia in human glioblastoma

Matthew R. Sarkisian,
Department of Neuroscience, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Dorit Siebzehnrubl,
Department of Neuroscience, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Lan Hoang-Minh,
Department of Neuroscience, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Loic Deleyrolle,
Department of Neurosurgery, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Daniel J. Silver,
Department of Neurosurgery, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Florian A. Siebzehnrubl,
Department of Neurosurgery, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Sarah M. Guadiana,
Department of Neuroscience, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Gayathri Srivinasan,
Department of Neuroscience, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Susan Semple-Rowland,
Department of Neuroscience, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Jeffrey K. Harrison,
Department of Pharmacology and Therapeutics, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

msarkisian@ufl.edu.

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Dennis A. Steindler, and
Department of Neurosurgery, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Brent A. Reynolds
Department of Neurosurgery, McKnight Brain Institute, University of Florida College of Medicine, Gainesville, FL 32610, USA

Abstract

Glioblastoma (GBM) is the most common malignant adult brain tumor and carries a poor prognosis due to primary and acquired resistance. While many cellular features of GBM have been documented, it is unclear if cells within these tumors extend a primary cilium, an organelle whose associated signaling pathways may regulate proliferation, migration, and survival of neural precursor and tumor cells. Using immunohistochemical and electron microscopy (EM) techniques, we screened human GBM tumor biopsies and primary cell lines for cilia. Immunocytochemical staining of five primary GBM cell lines revealed that between 8 and 25 % of the cells in each line possessed gamma tubulin-positive basal bodies from which extended acetylated, alpha-tubulin-positive axonemes. EM analyses confirmed the presence of cilia at the cell surface and revealed that their axonemes contained organized networks of microtubules, a structural feature consistent with our detection of IFT88 and Arl13b, two trafficked cilia proteins, along the lengths of the axonemes. Notably, cilia were detected in each of 23 tumor biopsies (22 primary and 1 recurrent) examined. These cilia were distributed across the tumor landscape including regions proximal to the vasculature and within necrotic areas. Moreover, ciliated cells within these tumors co-stained with Ki67, a marker for actively dividing cells, and ZEB1, a transcription factor that is upregulated in GBM and linked to tumor initiation, invasion, and chemoresistance. Collectively, our data show that subpopulations of cells within human GBM tumors are ciliated. In view of mounting evidence supporting roles of primary cilia in tumor initiation and propagation, it is likely that further study of the effects of cilia on GBM tumor cell function will improve our understanding of GBM pathogenesis and may provide new directions for GBM treatment strategies.

Keywords

Glioma; Cilium; Intraflagellar transport; Cancer

Introduction

The intrinsic and acquired resistance of glioblastoma (GBM) tumors to aggressive treatments that combine surgery, chemotherapy, and radiation, limits the post-diagnosis median survival of GBM patients to ~12–18 months [1]. Improvements in these patients' prognoses will require a better understanding of the factors that permit survival, migration, and proliferation of GBM cells within the brain. The GBM microenvironment contains a diverse mixture of cells that includes brain tumor stem cells, microglia, astrocytes, stromal cells, endothelial cells, and pericytes (for reviews see [2–4]). Successful proliferation and survival of GBM cells within this microenvironment have been proposed to be highly

dependent on signaling between these cells. Thus, improving our understanding of intra- and inter-cellular communication within the tumor and its microenvironment may provide important new insights that could lead to the development of more effective GBM treatments.

Primary cilia are non-motile, hair-like organelles that play a key role in promoting neural cell proliferation [5, 6], migration [7, 8], and survival [9] in the developing and adult CNS. Their ability to influence these important cellular events is linked to their ability to detect various mitogens [10, 11], neuropeptides [12], and secreted growth factors [13, 14] in the extracellular environment. Previous analyses of five commonly studied astrocytoma and GBM cell lines (U87-MG, T98G, U-373G, U-138MG, U-251 MG) have suggested that these cell lines, which have been in existence for about 30–50 years [15, 16], typically fail to elaborate primary cilia [17]. Given the important roles cilia signaling pathways may play in regulating neural cell behavior in developing and adult CNS, and recent reports showing that basal cell carcinomas and medulloblastoma tumors contain ciliated cells that influence the formation and maintenance of these tumors [18–20], we decided to revisit the question of GBM cell ciliogenesis by examining primary human GBM specimens and derivative primary cell lines using immunostaining and EM to determine whether they contain ciliated cells.

Methods

GBM biopsies and primary GBM cells

GBM biopsies—The brain tumor biopsies analyzed in this study were collected from archived tumor samples housed within the Florida Center for Brain Tumor Research Repository. All tissue samples were obtained with informed consent and were collected using protocols approved by the University of Florida Institutional Review Board approval (to M.R.S, B.A.R, and D.A.S). Biopsies were classified by neuropathologists as either glioblastoma or grade III glioma according to WHO guidelines [21]. For immunohisto-chemistry, we examined a total of 22 biopsies that were fixed overnight at 4 °C in 4 % paraformaldehyde in 0.1 M phosphate buffer (4 % PFA). There were some differences in how the 22 biopsies were handled prior to fixation overnight. Four of the biopsies were immersed in 4 % PFA immediately following collection, which in our hands proved to be optimal for immunohistochemistry. Six biopsies underwent cryopreservation in supplemented NeuroCult™ media (StemCell Technologies, Inc.) containing 10 % DMSO at collection and were then thawed into 4 % PFA for fixation overnight. Finally, 12 biopsies were snap frozen in liquid N_2 at collection and then thawed into 4 %PFA for fixation overnight. After fixation, biopsies were rinsed in PBS, placed in 30 % sucrose, frozen in OCT compound over liquid N_2, and cryosections (20 μm) were collected onto coated slides. For EM, we fixed one biopsy (~1 h post-surgery) in a mixture of 3 % PFA and 2 % glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4 °C and processed the tissue as described below.

Primary GBM cells and spheres—We examined five GBM primary cell lines in this study (Line 0, Line 1, Line 2, SN179 (S2), and SN186 (S3)) that were isolated from human

GBM tumors [22, 23]. Spheres of cells were cultured in Neurobasal media supplemented with 20 ng/ml human epidermal growth factor (hEGF), 10 ng/ml basic fibroblast growth factor (bFGF), and 2 μg/ml heparin. When the spheres reached approximately 150 μm in diameter, they were enzymatically dissociated by digestion with a solution containing trypsin/EDTA (0.05 %) for 3–5 min at 37 °C. Finally, cells were washed, counted using trypan blue to exclude dead cells, and re-plated in fresh media supplemented with hEGF and bFGF. For spheres or cells grown on glass coverslips, Neurobasal media was supplemented with 5 % fetal bovine serum. Spheres or cells after 2 or 24 h of growth, respectively, were fixed for 15 min in 4 % PFA and washed with PBS.

Serum starvation—For serum starvation experiments, S2 and S3 cells were seeded onto glass coverslips in 24 well plates in Neuro-basal media containing FBS but no growth factors. Twenty-four hours later, the media in all cultures was changed and in selected wells was replaced with media lacking FBS. After 24 or 72 h, the cultures were fixed in 4 % PFA and the percentage of ciliated cells was determined as described below.

Antibodies

The primary antibodies used for immunocytochemistry (ICC) or immunohistochemistry (IHC) included mouse anti-acetylated alpha-tubulin (1:3000(ICC/IHC); Sigma), rabbit anti-Arl13b (1:1500 (ICC); gift from T. Caspary), mouse anti-gamma tubulin (1:2000 (ICC/IHC); Sigma), rabbit anti-IFT88 (1:200 (ICC); Proteintech), rabbit anti-Ki67 (1:200 (ICC/IHC); Vector), and rabbit anti-ZEB1 (1:1000 (ICC/IHC); Sigma). Sections were blocked with anti-mouse Fab fragments (20 μg/ml: Jackson Immunore-search) when stained sequentially with mouse antibodies against gamma- and acetylated alpha-tubulin. Secondary antibodies were species-specific and were conjugated with fluorescent tags (1:400 (ICC/IHC); Jackson Immunore-search). Slides were coverslipped with Prolong Gold antifade media containing DAPI (Invitrogen).

Immunostaining and quantification of ciliated cells

GBM tumor biopsies and primary cell lines were immunostained with anti-acetylated alpha-tubulin and gamma-tubulin antibodies to label the axoneme and basal bodies, respectively. Primary cilia can be identified by searching for G-tubulin-positive basal bodies proximate to AA-tubulin-positive axonemes [5, 19, 20]. The stained sections were examined using an Olympus IX81-DSU confocal microscope fitted with a 60× water objective and all images were captured as z-stacks (0.5 μm steps). Because we did not observe multi-ciliated cells during our analyses, we quantified the numbers of ciliated cells in dissociated cultures of the primary cell lines by randomly selecting 4–6 microscopic fields per coverslip (3–5 coverslips/cell line) and then counting the number of DAPI-labeled nuclei and cilia in each of the fields/z-stacks. The percentage of ciliated cells was calculated as the number of cilia/number of DAPI-labeled nuclei for each field. To quantify the numbers of ciliated cells in cultured spheres we counted the total number of DAPI-labeled nuclei and cilia within z-stacks of spheres averaging 40–50 cells in size (8–10 spheres/group). The percentage of ciliated cells was calculated as the number of cilia/number of DAPI-labeled nuclei for each sphere. For analyses of GBM biopsies, biopsies were considered to contain ciliated cells if cilia were observed within the first 2–3 z-stacks of each stained section. For analyses of the

distribution of ciliated cells within the tumor biopsies, we focused our analyses on four tissue samples that had been immersed in fixative immediately following harvest. The percentage of ciliated cells located within blood vessels, which were identified using rabbit anti-laminin antibody (not shown), or within either a 30 μm or >50 μm radius of a blood vessel was determined by examining z-stack images of randomly selected microscopic fields.

Electron microscopy

Spheres of Line 0 cells were grown in growth-factor supplemented, serum-free Neurobasal media (described above) and were then fixed in a mixture of 3 % PFA and 2 % glutaraldehyde for 1 h. The fixed spheres and biopsy tissues were washed in 0.1 MPB, post-fixed in 1 % osmium tetroxide, dehydrated in ethanol, and embedded in resin. Semithin sections were stained with toluidine blue. Ultrathin sections (70 nm) were collected onto grids, stained with uranyl acetate and lead citrate, and viewed using a Hitachi H-7600 transmission electron microscope at 80 kV. Images were captured with a Hitachi digital camera and proprietary software.

Statistical analyses

Data obtained from the serum starvation experiments were analyzed using two-way ANOVA with treatment and time as main factors. The groups compared (n = 4 coverslips/group) were as follows: 24 h (+FBS), 24 h (–FBS), 72 h (+FBS) and 72 h (–FBS). The significant ($p < 0.05$) treatment × time interaction identified using two-way ANOVA was followed up with Fisher's PLSD post hoc comparisons of treatment at each time point. A p-value less than 0.05 was considered significant. All analyses were carried out using StatView 5.0 software (SAS Institute Inc, NC).

Results

GBM primary cell lines contain subpopulations of ciliated cells

Acetylated alpha-tubulin (AA-tubulin) and gamma-tubulin (G-tubulin) are microtubule proteins that are enriched in the axonemes and anchoring basal bodies of most cilia, respectively, and are critical for maintaining cilia structure [5, 19, 24]. Examination of primary GBM cells and spheres co-stained with antibodies recognizing AA-tubulin and G-tubulin revealed subpopulations of cells that stained positively for both G- and AA-tubulin in a manner consistent with the presence of well-formed primary cilia (Fig. 1a–c). Analyses of the number of ciliated cells in dissociated cell cultures revealed that 23.7 ± 2.4 % of Line 0, 16.7 ± 2.3 % of Line 1, 11.9 ± 1.4 % of Line 2, 15.7 ± 2.6 % of S2, and 15.7 ± 2.0 % of S3 cells were ciliated (mean ± SEM; Fig. 1d). Analyses of the number of ciliated cells in spheres generated from two cell lines revealed that 14.4 ± 2.7 % of Line 0 and 7.6 ± 0.8 % of Line 2 cells in these spheres were ciliated (Fig. 1e). These results show that primary cell lines derived from GBM tumors contain subpopulations of cells that retain their ability to synthesize cilia.

Serum starvation has been used as a strategy to induce ciliogenesis in various cell types [17, 25]. Thus, we asked whether such treatment could increase the number of ciliated cells in

dissociated S2 or S3 GBM cells. Our results showed that serum starvation for either 24 or 72 h did not significantly increase the relative numbers of ciliated S2 cells ($F_{(1,12)} = 0.014$, $p = 0.908$) or S3 cells ($F_{(1,12)} = 0.403$, $p = 0.537$) in our cultures (Fig. 1f). These results suggest that GBM cells may not respond to traditional measures of inducing ciliogenesis.

Next, we examined the ultrastructure of the cilia elaborated by cells in Line 0 spheres using EM (Fig. 2a). We detected several cells with docked basal bodies and elon-gated axonemes (Fig. 2b–g). The microtubules within these axonemes were well-organized and extended along the entire length of the visible axoneme (Fig 2c, e). The well-formed, microtubular axoneme backbone observed in these cilia would be expected to support intraflagellar transport (IFT), that is essential for the formation and function of cilia ([26]; for review see [27, 28]). This prediction is supported by our data showing IFT88, a complex B protein that carries cargo to the cilia tip, was localized to the ciliary base, axoneme, and tips of AA-tubulin + cilia (Fig. 2h). In addition, we detected Arl13b, a small GTPase reported to be trafficked into neural cilia [29], localized along the length of the ciliary axonemes of the Line 0 cells, (Fig. 2i). These results suggest that the structure of the cilia elaborated by GBM cells is normal and that they are capable of trafficking cargo along their axonemes.

Surgically resected primary GBM tumors contain ciliated cells

We next asked whether ciliated cells persist in surgically resected specimens. We examined sections of 22 different GBM biopsies (21 primary and 1 recurrent) stained with antibodies to AA-tubulin and G-tubulin to determine if these biopsies contained ciliated cells. We detected G- and AA-tubulin + cilia in all 22 biopsies, including the tumor resected from a patient with recurrent GBM (data not shown). Moreover, cilia appeared to be distributed throughout the GBM microenvironment. Examination of a tumor removed from a 49-year-old male revealed that ciliated cells were present within and surrounding the necrotic zones of the tumor (Fig. 3a). Examination of another tumor from an 84-year-old male revealed subsets of ciliated cells within and immediately surrounding the blood vessels (Fig. 3b and b', boxed areas show zoomed views of cilia). Ciliated cells were also found adjacent to and within pseudopalisades (data not shown). The presence of cilia around the vasculature prompted an analysis of the spatial distribution of cilia in four GBM biopsies. Overall, we found cilia on 14.1 ± 4.5 % of cells within blood vessels, 24.6 ± 4.2 % of cells within a 30 μm radius of blood vessels, and 18.2 ± 5.2 % of cells 50 μm or further away from blood vessels. Together, these results suggest that ciliated cells can be detected in different regions of the tumor microenvironment.

To further confirm our immunohistochemical detection of cilia in GBM, we also performed an EM analysis of a GBM biopsy freshly extracted from the right occipital lobe of a 61-year-old male. Ultrastructurally, this tumor was characterized bydense connective/collagen-like fibers,blood vessels and mitotic cells (Suppl. Fig. 1). Within this environment, we identified cells extending cilia. The cells possessed docked basal bodies and axonemes containing microtubules that appeared well-organized and extended along the length of the visible axoneme (Fig. 4a, b). It is noteworthy that we also observed cells that displayed abnormal basal bodies/centrioles which did not appear to project cilia (Fig. 4c). Together,

these results provide ultrastructural confirmation that ciliated cells are present within GBM, and suggest that some GBM cells may be defective in their ability to extend cilia.

Ciliated cells within GBM tumors also express Ki67 and ZEB1

It has been suggested that cilia are required for normal and tumorigenic cell proliferation [5, 6, 18, 19]. Thus, we sought to determine whether actively dividing GBM tumor cells are ciliated by co-staining GBM Line 0 cells with antibodies against the proliferating antigen, Ki67, [30] and AA-tubulin. We found that Ki67+ Line 0 cells possessed AA- tubulin + cilia (Fig. 5a). We then co-immunostained sections of all of our GBM biopsies (n = 22) that contained G-tubulin/AA-tubulin + cilia (Fig. 3) with the Ki67 antibody and found that 17/22 biopsies contained Ki67+ cells that were also ciliated (Fig. 5b, e). This finding prompted us to determine whether ciliated cells in the GBM tumors were expressing other markers of tumorigenesis. We recently reported that levels of the transcription factor, ZEB1, in GBM are significantly elevated and that increased expression of ZEB1 is associated with cells that are invasive and chemo-resistant [31]. We found that ZEB1+ S2 cells possessed G- and AA-tubulin + cilia (Fig. 5c). Staining of GBM sections with a ZEB1 antibody revealed that 19/22 of the biopsies contained subsets of cells bearing G- and AA-tubulin + cilia that also stained for ZEB1 (Fig. 5d, e). Together, these results suggest that the cilia present in GBM tumors are associated with cells displaying proliferative and potentially invasive and chemoresistant capabilities.

Discussion

Our immunohistological and EM analyses of GBM tumors and primary cell lines consistently show that subsets of cells within these tumors possessed well-formed primary cilia. Moreover, these cilia were associated with cells expressing protein markers indicative of proliferative and invasive/chemoresistant capabilities. While the numbers of ciliated cells associated with various cancers, including GBM, are low [17, 32–36], we suggest that the presence of subpopulations of ciliated cells in GBM may be clinically relevant given that primary cilia have been shown to play roles in the proliferation, migration, and survival of some cell types.

We consistently identified ciliated cells in GBM tumors and primary GBM cell lines, a result that differs from that reported by Moser et al. (2009), who reported that fully formed primary cilia were either rare, absent, or abnormal in five highly propagated GBM cell lines. Differences in cell lines and their growth conditions may contribute to the disparities between these results. The cell lines examined by Moser et al. (2009) were well-established, older GBM cell lines, whereas we examined recently derived primary tumor cell lines and tumor biopsies. Unlike the culture media used by Moser et al. (2009), we used media supplemented with bFGF and EGF in an attempt to mirror conditions resembling those found in primary glioblastomas [37]. Alternatively, since we observed that only 8–25 % of the cells examined in vitro and in the biopsies were ciliated, an estimate that may be low because not all primary cilia are positive for gamma- or acetylated alpha-tubulin [38–40], it is possible that the majority of GBM cells are unable to grow cilia, a state that would be expected to lead to dysregulation of the cell cycle [41]. Our observations that serum

starvation did not increase the numbers of ciliated GBM cells in our cultures and that nonciliated cells were identified by ultrastructural analysis of a GBM biopsy are consistent with this possibility, and are findings consistent with those reported by Moser et al. (2009). It is noteworthy that a recent study of U-251 GBM cells, which typically lack cilia [17], reported that expression of cell cycle-related kinase (CCRK), a protein often upregulated in GBM, is abnormally high in these cells [42]. These authors found that knockdown of CCRK levels increased ciliogenesis and inhibited U-251 proliferation, results suggesting GBM cells that fail to elaborate cilia may contribute to GBM tumor growth. Future studies will be needed to determine what factors determine whether GBM cells generate cilia and whether failure of GBM cells to generate cilia contributes to tumor cell propagation.

Could subpopulations of ciliated GBM cells trigger and/or maintain tumor growth+ There is growing evidence that cilia support cell tumorigenesis. For example, knockout of Kif3a, a protein required for cilia formation and function, in cerebellar granule neuron precursors [18, 19] and in human basal cell carcinoma [20] has been reported to inhibit the formation and growth of medulloblastoma and basal cell carcinoma, respectively. Our observations that KIF3A (data not shown) and ciliated cells are present in GBM tumors suggest the possibility that cilia may also play a role in regulating growth of these tumors. We also identified ciliated cells that were actively dividing (Ki67+) and expressing a transcription factor (ZEB1+) reported to mediate GBM initiation, invasion and chemoresistance [31], throughout the tumor biopsies. The close proximity of Ki67+ and ZEB1+ ciliated cells to blood vessels could facilitate their responses to proliferation, migratory or survival cues originating from the bloodstream, cues that could promote growth of the tumor. Clearly, the ability of cilia to transduce changes in the extracellular environment into adaptive cellular responses would be advantageous to tumors. Finally, GBM tumors are notoriously invasive with cells infiltrating both adjacent and remote brain regions. It has recently been shown that migrating cells in the developing brain use cilia to navigate across the forebrain, and that Arl13b is involved in this process [7, 8]. This is noteworthy since we detected Arl13b in the cilia of Line 0 cells, cells that form lethal and invasive tumors when xenografted into mice [22, 31, 43]. Thus, it is conceivable that cilia could also play a role in GBM invasion by guiding tumor cell migration. Taken together, our results suggest several potential lines of investigation that may lead to improved understanding of how ciliated GBM cells might contribute to the characteristic aggressiveness and treatment resistance of this cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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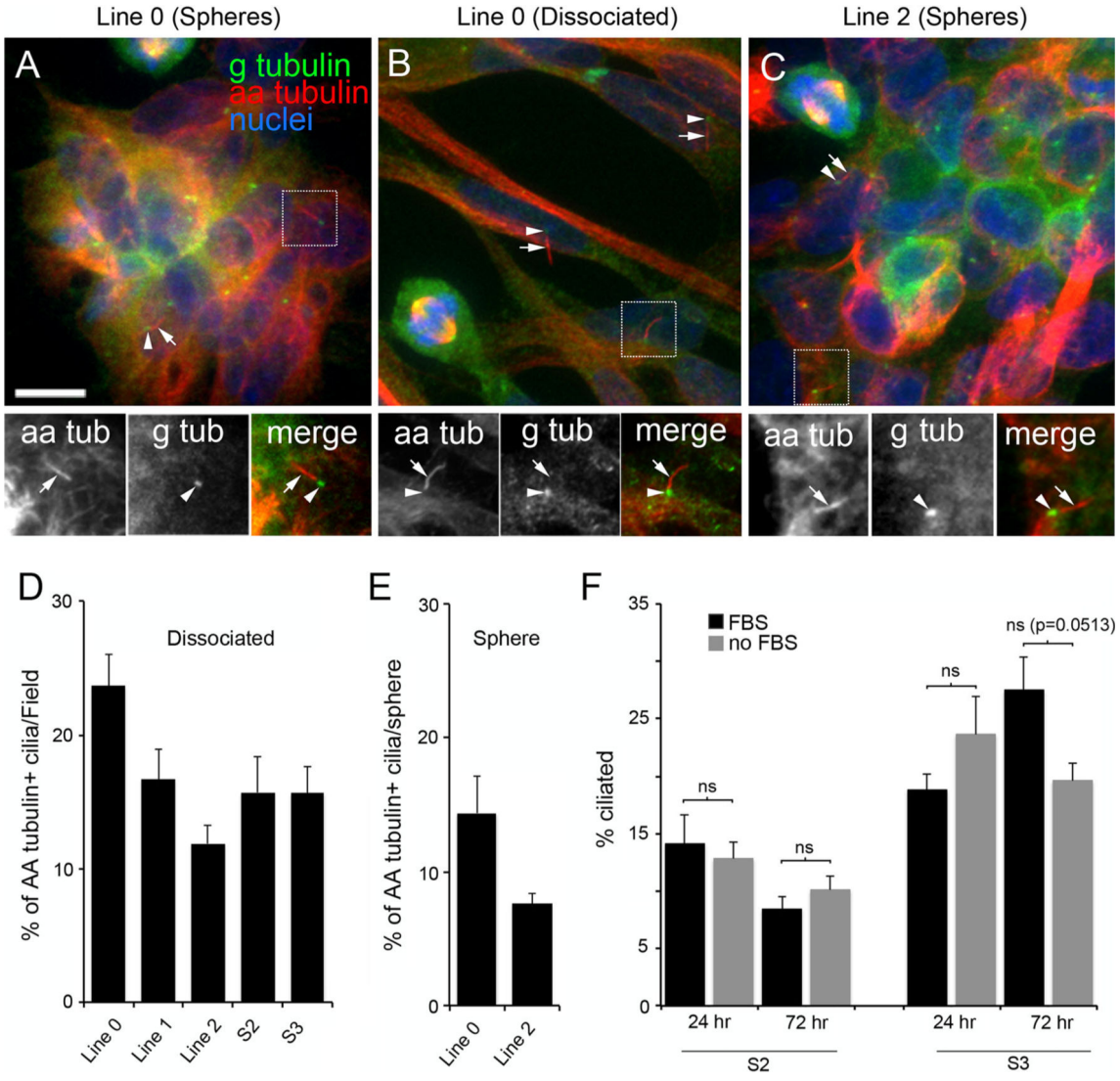

Fig. 1.

Subsets of cultured GBM cell lines derived from human GBM specimens stain positively for primary cilia. (**a–c**) Sphere aggregates and dissociated Line 0 (**a, b**) and Line 2 (**c**) cells were immunostained for gamma-tubulin (G-tubulin or g tub) (*green*) and acetylated alpha-tubulin (AA-tubulin or aa tub) (*red*). G-tubulin is enriched in basal bodies and spindle poles, and AA-tubulin is enriched in spindle fibers and the cilia axoneme but can also be detected throughout the cell. The spatial relationship of the staining of these two proteins was used to identify cilia. All images are maximum projections from confocal z-stacks. *Scale bar* (**a**) = 10 lm. In **a–c**, magnified images of the boxed regions showing the staining patterns for aa tub (*arrows*) and g tub (*arrowheads*) are displayed below the larger image. The adjacent localization of g tub and aa tub is clearly evident in the merged images. Additional cilia are indicated in the larger images using *arrows*/*arrowheads*. **d** Percentage (±SEM) of AA-tubulin + ciliated cells/field for the indicated cell lines, 24 h after seeding onto glass coverslips. **e** Percentage (±SEM) of AA-tubulin + ciliated cells/sphere for indicated lines, 2 h after seeding onto glass coverslips. **f** Percentages (±SEM) of S2 or S3 cells that were

ciliated at either 24 or 72 h after serum removal compared to control cells maintained in FBS. Groups were compared using a 2-way ANOVA followed by Fisher's PLSD post hoc analysis. *ns* not significant

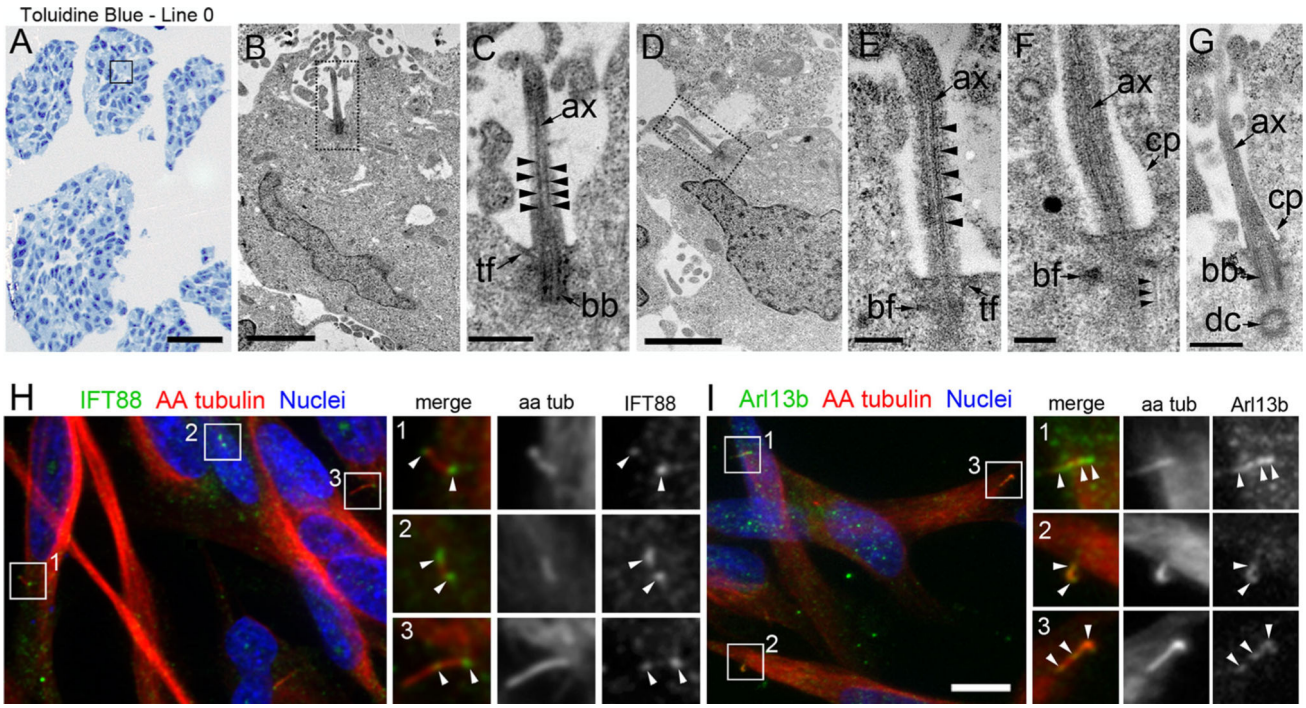

Fig. 2.

Ultrastructure of Line 0 cilia and detection of IFT88 and Arl13b along their axonemes. **a** Semithin sections of Line 0 spheres stained with toluidine blue. The box approximates regions where EM analysis was performed. **b** Low magnification of a cilium (*dotted box*) extending out of a cell. **c** Higher magnification of the cilium in (**b**) reveals a docked basal body anchored by transition fibers and an axoneme comprised of organized, parallel arrangements of microtubules (*arrowheads*). **d** Another example of a cilium (*dotted box*) extending out of the cell body, whose axoneme contained organized, parallel arrangements of microtubules (**e**, *arrowheads*). **f, g** Additional high magnification images of cilia showing axonemes extending out of the ciliary pocket. Typical parallel arrangements of microtubules that impinged on the basal foot (**f**, *arrowheads*) and daughter centrioles arranged perpendicularly to basal bodies were observed (**g**). **h** Immunostaining for IFT88 (*green*) revealed localization to the bases, axonemes, and tips of Line 0 cell cilia. Boxes 1–3 highlight IFT88 + cilia that are shown in the magnified images on the right (*arrowheads*). **i** Immunostaining for Arl13b (**green**) revealed localization along the ciliary axonemes and tips. Boxes 1-3 highlight Arl13b + cilia that are shown in the magnified images on the right (arrowheads).ax axoneme, *tf* transition fibers, *bb* basal body, *bf* basal foot, *dc* daughter centriole, *cp* ciliary pocket. *Scale bars* in A = 100 μm, B, D = 2 μm, C = 500 nm, E = 250 nm, F = 200 nm, G = 500 nm, I = 10 μm

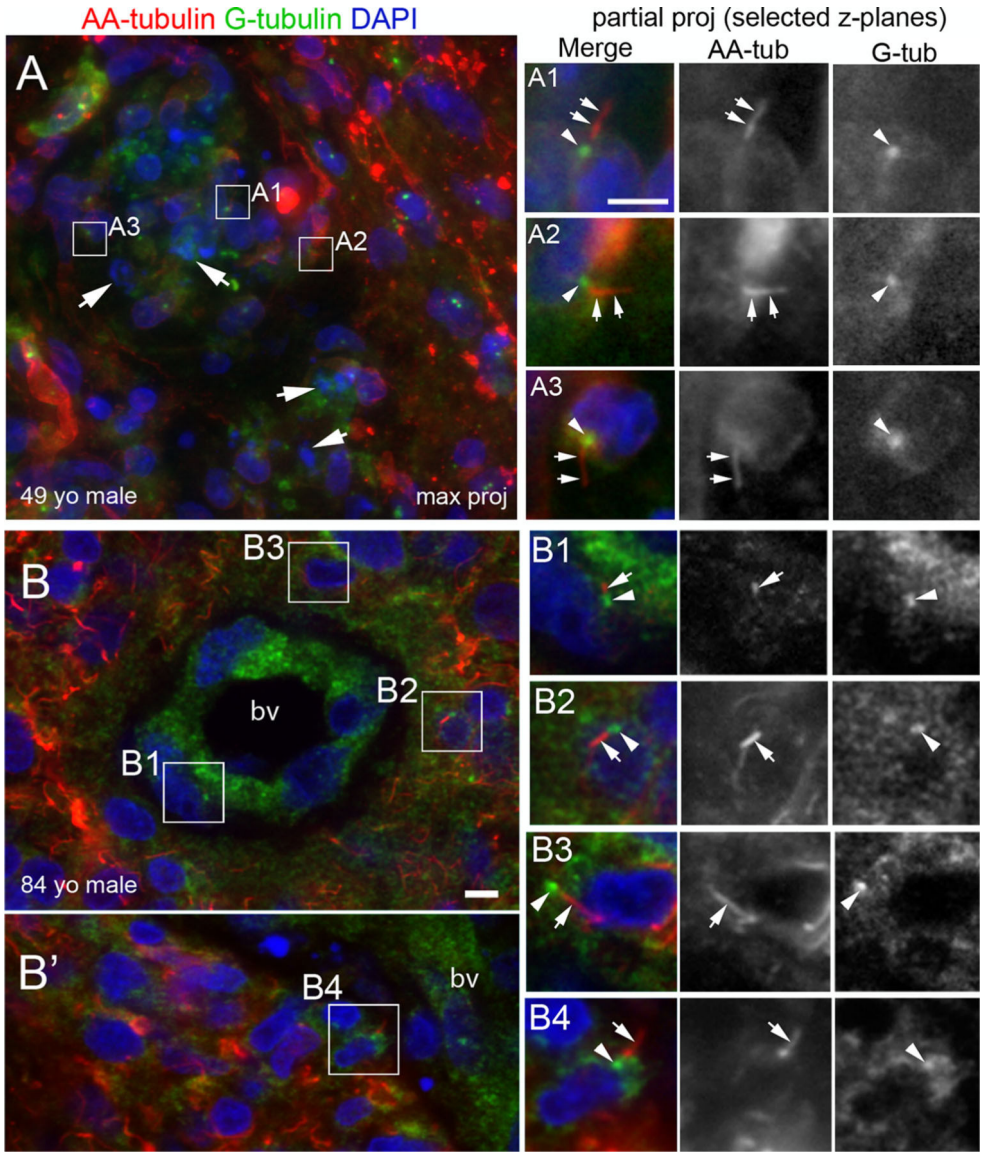

Fig. 3.

Identification of cilia in GBM biopsies. (**a, b**) Representative examples of G-tubulin/AA-tubulin + cilia in 2 out of 22 stained GBM biopsies. Cilia were detected throughout the tumor microenvironment. **a** Biopsy from a 49-year-old male immunostained for G-tubulin (*blue*) and AA-tubulin (*red*). Nuclei were stained with DAPI (*green*). This region contained nuclear debris and several apoptotic nuclei (*arrows*). Ciliated cells are indicated in boxed regions. **A1–A3** Magnified images of *boxed* regions in A (partial projections representing 2–3 collapsed z-stacks within the maximum projected) show the G-tubulin positive basal bodies (*arrowheads*) and AA-tubulin positive axonemes (*arrows*) of the cilia. Bars (μm) = 10 (A), 5 (A1). **B** and **B'** Biopsy from an 84 year-old male stained with G-tubulin (*blue*) and AA-tubulin (*red*) revealed subsets of ciliated cells within and surrounding blood vessels (bv). **B1–B4** Magnified images of *boxed regions* in **B** and **B'** clearly show adjacent localization of G- and AA-tubulin staining on the cilia. Cilium in B1 is located on a cell

within the bv wall. Cilia in *B*2–*B*4 are found on cells surrounding the bv. *Scale bar* B = 5 μm

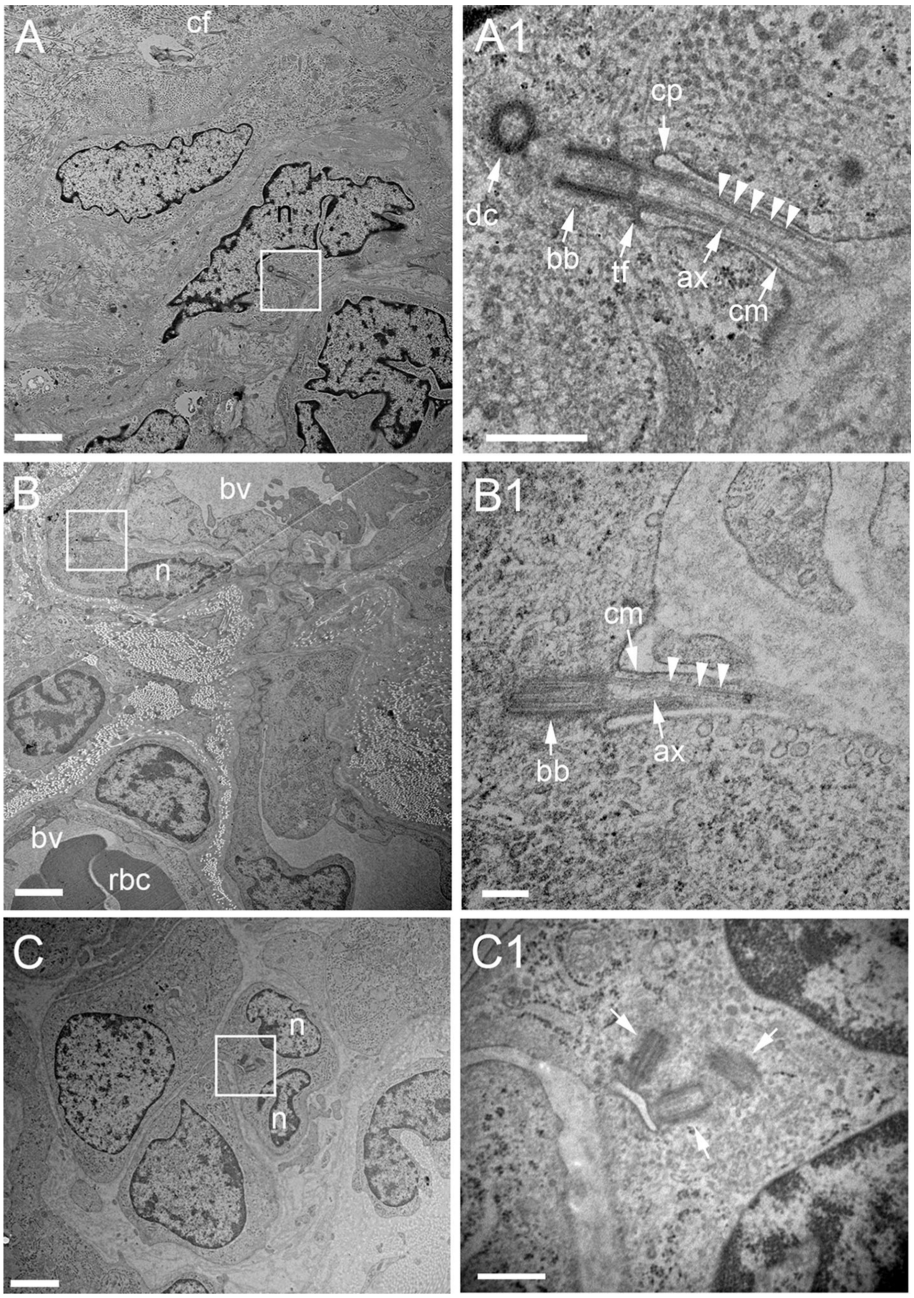

Fig. 4.

Ultrastructural detection of cilia in a GBM biopsy from a 61 year-old male. **a** Example of a cell close to densely packed collagen-like fibers (also see Suppl. Fig. 1) extending a cilium (boxed region). **a1** Higher magnification of the cilium in (**a**) reveals a docked basal body anchored by transition fibers and an axoneme comprised of organized, parallel arrangements of microtubules (*arrowheads*). **b** Example a cell adjacent to blood vessels (bv) projecting a cilium (boxed region, magnified in B1). **c** Example of cell that appears to contain multiple nuclei and abnormal numbers of basal bodies/centrioles (*arrows*). *ax* axoneme, *tf* transition fibers, *bb* basal body, *dc* daughter centriole, *cf* collagen-like fibers, *cp* ciliary pocket, *cm* ciliary membrane, *n* nucleus. *Scale bars* in A, B, C = 2 μm, A1, C1 = 500 nm, B1 = 200 nm

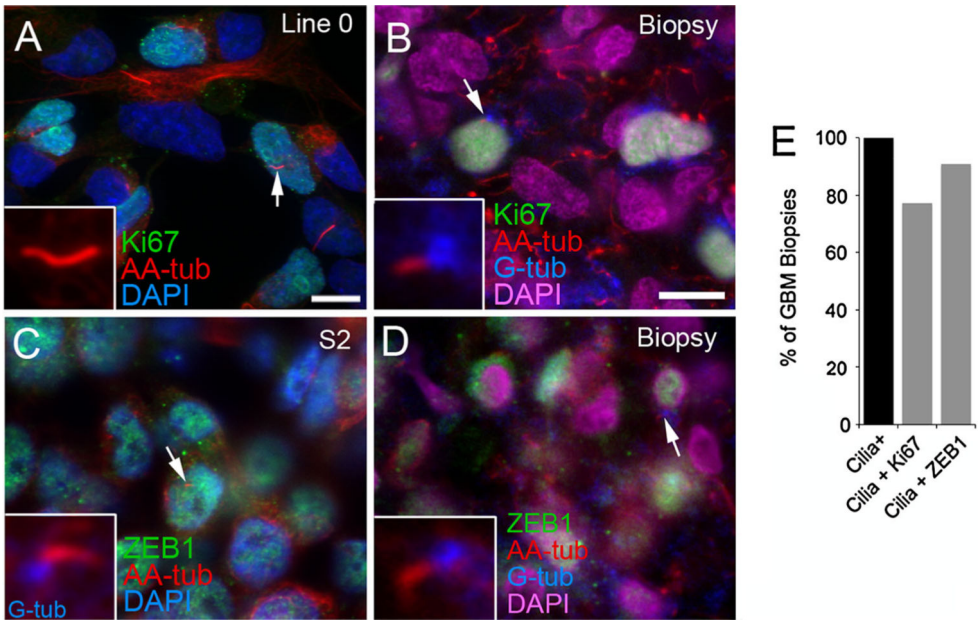

Fig. 5.

Detection of primary cilia on Ki67+ and ZEB1+ GBM cells. **a** Line 0 cells were immunostained for Ki67 (*green*) and AA-tubulin (*red*) and nuclei were labeled with DAPI (*blue*). AA-tubulin + cilia (*arrow*, enlarged in *inset*) could be readily identified on Ki67+ cells. **b** Immunostaining of a biopsy (same as Fig. 3b) shows a G-tubulin+/AA-tubulin + cilium (*arrow*, enlarged in *inset*) associated with a Ki67+ cell in the tumor. **c** S2 cells were immunostained for ZEB1 (*green*), AA-tubulin (*red*), G-tubulin (*blue* in *inset*) and nuclei were labeled with DAPI (*blue*). AA-tubulin+/G-tubulin + cilia (*arrow*, enlarged in *inset*) could be identified on ZEB1 + cells. **d** Immuno-staining of a different biopsy shows an example of a ZEB1 + nucleus associated with a G-tubulin+/AA-tubulin + cilium (*arrow*, enlarged in *inset*). **e** Percentage of GBM biopsies that contained cells positive for cilia, both cilia and Ki67, or both cilia and ZEB1. *Scale bars* in A, B = 10 μm