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### **Citation**

Andrea, C. E. de, Wiweger, M., Prins, F., Bovee, J. V. M. G., Romeo, S., & Hogendoorn, P. C. W. (2010). Primary cilia organization reflects polarity in the growth plate and implies loss of polarity and mosaicism in osteochondroma. *Laboratory Investigation*, 90(7), 1091-1101. Retrieved from <https://hdl.handle.net/1887/109197>

Version: Not Applicable (or Unknown)

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# Primary cilia organization reflects polarity in the growth plate and implies loss of polarity and mosaicism in osteochondroma

Carlos E de Andrea<sup>1</sup>, Malgorzata Wiweger<sup>1</sup>, Frans Prins<sup>1</sup>, Judith VMG Bovée<sup>1</sup>, Salvatore Romeo<sup>2</sup> and Pancras CW Hogendoorn<sup>1</sup>

Primary cilia are specialized cell surface projections found on most cell types. Involved in several signaling pathways, primary cilia have been reported to modulate cell and tissue organization. Although they have been implicated in regulating cartilage and bone growth, little is known about the organization of primary cilia in the growth plate cartilage and osteochondroma. Osteochondromas are bone tumors formed along the growth plate, and they are caused by mutations in *EXT1* or *EXT2* genes. In this study, we show the organization of primary cilia within and between the zones of the growth plate and osteochondroma. Using confocal and electron microscopy, we found that in both tissues, primary cilia have a similar formation but a distinct organization. The shortest ciliary length is associated with the proliferative state of the cells, as confirmed by Ki-67 immunostaining. Primary cilia organization in the growth plate showed that non-polarized chondrocytes (resting zone) are becoming polarized (proliferating and hypertrophic zones), orienting the primary cilia parallel to the longitudinal axis of the bone. The alignment of primary cilia forms one virtual axis that crosses the center of the columns of chondrocytes reflecting the polarity axis of the growth plate. We also show that primary cilia in osteochondromas are found randomly located on the cell surface. Strikingly, the growth plate-like polarity was retained in sub-populations of osteochondroma cells that were organized into small columns. Based on this, we propose the existence of a mixture ('mosaic') of normal lining (*EXT*<sup>+/-</sup> or *EXT*<sup>wt/wt</sup>) and *EXT*<sup>-/-</sup> cells in the cartilaginous cap of osteochondromas.

*Laboratory Investigation* (2010) 90, 1091–1101; doi:10.1038/labinvest.2010.81; published online 26 April 2010

**KEYWORDS:** bone tumor; cartilage; cell polarity; growth plate; hereditary multiple exostoses; osteochondroma

The growth plate is a highly organized, cartilaginous template needed for the longitudinal growth of long bones. Structurally, the growth plate can be divided into three distinct zones.<sup>1</sup> The mitotic spindles of proliferating chondrocytes are aligned perpendicular to the long axis of the growing bone. The chondrocytes have to undertake a series of cell movements/rotations and shape changes to align one on top of the other to generate the typical columns of the growth plate.<sup>2–4</sup> Once acquired, this columnar organization is maintained. On the metaphyseal side of the growth plate, the chondrocytes form the hypertrophic zone. The cells stop proliferating and change their expression profile to synthesize type X collagen and to prepare for programmed cell death and mineralization.<sup>1</sup> Regulation of the growth

plate is tightly maintained by signaling pathways in which heparan sulfate proteoglycans have a crucial role in facilitating the transport of signaling molecules within the extracellular matrix and in modulating the receptor-ligand interactions.

Mutations in *EXT1* and *EXT2* genes, which are involved in synthesis of heparan sulfate chains, lead to hereditary multiple osteochondromas (previously known as hereditary multiple exostoses), most likely by deregulating signaling pathways. Osteochondromas are the most common benign bone tumors<sup>5</sup> and usually arise from the juxta-epiphyseal region of the growth plate. Although the cells seem somewhat disorganized, the histological features of osteochondromas are similar to those of the growth plate.<sup>6</sup>

<sup>1</sup>Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands and <sup>2</sup>Department of Pathology, Treviso Regional Hospital Treviso, Treviso, Italy  
Correspondence: Dr PCW Hogendoorn, MD, PhD, Department of Pathology, Leiden University Medical Center, PO Box 9500, L1-Q, 2300 RC Leiden, The Netherlands.  
E-mail: P.C.W.Hogendoorn@lumc.nl

Received 6 November 2009; revised 12 February 2010; accepted 19 February 2010

Primary cilia are specialized cell surface projections present on most eukaryotic cells.<sup>7,8</sup> The primary cilium can be viewed as the antenna of the cell, which receives and transduces mechanical and chemical signals from the surrounding cells and the extracellular matrix.<sup>9</sup> Primary cilia are formed during interphase, when the centriole pair moves together to the plasma membrane. The mother centriole becomes the basal body of the cilium by generating a 9 + 0 microtubular doublet symmetry that represents the structural core of the axoneme.<sup>9</sup> Construction of the axoneme requires effective intraflagellar transport, a bidirectional drive system for molecules run by motor protein complexes. Anterograde transport, from the base to the tip of the primary cilium, is driven by heteromeric kinesin-II motor proteins, which are composed of KIF3A and KIF3B motor subunits. Retrograde transport, from the tip to the base of the cilium, is mediated by dynein 1B.<sup>10</sup>

The link between cilia function and growth plate development is described in several recent studies. Primary cilia have been implicated as recipients of some signaling pathways essential for the regulation of the growth plate, such as the non-canonical Wnt and Indian Hedgehog pathways.<sup>3,11–14</sup> KIF3A deficiency has been shown to cause abnormal topography of Hedgehog signaling and growth plate dysfunction.<sup>15</sup> In several tissues, the organization of primary cilia has been shown to reflect cell polarity.<sup>16–18</sup> In cartilage, it has been reported that primary cilia have a specific orientation pointing away from the articular surface in articular chondrocytes or located at the center of the columnar cells in the growth plate.<sup>3,19,20</sup> In the extensor tendon, cilia are aligned parallel to the collagen fibers and the long axis of the tendon.<sup>8,21,22</sup>

In this study we show the organization of primary cilia within and between the zones of the growth plate and osteochondroma. In both tissues, cilia were found to have similar forms and ultrastructures but distinct organization. In the growth plate, non-polarized chondrocytes (resting zone) are becoming polarized (proliferating and hypertrophic zones), orienting the cilia parallel to the longitudinal axis of the bone. This reflects cell polarity. In osteochondromas, primary cilia are randomly located on the cell surface. Strikingly, the growth plate-like polarity was retained in sub-populations of osteochondroma cells that were organized into small columns. We also show that the shortest ciliary length was related to the proliferative state of the cells.

## MATERIALS AND METHODS

### Growth Plate and Osteochondroma Samples

Five postnatal growth plates were sampled from orthopedic resections for pathological conditions not related to osteochondroma. Five osteochondroma cases were retrieved from the surgical pathology files. Patient data were obtained by reviewing pathological reports or clinical charts (Supplementary Table 1). All samples were obtained from the Leiden University Medical Center and handled according to ethical

guidelines as described in the *Code for Proper Secondary Use of Human Tissue* in the Netherlands of the Dutch Federation of Medical Scientific Societies.

### Histopathological and Immunohistochemistry Evaluation

All the samples were fixed in formalin, decalcified and embedded in paraffin as previously described.<sup>23</sup> For histopathological evaluation, 4  $\mu$ m-thick sections were stained with hematoxylin and eosin (H&E).

To compare osteochondromas with growth plates, three zones were identified in the cartilaginous cap of the tumor (Figure 1) according to the following morphologic features:

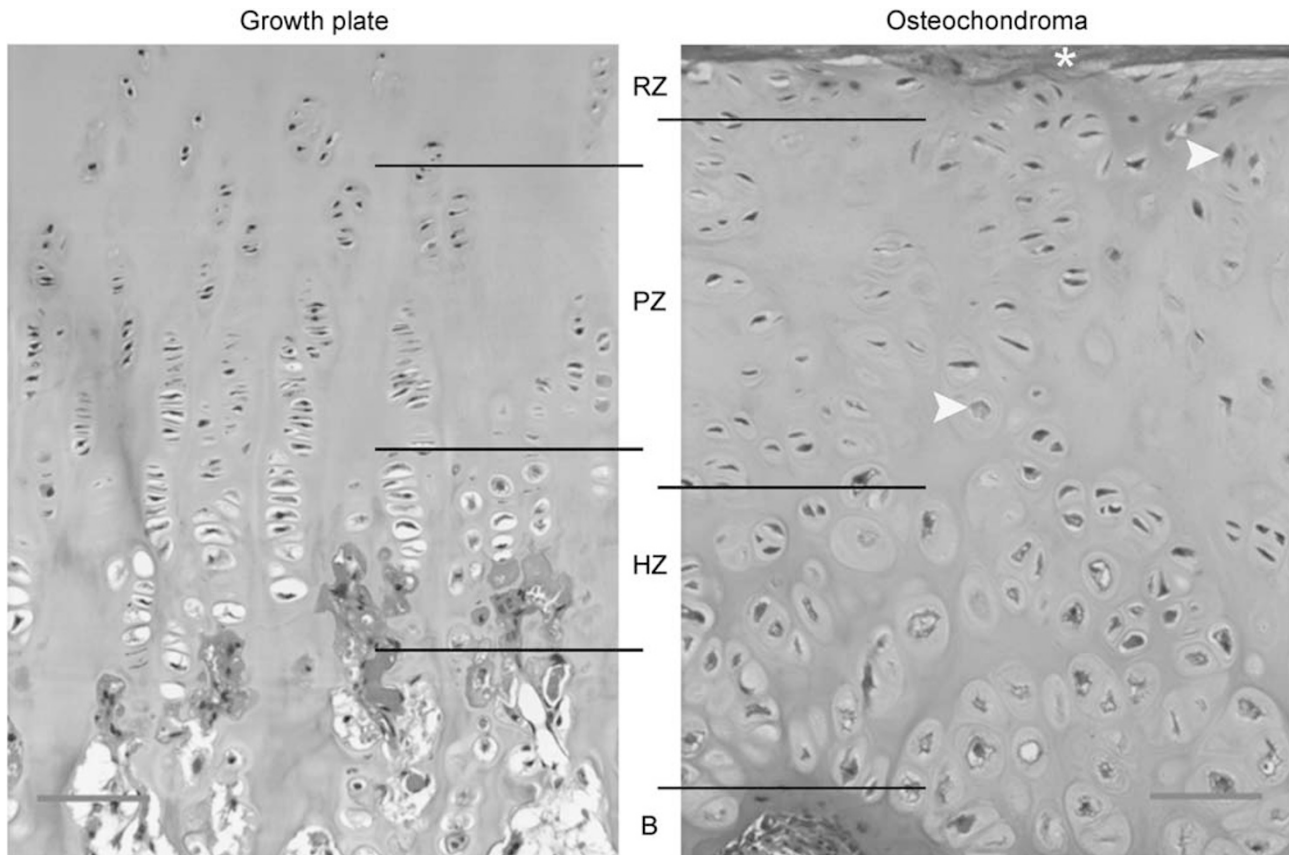
- Resting zone: spherical chondrocytes, single or in pairs, irregularly arranged;
- Proliferating zone: flattened chondrocytes arranged in columns or forming clusters;
- Hypertrophic zone: medium/large and rounder chondrocytes.

Pre-hypertrophic and hypertrophic zones were counted as a single zone because morphological distinction of the two is not reliable.<sup>24</sup>

Immunohistochemistry was performed for proliferation rate analysis using a monoclonal antibody against Ki-67 (clone MIB1, 1:100; Dako, Glostrup, Germany) according to standard laboratory procedures.<sup>25</sup>

### Immunofluorescence Staining

For immunofluorescence studies, 20  $\mu$ m-thick sections of growth plates and osteochondromas were cut parallel to the longitudinal axis of the bone or the tumor. The sections were incubated with testicular hyaluronidase (2 mg/ml in 0.1 M Tris saline, pH 5.0; Sigma-Aldrich, St Louis, MO, USA) at 37 °C for 2 h and with 0.5% Triton X-100 in PHEM buffer (PIPES 0.05 M, HEPES 0.025 M, EGTA 0.01 M and MgCl<sub>2</sub> 0.01 M) at room temperature for 5 min as previously described.<sup>7,26</sup> In addition, to increase antibody penetration, proteinase K (5  $\mu$ l/ml in 0.1 M Tris-buffered saline, pH 5.0; DakoCytomation, Carpinteria, CA, USA) was applied at room temperature for 3 min. The sections were stained with primary monoclonal antibodies against acetylated  $\alpha$ -tubulin (clone 6-11b-1, 1:1000; Sigma-Aldrich, Steinheim, Germany),  $\gamma$ -tubulin (clone GTU-88, 1:1000; Sigma-Aldrich, USA) and/or Ki-67 (1:100) and/or a polyclonal antibody against KIF3A (1:100; Abcam, Cambridge, UK) at 4 °C overnight. Secondary antibodies conjugated with Alexa Fluor 488 (1:200; Invitrogen Molecular Probes, Eugene, OR, USA) or Alexa Fluor 647 (1:200) were applied for 30 min to detect the primary antibody. Finally, the sections were mounted in Vectashield with propidium iodide (Vector Laboratories, Burlingame, CA, USA). Negative controls were sections stained without adding the primary antibody.



**Figure 1** Histology of the growth plate and osteochondroma. The growth plate is formed by three zones: resting (RZ), proliferating (PZ) and pre/hypertrophic (HZ) that over time turn into bone (B). Along the growth plate, the chondrocytes undergo proliferation (PZ) and differentiation (HZ and B). During these processes, the cell columnar organization is maintained. Osteochondromas are formed by three layers: outer perichondrium (asterisk), cartilaginous cap and underlying bone. The histological features of osteochondromas are to some extent similar to the ones of the growth plate. The zonation is less defined (RZ, PZ and HZ), and the cells are irregularly arranged and rarely acquire columnar organization. Hypertrophic-like chondrocytes (arrowhead) are observed within the RZ and PZ. The osteochondroma cells also undergo terminal ossification (B). (RZ, resting zone; PZ, proliferating zone; HZ, hypertrophic zone; Scale bars, 100  $\mu$ m).

### Electron Microscopy

Electron microscopy studies were performed on two growth plates and two osteochondromas as previously described.<sup>27</sup> To evaluate the ultrastructure of primary cilia, ultrathin sections were examined in a JEOL JEM-1011 electron microscope equipped with a MegaView III digital camera.

### Confocal Microscopy and Analysis of Primary Cilia

Fluorescently labeled primary cilia were imaged using a confocal laser scanning microscope (LSM 510; Zeiss, Jena, Germany) and a plan apochromat  $\times 63/1.40$  oil or a C-Apo  $\times 40/1.2$  water immersion objective lens (both from Zeiss). All images were acquired at a resolution of  $1024 \times 1024$  or  $512 \times 512$  pixels, a pinhole of 1 Airy unit, and an average of two successive scans at a speed of 6. After initial scanning to orient the section parallel to the longitudinal axis of the bone/tumor, z-series were imaged at  $0.5 \mu$ m intervals to depths as great as  $20 \mu$ m. A total of 10 fields of randomly selected views from each zone of the growth plate and

osteochondroma were scanned in their full depth and used to evaluate (ImageJ software; NIH Image, Bethesda, MD, USA) the presence of the cilium in each z-stack series as well as its characteristics (length, location, orientation and position). Only viable cells (with cytoplasm and a nucleus stained with propidium iodide) were selected. In addition, to avoid considering centrioles or auto-fluorescence of the tissue as a cilium, only cilia that appeared on at least three consecutive images and approximately  $90^\circ$  to the incident light were analyzed. Colocalization of the KIF3A subunit and  $\alpha$ -tubulin was used to correctly identify primary cilia. The  $\gamma$ -tubulin antibody was used to analyze the orientation of the centrioles. In addition, the cell nuclei stained with propidium iodide were ‘pseudo-colored’ in blue by changing the color of the channel in the confocal microscope settings.

### Statistical Analysis

Data are expressed as mean  $\pm$  s.e.m. Statistical significance was computed by one-way ANOVA followed by *post hoc*

Tukey's HSD test using the SPSS 16.0 software package. Results were considered significant when  $P < 0.05$ .

## RESULTS

### Morphology of the Growth Plate and Osteochondroma

All three zones (resting, proliferating and hypertrophic) were identified in all analyzed samples. Although present, the 'zonation' in the osteochondromas was less defined (Figure 1) and the cells were irregularly arranged and rarely acquired columnar organization. In addition, hypertrophic-like chondrocytes were often observed within the resting and proliferating zones in osteochondromas but not in the growth plate (Figure 1).

### The Presence of Primary Cilia

Primary cilia were found on cells at all stages of differentiation in both the growth plate and osteochondromas (Figure 2a and b). All the three zones of osteochondroma had a significantly higher percentage of cells with primary cilia when compared with the growth plate (resting: 94.9 vs 89.4%,  $P = 0.044$ ; proliferating: 93.2 vs 71.9%,  $P < 0.001$ ; and hypertrophic: 94 vs 71.6%,  $P < 0.001$ , respectively). In the growth plate analyzed with respect to zones, the percentage of ciliated cells was significantly higher in the resting (89.4%) than in the proliferating and hypertrophic zones (71.9 and 71.6%, respectively;  $P < 0.001$ ). No significant differences were observed between the three zones in osteochondroma samples ( $P = 0.532$ ).

### Proliferation Rate in the Growth Plate and Osteochondroma

A lower proliferation rate was observed in osteochondroma, as shown by Ki-67 immunostaining, a known marker of cell proliferation. Fewer Ki-67-positive cells were identified along the tumor (Supplementary Figure 1b). In contrast, a difference in proliferation was observed between the zones of the growth plate. A higher proliferation rate was found in the proliferating than in the resting and hypertrophic zones (Supplementary Figure 1a).

Double staining for Ki-67 and primary cilia could not be evaluated because of tissue detachment from the glass slides during antigen retrieval procedures.

### Length of Primary Cilia

The measurement of the length of primary cilia was performed in the three zones of the growth plate and in osteochondromas. A high variability of primary cilium length within and between zones was observed (Figure 2c). In the growth plate, primary cilia of the proliferating zone (mean length 1.62  $\mu\text{m}$ ) were significantly shorter than those in the resting (mean length 2.39  $\mu\text{m}$ ) and the hypertrophic zones (mean length 2.23  $\mu\text{m}$ ) (in both,  $P < 0.001$ ; Figure 2c). In the osteochondromas, similar results were found. However, cilia were significantly shorter only in the proliferating zone

(mean length 2.13  $\mu\text{m}$ ) than the hypertrophic zone (mean length 2.38  $\mu\text{m}$ ) ( $P < 0.001$ ; Figure 2c).

The ciliary axonemes were found projecting into the extracellular matrix in both tissues. Undulations along the axoneme length were observed (Figure 2a, arrowheads), as previously described in chondrocytes.<sup>8</sup>

### The Ultrastructure of Primary Cilia

Electron microscopy revealed the presence of the centriole with a typical 9 + 0 microtubule doublet organization in the growth plate (Figure 3c) and osteochondromas (Figure 3f).

The basal body of the cilium, which is formed by one centriole, was found supporting the axoneme (Figure 3a, b, d and e). At higher magnification, the ciliary axoneme was observed retracted within a plasma membrane invagination (Figure 3a, b and e).

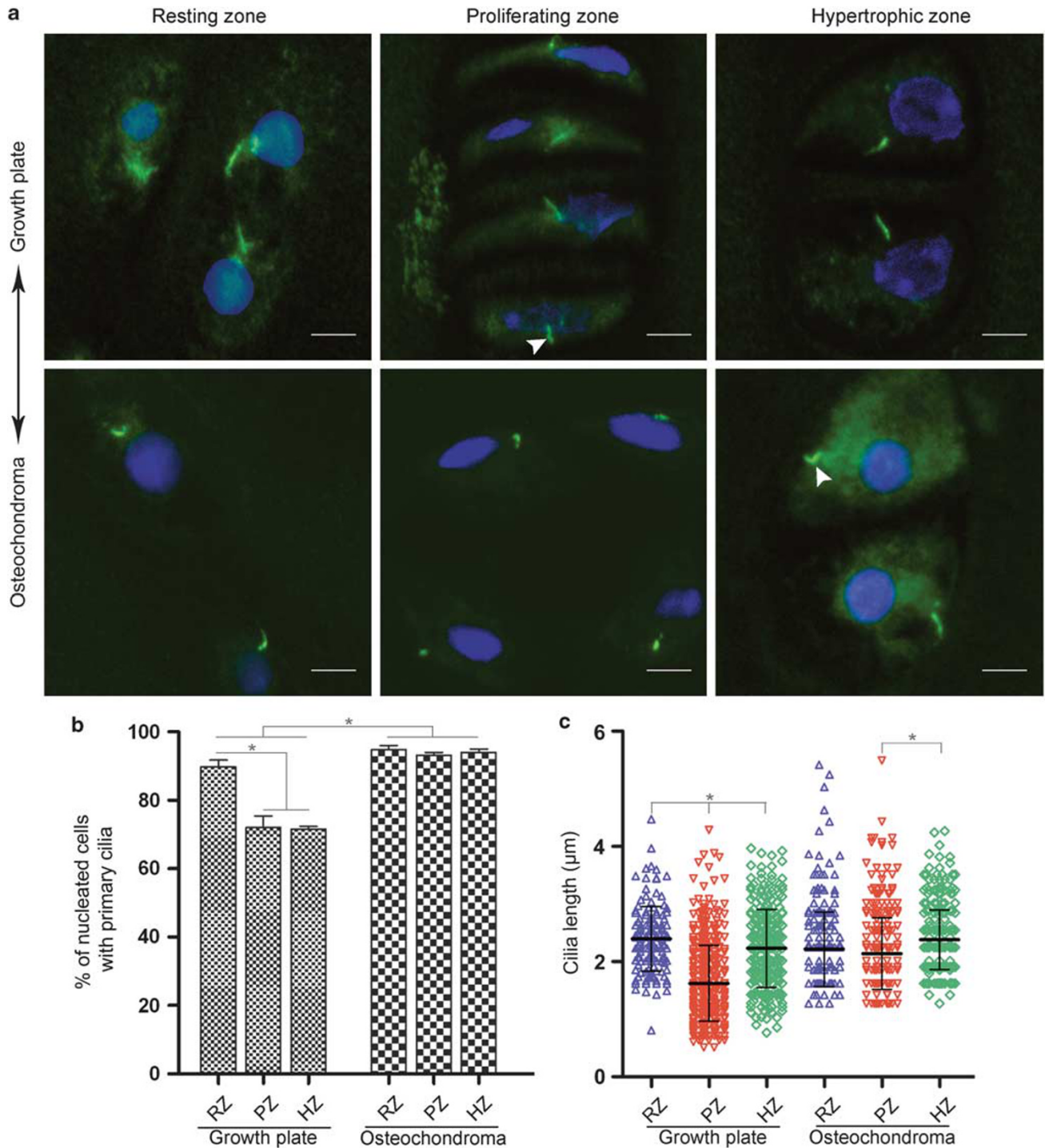
Numerous small clusters of glycogen were found in large areas of the cytoplasm in osteochondroma cells (Figure 3d), but not in the growth plate.

### Localization of KIF3A Kinesin II Subunit

The KIF3A subunit has been shown to have a critical role in growth plate formation.<sup>15</sup> Using an antibody against the KIF3A subunit, positive cells were found in the growth plate and osteochondromas. One strongly stained spot (Figure 4) could be observed, although the staining was diffuse throughout the cytoplasm in both tissues. We next analyzed whether the strongly stained KIF3A spot was properly located in the primary cilia, and we were able to colocalize the KIF3A spot with the cilia (Figure 4).

### Primary Cilia Organization in the Growth Plate and Osteochondroma

The typical architecture of the growth plate may reveal a specific location of the organelles within the chondrocytes.<sup>28</sup> To address this hypothesis, we studied the organization of primary cilia along the  $x$ - $y$  axis of the growth plate and osteochondromas. In the growth plate, we found that primary cilia in the resting zone do not acquire a clear pattern of orientation (Figure 5c). However, in the proliferating and hypertrophic zones, primary cilia were oriented parallel to the longitudinal axis of the bone pointing either toward the epiphyseal side or the metaphyseal side of the growth plate (Figure 5a and d). At lower magnification, one virtual axis that is formed by the alignment of primary cilia and crosses the center of each column of chondrocytes could be identified (Figure 5a and 6a, left panel). The parallel axes seem to represent the axis of chondrocyte polarity inside the growth plate. To confirm this specific positioning of the cell region containing the cilium, we immunolabeled the centrioles using an antibody against  $\gamma$ -tubulin (Figure 5e). The centrioles are located in the centrosome of the cell next to the plasma membrane. The centrioles were found aligned inside the columns of cells, delineating one virtual axis (Figure 5e). In osteochondromas, primary cilia were randomly located



**Figure 2** Primary cilia are found on cells at all stages of differentiation in both the growth plate and osteochondroma. Fluorescently labeled primary cilia (green) are found on the cell surface projecting into the extracellular matrix (a). Undulations along the axoneme length are observed (a; arrowheads). The percentage of nucleated cells with primary cilia is depicted (b). A high number of ciliated cells are observed among the proliferating cells of the growth plate. The average length of the cilium in each zone of both tissues is shown (c). High variability in length of the cilium was observed within and between the zones. Nuclei are stained with propidium iodide and pseudocolored in blue. Error bars represent s.e.m. \* $P < 0.05$ , as determined by one-way ANOVA followed by *post hoc* Tukey's HSD test. (RZ, resting zone; PZ, proliferating zone; HZ, hypertrophic zone; Arrow-bar, longitudinal axis of the bone or tumor; Scale bars, 5  $\mu\text{m}$ ).

along the  $x$ - $y$  axis of the tumor either on the center or on the lateral-medial region of the cells in relation to the longitudinal axis of the tumor (Figure 5b and 6a, right panel).

In 5 osteochondromas analyzed, 12 sub-populations of cells were found organized into small columns. Interestingly, in 10 of the 12 small columns identified, the cilia were

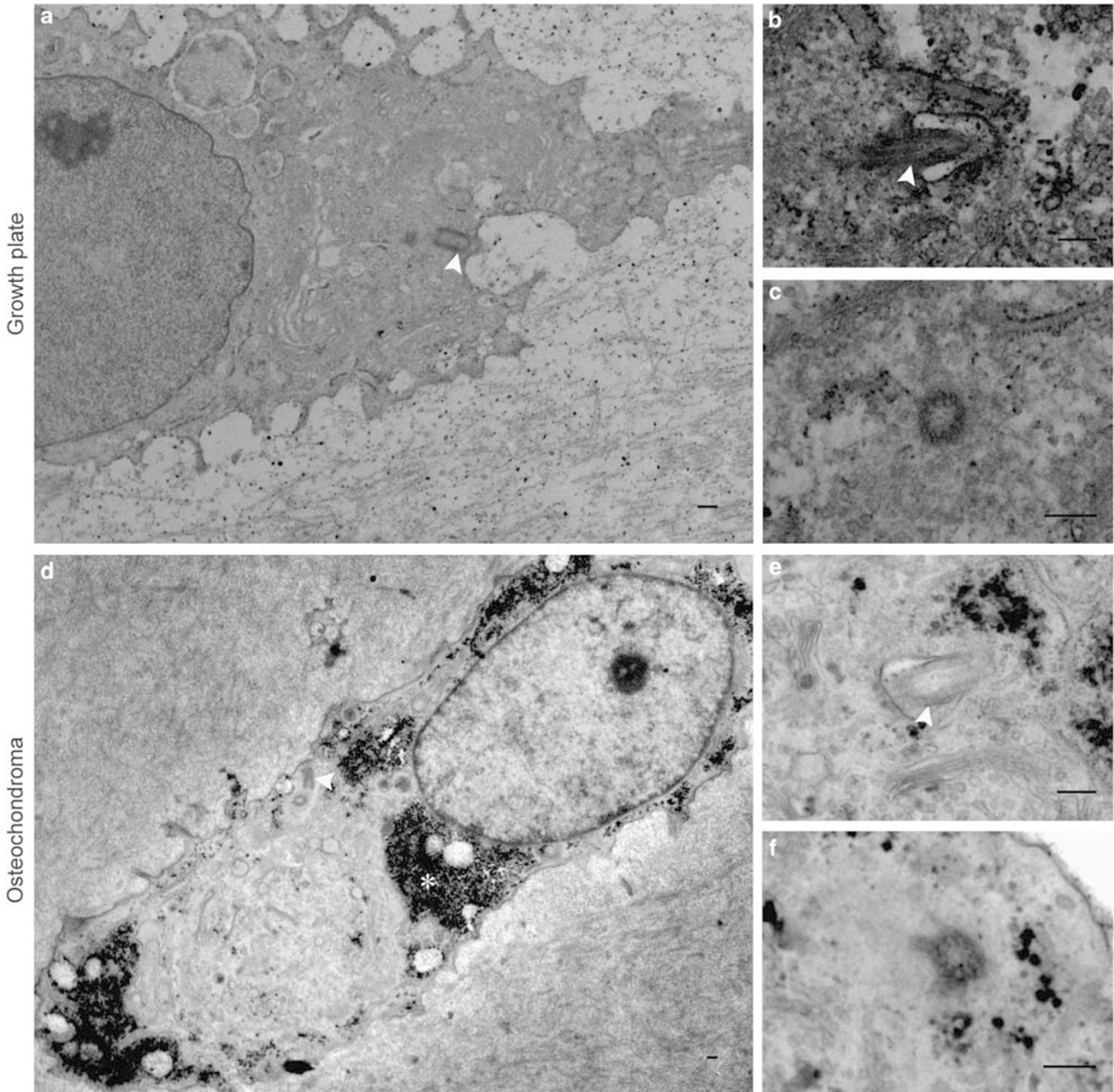


aligned in one axis parallel to the axis of tumor growth (Figure 5h).

**DISCUSSION**

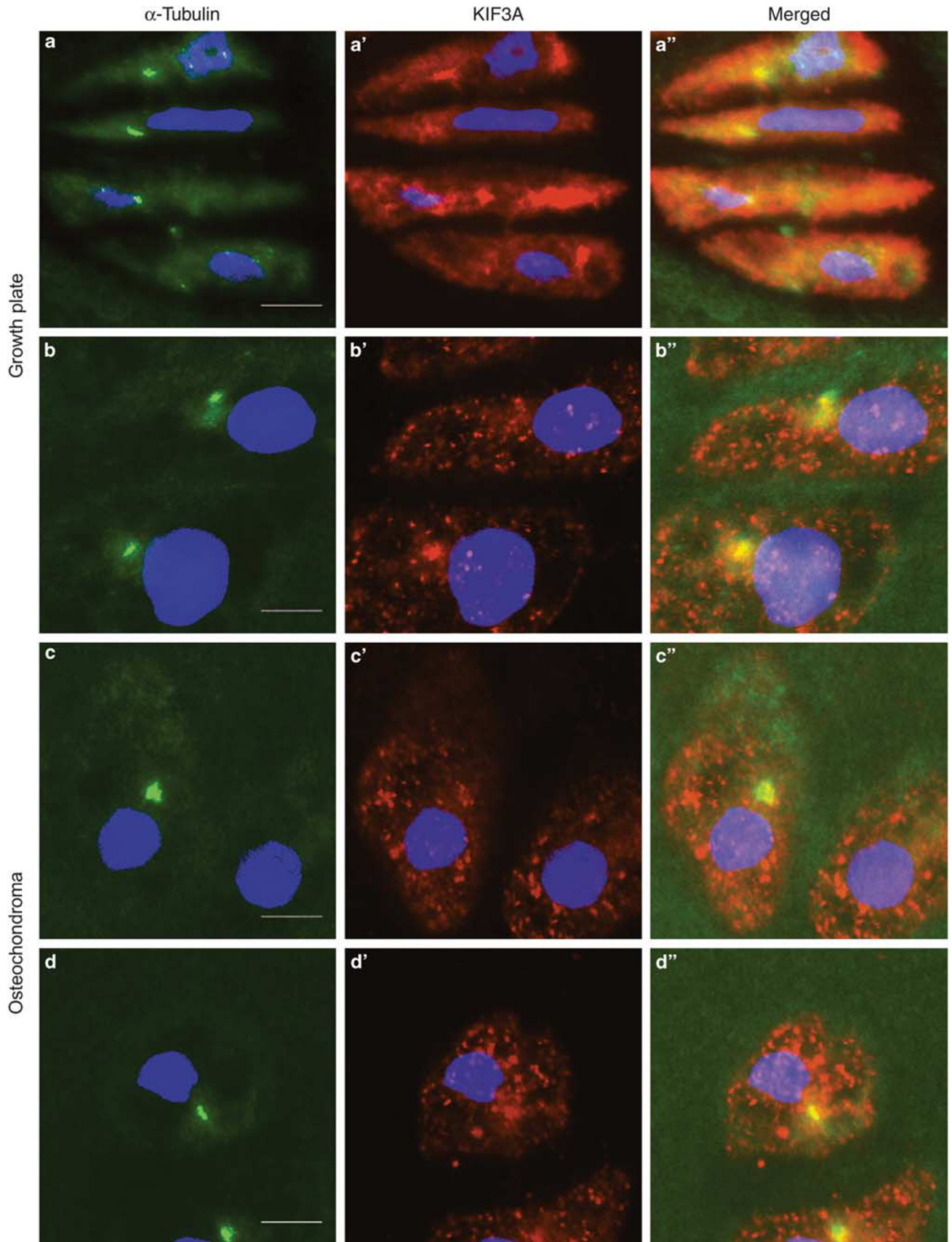
Primary cilia were found to reflect cell polarity in several tissues.<sup>16,17,29</sup> It has been shown that primary cilia have a

critical role in the development of the growth plate. Disturbance of the intraflagellar transport system leads to a depletion of cells with primary cilia and defects in the growth plate such as disruption of cell shape and lack of columnar orientation, which suggests defects in cell movement/rotation.<sup>3,11,12</sup>



**Figure 3** Electron micrographs of the primary cilium and the centrioles. The cilium is observed with a similar ultrastructure in the cells of the growth plate (a–c) and osteochondroma (d–f). The centriole with a typical 9 + 0 microtubule doublet organization is found in both tissues (c, f). One centriole forms the basal body of the cilium that supports the axoneme (arrowheads) (b, e). The axoneme is observed retracted within a plasma membrane invagination (a, b, e). Numerous small clusters of glycogen (asterisk) are found in large areas of the cytoplasm in osteochondroma cells but not in the growth plate (d). (Scale bars, 0.25 μm).

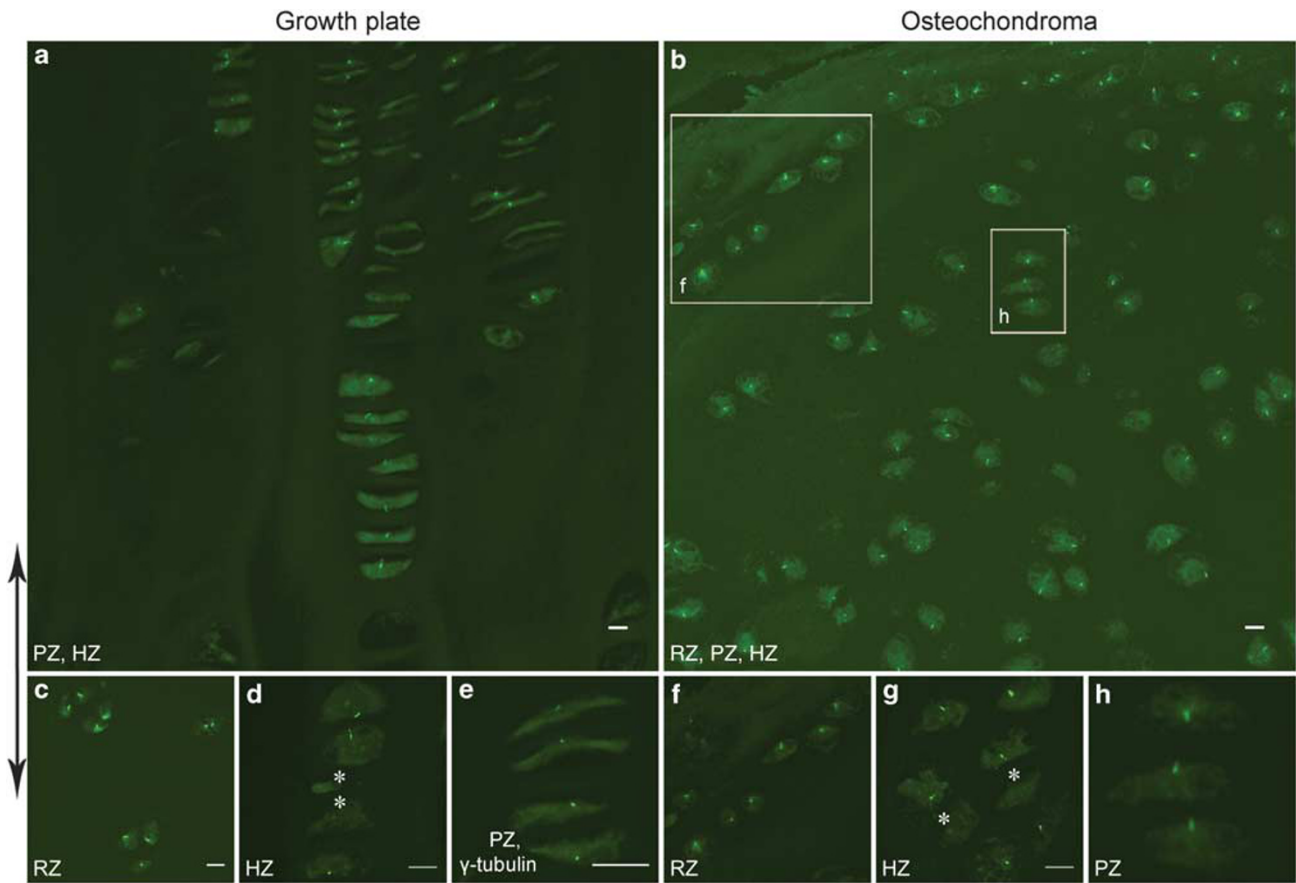
**Figure 4** KIF3A subunit, which facilitates intraflagellar transport, is present in the cells of the growth plate and osteochondroma. Immunolabeling with antibodies against acetylated α-tubulin (a–d; green) and KIF3A (a'–d'; red) shows colocalization of both proteins (merged) in the primary cilium in the growth plate (a'' and b'') and osteochondroma (c'' and d''). Nuclei are stained with propidium iodide and pseudo-colored in blue. (Scale bars, 5 μm).





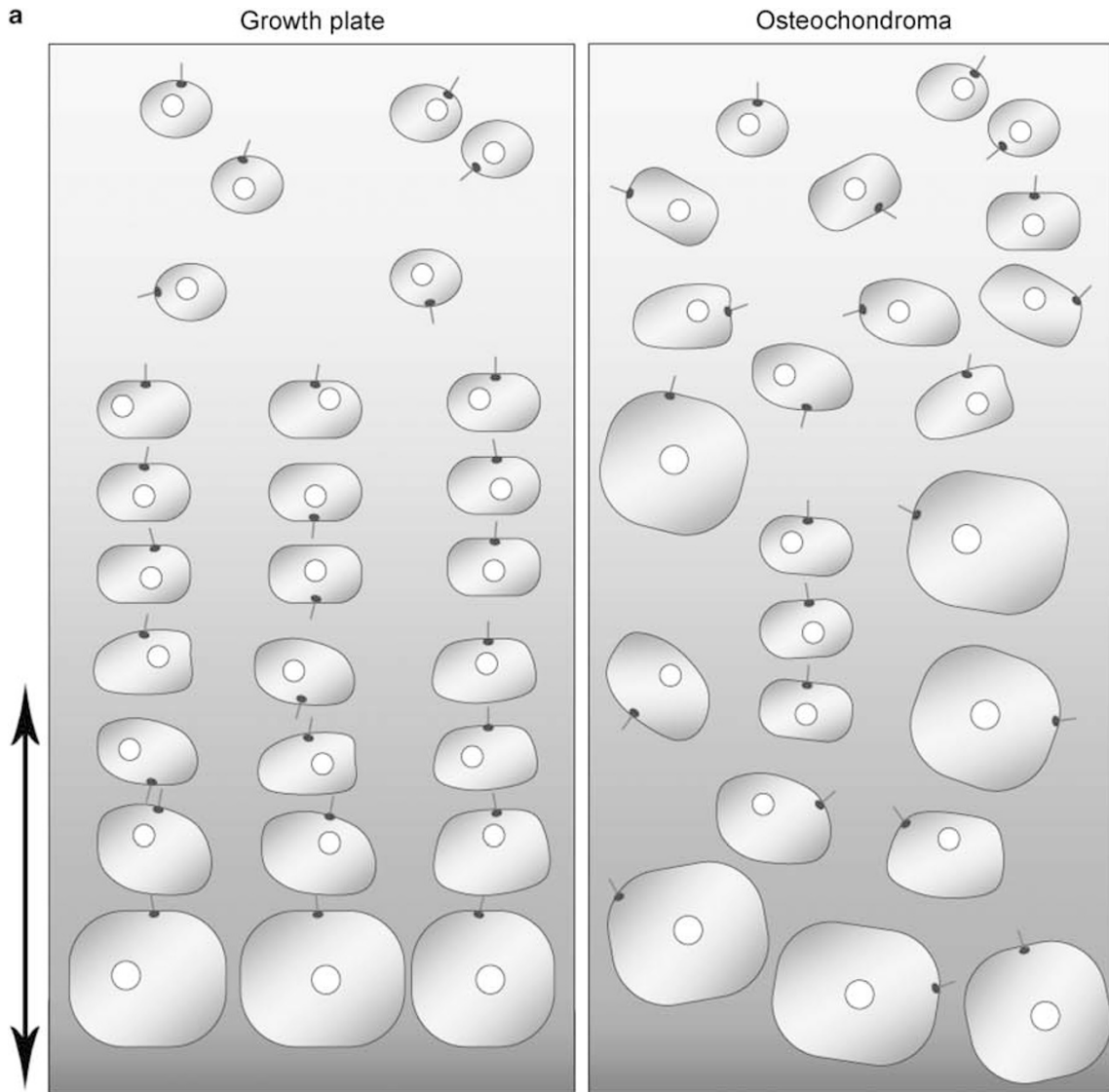
In this study, we showed that, in the proliferating and hypertrophic zones of the growth plate, the organization of primary cilia forms virtual axes parallel to the longitudinal axis of the bone. The fact that this orientation pattern of the cilium was not detected in the resting zone implies that polarization gradients exist inside the growth plate, driving the cells to their specific positions and orientations. It has been suggested that the polarization gradients are generated and maintained by the cartilage itself and that the resting zone establishes the organization of the growth plate.<sup>30</sup>

Several morphogens or signaling molecules are secreted from the two opposite poles of the growth plate, having an important role in chondrocyte proliferation and differentiation.<sup>1</sup> The normal diffusion and function of morphogens regulate the formation of zones in the growth plate.<sup>31</sup> Inside each zone of the growth plate, the chondrocytes respond in an orchestrated manner to the signaling molecules, which reflects a collective cell behavior that is typical for polarized tissues in which the cells adopt the same polarity axis and identical positioning of their organelles.<sup>32</sup> In conclusion, the

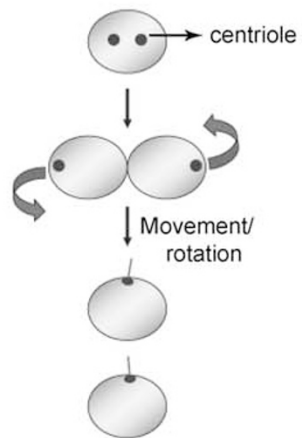


**Figure 5** The organization of primary cilia in the growth plate and osteochondroma. Primary cilia (green) in the growth plate (a) are organized in such a way that they form a virtual axis crossing the center of the columns of proliferating and hypertrophic chondrocytes. The ciliary axis is always parallel to the longitudinal axis of the bone (arrow-bar).  $\gamma$ -Tubulin marks the location of the centrioles and confirms the existence of one virtual axis (e). Primary cilia in osteochondromas are randomly located along the x-y axis of the tumor (b). On rare occasions when osteochondroma cells are organized into small columns (h), primary cilia are aligned parallel to the longitudinal axis of the tumor (arrow-bar). In the resting zones of both tissues, primary cilia do not acquire any clear orientation (c, f). Some hypertrophic cells of the growth plate and osteochondroma with a well-preserved morphology are found without cilia (d, g). (RZ—resting zone; PZ—proliferating zone; HZ—hypertrophic zone; asterisks—cells without primary cilia; f and h, inserted from b; arrow-bar—longitudinal axis of the bone or tumor; scale bars, 10  $\mu$ m).

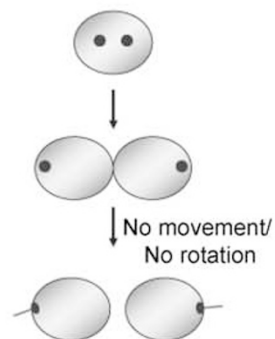
**Figure 6** Primary cilia organization reflects cell polarity in the growth plate and in sub-populations of osteochondroma cells. Schematic representation shows the virtual axis that is formed by the alignment of primary cilia and crosses the center of each column of chondrocytes (a, left and right panels). Chondrocytes of the growth plate are known to divide parallel to the longitudinal axis of the bone (arrow-bar) (b, left panel). The daughter chondrocytes must undertake a series of cell movements/rotations and shape changes to generate the typical columns of the growth plate and align primary cilia on one common axis (a, b, left panel). A model is proposed in which an osteochondroma forms as a consequence of chondrocytes losing their ability to move/rotate to re-orient themselves with respect to the growth axis (arrow-bar) (b, right panel). Interestingly, some sub-populations of osteochondroma cells are able to form columns and to orient the cilium parallel to the longitudinal axis of the tumor (arrow-bar) (a, right panel).



**b** Classical growth plate cell division



Putative osteochondroma cell division



results provide evidence that normal growth plate formation depends on the establishment and maintenance of individual and collective chondrocyte polarity.

We also showed that the osteochondroma cells have a primary cilium and its formation is similar to that of the growth plate cilia. However, no clear orientation of the cilium was observed in the osteochondromas, with primary cilia often randomly located along the  $x$ - $y$  axis either organized on the center or on the lateral-medial region of the cells in relation to the growth axis of the tumor. This suggests that the presence of primary cilia is not sufficient to direct proper organization of the chondrocytes. The formation of the ciliary axis seems to be a final result of several factors (eg, signaling molecules and their effective transduction) that determine the final orientation of the cells.

Primary cilia have been shown to mediate Indian Hedgehog signaling.<sup>9,15</sup> Indian Hedgehog is secreted by pre-hypertrophic chondrocytes, and its diffusion generates a signal gradient inside the growth plate that coordinates cell proliferation and differentiation.<sup>1</sup> The alignment of primary cilia might be critical for transduction of this signaling molecule along the growth plate. Homogeneous expression of Indian Hedgehog has been shown in osteochondromas, which is suggestive of autonomous cell signaling.<sup>6</sup> Therefore, correct orientation of the cilium is no longer needed to receive and transduce the diffusion gradient of Indian Hedgehog signaling. Other deregulated signaling pathways might also contribute to the misorientation of primary cilia. Intriguingly, some sub-populations of osteochondroma cells, which were organized into small columns, appeared to keep the growth plate cell polarity. This leads us to hypothesize that a mixture ('mosaic') exists of normal lining ( $EXT^{+/-}$  or  $EXT^{wt/wt}$ ) and  $EXT^{-/-}$  cells in the cartilaginous cap. In this mixture, the normal lining cells would produce heparan sulfate proteoglycans and the receptor-ligand interactions would be modulated regularly. Consequently, these cells would respond to the polarization signals and align primary cilia in one axis. Further analysis will be required to identify mosaicism in osteochondromas.

This study sheds light on the events related to primary cilia formation. For example, the smallest ciliary length was associated with the proliferative state of the cells. In addition, several proliferating cells were found to have primary cilia. Knowing that the cell turnover is increased in the proliferating zone and that primary cilia are absent in replicating cells,<sup>10</sup> it is possible that only a subset of proliferating cells are in mitosis. This suggests that the cilia length is directly related to cell cycle duration. Further studies on cilia function are necessary. Interestingly, several hypertrophic cells with well-preserved morphology were found without cilia (Figure 5d and g, asterisks). As most of them undergo apoptosis,<sup>1</sup> this observation might indicate that primary cilia were disassembled in the early stages of apoptosis. In addition, the osteochondroma cells were found to have a higher percentage of cells with primary cilia. The presence of primary cilia

might be associated with the lower proliferation rate of the tumor, which was confirmed by Ki-67 immunostaining. Taken together, the primary cilia length observed in osteochondromas is similar to that in the growth plate, suggesting that ciliary formation is normal in these tumors. This also enforces the hypothesis that deregulation of cell-cell and/or cell-matrix signaling pathways is instrumental to the pathogenesis of osteochondromas.

The alignment of primary cilia along the growth plate supports the idea that the chondrocytes of the growth plate have the ability to move/rotate (Figure 6b).<sup>2,4,33</sup> Integrins are the major class of cell adhesion molecules that mediate cell-matrix signaling pathways.<sup>33</sup> Integrins have heparan-binding domains, which indicate that heparan sulfate proteoglycans may modulate integrin signaling and cell adhesion.<sup>34</sup> Movement/rotation problems in the chondrocytes of the growth plate have been reported in  $\beta 1$ -integrin-null mice.<sup>33</sup> The  $\beta 1$ -integrin-null growth plates show larger and round-shaped proliferating chondrocytes arranged side by side, possibly reflecting impaired movement and inability to form columns.<sup>33</sup> Strikingly,  $\beta 1$ -integrin receptors have been reported to colocalize with  $\alpha$ -tubulin in the primary cilia of chondrocytes.<sup>35</sup> The loss of *EXT* expression results in disordered distribution of heparan sulfate proteoglycans. Recently, we showed that in osteochondromas, heparan sulfate proteoglycans are no longer present at the cell surface but accumulate in the cytoplasm.<sup>36</sup> Therefore, it is possible that the lack of heparan sulfate proteoglycans at the cell surface might influence cell adhesion and motility. The random orientation of the cilium in osteochondromas leads to the assumption that osteochondroma cells have impaired movement (Figure 6b) that may underlie the lack of orientation of the cells, which is a morphological characteristic of this tumor. The impaired movement might be caused by diminished heparan sulfate proteoglycans, which in turn could reduce integrin signaling activity and cell adhesion to the surrounding matrix.

In conclusion, we showed that the organization of primary cilia reflects cell polarity in the growth plate and in sub-populations of osteochondroma cells. We also showed that the ciliary formation in osteochondroma seems to be similar to that of the growth plate. The growth plate-like polarity retained in sub-populations of osteochondroma cells (organized into small columns) leads us to propose the existence of a mixture ('mosaic') of normal lining ( $EXT^{+/-}$  or  $EXT^{wt/wt}$ ) and  $EXT^{-/-}$  cells in the cartilaginous cap of osteochondromas.

#### Note added in proof

Since the work described in this paper was completed and submitted for publication, the mixture ("mosaic") of normal lining ( $EXT^{+/-}$  or  $EXT^{wt/wt}$ ) and  $EXT^{-/-}$  cells in the cartilaginous cap of osteochondromas has been elegantly reported by Jones et al (*Proc Natl Acad Sci U S A* 2010;107:2054–2059) using a conditional mouse model for *Ext1*.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

#### ACKNOWLEDGEMENTS

We thank Annemieke M. van der Wal for the expert technical help. This study was supported by EuroBoNet, a European Commission-granted Network of Excellence for studying the pathology and genetics of bone tumors (Grant LSHC-CT-2006-018814).

#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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