

# The differential distribution of acetylated and detyrosinated alpha-tubulin in the microtubular cytoskeleton and primary cilia of hyaline cartilage chondrocytes

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## ABSTRACT

The primary cilium is a ubiquitous cytoplasmic organelle of unknown function. Ultrastructural evidence of primary cilia in chondrocytes, and their colocalisation with the Golgi apparatus, has led to speculation that these structures are functionally linked. To investigate the relationship between these organelles, we examined the molecular anatomy of the microtubular cytoskeleton in the chondrocytes of chick embryo sterna. Thick cryosections were immunolabelled with antibodies directed against acetylated  $\alpha$ -tubulin (C3B9), detyrosinated  $\alpha$ -tubulin (ID5) and total  $\alpha$ -tubulin (TAT), and imaged at high magnification using confocal laser scanning microscopy. Transmission electron microscopy confirmed the ultrastructure of the chondrocyte primary cilium and its structural relationship to the Golgi apparatus. Detyrosinated and acetylated  $\alpha$ -tubulins were concentrated in the centrioles, centrosome and microtubule organising centre adjacent to the nucleus, with total  $\alpha$ -tubulin distributed throughout the cytoplasm. ID5 stained the primary cilium at an incidence of 1 per cell, its colocalisation with C3B9 identifying the primary cilium as one of the most stable features of the microtubular cytoskeleton. Primary cilia varied from 1 to 4  $\mu\text{m}$  in length, and 3 patterns of projection into the extracellular matrix were identified; (1) full extension and matrix contact, with minor undulations along the length; (2) partial extension and matrix contact, with a range of bending deflections; (3) cilium reclined against the cell surface with minimal matrix contact. Ultrastructural studies identified direct connections between extracellular collagen fibres and the proteins which decorate ciliary microtubules, suggesting a matrix–cilium–Golgi continuum in hyaline chondrocytes. These results strengthen the hypothesis that the primary cilium acts as a ‘cellular cybernetic probe’ capable of transducing environmental information from the extracellular matrix, communicating this information to the centrosome, and regulating the exocytosis of Golgi-derived secretory vesicles.

*Key words:* Chick embryo sterna; immunohistochemistry; microtubules; confocal microscopy; electron microscopy.

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## INTRODUCTION

Hyaline cartilage performs a critical role in bone formation and load bearing during skeletal development (Hirsch & Hartford-Svoboda, 1993; Poole, 1996). In chick embryo sterna, the hyaline cartilage is composed of small ovoid chondrocytes, spatially separated within a complex extracellular matrix composed of collagens, proteoglycans, glycoproteins and fluid (Hirsch & Hartford-Svoboda, 1993; Muir, 1995). Chondrocytes are known to be responsive to

biomechanical load, synthesising and secreting the appropriate mixture of extracellular matrix macromolecules necessary to absorb and transmit the physiological load applied (Kim et al. 1995; Lee & Bader, 1997; Toyoda et al. 1999).

The mechanisms by which chondrocytes respond to biomechanical force, and the changing physicochemical properties of the matrix, are currently the focus of intense research interest (see Banes et al. 1995). In particular, the  $\beta 1$  integrins are thought to play a major role in this process (for review see

Loeser, 2000), linking extracellular matrix components with the actin and intermediate filament networks that form the tensegrity signalling system within the cell (Ingber, 1993; Banes et al. 1995; Maniotis et al. 1997). Studies on the differential distribution of the actin and vimentin networks in chondrocytes clearly suggest a role for these cytoskeletal elements in transducing mechanical deformation of the matrix into a cellular response (Durrant et al. 1999; Idowu et al. 2000).

The role of the microtubular cytoskeleton in mediating the chondrocyte response to biomechanical load has been less thoroughly investigated (Jortikka et al. 2000). Most studies have been restricted to de-differentiated chondrocyte monolayers (Moskalewski et al. 1980; Brown & Benya, 1988; Farquharson et al. 1999) or chondrocytes cultured in agarose gel (Idowu et al. 2000), with little attention given to the microtubular configuration in intact tissue. It is well established that microtubular integrity is vital for the assembly and maintenance of the Golgi apparatus (Thyberg & Moskalewski, 1985; 1999; Kreis, 1990), and for secretion of matrix components that are posttranslationally modified and packaged by the Golgi apparatus (Vertel et al. 1985; Silbert & Sugumaran, 1995). Studies which examined microtubular disruptive agents, or the redistribution of the Golgi elements during cell division, were consistent in showing that the Golgi apparatus dissociated following microtubular disruption or mitotic spindle formation (Thyberg & Moskalewski, 1985, 1992, 1999; Moskalewski & Thyberg, 1992; Moskalewski et al. 1994; Zaal et al. 1999). Consequently, Golgi-mediated secretion was significantly reduced and only resumed normal secretory activity in association with the reassembly of the microtubular network (Thyberg & Moskalewski, 1985, 1999).

The  $\alpha$ - and  $\beta$ -tubulin subunits that form the metastable structure of the microtubule are known to exist in a number of posttranslationally modified isoforms which confer specific characteristics to the microtubules (Schulze et al. 1987; Wehland & Weber, 1987; Piperno et al. 1987; Bulinski et al. 1988; Panda et al. 1994; Ludueña, 1998; Rudiger et al. 1999). Tyrosinated  $\alpha$ -tubulin forms the major cytoplasmic pool of labile tubulin, and is in a state of 'dynamic instability' with assembled microtubules (Geuens et al. 1986; Schulze & Kirschner, 1987; Alberts et al. 1994). Acetylation of  $\alpha$ -tubulin in assembled microtubules (Piperno & Fuller, 1985; Piperno et al. 1987) produces a subset of more stable microtubules associated with the Golgi apparatus (Thyberg & Moskalewski, 1993; Le Tourneau & Wire, 1995) and

the primary cilium (Poole et al. 1997; Alieva et al. 1999). Detyrosination of the  $\alpha$ -tubulins in assembled microtubules results in the most stable subset of microtubules, and is generally restricted to the centrosome, the diplosomal centrioles and the primary cilium (Geuens et al. 1986; Skoufias et al. 1990; Thyberg & Moskalewski, 1993; Wheatley et al. 1994; Poole et al. 1997).

Almost all animal cells contain a single primary cilium, yet its function remains speculative and enigmatic (Wheatley, 1982, 1995; Poole et al. 1985). Primary cilia are minute structures measuring 0.2  $\mu\text{m}$  in diameter by 1–5  $\mu\text{m}$  in length, and they differ fundamentally from motile (9+2) cilia in several respects. First, the axoneme of the primary cilium lacks a central microtubular doublet, its 9+0 microtubular symmetry sharing characteristics with many sensory cilia (Barber, 1974; Moran & Rowley, 1983; Keil, 1997). Second, the ciliary axoneme is not considered motile, particularly in dense connective tissues like cartilage, but it does project into the extracellular microenvironment adjacent to the cell (Poole et al. 1985; Handel et al. 1999). Third, the basal body of the primary cilium is formed by the most mature of the two centrioles which form the diplosome (Lange & Gull, 1995). Finally, this basal diplosome and its associated centrosome, together function as the microtubule organising centre (MTOC) of the cell (Brinkley, 1985; Kreis, 1990; Kimble & Kuriyama, 1992; Rose et al. 1993), and the focal point for Golgi assembly prior to the formation of a primary cilium (Jensen et al. 1987; Moskalewski & Thyberg, 1992).

Ultrastructural studies have reported primary cilia in a variety of hyaline cartilage chondrocytes (Wilsman, 1978; Meier-Vismara et al. 1979; Poole et al. 1985; Kouri et al. 1996), at an incidence of one per cell (Wilsman & Fletcher, 1978). Our laboratory has also demonstrated a structural correlation between the functional properties of the extracellular matrix, the projection and angularity of the primary cilium, and its juxtannuclear localisation with the Golgi apparatus (Poole et al. 1985). Cilia-specific tubulin antibodies, Golgi lectin binding and multichannel confocal microscopy confirmed the structural relationship between the primary cilium and the Golgi apparatus in articular cartilage chondrocytes and cultured aortic smooth muscle cells (Poole et al. 1997). We proposed that the primary cilia of connective tissue cells could act as 'cybernetic probes', transducing biomechanical and/or physicochemical information from the extracellular matrix, communicating this information to the centrosome and Golgi apparatus, and regulating the cellular synthetic

and secretory responses necessary to maintain a functionally effective extracellular matrix (Poole et al. 1985, 1997).

Chick embryo sternal cartilage was chosen as a tissue model to investigate the relationship between the extracellular matrix, the primary cilium, the cytoplasmic microtubules and the Golgi apparatus of chondrocytes. In this tissue, each cell has a primary cilium, is actively secretory, and is spatially separated in a transparent extracellular matrix. We identified a differential distribution of  $\alpha$ -tubulin isoforms in the primary cilium and cytoplasm of hyaline chondrocytes, and an ultrastructural continuum between the matrix, the cilium and the Golgi complex. The results support the proposition that the primary cilium plays a cellular cybernetic role in facilitating the functional interaction between the chondrocyte and its extracellular matrix.

#### MATERIALS AND METHODS

ID5 antibody raised against detyrosinated  $\alpha$ -tubulin was a gift from J. Wehland (Wehland & Weber, 1987); C3B9 raised against acetylated  $\alpha$ -tubulin and TAT raised against total  $\alpha$ -tubulin were kindly provided by K. Gull (Woods et al. 1989). Goat antimouse-Alexa488 was obtained from Molecular Probes, USA; goat antimouse-Cy3 was obtained from Amersham Pharmacia Biotech, UK. Antiparvalbumin was a gift from R. Faull. Dulbecco's phosphate buffered saline (DPBS) and bovine serum albumin were obtained from Life Technologies Inc. (NZ) Ltd. Triton-X100 and testicular hyaluronidase were obtained from Sigma Chemical Co, Australia. Vectashield was obtained from Vector Laboratories, USA. Fixatives and all electron microscopy processing materials were obtained from Probing and Structure, Australia.

#### *Tissue sampling*

Fertile Shaver Brown eggs were maintained in an incubator at 39 °C for 16–17 d prior to sacrifice of the embryo. A total of 6 sterna were immediately dissected and the perichondrium removed prior to immersion fixation in either fresh 2% glutaraldehyde in 0.1 M sodium cacodylate buffer for electron microscopy, or fresh 4% paraformaldehyde in DPBS for immunohistochemistry and confocal microscopy. After 2 h of fixation at room temperature, the tissues were washed extensively and divided into caudal and cephalic portions for further processing.

#### *Electron microscopy*

Sternal samples were treated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour, rinsed extensively and processed into resin using established techniques (Poole et al. 1985). Resin blocks were sectioned at 90–100 nm, collected on to copper grids and stained with saturated uranyl acetate at 60 °C for 1 h. Grids were examined and photographed on an Hitachi H7000 transmission electron microscope.

#### *Cryosections*

Samples were orientated, embedded and frozen in OCT compound to allow sectioning perpendicular to the long axis of the sternum. Cryosections were prepared at 10–15  $\mu$ m to ensure a significant number of intact cells were included in each section, and dried on to silanated slides.

#### *Immunohistochemistry: single labelling*

Sections were rehydrated in DPBS, washed in Tris-HCl (pH 5.5) and predigested with testicular hyaluronidase (2 mg/ml in 0.1 M Tris-buffered saline, pH 5.5) for 2 h at 37 °C to remove matrix proteoglycans and assist antibody penetration (Poole et al. 1997). The chondrocytes were then permeabilised with 0.5% Triton-X100 in PHEM buffer (Wheatley et al. 1994) for 1 h and washed extensively. Goat serum (1:50) was added for 30 min to block nonspecific staining. Monoclonal antibodies ID5, C3B9 and TAT were added neat to separate slides, and the sections incubated overnight at 4 °C. After thorough washing, goat-antimouse-Alexa488 (1:200) was applied to the sections and incubated for 4 h in the dark at room temperature. Staining was completed with two buffer washes, and the sections mounted in Vectashield to prevent photo-bleaching of the fluorochrome.

#### *Immunohistochemistry: double labelling*

The antibodies ID5 and C3B9 were used for double labelling experiments since they target separate subsets of microtubules. Individual slides were first pretreated and immunolabelled with ID5 as described for single labelling. Each slide was then incubated overnight at 4 °C with antiparvalbumin (1:2500) to block any remaining free binding sites on the Alexa488 antibody. The slides were then immunolabelled with C3B9 overnight at 4 °C, and goat-antimouse-Cy3 used as the second fluorochrome. Negative controls

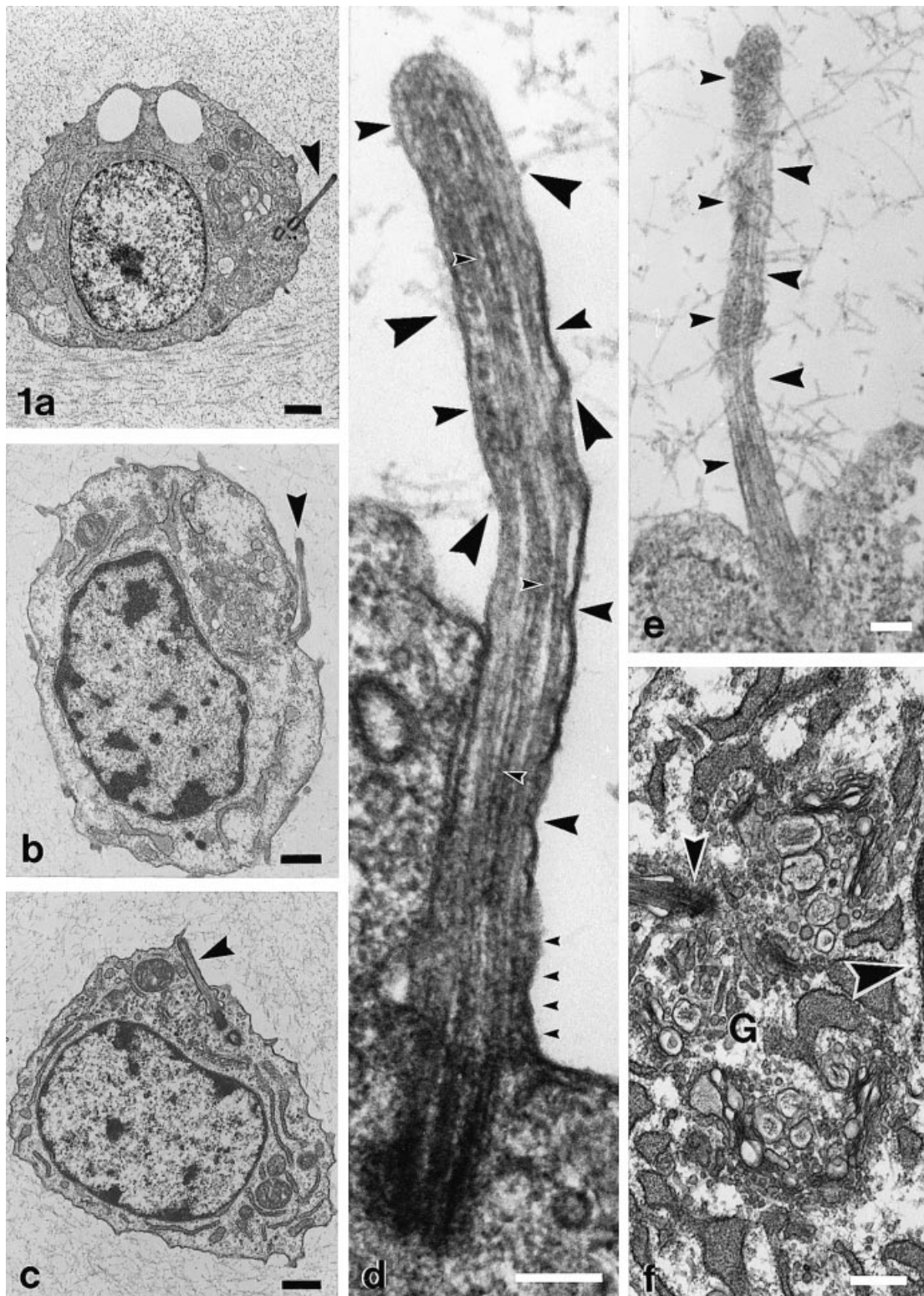


Fig. 1. Electron micrographs of chick embryo sternal chondrocytes showing cells in which the primary cilia (arrows) were fully extended into the matrix (*a*), were bent or reflected towards the cell surface (*b*), or were reclined against the cell membrane (*c*). Note the juxtannuclear location of the Golgi apparatus and its relationship to the base of the primary cilium. (*d*) Detail of a primary cilium showing microtubules extending from the basal body to the distal tip. Microtubule-associated proteins linked adjacent microtubules to each other (small arrows), or to the ciliary membrane which was often 'dimpled' at the linkage points (medium arrows). Extracellular collagen fibres impinging upon the ciliary membrane were matched by protein linkages to the ciliary microtubules (large arrows). Electron-dense proteins, equivalent to the ciliary necklace, were evident at the base of the axoneme (four arrows). (*e*) Detail of an extended cilium and its interaction with extracellular collagen fibres. Differences in ciliary ultrastructure between the regions where collagen fibres traverse the axoneme within the depth of section (large arrows), and where they do not (small arrows), suggests small undulations exist along the length of the primary cilium. (*f*) Detail of the Golgi apparatus (G) showing its eccentric location between the nucleus (large arrow) at its cis-face, and the basal body of the primary cilium (small arrow) at its trans-face. Bars, 1  $\mu\text{m}$  (*a-c*); 0.2  $\mu\text{m}$  (*d, e*); 0.5  $\mu\text{m}$  (*f*).

included substitution of the primary antibodies with PBS or normal mouse serum. No labelling of cartilage was observed with mouse antiparvalbumin alone.

### *Confocal microscopy*

Sections were imaged using a Leica TCS 4D confocal laser scanning microscope and a  $\times 100$  (NA1.3) oil immersion objective lens. Digital images of each fluorochrome were captured using a range of electronic zoom factors, and optical sections ( $\sim 0.5 \mu\text{m}$  thick) were collected serially at  $0.3\text{--}0.5 \mu\text{m}$  steps to ensure complete sampling of all cilia. Data sets from each antibody were prepared as *z*-series projections and saved as TIFF files for image processing and reconstruction.

### *Image processing*

All images were processed and reconstructed using Adobe Photoshop 4.0 (Adobe Systems, USA). For double-labelled colocalisation studies, *z*-series projections of ID5 staining were assigned a green colour, while projections of C3B9 staining were assigned a red colour. The 2 images were combined in layers and assembled in PageMaker 6.5 (Adobe Systems, USA).

## RESULTS

### *Electron microscopy*

A total of 42 primary cilia from chick embryo sternal chondrocytes were examined ultrastructurally, with three examples illustrated in Figure 1(*a–c*). Each cell was characterised by its small size and ovoid shape, the dominance of the nucleus, the juxtannuclear position of the Golgi apparatus, and a primary cilium which varied from 1 to  $4 \mu\text{m}$  in length. The projection of the cilium into the extracellular matrix varied from cell to cell, but could be grouped into 3 distinct patterns. In the first group, the axoneme of the primary cilium was fully extended into, and formed maximum contact with, the extracellular matrix (Fig. 1*a*). In the second group, the axoneme was partially extended into the matrix with a range of bending deflections along the ciliary shaft (Fig. 1*b*). In the third group, the primary cilium was reclined against the cell surface, with minimal matrix contact (Fig. 1*c*).

The detail of a primary cilium sectioned along its length is shown in Figure 1*d*. Axonemal microtubules originated from the basal body and extended the

length of the axoneme before terminating in a 'cap-like' region of electron-dense granules which commonly accumulated at the distal tip (cf. Fig. 1*a–e*). All microtubules in the axoneme were decorated to varying degrees along their length with microtubule-associated proteins which appeared to form linkages with both adjacent microtubules and the ciliary membrane, particularly at the distal tip. The ciliary membrane had an undulating contour, and was dimpled at the points of contact between the microtubule-associated proteins and the membrane. Direct contact between extracellular collagen fibres and the ciliary membrane was evident along the axoneme, and was matched by electron-dense protein linkages between the membrane and the microtubules.

The variable nature of the interaction between the primary cilium and the extracellular matrix is further illustrated in Figure 1(*e*). The axoneme of the cilium appeared to be threaded amongst the fine collagen fibres of the extracellular matrix, creating minor variations in ultrastructure along its length. Regions of the axoneme traversed by collagen fibres contrast with other regions of electron-dense granules where fibres are absent, suggesting small undulations along the length of the axoneme.

The ultrastructural relationship between the basal body and the Golgi apparatus is illustrated in Figure 1(*f*). The flattened cisternae of the cis-Golgi network define the convex nature of the Golgi apparatus in chondrocytes, while engorged secretory vesicles characterise the trans-Golgi network. In addition to its characteristic density, the diplosomal basal body was consistently located in close proximity to the trans-Golgi network (cf. Fig. 1*a–c*). Numerous microtubules were interspersed throughout the Golgi apparatus and impinged upon the diplosome (not illustrated).

### *Immunohistochemistry and confocal microscopy*

*Detyrosinated  $\alpha$ -tubulin (ID5)*. The distribution of ID5 is shown in Figure 2(*a–c*). Field views show ID5 concentrated in the primary cilium (Fig. 2*a*), with weak staining throughout the cytoplasm. Chondrocytes imaged through their entire depth all contained a primary cilium that was either extended, deflected or reclined with respect to the cell surface. While not yet quantified, the orientation of primary cilia appeared completely random with respect to the axis of the sterna.

The majority of cilia selected for specific examination were orientated at right angles to the confocal

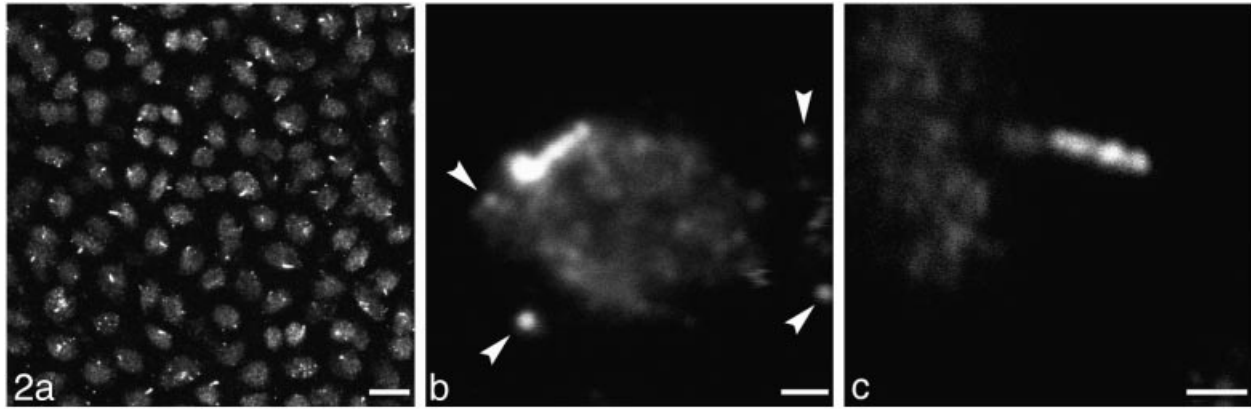


Fig. 2. Chick embryo sternal chondrocytes labelled with ID5 for detyrosinated  $\alpha$ -tubulin. (a) Field view ( $\times 100$  objective) showing discrete staining of primary cilia in most cells, their random orientation and weak staining in the cytoplasm. A z-series projection of 15 optical sections through  $7.53 \mu\text{m}$  (z-series,  $15/7.53 \mu\text{m}$ ). (b) Electronic zoom ( $\times 11.36$ ) of a single cell showing a primary cilium reclined against the cell surface. Note the discontinuity of staining in the distal axoneme, bright punctate spots at the cell periphery (arrows) and weak cytoplasmic staining. (z-series,  $8/2.32 \mu\text{m}$ ). (c) Detail (electronic zoom,  $\times 15.03$ ) of an extended primary cilium showing distinct variability in staining along the axoneme and weak cytoplasmic staining at its base (z-series,  $6/1.68 \mu\text{m}$ ). Bars,  $10 \mu\text{m}$  (a);  $1 \mu\text{m}$  (b, c).

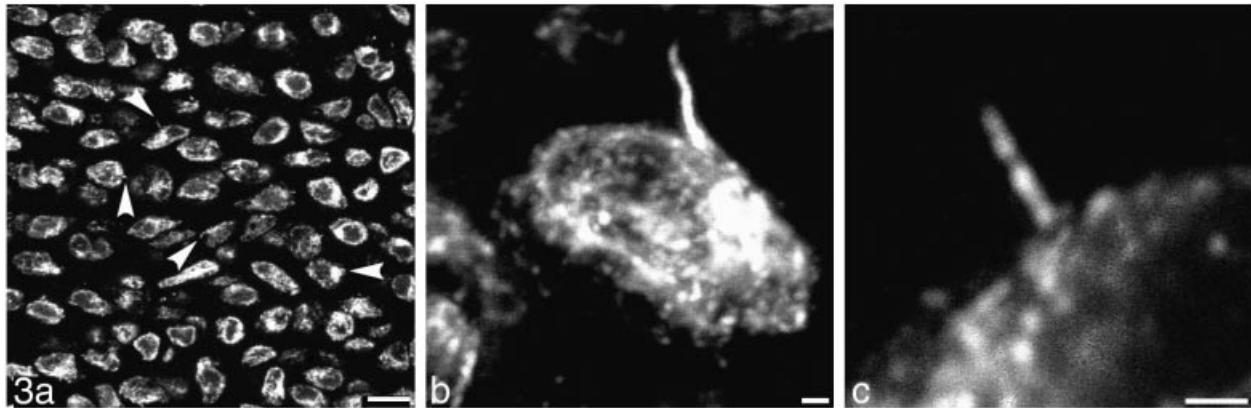


Fig. 3. Chick embryo sternal chondrocytes labelled with C3B9 for acetylated  $\alpha$ -tubulin. (a) Field view showing intense cytoplasmic staining which obscures all but the fully extended primary cilia (arrows). (z-series,  $10/6.38 \mu\text{m}$ ). (b) Electronic zoom ( $\times 6.47$ ) of a single cell showing an extended primary cilium with undulations along its length, and intense cytoplasmic staining in the region of the MTOC (z-series,  $13/7.54 \mu\text{m}$ ). (c) Detail (electronic zoom,  $\times 15.03$ ) of an extended primary cilium showing distinct variations in staining along the axoneme, and strong staining in the MTOC. (z-series,  $6/2.10 \mu\text{m}$ ). Bars,  $10 \mu\text{m}$  (a);  $1 \mu\text{m}$  (b, c).

light path. Figure 2(b) shows a brightly stained primary cilium reclined against the chondrocyte surface (cf Fig. 1c). A small discontinuity of staining was evident at the distal tip, while punctate spots of moderate staining were often found at the periphery of the cell. Weak amorphous staining persisted in the cytoplasm. A fully extended cilium is shown at greater magnification in Figure 2(c). ID5 staining was not uniform along the length of the axoneme, and irregular gaps of weaker fluorescence were common in most of the cilia examined.

**Acetylated  $\alpha$ -tubulin (C3B9).** The distribution of C3B9 is shown in Figure 3(a–c). In contrast to ID5, field views show C3B9 concentrated in the primary cilia and specific regions of the cytoplasm (Fig. 3a). At higher magnifications, intense staining for acetylated tubulin was evident in the primary cilium and a juxtannuclear region corresponding to the

centrosome and MTOC (Fig. 3b). Staining declined to moderate levels throughout the remainder of the cytoplasm. While some primary cilia displayed fairly uniform C3B9 staining (Fig. 3b), the majority showed random discontinuities of staining along the axoneme (e.g. Fig. 3c).

**Total  $\alpha$ -tubulin (TAT).** The distribution of TAT is shown in Figure 4(a–c). Field views show the strongest staining of the 3 antibodies employed, with intense cytoplasmic labelling obscuring all but fully extended primary cilia (Fig. 4a). At higher magnifications (Fig. 4b), strong but irregular staining persisted in the axoneme, with bright punctate staining in the MTOC and perinuclear cytoplasm. Bending of the ciliary axoneme was clearly evident in some cilia (Fig. 4c).

**Colocalisation of detyrosinated and acetylated  $\alpha$ -tubulins.** The differential distribution of detyrosinated and acetylated  $\alpha$ -tubulins was examined by double

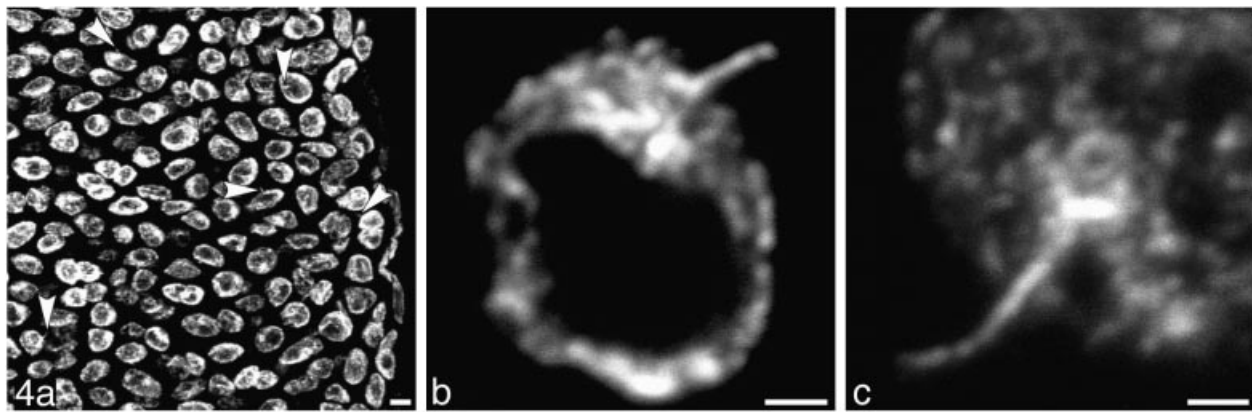


Fig. 4. Chick embryo sternal chondrocytes labelled with TAT for total  $\alpha$ -tubulin. (a) Field view showing intense staining of the perinuclear cytoplasm and extended primary cilia (arrows) (z-series, 10/4.56  $\mu\text{m}$ ). (b) Electronic zoom ( $\times 15.03$ ) of a single cell showing strong staining of the primary cilium, intense staining in the MTOC and variable staining throughout the cytoplasm. (z-series, 6/1.08  $\mu\text{m}$ ). (c) Detail (electronic zoom,  $\times 15.03$ ) showing bending and narrowing in the distal region of a primary cilium, variable staining along its length, and its proximal relationship to the centrosome and MTOC (z-series, 6/1.99  $\mu\text{m}$ ). Bars, 10  $\mu\text{m}$  (a); 1  $\mu\text{m}$  (b, c).

labelling in order to better define the composition and organisation of the microtubular network. Immunostaining of sternal chondrocytes with ID5 (Fig. 5a) and C3B9 (Fig. 5b) was consistent with single labelling studies. However, the composite image (Fig. 5c) revealed regions of separate staining along the axoneme and within the cytoplasm, with some areas of colocalisation (shown in yellow). Three composite images of immunolabelled chondrocytes are shown in Figure 5(d–f). They illustrate the marked variability in staining and colocalisation of detyrosinated and acetylated  $\alpha$ -tubulins in the primary cilia of chondrocytes.

## DISCUSSION

Chick embryo sternal cartilage has proved a highly successful tissue model to investigate the relationship between the extracellular matrix, the primary cilium and the microtubular cytoskeleton of intact chondrocytes. Four key findings emerged from this study. We define the differential distribution of  $\alpha$ -tubulin isoforms in the cytoplasmic microtubules of intact hyaline chondrocytes. We demonstrate that all chondrocytes have a primary cilium containing both acetylated and detyrosinated  $\alpha$ -tubulin, and that it is directly associated with the centrioles and centrosome in the MTOC. We show that primary cilia project, to various degrees, into the pericellular matrix, and are often bent while interacting with the mechanically robust matrix. Finally, we confirm a structural continuum between the extracellular matrix, the primary cilium and the MTOC associated with the Golgi apparatus.

### *The differential cytoplasmic distribution of $\alpha$ -tubulin isoforms*

Intact hyaline chondrocytes showed a clear differentiation of  $\alpha$ -tubulin isoforms within the cytoplasm. Detyrosinated microtubules labelled with ID5 were restricted in chondrocytes to the centrioles and the region of the centrosome, with punctate but variable staining at the periphery of the cytoplasm. Several studies have reported a similar distribution of detyrosinated  $\alpha$ -tubulin in a range of cell types (Wheatley et al. 1994; Poole et al. 1997), although the significance of the punctate staining near the cell membrane is not known. Tubulin heterodimers are thought to function in the membrane environment as polymers arranged into short protofilaments (Ravindra, 1997), and if true of chondrocytes, could explain this cortical punctate staining. ID5 is located within the most stable subset of cytoplasmic microtubules (Geuens et al. 1986; Skoufias et al. 1990; Thyberg & Moskalewski, 1993), the differentiation and stability of these structures reflecting their importance to the integrity and function of the microtubular cytoskeleton.

Acetylated  $\alpha$ -tubulin had a much broader cytoplasmic distribution, but was concentrated in the region of the MTOC and centrosome. This differentiation was consistent with previous studies in which the appearance and persistence of C3B9 in the MTOC correlated directly with the spatial integration of the Golgi complex following cytokinesis (Thyberg & Moskalewski, 1993). Ultrastructural data confirmed previous studies reporting a physical relationship between the centrosome and the trans-Golgi network (Poole et al. 1985, 1997; Tenkova & Chaldakov,

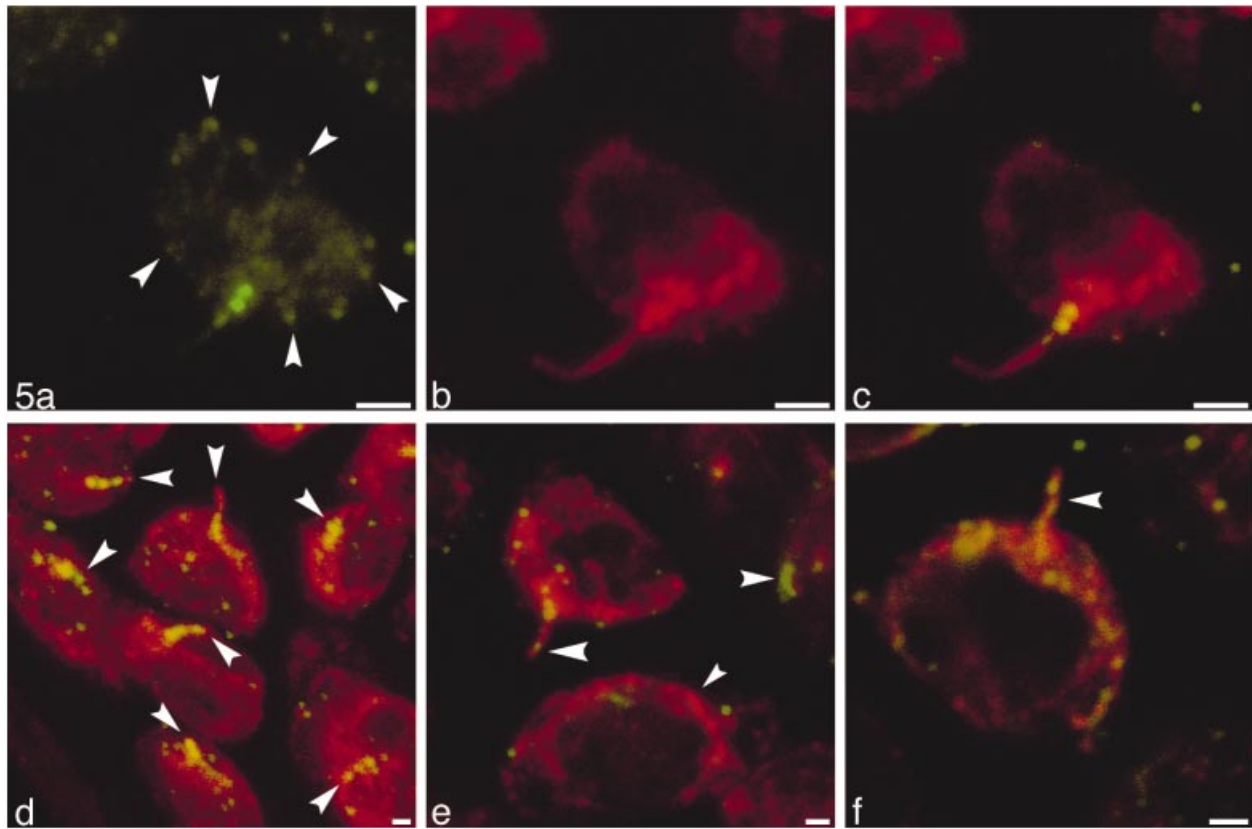


Fig. 5. Chick embryo sternal chondrocytes double-labelled for detyrosinated and acetylated  $\alpha$ -tubulins. (a–c) Electronic zoom ( $\times 13.64$ ) of a single chondrocyte stained for ID5 (a), C3B9 (b) and combined (c). Note strong staining of the basal diplosome and proximal axoneme for ID5 (a), while the bent segment of the distal axoneme is dominated by C3B9, which also defines the MTOC (b, c). Punctate staining for ID5 at the periphery of chondrocytes (arrows) forms an integral part of the microtubular cytoskeleton when combined (c) (z-series,  $6/2.69 \mu\text{m}$ ). (d–f) Examples of chondrocytes double-labelled for detyrosinated (green) and acetylated (red) tubulin. (d) Each chondrocyte in the field had one primary cilium, which varied uniquely in its pattern of tubulin colocalisation (arrows) (z-series,  $10/6.71 \mu\text{m}$ ; electronic zoom,  $\times 4.87$ ). (e) Chondrocytes showing primary cilia stained predominantly for acetylated tubulin (small arrow), detyrosinated tubulin (medium arrow) and both tubulins (large arrow) (z-series,  $10/4.24 \mu\text{m}$ ; electronic zoom,  $\times 4.05$ ). (f) Single chondrocyte illustrating the colocalisation of detyrosinated and acetylated tubulin in both the MTOC and the primary cilium which had a distinct ‘kink’ in its midregion (arrow) (z-series,  $7/2.30 \mu\text{m}$ ; electronic zoom,  $\times 9.64$ ). Bars,  $1 \mu\text{m}$ .

1988), and this interaction is conceptually illustrated in Figure 6. Microtubules containing C3B9 are also relatively stable (Piperno et al. 1987; Schulze et al. 1987; Bulinski et al. 1988), and their colocalisation with detyrosinated microtubules in the centrosome and MTOC suggests a role in stabilising the Golgi apparatus and polarising its secretory function (Allan, 1996). Strong cytoplasmic staining for TAT, which includes the labile tyrosinated  $\alpha$ -tubulin pool, confirms that intact chondrocytes possess the full complement of  $\alpha$ -tubulin isoforms necessary to maintain the synthetic and secretory functions of the cell.

#### *The microtubular composition of the chondrocyte primary cilium*

Ultrastructural data from this and previous studies have shown a constant microtubule morphology along the length of the primary cilium (Wilsman, 1978; Poole et al. 1985). However, the introduction of

antitubulin antibodies like ID5 has proved highly specific for detyrosinated ciliary microtubules (Wehland & Weber, 1987; Wheatley et al. 1994), and in chondrocytes, was equally specific for the primary cilium and basal diplosome. Discontinuities of axonemal staining were common to most intact chondrocytes, in keeping with studies on cultured cells (Wheatley, 1995; Poole et al. 1997).

Discontinuities of primary cilia staining have also been reported with antibodies directed against acetylated  $\alpha$ -tubulin (Piperno & Fuller, 1985; Poole et al. 1997; Alieva et al. 1999), and were a consistent feature of the current study. Variability in the distribution of detyrosinated and acetylated  $\alpha$ -tubulins was also evident in the double labelling studies. The colocalisation of these  $\alpha$ -tubulins was most common in the basal diplosome and proximal region of the axoneme, with greater variability towards the distal region of the ciliary shaft. Frequently, areas of weak labelling for ID5 were



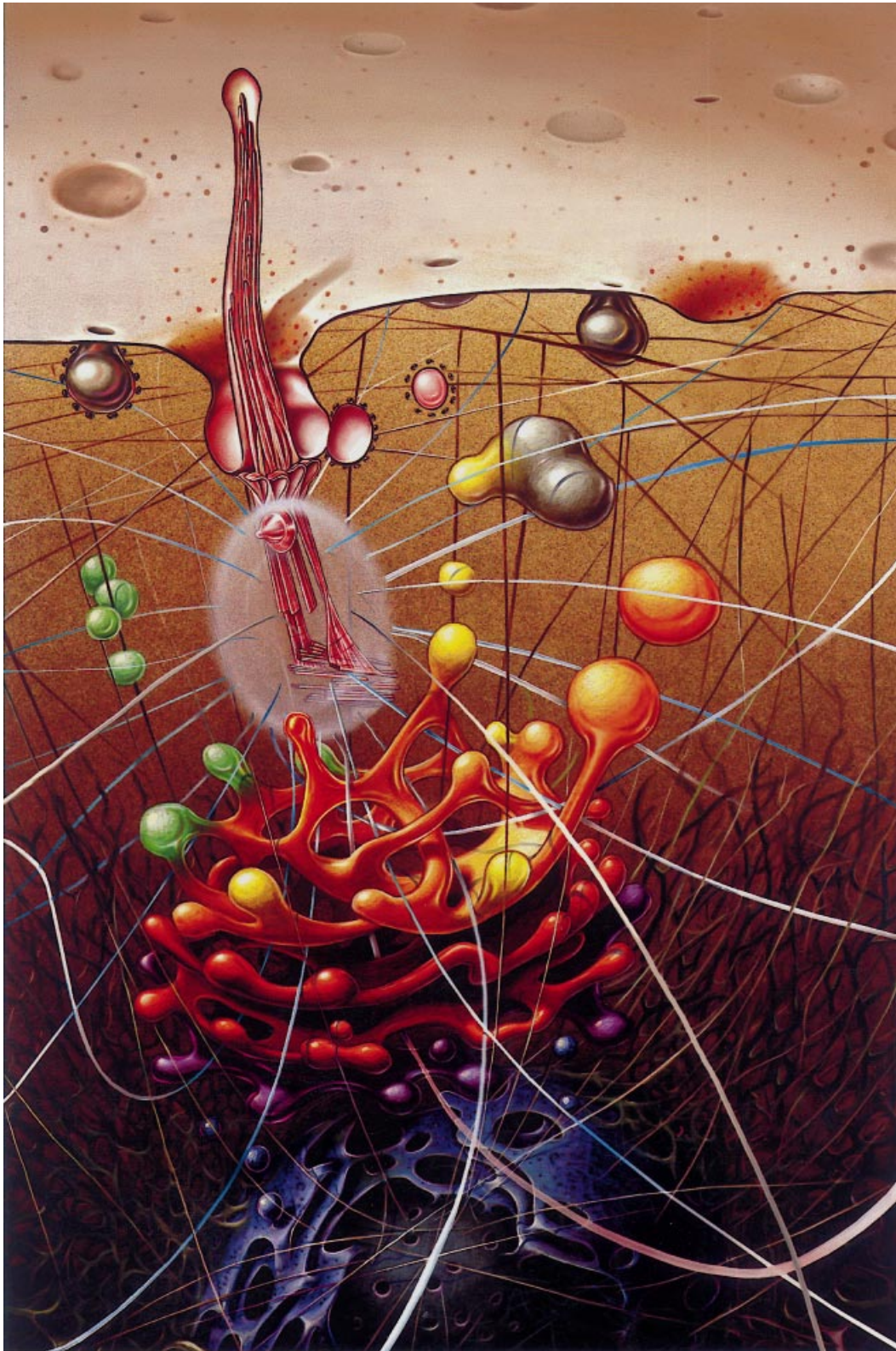


Fig. 6. Conceptual illustration of the primary cilium and the microtubular cytoskeleton in relation to the Golgi-endoplasmic reticulum-lysosomal system as previously illustrated by Cormack (1993). The structure of the primary cilium in red was based on Wilsman (1978), and positioned to show its relationship to the *trans*-Golgi network in orange (unregulated secretion), green (regulated secretion) and yellow (lysosomal system). The diplosomal centrioles at the base of the cilium are surrounded by an amorphous region termed the centrosome, illustrated in pale white. The centrosome represents the nucleating centre for the microtubular network (white and blue), which maintains Golgi integrity and radiates throughout the cytoplasm. Modified by permission of David H. Cormack, from *Essential Histology* (J. B. Lippincott, 1993).

complemented by strong labelling with C3B9, although this was not consistent in all cilia. Often, the full length of the chondrocyte primary cilium could only be visualised when both antibodies were used, and variations in staining intensity were common even when the TAT pool was labelled. Although they varied significantly in composition and organisation, the acetylation and detyrosination of ciliary microtubules clearly defines the primary cilium as one of the most stable components of the microtubular cytoskeleton. Confined and restricted within the ciliary membrane, and decorated with electron-dense proteins which mediate microtubular and membrane interactions (Dentler, 1981; Bloodgood, 1991), it is conceivable that the primary cilium could provide the flexural rigidity necessary to function as a matrix mechanotransducer.

*The projection of the primary cilium influences its exposure to the matrix*

All chondrocytes had one primary cilium, but the angularity of the axoneme, and the extent to which it projected into the extracellular matrix, varied from cell to cell. Three patterns of ciliary projection were identified ultrastructurally and confirmed by confocal microscopy. In the first group, the primary cilium was fully extended into the extracellular matrix, and often showed small undulations along its length. In the second group, the primary cilium was partially extended into the matrix, with deflection patterns which varied from slight bending in the distal region, through to acute deflections in the proximal region of the axoneme. In the third group, the primary cilium was reclined against the cell surface, with minimal matrix interaction. These patterns appeared randomly throughout the sterna, suggesting that chondrocytes might be able to alter ciliary projection and modify the degree of interaction with the mechanically functional extracellular matrix.

Random ultrastructural sections which include the full length of a primary cilium are rare, while confocal imaging showed that ciliary bending and undulations are relatively common and in need of further investigation. Previous studies have reported similar ciliary bending in a range of connective tissues including cartilage, and correlated this deflection with the biomechanical properties of the matrix (Poole et al. 1985). In hyaline cartilage, we suggest that tensioned collagen fibres traversing the axoneme could apply a local lateral force to the axoneme, causing it to bend slightly. If this bending fulcrum were counteracted by similar collagen fibre interactions on the opposing

side of the axoneme, the ciliary shaft would undulate slightly but remain essentially straight. Conversely, if the sum of the collagen-induced bending forces were greater on one side, the cilium would be deflected according to the magnitude of the force.

Bending responses have been reported in primary cilia under fluid shear (Schwartz et al. 1997). The bending of modified primary cilia has also been adopted across different phyla as the preferred mechanism of mechanosensory transduction (Barber, 1974; Moran & Rowley, 1983; Keil, 1997). The proximal region of these modified sensory cilia are characterised by a spiral of integral membrane proteins which form a 'ciliary necklace' around the base of the ciliary shaft (Gilula & Satir, 1972; Dentler, 1981; Menco, 1988). It has been proposed that ciliary necklace proteins act as stretch-activated cation channels (Gilula & Satir, 1972; Dentler, 1981), which serve to move calcium through the ciliary membrane and provide second messenger regulation for a number of cellular processes including the exocytotic step of secretion (Burgoyne & Morgan, 1993). Cyclic-AMP-dependent protein kinase II (A-Kinase), the principal mediator of the second messenger effects of cAMP, has also been localised to the trans-Golgi network, the MTOC and the diplosomal centrioles of connective tissue cells and modified sensory cilia (De Camilli et al. 1986). It will be of interest to determine if a functional relationship exists between these potential regulatory mechanisms and the transduction of the biomechanical forces that cause ciliary bending in hyaline chondrocytes.

*The extracellular matrix-primary cilium-Golgi continuum*

Ultrastructural studies of primary cilia have tended to concentrate on the axonemal microtubules, the basal diplosome and associated cytoplasmic structures (Wheatley, 1982). In contrast, relatively few studies have reported on the interaction between the ciliary membrane and the extracellular microenvironment into which it projects (Poole et al. 1985; Handel et al. 1999). This is particularly important in connective tissues like cartilage, where the primary cilium of the chondrocyte projects into a biomechanically responsive extracellular matrix of tensioned collagen fibres, osmotically active proteoglycans and adhesive glycoproteins (Muir, 1995).

Much of our understanding of ciliary membranes derives from studies on *Chlamydomonas* and *Paramecium*, or specialised structures like the vertebrate

photoreceptors and olfactory cilia. (Dentler, 1981; Moran & Rowley, 1983; Menco 1988; Bloodgood, 1991). The ciliary membrane represents a unique plasma membrane domain with several overlapping functions including adhesion, force transduction, sensory reception and regulation of axonemal calcium concentration (Bloodgood, 1991). In hyaline chondrocytes, the ciliary membrane was dimpled at the point of interaction with microtubule-membrane linkages, which were most numerous at the distal tip. On the extracellular surface, collagen fibres terminating at the ciliary membrane were matched by electron-dense proteins linking the ciliary membrane to the microtubules. While the receptors anchoring these collagen fibres, and the nature of the microtubule-membrane linkages, have yet to be identified, the data presented provides compelling evidence for a structural continuum between the extracellular matrix, the primary cilium, the microtubular cytoskeleton and the Golgi apparatus of hyaline chondrocytes.

#### *Primary cilia – a cybernetic probe for chondrocytes?*

In the hyaline cartilage model presented, the chondrocyte primary cilium interacts with, and is deflected by, the extracellular matrix. Matrix macromolecules form transmembrane linkages with acetylated and detyrosinated ciliary microtubules, which are structurally linked to the diplosomal centrioles and determine the position of the centrosome and MTOC within the cytoplasm. Microtubules are known to participate in cellular signalling by communicating physical changes in microtubular conformation from one locus in the cell to other regions of the cytoplasm (Ravindra, 1997). It is therefore possible that the primary cilium could 'sense' the biomechanical and/or physicochemical properties of the matrix and transduce this information into a cellular response mediated via the microtubular cytoskeleton. The stable microtubules of the MTOC are vital for the polarised assembly of the Golgi apparatus, with the basal diplosome positioned adjacent to the trans-Golgi network where vesiculated matrix macromolecules assemble prior to secretion (see Fig. 6). This structural relationship positions the Golgi apparatus close to the cell membrane at its junction with the ciliary membrane, and must polarise secretory activity towards this region of the plasma membrane. If intrinsic ciliary-membrane proteins which regulate intracellular calcium levels or cAMP production can be identified in the chondrocyte primary cilium, then mechanical

deflection of the cilium could provide a transducing mechanism for a number of processes regulated by second messenger cascades, including the microtubular transport of secretory vesicles and exocytosis. We believe the chick embryo sterna model will facilitate more systematic challenges to the concept of the primary cilium as a cybernetic probe which transduces environmental information from the matrix, communicates this information to the centrosome, and directs exocytosis of the Golgi-processed matrix macromolecules essential for skeletal development.

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