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Primary cilia in fibroblast-like type B synoviocytes lie within a cilium pit: a site of endocytosis

Jerome B. Rattner[1], Paul Sciore[2], Young Ou[3], Frans A. van der Hoorn[3] and I.K.Y. Lo[2]

[1]Department of Cell Biology and Anatomy, [2]Department of Surgery, Faculty of Medicine, and [3]Department of Molecular Biology and Biochemistry, University of Calgary, Calgary, Alberta. Canada

Summary. The synovium is a thin connective tissue that lines the joint space of free moving articulations. In this report, the expression, structure, and composition of non-motile (primary) cilia in fibroblast-like synoviocytes (FLS) that populate the synovium have been studied. Primary cilia are non-motile, microtubule-based organelles that have been found in a variety of vertebrate cell types. We document that primary cilia are expressed in normal human synovium FLS, cultured human FLS, and FLS cells present in human synovial fluid, and that the cellular region occupied by the primary cilium shows a similar and highly defined architecture within these FLS. This architecture includes the presence of a unique structure that surrounds the lower portion of the cilium shaft. This structure, given the term cilium-pit, includes a space, the pit reservoir. Actin filament bundles surround the cilium-pit, and when these bundles are removed experimentally the volume of the cilium-pit and its continuity with the extracellular environment changes. Finally, this study documents that the cilium-pit is a site of endocytosis and is also the site for the localization of receptors (TNF receptors TNFR1 and TNFR2) associated with synoviocyte function. Taken together, the results of the present study suggest that the FLS cilium-pit functions to regulate the exposure of the primary cilium, both spatially and temporally to extracellular molecules and to couple primary cilium based signaling pathways with those linked to endocytosis.

Key words: Primary cilia, Endocytosis, TNF, TNF receptors, Synoviocytes

Offprint requests to: Dr Jerome B. Rattner, Department of Cell Biology and Anatomy, University of Calgary, 3280 Hospital Drive NW, Calgary, Alberta T2N 4Z6, Canada. e-mail: rattner@ucalgary.ca

Introduction

The synovium (also described as the synovial membrane or synovial lining) is a thin connective tissue that lines the joint space of free moving articulations. Histologically, the normal synovial membrane, generally 1-2 cell layer(s) thick, has been divided into intima and subintima layers. In both of these layers, fibroblasts, specialized macrophage populations, as well as endothelial cells that form the vascular compartment of the tissue are present (Krey and Cohen 1973; Graabaek, 1982, 1984; Edwards, 1994). Two main types of synoviocytes have been identified, and have been classified as the macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes (FLS). FLS synthesize and secrete a series of molecules that include hyaluronan, collagens, fibronectin, glycans, cytokines, arachidonic acid metabolites and metalloproteinases (Fell et al., 1976; Matsubara et al., 1983; Mapp and Revell, 1989). Together, these components form a complex fluid (i.e. synovial fluid) that is secreted into the joint space where it functions in both joint lubrication and joint homeostasis.

One characteristic of FLS often noted but not defined in any detail is the presence of a single cilium (Wyllie et al., 1964; Schumacher, 1975; Graabaek, 1984; Meek et al., 1991; Shikichi et al., 1999). This type of cilium, also known as a primary cilium, is non-motile and is a microtubule-based organelle that has been found as a component of a variety of vertebrate cell types. Work over the past several years has shown that this class of cilium is capable of playing a number of roles in mechano-, chemo-, and osmo-sensory functions, cell cycle regulation, left-right axis development determination, neurogenesis, and photoreceptor maintenance (for example see Davis et al., 2006; Marshall and Nonaka, 2006). Of particular interest,

recent studies have identified Wnt signaling and platelet-derived growth factor receptor signal transduction cascades at the primary cilia (reviewed in Christensen et al., 2007, 2008). These studies suggest that growth factor mediated mitogenic cascades may be localized to the primary cilia.

Mutations that affect proteins associated with primary cilia have been found to be associated with a number of human diseases, including polycystic kidney disease, as well as Alstrom, Bardet-Biedl, Joubert, Meckel-Gruber and Oral-facial-digital type 1 syndromes (for review see Sharma et al., 2008). Recent investigations have also identified primary cilia in cells populating skeletal connective tissues. For example, primary cilia have been found in osteocytes of bone (Whitfield, 2003, 2008), fibroblasts of dense connective tissues (e.g. ligaments, tendons, menisci, intervertebral disc) (Donnelly et al., 2008), as well as chondrocytes of growth plate cartilage and articular cartilage (McGlashan et al., 2006, 2008; Ascenzi et al., 2007. Research models, such as knockout mice, have shown that defects in primary cilia are associated with the development of skeletal abnormalities (McGlashan et al., 2007; Haycraft and Serra, 2008; Kaushik et al., 2009). These studies provide a link between primary cilia function and connective tissue homeostasis.

As mentioned above, the synovial membrane lines the cavity of the synovial articulation and functions in the lubrication and homeostasis of the joint. Fibroblast cells that reside in the intimal layer of the synovial membrane are in direct contact with the synovial fluid. During normal and pathological conditions, these cells are exposed to a number of mechanical stresses (e.g. shear stresses) that are induced by the motion of synovial fluid within the joint space (Gomez and Thurston, 1993). In addition, these cells respond to the presence of proinflammatory factors present in the synovial fluid that affect processes involved in disease pathology including cell activation, cell proliferation, and tissue degradation and destruction. It has been hypothesized that primary cilia in epithelial and connective tissue cells are acting as mechano- or chemosensors to their extracellular environment. Since the synovium contacts the synovial fluid that bathes the joint cavity, it is possible that primary cilia have a similar sensory function monitoring the synovial fluid. Unfortunately, other than documentation of the presence of primary cilium, detailed information on the structure, composition, or function of this organelle in FLS has not been investigated in any detail. Furthermore, it is not known if this structure is expressed in FLS suspended in synovial fluid. To address this deficiency, and to begin to explore the function of the primary cilium in FLS, we first carried out a study to detect primary cilia in human synovial tissue, cultured FLS derived from synovial tissue and FLS from human synovial fluid. Subsequently, an ultrastructural study of this region was undertaken using transmission electron microscopy (TEM), scanning electron microscopy (SEM), and indirect immunofluorescence microscopy (IFF). This study revealed a complex structure, the cilium-pit, enclosing a major portion of the primary cilium in these cells. The walls of this structure are shown to be a focus of endocytosis and contain a subset of tumor necrosis factor alpha (TNF-α) receptors, TNFR1 and TNFR2. In addition, the cilium-pit was found to contain a space, the pit reservoir, surrounding the proximal end of the cilium shaft. The functional implications of the cilium-pit, the pit reservoir, and the association of TNF-α receptors with this region are discussed.

Materials and methods

Antibodies

Primary cilia were marked by two separate antibodies: rabbit anti-adenylyl cyclase III (ACIII) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and rabbit anti-glu tubulin (Chemicon, Temecula AC). Mouse monoclonal antibodies to Golgin 97 (CDF4 clone) were from Invitrogen (Burlington, ON, Canada). Mouse monoclonal antibodies to EEA1 and LAMP-1 were from Abcam Inc. (Cambridge, MA, USA) and antibodies to TNFR1 and TNFR2 were from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA); the specificity of these antibodies have been previously documented (Zhang et al., 2008, Ding et al., 2009). Polyclonal goat antibodies to TNF-α were from Abcam Inc. (Cambridge, MA, USA). All antibodies were used according to the manufacturer's specifications.

Sample collection, culturing, cryopreservation and sectioning

Human FLS derived from normal human synovium were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured at 37ºC and 5% CO_2 following the suppliers suggested protocol. Culture media for these cells were supplied by ScienCell. Human synovium tissue samples and synovial fluid samples were obtained during clinical procedures and approved by the Calgary Health Region Ethics Review Committee. Synovial fluid was aspirated into heparinized syringes and the fluid was subsequently placed in a 35mm culture dish in the presence of DMEM supplemented with 10% fetal calf serum and antibiotics and cultured at 37ºC and 5% CO_2. Samples of synovial lining were either fixed and processed for electron microscopy or placed in OCT and frozen for cryo-sectioning. Cryo-sections (6-8 μm thickness) were collected on glass slides and fixed in 100% cold methanol before processing for Indirect Immuno-fluorescence (IIF) as described in the following section.

Indirect Immunofluorescence (IIF)

FLS from synovial fluid or cultured normal human FLS were plated on poly-L-lysine coated coverslips for

approximately 24 hours at 37°C and fixed in 100% ice cold methanol for 10 minutes. In some experiments the cultures were pretreated prior to fixation. To test for the impact of actin on the structure of the cilium pit, cells were grown in the presence of Cytochalasin D (0.3-0.9 μg/ml (Sigma Chemical Co., St Louis, MO) for either 30, 60, or 90 minutes and then fixed as described above. In separate experiments, to determine if TNF localizes to the cilium pit cultures were grown in the presence of 10 ng/mL recombinant human TNF-α (R&D systems, Minneapolis, MN) for either 1, 2, 3, or 4 hours prior to fixation and processing.

Fixed cells were incubated in a blocking buffer containing 10% normal goat serum (Antibodies Incorporated, Davis, CA) and 2% bovine serum albumin (Sigma-Aldrich) to minimize non-specific binding of the antibodies. For co-localization studies, cells were incubated with primary antibodies at their appropriate working dilutions for 30 minutes at room temperature (RT). After washing with phosphate buffered saline (PBS), cells were incubated for 30 minutes in a dark chamber with the corresponding secondary goat fluorochrome-conjugated antibodies. Alexa Fluor (AF) 488 (green) or 568 (red) secondary antibodies were purchased from Invitrogen (Burlington, ON, Canada). After washing with PBS, the cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined for IIF using a 100x objective on a Leica DMRE microscope equipped with epifluorescence and an Optronics camera. Appropriate IIF controls with no or only one primary antibody or both secondary antibodies alone or in combination revealed no observable non-specific background staining and no detectable bleed-through between microscope filter sets.

Electron microscopy

Cells were seeded into 35 mm dishes two days before fixation and grown to 80% confluence. In some experiments, the cells were experimentally manipulated prior to fixation (see previous section). For transmission electron microscopy (TEM) studies, cell monolayers where washed in PBS and fixed in 3% glutaraldehyde in Millonig's phosphate buffer for 1 hour at RT. Tissue samples were fixed directly in 3% glutaraldehyde in Millonig's phosphate buffer. Post-fixation was in 2% OsO_4 for 20 minutes. The cells were dehydrated in ethanol, and then embedded using Polybed 812 resin (Polysciences, Warrington, PA). Polymerization was performed at 37°C for 24 hours. Silver-gray sections were cut with an ultramicrotome (Leica) equipped with a diamond knife, stained with uranyl acetate and lead citrate, and then examined in a H-7000 Hitachi electron microscope. For scanning electron microscopy (SEM), cells were fixed in 2% glutaraldehyde and post-fixed in 1% OsO_4. The samples were then dehydrated in a graded ethanol series and critical point dried. After coating with a layer of colloidal gold, samples were examined in a Hitachi XL30 SEM.

To study endocytosis in FLS we used horseradish peroxidase(HRP) to map this process (Steinman et al., 1976; Griffiths et al., 1989). One day before the assay, FLS cells were trypsinized and plated in 35 mm culture dishes to allow for 90% confluency on the following day. 24 hours later, the cells were washed with serum free medium twice and then incubated with serum-free medium containing 2 mg/ml HRP for 30 min. After washing twice with serum-free medium, cells were processed for EM as described by Robbins et al. (1971). Briefly, cells were fixed for 30 min with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH7.4. Cells were then washed 3 times with the same cacodylate buffer (without glutaraldehyde) and twice with 50 mM Tris-HCl, pH7.5. For dye precipitates formation, cells were incubated first for 30 min at room temperature in a T-DAB buffer (50 mM Tris, pH7.5, 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis)) in the absence of hydrogen peroxide, and then for 60 min in the same T-DAB buffer in presence of 0.01% hydrogen peroxide. Cells were washed again three times with 50 mM Tris, pH7.5 and twice with distilled water, post-fixed for 90 min in 2% OsO_4in distilled water, dehydrated in ethanol and embedded in Epon. Thin sections were stained with lead citrate and examined under electron microscopy.

Results

Primary cilia are expressed by human FLS derived from a variety of sources and have a common ultrastructural organization

A single primary cilium was detected in human synovial lining FLS (Fig. 1A), cultured FLS derived from normal human synovial lining (Fig. 1B), and cultured FLS derived from normal human synovial fluid (Fig. 1C) when fixed samples were overlayed with antibodies to detyrosinated tubulin (glu tubulin), a verified marker of primary cilium (Gundersen and Bulinski, 1986) and examined by indirect immunofluorescence (IIF) using fluorochrome conjugated secondary antibodies to identify this organelle. Since previous reports have shown that antibodies to adenylyl cyclase III (AC III) react with primary cilia in the brain (Bishop et al., 2007), we investigated whether these antibodies also identify FLS primary cilia. Fig. 1D illustrates that AC III antibodies react with primary cilia in cultured FLS. Similar staining with ACIII was also obtained with FLS of the intact synovial lining, as well as cultured FLS from synovial fluid (data not shown), indicating that AC III is a component of FLS primary cilia. Synoviocyte primary cilia were also stained with antibodies to acetylated tubulin (data not shown). Examination of cycling cell

populations revealed that FLS primary cilia are expressed in a cell cycle specific manner, appearing first in G_1 and regressing in late S/G_2 (data not shown). In IIF images, cilia in cells from each source displayed a similar maximum length of 5 μm (±0.5 μm; n=300).

Using TEM, it was determined that FLS primary cilia from each of the three sources described above exhibited a similar ultrastructure. Unlike primary cilia found in other connective tissues such as chondrocytes (Kouri et al., 1996), where the basal body is positioned at the surface of the cells and the entire shaft of the cilium extends out into the extracellular matrix, the basal body of a FLS cilium remains submerged within the interior of the cell, with a major portion of the cilium shaft lying within a pit that appears as a deep invagination of the plasma membrane (Fig. 2A). We have termed this structure the *cilium-pit*, and the area within the pit between the primary cilium and the plasma membrane pit walls, the *pit reservoir*. The remainder of the shaft extends past the cell surface into the extracellular environment as indicated in TEM (Fig. 2A) and SEM (Fig. 2A lower left inset) image. The failure to detect primary cilia at the surface of FLS in synovial tissue as well as cultured cells suggests that the pit morphology is not an intermediate stage in cilium development but rather a feature of a mature primary cilium.

Examination of the walls of the pit revealed the presence of a large number of vesicles, many of which appeared to be fused with the plasma membrane (Fig. 2A). This feature is also apparent in cross-sections of the pit (Fig. 2A upper right inset). Cross-sections also reveal the 9+0 arrangement of microtubules within the axoneme of the primary cilium. An additional feature of the cytoplasm surrounding the cilium-pit is Golgi complexes (labeled G in Fig. 2A and 2B) and bands of cytoplasmic filaments (present within the box of Fig. 2B). Staining of the primary cilium region with the Golgi complex marker Golgin 97 (Griffith et al., 1997) by IIF further confirmed that Golgi complexes line the region surrounding the primary cilium (Fig. 3C). The bands of cytoplasmic filaments found in this area were found to react with actin antibodies (data not shown).

The cilium-pit is a focus of endocytosis

The ultrastructure of the region surrounding the cilium-pit suggests that it is a focus for endocytosis. To verify this supposition, cultured FLS were double stained with glu tubulin antibodies to mark primary cilia and antibodies directed to an early endosome component EEA1 (early endosome antigen 1) (Mu et al., 1995). The early endosome is the major sorting compartment for the endocytic pathway, where many ligands dissociate from their receptors and also recycle to the cell surface. We found that a subset of early endosomes co-localize and align with the region surrounding the primary cilium (Fig. 3A). Similarly, antibodies reactive with late endocytic structures such as lysosomes detected by LAMP-1 (lysosomal-associated membrane protein-1) (Richardson et al., 2008) surrounded and co-localized to the primary cilium-pit (Fig. 3B). Further, staining of the primary cilium region with the Golgi complex marker Golgin 97 (Griffith et al., 1997) confirmed that Golgi complexes line the region surrounding the primary cilium (Fig. 3C).

To further verify and map the relationship between the cilium pit and endocytosis we cultured cells in the presence of HRP and then prepared them for electron microscopic analysis. HRP is rapidly internalized, appearing first in early endosomes and subsequently in late endosomes (Steinman et al., 1976; Griffiths et al., 1989). Figure 3D,E illustrate that labeling could be found at the plasma membrane but was most pronounced along the margins of the cilium pit. Using micrographs that demonstrated labeling at both the primary cilium region and the plasma membrane we counted the number of labeled structures in a 1.7x1.7 μm area along the plasma membrane and within a region centered at the primary cilium. The basal body region had an average of

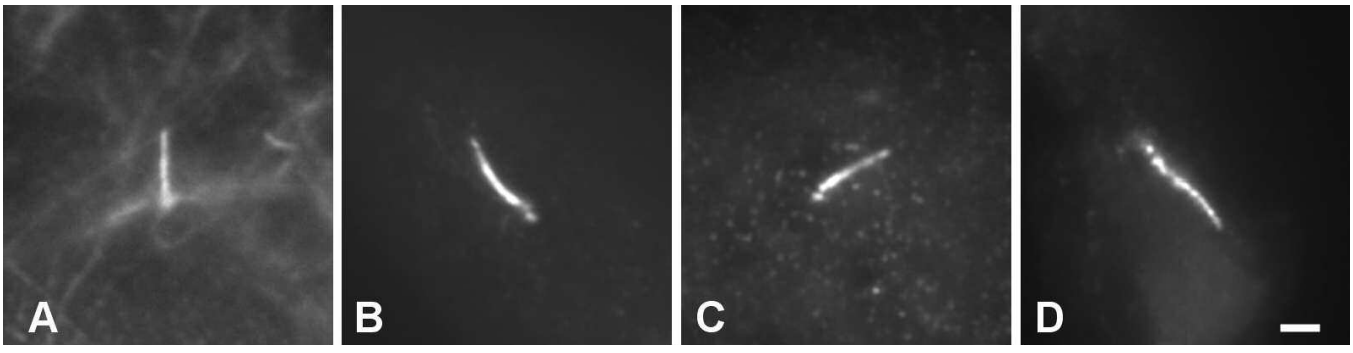


Fig. 1. Synoviocytes display a single primary cilium. A single primary cilium is displayed by FLS from normal human synovium **(A)**, normal human synoviocytes in culture (**B)**, and FLS derived from synovial fluid **(C)** stained with antibodies to glu tubulin. Antibodies to ACIII **(D)** also highlight these cilia. Bar: 5 μm.

29.5 labeled structures with a standard deviation of 6.8 (n=4). In contrast, along the plasma membrane an average of only 3.4 labeled structures were observed with a standard deviation of 2.59 (n=27). Thus, our data as a whole illustrates for the first time that the primary cilium in FLS is associated with a highly structured region, the cilium-pit, which is a focus of endocytosis.

We also investigated whether caveoli-mediated endocytosis is active in the cilium pit by staining cells with and antibody to caveolin. This study failed to show any clustering of caveolin in the cilium pit region (data not shown).

The diameter of the pit reservoir and its continuity with the extracellular environment is influenced by actin

In the course of studying the fine structure of the cilium pit it was noticed that the plasma membrane and

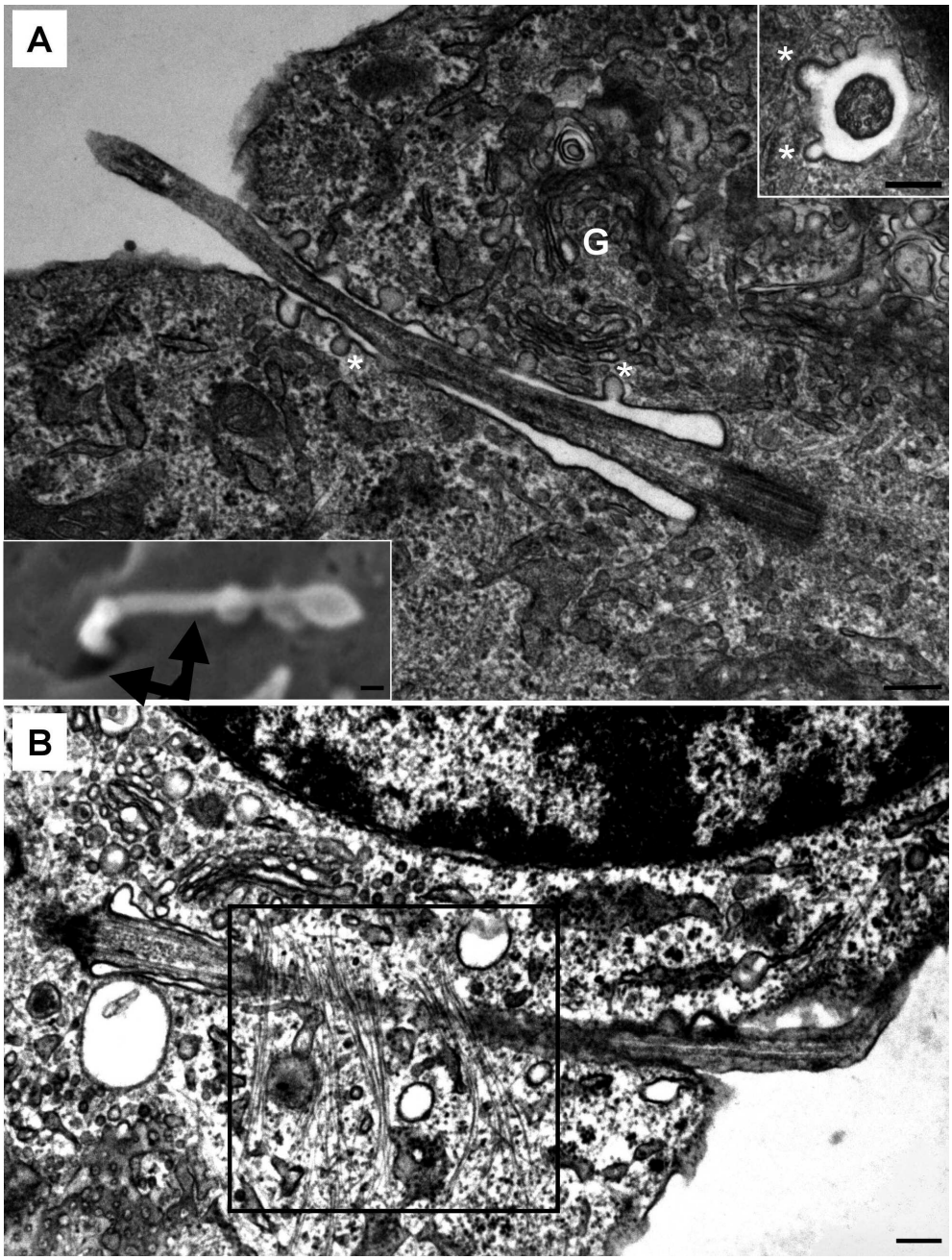


Fig. 2. FLS primary cilium lies within a cilium-pit, a site for endocytosis, and is surrounded by a pit reservoir. **A.** TEM micrograph of the primary cilium region of a FLS in culture. The single cilium lies within a deep invagination (cilium-pit) continuous with the plasma membrane and a space, the pit reservoir, which can be seen between the shaft of the cilium and the walls of the pit. The cilium-pit is lined with endocytotic vesicles (some are denoted by stars) and Golgi complex (G). The cilium-pit, the pit reservoir, the 9+0 axoneme, as well as sites of endocytosis are also illustrated in cross-sections of this region (upper right inset). The lower left inset image shows the extension of the cilium (right arrow) out of the cilium-pit (left arrow) as illustrated in SEM micrographs (low left inset). Bar: 350 nm. **B.** The cilium-pit is encircled by actin fiber bundles. TEM micrograph of a section transecting the region immediately adjacent to the walls of the cilium-pit. The boxed in area denotes a region populated by actin filament bundles that encircle the cilium-pit. Bar: 350 nm.

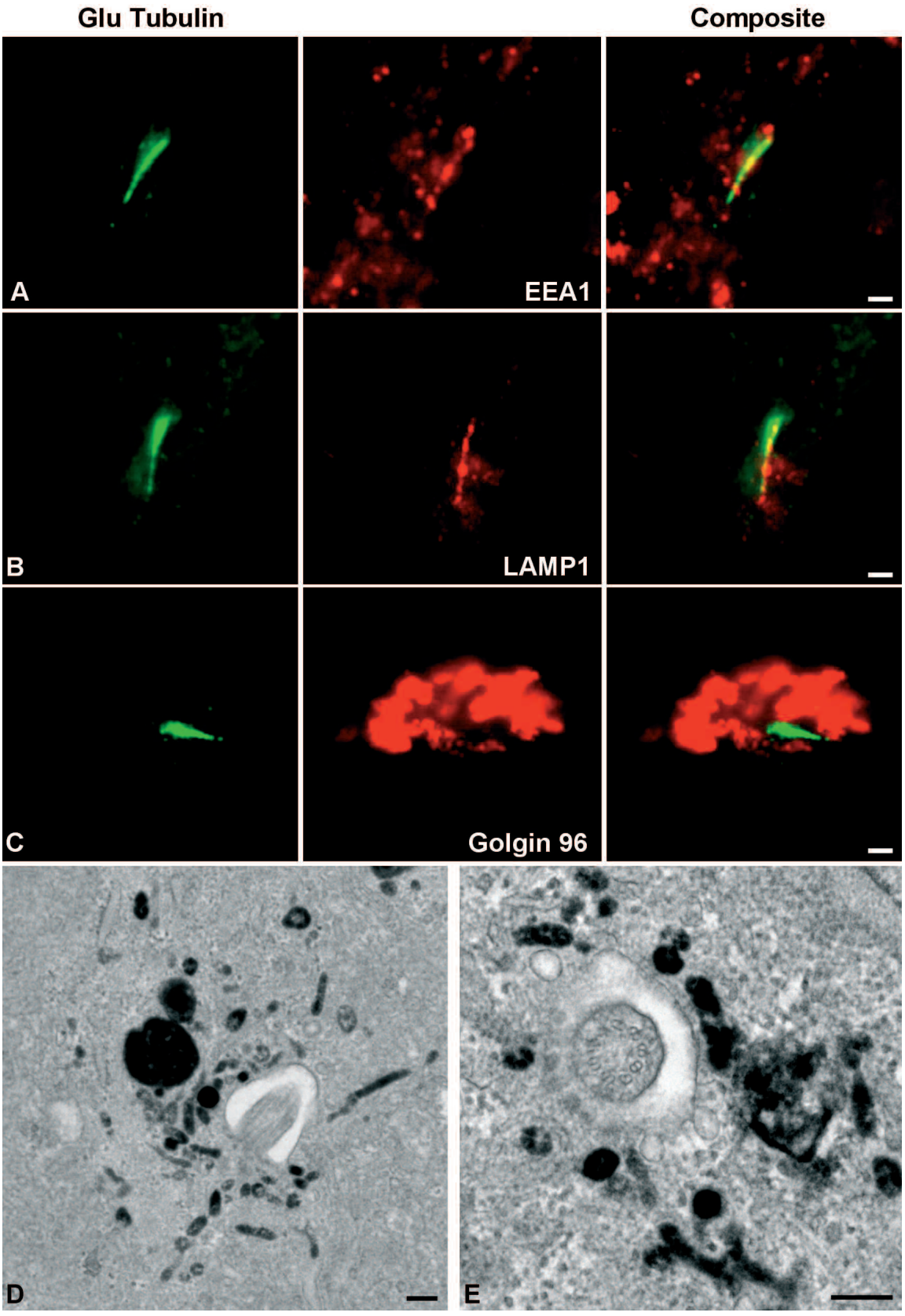


Fig. 3. Early endosomes, late endosomes/ lysosomes, and Golgi complexes populate the cilium-pit region. Cultured synoviocytes stained glu-tubulin (green; left panels), and either **(A)** EEA1, **(B)** LAMP-1, or **(C)** Golgin-97 (red: central panel from top to bottom, respectively). The right panel shows overlay of the two images and the localization of early endosomes, late endosomes/lysosomes, and Golgi complexes with the cilium-pit. Bar: 5 μm. **D,E.** Electron micrographs of sections through the primary cilium region of two different synoviocytes grown in the presence of HRP that labels early and late endosomes. Bar: 200 nm.

the ciliary membrane were situated some distance from one another or situated closely apposed, suggesting that the volume of the pit reservoir could change and restrict the continuity between the pit reservoir and the extracellular environment (Fig. 4A). The constricted region was common to the distal portion of the cilium-

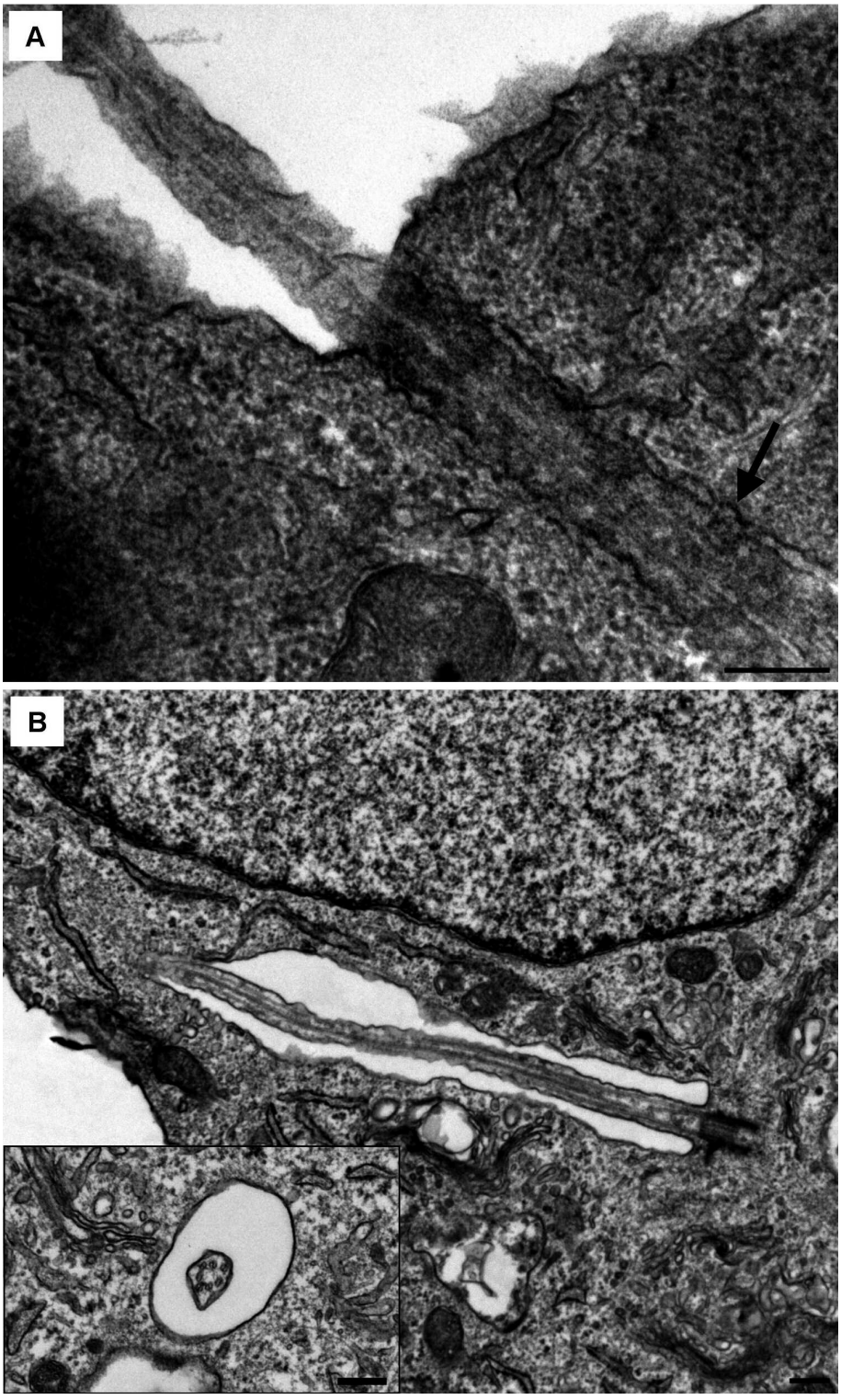
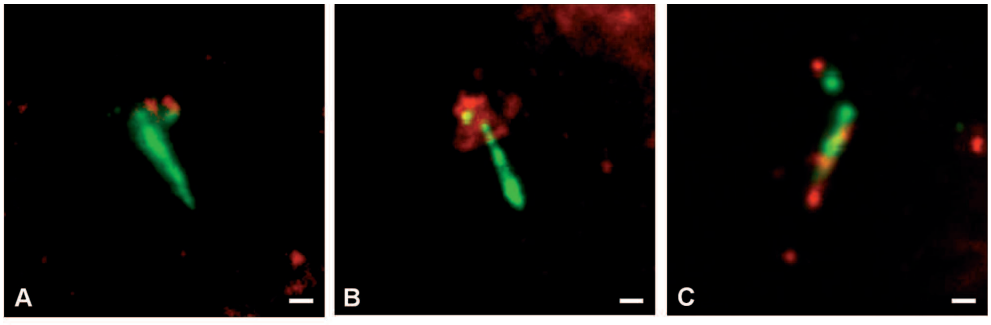


Fig. 4. Actin influences cilium-pit structure. **A.** Electron micrograph illustrating the close association often seen between the ciliary membrane and the cilium-pit membrane (arrow) in untreated cells. **B.** Following treatment with Cytochalasin D the actin filaments are lost from the primary cilium region and the pit reservoir expands. This expansion is also seen in cross-section of this region (lower left inset). Also compare this image with Fig. 2A upper right inset and note the increase in the volume of the cilium reservoir. Bar: 350 nm.

pit (Fig. 4A) and this region coincided with the region displaying bundles of cytoplasmic actin filaments (Fig. 2B). Such observations suggest that the walls of the pit reservoir may have the ability to expand and contract, thus regulating the flow of material into and out of the pit reservoir. To determine if actin filament bundles could regulate the expansion/contraction of the pit reservoir, FLS cultures were treated with an actin-depolymerizing drug, cytochalasin D. The impact of actin loss on pit structure was evaluated by TEM. It was found that after 30 minutes of cytochalasin D treatment at a concentration of 0.3 μg/ml, actin fiber bundles in the region of the cilium pit disappeared (compare Fig. 4B with Fig. 2B). This loss was detected by staining similar preparations with actin antibodies (data not shown). Electron micrographs of treated cells revealed that the cilium-pit displayed an open conformation along its length and the pit reservoir had a large internal volume (compare Fig. 4B with Fig. 4A). This large internal volume of the pit reservoir can also be seen in cross section (Fig. 4B inset). To verify these findings we measured and compared the cross-sectional diameter of the cilium pit before and after cytochalasin D treatment. The diameter of the cilium pit in untreated cells had a mean diameter of 300.8 nm (n=10) while the diameter of this structure in treated cells had a mean of 1095.3 nm (n=10) (Student t test $p < 0.0001$). These findings suggest that the volume of the pit reservoir and the continuity of the reservoir with the extracellular environment are influenced by actin, either directly or indirectly.

The TNF-α receptors TNFR1 and TNFR2 are associated with the primary cilium region in FLS

Given that the plasma membrane walls of the cilium-pit are a focus for endocytosis, it was decided to search for receptors that are dependent on endocytosis and therefore may be linked to this region. TNF-α is a cytokine that elicits diverse cellular responses via two receptors (namely TNFR1 and TNFR2) that function to mediate the cellular trafficking of TNF and facilitate its



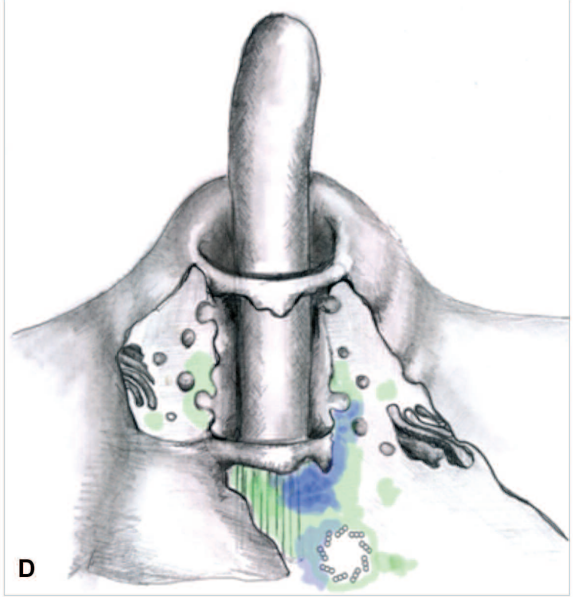


Fig. 5. TNF-α, TNFR1, and TNFR2 populate the primary cilium region. FLS cilium reacted with glu tubulin (green) and antibodies to TNFR1 **(A)**, TNFR2 **(B)** and TNF **(C)** (red). Note that these antibodies stain the basal body and proximal end of the cilium-pit (**A and B)** as well as the length of the cilium-pit **(C)**. In **A, B, and C**, the proximal end of the primary cilia is located at the top of the image. **D.** Schematic diagram of the primary cilia region in FLS summarizing the distribution of TNFR1 (green shading) and TNFR2 (blue shading). Bar: 5 μm.

cellular effects. Importantly, TNF receptor function has been shown to require endocytosis (Pan et al., 2007). To determine if the cellular trafficking of TNF was associated with the cilium-pit region, synoviocytes were stained with mono-specific antibodies to TNFR1 and TNFR2. IIF images revealed that a subset of both TNFR1 and TNFR2 localize to the basal body region of the primary cilium and the proximal end of the cilium pit, respectively (Fig. 5A,B). In the presence of TNF-α, mono-specific antibodies to TNF-α revealed that this protein collected along the length of the cilium-pit (Fig. 5C). Thus, the cilium-pit acts a site that brings together endocytosis, TNF receptors, TNF-α, and the primary cilium.

Discussion

Studies of the synovium in a variety of species have remarked on the presence of primary cilia but there has been little impetus to study these structures further until recently, for it is only within the past few years that the overall importance of this structure has been established. Not only do primary cilia function in a multitude of signaling pathways elicited by chemo-, mechano- and osmo- sensation, but dysregulation or knockout of primary cilia lead to ciliopathic diseases (e.g. polycystic kidney disease and obesity). In addition, it has been known for some time that primary cilia are either found at the surface of cells or located within the cell interior, but the significance of this variability has not been investigated. This study establishes several important features of FLS primary cilia. First, FLS primary cilia are expressed in normal synovial tissue, cultured FLS, and in synovial fluid FLS. Second, all these FLS display a primary cilium region with a similar architecture that includes a cilium pit and cilium reservoir which surrounds and encloses a portion of the cilium shaft. Third, the cilium pit region is a site for endocytosis. Fourth, actin disruptive agents can influence the diameter and volume of the cilium-pit. Fifth, the region around the proximal end of the cilium-pit functions as a site for TNFR1 and TNFR2 localization and TNF-α can bind to these receptors in the cilium-pit region. The organization of the primary cilium region in FLS is summarized in figure 5D.

To date, studies of primary cilia have focused on the cilium itself with little attention to its cellular context. As illustrated for the FLS primary cilium, the primary cilium is often integrated with a highly structured cellular region that includes the cilium-pit. Such structures have also been detected in human astrocytes, as well as connective tissue cells from the knee meniscus and the medial collateral ligament (JBR unpublished data). Thus, this organization is not restricted to FLS, suggesting that our findings have broader applications. Why do some cell types express primary cilia positioned within a cilium-pit while others express primary cilia that extend completely free of the cell body? The present study may provide some important clues to the answer to this question. First, we show that the cilium-pit has, as one of its main features, the pit reservoir where the volume of this reservoir can change due to the presence of actin. It is proposed that the cilium-pit is a dynamic structure that is capable of controlling the volume and duration of proximal cilium exposure to the extracellular environment. By directly affecting the flow of extracellular ions and cytokines, the cilium-pit may play a role in modulating the sensory function of primary cilia. Second, we show that the walls of the pit are a site of endocytosis and are a site for endocytotic linked TNF receptors. Only recently has it been appreciated that endocytosis not only functions as a mechanism to switch off membrane-derived receptor signaling, but that signaling can continue within the endocytic pathway vesicles, and in some cases signaling events require endocytosis for full activation to occur (see review by Di Flore and DeCamilli, 2001). The present study shows that the walls of the cilium-pit can function in endocytosis-linked cell signaling. Thus, it is proposed that the association of the primary cilium with the cilium-pit serves to bring together and coordinate endocytosis and primary cilium based cell signaling pathways. This type of coordination may be essential to specific functional roles of the primary cilium in certain subsets of cells.

The association of a cilium pit with the primary cilium has some similarities to the flagellar pocket that is found in association with the single flagellum of trypanosomes. Like the cilium pit, the flagellar pocket forms a structure that encases a portion of the flagellar shaft and is also a site of endocytosis (Hung et al., 2004). However, the cilium pit is associated with a cilium rather than a flagellum and is more extensive, while lacking a highly structured region that is characteristic of the flagellar pocket's distal end.

TNF is known to play a major role in rheumatoid arthritis (RA) and the finding that TNFR1 and TNFR2 are localized to the basal body and cilium-pit in FLS suggests that the cilium-pit is central to synoviocyte function. It has been established that TNF-α binding induces endocytosis of the activated TNFR1 complex and that the inhibition of TNFR1 endocytosis prevents TNF-inducted apoptosis (Schutze et al., 1999), suggesting that TNFR1 compartmentalization and internalization is involved in initiation of pro-apoptotic events compared to non-internalized TNFR1 signaling (Schneider-Brachert et al., 2004, 2006; Schutze et al., 2008). In addition, TNFR2 has been shown to share components of the TNFR1 signaling cascades (apoptotic and gene activation) (Carpentier et al., 2004). The finding that both TNFR1 and TNFR2 are present at the cilium pit suggests that they may be acting cooperatively in this region. Interestingly, we have found that TNFR1 and TNFR2 remain associated with the centrosome throughout the cell cycle (unpublished observations). The colocalization of TNFRs with the centrosome may serve to link the proliferative functions of both the TNFRs and the centrosome. This linkage may be

particularly important since FLS found in RA synovium have been reported to be resistant to TNF-α mediated apoptosis, and are activated and proliferate upon TNF-α stimulation (Youn et al., 2002). In future, it will be important to determine both the structure and composition of the primary cilium region in RA FLS, since loss or changes in the organization of the cilium-pit or alterations in the type of TNF receptors associated with this region may impact the function of these receptors and thus may contribute to shifting the balance of TNFR cascades toward cell activation and proliferation.

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