研究简报

腺苷酸环化酶III(AC3)在非神经细胞内的定位

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摘要 腺苷酸环化酶III(adenylate cyclase III, AC3)最早发现于小鼠主要嗅觉表皮, 是cAMP信号通路的组成成分之一, 它的缺失可导致小鼠嗅觉丧失、体重增加、雄性不育、母性关爱行为丧失和学习与记忆能力下降等。脊椎动物体内绝大多数细胞具有纤毛, 在小鼠大脑神经元中AC3定位于初级纤毛, 许多非神经类型细胞也有AC3的表达, 然而其与非神经类细胞纤毛的关系尚待确定。该文选择了8种细胞株(2种神经类细胞株、6种非神经类细胞株), 对上述问题进行了研究。RT-PCR及其产物DNA测序结果显示, 这些细胞中均有AC3的表达。免疫荧光染色结果显示, 在IMCD3、293T和BMSCs等细胞株中, AC3与细胞初级纤毛的荧光信号重叠; 在A549和HeLa两种癌细胞株中, AC3也定位在初级纤毛上。该研究结果表明, AC3在多数非神经细胞中也表达于纤毛部位。

关键词 腺苷酸环化酶; 初级纤毛; 非神经细胞; 免疫荧光染色; 癌细胞

Subcellular Localization of Adenylate Cyclase III in Non-Neuronal Cells

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Abstract Adenylate cyclase III (AC3), which was initially discovered in the main olfactory epithelium of mouse, is an important component of the cyclic adenosine monophosphate signaling pathway. Deletion of AC3 leads to olfactory loss, weight gain, male sterility, loss of maternal care behaviors, and declines in learning and memory abilities in mice. The majority of vertebrate cells have cilia, and AC3 is localized to the cilia of neurons in the mouse brain. Although many types of non-neuronal cells also express AC3, whether it is localized in the ciliary region of non-neuronal cells is yet to be determined. In this study, eight cell lines (two neuronal cells and six non-neuronal cells) were selected to investigate the localization of AC3 in non-neuronal cells. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequencing indicated that AC3 was expressed in all of

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the test cells. Immunofluorescence staining showed that fluorescence signal of AC3 overlapped with that of cilia in the non-neuronal cell lines including IMCD3, 293T and bone marrow stromal cells. Additionally, AC3 was localized to the cilia of both A549 and HeLa cancer cells. These data indicate that AC3 is expressed in the ciliary region in non-neuronal cells.

Keywords adenylyl cyclase; primary cilium; non-neuronal cells; immunofluorescence staining; cancer cells

Cyclic adenosine monophosphate (cAMP) is one of the most important intracellular second messengers and is involved in the regulation of many cellular metabolic processes, including oogenesis, embryogenesis, paedomorphosis, hormone secretion, glucose metabolism, smooth muscle contraction, cardiac contraction, olfactory sensation, learning and memory. Adenylyl cyclase (AC) is an important component of the cAMP signal transduction pathway, mainly playing a role in catalyzing cAMP production from ATP[1-2]. To date, ten different isoforms of AC have been identified, i.e., AC1~AC10. Of these, AC1~AC9 are membrane-bound proteins while AC10 is a soluble protein[3].

AC3, which plays an important role in olfactory signal transduction, was initially thought to be a unique isoform of AC that was only expressed in the cilia of olfactory receptor neurons in the main olfactory epithelium[4]. It was later found that AC3 is expressed in neuronal cilia throughout the brain. Bishop and Wang *et al*.[5-7] showed that AC3 is generally expressed in the cilia of neuronal cells in the hippocampus and hypothalamus of the mouse brain, indicating that AC3 can be used as a marker of the cilia in neuronal cells[5-7].

Cilia are hair-like organelles projecting from the surface of eukaryotic cells, with microtubules as the basic structure. Cilia can sense mechanical and chemical stimuli in the periphery of cells[8], and harbor a variety of receptors, ion channel proteins and signal transduction proteins. Cilia are known to be involved in many biological processes such as intracellular signal transduction, regulation of animal development and the maintenance of normal physiological function of various tissues and organs[9-12].

In addition to neuronal cells in the brain, AC3 is widely expressed in many tissues and organs, such as spinal cord, adrenal medulla and cortex, atrial myocardium, aortic smooth muscle, lung, kidney, pancreatic island, testis and ovary, and is closely associated with a variety of physiological functions[13-20]. Wong *et al*.[21] found that AC3 mediated the formation of prostaglandin E2 in aortic smooth muscle cells, inhibited the growth of smooth muscle cells and promoted atherosclerosis in blood vessels. Pluznick *et al*.[22] reported that AC3 was expressed in the kidney, and was involved in the regulation of glomerular filtration rate (GFR) and renin release. In addition, AC3 plays an important role in maintaining normal physiological function of sperm and promoting male fertility[23].

Despite the knowledge that AC3 is expressed in a variety of non-neuronal cells and performs important physiological functions, its subcellular localization in non-neuronal cells remains to be determined. In the present study, different types of cell lines were selected and AC3 expression in these cell lines was clarified by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) combined with DNA sequencing analysis. Subcellular localization of AC3 was performed using immunofluorescence staining, and the results confirmed AC3 expression in the ciliary region of the non-neuronal cells.

1 Materials and methods

1.1 Cell lines

Rat pheochromocytoma cells PC12 (ATCC® CRL-172™) were purchased from CHI Scientific, Inc., Jiangyin, Jiangsu Province, China. Human glioma cells U251 were kindly provided by the Affiliated Hospital of Hebei University, Baoding, Hebei Province, China. Mouse renal collecting duct epithelial cells IMCD3 (ATCC® CRL-2123™) and human renal epithelial 293T cells (ATCC® CRL-11268™) were purchased from BioLeaf, Shanghai, China. The human lung ad-

enocarcinoma cell line A549 (ATCC® CCL-185™) and the human cervical cancer cell line HeLa (ATCC® CCL-2™) were purchased from the Research Center of Hebei Cancer Hospital, Shijiazhuang, Hebei Province, China. Bone marrow stromal cells (BMSCs) and osteoblasts (OBs) were obtained from primary culture: the former were taken from 6-week-old female C57BL/6J mice in accordance with methods described in the literature[24], and the latter were taken from 3-day-old newborn C57BL/6J mice using a combined-enzyme digestion method[25]. The experimental procedures were conducted according to the guidelines of the Animal Care and Ethics Committee of Hebei University, China.

1.2 Cell culture

Normal cells were cultured in high-glucose Dulbecco′s Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, Zhejiang, China). Cancer cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Life Technologies, NY, USA). All cell cultures were incubated at 37 °C in an atmosphere of 5% CO_2. Cells of the experimental group were induced by FBS to starvation to induce cilia development.

1.3 RT-PCR and nucleotide sequence analysis

Log-phase cells were adjusted to the density of 1×10^5 cells/mL. The resultant suspensions were added into 25 cm^2 flasks (3 mL per container) and incubated overnight at 37 °C in an atmosphere of 5% CO_2. When cells were wall-adherent, the old medium was drained and cells were subjected to 24 h of starvation treatment in fresh medium containing 0.5% FBS. Then, cells were digested with trypsin, centrifuged at 1 000 ×*g* for 15 min and harvested.

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA was removed using the PrimeScript™ RT reagent kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) with gDNA Eraser. The extracted RNA was reverse-transcribed into cDNA. The PCR conditions were: predenaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 40 s, and a final extension step at 72 °C for 7 min. Nucleotide sequences of *β-actin* internal reference and *AC3* amplification primers are shown in Table 1, all primers are designed by Premier 5.0 (http://www.premierbiosoft.com). The PCR products were subjected to forward DNA sequencing (Sangon Biotech, Shanghai, China), and the obtained sequences were compared with the corresponding known human and mouse sequences using DNAMAN 6.0 (http://www.lynnon.com).

1.4 Immunofluorescence staining and microscopic examination

Log-phase cell suspensions were adjusted to the density of 1×10^5 cells/mL, spread to six-well plates containing coverslips (2 mL per well) and incubated overnight at 37 °C in an atmosphere of 5% CO_2. When cells became wall-adhered, the old medium was drained for FBS starvation treatment. After another 24 h of incubation, cells were washed twice (5 min each time) with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde for 30 min. The medium was drained and cells were washed twice with PBS. Rupture of cell membrane was achieved with 0.2% Triton X-100 for 10 min, followed by two stages of wash-

Table 1 Primer sequences for PCR amplification of the adenylate cyclase III (*AC3*) gene

Target gene	Orientation	Size (bp)	Sequence (5′→3′)
Rodent *AC3*	S	448	CAT CGA GTG TCT ACG CTT C
	AS		GGA TGA CCT GTG TCT CTT CT
Human *AC3*	S	451	CCG ACA TCG TGG GCT TTA
	AS		CCT GGC TCC ACA TCA AAC
β-actin	S	173	AAA TCG TGC GTG ACA TCA AA
	AS		AAG GAA GGC TGG AAA AGA GC

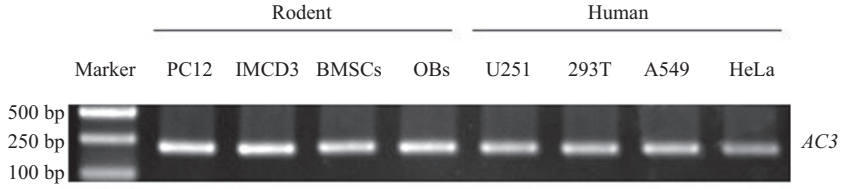
S: sense; AS: antisense; *β-actin* served as an interval reference.

ing with PBS. Ruptured cells were blocked at 37 °C for 1 h in a blocking buffer containing 1% bovine serum albumin (Biotopped, Beijing, China), 5% FBS and 0.05 mol/L of glycine, then double-labeled with the primary antibodies against AC3 (1:1 000, Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat. No.sc-588) and acetylated α-tubulin (1:1 500, Sigma-Aldrich, St. Louis, MO, USA; Cat. No.T6793), and incubated overnight at 4 °C in a wet box. The corresponding fluorescent secondary antibodies were Alexa Fluor488 goat-anti-mouse IgG (H+L, 1:1 000, Invitrogen, San Diego, CA; Cat. No.A11029) and Alexa Fluor594 (1:1 000, Invitrogen, San Diego, CA; Cat. No.A11037), respectively. The nucleus was stained with 4',6-diamidino-2-phenylindole (Roche, Basel, Switzerland Plum; Cat. No.236276). Specimens were mounted using Fluor-Gel With Tris Buffer (Electron Microscopy Sciences, Hatfield, PA, USA)[26] and then examined under a laser scanning confocal microscope (Olympus IX81, Tokyo, Japan). Images were acquired using Olympus Fluoview1000.

2 Results

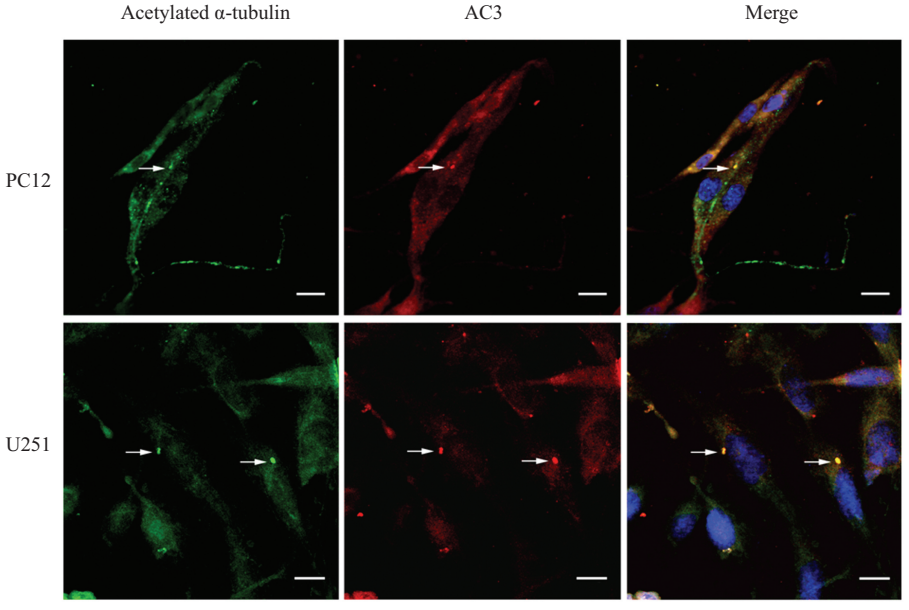
2.1 *AC3* gene expression in various types of cells

This study was conducted in a variety of cell lines, including PC12, U251, IMCD3, 293T, BMSCs, OBs, A549 and HeLa. Rodent and human *AC3*-specific primers were used to amplify the target gene from rodent-derived (PC12, IMCD3, BMSCs and OBs) and human-derived (293T, U251, A549 and HeLa) cells, respectively, via RT-



After 24 h of starvation with 0.5% fetal bovine serum, total RNA was extracted from different cells and used for reverse transcription and *AC3*-specific PCR amplification. From left to right: *AC3* expression in rodent-derived PC12, IMCD3, BMSCs and OBs, and human-derived U251, 293T, A549 and HeLa cells.

Fig.1 Adenylate cyclase III (*AC3*) expression in different types of human and rodent cells



Upper panel: representative images of immunoreactive cilia in PC12 cells labeled with antibodies to acetylated α-tubulin (green) and AC3 (red), and co-localization of acetylated α-tubulin and AC3 (merge); Lower panel: representative images of immunoreactive cilia in U251 cells labeled with antibodies to acetylated α-tubulin (green) and AC3 (red), and colocalization of acetylated α-tubulin and AC3 (merge). Scale bars=20 μm. Nuclei were stained with DAPI (blue). Representative cilia were highlighted by arrows.

Fig.2 Adenylate cyclase III (AC3) expressed in the cilia of neuronal cells

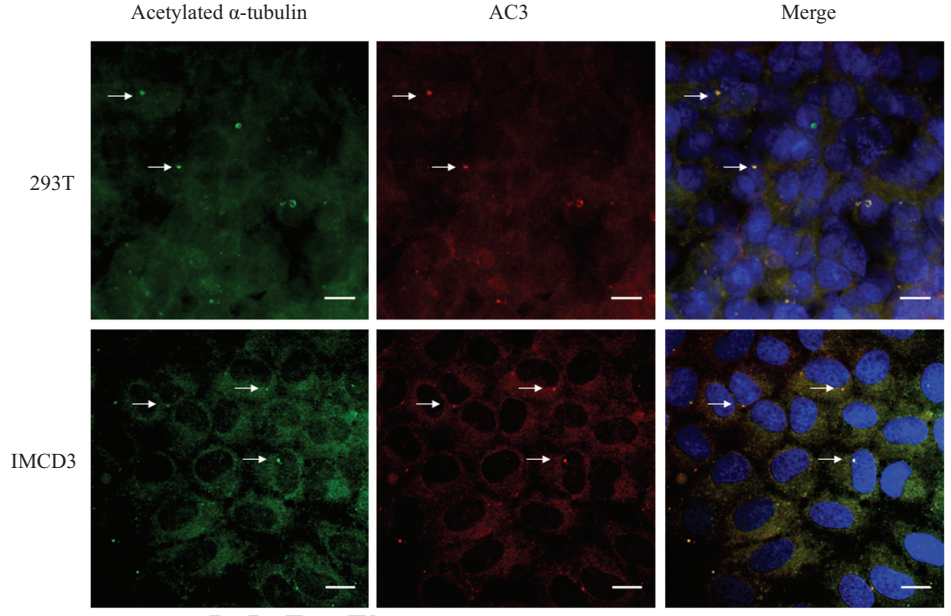
PCR. All the RT-PCR experiments yielded positive results (Fig.1). After DNA sequencing, the obtained sequences were compared with known human and rodent cDNA sequences. The PCR products of all test cells fully matched the corresponding cDNA sequences of human and rodent (data not shown). These results were indicative of endogenous expression of *AC3* in these non-neuronal cells derived from human and rodent.

2.2 Localization of AC3 in the cilia of neuronal cells

Acetylated α-tubulin is a common marker of cellular cilia and its immunofluorescent staining can be used as a sign of the presence of cilia. In this study, immunofluorescence was used to detect whether AC3 was located in the cilia of PC12 or U251 cells. After staining, AC3-positive labeling was found in both PC12 and U251 cells, which overlapped with acetylated α-tubulin staining (Fig.2). These observations indicated that AC3 and acetylated α-tubulin were both expressed in the ciliary regions of PC12 and U251 cells, which thereby once again confirmed that AC3 could be used as a marker for the cilia of neuronal cells[27-28].

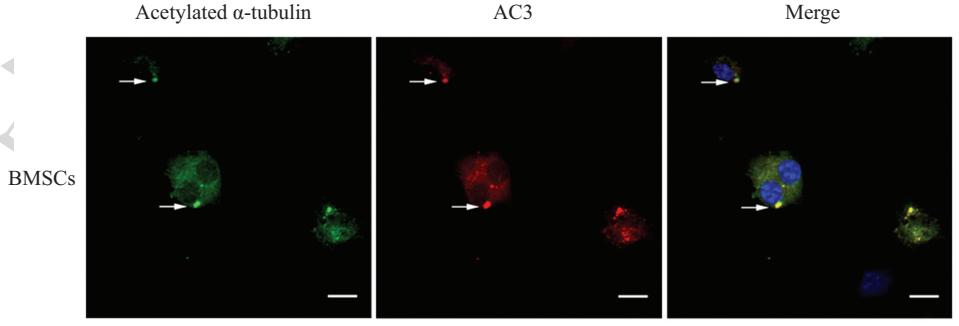
2.3 Localization of AC3 in the cilia of non-neuronal cells

Subcellular localization patterns of AC3 in non-neuronal cells including IMCD3, 293T, OBs and BMSCs were further investigated. Immunofluorescence



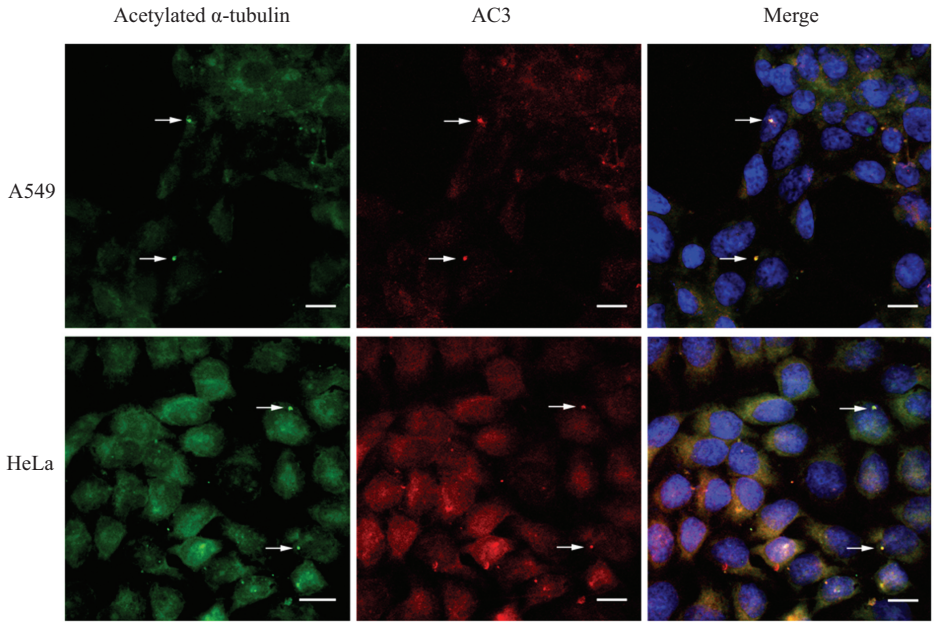
Upper panel: representative images of immunoreactive cilia in 293T cells labeled with antibodies to acetylated α-tubulin (green) and AC3 (red), and co-localization of acetylated α-tubulin and AC3 (merge); Lower panel: representative images of immunoreactive cilia in IMCD3 cells labeled with antibodies to acetylated α-tubulin (green) and AC3 (red), and colocalization of acetylated α-tubulin and AC3 (merge). Scale bars=20 μm. Nuclei were stained with DAPI (blue). Representative cilia were highlighted by arrows.

Fig.3 Adenylate cyclase III (AC3) expressed in the cilia of kidney cells



Representative images of immunoreactive cilia in mouse BMSCs labeled with antibodies to acetylated α-tubulin (green) and AC3 (red), and colocalization of acetylated α-tubulin and AC3 (merge). Scale bars=20 μm. Nuclei were stained with DAPI (blue). Representative cilia were highlighted by arrows.

Fig.4 Adenylate cyclase III (AC3) expressed in the cilia of mouse bone marrow stromal cells (BMSCs)



Upper panel: representative images of immunoreactive cilia in A549 cells labeled with antibodies to acetylated α-tubulin (green) and AC3 (red), and co-localization of acetylated α-tubulin and AC3 (merge); Lower panel: representative images of immunoreactive cilia in HeLa cells labeled with antibodies to acetylated α-tubulin (green) and AC3 (red), and colocalization of acetylated α-tubulin and AC3 (merge). Scale bars=20 μm. Nuclei were stained with DAPI (blue). Representative cilia were highlighted by arrows.

Fig.5 Adenylate cyclase III (AC3) expressed in the cilia of cancer cells

staining showed that mouse IMCD3 cells had a ciliated substructure (Fig.3) and AC3 was localized to the ciliary region of these cells. Similarly, 293T cells showed AC3-positive staining. The costaining of AC3 and acetylated α-tubulin, the cilium marker, further desmonstrated that AC3 was localized to the cilia of these cells. Thus, AC3 was expressed in the ciliary region of both 293T and IMCD3 renal cells.

To clarify the localization of AC3 in the cilia of more non-neuronal cells, this study also examined the presence of AC3 in the cilia of BMSCs. BMSCs, as the progenitor cells, can be induced to differentiate into OBs[29]. After 24 h of FBS starvation, BMSCs were subjected to double staining with anti-AC3 and anti-acetylated α-tubulin antibodies (Fig.4). In these experiments, AC3 was expressed in the majority of the cilia in BMSCs, suggesting the localization of AC3 in the cilia of these cells. Despite AC3-positive immunofluorescence staining was observed in OBs, there was no clear acetylated α-tubulin positive immunofluorescence staining in OBs (data not shown). Thus, the subcellular localization of AC3 in OBs was needed to be determined further.

2.4 Localization of AC3 in the cilia of cancer cells

Cilia-based signaling transduction pathways are closely related to cancer cells, and cilia have a crucial role in tumor cell invasion and metastasis[30]. Upregulation of AC3 in gastric cancer cells increased the potential for the occurrence of gastric cancer[31]. To determine whether AC3 is also expressed in the cilia of cancer cells, immunofluorescence staining was performed on HeLa and A549 cells. Double staining with anti-AC3 and anti-acetylated α-tubulin antibodies showed that AC3 completely overlapped with the cilia marker acetylated α-tubulin in A549 cells. This indicated that AC3 was localized to the cilia of A549 cells (Fig.5). Similarly, AC3 distribution was observed in HeLa cells, i.e., in the ciliary region. Thus, it could be seen that AC3 was localized to the cilia of both A549 and HeLa cancer cells.

3 Discussion

In neuronal cells of the brain, it has been shown that AC3 is localized to the cellular cilia[5,27-28]. In the present study, acetylated α-tubulin—a universal marker of cilia, and AC3 were found to co-localize in two types of neuronal cells (U251 and PC12, Fig.2). These

observations were consistent with the results of previous studies, which confirmed that AC3 is a specific marker for the cilia of neuronal cells. On this basis, we further investigated the expression and distribution of AC3 in non-neuronal cells (IMCD3, 293T, BMSCs and OBs) as well as in cancer cells (A549 and HeLa). Results were indicative of endogenous expression of AC3 in all of these non-neuronal cells. AC3 was localized to the cilia of most of the non-neuronal cells tested (IMCD3, 293T, BMSCs, A549 and HeLa). Thus, the expression of AC3 in the cellular cilia in the majority of non-neuronal cells mirrors it's expression in neuronal cells.

As in the olfactory signaling pathway, AC3 and G_{olf} may play important roles in renal tissues, where they are expressed. Supporting this notion is that *AC3* knockout mice are unable to properly regulate GFR and renin levels[22]. However, the exact cellular location of AC3 in renal cells is yet to be clarified. In the present study, AC3 was found to be expressed and localized to the cilia of both IMCD3 and 293T cells (Fig.3). Studies have shown that polycystic kidney disease (PKD) is a cilia-related syndrome, and that cAMP is an important regulator of cyst generation[32-34]. Although there is no direct evidence for the association between *AC3* deletion and PKD, our study found that AC3 was localized in the cilia of renal cells whose function and growth might be affected by *AC3* deletion.

BMSCs, as progenitor cells, can be induced to differentiate into osteocytes, adipocytes, fibroblasts, and neuronal cells[29]. The cAMP signaling pathway plays an important role in directed differentiation of BMSCs[35-37], and it has been found that cilia have an important role in directed differentiation of BMSCs[38]. In the present study, we provided the first evidence for endogenous expression of AC3 in BMSCs and its localization in the cilia (Fig.4). BMSCs are single-mesoderm pluripotent stem cells derived from bone marrow cells, which have multipotent differentiation potentials. A number of studies have shown that multiple signaling pathways are involved in the differentiation of BMSCs, including the Wnt, Notch, BMP, MAPK and cAMP signaling pathways[35,39-40]. Forskolin stimulates the cAMP signaling pathway, and thus can induce osteogenic differentiation and inhibit adipogenic differentiation of BMSCs[35]. As one of the major components of the cAMP signaling pathway, AC3 may play an important role in the differentiation of BMSCs.

Cilia have a dual regulatory effect on cancer cells and they can act as tumor suppressors to inhibit cancer cell growth or as promoters to promote the growth of cancer cells[41-42]. The occurrence of a variety of cancers such as breast, bile duct and ovarian cancer is closely associated with ciliary abnormality[43-45]. It has been demonstrated for the first time that AC3 is localized to the cilia of A549 and HeLa cells (Fig.5). We inferred from this expression that AC3 and relevant cAMP signaling pathways might have regulatory effects on the growth or function of the cilia in cancer cells.

In short, AC3 serves as a marker for the cilia of neuronal cells and plays an important role in brain tissues[6-7,46-47]. In addition to neuronal cells, AC3 is widely present in other tissues and organs. The present study demonstrates that AC3 is also expressed in the cilia of several types of non-neuronal cells. This finding lays a solid foundation for further study on the function of AC3 in these non-neuronal cells.

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