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BIX-01294-induced autophagy regulates elongation of primary cilia

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ABSTRACT

Previously, we showed that BIX-01294 treatment strongly activates autophagy. Although, the interplay between autophagy and ciliogenesis has been suggested, the role of autophagy in ciliogenesis is controversial and largely unknown. In this study, we investigated the effects of autophagy induced by BIX-01294 on the formation of primary cilia in human retinal pigmented epithelial (RPE) cells. Treatment of RPE cells with BIX-01294 caused strong elongation of the primary cilium and increased the number of ciliated cells, as well as autophagy activation. The elongated cilia in serum starved cultured cells were gradually decreased by re-feeding the cells with normal growth medium. However, the disassembly of cilia was blocked in the BIX-01294-treated cells. In addition, both genetic and chemical inhibition of autophagy suppressed BIX-01294-mediated ciliogenesis in RPE cells. Taken together, these results suggest that autophagy induced by BIX-01294 positively regulates the elongation of primary cilium.

1. Introduction

Autophagy is a lysosome-dependent degradation event for the removal of cytoplasmic proteins, certain pathogens and organelles. Autophagosomes engulf target substrates and deliver them to lysosomes for degradation [1]. The formation of autophagosome is regulated by autophagy-related (ATG) proteins [1]. The phosphorylation of Belcin-1/ATG6 complex by unc-51 like autophagy activating kinase (ULK1) complex, which is regulated by mammalian target of rapamycin (mTOR), promotes autophagosome formation [2,3]. Cleavage of microtubule-associated protein 1A/1B-light chain 3 (LC3) by ATG4 protease is required for the terminal fusion of an autophagosome with its target membrane [4,5]. Since type 3 phosphatidylinositol 3-kinases (PI-3K) regulates mTOR, the PI-3K inhibitor, 3-Methyladenine (3-MA) is wildly used as an inhibitor of autophagy activation [1]. Autophagy basically functions as a protective response of cell under cellular conditions of stress such as starvation, organelle damage, pathogen invasion, and oxidative stress [6–8]. Nonetheless, excessive activity of autophagy can contribute to programmed cell death (autophagy-associated cell death) under certain conditions [9,10].

Recently, we identified BIX-01294 as an inducer of autophagy in multiple cancer cells [11]. BIX-01294 is known to be a selected inhibitor of euchromatin histone lysine N-Methyltransferase-2 (EHMT2), which has a primary role in catalyzing mono- and dimethylation of H3K9 in euchromatin. Generally, the EHMT2-dependent methylation of lysine residues is implicated in epigenetic gene repression and silencing [12]. In addition, EHMT2 was found to be up-regulated in various cancer tissues including breast, prostate, colon, bladder, ovarian, lung and liver [13–15], while knock-down inhibits the proliferation of cancer cells [15]. Previously, we demonstrated that treatment with BIX-01294 strongly induced autophagy and cell death by increasing the cellular production of reactive oxygen species (ROS) in cancer cells. In addition, the inhibition of autophagy suppressed BIX-01294-mediated cancer cell death [11].

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Primary cilia, microtubule-based structures, are the major cellular sensory organelles, of which the structure and length are tissue dependent [16]. Abnormal phenotypes of cilia have often been associated with pathological conditions (ciliopathies), including Meckel syndrome, Bardet Biedl syndrome, and tuberous sclerosis [17,18]. Interflagellar transport (IFT) protein complexes, IFT-A and IFT-B, are essential for the production and maintenance of primary cilia [19,20]. Recent accumulating evidences indicate that various signaling pathways including the sonic hedgehog (Shh) pathway, Wnt pathway, platelet-derived growth factor (PDGF) pathway, and calcium signaling pathway are coordinated via the primary cilia during development, cell cycle, cell differentiation and apoptosis [16,19,21,22]. However, the molecular mechanisms behind the regulation of ciliogenesis remain unclear. Smoothened (Smo) protein transduces Shh signal, which may be activated in the ciliary membrane [23]. Since Smo is localized and accumulated at the primary cilia, it is widely used as a cilium monitoring system.

Although interplay between autophagy and cilliogenesis has been suggested, the role of autophagy on ciliogenesis is controversial [24,25]. Furthermore, the role of epigenetic regulation in ciliogenesis regarding autophagy is largely unknown. In present study, we investigated the effects of autophagy induced by treatment of BIX-01294, on the formation of primary cilium in human retinal pigmented epithelial (RPE) cells. Treatment with BIX-01294 in RPE cells strongly induced ciliogenesis as well as autophagy. In addition, inhibition of autophagy significantly suppressed the formation of primary cilium in the RPE cells treated with BIX-01294.

2. Materials and methods

2.1. Reagents

BIX-01294, 3-methyladenine, bafilomycin A1, and cytochalasin D were purchased from Sigma–Aldrich (St. Louis, MO). Ciliobrevin A1 was purchased from TOCRIS (St. Louis, MO). The expression plasmid pEGFP-Smo was provided by Dr. Joon Kim (KAIST, Korea). The previously validated small interfering RNA (siRNA) for human autophagy related gene 6 (ATG6)/Beclin-1 siRNA (5′-CAGUUUGG-CACAAUCAAUA) [26] and scrambled siRNA (5′-CCUACGCCAC-CAAUUUCGU-3′) were synthesized from Genolution (Seoul, Korea).

2.2. Cell culture and stable cell line

Both human telomerase-immortalized retinal pigmented epithelial (RPE) cells and RPE/Smo-GFP cells stably expressed Smo-GFP proteins were provided by Dr. Joon Kim (KAIST, Korea) [27]. The RPE cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). To generate a GFP-LC3 stable cell line (RPE/GFP-LC3), RPE cells transfected with pGFP-LC3 were selected by G418 resistance following seven days.

2.3. Determination of autophagic cells and confocal microscopy

The cells were treated with BIX-01294 (1 μM), and the number of autophagic cells were determined by counting the number of cells with GFP-LC3 punctuate structures under a fluorescence microscope. For confocal microscopy, htRPE/smo-GFP cells were plated on glass-bottom dishes. RPE cells pre-treated with 3-MA (5 mM) and ciliobrevin A1 (10 μM) were further incubated with BIX-01294 (1 μM) and cytochalasin D (50 nM). After 24 h, the cells were fixed and immune-stained with poly-glutamylated tubulin antibody (Adipogen, San Diego, CA). The fluorescence images were captured using a confocal laser scanning microscope.

2.4. LDH cytotoxicity assay

Cell death was measured by LDH (Lactate dehydrogenase) assay (Takara). LDH cytotoxicity detection kit was determined by measuring LDH activity released into the culture medium from damaged Cells. LDH activity in culture media was assayed by the addition of reaction buffer containing diaphorase/NAD+ and dye solution, and was monitored at 490–492 nm in fluorescence micro plate reader (PerkinElmer).

2.5. Measurement of increased cilium number and cilium length

Changes in the cilium numbers were measured by counting the cilia with RPE/Smo-GFP cells under a fluorescence microscope (IX71, Olympus, Japan). The cilium length was determined with the 'cellSense' Standard software (Olympus). The average cilium length was calculated using the Free-hand Line Selection Tool. The length of an individual cilium was examined from randomly selected cells. And the images were captured and digitized using the cell-Sense Standard software ($\geq$15 cells per experiments, n = 3).

2.6. Western blot analysis

Whole cell lysates were prepared with protein sample buffer (62.5 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% Bromophenol blue) (BioRad, Hercules, CA). After separation in 10–15 % SDS-PAGE, proteins were transferred onto polyvinylidene fluoride membrane and (Bio-Rad, Hercules, CA). After blocking with skim milk, the membranes were treated with primary antibodies. Anti-ATG6 antibody was purchased from Abcam (Cambridge, UK); anti-LC3 antibody was purchased from NOVUS Biologicals (Littleton, CO); anti-IFT20, anti-IFT88 and anti OFD1 antibodies were obtained from Proteintech; anti-Actin antibody was purchased from Millipore (Temecula, CA). For protein detection, the membranes were incubated with HRP-conjugated secondary antibodies and signals were detected with Super-signal West Dura HRP detection kit (Pierce, Rockford, IL).

2.7. DCFH-DA assay

Intracellular ROS levels were assayed using the fluorescent dye, 2,7-dichlorofluorescein diacetate (DCFH-DA) (Invitrogen), which is converted to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of oxidant. Briefly, cells plated in 96-well plate were treated with BIX-01294. After 24 h, the cells were washed out and incubated with DCFH-DA (0.4 nM) in serum free medium for 30 min, ROS production was analyzed with a fluorescence micro plate reader (wave length 358/485) (PerkinElmer). Relative ROS ratio was presented as the change in fluorescence of drug treated samples compared with that of control samples.

2.8. Statistical analysis

Data were analyzed from least three independent experiments, and presented as means ± S.E.M. Statistical evaluation of the results was performed with one-way ANOVA ($^*p < 0.05$).

3. Results and discussion

3.1. BIX-01294 increases autophagy activation in RPE cells

Previously, we showed that treatment of BIX-01294 induces the activation of autophagy in various cancer cells including MCF-7, a breast cancer cell line and HCT116, a colon cancer cell line [11]. To confirm the autophagy-inducing activity of BIX-01294 in human

retinal pigment epithelial (RPE) cells, a stable RPE cell line (RPE/GFP-LC3) was generated with GFP-fused LC3, which is widely used as an autophagy monitoring system. In accordance with the previous results, BIX-01294 treatment strongly induced the formation of punctate dot structures by GFP-LC3, and a dose-dependent increase in the number of cells with autophagic punctate structure was observed (Fig. 1A and B). Treatment of BIX-01294 strongly induced cell death in various cancer cells (Fig. 1C) [11]. However, BIX-01294 did not induced cell death in low concentration (~1 μM) but slightly increased cell death at high concentration (~10 μM) in RPE cells (Fig. 1C). The conversion of LC3I to LC3II is considered to be another marker of activation of autophagy. Consistent with this notion, treatment of RPE cells with BIX-01294 highly enhanced the conversion of LC3 protein which was inhibited by treatment with the autophagy inhibitor, 3 MA (Fig. 1D). We previously showed that the expression of ATG6/Beclin1 was increased in BIX01294-treated MCF-7 cells during autophagy induction [11]. Thus, we next examined that the effects of BIX-01294-induced autophagy on ATG6 in RPE cells. Unlike in the MCF-7 cells (10 μM), treatment with a low dosage of BIX-01294 (1 μM) did not cause efficient enhancement of the expression of ATG6 in RPE cells (Fig. 1E). However, down-regulation of ATG6 by RNAi considerably decreased the conversion of LC3I to LC3II in the BIX-01294-treated RPE cells (Fig. 1E). In addition, combined treatment with a lysosome inhibitor, bafilomycin A1, confirmed the activation of autophagic flux by BIX-01294. Co-treatment with BIX-01294 and bafilomycin A1 resulted in higher accumulation of LC3II protein than that of BIX-01294 alone (Fig. 1F), suggesting that BIX-01294 induces the activation of autophagy in RPE cells.

3.2. BIX-01294 increases elongation of primary cilia in RPE cells

Both autophagy and primary cilia formation are enhanced under condition of serum deprivation. Additionally, a connection between autophagy and ciliogenesis has been reported [24,25]. Since we demonstrated that BIX-01294 is a strong autophagy inducer, we further evaluated the effects of BIX-01294 on ciliogenesis. Smo is widely used as a cilium marker, hence Smo-GFP stably expressing RPE cell line (RPE/Smo-GFP) was employed for analysis. Treatment of BIX-01294 dramatically increased both the formation and elongation of primary cilium in RPE cells (Fig. 2A–C). Cytochalasin D (Cyto. D) acts as a positive inducer of cilium formation by inhibiting actin polymerization [27]. Ciliary tubulin is extensively modified by post-translational modifications (PTM) [28]. The poly-glutamylation of tubulin is the predominant PTM taken in the ciliary axoneme [29]. In accordance with this notion, treatment of BIX-01294 also induced high levels of poly-glutamylated tubulin in RPE cells (Fig. 2D). IFT20 functions in the delivery of ciliary membrane proteins, and suppression of IFT20 blocks ciliary assembly [30]. Treatment of RPE cell with BIX-01294 also increased the expression of IFT20 (Fig. 2E). Moreover, treatment of ciliobrevin A1 (Cilio A1), an inhibitor of ciliogenesis significantly reduced the increase of primary cilium formation in the cells treated with BIX-01294 (Fig. 2F). Taken together, these results suggest that BIX-01294 increases the elongation of primary cilia in RPE cells.

3.3. BIX-01294 suppresses cilia disassembly in RPE cells

Cilia are microtubule-based dynamic structures. The growth and shrinkage of primary cilia are dynamically regulated by microtubules. Not only increase of assembly but also decrease of disassembly of cilia can influence the elongation of primary cilia. Therefore, we further assessed the effects of BIX-01294 on cilia disassembly. Serum deprivation leads to elongation of primary cilium, whereas stimulation of cells with serum initiates the disassembly of primary cilia [31]. Consistently, increase in ciliated cells was observed by culturing the RPE/Smo-GFP cells without serum for 48 h, while gradual disassembly was observed when re-

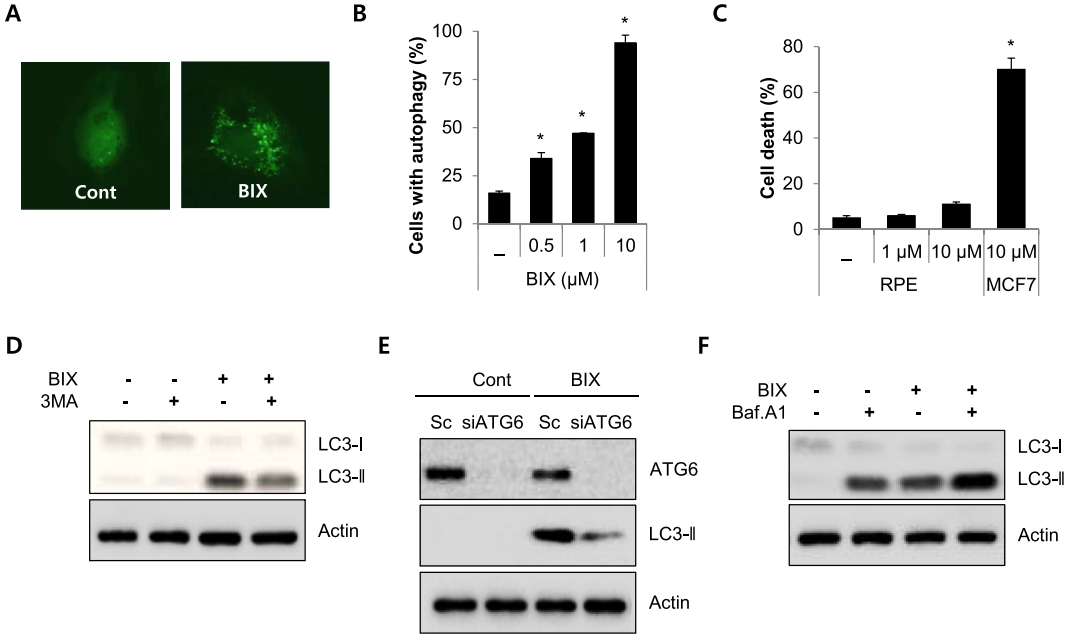

Fig. 1. BIX-01294 activates autophagy in RPE cells. (A) RPE/GFP-LC3 cells were treated with BIX-01294 (BIX, 1 μM) and imaged by confocal microscopy. (B) RPE/GFP-LC3 cells treated with BIX (0.5, 1 μM) for 24 h were fixed and cells with autophagic punctuate structures were counted (B). (C) RPE and MCF-7 cells were treated with BIX (1, 10 μM) for 24 h and cell death was measured by LDH assay. (D) RPE cells were treated with BIX (1 μM) or without 3 MA, then the conversion of LC3 protein was detected by Western blotting. (E) RPE cells were transfected with scrambled siRNA (Sc) or a specific siRNA against *ATG6* (siATG6). After 3 days, the cells were incubated with or without BIX (1 μM) for additional 24 h. The protein expression levels of LC3 and ATG6 were examined by Western blot analysis. (F) RPE cells were treated with BIX (1 μM) in the presence or absence of with bafilomycin A1 (Baf). Then, the conversion of LC3 protein was detected by Western blotting. Data were obtained from at least three independent experiments and values are presented as the means ± S.E.M. ($n > 3$, $^{*}p < 0.02$).

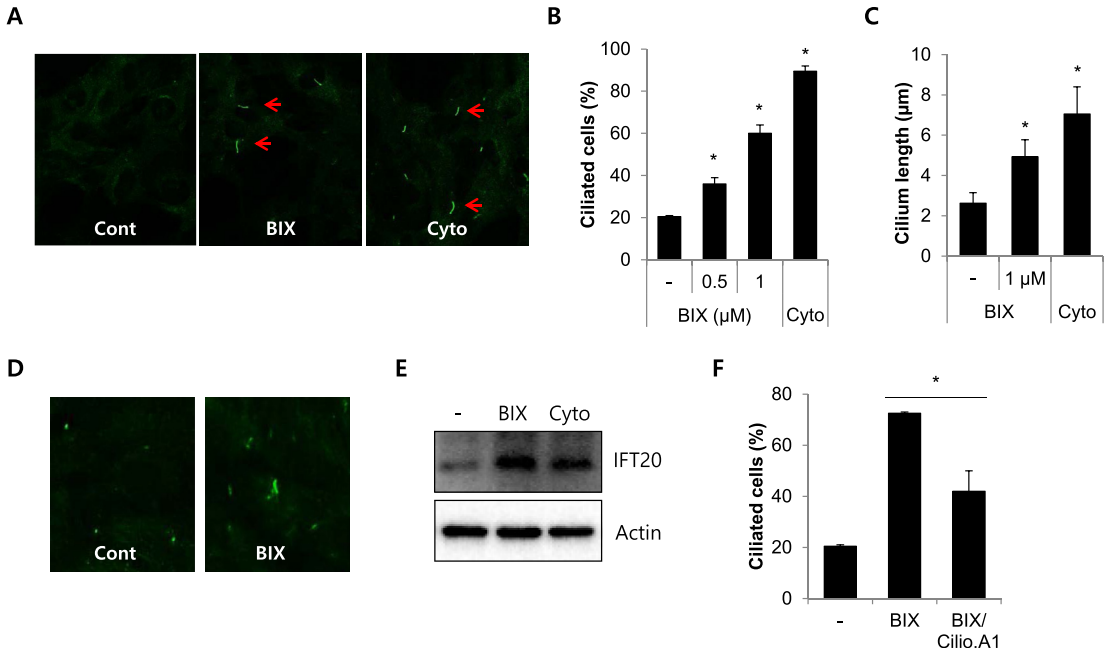

Fig. 2. BIX-01294 induces formation of primary cilium in RPE cells. (A) RPE/Smo-GFP cells were treated with BIX-01294 (BIX, 1 μM) or cytochalasin D (Cyto, 50 nM) for 24 h. The cells were imaged by confocal microscopy. Red arrows indicate the primary cilia. (B and C) RPE/Smo-GFP cells were treated with BIX (0.5, 1 μM) or Cyto (50 nM). The cilated cells and cilium length of the cells were measured under a fluorescence microscope. (D and E) RPE cells treated with BIX-01294 (BIX, 1 μM) or cytochalasin D (Cyto, 50 nM) for 24 h were analyzed by immune-staining with poly-glutamylated tubulin antibody (D) and by Western blotting with IFT20 antibody (E). (F) RPE/Smo-GFP cells pre-treated with ciliobrevine A1 (Cilio. A) (10 μM) for 1 h were further incubated with BIX (1 μM) for 24 h. Then the cilated cells were counted. Data were obtained from at least three independent experiments and values are presented as the means ± S.E.M. (n = 3, $^{*}p < 0.02$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

feeding the cells with normal growth medium over a period of 24 h (Fig. 3A). However, the disassembly of cilia was almost completely blocked in the BIX-01294-treated cells (Fig. 3B). These results suggest that BIX-01294 induces elongation of primary cilium by both increasing assembly and reducing disassembly of the primary cilium in RPE cells.

3.4. Inhibition of autophagy suppresses BIX-01294-mediated elongation of cilia

Our results indicated that BIX-01294 induces autophagy as well as cilium formation. Thus, the effect of BIX-01294-mediated autophagy on ciliogenesis was further evaluated using in htRPE cells. Interestingly, the down-regulation of ATG6/Beclin-1 significantly inhibited the BIX-01294-meditad increase of primary cilium formation in RPE cells (Fig. 4A). Accordingly treatment of the autophagy inhibitor, 3-MA, also suppressed the BIX-01294-mediated formation of primary cilia (Fig. 4B). As a regulatory mechanism, Tang et al., recently suggested that autophagy degrades oral-facial-digital syndrome 1 (OFD1), a ciliopathy protein and promotes the biogenesis of primary cilium [24]. Knock-down of OFD1 was suggested to promote ciliogenesis in breast cancer MCF-7 cells. Therefore, we further investigated the expression of OFD1 in BIX-01294-treated cells. As shown in Fig. 4C, treatment of BIX-

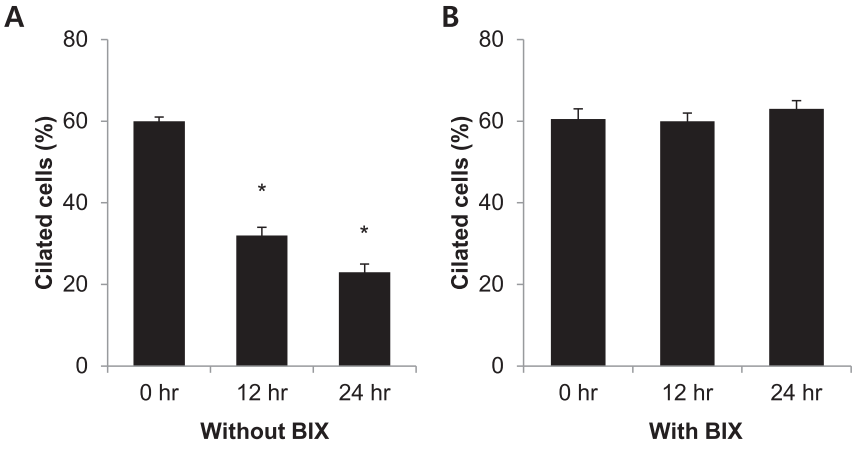

Fig. 3. BIX-01294 blocks the disassembly of primary cilia in RPE cells. (A and B) RPE/Smo-GFP cells were plated in a medium without serum for 48 h. Then, the cells were further incubated with normal growth medium in the absence (A) or presence of BIX-12094 (BIX, 1 μM) (B). The percentage of cells with primary cilia was measured after 12 and 24 h. Data represent ± standard error of the mean (S.E.M.) from three independent experiments (n = 3,$^{*}p < 0.02$).

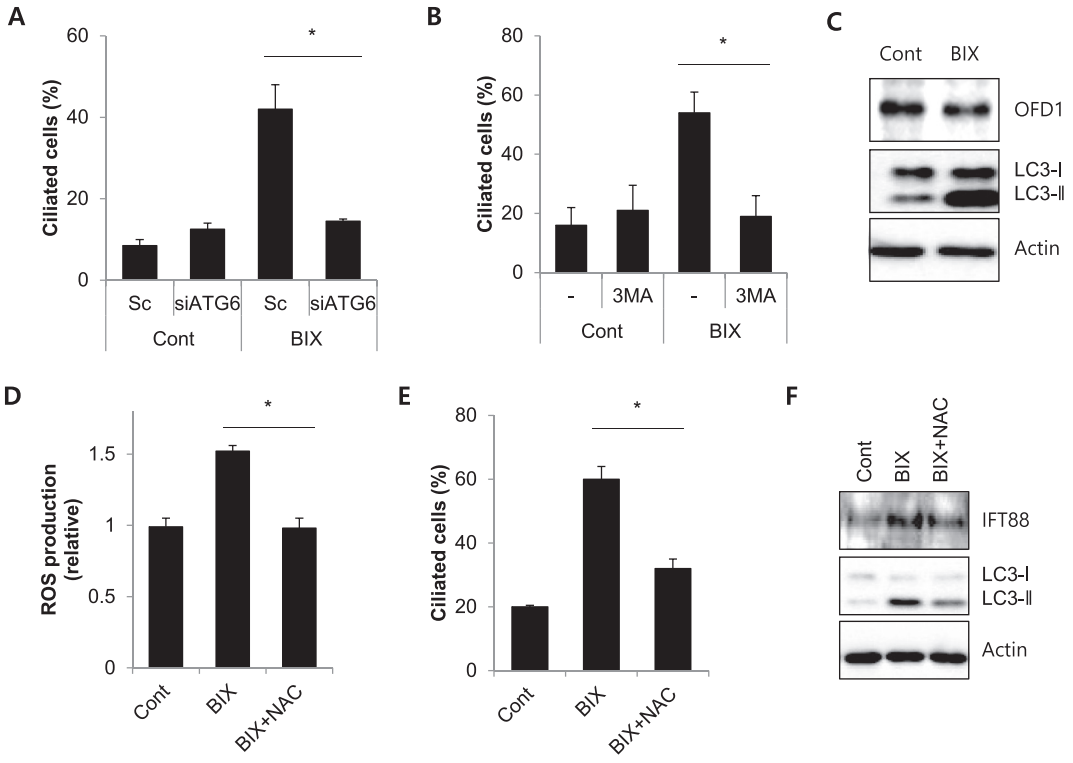

Fig. 4. Inhibition of autophagy suppresses elongation of primary cilium formation in BIX-01294-treated cells. (A) RPE/Smo-GFP cells, which were transfected with scrambled siRNA (Sc) or siRNA against ATG6 (siATG6) were further treated with BIX-01294 (BIX, 1 μM) for 24 h. Ciliated cells were counted under a fluorescence microscopy. (B) RPE/Smo-GFP cells pre-treated with 3-MA (5 mM) for 12 h were additionally incubated with BIX-01294 (1 μM) for 24 h. The ciliated cells were counted under a fluorescence microscope. (C) RPE cells were treated with BIX-01294 (1 μM) for 24 h and OFD1 expression was analyzed by Western blotting. (D) RPE cells were treated with BIX-01294 (1 μM) in the presence or absence of NAC (1 mM) for 24 h, and ROS production was measured by DCF-DA ROS detection assay. (E, F) RPE/Smo-GFP cells were treated with BIX-01294 (1 μM) in the presence or absence of NAC (1 mM) for 24 h, then ciliated cells (E) and IFT88 expression (F) were analyzed. Data were obtained from at least three independent experiments and values are presented as the means ± S.E.M. (*$p < 0.05$).

01294 slightly decreased ODF1 expression in RPE cells, suggesting that autophagy induced by BIX-01294 positively regulates the elongation of cilia in RPE cells. Recently, our group showed that BIX-01294 induces autophagy-associated cell death in cancer cells by causing the over-production of reactive oxygen species (ROS) [11]. Inhibition of ROS blocked BIX-01294-mediated autophagy and autophagy-associated cell death, suggesting that ROS is a key mediator in autophagy activation. Thus, we addressed the effect of ROS on ciliogenesis in BIX-01294-treated RPE cells. Consistently, treatment of BIX significantly increased ROS production which was blocked by NAC treatment, a ROS scavenger in RPE cells (Fig. 4D). We also found that inhibition of ROS by NAC treatment also efficiently suppressed the formation of primary cilium in BIX-01294-treated cells (Fig. 4E and F), suggesting that ROS is involved in the elongation of cilia by BIX-01294 in RPE cells.

The assembly and disassembly of primary cilia is a very dynamic process, which involves various cellular signaling pathways [21,16]. Despite its importance, the relationship between autophagy and ciliogenesis remains largely unknown. Recently, Huang et al. showed that resveratrol (RSV) enhances the Shh signaling pathway in the primary cilia to cause differentiation of mesenchymal stem cells (MSCs) into neuronal like cells [32]. Interestingly we reported that treatment of RSV strongly increases activation of autophagy in melanocytes [33], while controversial reports about the relationship between autophagy and ciliogenesis have also been suggested [24,25]. Both autophagy and assembly of primary cilium are induced by condition of serum deprivation. Regarding this, Tang et al. demonstrated that autophagy triggers the biogenesis of primary cilium [24]. On the other hand, Pampliega et al. suggested that autophagy suppresses ciliogenesis by limiting ciliary trafficking of the components required for formation of primary cilium [25]. Therefore, these apparently contradictory results require further investigation into the role of autophagy in ciliogenesis. In the present study, we addressed the relationship between autophagy and ciliogenesis in BIX-01294-treated cells. Treatment of RPE cells with BIX-01294 strongly increased the activation of autophagy, as well as the formation of primary cilium. Moreover, both genetic and chemical inhibition of autophagy significantly reduced the formation of primary cilium in the BIX-01294-treated cells. Our results further support that autophagy positively regulates the formation of primary cilium.

ROS is associated with alteration of the primary cilium length in tubular epithelial cells. Inhibition of ROS, which is enhanced in ischemic injury, reduces the elongation of cilia by oxidative stress [34]. According to this notion, we also found that treatment of a ROS scavenger, NAC efficiently suppressed the elongation of primary cilium by treatment of BIX-01294 (Fig. 4D–F). MAPK proteins such as ERK, JNK, and p38 are responsible for ROS production. Consistent with these notions, an ERK inhibitor was reported to block both the activation of autophagy and cilia elongation during oxidative stress [35,36]. These evidences also suggest a linkage of autophagy and ciliogenesis. Therefore, exploration of the ROS-ERK pathway may help to extend our understanding of autophagy in ciliogenesis.

BIX-01294 is a known inhibitor of EHMT2, which is involved in various cellular processes, including proliferation, development, differentiation, and cell death [12,37]. Although EHMT2 plays an essential role in histone modification, the epigenetic regulation of ciliogenesis has largely not been elucidated. BIX-01294 treatment induces epigenetical modulation and regulates multiple events. It is known that BIX-01294 enhances the cardiac potential of bone

marrow cells through the up-regulation of cardiac-associated genes [36], and increases the endothelial differentiation of adipose-derived MSCs [38]. We found that the expression of regulatory genes of autophagy, including ATG6/Belin-1, was increased by BIX-01294 treatment [11]. In addition, other autophagy regulating proteins such as WIPI1 could be induced by BIX-01294 treatment [39]. The expression levels of cilia proteins, such as MKKS, BBS-2, -4, -5, -7, and -9, were not significantly altered by conditional knockdown of DNA methyltransferase in the mouse retina [40]. Nonetheless, further investigations are necessary to delineate the epigenetic effects on ciliogenesis.

Conflict of interest

There is no conflict of interest.

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Transparency document

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