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Autophagy Regulates Formation of Primary Cilia in Mefloquine-Treated Cells

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Abstract

Primary cilia have critical roles in coordinating multiple cellular signaling pathways. Dysregulation of primary cilia is implicated in various ciliopathies. To identify specific regulators of autophagy, we screened chemical libraries and identified mefloquine, an anti-malaria medicine, as a potent regulator of primary cilia in human retinal pigmented epithelial (RPE) cells. Not only ciliated cells but also primary cilium length was increased in mefloquine-treated RPE cells. Treatment with mefloquine strongly induced the elongation of primary cilia by blocking disassembly of primary cilium. In addition, we found that autophagy was increased in mefloquine-treated cells by enhancing autophagic flux. Both chemical and genetic inhibition of autophagy suppressed ciliogenesis in mefloquine-treated RPE cells. Taken together, these results suggest that autophagy induced by mefloquine positively regulates the elongation of primary cilia in RPE cells.

Key Words: Mefloquine, Autophagy, Primary cilia, Retinal pigmented epithelial cells

INTRODUCTION

Primary cilia are highly conserved, dynamic, microtubule-based organelles that emanate from the surface of many human cell types. The major role of cilia is to sense extracellular signals such as hormones, growth factors, and nutrients (Singla and Reiter, 2006; Berbari *et al.*, 2009). Ciliary defects have been implicated in many human diseases called ciliopathies, including primary ciliary dyskinesia, hydrocephalus, polycystic liver and kidney disease, and some forms of retinal degeneration (Goetz and Anderson, 2010). Therefore, the targeting the mechanism of regulation of ciliogenesis may represent a new therapeutic strategy for treatment of various ciliopathies.

Cilia are generated through ciliogenesis, which regulates the intraflagellar transport (IFT) mechanism. The IFT not only helps in the transport of building material from the cell body to the growing cilium but also carries disassembled material from the cilium assembly site back to the cell body for recycling (Hao *et al.*, 2011). Primary cilia play an important role in development, cell migration, the cell cycle and apoptosis by signal transduction, including sonic hedgehog (SHH), the Wnt pathway and calcium signaling (Quinlan *et al.*, 2008; Ishikawa and Marshall, 2011). The mammalian SHH signaling pathway has major signaling components localized in the cilium, and IFT proteins are important for trafficking of SHH molecules (Goetz and Anderson, 2010). SHH signaling is inhibited by a lack of HH ligand through the inhibitory effect of Patched 1(Ptc1), on Smoothened (Smo), 7-transmembrane-domain protein (Goetz and Anderson, 2010). In addition, the primary cilium regulates canonical and non-canonical Wnt signaling pathways (Gerdes and Katsanis, 2008). It was recently demonstrated that autophagy promotes ciliogenesis by degrading proteins involved in cilia formation (Tang *et al.*, 2013). Furthermore, both autophagy and ciliogenesis are regulated by the mammalian target of rapamycin (mTOR) signaling (DiBella *et al.*, 2009), suggesting that autophagy is associated with ciliogenesis.

Autophagy is a lysosome-dependent, degradation mechanism for the removal of long-lived cytoplasmic constituents,

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cellular organelles, and protein aggregates (Rubinsztein, 2006; Klionsky, 2007). Under basal autophagy, ciliary growth is prevented through degradation of proteins such as IFT20. In early-starvation autophagy, the endogenous inhibitor of ciliogenesis OFD1 is degraded, and ciliogenesis is promoted through the delivery of IFT20. Ciliary growth, in turn, leads to the recruitment of autophagy-related (ATG) proteins, such as ATG16L. Excessive activation of autophagy, however, leads IFT20 degradation to prevent unlimited growth of the cilia (Tang *et al*., 2013). The critical role of autophagy is to help cells survive under stressful conditions by enhancing cellular homeostasis. In contrast, defective autophagy leads to many diseases such as ciliopathies, neurodegenerative diseases, and cancer (Kiprilov *et al*., 2008; Mizushima *et al*., 2008; Lista *et al*., 2011). However, the precise mechanism of autophagy in ciliogenesis is not fully understood.

We previously demonstrated that mefloquine, a medicine for malaria, induces autophagy and autophagy-associated cell death in SH-SY5Y neuroblastoma cells (Shin *et al*., 2012b). In the present study, we showed that mefloquine induces autophagy and ciliogenesis in human telomerase-immortalized retinal pigmented epithelial (htRPE) cells. We also showed that inhibition of autophagy suppressed the elongation of primary cilia in mefloquine-treated htRPE cells. These findings suggest that regulation of autophagy may provide a new strategy to minimize ciliopathies.

MATERIALS AND METHODS

Cell culture and stable cell line

htRPE cells and htRPE cells stably expressing a Smo-green fluorescence protein (GFP) fusion protein (htRPE/Smo-GFP) were kindly provided by Dr. J. Kim (KAIST, Daejeon, South Korea) (Kim *et al*., 2010). To generate a GFP-LC3 stable cell line (htRPE/GFP-LC3), htRPE cells were transfected with pGFP-LC3 and selected by G418 (1 mg/mL) for 7 days. The stable transfectants were identified by a fluorescence microscopy. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and cultured at 37°C in a 5% CO_2 incubator.

Chemical screening for regulators of autophagy

The htRPE/Smo-GFP cells were seeded in 96-well plates (2,000 cells per well). After 24 hour, each chemical from the Lopac® 1280 compound library (Sigma-Aldrich Co., St. Louis, MO, USA) and the Prestwick Chemical Library® (Prestwick Chemical, Illkirch, France) was separately added to a well at a final concentration of 10 μM. After further incubation for 24 h, the ciliated cells were observed under a fluorescence microscope.

Reagents

Mefloquine, bafilomycin A1, 3-methyladenine, and cytochalasin D were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ciliobrevin A1 was purchased from Tocris (St. Louis, MO, USA). Previously validated siRNA targeting for human ATG5 (5'-GCAACUCUGGAUGGGAUUG-3') and negative scrambled siRNA (5'-CCUACGCCACCAAUUUCGU-3') were synthesized from Genolution (Seoul, Korea) (Shin *et al*., 2012a).

Measurement of autophagy activation and length of primary cilia

Autophagic cells were determined by counting the number of cells with GFP-LC3 punctate structure under a fluorescence microscope (IX71, Olympus, Japan). Ciliated cells were determined by counting the number of cells with elongated Smo-GFP under a fluorescence microscope (IX71, Olympus, Japan). The cilium length was determined with the cellSens® standard digital imaging software (Olympus Imaging Corp., Tokyo, Japan). The average cilium length was calculated using the cellSens® freehand line selection tool. The lengths of cilia from randomly selected cells were examined, and the images were captured and digitized using the cellSens® standard digital imaging software (≥15 cells per experiments, n=3).

Western blot analysis

Cell lysates were prepared with 2×Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) (BioRad, Hercules, CA, USA). Proteins (approximately 50 μg) were quantitated by using the Bradford solution (BioRad) according to the manufacture's instruction. Then the samples were separated by 10-15% SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membrane (BioRad). After blocking with 4% skim milk in TBST (25 mM Tris, 3 mM 140 mM NaCl, 0.05% Tween20), the membranes were incubated over-night with specific primary antibodies at 4°C. Anti-ATG5 (ab54033, 1:2000) was from Abcam (Cambridge, UK); anti-LC3 (NB100-2220, 1:10,000) antibody was from NOVUS Biologicals (Littleton, CO); anti-IFT20 antibody was obtained from Proteintech (13615-01-AP, 1:2,000); anti-Actin (MAB1501, 1:10,000) antibody was from Millipore (Temecula, CA, USA). For protein detection, the membranes were incubated with HRP-conjugated secondary antibodies (Pierce, Rockford, IL, USA).

Statistical analysis

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

RESULTS

Mefloquine regulates formation of primary cilia by suppressing disassembly in htRPE cells

Ciliary defects are associated with various diseases. Recently, several groups have independently identified molecules regulating ciliogenesis as new therapeutic targets. The Smo protein is accumulated on the primary cilium and is widely used as a cilium marker (Kim *et al*., 2010). To identify chemical modulators of ciliogenesis, we developed a cell-based screening system by using htRPE cells that stably express GFP fused Smo (htRPE/Smo-GFP). Using this assay, we screened the LOPAC® 1280 compound library (a collection of 1,280 pharmacologically active compounds) and the Prestwick Chemical Library® (a collection of 1,120 chemicals, 85% of which are marketed drugs). Based on the screening results, we selected mefloquine ($C_{17}H_{16}F_6N_2O$) for further analysis as a potent ciliogenesis regulator (Fig. 1A). Mefloquine is an orally administered medication used in the prevention and treatment of malaria. However, the effect of mefloquine on ciliogenesis has not been examined. Therefore, we studied the effect of this drug

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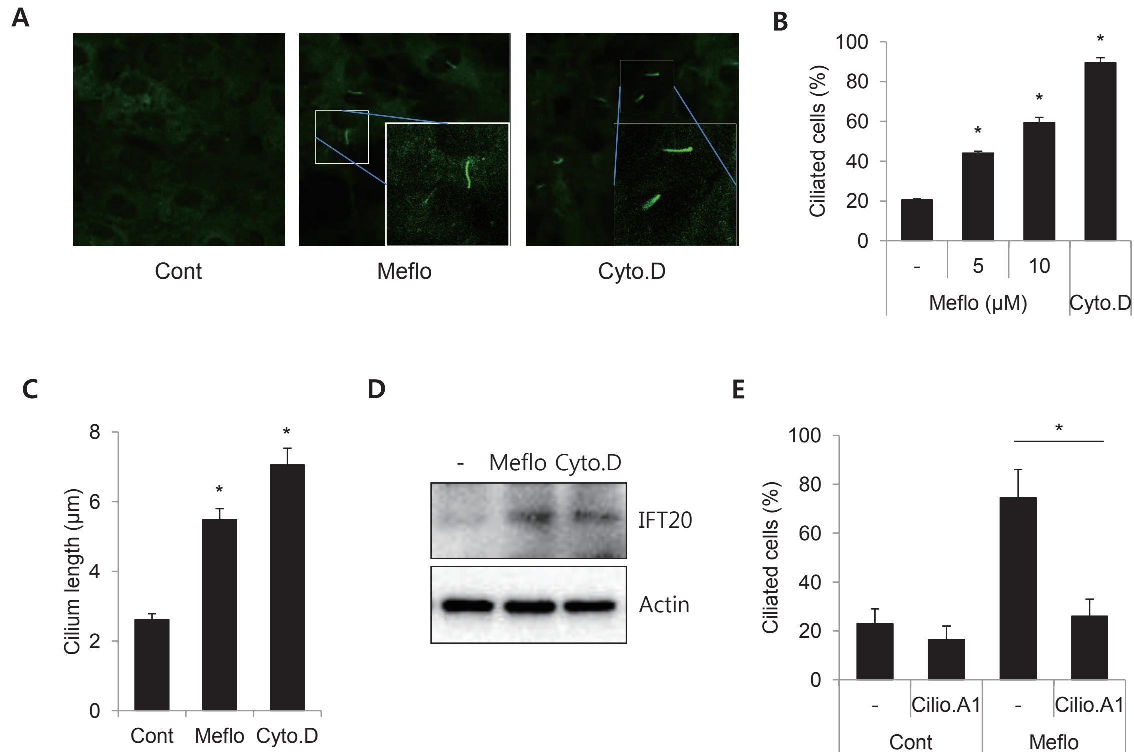

Fig. 1. Mefloquine induces cilia formation in htRPE cells. (A, B) htRPE/Smo-GFP cells were treated with 10 μM mefloquine (Meflo) or 50 nM Cytochalasin D (CytoD) for 24 h. The ciliated cells were imaged with a confocal microscope. (C, D) htRPE/Smo-GFP cells were treated with either Meflo (5 and 10 μM) or CytoD (50 nM). Cilium length and IFT20 expression were analyzed. (E) htRPE/Smo-GFP cells were treated with 10 μM Ciliobrevin A1 (CilioA1) in the presence or absence of Meflo (10 μM). After 24 h, the ciliated cells were observed and counted under a fluorescence microscope. Data were obtained from at least three independent experiments, and values are presented as the means ± S.E.M. (**p*<0.05).

on ciliogenesis. To confirm the screening results, htRPE/Smo-GFP cells were treated with mefloquine, and ciliogenesis was observed. Treatment of htRPE cells with mefloquine strongly increased the formation of primary cilia as much as cytochalasin D (Cyto D), which is an inducer of ciliogenesis (Fig. 1A). In addition, both the number of ciliated cells and the length of the cilia were significantly increased in mefloquine-treated cells (Fig. 1B, C). Similar to Cyto D, treatment of htRPE cells with mefloquine also increased the level of IFT20, which participates in trafficking of ciliary membrane proteins from the Golgi apparatus to the base of the cilium (Follit *et al.*, 2009), indicating that mefloquine induces ciliogenesis in htRPE cells (Fig. 1D). We further examined the cilium elongation activity of mefloquine with an inhibitor of ciliogenesis, Ciliobrevin A1 (Cilio A1), which suppresses ciliogenesis by inhibiting cytoplasmic dynein. Consistently, treatment of mefloquine-treated htRPE cells with CilioA1 significantly reduced the number of ciliated cells induced by mefloquine (Fig. 1E). Taken together, these results suggested that mefloquine induces the formation of primary cilia in htRPE cells.

Not only increased assembly but also decreased disassembly of cilia results in elongation of primary cilium. To determine the effect of mefloquine on cilial dynamics, we employed serum-deprivation conditions, which promote elongation of cilia (Tang *et al.*, 2013). According to previous reports, cilium elongation was increased in serum-starved cells (Fig. 2A). After 48 h of incubation under such serum-deprived conditions, the cells were further incubated in a normal growth medium in the presence or absence of mefloquine. Notably, re-feeding the cells with serum gradually disassembled the cilia over a period of 24 h, while the disassembly of the cilia was almost completely prevented in the mefloquine-treated cells (Fig. 2A, B). These results suggested that mefloquine regulates the ciliogenesis by suppressing the disassembly of the primary cilia in htRPE cells.

Mefloquine induces autophagy in htRPE cells

We previously demonstrated that mefloquine induces autophagy and autophagy-associated cell death in a neuroblastoma cells. To examine whether mefloquine induces autophagy in htRPE cells, we generated the cell line htRPE/GFP-LC3, which stably expresses LC3, a molecular marker for activation of autophagy as a fusion protein with GFP. In accordance with previous results, treatment of mefloquine in htRPE cells significantly increased the punctate structure of GFP-LC3 in a dose- and time-dependent manner (Fig. 3A, B). However, Cyto D did not increase autophagy suggesting that induction of ciliogenesis does not directly activate autophagy in htRPE cells (Fig. 3B). In addition, autophagy related gene such as Beclin-1/ATG6 was not notably increased by in htRPE cells treated with mefloquine (Fig. 3C). We further examined autophagy flux by mefloquine treatment in htRPE cells. Com-

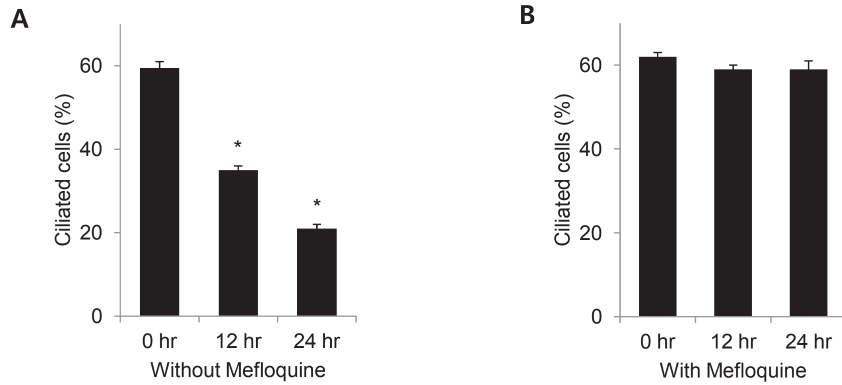

Fig. 2. Mefloquine inhibits primary cilia disassembly in htRPE cells. (A, B) htRPE/Smo-GFP cells cultured in a serum-deprived medium for 48 h were further incubated with normal growth medium in the absence (A) or presence (B) of Meflo (10 μM) for the indicated times; thereafter, ciliated cells were counted. Data were obtained from at least three independent experiments, and values are presented as the means ± S.E.M. (*p<0.05).

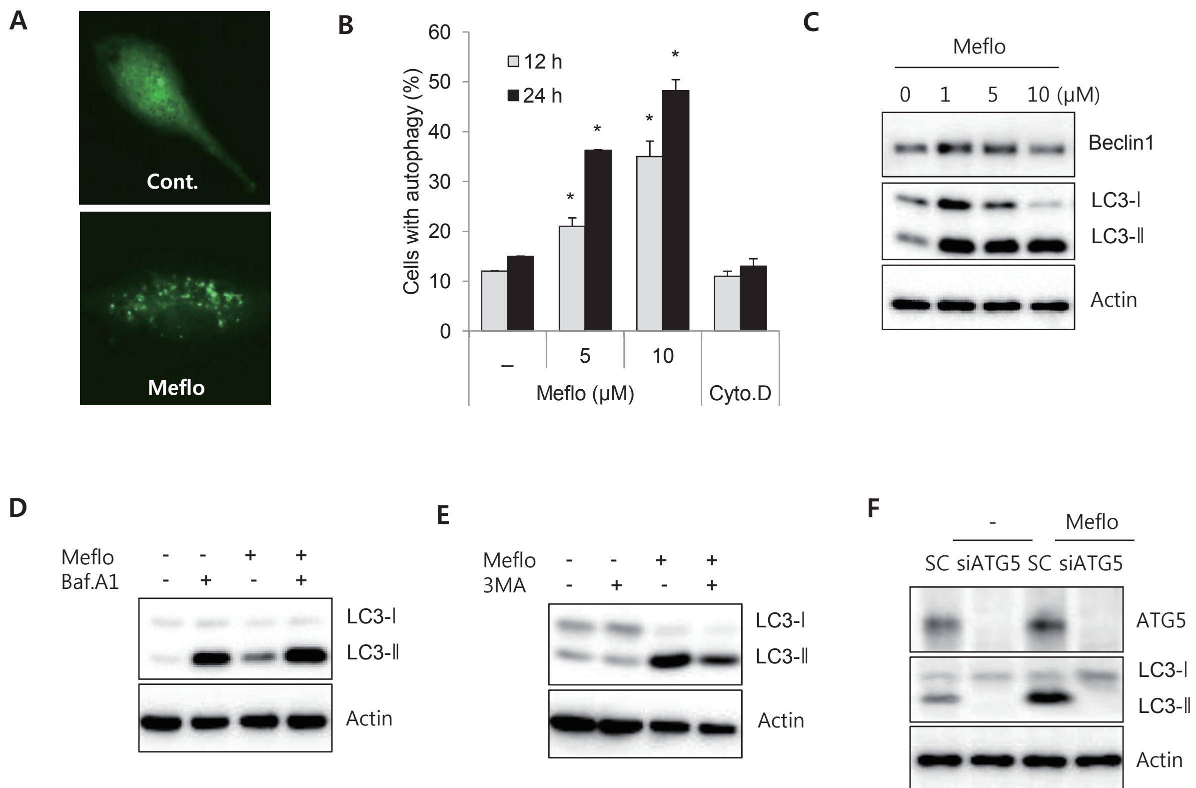

Fig. 3. Mefloquine induces autophagy in htRPE cells. (A) htRPE/GFP-LC3 cells were treated with Meflo (10 μM) and imaged by confocal microscopy. (B) htRPE/GFP-LC3 cells treated with Meflo (5, 10 μM) or Cyto D (50 nM) does and time dependent manner were fixed, and cells with autophagic punctate structures were counted under a fluorescence microscopy. (C) htRPE cells were treated with Meflo with different concentration and protein expression was detected by Western blotting with indicated antibodies. (D) htRPE cells were treated with Meflo (10 μM) in the presence or absence of bafilomycin A1 (Baf). The conversion of LC3 protein was detected by Western blotting. (E) htRPE cells treated with 3-methyladenine (3MA) were further incubated with Meflo (10 μM); the conversion of LC3 protein was detected by western blotting. (F) htRPE cells were transfected with scrambled siRNA (Sc) or a specific siRNA against *ATG5* (siATG5). Three days later, the cells were incubated with or without Meflo (10 μM) for an additional 24 h. LC3 and ATG5 expression levels were examined by western blot analysis. Data were obtained from at least three independent experiments, and values are presented as the means ± S.E.M. (n>3, *p<0.05).

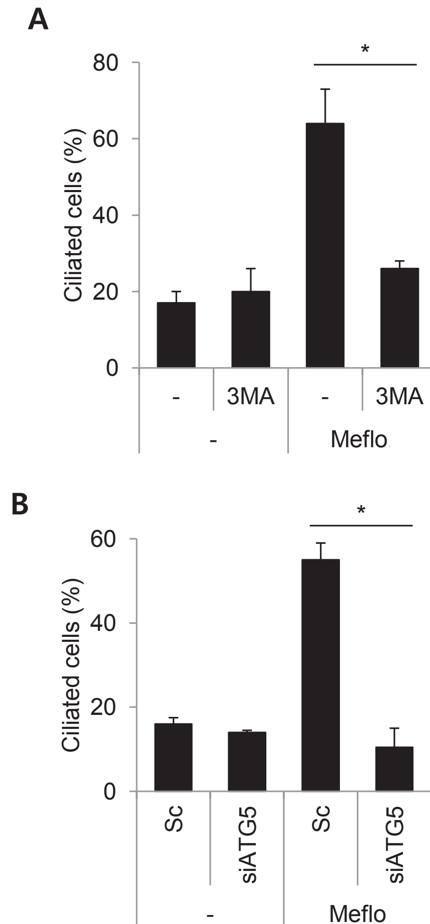

Fig. 4. Autophagy mediates mefloquine-induced elongation of primary cilia in htRPE cells. (A) htRPE/Smo-GFP cells pre-treated with 3MA (5 mM) for 12 h were further incubated with Meflo (10 μM) for 24 h. Ciliated cells were counted under a fluorescence microscope. (B) htRPE/Smo-GFP cells transfected with Sc or siATG5 were further treated with Meflo (10 μM) for 24 h. Ciliated cells were counted under a fluorescence microscope. Data were obtained from at least three independent experiments, and values are presented as the means ± S.E.M. (*p<0.05).

bined treatment of mefloquine with bafilomycin A1, a lysosomal inhibitor, enhanced the level of LC3 II than that of Mefloquine alone (Fig. 3D). Next we investigated the effect of autophagy inhibition in mefloquine-treated cells. 3-methyladenine (3MA) can inhibits autophagy by blocking autophagosome formation via the inhibition of class 3 PI3K. Therefore, 3MA has been applied to block the autophagy activation (Klionsky *et al*., 2012). As shown in Fig. 3E and 3F, both chemical and genetic inhibition of autophagy by treatment with 3MA or ATG5 knock-down efficiently reduced mefloquine-mediated autophagy in htRPE cells treated with Mefloquine (Fig. 3E, 3F). These results suggested that mefloquine also strongly induces autophagy in htRPE cells.

Mefloquine-induced autophagy regulates elongation of primary cilia in htRPE cells

Because mefloquine induces both autophagy and elongation of primary cilia in htRPE cells, we further investigated the effect of autophagy on the formation of primary cilia in mefloquine-treated cells. Interestingly, inhibition of autophagy by 3MA significantly suppressed the number of ciliated cells induced by treatment with mefloquine (Fig. 4A). Consistently, down-regulation of ATG5 also markedly blocked the mefloquine-induced formation of primary cilia in htRPE cells (Fig. 4B). These results suggested that autophagy positively regulates the elongation of primary cilia in mefloquine-treated RPE cells.

DISCUSSION

Dysregulation of primary cilia is implicated in human diseases, including various ciliopathies (Goetz *et al*., 2010). Thus, chemical modulators of ciliogenesis may offer some potential for the treatment of ciliopathies. We screened approximately 2,400 bioactive chemicals to identify novel regulators of ciliogenesis and selected mefloquine as a ciliogenesis inducer. Mefloquine has been used as an anti-malarial drug. However, its exact mode of action is not fully elucidated (Toovery, 2009). Mefloquine binds to a number of receptors in the brain and has a high affinity to serotonin receptors (Janowsky *et al*., 2014). In cortical neurons, mefloquine also induces oxidative stress which is involved in many diseases, such as neurodegenerative diseases, cancer, diabetes, and ciliopathies (Hood *et al*., 2010; Kim *et al*., 2013). In this study, we found that treatment with mefloquine elongates primary cilia in htRPE cells. It was recently reported that ROS regulates the length of primary cilia through ERK activation in ischemic damaged kidney cells (Kim *et al*., 2013). Subsequently, we showed that mefloquine increases ROS production in neuroblastoma cells (Shin *et al*., 2012b). Therefore, the effect of oxidative stress on mefloquine-mediated ciliogenesis remains to be further elucidated.

Previously, we demonstrated that autophagy attenuates mefloquine-mediated cytotoxicity in neuroblastoma cells. Inhibition of autophagy aggravates cell death in mefloquine-treated cells (Shin *et al*., 2012b). Autophagy basically is a cellular protective event in response to various stress conditions (Kroemer *et al*., 2010). Therefore, dysregulation of autophagy is also strongly linked to many pathophysiological conditions, including certain ciliopathies, neurodegenerative diseases and cancer (Tang *et al*., 2013). Recently, it was shown that rapamycin, a well-known autophagy inducer has a therapeutic effect in rats and mice on polycystic kidney disease, which is an example of ciliopathies (Huber *et al*., 2012; Ravichandran and Edelstein, 2014). Despite its potential relevance, the underlying molecular mechanism between autophagy and ciliogenesis is largely unknown. Tang *et al*. recently showed an interesting result that autophagy might promote ciliogenesis by accelerating the autophagic degradation of a ciliopathic protein, oral-facial-digital syndrome-1 (OFD1) (Tang *et al*., 2013). Here, we found that mefloquine increases activation of autophagy as well as enhances formation of primary cilia in htRPE cells. Furthermore, we also showed that inhibition of autophagy significantly suppressed the formation of primary cilia in cells treated with mefloquine. These results further implicates that autophagy positively regulates ciliogenesis. In contrast, Pampliega *et al*. suggested a controversial result that inhibition of autophagy enhances the growth of primary

cilia and cilia-associated signaling while activation of autophagy reduces the growth of cilia (Pampliega *et al*., 2013). Hence, further studies are needed to address the complex functional relationship between autophagy and primary cilia and illustrate the molecular mechanisms of ciliogenesis. In summary, present findings show that autophagy positively regulates ciliogenesis in mefloquine-treated htRPE cells.

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