Mitotic control by mRNA splicing regulators ensures primary cilia formation

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ABSTRACT
The biogenesis of the primary cilium is coordinated with cell cycle exit/re-entry in most types of cells. After serum starvation, the cilia-generating cells enter quiescence and produce the primary cilium; upon re-addition of serum, they re-enter the cell cycle and resorb the cilium. We previously identified novel mechanisms to link cell cycle progression and ciliogenesis by high-content genome-wide RNAi cell-based screening. In the present study, we pay attention to reveal the impact of mRNA splicing on cilia assembly after mitosis of cell cycle. We demonstrate that splicing regulators such as SON and XAB2 play an important role in mitosis exit, and thus affect ciliogenesis in G1/G0 phases. Knockdown of the splicing regulators in hTERT-RPE1 cells caused abnormal G2/M arrest under both serum addition and serum starvation, indicating defects in mitosis exit. Moreover, the knockdown cells failed to assemble the cilia under serum starvation and an inhibition of mRNA splicing using SSA, a spliceosome inhibitor, also revealed ciliogenesis defect. Finally, we show that the SSA-treated zebrafish display abnormal vascular development as a ciliary defect. These findings suggest the pivotal role of mRNA splicing regulators in cilia assembly and underscore the importance of mitotic regulation in ciliogenesis.

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Introduction
The primary cilium is a microtubule-based organelle that extends from a differentiated mother centriole, termed the basal body. In the cultured cells, the primary cilium is readily observed under serum starvation or 100% confluences that induce quiescent state (G0 phase) of the cell cycle. Previous studies have reported that complete resorption of the cilia at the G2/M phases is a prerequisite for mitotic progression (Pan et al. 2013). More recent works have suggested that ciliary resorption also occurs at earlier stages (G1/S phases) in the cell cycle (Kim et al. 2011; Li et al. 2011; Pan et al. 2013; Kim et al. 2016). Given these findings, several studies demonstrated that cilia disassembly occurs in two waves: the first wave occurs within during G1 to reenter cell cycle and the second wave occurs during G2 to enter mitosis (Pugacheva et al. 2007). Because of mutual relationship between cilia biogenesis and cell cycle progression, the debate as to whether cell cycle progression contributes to cilia assembly/disassembly or vice versa has been longstanding.

In the previous studies, we performed genome-wide high-content screening and identified novel mechanisms whereby ciliary resorption at the G1/S transition influenced cell cycle reentry (Kim et al. 2016). As our main focus on a promoting mechanism of cilia disassembly during cell cycle, we demonstrated that mRNA processing and ubiquitin-proteasome system (UPS) mechanisms are required for cilia absorption to stimulate cell cycle reentry (Kim et al. 2016). Noticeably, mRNA-processing regulators containing the spliceosome subunits were required for expression of representative cilia disassembly factors such as AURKA, PLK1, and NEK2 and committed to reentry of the cell cycle. However, other types of splicing regulators within the mRNA processing-related hits affected cilia assembly rather than cilia disassembly. Given the results, we focused on the mechanisms by which cell cycle cues control ciliogenesis in the current study. Recent reports noted that proteolysis of ciliary proteins including Trichoplein contributes cell cycle exit and cilia formation (Tang et al. 2013; Kasahara et al. 2014). Therefore, it is conceivable that the defective formation of the cilia caused by the depletion of mRNA splicing regulators is due to deregulation of the cell cycle exit. Although there are clues to the role of mRNA splicing regulators during mitosis and the involvement of mitotic regulators in cilia assembly/disassembly, the direct evidence that the mRNA splicing regulators-mediated mitotic control serves as a checkpoint for the cilia formation is currently unavailable.

In the present study, we selected splicing regulators such as SON (Ahn et al. 2011) and XAB2 (Andersen & Tapon 2008), which were associated with mitosis regulation and ciliogenesis in the previous study (Kim et al. 2016).
Our results from knockdown experiments reveal that these regulators are essential for G2/M transition and mitosis exit of the cells under serum addition, and for cilia assembly of the cells under serum starvation. In addition, we demonstrate that treatment of a splicing inhibitor blocks ciliogenesis of the cells under serum starvation and results in dysfunctional ciliary phenotypes in zebrafish. Therefore, these data suggest that tight control of mRNA splicing regulators in mitosis is required for the cells to commit to cell cycle exit and cilia assembly.

Materials and methods

Cell culture

hTERT-RPE1 cells and the stable cell line Smo-EGFP-mCherry-Geminin/hTERT-RPE1 (SEMG) were cultured in the DMEM/F12 medium supplemented with 10% FBS under standard conditions (37°C, 5% CO₂). To induce ciliogenesis, the cells were serum-starved in the serum-free DEMEM/F12 medium for 48 h before fixation.

siRNA transfections

Reverse transfection procedures were set up using Lipofectamine RNAiMAX (Invitrogen). The final siRNA concentration was 20 nM, and SEMG cells were resuspended in DMEM/F12 supplemented with 10% FBS and then seeded on Lab-Tek™ 8-well chamber slides (Nunc). The siRNAs were diluted in Transfectagro™ (Corning) and mixed with Lipofectamine RNAiMAX in Transfectagro™ at the 1:1 ratio by volume. The medium was refreshed for the transfected cells after 24 h of incubation, and the cells were assayed after 48–72 h, according to the experimental conditions.

Cellular image analysis

After the siRNA transfection, the SEMG cells at 90–100% confluence were fixed with 4% paraformaldehyde (PFA) and stained with DAPI. To monitor the primary cilia and cell cycle state, we used an LSM700 confocal microscope (Carl Zeiss). The total numbers of ciliated cells (green) and cycling cells expressing Geminin (red) were counted using the Image J software (NIH) and were statistically analyzed by student’s t-test for at least three independent experiments.

Cell cycle analysis

siRNA transfection was performed on SEMG cells seeded in 100 mm dishes and grown to approximately 70% confluence. The cells were trypsinized and fixed in 70% ethanol, followed by incubation with 100 g/ml RNase A (Mentos) for 15 min at room temperature. To determine cell cycle status of each cell, the cells were incubated with 50 g/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature, and the DNA contents were analyzed by FACS analysis using a BD FACSVers™ flow cytometer and BD FACSuite software (Becton Dickinson). The statistical analysis involved student’s t-test for at least three independent experiments.

Zebrafish housing and manipulations

Adult zebrafish were maintained at 28.5°C and pH 7.0–7.9 with a cycle of light (13 h) and dark (11 h) per day in the automatic system (Constructed by Genomic-Design, Korea). The zebrafish embryos were collected by natural breeding and incubated in the E3 medium (297.7 mM NaCl, 10.7 mM KCl, 26.1 mM CaCl₂, and 24.1 mM MgCl₂) containing 1% methylene blue (Sigma-Aldrich) at 28.5°C. To inhibit formation of melanin, we raised the zebrafish larvae (after 24 hpf) in the E3 medium containing 0.2 mM N-phenylthiourea (PTU; Sigma-Aldrich Chemistry, cat. # P7629).

Imaging of zebrafish larvae

To induce ciliary defects, we treated Tg (flk1:eGFP) zebrafish embryos with 30–50 µM spliceostatin A (AdooQ Bioscience, cat. # A12700) at 24 hpf, and the control embryos were treated with 1% dimethyl sulfoxide (DMSO). The drug-treated embryos were raised at 28.5°C until three days post-fertilization (dpf) and observed under a fluorescent microscope (SMZ1270, NIKON), and the images were captured using a monocamera (DS-Qi2, Nikon) and analyzed using the NIS-Elements software (Nikon).

Results

Identification of mRNA splicing regulators functioning in ciliogenesis

In the previous high-content screening (Kim et al. 2016), Group C, which was involved in dual regulation of cell cycle progression and ciliogenesis, was focused to analyze. Control cells revealed ~60% of EGFP-positive cilia and ~5% of mCherry-positive nuclei under serum deprivation for 24 h, while Group C hits-silenced cells had a lower than normal ciliated cells and higher than normal cycling cells (Figure 1(a)). Besides the previous analysis that found genes involving G1/S transition and
cilia disassembly, we further analyzed the hits to identify genes involving mitosis and cilia assembly here. To find genes regarding the mechanism associated with the coupling of cell cycle progression and ciliogenesis, we analyzed the mRNA processing-related hits by means of Gene ontology and DanioNet (Shim et al. 2016). The functional network analysis revealed that SON DNA binding protein (SON) and XPA binding protein 2 (XAB2) within the hits are linked via Polo like kinase 1 (PLK1) and Cyclin-dependent kinase 1 (CDK1), mitosis regulators (Figure 1(b) and (c)). The data implicated the impact of the mitotic role of these genes on ciliogenesis.

**The effect of mRNA splicing regulators on mitosis**

To validate the role of SON and XAB2 in the cell cycle progression, we performed fluorescent imaging and fluorescence-activated cell sorting (FACS) analyses on knockdown cells generated by means of individual siRNAs. We counted Smo-EGFP-positive ciliated cells (G1 phase indicator) and mCherry-geminin-positive cycling cells (S, G2, M phase indicator) in the imaging data and compared the results to the FACS data. The SEMG-RPE1 cells were transfected with siRNAs and cultured under serum starvation for 48 h before fixing for imaging analysis (Figure 2(a)). The imaging results revealed that both SON- and XAB2-depleted cells caused an insignificant change in the numbers of cycling cells and ciliated cells compared to controls (Figure 2(b) and (c)). However, the FACS analysis showed that the knockdown resulted in a noticeable increase of G2/M phase cells (Figure 2(d)). Therefore, this result suggests that SON and XAB2 play a role to control the mitosis during cell cycle progression.

**The effect of mRNA splicing regulators on ciliogenesis**

In order to determine whether the G2/M arrest shown in the knockdown cells is correlated with ciliogenesis defect, we examined the effects of downregulation of SON and XAB2 on cilia formation with serum deprivation. The SEMG-RPE1 cells were transfected with siRNAs and cultured under serum starvation for 48 h before fixing for imaging analysis (Figure 3(a)). The imaging data revealed that the control siRNA-transfected SEMG-RPE1 cells exit the cell cycle (cycling cells ≤ 5%) and assembled cilia (cilia-containing cells ≥ 40%) (Figure 3(b) and (c)). In contrast, a knockdown of the SON and XAB2 repressed the cell cycle exit (cycling cells ≥ 10%) and cilia assembly...
Figure 2. The depletion of mRNA splicing regulators induces G2/M arrest. (a) A schematic diagram shows the time point for serum addition of siRNA-transfected cells. (b) The result of fluorescent image analysis in the SON- and XAB2-depleted cells. Scale bars, 20 μm. (c) The quantified data of imaging analysis by student’s t-test. (d) The result of FACS analysis in the knockdown cells cultured with serum.

Figure 3. The depletion of mRNA splicing regulators inhibits ciliogenesis. (a) A schematic diagram shows the time point for serum starvation of siRNA-transfected cells. (b) The result of fluorescent image analysis in the SON- and XAB2-depleted cells. Arrowheads indicate the cilia. Scale bars, 20 μm. (c) The quantified data of imaging analysis by student’s t-test. The data are presented as mean ± SD (n = 3). ***p < .001. (d) The result of FACS analysis in the knockdown cells cultured without serum.
Inhibition of mRNA splicing causes ciliary defects

To ensure whether the mechanism of mRNA splicing is necessary for ciliogenesis, we induced functional inhibition of the spliceosome by means of spliceostatin A (SSA) in SEMG-RPE1 cells. The plated cells were treated with either DMSO or SSA for 24 h and cultured without serum for 48 h to induce cilia generation (Figure 4(a)). The SSA-treated cells showed similar defects to SON- and XAB2-siRNAs-transfected cells such as more cycling cells and less ciliated cells than DMSO-treated cells under serum starvation (Figure 4(b)). In addition, we treated zebrafish with SSA to confirm the mRNA splicing effect on ciliogenesis in vivo. Besides the typical ciliary phenotypes reported in the previous study (Kim et al. 2016), we further found that the mRNA splicing inhibited-zebrafish had disrupted vasculature systems (Figure 4(c)). These malformed blood vessels are also known ciliary defects (Lamont et al. 2010; Goetz et al. 2014), and thus our data suggest that mRNA splicing is involved in a ciliogenesis control.

Discussion

Although the coupled biological processes between ciliary dynamics and cell cycle progression have been largely accepted, the mechanisms governing the establishment of the quiescent state, associated with cilia assembly and of the cell cycle reentry, associated with cilia disassembly are still incomprehensive. In the present study, we have focused to reveal the novel role of mRNA splicing regulators that were identified from the previous whole-genome HCS to target the link between ciliogenesis and cell cycle. We show that the mRNA splicing regulators such as SON and XAB2 are novel ciliogenesis regulators and suggest that their function in the mitosis is associated with the control of cilia assembly (Figure 4(d)). In addition, the G2/M arrest shown in the knockdown cells under serum starvation implicates that the mRNA splicing may be involved in the mitotic exit and subsequent cilia formation in the quiescent state of cell cycle.

Given that the correct mitotic control is essential for ciliogenesis, we speculate the involvement of genome
stability in the cilia formation. Previous studies have suggested that the control mechanism of genome stability is tightly associated with cytokinesis including ciliogenesis (Nigg & Stearns 2011; Bornens 2012). We also found that some kinases including PLK1 and CDK1, which are related to regulate the genome stability, are linked with both SON and XAB2 by functional network analysis. Therefore, whether the mRNA splicing mechanism is involved in the genome stability whereby cilia assembly is influenced in the quiescent state of cell cycle would be uncovered in the further studies. Moreover, the identification of target genes modulated by SON- and XAB2-mediated mRNA splicing complex could be helpful to understand the molecular mechanism of the mitotic control-linked ciliogenesis.

Disclosure statement
No potential conflict of interest was reported by the authors.

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