Research Article

Keywords: Super elongation complex (SEC), inner ear, expression, proliferation, differentiation

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Abstract

**Background:** The super elongation complex (SEC) has been reported to play a key role in the proliferation and differentiation of mouse embryonic stem cells. However, the expression pattern and function of the SEC in the inner ear has not been investigated.

**Methods:** Here, we measured the expression of the three key SEC subunits AFF1, AFF4, and ELL3. We also used Lgr5-EGFP-Ires-CreERT2 mice to isolate the Lgr5+ progenitors via flow cytometry to evaluate the effect of the SEC on the proliferation and differentiation ability of Lgr5+ progenitors.

**Results:** We studied the inner ear expression pattern of three key SEC components, AFF1, AFF4 and ELL3, and found that these three proteins are all expressed in both cochlear hair cells and supporting cells. We also cultured Lgr5+ inner ear progenitors *in vitro* for sphere-forming assays and differentiation assays in the presence of the SEC inhibitor flavopiridol. We found that flavopiridol treatment decreased the proliferation ability of Lgr5+ progenitors, while the differentiation ability of Lgr5+ progenitors was not affected.

**Conclusions:** Our results suggest that the SEC might play important roles in regulating inner ear progenitors and thus regulating hair cell regeneration. Therefore, it will be very meaningful to further investigate the detailed roles of the SEC signaling pathway in the inner ear *in vivo* in order to develop effective treatments for sensorineural hearing loss.

Introduction

Hearing loss occurs mainly due to noise exposure, aging, ototoxic drugs, and genetic factors\(^1\). There were around 466 million people worldwide with disabling hearing loss in 2020, and the World Health Organization estimates that by 2050 over 900 million people will have disabling hearing loss\(^2\). Deafness has become a major global health problem, and sensorineural hearing loss is the most common type of hearing impairment\(^3\). However, due to the lack of effective drugs and a non-invasive method for targeted delivery of drugs to the inner ear, the treatment options for sensorineural hearing loss are limited\(^4\).

Cochlear hair cells (HCs) in adult mammals lose the ability to regenerate, thus hearing deficits caused by HC loss are permanent\(^5-9\). Therefore, induction of HC regeneration after injury by stimulating quiescent inner ear progenitor cells has been a main focus of auditory research in recent years.

The super elongation complex (SEC) is extremely important in the transcriptional elongation checkpoint control stage of transcription and is composed mainly of P-TEFb (positive transcription elongation factor), ELL (eleven–nineteen lysine-rich leukemia gene) family proteins, AFF (AF4/FMR2) family proteins, ENL (eleven-nineteen leukemia), AF9 (ALL1-fused gene from chromosome 9), and many other transcription factors\(^10\). P-TEFb and ELL are RNA polymerase II (Pol II)-related elongation factors\(^11\). AFF family proteins act as transcriptional activators with a positive action on RNA elongation\(^12\). ENL and AF9 are homologous, and they can connect the SEC to RNA Pol II-related factors\(^13\). P-TEFb is composed of
cyclin-dependent kinase 9 (CDK9) and cyclin T (CycT), and it promotes the transition into productive elongation by phosphorylating RNA polymerase II\textsuperscript{14}. It has been reported that the SEC plays an important role in regulating mouse embryonic stem cell proliferation and differentiation\textsuperscript{15}, and mis-regulation of the SEC leads to the uncontrolled regulation of gene expression during the differentiation of embryonic stem cells, which results in a variety of diseases such as acute lymphoblastic leukemia, cerebellar ataxia, and diffuse midline glioma\textsuperscript{16, 17}. ELL3, one of the key factors of the SEC, can protect differentiated cells from apoptosis by promoting the degradation of p53, enhancing the differentiation of mouse embryonic stem cells, and regulating the proliferation and survival of embryonic stem cells\textsuperscript{18}. However, the roles of the SEC in the inner ear remain unclear.

Flavopiridol is a semi-synthetic flavonoid that has been used in the treatment of acute myeloid leukemia\textsuperscript{19}, chronic lymphocytic leukemia\textsuperscript{20}, and other chronic diseases. Flavopiridol binds directly to CDK9, which is a component of P-TEFb, and inhibits its kinase activity\textsuperscript{21}. In turn, P-TEFb, as an important component of the SEC, can activate RNA polymerase II and transcriptional elongation\textsuperscript{22}. Thus, the most common method for blocking SEC function is to directly inhibit CDK9 with flavopiridol\textsuperscript{23}, and we used flavopiridol to inhibit the function of the SEC as previously reported\textsuperscript{15}.

Recent studies have shown that Lgr5\textsuperscript{+} supporting cells (SCs) are inner ear progenitors and that they have the ability to regenerate new HCs in the neonatal stage\textsuperscript{24}. The activation of Wnt/\(\beta\)-catenin signaling and inhibition of Notch signaling can induce Lgr5\textsuperscript{+} progenitors to regenerate Myo7a\textsuperscript{+} HCs\textsuperscript{25-27}, and several recent studies have also shown that Lgr5\textsuperscript{+} progenitors can be regulated by many other factors and signaling pathways such as Shh, Foxg1, and Hippo\textsuperscript{28-30}. However, the regeneration efficiency of Lgr5\textsuperscript{+} progenitors is still very limited, which suggests that there are other factors or signaling pathways involved in the HC regeneration process. Because the transcription extension stage is the main stage of gene expression regulation, transcriptional regulation of developmental regulatory genes is the core link between embryonic stem cell differentiation and organ formation\textsuperscript{31, 32}. Therefore, we speculate that the SEC may also play important roles in cochlear progenitor cells.

Here we measured the expression of the key SEC factors AFF1, AFF4, and ELL3 in the neonatal mouse cochlea, the function of SEC inhibitor flavopiridol in HEI-OC1 cell line, and we assessed the proliferation and differentiation ability of Lgr5\textsuperscript{+} progenitors after treatment with the SEC inhibitor flavopiridol. Our results suggest important roles for the SEC in Lgr5\textsuperscript{+} progenitors \textit{in vitro}, and further \textit{in vivo} studies need to be done to elucidate the roles of the SEC in the inner ear. These studies will form the experimental basis for using cochlear progenitors to regenerate functional HCs in order to treat patients with sensorineural hearing loss.

\section*{Material And Methods}

\subsection*{Experimental Animals}
Lgr5-EGFP-Ires-CreERT2(Lgr5-EGFP) mice\(^{33}\)(Jackson Laboratory, Stock No. 00887) and wide-type mice were raised in a comfortable environment with suitable temperature and light and fed with standard laboratory food and water ad libitum. We are approved by the Animal Care and Use Committee of Southeast University and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All the operations were carried out in accordance with the procedures.

**RNA extraction and RT-PCR**

About 20 wild-type mouse cochleae were dissected to extract total RNA, which was reverse transcribed into cDNA with the cDNA Synthesis Kit (Thermo Fisher Scientific, K1622). Gene expression was measured by RT-PCR with GAPDH as the endogenous reference gene. The primers were as follows: GAPDH: (F) 5′-AGG TCG GTG TGA ACG GAT TTG-3′; (R) 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′; AFF1: (F) 5′-GAA GGA AAG ACG CAA CCA AGA-3′; (R) 5′-TAG CTC ATC GCC TTT TGC AGT-3′; AFF4: (F) 5′-ATG AAC CGT GAA GAC CGG AAT-3′; (R) 5′-TGC TAG TGA CTT TGT ATG GCT CA-3′; ELL3: (F) 5′-GAC CAG CCT CCT GAT GCT AAG-3′; (R) 5′-GCC ACC ATT AGT AGT GCC CTC TTG-3′.

**Western blotting**

About 10 cochleae from postnatal day (P)3 mice were dissected in order to extract proteins. GAPDH was used as the reference protein. The primary antibodies were anti-AFF1 (Sigma-Aldrich, #SAB2106246), anti-AFF4 (Santa Cruz, #sc135337), and anti-ELL3 (Abcam, #ab67415). Peroxidase-conjugated goat anti-rabbit (Life, A-31572) and goat anti-mouse (Invitrogen, A21202) were used as the secondary antibodies.

**Cell Culture**

HEI-OC1 cells were cultured in DMEM with 10% fetal bovine serum and 1% ampicillin at 37°C and 5% CO2. The cells were divided into two groups. The experimental group was treated with flavopiridol (AbMole M1710) at the concentration of 10 µM. Control cells were treated with DMSO in the same culture medium. After 12-hour culture, cells were treated with 0.25% trypsin/EDTA and then ultrasonicated (Bioruptor\(^{\text{TM}}\) UCD-200) for CDK9 kinase detection.

**CDK9 kinase assay**

**Isolation of Lgr5+ progenitors via flow cytometry**

About 50–60 cochleae were isolated from P0–P3 Lgr5-EGFP mice and then treated with 0.125% trypsin/EDTA (Invitrogen, 25200114) at 37°C. Trypsin inhibitor (10 mg/ml, Worthington Biochem) was added after 10 minutes to terminate the reaction. The trypsinized cochleae were pipetted up and down 80–100 times to obtain single cells, and the cells were then filtered through a 40 µm cell strainer (BD Biosciences, 352340). Dissociated cells were sorted on a flow cell sorter (BD FACS Aria III). The EGFP+ cells were collected as Lgr5+ progenitors for further in vitro cell culture experiments.

**Sphere-forming assay and differentiation assay**
Sorted Lgr5+ cells were cultured in DMEM/F12 medium at a density of 2 cells/μl (200 cells per well) for 5 days for sphere forming. The formula of DMEM/F12 medium was the same in previous study. Spheres were identified with the Live Cell Imaging System and quantified using Image J. For differentiation, cells were cultured in the DMEM/F12 medium described above at a density of 20 cells/μl (2,000 cells per well) for 10 days. EdU (10 µM, Invitrogen, C10420) was added to label proliferating cells from day 4 to day 7. Flavopiridol (AbMole, M1710) was added to the experimental group from day 1 to day 10 at a concentration of 10 µM, while DMSO was added to the control group. Differentiated neurospheres were analyzed by immunofluorescent staining.

**Immunofluorescent staining**

The cochleae were dissected in cold HBSS in order to prevent protein degradation and then fixed with 4% PFA for 1 h at room temperature. In vitro cultured neurospheres were also fixed with 4% PFA for 1 h at room temperature. After washing with PBST three times, the cochleae or neurospheres were blocked with blocking solution for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies. The primary antibodies used were anti-Myosin7a (Myo7a; Proteus Bioscience, #25-6790; 1:1000 dilution), anti-Sox2 (1:400 dilution), anti-AFF1 (1:400 dilution), anti-AFF4 (1:50 dilution), and anti-ELL3 (1:400 dilution). After washing again three times, the cochleae or neurospheres were further incubated with secondary antibodies (Invitrogen, A21131, A21124) diluted 1:400 in PBT2 for 1 h at room temperature. After washing three times, the cochleae or neurospheres were mounted on slides with anti-fade fluorescence mounting medium (DAKO, S3023). Images were captured by Zeiss LSM 710 confocal microscope and analyzed by Image J software.

**Statistical analysis**

All the data in this research are presented as means ± SEM, and all experiments were repeated at least three times. All statistical analyses were performed in GraphPad Prism 5. P-values were calculated using a two-tailed, unpaired Student’s t-test, and a p-value < 0.05 was considered statistically significant.

**Results**

**AFF1, AFF4, and ELL3 are expressed in the cochlea**

We first measured the expression of the three key SEC subunits AFF1, AFF4, and ELL3 by RT-PCR (Figure 1a) and Western blotting analysis (Figure 1b), and we found that AFF1, AFF4, and ELL3 were all highly expressed in the cochlea. We further studied the expression pattern of AFF1, AFF4, and ELL3 in the cochlea of P3 mice and found that AFF1, AFF4, and ELL3 were all expressed in the cochlear HCs and SCs at P3 (Figure 2a-c). However, the immunostaining intensities of these three subunits in the SCs were weaker than in the HCs.

**Flavopiridol treatment inhibited the activity of CDK9 in HEI-OC1 cells**
Flavopiridol has been reported to be an inhibitor of CDK9 which is an indispensable part of SEC and the low level of its kinase activity prevents the recruitment of other elongation factors in SEC\textsuperscript{14}. However, the function of flavopiridol has not been verified in inner ear. Here, we used ADP-Glo Kinase Assay to detect the activity of CDK9. After the kinase reaction, the remaining ATP was depleted and the ADP was converted to luminescent ATP (Figure 3a). HEI-OC1 cells were cultured for 3 days, and then treated by 10\(\mu\)M flavopiridol which was diluted in the culture medium for 12 hours. Images of cells were taken before and after flavopiridol treatment (Figure 3b). The luminescent ATP was recorded and the relative light units was calculated to represent the activity of CDK9 (Figure 3c). The results showed that flavopiridol could also function as the CDK9 inhibitor to inhibit SEC activity in HEI-OC1 cells.

**Flavopiridol treatment decreased the sphere-forming ability of Lgr5+ progenitors \textit{in vitro}**

Flavopiridol was previously used to inhibit SEC transcription activity \textsuperscript{17}. Here we also used flavopiridol to inhibit SEC activity in Lgr5+ progenitors in order to determine whether the SEC plays roles in the proliferation and differentiation ability of Lgr5+ progenitors. In order to determine the effect of the SEC on the sphere-forming ability of Lgr5+ progenitors, Lgr5+ cells were isolated from Lgr5-EGFP mice by flow cytometry and then cultured \textit{in vitro} for 5 days to form spheres with or without 10 \(\mu\)M flavopiridol treatment (Figure 4a, b). The flavopiridol treatment decreased both the number (Figure 4c) and diameter of the spheres (Figure 4d), which suggested that inhibition of the SEC could decrease the sphere-forming ability and proliferation ability of Lgr5+ progenitors \textit{in vitro}.

**No difference was observed in the differentiation assay after flavopiridol treatment**

In order to further evaluate the effect of the SEC on the differentiation ability of Lgr5+ progenitors, we isolated Lgr5+ cells by flow cytometry and cultured them \textit{in vitro} for the differentiation assay with or without 10 \(\mu\)M flavopiridol treatment (Figure 5a). The cells were immunostained with Myo7a, EdU, and DAPI (Figure 5b), and the Myo7a+ cells and EdU+ cells inside and outside the colonies were quantified. There were more EdU+ cells in the flavopiridol treatment group than in the control group (Figure 5c), while the numbers of Myo7a+ cells were almost the same in the flavopiridol treatment group and the control group (Figure 5d). These results suggested that inhibition of the SEC did not affect the differentiation ability of Lgr5+ cells \textit{in vitro}.

In summary, our results showed that the key SEC factors AFF1, AFF4, and ELL3 are all highly expressed in the cochlea. And we verified that flavopiridol could inhibit SEC by inhibiting CDK9 activity in HEI-OC1 cell line. We used flavopiridol as an SEC inhibitor to investigate the effect of the SEC on the proliferation and differentiation ability of Lgr5+ progenitors and found that the number and diameter of spheres of Lgr5+ progenitors were both decreased after SEC inhibitor treatment, while the differentiation ability of Lgr5+ progenitors was not affected. Therefore, the SEC appears to promote the proliferation ability of Lgr5+ progenitors, but not their differentiation ability.

**Discussion**
Irreversible damage to HCs in the mammalian cochlea is the main cause of sensorineural hearing loss. Previous studies have reported that Lgr5+ cells are cochlear progenitors with the ability to regenerate HCs, and it has been documented that the SEC plays an important role in the process of gene transcription and extension and that it is necessary for the differentiation of mouse embryonic stem cells. Therefore, we speculated that the SEC also plays an important role in the proliferation and differentiation of mouse cochlear progenitors. Besides, it has also been reported that AFF proteins and ELL proteins increase the diversity and regulatory potential of the SEC family in mammals. Furthermore, AFF1 and AFF4 are the backbones of the SEC and ELL3 has the ability to associate with other translocation partners of the SEC. Thus we chose to investigate the expression of AFF1, AFF4, and ELL3 in the inner ear. Our results showed that AFF1, AFF4, and ELL3, were all highly expressed in the cochlea and that the SEC inhibitor flavopiridol could induce the proliferation of Lgr5+ progenitors in vitro, but not their differentiation. This study thus provides an experimental foundation for the clinical application of HC regeneration for treating hearing loss.

The SEC is known to be widely expressed in most tissues, and it plays important roles during development. However, its expression in the inner ear has not been studied. We found that AFF1, AFF4, and ELL3 were highly expressed in the cochlea, and this suggests that the SEC functions in the inner ear. Furthermore, we found that AFF1, AFF4 and ELL3 were all expressed in the cochlear HCs and SCs by immunostaining, but the expression of SEC proteins in SCs was lower than that in HCs. In addition to its role in Lgr5+ progenitors, the SEC likely plays an essential role in cochlear HCs. However, due to the lack of studies of the SEC in the inner ear and the lack of mouse models, the specific role of the SEC in HCs awaits further study.

Flavopiridol, a potent inhibitor of CDK9, is reported to inhibit transcription. CDKs combine with cyclins to play important roles in transcription and stem cell self-renewal, and P-TEFb (composed of CDK9 and CycT) is an indispensable part of the SEC that phosphorylates RNA polymerase II and thus activates transcription elongation of important genes involved in cell proliferation and survival. Flavopiridol can inhibit the function of the SEC by inactivating CDK9, and thus we chose flavopiridol as the SEC inhibitor as previously reported.

In addition, our results suggest that inhibition of the SEC by flavopiridol could reduce the sphere-forming ability and proliferating cell number of Lgr5+ progenitors in the differentiation assay, which is consistent with previous reports that AFF1 promotes leukemia cell proliferation, that AFF4 enhances the sphere-forming capacity and tumor-initiation capacity in head and neck squamous cell carcinoma, and that ELL3 stimulates the proliferation and stem cell properties of breast cancer cells. In our results, inhibition of the SEC did not affect the differentiation ability of Lgr5+ progenitors in vitro.

ELL3 has also been shown to promote the differentiation of mouse embryonic stem cells by regulating the epithelial-mesenchymal transition and apoptosis, and the overexpression of AFF1 impairs the differentiation of mesenchymal stem cells, while overexpression of AFF4 enhances their differentiation. In our results, inhibition of the SEC did not affect the differentiation ability of Lgr5+ progenitors in vitro.
vitro, which might be because of the different regulatory roles of these three proteins in cell differentiation. The detailed mechanisms behind this need further exploration.

Conclusions

We show here that the SEC is expressed in cochlear HCs and SCs in neonatal mice and that the SEC can induce the proliferation ability of Lgr5+ progenitors but not their differentiation. Our study thus provides new candidates for regulating inner ear progenitor cells as a step towards HC regeneration.

Abbreviations

SEC: super elongation complex; HCs: hair cells; P-TEFb: positive transcription elongation factor; ELL: eleven–nineteen lysine-rich leukemia gene; AFF: AF4/FMR2; ENL: eleven-nineteen leukemia; AF9: ALL1-fused gene from chromosome 9; Pol II: polymerase II; CDK9: cyclin-dependent kinase 9; CycT: cyclin T; SCs: supporting cells; Lgr5-EGFP: Lgr5-EGFP-Ires-CreERT2

Declarations

Ethics approval and consent to participate

We are approved by the Animal Care and Use Committee of Southeast University and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All the operations were carried out in accordance with the procedures.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

YC, HL, LL, SZ, and RC conceived and designed the experiments. YC, RQ, YZ, WC, YD, LW, PJ, JA, and XM performed the experiments. YC, RQ, YZ, WC, XG, LL, SZ, and RC analyzed the data. YC, HL, LL, SZ, and RC wrote the paper. All authors read and approved the final manuscript.

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