Polycomb repressive complex 2 facilitates the nuclear export of the influenza viral genome through the interaction with M1

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The organization of nuclear domains is crucial for biological events including virus infection. Newly synthesized influenza viral genome forms viral ribonucleoprotein (vRNP) complexes and is exported from the nucleus to the cytoplasm through a CRM1-dependent pathway mediated by viral proteins M1 and NS2. However, the spatio-temporal regulation of the progeny vRNP in the nucleus is still unclear. Here we found that polycomb repressive complex 2 (PRC2), which contains a methyltransferase subunit EZH2 and catalyzes histone H3K27me3 for the formation of facultative heterochromatin, is a positive factor for the virus production. Depletion of PRC2 complex showed the nuclear accumulation of vRNP and the reduction of M1-vRNP complex formation. We also found that PRC2 complex directly binds to M1, and facilitates the interaction of M1 with vRNP. In conclusion, we propose that the progeny vRNP could be recruited to facultative heterochromatin and assembled into the export complex mediated by PRC2 complex.

The genome of influenza A virus consists of eight-segmented and single-stranded RNAs of negative polarity (vRNA). vRNA forms viral ribonucleoprotein complexes (vRNP) with viral RNA-dependent RNA polymerases and nucleoprotein (NP). After the nuclear import of incoming vRNP, nuclear structures and functions are remodelled possibly for the replication and transcription of vRNA, and post-replicative processes1.

The nucleus is highly compartmentalized into chromosome territories and numerous distinct nuclear compartments such as nucleolus, promyelocytic leukemia nuclear body (PML NB), spliceosome, and so on. Chromatin forms either a condensed and transcriptionally inactive structure called heterochromatin or a relaxed and transcriptionally active structure called euchromatin. Heterochromatin is classified into two groups based on histone modifications. One is constitutive heterochromatin (cHC) marked by histone H3 lysine 9 trimethylation (H3K9me3) mediated by a variety of factors including, RIZ1, SUV39H1, SUV39H2, SETDB, and so on2. The other is facultative heterochromatin (fHC) marked by histone H3 lysine 27 trimethylation (H3K27me3) mediated by polycomb repressive complex 2 (PRC2)3 and histone H2A lysine 119 (H2AK119Ub) ubiquitination mediated by polycomb repressive complex 1 (PRC1)4.

After transport of incoming influenza virus vRNP into the nucleus, viral polymerases interact with cellular RNA polymerase II machineries for the viral transcription at euchromatin5–8. vRNP also interacts with heterochromatin through the histone tails containing H3K9me3 and H3K27me39–12. However, the functional significance of heterochromatin localization of vRNP is unknown. After viral genome replication, newly synthesized vRNA is assembled into vRNP, followed by export from the nucleus to the cytoplasm mediated by the CRM1-dependent pathway13–15. A current model of the nuclear export of vRNP suggests that M1 directly binds to vRNP16,17, and M1 is then linked to CRM1 by NS2 through its nuclear export signal (NES)15,18. It has been suggested that heterochromatin functions as a scaffold to form vRNP export complexes due to the localization of vRNP export complexes at heterochromatin19. It is also reported that M1 and NS2 are localized at PML NB with vRNA in cells treated with a potent CRM1 inhibitor, leptomycin B20. However, the detail of the spatio-temporal dynamics of the progeny vRNP in the nucleus is unclear.

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Here we found that a punctate localization pattern of EZH2, a catalytic subunit of PRC2 complex, becomes dispersed after influenza virus infection. vRNP was found to interact with PRC2 complex in infected cells. EZH2, EED, and SUZ12 are required for a minimal methyltransferase activity of PRC2 complex. EED recognizes histone H3K27me3 to recruit PRC2 complex onto 5'HC to maintain the methylation level. SUZ12 binds to EZH2 through EED and stimulates the methyltransferase activity. Knockdown (KD) analyses indicated that EZH2 stimulates the vRNP export in a methyltransferase activity-independent manner. Further, we showed that PRC2 complex facilitates the association of M1 with vRNP. Taking these findings together, we propose that PRC2 complex promotes the nuclear export of vRNP by recruiting M1 onto vRNP.

Results

PRC2 complex is a positive factor for the virus production. To analyze the effect of the virus infection on the nuclear compartments, we carried out indirect immunofluorescence assays with either mock-infected or infected cells using antibodies against cellular factors related to the nuclear structures such as euchromatin, heterochromatin and nuclear matrix (Fig. 1a). We found that punctate foci of EZH2 becomes dispersed through the nucleus in response to the infection, although the function of EZH2 foci is unclear. In contrast to the case for EZH2, we did not observe localization changes for other proteins. EZH2 is a catalytic subunit of PRC2 complex methyltransferase. It is known that PRC2 complex induces the 5'HC formation through the methyltransferase activity of histone H3K27me3 and represses cellular gene expression. To examine whether PRC2 complex is involved in a process of progeny virion production, we examined the effect of EZH2 KD on the virus titer. The expression level of EZH2 in KD cells decreased to approximately 15% of that in control cells at 72 h post-transfection (Fig. 1b and Supplementary Fig. 1). EZH2 KD reduced the amounts of SUZ12 due to the degradation as previously reported (Fig. 1c and Supplementary Fig. 3). The amount of infectious progeny virions produced from EZH2 KD cells was reduced to approximately 30% of that produced from control cells (Fig. 1d). Thus, we concluded that PRC2 complex stimulates the production of infectious viruses.

EZH2 and other PRC2 complex components are predominantly localized in the nucleus (Fig. 1a). We assumed that PRC2 complex affects the viral genome functions in the nucleus, such as the viral genome replication, viral transcription, and nuclear export of vRNP. Thus, we examined the amounts of vRNA and viral mRNA in EZH2 KD cells at 6 h post infection. We found that the expression levels of vRNA and viral mRNA in EZH2 KD cells were unchanged compared with those in control cells (Fig. 1e). In addition, the expression levels of viral proteins were not affected by EZH2 KD (Fig. 1f and Supplementary Fig. 3). Therefore, it is possible that PRC2 complex is not involved in the replication and transcription of the viral genome.

PRC2 complex is required for the nuclear export of vRNP. Next, we examined the nuclear export of vRNP by fluorescent in situ hybridization (FISH) assays. In control cells, more than 90% of cells showed the cytoplasmic localization pattern of vRNA at 6 h post infection (Fig. 2a,b). On the other hand, approximately 43% of EZH2 KD cells exhibited the nuclear localization of vRNA, suggesting that PRC2 complex is required for the nuclear export of vRNP (Fig. 2a,b). NF-κB is a nucleocytoplasmic shuttling protein mediated by CRM1, and showed the nuclear localization by inhibiting CRM1. In contrast to vRNA, NF-κB was successfully exported to the cytoplasm in control and EZH2 KD cells, suggesting that EZH2 KD does not impair the CRM1 function (Fig. 2a). The expression levels of M1 and NS2, which are viral proteins and involved in the nuclear export of vRNP, were not different between control and EZH2 KD cells (Fig. 1f and Supplementary Fig. 3).

It has been reported that CRM1 and other viral proteins were recruited to heterochromatin, and heterochromatin functions as a scaffold to assemble the export complex of vRNP. Thus, it is possible that EZH2 KD disrupts the 5'HC structure, thereby inhibiting the vRNP export. Hence, we next examined whether the methyltransferase activity of PRC2 complex is required for the vRNP export using GSK126, a potent methyltransferase inhibitor. EZH2 KD was successfully exported to the cytoplasm in control and EZH2 KD cells, suggesting that EZH2 KD does not impair the CRM1 function (Fig. 2a). From the results shown in Fig. 2, it is possible that PRC2 complex binds to vRNP and stimulates the nuclear export of vRNP. To address this, we carried out immunoprecipitation assays with infected cell lysates using anti-NP antibody to detect the interaction of NP/vRNP with PRC2 complex. We found that not only EZH2 but also EED and SUZ12 were co-immunoprecipitated with vRNP (Fig. 3a and Supplementary Fig. 5). Further to examine whether these chromatin proteins interact with NP associated with viral RNAs, the cell lysates prepared from infected cells were subjected to immunoprecipitation assays with anti-EZH2 and anti-SUZ12 antibodies, respectively. The amounts of co-precipitated viral RNAs were quantitatively determined by quantitative RT-PCR. The amount of vRNA co-immunoprecipitated with endogenous EZH2 and SUZ12 was 17.31 ± 0.57 and 10.36 ± 0.64 times more than cRNA, respectively (Fig. 3b). These results indicate that PRC2 complex interacts with vRNP in infected cells. Next, we carried out immunoprecipitation assays with cell lysates prepared from infected HeLa cells expressing 3xFLAG-EZH2. We found that viral polymerases, NP, and M1 were co-immunoprecipitated with 3xFLAG-EZH2, but NS2 was not (Fig. 3c and Supplementary Fig. 6).

PRC2 complex is required for the interaction between M1 and vRNP. vRNP is exported from the nucleus to the cytoplasm mediated by the CRM1-dependent pathway through the interaction of M1 and NS2 with vRNP. To test whether PRC2 complex regulates the association of M1 with vRNP, we carried out proximity ligation assays (PLA) using rabbit anti-M1 and mouse anti-NP (mAb61A5) antibodies. It is reported that
Figure 1. PRC2 complex is involved in the viral life cycle. (a) Remodeling of nuclear compartments by influenza virus infection. At 6 h post infection, mock-infected (upper panel) or infected (lower panel) HeLa cells were subjected to indirect immunofluorescence assays with anti-EZH2, anti-Pol II, anti-HP1β, anti-RAD21, anti-HDAC1, anti-Brg1, anti-Topo II, and anti-CTCF antibodies, respectively (green). Nuclei were counterstained with DAPI (blue). Punctate foci of EZH2 are indicated by arrowheads. (b) Knockdown efficiency of EZH2. HeLa cells were transfected with either control siRNA or siRNA against EZH2. At 60 h post transfection, 1 × 10⁵ cells (lane 1), 5 × 10⁴ cells (lane 2), 2.5 × 10⁴ cells (lane 3), 1.25 × 10⁴ cells (lane 4) of control cells, and 1 × 10⁵ cells of EZH2 KD cells (lane 5) were subjected western blotting with anti-EZH2 antibody. The original blot is presented in Supplementary Fig. 1. (c) The amounts of PRC2 complex components in EZH2 KD cells. Control or EZH2 KD cells were subjected to western blotting with anti-EZH2, anti-SUZ12, anti-EED, and anti-β-actin antibodies. The original blots are presented in Supplementary Fig. 2. (d) Production of infectious virions. At 60 h post transfection, control and EZH2 KD cells were infected with influenza virus at MOI of 0.3. The culture supernatants were collected at 24, 28, 32, and 36 h post infection, and subjected to plaque assays. The average titers and standard deviations determined from three independent experiments are shown. (e) Expression levels of vRNA and mRNA in EZH2 KD cells. Total RNAs prepared from control and EZH2 KD cells at 6 h post infection were subjected to the quantitative real-time PCR. The amounts of vRNA and mRNA of segment 5 in control cells were indicated as 100%, respectively. The average expression levels and standard deviations determined from three independent experiments are shown. (f) Expression levels of M1 and NS2 in control and EZH2 KD cells. At 6 h post infection, control and EZH2 KD cells were subjected to SDS-PAGE, and carried out western blotting with anti-EZH2, anti-M1, anti-NS2, and anti-β-actin antibodies. The original blots are presented in Supplementary Fig. 3.
the mAb61A5 antibody recognizes NP on the viral genome, and thus the interaction of M1 with vRNP can be detected by the in situ PLA system. The number of PLA signals between vRNP and M1 in EZH2 KD cells decreased to approximately 50% of that in control cells at 6 h post infection (Fig. 4a, b). These results suggest that PRC2 complex is involved in the interaction between M1 and vRNP.

PRC2 complex facilitates M1-vRNP complex formation through the direct interaction with M1. To examine the molecular mechanism of the assembly of M1-vRNP complex stimulated by PRC2 complex, we performed immunoprecipitation assays using recombinant PRC2 complex and M1. 3xFLAG-EZH2 and SUZ12 were purified from 293T cells using anti-FLAG antibody-conjugated agarose (Fig. 4c, lanes 2 and 3, Supplementary Fig. 7). Other PRC2 subunits were also co-purified with 3xFLAG-EZH2 and 3xFLAG-SUZ12, respectively (Fig. 4d and Supplementary Fig. 8), which suggests that part of purified 3xFLAG-EZH2 and 3xFLAG-SUZ12 forms PRC2 complexes. We found that M1 was co-immunoprecipitated with PRC2 complexes purified with 3xFLAG-EZH2 and 3xFLAG-SUZ12, respectively (Fig. 4e, lanes 4–6 and Supplementary Fig. 9). Next, we tested the interaction of M1 with vRNP in the presence or absence of PRC2 complex by immunoprecipitation assays with anti-NP antibody using purified recombinant proteins. The amount of M1 co-immunoprecipitated with vRNP was increased in the presence of PRC2 complex (Fig. 4f, lanes 4–6 and Supplementary Fig. 10). These findings suggest that PRC2 complex could recruit M1 onto vRNA for the nuclear export of vRNP through the direct interaction of PRC2 complex with M1.

Discussion

PRC2 complex has the methyltransferase activity of histone H3K27me3, which is one of the markers of fHC. Recently, it was reported that CRM1 interacts with a particular focus of fHC, and recruits Nup98-HoxA9 and CALM-AF10 fusion proteins on fHC through the recognition of NES. These findings suggest that NS2 could...
be recruited to CRM1-bound fHC through the NES. NP also binds to histone H3K27me311, suggesting that vRNP localizes at fHC. Thus, it is possible that M1 is recruited to fHC through its interaction with PRC2 complex, and the export complex of vRNP is formed at fHC. CHD3, one of the deacetylase components of NuRD complex, also promotes the nuclear export of vRNP through its interaction with NS222. It has been reported that NuRD complex binds to histone H3K27me3 and cooperates with PRC2 to suppress cellular gene expression33–35. Taken together, these findings suggest that fHC functions as an assembly site for the export complex of vRNP.

Figure 3. Interaction of PRC2 complex with vRNP complex in infected cells. (a) Interaction of vRNP with PRC2 complex. At 6 h post infection, infected cells were subjected to immunoprecipitation assays with either non-specific IgG (lane 2) or anti-NP (lane 3) antibody-conjugated protein A Sepharose. Co-immunoprecipitated proteins were separated by 10% SDS-PAGE, and analyzed by western blotting with anti-EZH2, anti-EED, anti-SUZ12, and anti-NP antibodies. The original blots are presented in Supplementary Fig. 5. (b) Interaction of PRC2 complex with vRNA. At 6 h post infection, infected HeLa cells were subjected to immunoprecipitation assays with non-specific IgG, anti-EZH2, and anti-SUZ12 antibodies, respectively. The absolute amounts of precipitated RNAs were quantified by quantitative real-time PCR as described in Methods. (c) Interaction of EZH2 with vRNP complex. HeLa cells were transfected with a plasmid expressing 3xFLAG-EZH2, and then infected with influenza virus at MOI of 5. At 6 h post infection, these cells were subjected to immunoprecipitation assays with either non-specific IgG (lane 2) or anti-FLAG (lane 3) antibody-conjugated Sepharose. Co-immunoprecipitated proteins were separated by 7.5% or 12.5% SDS-PAGE, and analyzed by western blotting with anti-FLAG, anti-PB1, anti-PB2, anti-NP, anti-M1, and anti-NS2 antibodies. The original blots are presented in Supplementary Fig. 6.
We demonstrated that EZH2 KD results in the accumulation of vRNP in the nucleus (Fig. 2a). Proteomics analyses revealed that a number of viral proteins are subjected to post-translational modifications for virus growth36. However, the treatment of GSK126 does not affect the localization of the viral genome (Fig. 2d). This suggests that the methyltransferase activity of PRC2 complex and histone H3K27me3 are not important for vRNP export. Previous reports showed that histone H3K27me3 is required for the assembly of heterochromatin, but not essential for the maintenance of the chromatin compaction3,37,38. Therefore, it is possible that the fHC region is important for the formation of vRNP export complex.

A number of different RNA and DNA viruses inhibit the formation of PML NB to prevent antiviral responses, and certain viruses hijack PML NB for their viral replication and transcription39,40. It is also reported that the proviral promoter of HIV is assembled at fHC near PML NB and that the promoter is activated in response to the disruption of PML NB41. Further, it is worthy to note that some parts of M1, NS2, and vRNA are found at PML NB20,42,43. Based on these reports, it is possible that PML NB is in close proximity to fHC, and that vRNP might

Figure 4. PRC2 complex interacts with M1, and facilitates the interaction between M1 and vRNP (a, b) in situ PLA assays. At 60 h post transfection of siRNA, control and EZH2 KD cells were infected with influenza virus at MOI of 5. At 6 h post infection, cells were subjected to in situ PLA assays with rabbit anti-M1 and mouse anti-NP antibodies (red), then indirect immunofluorescence assays were carried out to detect the intracellular localization of M1 (green). Nuclei were counter-stained with DAPI (blue). The number of PLA signals was obtained from three-dimensional reconstruction images by IMARIS software (n = 11). The level of significance was determined by Student’s t test. (c) Purification of recombinant proteins. Purified 3xFLAG-EZH2, 3xFLAG-SUZ12, and His-M1 proteins were applied to 10% SDS-PAGE, and visualized by CBB staining. The original blot is presented in Supplementary Fig. 7. (d) Western blotting of purified proteins. Purified proteins were subjected to western blotting with anti-EZH2, anti-SUZ12, and anti-EED antibodies. The original blot is presented in Supplementary Fig. 8. (e) Interaction of PRC2 complex with M1. Recombinant His-M1 (12 pmol) was incubated without (lanes 1 and 4) or with either 8 pmol of 3xFLAG-EZH2 (lanes 2 and 5) or 8 pmol of 3xFLAG-SUZ12 (lanes 3 and 6) for 1 h. Then, the proteins were subjected to immunoprecipitation assays with anti-FLAG antibody, followed by western blotting with anti-FLAG and anti-M1 antibodies (lanes 4–6). The original blots are presented in Supplementary Fig. 9. (f) PRC2 complex facilitates the interaction of M1 with vRNP. Recombinant His-M1 (12 pmol) and vRNP purified from influenza virions were incubated without (lanes 1 and 4) or with either 8 pmol of 3xFLAG-EZH2 (lanes 2 and 5) or 8 pmol of 3xFLAG-SUZ12 (lanes 3 and 6) for 1 h. Then, vRNP was immunoprecipitated with anti-NP antibody, and then the co-immunoprecipitated M1 was detected by western blotting with anti-M1 antibody (lanes 4–6). The original blots are presented in Supplementary Fig. 10.
translocate from PML NB to IF. To further understand the regulatory mechanism of vRNP nuclear export, the significance of the accumulation of vRNP and viral proteins at PML NB requires further analysis.

**Methods**

**Biological materials.** Rabbit polyclonal antibodies against M1, PB1, PB2 NP and CTCF were prepared as previously described. Rabbit polyclonal antibodies against EZH2 (Abcam), EED (Proteintech), SUZ12 (Proteintech), HDAC1 (Upstate), pol II (Santa Cruz), RAD21 (Abcam), and mouse monoclonal antibody against Brd1 (Santa Cruz), and rat polyclonal antibody against HP1α (Abcam) were purchased. Mouse monoclonal antibodies against NP (mAb61A5) and Topoisomerase II were a gift from Dr. F. Momose (Kitasato University) and Dr. A. Kikuchi (Nagoya University), respectively. vRNP was prepared from purified influenza virus (A/Puerto Rico/8/34) as previously described. For the construction of the plasmids expressing 3xFLAG-EZH2 or 3xFLAG-SUZ12, cDNA was generated from total RNA extracted from HeLa cells using oligo dT primer (5′-TTTTTTTTTTTTTTT-3′), then amplified with following specific primer sets: 5′-AGATCTACGGTATGGCCGGCAGCTGGAGAAA-3′ and 5′-AGATCTACGCCTAAGGGATTTCTCTCCTCGAG-3′ for EZH2; 5′-CAGGAATTCTCCTCGAGATGGCGCTCAGAAGC-3′ and 5′-CAGGAATTCCTCGAGATCTTTCGAGCTCAGAC-3′ were inserted into pCAGGS digested with EcoRI and EcolI with and Klenow fragment. The amplified cDNAs of EZH2 and SUZ12 were cloned into pCAGGS-3xFLAG digested with EcoRI, respectively. HeLa cells (a kind gift from Dr. MA. Yamada, The University of Tokyo) and 293T cells (a kind gift from Dr. Y. Kawaoka, The University of Tokyo) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), and MDCK cells (a kind gift from Dr. P. Palese, Icahn School of Medicine at Mount Sinai) were cultured in minimal essential medium (MEM) containing 10% FBS. GSK126 was purchased from Xcessbio.

**Immunoprecipitation.** At 6 h post infection, cells were cross-linked with 0.5% of formaldehyde at room temperature for 10 min. Fixed cells were lysed by sonication in a lysis buffer containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.1% Triton X-100. The supernatants were subjected to the immunoprecipitation assays with antibodies as indicated in figure legends. After washing with lysis buffer, co-immunoprecipitated proteins were eluted with 0.1 M glycine (pH 3.0). Co-immunoprecipitated proteins were separated by SDS-PAGE and analyzed by western blotting with antibodies as indicated in each figure legend.

**Gene silencing mediated by siRNA.** Cells (7×10^5) were transfected with 30 pmol of either control siRNA or siRNA against EZH2 (Invitrogen) using lipofectamine RNAi Max (Life Technologies) according to the manufacturer’s protocol.

**Quantification of viral RNA.** Total RNAs were purified from infected cells using RNA purification kit (Nacalai). For RNP immunoprecipitation assays, infected cells were cross-linked with 0.5% of formaldehyde for 10 min at room temperature. Fixed cells were lysed by sonication in a buffer containing 12.5 mM Tris-HCl (pH 7.9), 150 mM NaCl, 5 μg/ml heparin, and 1% Triton X-100. The supernatants were subjected to the immunoprecipitation assays with antibodies as indicated in figure legends. After washing with lysis buffer, co-immunoprecipitated proteins were eluted with 0.1 M glycine (pH 3.0). Co-immunoprecipitated proteins were separated by SDS-PAGE and analyzed by western blotting with antibodies as indicated in each figure legend.

**Indirect immunofluorescence assays and fluorescent in situ hybridization assays.** Indirect immunofluorescence assays and fluorescent in situ hybridization assays were carried out as previously described. Briefly, infected cells were fixed with 4% paraformaldehyde for 1 min at room temperature, permeabilized with PBS containing 0.5% Triton X-100 for 5 min at room temperature, and incubated with PBS containing 1% bovine serum albumin (BSA) for 1 h. Cells were incubated with primary antibodies as indicated in each figure legend for 1 h, and then incubated with Alexa Fluor488-conjugated secondary antibodies, respectively (Thermo Fisher Scientific). FISH assays were carried out using an RNA probe complementary to the segment 1 vRNA polymerase in vitro (Promega) were used as a standard for quantitative RT-PCR to quantify the absolute amount.

**In situ proximity ligation assays (PLA).** In situ PLA was carried out as previously described. Infected cells were fixed with 4% PFA for 10 min, followed by permeabilization with PBS containing 0.5% Triton X-100 at room temperature for 5 min. The cells were incubated with PBS containing 1% skim milk for 30 min, and incubated with rabbit anti-M1 and mouse anti-NP antibodies for 1 h. PLA assays were performed using Duolink in situ PLA Kit (Olink Bioscience) according to the manufacturer’s protocol. Counting of the PLA foci was carried out using IMARIS software (Carl Zeiss).

**Purification of recombinant proteins.** Purification of His-M1 was performed as previously described. To purify the PRC2 complex, 293T cells were transfected with plasmids expressing either 3xFLAG-EZH2 or 3xFLAG-SUZ12, and then incubated for 48 h. Transfected cells were lysed by sonication in a buffer containing 20 mM Tris–HCl (pH 7.9), 100 mM NaCl, and 0.1% Triton X-100. Lysates were incubated with FLAG antibody-conjugated agarose (Sigma) at 4°C for 1 h. After washing with a buffer (Wash buffer) containing 50 mM...
Tris-HCl (pH 7.9) and 175 mM KCl, 0.2 mM EDTA, 0.1% Triton X-100, and 10% glycerol, PRC2 complex was eluted with Wash buffer containing 0.5 mg/ml 3xFLAG peptide.

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Author Contributions
M.N.A., Y.S. and A.K. performed experiments. M.N.A., A.K., K.M. and K.N. conceived the research strategies and wrote the paper.

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