Extracellular vesicles derived from endothelial cells in hypoxia contribute to pulmonary artery smooth muscle cell proliferation in vitro and pulmonary hypertension in mice

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Abstract

In the lung, communication between pulmonary vascular endothelial cells (PVEC) and pulmonary artery smooth muscle cells (PASMC) is essential for the maintenance of vascular homeostasis. In pulmonary hypertension (PH), the derangement in their cell–cell communication plays a major role in the pathogenesis of pulmonary vascular remodeling. In this study, we focused on the role of PVEC-derived extracellular vesicles (EV), specifically their microRNA (miRNA, miR-) cargo, in the regulation of PASMC proliferation and vascular remodeling in PH. We found that the amount of pro-proliferative miR-210-3p was increased in PVEC-derived EV in hypoxia (H-EV), which contributes to the H-EV-induced proliferation of PASMC and the development of PH.

Keywords: extracellular vesicles, microRNAs, pulmonary artery smooth muscle cells, pulmonary hypertension, pulmonary vascular endothelial cells

INTRODUCTION

Pulmonary vascular endothelial cells (PVEC) and pulmonary artery smooth muscle cells (PASMC) are two key cell types involved in the pathogenesis of pulmonary vascular structural remodeling in pulmonary hypertension (PH) in all forms of PH.¹,² PVEC often act as the “signal initiator” in the process of vascular remodeling, affecting the function of PASMC via secretion of bioactive agents,¹,³–⁷ transfer of signals...
through myoendothelial injunctions (MEJ)\(^8\) and/or by releasing extracellular vesicles (EV),\(^9\) in both health and disease.

EV are a heterogeneous family of membrane-limited vesicles originating from the endosome or plasma membrane\(^10\) and are classified into three types based primarily on their size and presumed biogenetic pathways: exosomes (30–100 nm),\(^11–14\) microvesicles (MV) or ectosomes (100–1000 nm),\(^12,15–18\) and apoptotic bodies (1–5 µm),\(^12,19\) and they carry complex cargo including proteins, lipids, and nucleic acids, and so on.\(^10,20,21\)

In this study, we first showed that EV released from PVEC in culture in hypoxia (H‐EV) induced PASMC proliferation and when given to mice in room air intronously (i.v.), induced pulmonary vascular remodeling and PH. Next, we investigated the role of the microRNA \(^{22–25}\) (miRNA, miR‐) cargo in H‐EV in mediating these effects. Hypoxia significantly increased the amount of a known “pro‐proliferative” miRNA, miR‐210‐3p in PVEC‐derived EV, while inhibition of miR‐210‐3p in H‐EV significantly attenuated the ability of H‐EV to induce PH in mice in room air in‐vivo, suggesting that miR‐210‐3p in H‐EV plays a major role in H‐EV‐induced PH in mice in room air. Our study provided the first evidence of the role of endothelium‐derived EV in PH as well as the underlying mechanisms.

**METHODS AND MATERIALS**

**Cell culture**

Mouse and rat PVEC (mPVEC and rPVEC) were purchased from Cell Biologics and maintained in Endothelial Cell Medium containing 5% fetal bovine serum (FBS), endothelial cell growth factors, and antibiotics (Cell Biologics). Mouse and rat PASMC (mPASMC and rPASMC) were isolated from mouse lungs as we described previously\(^26\) and were maintained in SmGM‐2 medium (Lonza) containing 5% FBS, growth factors, and 1% penicillin‐streptomycin. We also isolated mPASMC from mice exposed to 10% O\(_2\) for 3 weeks and from mice exposed to room air for 3 weeks (controls).

Human pulmonary artery endothelial and smooth muscle cells (hPVEC and hPASMC) were purchased from Lonza and were maintained in EBM‐2 and SmGM‐2 medium containing FBS and growth factors (Lonza), respectively.\(^26\)

All cells were maintained in a humidified incubator with a constant supply of 5% CO\(_2\) at 37°C.

**Isolation of EV**

EV were isolated from cultured mPVEC using a multi‐step centrifugation method.\(^15,17,18,27\) Briefly, after obtaining confluent monolayers of mPVEC, the medium was refreshed with a medium free of FBS or vesicles, and mPVEC were then exposed to room air (normoxia, N) or hypoxia (1% O\(_2\), H). Conditioned medium was collected 24 hours (h) later and cell debris pre‐clear by centrifugation at 2000g for 15 min at 4°C. The supernatant was centrifuged at 20,500g for 1 h at 4°C. Pelleted vesicles (mainly MVs) were washed with ice‐cold vesicle‐free Dulbecco’s phosphate‐buffered saline (DPBS) and pelleted again by centrifugation at 20,500g for 1 h at 4°C. Finally, the pelleted vesicles were resuspended in vesicle‐free DPBS or lysed in QIAzol Lysis Reagent (Qiagen) for RNA isolation using the miRNeasy kit (Qiagen).

**Transmission electron microscope (EM)**

EV were mounted on 300‐mesh copper grids and stained with phosphotungstic acid (PTA). All specimens were examined with a JEM‐1220 transmission EM (JEOL USA) at Electron Microscopy Core of the University of Illinois at Chicago (UIC).

**Nanoparticle tracking analysis (NTA)**

The vesicle size and number were determined using NTA technology with a NanoSight LM10‐HS instrument (Malvern Instruments Ltd.) at the Flow Cytometry Core of UIC. EV were resuspended in vesicle‐free DPBS and diluted as needed (typically 1:100 to 1:1000 dilution), and then 1 ml samples were injected into the sample chamber using a sterile NORM‐JECT syringe (Henke‐Sass Wolf). Images of EV were captured and analyzed by Nanosight NTA 3.2 software. The camera level was set to 11–16 and the detection threshold at 2–5 to get the optimum brightness of images, and each sample was run for 30 s per time for three to four times. For samples in each experiment, the setting was kept constant.

**Imaging of EV in the pulmonary vessel wall**

We labeled EV with PKH26 dye (Sigma‐Aldrich) and then injected 5 × 10\(^6\) EV into mice through the tail vein. 10 min later, mice were sacrificed and lungs were collected for the frozen tissue section. PKH26‐labeled EV were imaged using Zeiss LSM 710 Confocal Microscope...
Western blot analysis

Isolated EV were lysed in mRIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% deoxycholate, 150 mmol/L NaCl, and protease inhibitors). 10 µg (for EV samples) or 30 µg (for cell samples) of protein lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to BA85 nitrocellulose membrane (PROTRAN; Whatman). Proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The following primary antibodies were used: CD144 (Santa Cruz Biotechnology), α-Tubulin, β-actin, α-smooth muscle actin (α-SMA), calponin, myosin heavy chain (MHC) (Sigma-Aldrich), smooth muscle protein 22-α (SM22α) (Abcam), Myocardin (R&D Systems), proliferating cell nuclear antigen (PCNA) (ProteinTech Group), CD31, protein disulphide isomerase (PDI), endoplasmic reticulum (ER) stress protein 72 (ERp72), receptor-binding cancer antigen expressed on SiSo cells (RCAS1), cytochrome c oxidase (COX) IV (Cell Signaling Technology, Danvers, MA). Detailed information about antibodies is in Table S1.

Flow cytometry analysis

For flow cytometry, EV were resuspended in PBS and incubated with Alexa-488 conjugated Annexin V (mouse, Enzo Lifesciences) for 15 min at 4°C in the dark. Non-specific isotype antibodies served as negative controls.

RNA isolation

Total RNA was isolated using a miRNeasy Mini Kit or miRNeasy Micro Kit (Qiagen) and treated with RNase-Free DNase I (Qiagen) quantified with Nanodrop 2000 spectrophotometer (Thermo Scientific) and then used for miRNA deep sequencing (LC Sciences) or quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

miRNA deep sequencing

Six batches of EV from mPVEC exposed to room air or hypoxia (1% O2, 24 h) (H-EV vs. N-EV) were collected for miRNA deep sequencing (LC Sciences). Briefly, a small RNA library was generated from RNA isolated from N-EV and H-EV using the Illumina Truseq™ Small RNA Preparation kit (Illumina). The purified cDNA library was used for cluster generation on Illumina’s Cluster Station and then sequenced on Illumina GAIIX (Illumina). Raw sequencing reads (40 nts) were obtained using Illumina’s Sequencing Control Studio software version 2.8 (SCS v2.8) following real-time sequencing image analysis and base-calling by Illumina’s Real-Time Analysis version 1.8.70 (RTA v1.8.70). The extracted sequencing reads were then normalized under a proprietary pipeline script of LC Sciences (ACGT101-miR v4.2) and used for statistical analysis (Research Informatics Core, UIC).

qRT-PCR

For qRT-PCR analysis of miRNA expression, a poly (A) tail was first added to the 3’-end of miRNAs using a Poly (A) Polymerase Tailing Kit (Eplicitr Biotechnologies). Poly (A) tailed-miRNAs were then reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen) with a poly (T) adaptor, which consists of a poly (T) sequence and a sequence complementary to the universal primer used in the following qRT-PCR analysis. For cell or tissue samples, SNORD44, SNORD47, SNORD48, and/or miR-16 were used as internal controls. qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on StepOnePlus or ViiA 7 Real-Time PCR System (Applied Biosystems). Primer sequences are in Table S2.

Bromodeoxyuridine (BrdU) cell proliferation assay

Cell proliferation was measured using a BrdU cell proliferation assay kit (EMD Millipore). Briefly, PASMC were plated into a 96-well plate at a density of 3000 cells/well and incubated overnight. BrdU label was added to the culture medium the next day and cells were cultured for another 16–18 h. Colorimetric measurements were carried out on a GloMax®—96 Microplate luminometer (Promega). For proliferation assays of PASMC with N- or H-EV, 2 × 10⁶ or 10⁷ EV were added per well and proliferation was measured 24 h later. For proliferation assay of PASMC transfected with miRNA oligos, BrdU label was added 24 h after transfection.

miRNA antagonist and mimics

All miRVana™ miRNA inhibitors and mimics for functional studies were purchased from Ambion, Thermo Fisher Scientific. Negative control miRNA inhibitor #1 or
Negative control miRNA mimic #1 was used as the miRNA inhibitor or mimic control, respectively.

**Animal studies**

All animals were cared for in accordance with the University of Illinois at Chicago Animal Care Policy. Animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Six- to eight-week-old male C57BL/6 mice were purchased from Jackson Laboratory. Details of each animal study were described below. For right ventricular systolic pressure (RVSP) measurements, animals were first anesthetized with ketamine (100 mg/kg body weight) and xylazine (5 mg/kg) via intraperitoneal (i.p.) injection. The anesthetics were given once before each procedure and depth of anesthesia was monitored by toe pinching to determine the loss of reflexes. After RVSP measurement, animals were euthanized under anesthesia followed by lung perfusion/exsanguination.

**Assessment of PH in animal studies (PH indices)**

RVSP, Fulton’s index, and lung vascular remodeling were used as indices of severity of PH.

**In-vivo RVSP measurements**

To measure RVSP, as a surrogate for pulmonary artery pressure, mice were anesthetized with ketamine/xylazine (100 mg/kg and 5 mg/kg body weight, i.p.) and placed on a heating pad to maintain body temperature. A Millar ultraminiature pressure transducer (1.0 Fr; Millar Instruments) was introduced into the right ventricle (RV) via the jugular vein to obtain pressure measurements.

**Measurement of ventricular weights to assess right heart hypertrophy**

The RV was dissected from the left ventricle and interventricular septum (LV+S), and the ratios of their weights (RV/[LV+S]) (Fulton’s index) was measured and calculated as an index of right ventricular hypertrophy.

**Lung vascular morphometry**

Lungs were inflated at 20 cm H₂O pressure and fixed with 10% buffered formalin via the trachea. Lungs were also perfused sequentially with PBS and 10% buffered formalin at the same pressure via the RV. Fixed lungs were paraffin-embedded, cut into 5 µm sections, and stained with hematoxylin and eosin (H&E) at the Research Histology and Tissue Imaging Core of UIC. The stained slides were scanned using Aperio ScanScope (Leica Biosystems). Pulmonary arterioles, typically 50–100 µm in diameter, adjacent to bronchioles, were measured for wall thickness, represented by the difference between the area of the entire vessel and the area of the lumen divided by the area of the entire vessel.

To determine if mPVEC-derived EV play a role in hypoxia-induced PH in-vivo, mice were injected with 5 × 10⁸ normoxic (N-EV) or hypoxic (H-EV) suspended in 100 µl sterile and vesicle-free DPBS per injection, once daily (i.v. via tail vein) for three consecutive days, while in room air. Control mice received an equal volume of DPBS. Three weeks after the first injection, mice were sacrificed and PH indices measured.

To study the role of miR-210-3p in H-EV-induced PH in mice, we first generated H-EV from mPVEC in which miR-210-3p level was inhibited (H-EV [anti-miR-210]). Briefly, mPVEC were transfected with miR-210-3p inhibitors (anti-miR-210; Ambion) or Negative control inhibitors (anti-Neg; Ambion) and then exposed to hypoxia (1% O₂) the next day, in a fresh medium free of FBS and vesicles. EV were collected after 24 or 48 h exposure (H-EV [anti-miR-210] or H-EV [anti-Neg]). Then, mice were injected with H-EV (anti-miR-210) or H-EV (anti-Neg) (10⁸ EV per daily injection for three consecutive days, via tail vein) while in room air. Three weeks later, mice were sacrificed and PH indices measured.

**Statistical analysis**

All experiments were repeated at least three times independently. For in-vivo experiments, at least five animals were used in each group, except for experiments with H-EV with anti-miR-210 when n = 3 (Figure 5). Student’s t tests were used when comparing two conditions and a one-way analysis of variance (ANOVA) with Bonferroni correction was used for multiple comparisons using GraphPad Prism 5 (GraphPad) and Microsoft Excel (Microsoft) when applicable. Data are presented as mean±SEM. For significant differences, p < 0.05, 0.01, and 0.001 was set. The power of in-vivo studies was calculated via https://www.stat.ubc.ca/~rollin/stats/s ssize/n2.html.
RESULTS

Characterization of PVEC-derived EV

EV collected from mPVEC were round and typically 100–200 nm in diameter under the electron microscope (EM, Figure S1A), suggesting that these particles are mostly MV (100–1000 nm in diameter). Hypoxia (1% O2, 24 h) did not alter the shape or size of this EV (Figure S1A). We analyzed the size and number of collected EV using NTA and confirmed that their number and size were unchanged in hypoxia (mode size: N- vs. H-EV: 153.3 nm vs. 139.3 nm, mean size: N- vs. H-EV: 237.6 nm vs. 209.6 nm) (Figure S1B). EV expressed specific endothelial cell marker proteins CD31 and CD144 (Figure S1C), confirming their endothelial origin, and they bound Annexin V, a feature of MV (Figure S1D).

Next, we investigated if hypoxia altered the expression of contractile proteins MHC, α-SMA, calponin, smooth muscle protein 22 α (SM22α), or myocardin in mPASMC (Figure S2). Similarly, we collected N- and H-EV from rat and human PVEC (rPVEC or hPVEC), and incubated rat and human PASMC (rPASMC or hPASMC) in room air with rPVEC and hPVEC-derived EV, respectively. We found that H-EV, and not N-EV, also induced rat and human PASMC proliferation in a dose-dependent manner (Figure 1c,d), indicating that these results are valid in different species.

Next, we injected N- or H-EV into C57BL/6 mice in room air through the tail vein daily for three consecutive days. Mice injected with PBS served as controls. Three weeks later, we measured PH indices (Figure 2a) and found that H-EV induced a modest but significant increase of RVSP (Figure 2b), RV hypertrophy (Figure 2c), and pulmonary arterial wall remodeling (Figure 2d), whereas N-EV did not. To determine if injected EV were reaching the pulmonary vessels, we injected mice with H-EV labeled with PKH26 Red Fluorescent Cell Membrane Labeling through the tail vein and their lungs were collected 10 min later for frozen section. Using confocal microscopy we confirmed that some of the PKH26-labeled EV localized in the pulmonary vessel wall (Figure S3). Taken together, our results suggest that EV released by PVEC in hypoxia can induce PASMC proliferation, vascular remodeling, and PH.

Hypoxia exposure alters the miRNA cargo in PVEC-derived EV and pulmonary vascular cells

As hypoxia did not alter the phenotype (size, number, shape, or surface markers) of mPVEC-derived EV (Figure S1), the functional effect of H-EV is likely due to a change in their cargo. We performed miRNA deep sequencing to compare the miRNA cargo in N- and H-EV. We set the cutoff for fold change of miRNA levels at 1.2 and identified eight miRNAs whose expression was significantly induced by hypoxia exposure (Figure 3a). Using qPCR analysis, we confirmed that the expression of miR-210-3p, 3057-5p, 212-5p, 34-3p, and 106b-3p were induced in H-EV (Figure 3b). We were unable to detect miR-505-3p using our qPCR analysis (data not shown).

Next, we investigated if hypoxia altered the expression of the above-identified miRNAs in mPVEC, mPASMC, and mouse lungs by qPCR analysis. We found that hypoxia exposure (1% O2) significantly induced miR-210-3p and 212-5p levels in mPVEC in culture after both 6 h and 24 h and miR-3057-5p levels after 24 h (Figure S4A). In mPASMC, hypoxia exposure only induced the expression of miR-210-3p, while the expression of miR-106b-3p was suppressed at 6 h (Figure S4B). Expression of miR-210-3p, as well as that of miR-212-5p, was induced in mPASMC isolated from mice exposed to hypoxia for 3 weeks (Figure S4C). Hypoxia (10% O2, 3 weeks) also induced the expression of miR-210-3p, 212-5p, and 34c-3p in mouse whole lungs (Figure S4D).
MiRNA in PVEC-derived EV regulate PASMC proliferation

To determine if the above-identified miRNAs contribute to the effect of H-EV on PASMC proliferation (Figure 1), we transfected mPASMC with mimics of the identified miRNAs (miR-210-3p, 3057-3p, 212-5p, 34c-3p, and 106b-3p) and then measured mPASMC proliferation using BrdU proliferation assay. We found that miR-210-3p was the only identified miRNA that induced mPASMC proliferation (Figure 4), consistent with the findings previously reported by our group and others.32,33 All of the remaining miRNAs either inhibited (miR-3057-5p, 212-5p, and 34c-3p) or showed no significant effect (miR-106b-3p) on mPASMC proliferation (Figure 4). These data suggest that although H-EV contains both “pro-proliferative” and “antiproliferative” miRNA cargo, the net effect of H-EV is increased proliferation in PASMC and induction of PH in vivo, indicating that miR-210-3p may play a predominant role in H-EV-induced PASMC proliferation and PH in mice. Our speculation can be supported by the finding that miR-210-3p was expressed at a higher level than other identified miRNAs in H-EV and hypoxic PVEC (Figure S5).

MiR-210-3p in H-EV is mainly responsible for H-EV-induced PH and pulmonary vessel remodeling in-vivo

To confirm if miR-210-3p plays a predominant role in H-EV-induced PASMC proliferation and PH in mice, we
generated H-EV containing decreased levels of miR-210-3p (H-EV [anti-miR-210]) by transfecting mPVEC with miR-210-3p inhibitors (anti-miR-210) and then collecting H-EV from these cells. mPVEC transfected with Negative control miRNA inhibitors (anti-Neg) were used to generate control H-EV (anti-Neg). Decreased levels of miR-210-3p in mPVEC and mPVEC-derived H-EV were validated by qPCR analysis (Figure S6). Then we injected mice in room air with H-EV (anti-Neg) or PBS for 3 days and measured PH indices 3 weeks later (similar to the protocol for Figure 2a). We found that mice injected with H-EV (anti-Neg) had increased RVSP (Figure 5a), RV hypertrophy (Figure 5b), and pulmonary vessel remodeling (Figure 5c) similar to mice injected with H-EV (Figure 2), while mice injected with H-EV (anti-miR-210), had no elevation in RVSP (Figure 5a), had significantly reduced RV hypertrophy (Figure 5b) and had attenuated pulmonary vessel wall remodeling (Figure 5c,d). These data indicate that
miR-210-3p in H-EV plays a major role in H-EV-induced PH in mice in room air.

DISCUSSION

In this study, we demonstrated for the first time that PVEC-derived EV in hypoxia induce PASMC proliferation in vitro and induce PH in mice in room air (Figures 1 and 2). Though many extrinsic and intrinsic mechanisms for PASMC proliferation exist and other constituents in EV cargo may be contributing to the increased PASMC proliferation and PH in hypoxia, we have demonstrated an important contributory role of EV and their miRNA cargo in PH.

Recent studies have shown an association between dysregulated circulating levels of endothelium-derived EV and pathogenesis of PH. Tual-Chalot et al. demonstrated that EV obtained from the blood of rats exposed to hypoxia inhibited endothelium-dependent relaxation of rat pulmonary arteries. Aliotta and colleagues found that healthy mice injected with EV (mostly exosomes) isolated from lung cells or plasma of MCT-treated mice developed significant RV hypertrophy and pulmonary vascular remodeling. These studies are largely focused on EV from whole lungs or those in circulation, the cellular origin of which has not been determined. A recent study by Zhu et al. showed that cigarette smoke enhances EV generation from the endothelium, with spermine enrichment on their outer surface and cytosol, and that they contribute to SMC constriction and proliferation, and consequently the development of PH. However, from these studies our knowledge about the specific cargo in EV that was responsible for inducing vascular remodeling and PH is very limited.

We focused on the profile of miRNA cargo in the EV and the change in this profile induced by hypoxia. We found that miR-210-3p was increased in hypoxic EV (H-EV) and that H-EV could induce PASMC proliferation. Although hypoxia can directly stimulate the expression of miR-210-3p in PASMC and its role in hypoxia-induced PH has been previously reported by us and others, EV may also deliver an additional load of miR-210-3p to PASMC in hypoxia and further contribute to hypoxia-induced PASMC proliferation and pulmonary vessel remodeling. In this study, we demonstrated that inhibition of miR-210-3p in H-EV significantly attenuated the ability of H-EV to induce PH in mice in room air in vivo (Figure 5), indicating that miR-210-3p in H-EV plays a major role in inducing pulmonary vascular remodeling and PH. Although we also identified anti-proliferative miRNAs, miR-3057-3p, 212-5p, 34c-3p, in their cargo (Figure 4), the net effect of H-EV is increased PASMC proliferation in vitro and PH in mice, which can be explained by the greater expression level of miR-210-3p in H-EV (Figure S5). The finding that EV cargo contains miRNAs that both stimulate as well as inhibit PASMC proliferation suggests that under certain conditions the relative amounts of these miRNAs in EV may be modulated to either exacerbate or inhibit PASMC proliferation and vascular remodeling. Certainly, it is
possible that other cargos (like proteins, etc.) in H-EV may also be transferred to induce PASMC proliferation and contributes to the development of PH. Further studies are warranted.

ACKNOWLEDGMENT
We thank UIC Electron Microscopy Core, Fluorescence Imaging Core, Research Informatics Core and Research Histology and Tissue Imaging Core (RHTIC) for their professional services. This study is partly supported by an NIH R01HL123804 (Raj and Zhou), an American Lung Association Biomedical Research Award RG-416135 (Chen), an American Heart Association Career Development Award 18CDA34110301 (Chen), and a Gilead Sciences Research Scholars Program in Pulmonary Arterial Hypertension (Chen), an NIH 1R56-HL141206-01 (Raj and Chen), a Chicago Biomedical Consortium Catalyst Award (Raj, Chen, and Hubbell).

CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

ETHICS STATEMENT
All animals were cared for in accordance with the University of Illinois at Chicago Animal Care Policy. Animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS
Tianji Chen and J. Usha Raj contributed to the conception and design of the work and interpretation of data; Tianji Chen, Miranda R. Sun, Qiuyuan Zhou, Alyssa M. Guzman, Ramaswamy Ramchandran, Jiwang Chen, and Balaji Ganesh contributed to the acquisition of data; Tianji Chen, Miranda R. Sun, Ramaswamy Ramchandran, and Balaji Ganesh contributed to the analysis of data. Tianji Chen and J. Usha Raj have drafted the work and Miranda R. Sun also contributed to editing the manuscript.

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