Genetics and Molecular Research

Hypoxia enhances periodontal ligament stem cell proliferation via the MAPK signaling pathway

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Genet. Mol. Res. 15 (4): gmr15048965
Received July 13, 2016
Accepted September 5, 2016
Published November 21, 2016
DOI http://dx.doi.org/10.4238/gmr15048965

ABSTRACT. There is high incidence of periodontal disease in high-altitude environments; hypoxia may influence the proliferation and clone-forming ability of periodontal ligament stem cells (PDLSCs). The MAPK signaling pathway is closely correlated with cell proliferation, differentiation, and apoptosis. Thus, we isolated and cultured PDLSCs under hypoxic conditions to clarify the impact of hypoxia on PDLSC proliferation and the underlying mechanism. PDLSCs were separated and purified by the limiting dilution method and identified by flow cytometry. PDLSCs were cultured under hypoxic or normoxic conditions to observe their cloning efficiency. PDLSC proliferation at different oxygen concentrations was evaluated by MTT

assay. Expression of p38/MAPK and MAPK/ERK signaling pathway members was detected by western blotting. Inhibitors for p38/MAPK or ERK were applied to PDLSCs to observe their impacts on clone formation and proliferation. Isolated PDLSCs exhibited typical stem cell morphological characteristics, strong abilities of globular clone formation and proliferation, and upregulated expression of mesenchymal stem cell markers. Stem cell marker expression was not statistically different between PDLSCs cultured under hypoxia and normoxia ($P > 0.05$). The clone number in the hypoxia group was significantly higher than that in the control ($P < 0.05$). PDLSC proliferation under hypoxia was higher than that of the control ($P < 0.001$). p38 and ERK1/2 phosphorylation in hypoxic PDLSCs was markedly enhanced compared to that in the control ($P < 0.05$). Either P38/MAPK inhibitor or ERK inhibitor treatment reduced clone formation and proliferation. Therefore, hypoxia enhanced PDLSC clone formation and proliferation by activating the p38/MAPK and ERK/MAPK signaling pathways.

Key words: Hypoxia; Periodontal ligament stem cells; Cell proliferation; Clone formation

INTRODUCTION

Periodontitis is one of the most common periodontal diseases. As the main cause of adult tooth loss, periodontitis is characterized as the lack of periodontal support tissue (Hackett and Roach, 2001; Dumitrescu, 2016). Periodontitis has a relatively high incidence; 46% of Americans exhibit chronic periodontitis (Eke et al., 2015), and the incidence is even higher in developing countries (Corbet, 2006). China exhibits a high incidence of periodontal disease; 80-97% of adults are affected (Wu et al., 2015). Periodontal health has therefore become a global health problem.

In recent years, tissue engineering repair technology based on the theory of pluripotent cells in periodontal tissue became a hot spot in treating periodontal ligament disease. As an important component of periodontal tissue, periodontal ligaments (PDLs) play a critical role in maintaining the integrity of periodontal tissue (Somerman et al., 1990). PDLs maintain self-renewal and repair capabilities through the function of PDL cells (Trubiani et al., 2016). Numerous evidence demonstrated that stem cells in the PDL are the reason for periodontal tissue regeneration. In 2004, Seo et al. (2004) identified periodontal ligament stem cells (PDLSCs) from periodontal tissue and proposed this concept for the first time. PDLSCs promote the development of tissue engineering because of their characteristic clone-forming ability, proliferation, and pluripotent stem cell differentiation potential (Mattioli-Belmonte et al., 2015; Vandana et al., 2015).

However, the function of PDLSCs is regulated by various factors, including external stimuli and immune status. Many epidemiological studies reported that the incidence of periodontitis in residents living in areas with altitudes over 4000 meters was significantly higher than that in residents living at sea level (Jian et al., 2014). Animal experiments also revealed that hypoxia may induce severe periodontitis (Xiao et al., 2012; Terrizzi et al., 2013). The abovementioned evidence showed that hypoxic microenvironments may contribute to

periodontal cells' inability to regenerate. Furthermore, a previous study also demonstrated that exposure of PDLSCs to hypoxia affected their osteogenic potential, mineralization, and paracrine release (Wu et al., 2013). However, whether hypoxia exerts an effect on PDLSC proliferation remains poorly understood. Thus, we speculated that hypoxia may obstruct the self-renewing ability of PDLSCs. In this study, we clarified the impact of hypoxia on PDLSC proliferation and clone formation by isolating PDLSCs and cultivating them under hypoxia.

The mitogen-activated protein kinase (MAPK) signaling pathway is one of the most important biological signaling pathways. Its activation regulates a variety of cellular behaviors such as cell proliferation, differentiation, and apoptosis. The MAPK signaling pathway, including members p38 and ERK, plays a key role in the ability of bone marrow mesenchymal stem cells to differentiate into osteogenic cells (Jaiswal et al., 2000; Liu et al., 2009), and is related to cell self-renewal (Wang et al., 2016; Xu et al., 2016). Hypoxia can activate the p38/MAPK and MAPK/ERK signaling pathways (Qiu et al., 2016). Therefore, this study explored the impact of hypoxia on PDLSC self-renewal and related mechanisms, by regulating the expression and activity of p38/MAPK and MAPK/ERK signaling pathway-related proteins.

MATERIAL AND METHODS

Main instruments and reagents

DMEM medium was obtained from Gibco. Fetal bovine serum was obtained from Every Green Co., Ltd. (China). Penicillin-streptomycin was obtained from Hyclone. PBS was obtained from ZSbio. Type II collagenase and MTT were obtained from Sigma. PE-labeled STRO-1, CD146, CD44, and CD45 antibodies were obtained from Bioscience. RIPA lysis buffer was obtained from Beyotime. BCA protein quantification kit was from Shanghai Shengneng Bocai Co., Ltd. HRP-labeled mouse anti-human GAPDH monoclonal antibody was obtained from KangChen. Protein marker was obtained from Tiangen Biotech Co., Ltd. PVDF membranes were obtained from Millipore. The FC 500 MPL flow cytometry system was from Beckman. The automatic model 680 microplate reader was from Bio-Rad. X-ray film was from Fuji. The dental X-ray machine 2200 was from Eastman Kodak. Vertical electrophoresis apparatus E5889 was from Sigma. The 3111 water jacketed CO_2 incubator was from Thermo.

Human PDLSC isolation and cultivation

The third molar from 15 healthy donors (Han Chinese nationality) with a median age of 39 years (range: 18-56 years) received dental orthodontic treatment in the Second Affiliated Hospital, Third Military Medical University. The tissue was washed with PBS containing penicillin-streptomycin and periodontal tissue was isolated from the root of tooth. The tissue was cut with sterile scissors to a size of 1-3 mm and placed to a centrifuge tube. After centrifugation at 1200 rpm for 5 min, the tissue was treated with type II collagenase and oscillation-digested in a 37°C constant temperature water bath for 1.5 h. Next, the tissue was filtered through a 70-μm mesh and centrifuged at 1000 rpm for 5 min to prepare a single cell suspension. The cells were washed in DMEM supplemented with 10% FBS and penicillin-streptomycin three times and seeded in cell culture dishes at 1 x 10^4 cells/mL. Cells were cultured at 37°C and 5% CO_2 and medium was changed every 3-5 days. All donors gargled

with chlorhexidine before tooth extraction. Preoperative X-rays showed no dental caries, periodontal disease, bone absorption, or root lesions. The experiment was approved by the Ethics Committee of Second Affiliated Hospital, Third Military Medical University, and informed consent was obtained from all subjects.

Flow cytometry

PDLSCs were seeded on six-well plates at 1 x 10^6 cells/well and then were assigned to hypoxia or normoxia groups. Cells in the hypoxia group were incubated in 37°C, 5% CO_2, and 5% O_2, whereas the cells in the normoxia group were maintained in 37°C, 5% CO_2, and 20% O_2. The cells were digested with 0.25% trypsin when the confluency reached 80% and were then resuspended at 1 x 10^6 cells/mL. PDLSCs in 100 μL PBS were stained with 5 μL PE-labeled antibodies against STRO-1, CD44, CD45, and CD146, and then incubated at 4°C in the dark for 30 min. After centrifugation at 2000 rpm for 5 min, cells were resuspended in 500 μL PBS and analyzed by flow cytometry. All experiments were repeated at least three times.

MTT assay

Cell proliferation was detected by an MTT kit (Sigma, St. Louis, MO, USA) according to the manufacturer protocol. PDLSCs were seeded on 96-well plates at 5 x 10^3 cells/well and incubated under hypoxia or normoxia. After intervention, 20 μL MTT at 5 mg/mL was added to each well and the plate was incubated at 37°C for 3 h. Finally, the plates were treated with 150 μL DMSO to resolve the crystals, and optical density at 570 nm was read. The experiments were repeated at least three times.

Clone formation assay

PDLSCs were seeded on six-well plates at 1 x 10^6 cells/well and subjected to either hypoxia or normoxia. The cells were digested with 0.25% trypsin and washed with PBS three times. Next, cells were seeded on 96-well plates at different concentrations (1 x 10^4, 1 x 10^3, and 1 x 10^2 cells/well). After 14 days of culture, the cells were fixed in 10% methanol and stained with 0.5% crystal violet. Then, cell clones was observed under the microscope. Clones containing more than 50 cells were considered positive. Cloning efficiency = clone number / seeded cell number x 100%.

Western blotting

After hypoxic intervention, PDLSCs were incubated with RIPA buffer on ice for 30 min and centrifuged at 12,000 rpm for 25 min. The protein contents of the collected supernatant were quantified by BCA kit according to the manufacturer protocol. A total of 20 μg protein was mixed with 105 μL water and 125 μL Laemmli loading buffer containing dithiothreitol at 54 mg/mL, and boiled at 100°C for 10 min. Proteins were separated by SDS-PAGE at 180 V for 1 h, and then transferred to PVDF membranes at 100 mA for 80 min. After blocking with 5% skim milk, the membrane was incubated with the appropriate primary antibody at 4°C overnight. Next, the membrane was washed with TBST five times and incubated in the appropriate HRP-labeled secondary antibody at 37°C for 1 h. Finally, the membrane was incubated with ECL reagent at 22°C for 2-3 min and exposed to X-ray film.

Statistical analysis

The SPSS19.0 software was utilized for data analysis. Normally distributed data are reported as means ± standard deviation and groups were compared by the Student *t*-test. A significant difference was considered when $P < 0.05$.

RESULTS

PDLSC isolation, cultivation, and identification

After 14 days of cultivation, cell morphology was observed as shown in Figure 1A. The cells exhibited long fusiform or irregular shapes, small volume, and spiral or radial alignment, similar to known characteristics of periodontal ligament cells.

PDLSCs were seeded on 96-well plates and cultured for 21 days to observe clone formation. As shown in Figure 1B, the clone number in PDLSCs was significantly higher than that observed in PDL cells ($P < 0.05$).

Flow cytometry was performed to test for stem cell surface marker expression on PDLSCs and PDL cells. The expression rates of STRO-1, CD146, CD44, and CD45 on PDLSCs were 93.4, 92.8, 93.6, and 2.4%, respectively, which was significantly higher than their expressions on the PDL cell surfaces at 1.8, 2.2, 2.8, and 1.6%, respectively ($P < 0.05$).

MTT assays were performed to determine cell proliferation, and the proliferative ability of PDLSCs on days 3-7 was markedly higher than that of PDL cells ($P < 0.05$) (Figure 1C and D).

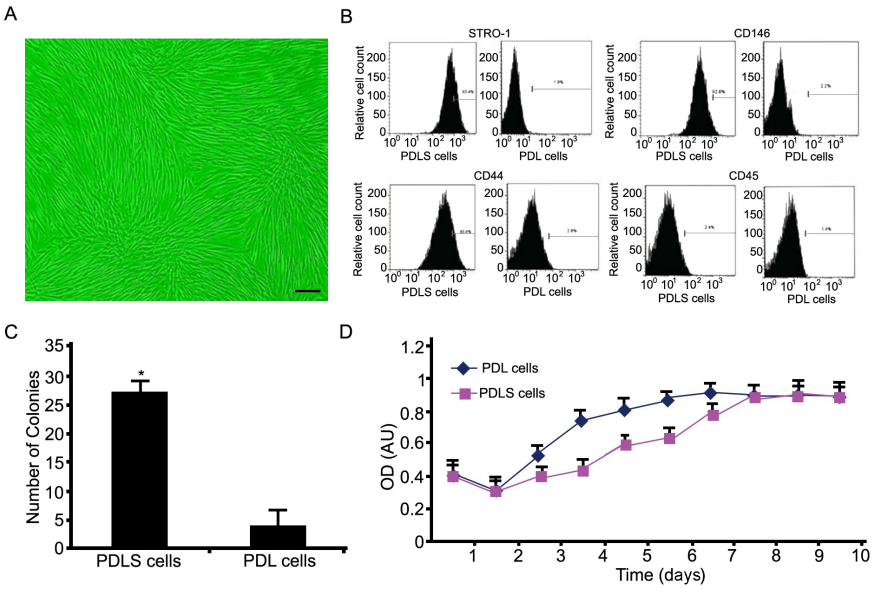

Figure 1. PDLSC isolation, cultivation, and identification. Tissues were extracted and a single cell suspension was prepared, followed by seeding onto cell culture dishes. **A.** Cells cultured under normal conditions for morphologic analysis of the isolated PDLSCs by microscopy. Bar = 100 µm. **B.** Cells collected for analysis of stem cell surface marker expression (STRO-1, CD146, CD44, and CD45) by flow cytometry. **C.** Clone formation and **D.** cell proliferation by MTT assay.

Impact of hypoxia on PDLSC surface marker expression

To explore the effect of hypoxia in maintaining PDLSC self-renewal ability and pluripotency, PDLSCs were cultured under hypoxia or normoxia for 7 days. Flow cytometry was performed to examine stem cell marker expression on PDLSCs. As shown in Figure 2, STRO-1, CD146, CD44, and CD45 levels showed no statistical difference between the hypoxia and normoxia groups ($P > 0.05$), indicating that PDLSCs can maintain pluripotency under hypoxic conditions.

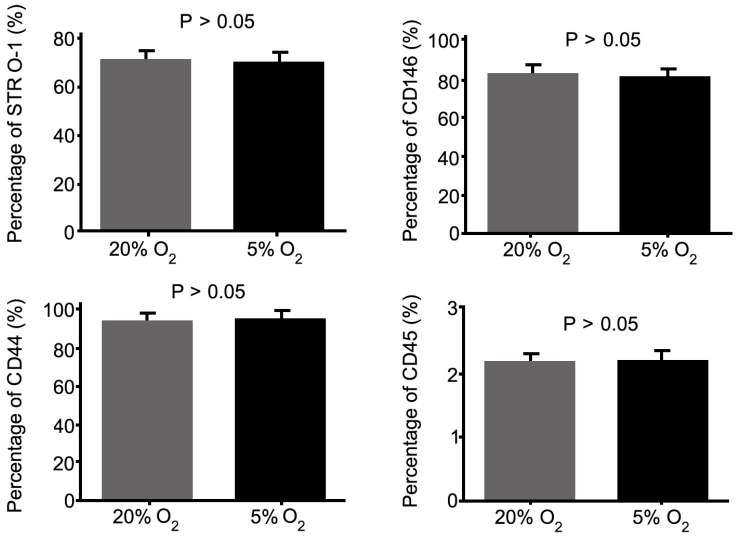

Figure 2. Impact of hypoxia on PDLSC surface marker expression.

Effect of hypoxia on PDLSC clone-forming ability

PDLSC clone-forming ability was evaluated after hypoxic culture for 14 days. As shown in Figure 3, the clone number of PDLSCs in the hypoxia group was significantly higher than that in the control ($P < 0.05$).

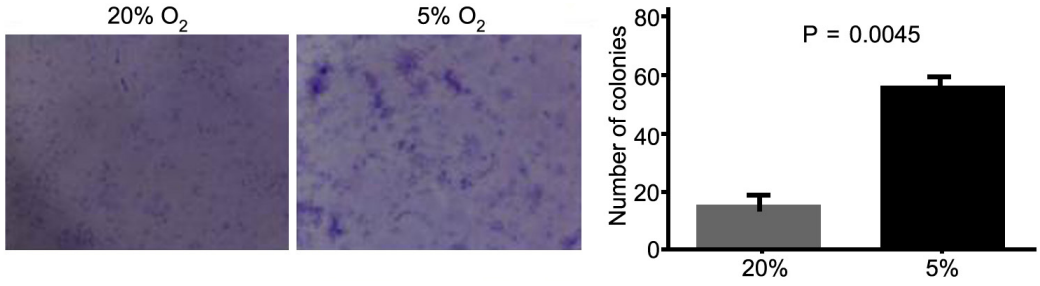

Figure 3. Effect of hypoxia on PDLSC clone formation ability.

Influence of hypoxia on PDLSC proliferation

MTT assays were adopted to determine PDLSC proliferation under different oxygen concentrations and time points. As shown in Figure 4, PDLSC proliferation rate in the hypoxic group was significantly different from that in the control. On days 3-6, the rate of PDLSC proliferation under hypoxia was obviously higher than that in the control ($P < 0.001$).

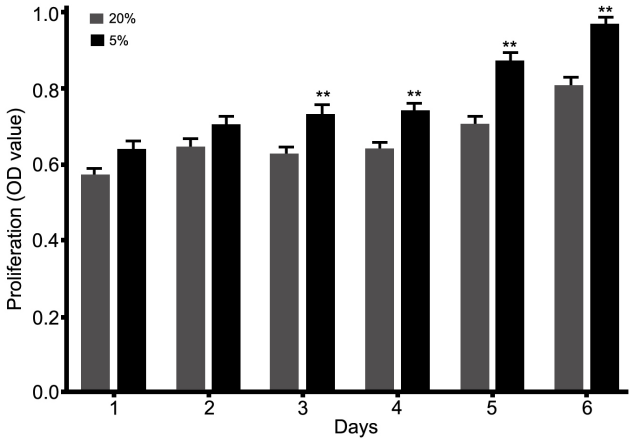

Figure 4. Influence of hypoxia on PDLSC proliferation. **P < 0.001 *vs* control.

Hypoxia activates p38/MAPK and MAPK/ERK signaling pathways in PDLSCs

It was reported that the p38/MAPK and MAPK/ERK signaling pathways are related to PDLSC self-renewal (Bernet et al., 2014; Sun et al., 2015; Rodríguez-Carballo et al., 2016). Therefore, western blotting was performed to detect related protein expression in PDLSCs after hypoxic culture for 48 h (Figure 5). Total p38 and ERK1/2 protein levels were not significantly different in PDLSCs grown under different oxygen concentrations, whereas p38 and ERK1/2 phosphorylation was markedly increased in the hypoxia group compared with that in the control ($P < 0.05$).

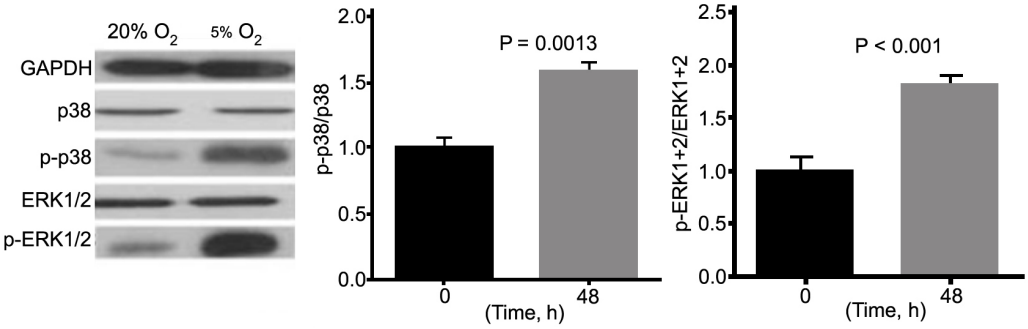

Figure 5. Hypoxia activated p38/MAPK and MAPK/ERK signaling pathways in PDLSCs.

Enhancement of PDLSC clone formation and proliferation is related to p38/MAPK and MAPK/ERK signaling pathway activation

To investigate the impact of the MAPK signaling pathway on PDLSC self-renewal under hypoxic conditions, p38/MAPK or ERK inhibitors were used to pretreat PDLSCs. As shown in Figure 6, PDLSC clone formation and proliferation in the p38/MAPK inhibitor group, ERK inhibitor group, and p38/MAPK + ERK inhibitor group were significantly lower than in the control ($P < 0.05$).

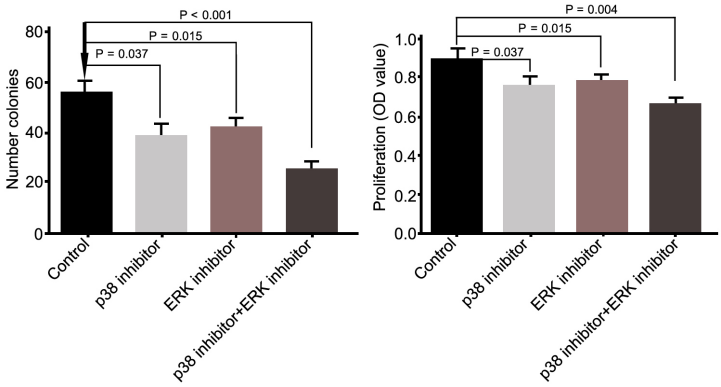

Figure 6. ERK and p38 inhibition suppressed PDLSC clone formation and proliferation under hypoxia.

DISCUSSION

The current methods for adult stem cell separation include immunomagnetic cell sorting, density gradient centrifugation, and continuous clonal cultivation. For PDLSCs, the main isolation method is limiting dilution. Therefore, in this study, we applied the limiting dilution method to separate and purify PDLSCs from molars of healthy adults. The typical ultrastructure of stem cells could be observed in these cells by optical microscopy. Another important feature of stem cells is their strong ability to form spherical clones. It was reported that PDLSCs formed spherical clones after culture for 10-15 days (Yang et al., 2009). Our study also revealed that the clone number in PDLSCs was significantly higher than that observed for PDL cells, in accordance with a previous report (Gay et al., 2007). This result indicated that these cells have strong self-renewal ability. In addition, PDLSCs show characteristics of mesenchymal stem cells. In addition to STRO-1 and CD146 expression, these cells also express CD105/14/44/106/166/106/166 (Tang et al., 2014; Park et al., 2015). Therefore, flow cytometry was applied to identify stem cell markers on PDLSCs. PDLSCs highly expressed the stem cell markers, including STRO-1, CD146, CD44, and CD45, indicating that PDLSCs were successfully isolated from the periodontal tissue.

Oxygen is an important element that maintains organ function and homeostasis. Hypoxia can promote osteoblast proliferation in the periodontal microenvironment (Li et al., 2015), as well as periodontal ligament fibroblast proliferation (Zhang et al., 2013), revealing that hypoxic environments may affect PDLSC proliferation. Therefore, PDLSCs were cultured under hypoxia and normoxia to examine the influence of hypoxia on PDLSCs. Hypoxia clearly enhanced PDLSC clone formation and proliferation, revealing that PDLSCs can maintain their

self-renewal ability *in vitro* under hypoxic conditions. Amemiya et al. (2008) reported that hypoxia can promote the proliferation of periodontal ligament cells. Consistent with this, a previous study (Zhang et al., 2014) also showed that upon exposing human PDLSCs to hypoxia for 7 days, the proliferation rate was increased and a higher osteogenic differentiation potential than that of control cells was observed. Twelve weeks after transplantation, hypoxia-treated PDLSCs differentiated into osteoblast-like cells that formed bone-like structures (Zhang et al., 2014).

In addition, we also explored the mechanism of hypoxia that promotes PDLSC clone formation and proliferation. p38 and ERK1/2 phosphorylation was significantly enhanced in PDLSCs after hypoxic intervention, whereas p38/MAPK or ERK/MAPK inhibition effectively suppressed PDLSC proliferation and clone formation. This result indicated that PDLSC self-renewal under hypoxia is regulated by the p38/MAPK and ERK/MAPK signaling pathways. Matsuda et al. (1998) reported that hypoxia can activate ERK1/2 to regulate the proliferation of periodontal ligament cells.

Therefore, we successfully separated and cultured PDLSCs and investigated the impact of hypoxia on their proliferation and clone-forming ability. Hypoxia enhanced clone formation and proliferation by activating the p38/MAPK and ERK/MAPK signaling pathways, indicating that PDLSC self-renewal is maintained under hypoxia. However, we were unable to provide an explanation for the high incidence of periodontal disease at high elevations; thus, further investigation is needed to clarify the effect of hypoxia on PDLSCs.

CONCLUSION

Hypoxia can enhance PDLSC clone formation and proliferation, which may be related to the activation of the p38/MAPK and ERK/MAPK signaling pathways.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

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