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Olig2+ single-colony-derived cranial bone-marrow mesenchymal stem cells achieve improved regeneration in a cuprizone-induced demyelination mouse model

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Abstract: Multiple sclerosis (MS) is a major demyelinating disease of the central nervous system, with no effective treatment available at present. This study investigated the effects of cranial bone-marrow mesenchymal stem cells (cBMMSC) and Olig2+ single-colony-derived cBMMSC (sc-cBMMSC) in a central nervous system demyelination mouse model, which was established by cuprizone induction. cBMMSC and Olig2+ sc-cBMMSC were administered to the mice via tail vein at 1×10^6 cells/100μL PBS once a week for two weeks. One week after the completion of treatment, the mice in each group were evaluated for the relevant indicators. The results showed that cBMMSC transplantation could significantly improve the performance of CPZ-induced demyelinated mice in Rotarod, suspension, and water-maze tests. The levels of inflammatory factors, such as TNFα, IL-1β, and IL-6, in the peripheral and central nervous system of the mice were suppressed. Furthermore, the pathological structure was improved, with significantly restored myelin and increased expression of Olig2 and Syn. Of note, Olig2$^+$ sc-cBMMSC achieved improved promotion of behavioral improvement, myelin repair, and nerve-cell regeneration in CPZ animals compared to cBMMSC. These findings suggest a reparative effect of cranial bone-marrow-derived stem cells in demyelination, which may be ascribed to the excellent immune regulation and protection of oligodendrocytes. The more pronounced effect of Olig2+ sc-cBMMSC transplantation further confirms the fact that heterogeneity of MSCs should be considered and utilized to improve the therapeutic effect in specific diseases.

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Key words: Cranial bone marrow; single colony; mesenchymal stem cells; oligodendrocytes; demyelination

Oligodendrocytes (OL) are the myelinating cells of the central nervous system. Brain injury and neurodegenerative disease often lead to OL cell death and subsequent demyelination-related pathological changes, resulting in neurological defects and cognitive impairment (Spaas et al., 2021; Zhang et al., 2022a). Multiple sclerosis (MS) is a major demyelinating disease of the central nervous system. The pathology of MS is characterized by the loss of myelin, oligodendrocytes and axons in the brain, brain stem, and spinal cord, as well as by white matter lesions (Lassmann et al., 2007). Unfortunately, no definitive cure for MS has been developed. Immunomodulatory and anti-inflammatory drugs are effective in the relapsing-remitting phase of MS because

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they reduce the frequency of relapses and the formation of inflammatory lesions; however, they do not alter the course of progressive MS and are insufficient to cure chronic neurological dysfunction (Xiao et al., 2015; Zhang et al., 2021). The treatment outcome is even worse for MS patients with primary and secondary progression. Mesenchymal stem cells (MSCs) are stromal cells that can self-renew and exhibit multilineage differentiation. MSCs are easy to expand *in vitro* and exhibit low immunogenicity, no tumorigenic risks, and ethical controversies, making them a promising candidate for regenerative medicine (Zhang et al., 2022b; Xu et al., 2023). Many studies have confirmed the neural differentiation potential of MSCs under certain conditions, making them a prime candidate for treating neurodegenerative diseases (Jang et al., 2010; Yan et al., 2013). The present study investigated the effects of cranial bone-marrow mesenchymal stem cells (cBMMSC) and Olig2$^+$ single colony-derived cBMMSC (sc-cBMMSC), isolated in our previous work (Yang et al., 2022), in a central nervous system demyelination mouse model.

First, we established the mouse model of central nervous system demyelination by feeding mice with 0.2% cuprizone (CPZ) for six weeks (see supplementary file for the specific methods) (Hedayatpour et al., 2013), and the animals were randomly divided into a model group, vehicle group, cBMMSC group, and Olig2$^+$ single colony-derived cranial bone-marrow mesenchymal stem-cell (sc-cBMMSC) group (N=7). The cBMMSCs and sc-cBMMSCs were isolated from the mononuclear cell content of cranial bones by adherent culture and passaging. To establish a sc-cBMMSC isolate, individual colonies were identified under a phase-contrast microscope and harvested using sterile cloning cylinders (see supplementary file for the specific methods). Cells at the 5th passage were used in the following treatments. The mice were administered cell isolates via tail vein at 1×10^6 cells/100 μL PBS once a week for two weeks. One week after the completion of treatment, mice in each group were evaluated for the relevant indicators. In the rod-rotating experiment, the rod-rotating time of CPZ model mice was significantly shorter than normal ($P<0.05$). Both cBMMSC and sc-cBMMSC transplantation substantially improved rod-rotating time ($P<0.05$, $P<0.01$, respectively), and the effect was more significant in the sc-cBMMSC group ($P<0.05$) (Figure 1a). The same change trend was observed in the suspension experiment; the hanging time of animals in the CPZ model group was significantly shorter than that of those in the normal group ($P<0.01$). Administration of cBMMSC slightly expanded mouse hanging time, but the difference was not significant ($P>0.05$). The animals in the sc-cBMMSC transplantation group exhibited significantly improved hanging time compared to the model group ($P<0.05$) (Figure 1b). We also conducted a water-maze experiment. During days 2-5 of positioning and navigation training, the time it took for mice in each group to find the platform gradually shortened with the increasing number of training days.The results on day 5 showed that compared with the model group, both the cBMMSC and sc-cBMMSC transplantation groups achieved significantly reduced times ($P<0.01$), and the effect was more significant in the sc-cBMMSC group ($P<0.05$) (Figure 1c). In terms of the number of crossings, those for the CPZ model group were significantly lower than the normal group ($P<0.01$), and those for the cBMMSC and Olig2$^+$ sc-cBMMSC transplantation groups were significantly higher ($P<0.05$, $P<0.01$) (Figure 1d). These findings suggest that cranial-derived MSC transplantation improves the movement coordination, muscle strength, and learning and memory abilities of CPZ mice, and that the effect of sc-cBMMSC transplantation is more pronounced.

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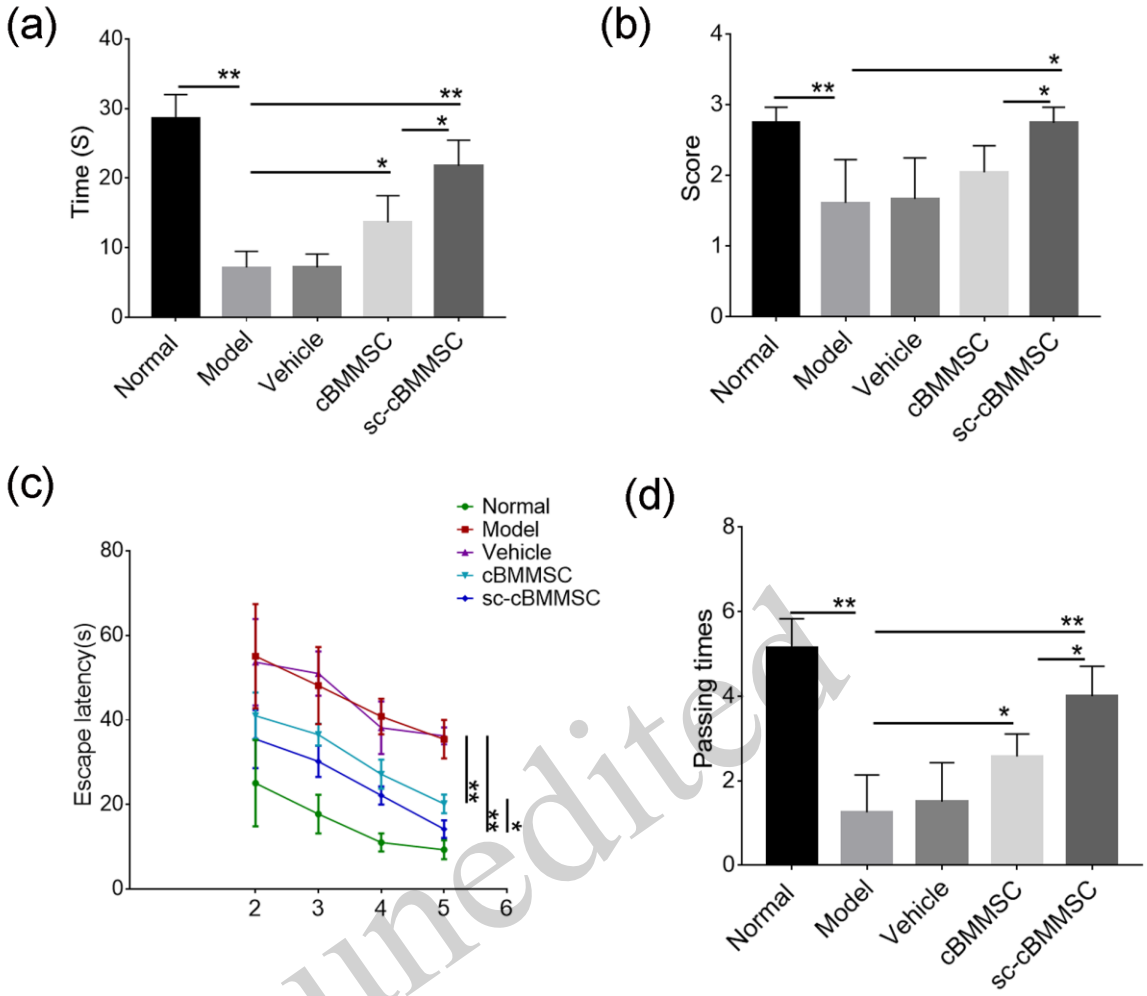

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Figure 1. Assessment of mouse behavior. (a): Rotation time for each group of mice in the Rotarod experiment; (b): Hanging time for each group of mice in the suspension experiment. (c): Positioning cruise training for the water-maze experiment, with the time it took for each group of animals to find the platform at different time points. (d): Number of times the animals in each group crossed the water maze on day 6. *, P<0.05; **, P<0.01.

Next, we assessed the levels of peripheral and central inflammatory factors in each group of mice (Avşar et al., 2021). The ELISA results showed that the levels of serum TNFα, IL-1β, IL-6, and other inflammatory factors in the peripheral blood of animals in the CPZ model group were significantly increased. MSC transplantation significantly suppressed levels of the above inflammatory factors (P<0.05) (Figure 2a). There was no significant difference between the cBMMSC and sc-cBMMSC groups (P>0.05). The brain-tissue protein-detection results were similar to those for peripheral blood (Figure 2b). T-cell subpopulations that were sensitive to CPZ were further analyzed (Solti et al., 2015). Compared with the normal group, the proportions of CD3+ and CD4+ T cells in the peripheral blood of the CPZ model group were significantly higher, as determined by flow assays (P<0.05). In the MSC transplantation groups, the proportions of CD3+ and CD4+ T cells were significantly suppressed (Figure 3), suggesting that these cells have excellent immune-regulatory capabilities.

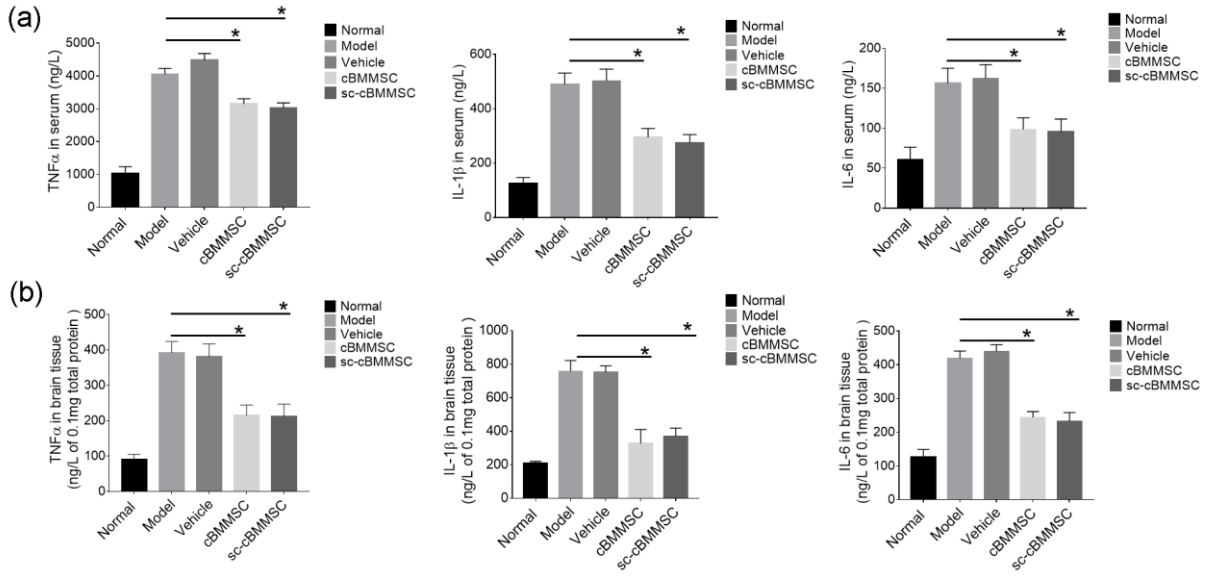

Figure 2. Expression levels of inflammatory factors. (a): ELISA detection results for serum inflammatory factors. (b): ELISA detection results for brain tissue inflammatory factors. *, P<0.05.

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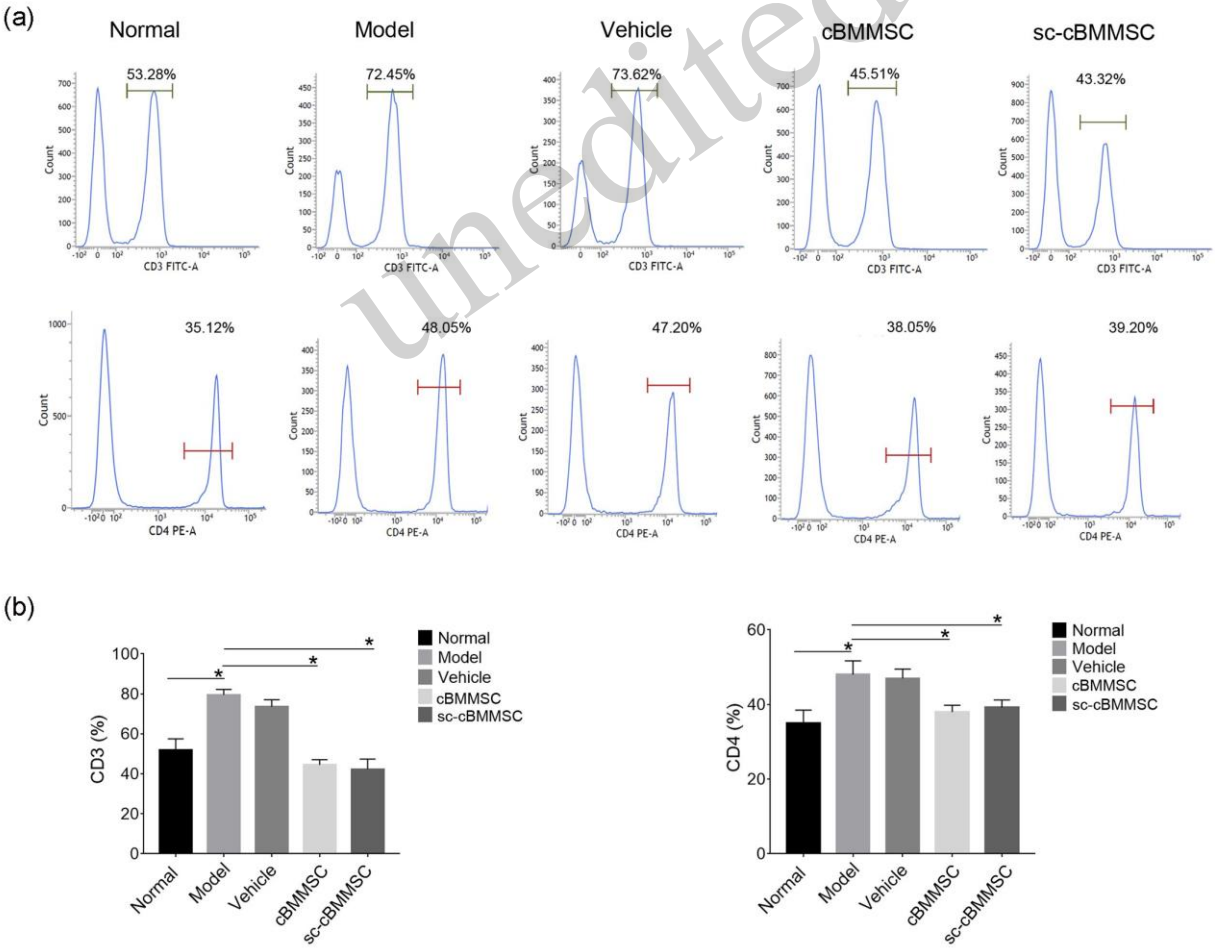

Figure 3. Detection of T-cell ratio in peripheral blood. (a): Flow cytometry showing the proportion of CD3+ and CD4+ cells. (b): Statistical results for the proportion of CD3+ and CD4+ T cells. *, P<0.05.

Myelin basic protein (MBP), a marker of mature oligodendrocytes, is widely present in myelin sheaths (Kashani et al., 2015). MBP is essential for maintaining the stability of CNS myelin-sheath structure and function (Vassall et al., 2015). Western blotting showed that MBP expression in CPZ model mice was significantly reduced (P<0.01), while MSC transplantation significantly increased MBP expression (P<0.05), especially in the sc-cBMMSC group (Figure 4a, b). HE staining and transmission electron microscopy were employed to observe the myelin-like structure in the corpus callosum of the brain tissue of experimental animals according to previous report (El Sharouny et al., 2022). The results showed that the myelin-like structure was largely diminished in CPZ model mice. We observed myelin-like structure in the MSC transplantation groups, among which recovery was more obvious in the sc-cBMMSC group (Figure 4c, Supplementary Figure1a). Immunohistochemistry staining of Olig2 and Syn was performed in the corpus callosum, showing that Olig2 and Syn signals were significantly decreased in CPZ model mice. The number of positive signals was increased in both MSC transplantation groups, with more obvious recovery in the sc-cBMMSC group (Figure 4d, Supplementary Figure1b). These results suggest that Olig2^{+} cBMMSC transplantation can promote the regeneration of oligodendrocyte progenitor cells and nerve cells, thereby promoting the repair of myelin structure, with a more significant effect of sc-cBMMSC.

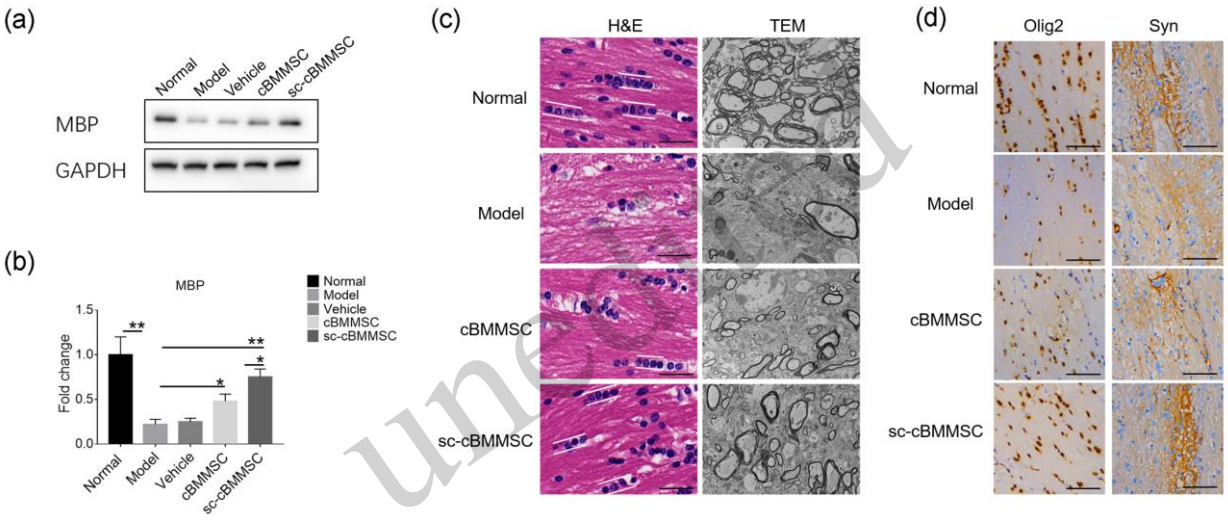

Figure 4. Analysis of demyelination and remyelination. (a, b): Western blotting detects MBP expression in brain tissue, *, P<0.05; **, P<0.01. (c): Pathological examination of brain-tissue corpus callosum. The nuclei of oligodendrocytes appear darkly stained and arranged in parallel longitudinal rows, as bracketed by lines. H&E, paraffin section, and HE staining; TEM, transmission electron microscopy. Scale bars=50 μm. (d): Immunohistochemical detection of Olig2 and Syn. Scale bars=100 μm.

The observed improvements in motor, strength, and cognitive functions following cBMMSC and sc-cBMMSC transplantation align with previous research demonstrating the potential of stem-cell therapy in various neurodegenerative diseases. For instance, studies exploring stem-cell applications in pre-clinical models of neurodegenerative disorders, such as Parkinson's disease and Huntington's disease, have reported positive effects on motor control, cognitive abilities, and neurofunctional outcomes (Biglari et al., 2023; Conner et al., 2023; Kandeel et al., 2023). These findings highlight the remarkable potential of stem-cell transplantation to promote functional recovery in the context of neurodegeneration.

The immunomodulatory effects of MSCs are increasingly recognized and have been studied extensively. MSCs have been shown to secrete a wide range of bioactive molecules that can exert potent immunosuppressive effects, such as reducing pro-inflammatory cytokines while promoting the release of anti-inflammatory factors (Eldaly et al., 2022; Lyamina et al., 2023). The reduction we observed in TNFα, IL-1β, and IL-6 levels in the

peripheral and central nervous system of the model mice in this study is in agreement with the known immunomodulatory capabilities of MSCs (Mo et al., 2022). By suppressing inflammatory response, cBMMSC and sc-cBMMSC transplantation may attenuate immune-mediated damage and promote a suitable environment for tissue repair.

The potential of MSCs to facilitate myelin repair has been a significant area of interest. Studies have also underscored the critical role of MSCs in disease modification and myelin restoration beyond symptomatic treatment in various demyelinating disorders, including multiple sclerosis (Allegretta et al., 2022; Islam et al., 2023; Zhang et al., 2023). The increase in myelin basic protein expression and the formation of myelin-like structures observed here further support the regenerative potential of cBMMSC and sc-cBMMSC transplantation. Our findings highlight the ability of these stem cells to promote the repair and formation of myelin sheaths, which are crucial for restoring normal neuronal communication and function.

Furthermore, the added value of using Olig2$^+$ sc-cBMMSCs that became apparent in this study corresponds with emerging research emphasizing the importance of enhancing the purity and specificity of stem-cell populations; recent studies have demonstrated that the enrichment or selection of specific cell subpopulations, such as Olig2$^+$ cells, can enhance the therapeutic efficacy of stem-cell-based therapies (Yang, et al., 2022). By focusing on specific stem-cell subtypes, researchers can potentiate the desired therapeutic effects for improved outcomes (Kashani et al., 2023).

Overall, the findings of this study contribute to the growing body of evidence supporting the therapeutic and immunomodulatory potential of stem cells, particularly cBMMSC and Olig2$^+$ sc-cBMMSC, in the context of demyelinating diseases like multiple sclerosis. Our results highlight the potential of this avenue for future clinical applications and underline the need for further research. This should include well-designed clinical trials to validate these findings and translate them into effective therapies for patients in need. The expanding knowledge in this field fosters optimism about harnessing the regenerative capabilities of stem cells to address the complex challenges posed by neurodegenerative diseases.

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Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author contributions

All authors contributed to the conception and design of this study. Deqing PENG and Ruijie LU designed the experiment and drafted the manuscript. Leyao LV, Qing YAO and Kaichuang YANG conducted the study. Yunfeng XU analyzed the data. Ruolang PAN revised the manuscript. Xiaoming FENG and Yuyuan MA designed the experiment and monitored the project progression. All authors read and approved the final manuscript, therefore, had full access to all the data in the study and took responsibility for the integrity and security of such data.

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Compliance with ethics guidelines

Deqing PENG, Ruijie LU, Leyao LV, Qing YAO, Kaichuang YANG, Yunfeng XU, Xiaoming FENG, Ruolang PAN, and Yuyuan MA declare that they have no conflict of interest.

All procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for inclusion in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article. All institutional and national guidelines for the care and use of laboratory animals were followed. This study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital (October 10, 2019; No. 2019KY15).

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Supplementary information

Materials and methods, Supplementary Figure1

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