miR-647 inhibits hepatocellular carcinoma cell progression by targeting protein tyrosine phosphatase receptor type F

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ABSTRACT
Hepatocellular carcinoma (HCC) is a kind of malignant tumor derived from hepatocytes and hepatobiliary cells, and its occurrence is prevalent worldwide. Although medical technology is developing rapidly, the therapeutic efficacy of HCC is still poor. Emerging evidence manifests that microRNAs (miRNAs) play a crucial role in various cancers and have been regarded as cancer suppressor gene. However, the regulatory mechanisms mediated by miR-647 involved in HCC remain unclear. Hence, to clarify the regulatory mechanisms mediated by miR-647 in HCC, we studied the independent effects of miR-647 and explored protein tyrosine phosphatase receptor type F (PTPRF) in the constructed HCC cell line (HCV-huh7.5). Thereafter, we used dual-luciferase gene reporting and Western blot to investigate the relationship between PTPRF and miR-647. Furthermore, we studied the mechanism of miR-647 on PTPRF in HCV-huh7.5. We found that miR-647 could not only promote the proliferation and invasion of HCV-huh7.5 cells but also facilitate cell migration, while PTPRF has the opposite effect. Besides, the results of cell function experiment implied that the overexpression of miR-647 or inhibition of PTPRF remarkably influenced the Erk signaling pathway, which could regulate cell proliferation, migration, and invasion. In addition, the dual luciferase reporting identified PTPRF as a direct target of miR-647. We further demonstrated that miR-647 inhibitor or PTPRF knockdown administration boosted HCV-huh7.5 cell proliferation, migration, and invasion by targeting PTPRF.

These findings provided clues for the mechanism of miR-647 in promoting the biology of HCV-huh7.5 cells by inhibiting the expression level of PTPRF.

1. Introduction
Hepatocellular carcinoma (HCC) is one of the most common cancers types in the world, and it is mainly caused by liver cirrhosis, aflatoxin, alcoholism, diabetes, and obesity [1,2]. Unfortunately, the incidence of HCC is increasing on the rise globally because of its poor prognosis [3–5]. Only 5–15% percent of patients are suitable for surgical resection, which applies only to patients in the early stages [6,7]. Therefore, there is an urgent need to elucidate the molecular pathogenesis of HCC should be determined urgently, and determine the optimal strategy for diagnosis and treatment should be determined.

Protein tyrosine phosphatase receptor type F (PTPRF) is a receptor protein tyrosine phosphatase that catalyzes the dephosphorylation of tyrosine residues [8]. Previous studies have demonstrated that PTPRF is considered to inhibit the carcinogenesis of liver cancer [9,10]. Once the expression of PTPRF decreases, the cancer cells will resume growth [11]. The inhibitory effect of PTPRF on the growth of cancer cells depends on its phosphatase activity. When the phosphatase element of PTPRF is mutated, the cell growth inhibition is abolished [12]. Therefore, this approach may serve as a useful strategy for the treatment of HCC through interfering with the expression of PTPRF.

microRNAs (miRNAs) have drawn increasing attention in recent years owing to their vital function in various biological processes, including human carcinogenesis, thus making them become promising targets for tumor diagnosis and treatments [13–15]. miRNAs are a class of small non-coding RNAs with a length of 21–24 nt in length. Some of miRNAs have been proved to regulate gene expression by degrading target mRNAs or...
inhibiting protein translation [16,17]. Currently, studies have suggested that miR-647 is involved in the growth and proliferation of tumor cells. For instance, it has been reported that miR-647, as a tumor suppressor gene, affects non-small cell lung cancer by directly down-regulating the NF-κB signaling pathway [18]. Besides, miR-647 can reduce the apoptosis of gastric cancer cells by changing the Bax/Bcl-2 protein ratio, indicating that miR-647 can able to regulate the mitochondrial apoptosis pathway in cancer cells [19]. More importantly, miR-647 can serve as a prognostic marker for ovarian cancer. Moreover, the expression levels of broad-spectrum miRNA in the serum of 10 patients with hepatitis C virus (HCV) viremia and 10 healthy volunteers were analyzed by miRNA polymerase chain reaction (PCR) chip. The results displayed that miR-647 expression was greatly up-regulated in HCV sera compared with that in healthy controls [20]. MicroRNA play vital roles in all stages of tumor, including invasion. Thus, miR-647 may be serve as a prognostic marker in cancer diseases. Based on the above results, how does the mechanism of miR-647 in HCC motivate us to conduct further studies.

Therefore, in order to clarify the regulatory mechanisms mediated by miR-647 in HCC, we studied the independent effects of miR-647 and PTPRF were explored in the constructed HCC cell line (HCV-huh7.5). Thereafter, we used dual luciferase gene reporting and Western blot to investigate the relationship between PTPRF and miR-647. Furthermore, we studied the mechanism of miR-647 on PTPRF in HCV-huh7.5. Through the above research, expect to clarify the regulatory mechanisms mediated by miR-647 in HCC can be clarified, providing new markers and targets for the diagnosis and treatment of HCC.

2. Materials and Methods

2.1 Reagents

Lipofectamine™ 3000 transfection reagent was obtained from Invitrogen (Carlsbad, USA). Cell counting kit-8 (CCK-8) was provided by Dojindo (Kumamoto, Japan). anti-ERK1/ERK2 (phospho T202/Y204, SP327), anti-c-Myc (phospho S62) antibody, anti-MNK1 (phospho T385, EPR2370), anti-ERK1+ ERK2, anti-MNK1 and anti-c-Myc antibodies were obtained from Abcam (London, United Kingdom). PTPRF/LAR (E6W4X) rabbit mAb was obtained from Cell Signalning Technology (Massachusetts, United States). BCA protein concentration assay kit and 5× protein loading buffer were provided by Beijing Solarbio life sciences (Beijing, China). NucleoZol was obtained from Gene Co., Ltd (Hongkong, China). ECL luminescence reagent was obtained from Thermo Scientific (Waltham, USA). qPCR Mix was provided by PROMEGA (Madison, MA, United States).

2.2 HCV infection and Cell culture

As described by Jiang, et al [21], the sequence of HCV Con1 was designed and the compound form Sangon Biotech (Shanghai, China), namely, BamHI/EcoRI was selected as the restriction site. The HCV Con1 gene sequence was amplified using pBRTM/HCV1-3011 plasmid as template, and the recombinant plasmid pcDNA3.1(+)/HCV Con1 was constructed by gene cloning technology. According to the description of Lipofectamine™ 3000 of Invitrogen Company, the recombinant plasmid was transfected into Huh7.5 cell line (BeNa Culture Collection, China), and the solution was changed 12 h after transfection. PCR was used to detect the expression of HCV-Con1 gene in Huh7.5 hepatoma cells 48 h after transfection. HCC cell line HCV-huh7.5 was cultured in DMEM (Gibco, United States) supplemented with 10% fetal bovine serum (FBS, PAN biotech, Germany), streptomycin (50 μg/mL), and penicillin (50 U/mL) at 37°C in a 5% CO₂ incubator.

2.3 Cell Transfection

The miR-negative control (miR-NC), miR-647 mimic, negative control inhibitor (NC inhibitor), miR-647 inhibitor, siRNAs for PTPRF (si-PTPRF), and siRNA-negative control (si-NC) were obtained from Sangon Biotech (Shanghai, China). PTPRF-overexpression vector was synthesized based on the pcDNA3.1(+) plasmid and sequenced confirmed by Sangon Biotech (Shanghai, China). Cultured cells were seeded at 1 × 10⁵ cells/well in 24-well plates (Corning Inc., United States) for
24 h. Cells were transfected until reaching a density of approximately 70%. The cells were transfected with 20 µM miR-647 mimic, 20 µM miR-647 inhibitor or the negative controls by using Lipofectamine™ 3000 (Invitrogen, United States) following the manufacturer’s protocol. si-PTPRF and si-NC were transfected at a final concentration of 50 pmol by using Lipofectamine™ 3000 (Invitrogen, United States) according to the manufacturer’s instructions. Cell, RNA and protein samples were collected 24 h or 48 h after transfection. The cell transfection efficiency was determined by qPCR assay. Primer sequences are shown in Table 1.

### 2.4 Quantitative Real-time PCR(qRT-PCR) analysis

According to the research method of Chen, et al [22], conduct qRT-PCR experiment. Total RNA was isolated from cells by using Nucleozol (Gene Co., Ltd, China) according to the manufacturer’s instructions and then reverse-transcribed into complementary DNA (cDNA) by using the reverse transcriptase. These cDNAs were analyzed for the expression of miR-647 and PTPRF by quantitative PCR by using 2x SYBR Green qPCR Master Mix (A6001, PROMEGA, United States). The real-time PCR reaction system (25 µL) consisted of 12.5 µL of 2x SYBR Green qPCR Master Mix, 1 µL of F-Primer and R-Primer (10 µM), 2.0 µL of cDNA, and 8.5 µL of ddH2O. The procedure settings are as follows: stage 1 pre-degeneration: 95°C for 5 min, stage 2 denaturation annealing extension: 95°C for 10 s, 60°C for 30 s, 72°C for 30 s, 40 cycles; Stage 3 dissolution curve: 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. U6 was used as the internal reference gene of miR-647 to detect the relative expression level of miR-647 gene. GAPDH was used as the internal reference gene. According to the $2^{-\Delta \Delta Ct}$ method, the relative quantitative analysis of the data was carried out. Primer sequences are shown in Table 2.

### 2.5 Luciferase reporter assay

As described by Chen et al [22], after the construction and identification of wild-type (PTPRF-WT) and mutant-type (PTPRF-MUT) pGL3 basic luciferase reporter plasmid, HCV-huh7.5 cells were co-transfected with miR-647 mimic or negative agent. HCV-huh7.5 cells in logarithmic growth phase were inoculated into 24-well plates before transfection. Lipofectaminetm 3000 was removed from the refrigerator at 4°C and co-transfected into HCV-huh7.5 cells with miR-647 mimic, mimic NC and wild-type and mutant-type PTPRF 3’UTR according to the operating procedures in the instructions. Correlation tests were performed 36 h after transfection to compare the differences between groups.

### 2.6 Cell proliferation assay

As described by Chen et al. [22], the viability of HCV-huh7.5 was assessed using a Cell Counting Kit-8 (CCK-8) assay kit obtained from Dojindo

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**Table 1. Sequences for si-PTPRF.**

| Name          | Sequences                      |
|---------------|--------------------------------|
| si-PTPRF-Homo1| Sense: 5’-GGACGACGAAUGUGUACG   |
|               | Antisense: 5’- UACGAAUGUGUCGACG|
| si-PTPRF-Homo2| Sense: 5’- CGUACAGGGGCUCAAAUC  |
|               | Antisense: 5’- UGGUGAAACCCGUAGA|
| si-PTPRF-Homo3| Sense: 5’- GCAACCGUUCUAAAUAG  |
|               | Antisense: 5’- UUAUAGAACGGUUUGCU|
| Negative control | Sense: 5’- UGGUGAAUUGGAAAUAAACUC |
|               | Antisense: 5’- UUAUUCGUUCACACAAAGAU |

**Table 2. The primers for PCR.**

| Primer name | Primer sequences                      |
|-------------|--------------------------------------|
| miR-647 Fwd | GCGGTGGGCTGCACTCACT                  |
| miR-647 Rev | ATGCCGAGGTCGGAGTTTT                  |
| miR-647 RT  | GTCGATCCAGTCCAGGTTGGTACGGATATTCCAGCA |
| U6 Fwd      | CTGCCTCGGCAACGAC                   |
| U6 Rev      | AAGCCTTCAGAATTTGCT                   |
| U6RT        | AAAATATGGAGCGGGCTCTCGGAATTG         |
| PTPRF Fwd   | CGACCAAGGCAATGACCGTTG               |
| PTPRF Rev   | GGAGGGATGGAGAAGAAGGAG                |
| GAPDH Fwd   | AGAAGGGTGTTGGCTCACATTG              |
| GAPDH Rev   | AGGGGGCATCAGACCTTCT                 |
(Kumamoto, Japan). In short, HCV-huh7.5 cells suspension was diluted to $1 \times 10^4$ cells/mL in 96-well plates at 37°C in a 5% CO₂ incubator for 24, 48, 72, and 96 h. Approximately 10 μL of CCK-8 solution was added to each sample and then incubated. Finally, the optical density was measured at 450 nm.

2.7 Western blot analysis
As described by Li et al [23], the transfected cells were harvested and lysed in a protein lysate containing PMSF (Thermo Scientific, Inc., Waltham, MA, USA). BCA kit (Beijing Solaibao Technology Co., Ltd, Beijing, China) was used to determine the total protein concentrations. Protein samples (40 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and blocked in 5% skim milk in TBST for 2 h at 25°C. Then the membranes were incubated at 4°C overnight with 1:1000 dilutions (v/v) of primary antibodies. Membranes were then washed and exposed to secondary antibodies for 2 h at 25°C, and visualized using the ECL chemiluminescence reagent kit (34,080, Thermo, United States) and photographed on a chemiluminescence apparatus (JS-1070, Peiqing Technology Co., Ltd, Shanghai, China).

2.8 Cell migration and invasion assay
As described by Chen et al. [22], the ability of migration and invasion was measured by Transwell assay. For the migration assay, cell suspension with a cell density of $1 \times 10^5$ cells/mL was added to the upper surface of Transwell chamber, and 600 μL of medium containing 20% FBS (Pan Biotech) was added to the lower chamber. For cell invasion assay, 50 μL of diluted Matrigel was coated with Transwell. Approximately $1 \times 10^5$ /mL cells of each group were seeded into the upper chamber of Transwell (100 μL), and then the Transwell chamber was placed into the well plate with 10% FBS medium for culture. After being cultured in an incubator at 37°C for 24 h, migrated or invaded cells were fixed and stained. Finally, the cells were observed under a microscope.

2.9 Cell clone formation assay
As described by Guo et al. [24], each experimental group was inoculated with 800 cells/wells in a six-well plate, and three multiple wells were employed for each experimental group. The inoculated cells were shaken well and gently placed in the incubator for further culture. When the number of cells in most single clones in the well was greater than 50, the cells could be fixed and stained. The number of cell clones was observed and counted under microscope ultimately.

2.10 Statistical analysis
Data of each group were expressed as mean ± standard deviation. SPSS 22.0 statistical analysis software package was used to conduct one-way ANOVA on the experimental data. $P < 0.05$ was considered statistically significant.

3. Results
In order to clarify the regulatory mechanisms mediated by miR-647 in HCC, we studied the independent effects of miR-647 and protein tyrosine phosphatase receptor type F (PTPRF) were explored in the constructed HCC cell line (HCV-huh7.5). Thereafter, the use of dual luciferase gene reporting and Western blot analysis was to investigate the relationship between PTPRF and miR-647. Furthermore, we studied the mechanism of miR-647 on PTPRF in HCV-huh7.5. The results are summarized below.

3.1 Expression of miR-647 and PTPRF in LO2 and HCV cell lines
SYBR green qPCR was used to detect the expression level of miR-647 and PTPRF in normal liver cells LO2 and HCV-huh7.5 cells. In comparison with LO2, the expression of miR-647 was significantly up-regulated, but the expression of PTPRF was markedly decreased in HCV-huh7.5 cells (Figure 1). Therefore, HCV-huh7.5 cells were selected for subsequent experiments.
3.2 Effects of miR-647 expression level on the proliferation, migration, and invasion of HCV-huh7.5 cells

To modulate miR-647 expression, we transiently transfected miR-647 mimic or inhibitor were transiently transfected into HCV-huh7.5 cells. As shown in Figure 2a, miR-647 mimic significantly increased miR-647 expression, whereas miR-647 inhibitor greatly suppressed miR-647 expression. Then, the proliferation of HCV-huh7.5 cells was determined by CCK-8 method. As shown in the Figure 2b, miR-647 mimic significantly enhanced the proliferation of HCV-huh7.5 cells, which is opposite as that of miR-647 inhibitor treatment.

The monoclonal ability of HCV-huh7.5 cells was detected by cell cloning assay. The cell monoclonal ability was markedly increased by miR-647 mimic, which is opposite as that of miR-647 inhibitor (Figure 2c). The cell migration and invasion ability were also determined after transfected with miR-647 mimic or inhibitor. As shown in Figure 2d, the experimental results of cell migration and invasion are the same as above.

3.3 PTPRF gene was targeted by miR-647

To explore the targeting relationship between miR-647 and PTPRF, we performed prediction using TargetScan7.0 was performed. The miR-647 sequences were predicted to interact with PTPRF 3’-UTR (Figure 3a) is displayed in Figure 3a. To confirm that miR-647 directly bound the 3’-UTR of the PTPRF, we carried out dual luciferase reporter assay was carried out. The results showed that miR-647 mimic significantly reduced the wild-type luciferase activity but had no effect on mutant PTPRF vector (Figure 3a).

In addition, Western blot assay was used to confirm the effect of miR-647 on PTPRF. We observed that miR-647 overexpression significantly decreased PTPRF expression. By contrast, the inhibition of miR-647 expression remarkably increased PTPRF protein level (Figure 3b).

3.4 Effects of miR-647 or PTPRF on Erk signaling pathway in HCV-huh7.5 cells

The effects of miR-647 expression on Erk pathway were determined by Western blot in HCV-huh7.5 cells. The results of Western blot analysis show that the phosphorylation of p-Erk1/2, p-Myc, and p-Mnk1 was increased by miR-647 mimic and reduced by miR-647 inhibitor (Figure 4a). To verify the effect of miR-647 on target genes, we overexpressed or knocked out PTPRF. Western blot assay was performed to detect the transfection efficiency. As expected, the expression of PTPRF protein was changed by pcDNA-PTPRF or si-PTPRF transfection (Figure 4b). As displayed in Figure 4c, the activation of Erk signaling pathway was reduced in PTPRF-silenced HCV-huh7.5 cells, but which was enhanced in PTPRF overexpression.
3.5 Effects of PTPRF expression on the biological function of HCV-huh7.5 cells

The effects of PTPRF expression on the biology of HCV-huh7.5 cells were also detected. As shown in Figure 5a, PTPRF overexpression significantly increased PTPRF expression, whereas PTPRF knockdown greatly suppressed PTPRF expression. The result of CCK-8 assay showed that PTPRF knockdown significantly enhanced the proliferation of HCV-huh7.5 cells, which is opposite as that of PTPRF overexpression treatment.
(Figure 5b). The monoclonal ability of HCV-huh7.5 cells after transfection was detected by cell cloning assay. The cell monoclonal ability was markedly increased by PTPRF knockdown, which is opposite as that of PTPRF overexpression (Figure 5c). The cell migration and invasion ability were also determined after transfected with PTPRF knockdown or overexpression. As shown in Figure 5d, the same cell migration and invasion results were obtained.

3.6 miR-647 regulates cell biological activity by targeting PTPRF

To further prove that miR-647 regulates the biology of HCV-huh7.5 cells by targeting PTPRF, we transfected the miR-647 inhibitor, negative agent or si-PTPRF was transfected into HCV-huh7.5 cells. The results displayed that the proliferation of HCV-huh7.5 cells was reduced by miR-647 inhibitor, which was abolished in miR-647 inhibitor + si-PTPRF treatment (Figure 6a). The cell monoclonal ability was increased by miR-647 inhibitor + siPTPRF treatment, which is opposite as that of miR-647 inhibitor (Figure 6b). Besides, the cell migration and invasion ability were inhibited by miR-647 inhibitor treatment, which that were abolished in miR-647 inhibitor + si-PTPRF treatment (Figure 6c).

3.7 miR-647 mediates Erk signaling pathway by targeting PTPRF

To detect whether the miR-647-mediated down-regulation of PTPRF expression affects the Erk signaling pathway, we carried out Western blot analysis to PTPRF-silenced HCV-huh7.5 cells. As expected, the expression levels of p-Erk1/2, p-MYC and p-MNK1 were significantly up-regulated (Figure 7).

Discussion

HCC is an aggressive malignancy that is associated with high mortality and poor prognosis [25,26]. Hence, a new therapeutic strategy should be developed for the treatment of HCC. miRNAs are a class of momentous endogenous small non-coding RNAs that play regulatory roles in a various basic biological processes, such as cell proliferation, apoptosis, and differentiation [27,28]. miRNAs can serve as negative feedback regulators of oncogenesis, metastasis, and
resistance to various therapies [29–31]. miR-647 is mainly involved in the modulation of tumor cell growth and acts as a tumor suppressor in several cancers [32–34]. By contrast, miR-647 could serve as a tumor promoter by downregulating nuclear factor IX in colorectal cancer [35]. However, limited information is available about miR-647’s role in HCC. Therefore, whether miR-647 has an anti-tumor effect in HCC is unclear. Accordingly, we explored its mechanism in HCC.

Figure 4. Effects of miR-647 or PTPRF on Erk signaling pathway in HCV-huh7.5 cells. (a). The effects of miR-647 expression on Erk pathway was determined by Western blot in HCV-huh7.5 cells. (b). The expression of PTPRF protein was detected by Western blot. (c). The effects of PTPRF expression on Erk pathway were determined by Western blot in HCV-huh7.5 cells. All the data above are expressed as the mean ± S.D of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
In this study, we found that the expression level of mir-647 in HCV-huh7.5 cells was dramatically higher than that in normal cells. Through cell experiments, we discovered that down-regulating the expression level of mir-647 could inhibit the biology of HCV-huh7.5 cells, such as proliferation, and invasion ability. Collectively, these results suggested that mir-647 might play a carcinogenic role in HCC, which is inconsistent with its role in gastric cancer [36,37]. Thus, mir-647 may be served as a potential agent in HCC treatment.

To explore the mechanism of mir-647 regulating HCC cells, we employed bioinformatics analysis tools and dual luciferase activity reporting assay were performed. The results implied that mir-647 could directly target PTPRF gene. Generally, PTPRF can

Figure 5. Effects of PTPRF expression on the biological function of HCV-huh7.5 cells. (a). The expression of PTPRF was detected by qRT-PCR after PTPRF overexpression or knockdown transiently transfected into HCV-huh7.5 cells. (b). CCK-8 assay was used to measure cell proliferation after transfection. (c). The monoclonal ability of cells was tested by cell cloning assay. (d). Cell migration and invasion ability after transfection. All the data above are expressed as the mean ± S.D of three independent experiments. **p < 0.01, ***p < 0.001.
restrain tumor activity in several malignancies. For example, PTPRF suppresses the metastasis of breast tumor cells by inhibiting the EGFR signaling pathway [38]. Besides, the knockdown of PTPRF inhibits xenograft tumor growth and decreases Wnt target gene expression in vivo [39]. In the present study, we demonstrated that PTPRF inhibited the proliferation, migration and invasion of HCV-huh7.5 cells. Therefore, the PTPRF expression level plays a vital role in the tumor disease.

As a member of the MAPK family, Erk is an important pathway of cell signal transduction, which can transmit various extracellular signals to the nucleus through phosphorylation and activation
step by step, and ultimately participate in cell growth, development, division and differentiation [40]. The activation of Erk1/2 into p-Erk1/2, plays an essential role in pathological processes such as malignant transformation of cells. Our studies revealed that miR-647 affected the development of HCC through the targeted regulation of Erk signaling pathway by PTPRF, thus laying a foundation for further molecular studies on the role of miR-647 in controlling PTPRF in HCC. Studies have shown that Erk1/2 could exhibit abnormal expression or enhanced activity in various tumor tissues such as liver cancer and prostate cancer, suggesting that the abnormal regulation of Erk is closely related to the occurrence and development of tumors [41,42]. In our study, we found that the miR-647-mediated downregulation of PTPRF expression affects the Erk signaling pathway in HCV-huh 7.5 cells, and the expression levels of p-Erk1/2, p-MYC and p-MNK1 were significantly up-regulated. This regulatory mechanism promotes the enhancement of proliferation, migration, and invasion of HCC cells.

**Conclusion**

Our study further proves that miR-647 can markedly regulate the biology of HCV-huh7.5 cells by targeting PTPRF. Further studies indicated that the inhibition of miR-647 expression could promote the expression of PTPRF, thereby affecting the Erk1/2 signaling pathway activation, and then inhibiting the development of HCV-huh 7.5 cells.

**Research highlights**

1. The functional role of mir-647 in liver cancer was identified for the first time.
2. The targeted binding relationship between mir-647 and PTPFRF was confirmed for the first time.
3. The effect and mechanism of mir-647/PTPFRF signal axis on the biological behavior of hepatocarcinoma C cells were clarified.

Ethical approval
This article does not contain any studies with human participants performed by any of the authors.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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