INVOLVEMENT OF MIR-125A-5P IN GINGIVAL FIBROBLAST-INDUCED M2-LIKE MACROPHAGE POLARIZATION

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Abstract

Peri-miniscrew implant inflammation (PMSII) is a common complication after orthodontic anchorage, causing chronic progressive inflammation. This study aimed to investigate the role of miR-125a-5p in the regulation of macrophage polarization. THP-1 cells were induced to differentiate into macrophages. We modulated miR-125a-5p levels in THP-1 cells through mimics and inhibitors and cocultured these with HGF-1 cells to simulate the physiological environment. Our results indicated that increased miR-125a-5p levels in THP-1 cells induced M2-like macrophage polarization, enhanced cell proliferation and stimulated cell migration, whereas these effects were counteracted by miR-125a-5p knockdown. Bioinformatics analysis predicted target genes of miR-125a-5p that were enriched in the HIF-1 pathway, and differential expression of miR-125a-5p regulated the activity of related proteins in this pathway. Crosstalk effect of miR-125a-5p-induced macrophage polarization on HGF-1 cells revealed that more cells lost their spindle shape and were closer to a round shape in miR-125a-5p high-expressing THP-1 cocultures with HGF-1. Further studies suggested that miR-125a-5p was involved in the growth and proliferation of gingival fibroblasts through macrophages. Collectively, our findings suggest that miR-125a-5p is a potential target for regulating macrophage polarization, which provides new insights for the clinical treatment of peri-implantitis.

Key-words: MIR-125A-5P, POLARIZATION, CROSSTALK, INFLAMMATION

INTRODUCTION

Peri-miniscrew implants provide a new option for orthodontic anchorage. It is an effective treatment for severe malocclusion and complex tooth movement and has many advantages, including small size, simple implantation process, limited trauma, a flexible implant site, and good patient tolerance. Peri-miniscrew implant inflammation (PMSII) is a risk factor for anchorage stability and a complication after treatment of peri-miniscrew implants1. PMSII is a chronic progressive inflammation that results in bleeding and swelling of the soft tissue around the anchorage, and soft tissue hyperplasia surrounding the implant anchorage, leading to bone resorption and destruction around the implant anchorage, decreased osseointegration rate, and implant anchorage loosening2. Approximately 30% of patients who received an implant were affected by PMSII3. Previous studies have found that the fibroblast-rich barrier tissue immediately surrounding the peri-miniscrew implant plays an important role in maintaining an adequate seal against the external environment4.

When the implant is inserted, macrophages will rapidly infiltrate the implant site and initiate the immune response around the implant site. Classical activation (M1) and

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alternative activation (M2) of macrophages play an important role in PMSII. M1 macrophages support the production of proinflammatory cytokines such as TNF-α and IL-1β, and M2 macrophages promote tissue repair by secreting cytokines including IL-10 and CD2065. Regulating the polarization of macrophages may have important therapeutic potential in treating inflammation-related diseases. Following macrophage polarization, human gingival fibroblasts migrate to the site and act as the major cell type responsible for creating a functional seal from the outside mucosa6. Human gingival fibroblasts are the most abundant cells in gingival tissue and are responsible for the maintenance of tissue structure and integrity 7. The basic functions of gingival fibroblasts, such as repair and regeneration are very important for gum health. In addition, gingival fibroblasts participate in the control of the inflammatory cascade in the microenvironment 8. HGF-1 is a widely used fibroblast model that has been established to study inflammatory responses 9.

MicroRNAs play important roles in the pathogenesis of various diseases, and differentially expressed miRNAs have been proven to have critical functions in inflammatory disease10. MiR-125a-5p ameliorated the macrophage-mediated inflammatory response and vascular dysfunction 11, 12. MiR-125a-5p induces M2 polarization in macrophages by targeting various transcription factors and adapter proteins13. Our previous research also found that miR-125a-5p promotes M2 macrophage polarization during orthodontic tooth movement 14.

This study mainly explores miR-125a-5p influencing the behavior and function of HGFs via differential macrophage polarization in vitro.

MATHERIAL AND METHOD

Cell lines and culture

Tohoku Hospital Pediatrics-1 cells (THP-1) were purchased from Procell Life Science & Technology Co., Ltd. (CL-0233, Procell, China), and human gingival fibroblastst (HGF-1) were obtained from Zhejiang Meisen Cell Technology Co., Ltd (CTCC-008-0001, MeisenCTCC, China). THP-1 cells were induced into macrophages using phorbol-12-myristate-13-acetate (PMA). All cells were incubated at 37 °C in a 5% CO2 atmosphere. MiR-125a-5p mimics and inhibitors were purchased from RiboBio Co., Ltd. (Guangzhou, China). The sequences were showed in Table 1. Mimics/inhibitor transfections were performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s protocol. Then, cells were seeded in a 96-well plate after 24 h of transfection for subsequent experiments. For coculture experiments, THP-1 cells were first transfected with either miR-125a-5p mimic or inhibitor and incubated for 6 hours to allow for efficient transfection. Subsequently, HGF-1 cells, which were plated in Transwell chambers, were cocultured with THP-1 cells in 24-well plates for a duration of 48 h to facilitate cell-cell interactions.

qPCR

Cell samples were lysed with TRIzol (Invitrogen, USA) for collection of total RNA according to the manufacturer's instructions. The cells and tissue samples were rinsed with phosphate-buffered saline (PBS) twice, TRIzol was added to the lysed cells and the total RNA was extracted following the phenol/chloroform method. cDNA was synthesized using the

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PrimeScript™ RT Reagent Kit (Takara, Japan). Real-time quantitative PCR was performed on the StepOne™ Real-Time PCR System (Life Technologies, USA). Each cDNA sample was run in triplicate, and target mRNA expression was normalized to GAPDH. The mRNA expression in each sample was calculated by using the 2-ΔΔCT analysis method. The primers are listed in Table 1.

Western blot

Cells were washed in ice-cold PBS and boiled in loading buffer, and the proteins were then separated by 8% SDS–PAGE, after which they were transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH00010, Millipore, Germany). The membranes were incubated overnight with the appropriate primary antibodies at 4 °C, followed by incubation with alkaline phosphatase-conjugated secondary antibodies. After three washes with TBST, chemiluminescence detection was performed with CLINX 6100 (Shanghai, China), and relative protein expression was analysed with Alpha Innotech software (AlphaEaseFC, CA, USA) and normalized to GAPDH. Antibody information: GAPDH (Abcam, ab181602, 1:10000); HIF-1α (CST, #14179, 1:500); VEGFA (Abcam, ab46154, 1:1000); EGFR (CST, #4267, 1:1000); Bcl-2(Abcam, ab196495, 1:2000); ERK2 (Abcam, ab32081, 1:2000).

CCK8 assay

Cells were inoculated in a 96-well plate (100 μL/well) and preincubated in an incubator (37 °C, 5% CO2). Then, 10 μL of Cell Counting Kit-8 (CCK-8) solution was added to each well and incubated in 37 °C and 5% CO2 for 2 hours. A microplate reader was used to measure the absorbance at 450 nm.

Transwell assay

Cell migration was assessed using Transwell chambers (Corning, USA) with a pore size of 8 μm. A total of 1 × 105 cells in 1 mL of serum-free medium were added to Transwell chambers, and 500 μL of complete medium containing 10% FBS was added to 24-well plates. After 24 h of incubation, the cells were fixed in paraformaldehyde for 20 min and stained with crystal violet for 10 min. The number of migrated cells was counted in random areas under a microscope (IX51, Olympus, Japan).

For coculture experiments, THP-1 cells transfected with miR-125a-5p mimics or inhibitors were inoculated and cultured for 6 h in a 24-well plate. Transwell chambers (8 μm, Corning) with THP-1 cells were then moved to HGF-1 cell plates and THP-1 cell culture medium was added and cultured for 48 h. The Transwell chambers was removed and the migration of HGF-1 cells was observed. The cells were fixed with paraformaldehyde and stained with crystal violet, and the number of migrated cells was counted.

Target gene prediction

MiRTarBase 15 was used to predict target genes of miR-125a-5p. The enrichment pathways of these target genes were then analysed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Immunofluorescence

THP-1 cells transfected with miR-125a-5p mimic or inhibitor were seeded on Transwell plates (3 μm, Corning) and cultured for 6 h. The Transwell plate with THP-1 cells was transferred to a 24-well plate with HGF-1 cells, and HGF-1 cell culture medium was added. After 48 h of coculture, HGF-1 cells were fixed with 4% paraformaldehyde for 30 min and then washed with PBS three times. Then, the cells were incubated with anti-F-actin antibody (Abcam, ab8226, 1:1200) and vinculin (PTG, 66035-1-Ig,

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1:200) with 5% BSA and incubated overnight in a humidified chamber at 4 ℃. On the following day, the fluorescent secondary antibodies cy3 (ASPEN Biotechnology, AS-1109) and coralite488 (Sanying, SA00013-2) were added at a dilution of 1:100 after washing, and incubated for 50 min. The cells were then washed three times with 1 × PBS, and nuclei were stained with DAPI (AS1075, ASPEN Biotechnology). Confocal microscopy and fluorescence analysis were performed using a fluorescence microscope (Leica DMI4000B, Buffalo Grove, IL, USA).

Statistical analysis

Statistical analysis was performed by GraphPad Prism 8.3. Unpaired T test was used to compare means between groups, with $p \leq 0.05$ determined to be statistically significant.

RESULTS

miR-125a-5p modulates THP-1 polarization

Previous studies have reported that miR-125a-5p exhibits an inflammatory response in periodontal tissue, but the function and role of miR-125a-5p in implants remain unclear. THP-1 cells were first induced into macrophages. miR-125a-5p mimics and inhibitors were constructed and transfected into THP-1 cells, and verified their expression efficiency by qPCR (Fig. 1A). Detection of inflammatory factor expression revealed that overexpression of miR-125a-5p increased the mRNA levels of IL-10 and Arg1 (Fig. 1B), of which IL-10 is an anti-inflammatory factor secreted by M1 macrophages, and Arg1 is a marker of M2 macrophages. In contrast, the mRNA levels of IL-12, TNF-α, and iNOS were elevated after treatment with the miR-125a-5p inhibitor (Fig. 1B). IL-12 and TNF-α are proinflammatory factors that can be secreted by M1 macrophages, and iNOS is a marker of M1 macrophages. To further explore the effect of miR-125a-5p on THP-1 cell proliferation and migration, the results of CCK8 and Transwell migration assays showed that miR-125a-5p overexpression promoted the proliferation and migration of THP-1 cells, while the effect was reversed with transfection of the miR-125a-5p inhibitor (Fig. 1C-D). These results suggested that miR-125a-5p mediates macrophage polarization of THP-1 cells, and promotes the shift process of macrophages to anti-inflammatory M2 macrophages and cell proliferation and migration.

To further explore the potential mechanisms of miR-125a-5p, the target genes were predicted using the miRTarBase database, and KEGG analysis was performed for all target genes. Notably, we found that many target genes were highly enriched in the HIF-1 pathway, suggesting that we focused on the connection between miR-125a-5p, the HIF-1 pathway, and cell polarization (Fig. 1E).

HIF-1 signaling involving miR-125a-5p regulated changes of THP-1 macrophages

To understand the regulatory mechanism of HIF-1, the expression of proteins related to the HIF-1 pathway was detected by western blot. The results showed that miR-125a-5p overexpression increased the expression of HIF-1α and the downstream protein Bcl-2 in THP-1 cells (Fig. 2A). The expression of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFA), key genes of the HIF-1 pathway, also showed a significant increase (Fig. 2B). In contrast, the protein expression of these molecules was reversed after transfection with the miR-125a-5p inhibitor. These results suggested that miR-125a-5p affects HIF-1α and may influence macrophage polarization by participating in the HIF-1 pathway.

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Crosstalk between macrophages and gingival fibroblasts

To explore the effect of miR-125a-5p-induced macrophage polarization on gingival fibroblasts, THP-1 cells and HGF-1 cells were cocultured on Transwell plates. Immunofluorescence staining showed that HGF-1 cells appeared to have a normal fibroblastic morphology with a spindle shape in the single cultivation (HGF-1) and coculture with THP-1 cell groups (THP-1 + HGF-1, Fig. 3A). The cells in the THP-1 and HGF-1 coculture group that overexpressed miR-125a-5p lost their spindle shape and appeared more nearly round. Furthermore, Transwell results of cell coculture also revealed that overexpression of miR-125a-5p promoted the migration of HGF-1 cells and increased cell activity (Fig. 3B-C). Western blotting for proliferation-associated proteins revealed that EGFR and VEGFA expression was significantly elevated in cocultured cells transfected with miR-125a-5p mimic (Fig. 3D-E). These effects were reversed after transfection with the miR-125a-5p inhibitor. Taken together, miR-125a-5p-mediated M2-type macrophages have an important role in cytoskeleton maintenance and gingival fibroblast.

DISCUSSION

Peri-implantitis is characterized by inflammatory damage around the implant that eventually leads to a loose implant. Macrophages are central participants in inflammatory and immune responses against periodontal pathogens and contribute to periodontal inflammation16. During bone remodeling, increased M2 macrophage polarization may contribute to the recovery and stability of tissues17. MiRNAs act as regulators that provide complex gene regulatory parameters that orchestrate a variety of biological processes, such as inflammation and bone metabolism. Previous studies have demonstrated that various miRNAs are involved in the pathogenesis of peri-implantitis. Our results suggested that miR-125a-5p is a valid candidate target in the development of peri-implantitis. More importantly, we found that high expression of miR-125a-5p promoted M2-like polarization of THP-1 cells and increased anti-inflammatory effects, which is consistent with previous studies 11, 13. Apart from that, high expression of miR-125a-5p also significantly increased THP-1 cell activity. Previous studies reported that HIF-1α promotes macrophage recruitment, while HIF-1α deficiency decreases the migration of macrophages18-20. This miR-125a-5p is beneficial in mediating the inflammatory response around the implant.

These results prompted us to further explore the mechanisms involved in the regulation of macrophage polarization by miR-125a-5p. We found that the HIF-1 signaling pathway was activated by analysing the target genes of miR-125a-5p, and HIF-1α is an important molecule in this pathway. HIF is a transcription factor that responds to changes in available oxygen in the cellular environment. DMOG, an activator of HIF-1α, has been reported to inhibit M1-like polarization of periodontitis, thereby contributing to the therapeutic effect21. We further found that overexpression of miR-125a-5p elevated HIF-1α expression. This suggests to us that miR-125a-5p may affect macrophage polarization by participating in the HIF-1 pathway.

Gingival fibroblasts are the most common connective tissue cell type in the gingiva. Fibroblasts produce extracellular matrix tissue that is important for tissue repair and wound healing22. The migration and proliferation of HGF in the injury area

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represents a rapid response to injury, as these cells can populate this region and generate the extracellular matrix components needed for wound healing23. Injured HGFs crosstalk with macrophages via the IL6-IL6R axis, secreting IL-6 and IL-8, recruiting neutrophils and macrophages and triggering the inflammation cascade24, 25. Therefore, we further explored the effect of the inflammatory environment simulated by coculturing miR-125a-5p-overexpressing THP-1 cells with HGF cells on the HIF-1 pathway. After HGF cocultured with THP-1 cells overexpressing miR-125a-5p, the cell morphology became square and round in size rather than spindle-shaped. Migration and proliferation were increased, suggesting that miR-125a-5p contributes to the wound healing process. Moreover, the expression of HIF-1α and its downstream target genes EGFR, and VEGFA was elevated, demonstrating that miR-125a-5p participates in the inflammatory response of macrophages to gingival fibroblasts by affecting the HIF-1 pathway.

CONCLUSIONS

Overall, our study demonstrated that miR-125a-5p is involved in promoting M2-like polarization of macrophages by affecting the HIF-1 pathway. Further investigation showed that macrophages overexpressing miR-125a-5p increased the proliferation of HGF-1 cells. However, the detailed mechanisms and more in-depth exploration of macrophage-gingival fibroblast crosstalk in PMSII will be required in the future. Nevertheless, our study still provides a foundation for the use of miR-125a-5p in the material application of implants and supports for the benefit of patients with peri-implantitis.

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CONSENT FOR PUBLICATION

All authors agreed to publish.

AVAILABILITY OF DATA AND MATERIALS

The author acknowledges responsibility for all aspects of the work. The data of the article can be obtained through the corresponding author.

CONFLICT OF INTEREST

All authors declared no conflict.

AUTHORS' CONTRIBUTIONS

WH performed the co-culture experiments and analyzed the data, and was primarily responsible for writing the manuscript. BD and ZW performed the THP-1 cell experiments and corresponding data analysis, and participated in the revision of the manuscript. XC designed and supervised this experiment and revised the manuscript. All authors read and approved the final manuscript.

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Figures

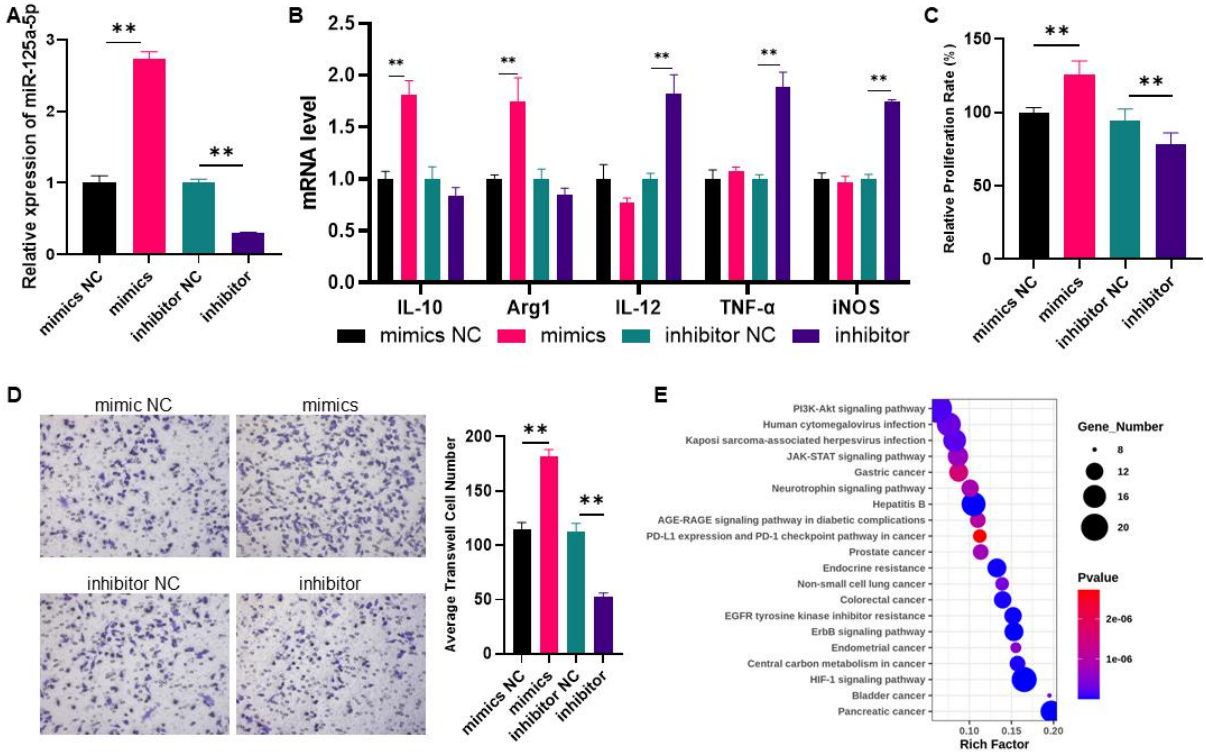

Figure 1. MiR-125a-5p modulates THP-1 polarization. A. Relative expression detected by qPCR in THP-1 cells after transfection with miR-125a-5p mimics or inhibitors. B. Relative expression of inflammatory factors detected by qPCR after transfection with miR-125a-5p mimics or inhibitors in THP-1 cells. C. CCK-8 proliferation assay of THP-1 cells after transfection with miR-125a-5p mimics or inhibitors. D. Migration assay of THP-1 cells after transfection with miR-125a-5p mimics or inhibitors. E. KEGG enrichment analysis of target genes for miR-125a-5p. Mimics NC: THP-1 cells transfected with mimics negative control; mimics: THP-1 cells transfected with miR-125a-5p mimics; inhibitor NC: THP-1 cells transfected with inhibitor negative control; inhibitor: THP-1 cells transfected with miR-125a-5p inhibitor. A–D. Data represent the mean ± SEM. Unpaired t test, * $p<0.05$, **$p<0.01$.

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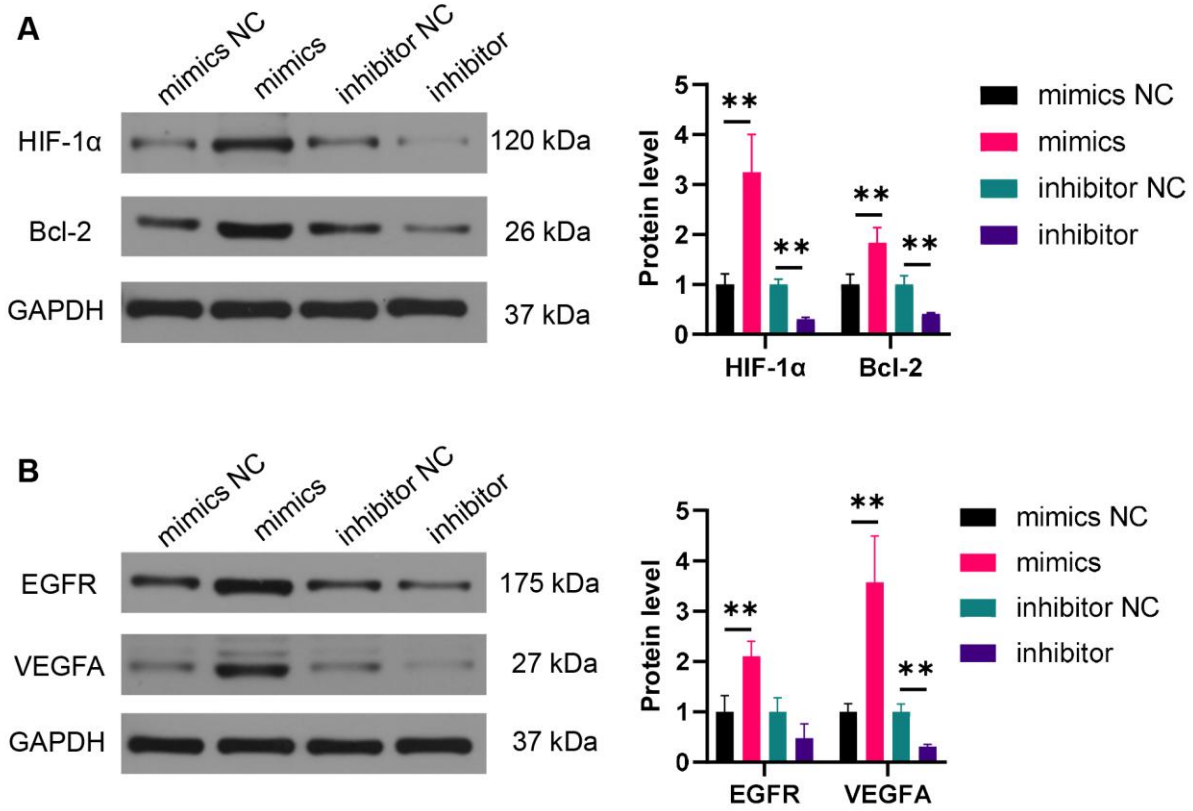

Figure 2. MiR-125a-5p modulates the HIF-1 pathway in THP-1 cells. A–B. Expression results of HIF-1 pathway-related proteins in THP-1 cells. Mimics NC: THP-1 cells transfected with mimics negative control; mimics: THP-1 cells transfected with miR-125a-5p mimics; inhibitor NC: THP-1 cells transfected with inhibitor negative control; inhibitor: THP-1 cells transfected with miR-125a-5p inhibitor. Data represent the mean ± SEM. Unpaired t test, * $p<0.05$, ** $p<0.01$.

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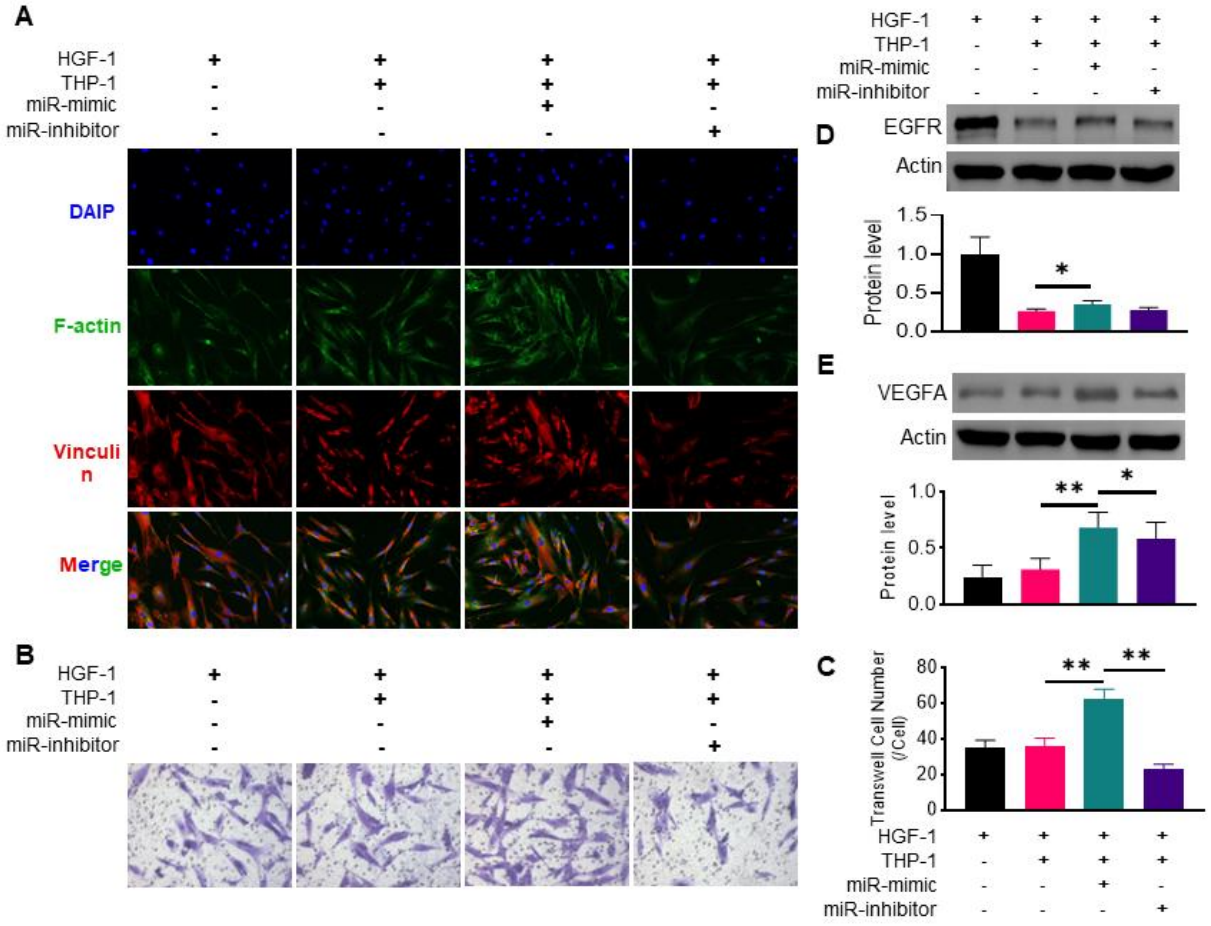

Figure 3. Effect of macrophage THP-1 interactions with HGF-1 gingival fibroblast. A. Immunofluorescence image of THP-1 cells co-cultured with HGF-1 cells. Blue: DAIP; Green: F-actin; Red: vinculin. B–C. Transwell assay of THP-1 cells co-cultured with HGF-1 cells. D–E. Expression of proliferation-related proteins of THP-1 cells co-cultured with HGF-1 cells. Data represent the mean ± SEM. Unpaired t test, * $p<0.05$, ** $p<0.01$.

Table 1. Primer information.

Primer	Sense	Antisense
GAPDH	CATCATCCCTGCCTCTACTGG	GTGGGTGTCGCTGTTGAAGTC
IL-10	AACCTGCCTAACATGCTTCG	GAGTTCACATGCGCCTTGAT
Arg1	AGACCACAGTTTGGCAATTGG	AGGAGAATCCTGGCACATCG
iNOS	GCAGGACTCACAGCCTTTGG	GGCTGGATGTCGGACTTTGT
TNF-α	CTCTTCTCCTTCCTGATCGTGG	CTTGTCACTCGGGGTTCGAG
ETV6	CTCTATACACACACAGCCGGAG	TTTGTCGTGATAGGTGACCTG
IL-12	TGAAGACTTGAAGATGTACCAGGT	TTTGTGGCACAGTCTCACTGTT
miR-125a-3p	GGACAGGTGAGGTTCTTGGG	CTCAACTGGTGTCGTGGAGTC
U6	AACGCTTCACGAATTTGCGT	CTCGCTTCGGCAGCACAT
miR-125a-5p mimics	GGACAGGUGAGGUUCUUGGG	CCUGUCCACUCCAAGAACCC
miR-125a-5p inhibitor	CCCAAGAACCUCACCUGUCC	

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NC mimics	UUUGUACUACACAAAAGUACUG	AAACAUGAUGUGUUUUCAUGAC
NC inhibitor	CAGUACUUUUGUGUAGUACAAA	
miR-125a-3p Reverse	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGCTCCCA	

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