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Adipose derived stem cells promote tumor metastasis in breast Cancer cells by stem cell factor inhibition of miR20b

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ABSTRACT

Breast cancer (BC) metastasis after surgery is associated with the tumor microenvironment and especially with adipose tissue-derived mesenchymal stem cells (ASCs) that have been shown to promote the BC progression. To better understand the role of ASCs in tumor metastasis, our study explored a novel mechanism that mediates the negative regulation of miR20b during ASC-induced tumor metastasis of BC cells. In this study, we found that the migration and invasion abilities of BC cells are markedly increased coculture with ASCs. By studying the regulatory mechanism, we found that miR20b biogenesis in BC cells can be attenuated by ASC-released stem cell factor (SCF) through the downstream c-Kit/MAPK-p38/E2F1 signaling cascade and that miR-20b acts as a tumor suppressor miRNA in the inhibition of BC migration and invasion. HIF-1α and VEGFA are the target genes of miR20b and miR20b downregulation activated HIF-1α-mediated VEGFA transcription and ASC-induced BC migration and invasion. The upregulation of miR20b abrogated the activation of EMT and lung metastasis of breast cancer cells cocultured with ASCs by the inhibition of N-cadherin, vimentin and Twist expression in vitro and in vivo. Collectively, our findings indicate that downregulation of miR20b by ASCs/SCF activates HIF-1α/VEGFA and induces BC cell EMT and metastasis, suggesting that this process is activated by the p-c-Kit/MAPK-p38/E2F1 pathway.

1. Introduction

Breast cancer (BC) is a common malignant tumor and a leading cause of death among women [1]. Although great achievement has been made, the high incidence of postsurgical recurrence and metastasis remain serious problems. Accumulating evidence suggests that ASCs may favor tumor progression by autocrine and paracrine signaling in different transplanted and metastatic tumor models [2]. However, the mechanism of interaction between ASCs and breast cancer in the tumor microenvironment remains to be determined.

The c-Kit signaling network is well studied in the tumor microenvironment [3]. SCF binds to the tyrosine kinase receptor c-kit (SCF/c-kit) and triggers the subsequent activation of the mitogen activated protein kinase (MAPK) pathways [4], inducing translocation of phosphorylated protein kinases to the nucleus and to stimulate transcriptional factor activities, including that of the E2F transcription factor 1 (E2F1), which is generally involved in cell proliferation, apoptosis and differentiation [5]. Abnormalities in E2F1 are frequently associated with tumor progression or metastasis and anticancer drug resistance [6]. E2F1-dependent progression can be mediated by the upregulation of EGFR and the activation of the cytoplasmic Ras/MAPK/ERK and PI3K/AKT signaling cascades [7]. Furthermore, E2F1 export from the nucleus and subsequent degradation are triggered by the stimulation of protein kinase C (PKC) and MAPK-P38 [8]. The phosphorylation of E2F1 at Ser403 and Thr433 by p38 is critical for the modulation of E2F1 stability and subcellular localization and for cell differentiation [8]. However, only a few studies have investigated whether E2F1 is regulated by the MAPK-p38 pathway to mediate the ASC-induced progression of breast cancer cells.

Tumor cell metastasis requires a coordinated sequence of multiple events, including tumor-host interactions and prometastatic molecular regulators. Many activated transcriptional factors can modulate the expression of posttranscriptional regulators in tumor cells to regulate tumor metastasis. For example, E2F transcription factor 1 (E2F1), modulates the expression of small non-coding RNAs in the tumor [9]. MicroRNAs(miRNAs), are endogenous non-coding small RNAs and miRNA involvement has been reported to be involved in numerous cancer cell processes, including proliferation, apoptosis, metabolism and angiogenesis [10]. Among the miRs, miR20b is encoded by the miR-106a-363 cluster located on human chromosome X and grouped

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with miR-17 located within the miR-17-92 cluster and the miR-106b-25 cluster [11]. It has been reported that an autoregulatory feedback loop between E2F1 and miR20b-5p is involved in cell proliferation and differentiation [12]. However, only a few studies have investigated whether miR20b mediates the epithelial to mesenchymal transition (EMT) and metastasis in breast cancer, especially in the context of regulation of breast cancer cell metastasis by ASCs.

Herein, we found that ASCs inhibited the biogenesis of miR20b in breast cancer cells through the paracrine release of SCF with the subsequent induction of breast cancer cell migration and invasion. Mechanistically, we demonstrated that the p-c-Kit/MAPK-p38/E2F1 cascade is involved in ASC/SCF-induced miR20b downregulation in BC cells. Furthermore, ASC induction of miR20b downregulation activated HIF-1α/VEGFA and induced EMT and metastasis of BC cells in vitro and in vivo.

2. Materials and methods

2.1. Cell lines and reagents

The breast cancer cell lines 4T1 were obtained from the American Type Culture Collection (Manassas, USA). All of the medium were purchased from HyClone and supplemented with 10% FBS (Gibco, USA). ASCs were isolated and identified as previously described [13]. SCF was purchased from R&D Systems and utilized at a final concentration of 100 ng/ml.

2.2. Animals

Four-week-old female nude mice (BALB/c) were obtained from the SLAC Laboratory Animal Center (Shanghai, China) and were housed in a specific-pathogen-free room. All the experimental protocols and animal handling procedures were approved by the Animal Committee of Harbin Medical University. All experimental procedures and postoperative animal care were conducted in accordance with the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals.

2.3. Wound healing assay

Breast cancer cells were plated into a 6-well plate. A scraped line was created using a 200 μl pipette tip when the cells had achieved 100% confluence. After incubation for 24 h, the wound closure was imaged with a microscope and the rate of closure was assessed.

2.4. Invasion assay

After coating the wells of a 24-well transwell culture plate (Corning, NY, USA) with 100 μl Matrigel (1 mg/ml) (BD, NY, USA), untransfected or transfected 4T1 cells were resuspended in FBS-free medium and plated in the upper chamber; the bottom chamber was filled with FBS-free medium with or without ASCs. The plates were incubated for 24 h at 37 °C and 5% CO_2. After removing the cells from the upper side of the membrane, the membrane was fixed with 4% formaldehyde solution and stained with 1% crystal violet solution. Then, the cells on the bottom side of the membrane were counted in five randomly chosen fields of view using a microscope at 200 × magnification.

2.5. Quantification of cell supernatant cytokines

Supernatant medium was collected after culturing for 1, 3, and 5 days by centrifugation for 10 min at 1000 rpm. The supernatant was immediately subjected to cytokine and chemokine detection with a commercially available Milliplex MAP kit (Cat.MAGPMAG-24 K, MMMP3MAG-79 K, Millipore Corporation, Billerica, MA, USA), according to the manufacturer's protocols.

2.6. RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using RNeasy Mini Kits (QIAGEN, Germany), according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed into cDNA using a QuantiTect Reverse Transcription Kit (QIAGEN). The primer sequences were provided in online supplementary table S4. qPCR was performed as described previously [3]. Amplification parameters were as follows: one cycle of 50 °C for 2 min and 95 °C for 3 min, followed by 35 cycles of 95 °C for 10 s and 60 °C for 30 s. Relative expression levels were determined using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$).

2.7. Western blot

All primary antibodies were obtained from Abcam (Abcam, USA). Proteins from cell and tissue lysates were analyzed using Western blotting as described previously [2], in which the nuclear protein extraction was performed according the protocol of CelLytic™ NuCLEAR™ Extraction Kit (Sigma, USA). In brief, samples were incubated overnight at 4 °C with primary antibodies against HIF-1α (1:1000), VEGFA (1:2000), c-Kit (1:500), p-c-Kit (1:1000), p-ERK1/2 (pT202/pY204, 1:1000), ERK1/2 (1:1000), P38(1:1000), E2F1 (1:2000), and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000) for 2 h at room temperature; protein-antibody complexes were visualized with an Enhanced Chemiluminescence Western Blotting Detection Kit (Beyotime Biotechnology, Shanghai, China) and an analysis system (Bio-Rad, USA).

2.8. RNA oligonucleotides and plasmid transfection

Breast cancer cells were respectively transfected with miR20b agomir, miR-20b inhibitor, MEK siRNA, P38 siRNA or nonspecific siRNA (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. The sequences of the siRNA oligonucleotides or shRNA were provided in online supplementary table S5. At 48 h after transfection, RNA and protein were isolated, and the relation between MAPK and E2F1 or E2F1 and miR20b promoter was tested.

2.9. Dual-luciferase reporter assay

4T1 breast cancer cells were co-transfected with the miR106a-363 (PGL-R1, PGL-R2) promoter-luciferase reporter plasmid (GenePharma, Shanghai, China) and E2F1 overexpression vector or control vector, and the pRL Renilla reporter (Promega, USA) as a control for the promoter assays of pri-miRNAs.

To validate HIF-1α and VEGFA as a direct target of miR20b, 4T1 cells were co-transfected with wild-type or mutant HIF-1α/VEGFA 3′UTR dual-luciferase reporter with miR20b agomirs or NC vector using Lipofectamine 2000 reagent in 96-well plates. After 24 h, cells were harvested, and luciferase activity was quantified using a Dual-Luciferase Assay Kit (Promega Corp., WI).

2.10. Immunofluorescence staining

Adherent cells plated on EZ Slides (Millipore, USA) were labelled with rat monoclonal anti-HIF-1α antibody (Abcam), followed by donkey anti-rat secondary antibody conjugated with Alexa Fluor 488 (Thermo Scientific, USA). After incubation with 4′6-diamidino-2-phenylindole (Thermo Scientific, USA) for 1 min, the cells were observed under a confocal microscopy system (Yokogawa, Tokyo, Japan).

2.11. Chromatin immunoprecipitation assay (ChIP)

ChIP was conducted using a ChIP assay kit (Active Motif, Rixensart,

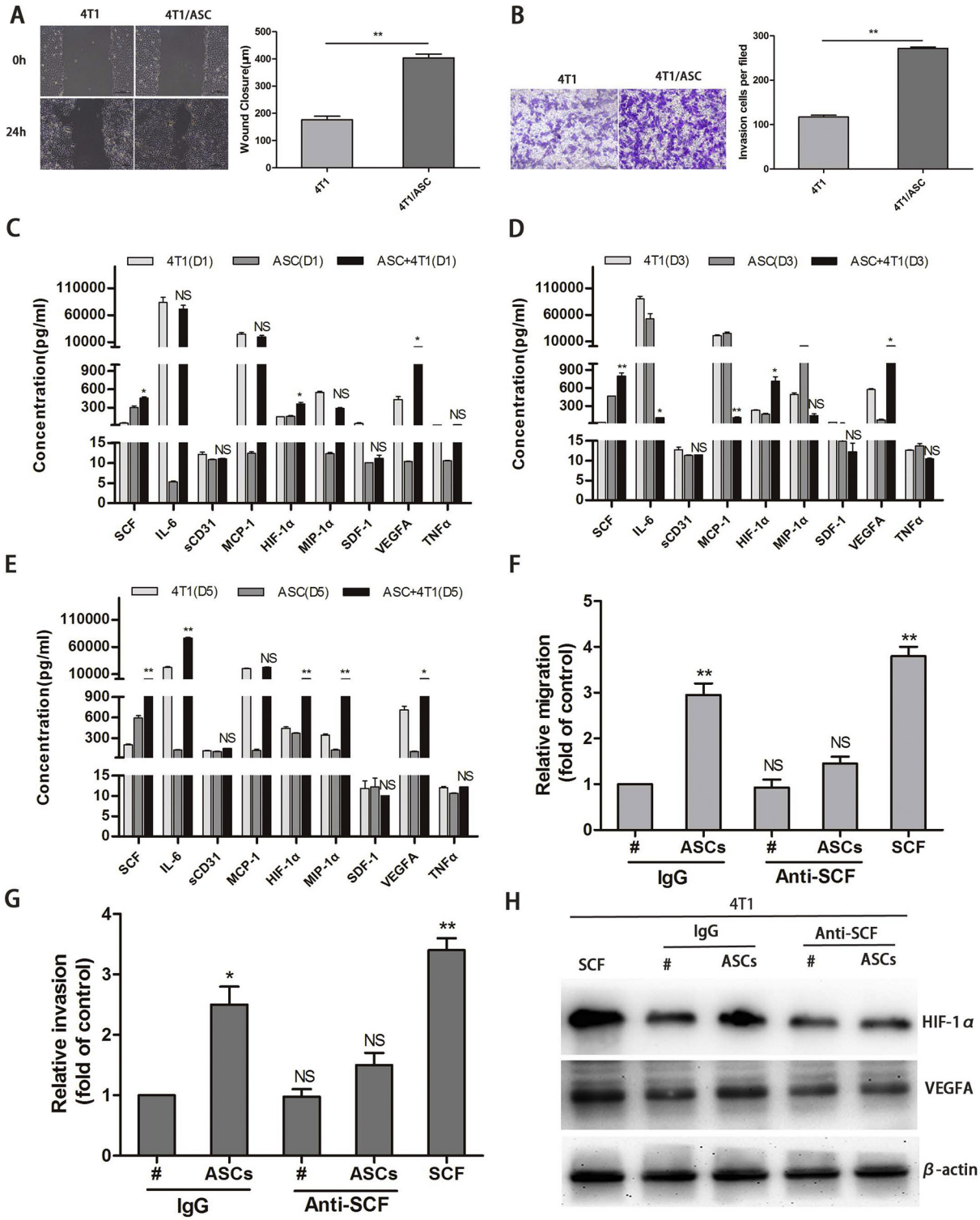


Fig. 1. ASC-induced migration and invasion of BC cells is mediated by SCF-dependent activation of HIF-1α and VEGFA. (A) Wound healing (B) and transwell assays were used to detect the migration and invasion of 4T1 cells treated with ASC culture supernatant. (C–E) The release of cytokines and chemokines including SCF, IL-6, sCD31, MCP-1, HIF-1α, MIP-1α, SDF-1, VEGFA and TNFα in 4T1 cells cocultured with ASCs was analyzed using a Milliplex MAP kit. (F) The migration (G) and invasion of 4T1 cells (H) and activation of HIF-1α and VEGFA induced by ASCs were assessed. The cells were pretreated with IgG or anti-SCF neutralizing antibody for 1 h. The cells stimulated with SCF for 2days were used as a positive control. *P < .05, **P < .01, ***P < .001, NS, not significant.

Belgium) following the manufacturer's instructions. Briefly, the cells were fixed with 1% formaldehyde for 10 min, after which chromatin was sonicated and immunoprecipitated with anti-HIF-1α antibodies, anti-E2F1 antibodies or non-immune rabbit IgG (Abcam, USA), and 10% chromatin was used as an input control. The immunoprecipitated DNA was assessed for the presence of the VEGFA promoter domain by PCR using the following primers: VEGFA forward 5′-CAGGAACAAGG GCCTCTGTCT-3′, reverse 5′-TGTCCCTCTGACAATGTGCCATC-3′; miR106a-363(1698 bp) forward: 5′- GAGAATGGAAAGATAAACA CGG-3′, reverse: 5′-TTTTGGCTGGTCGGTGC-3′; miR106a-363(2847 bp)

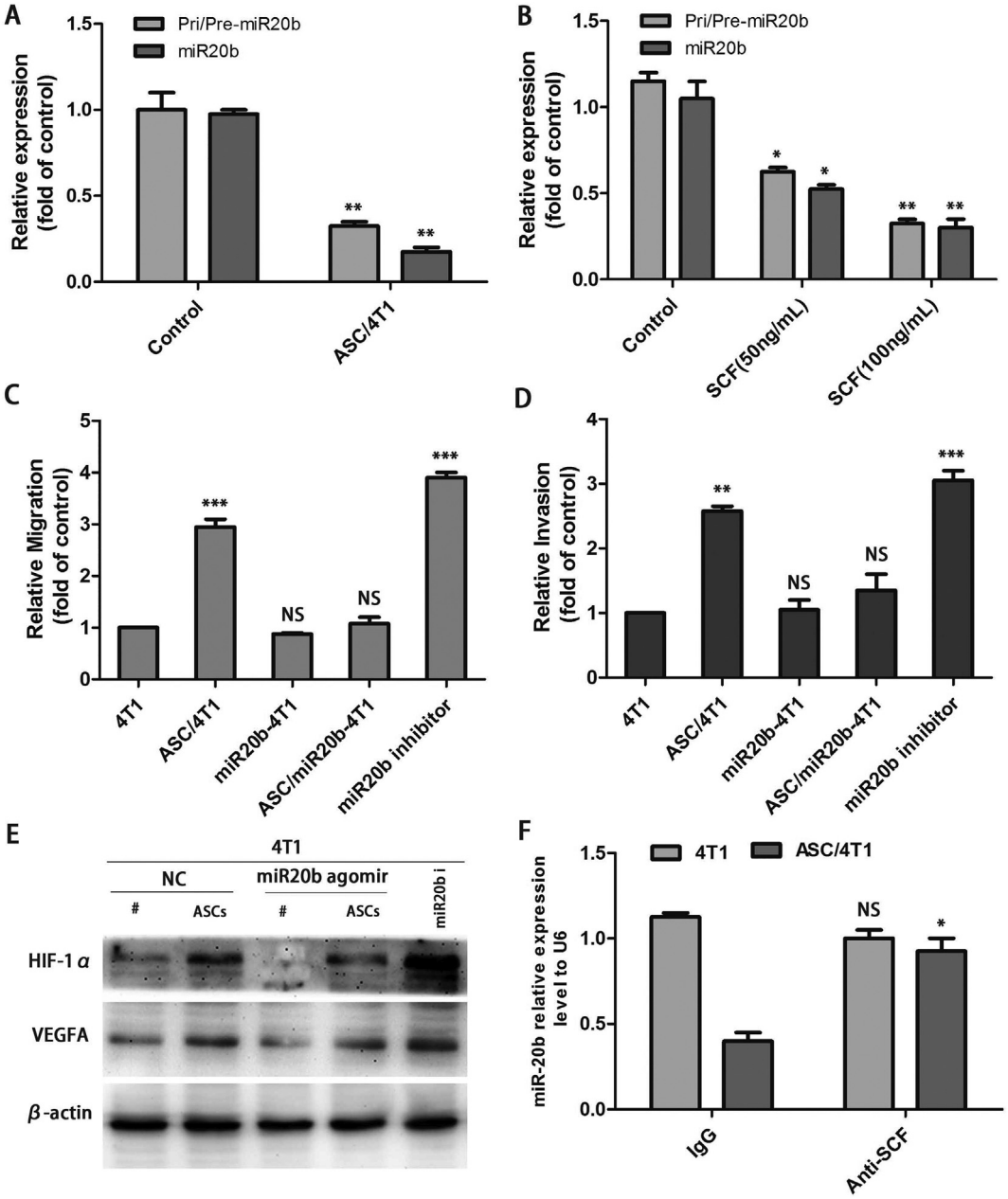


Fig. 2. ASC/SCF-induced BC cell migration and invasion are mediated by the inhibition of miR20b. (A) qPCR analysis of the expression levels of the primary/precursor and mature forms of miR20b in 4T1 cells cocultured with ASCs (B) or in 4T1 cells pretreated with SCF (50 ng/ml or 100 ng/ml) for 48 h. (C–E) 4T1 cells were transfected with a miR20b agomir or the corresponding control for 24 h and then cocultured with ASCs for 24 h. The cells pretreated with a miR-20b inhibitor were used as a positive control. The migration (C) and invasion (D) of the cells and the activities of HIF-1α and VEGFA (E) were assessed using a transwell assay and western blot, respectively. (F) The 4T1 cells pretreated with an anti-SCF antibody were cocultured with ASCs and then the expression levels of miR20b were determined by qPCR. *P < .05, **P < .01, ***P < .001, NS, not significant.

forward: 5′-CCGGTCCTGCCATGTTT-3′, reverse: 5′-ACGCTTCCACTGC TCCTG-3′. The amplified product was analyzed by agarose gel electrophoresis.

2.12. Animal model

Subcutaneous orthotopic injection in 4-week-old female nude mice (BALB/c) was performed under intraperitoneal injection anaesthesia (1.2% Avertin, 0.1 ml/10 g). 4T1 cells, and miR20b^{up} 4T1 cells were resuspended in 200 μl of PBS/Matrigel and injected into the mammary fourth fat pads of female nude mice with ASCs. In each group (n = 5), the tumor size was measured twice per week, and the tumor volume was calculated according to the following formula: tumor volume = 0.5 × (Dmax × Dmin^2). Three weeks after injection, the mice were sacrificed, and the primary tumours, livers and lungs were removed for further evaluation.

2.13. Statistical analysis

All experiments were performed in triplicate. All data were statistically analyzed using SPSS version 17.0, and graphs were made using GraphPad Prism version 5.0 software. One-way ANOVA and Newman-Keuls post hoc tests were used to compare variances between groups. P value < .05 was considered to be a significant difference.

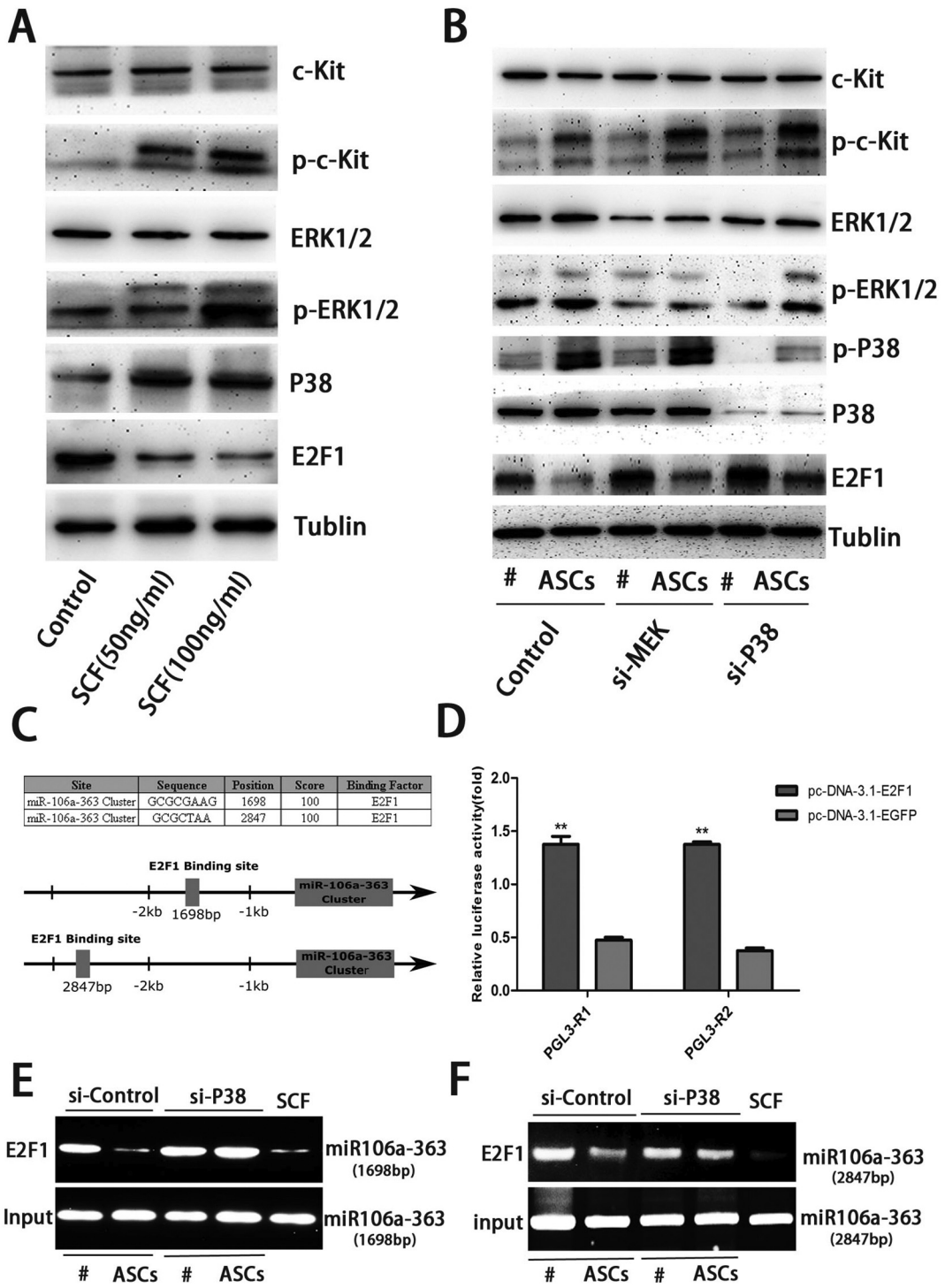
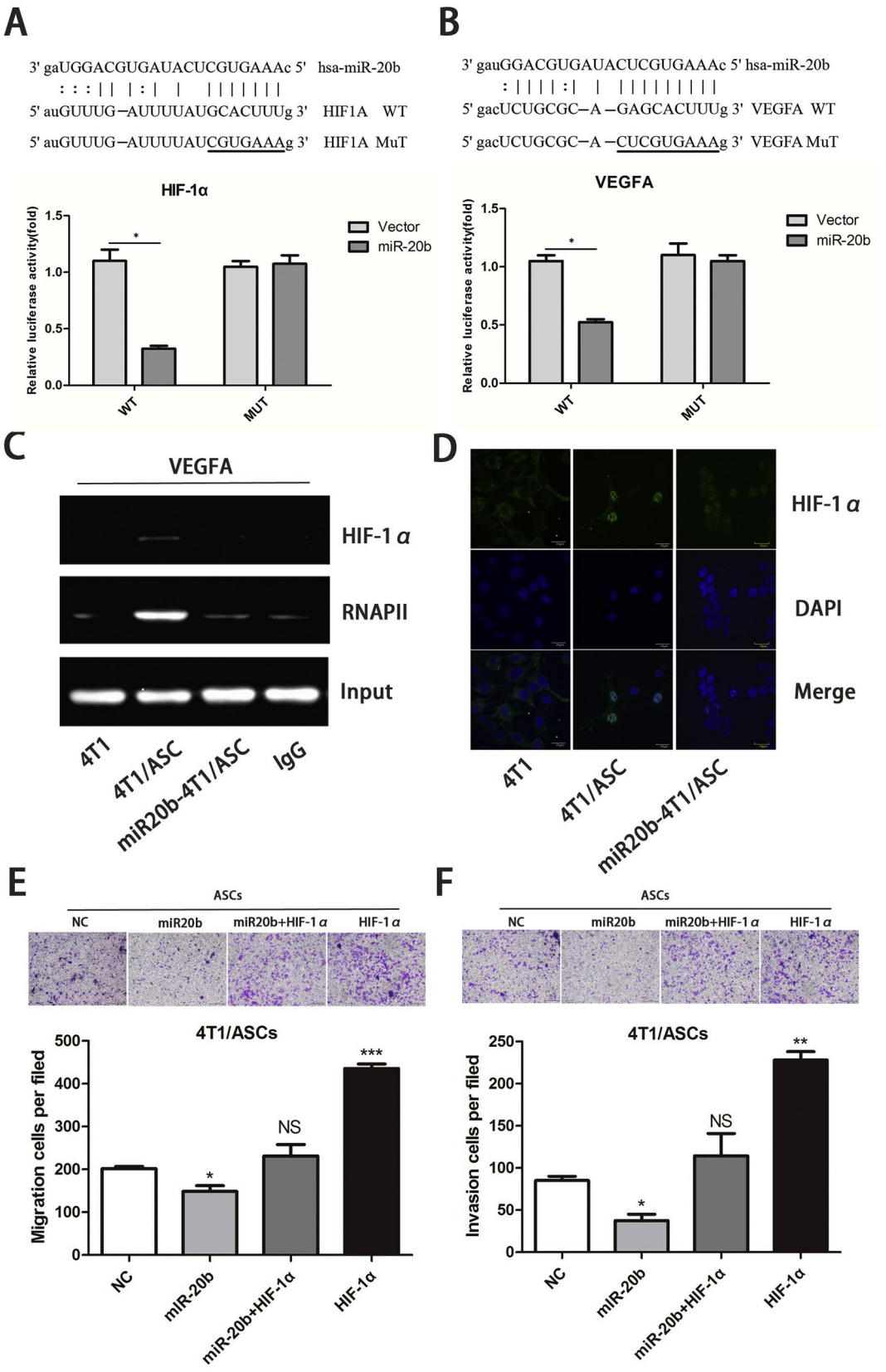


Fig. 3. ASCs/SCF inhibit miR20b transcription in BC cells through the p-c-Kit/p38 MAPK/E2F1 pathway. (A) 4T1 cells were stimulated with SCF (50 ng/ml or 100 ng/ml) or (B) 4T1 cells were transfected with an siRNA specific for MEK (si-MEK) or p38 (si-p38) or a control siRNA and then the indicated proteins were analyzed by western blot. (C) Basic information and location of the predicted E2F1 binding sites on the miR106a-363 cluster promoter. (D) Relative luciferase activity of pGL3-R1 and pGL3-R2 after pcDNA3.1-E2F1 or pcDNA3.1-EGFP transfection. (*E*-F) 4T1 cells transfected with p38-specific siRNA were cocultured with ASCs and then E2F1 protein binding to the miR106a-363 cluster promoter was analyzed using a ChIP assay. *P < .05, **P < .01, ***P < .001, NS not significant.

3. Results

3.1. SCF released by c-kit+ ASCs contributes to the induction of BC cell migration and invasion through HIF-1α and VEGFA activation

To confirm the effects of ASCs on the migration and invasion of BC cells, we cultured 4T1 breast cancer cells with the supernatant of ASC-conditioned medium. The results showed that the migration and invasion abilities of 4T1 cells were significantly enhanced by the ASCs (Fig. 1A–B). ASCs produce several cytokines and chemokines that affect cancer cells [2]. To explore the molecular mechanisms of the ASCs enhanced the migration and invasion of BC cells, we analyzed the levels of SCF, IL-6, sCD31, monocyte chemotactic protein-1 (MCP-1), hypoxia inducible factor 1-α (HIF-1α), macrophage inflammatory protein-1α



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(MIP-1α), stromal cell-derived factor-1 (SDF-1), vascular endothelial growth factor A (VEGFA) and tumor necrosis factor α (TNFα) in direct ASC/4T1 coculture (see online supplementary Table S1–3). Quantification analysis revealed that the levels of SCF, HIF-1α and VEGFA were significantly higher in the ASC/4T1 coculture group than in the single culture groups (Fig. 1C–E). Pretreatment of ASCs with an

Fig. 4. ASCs activates HIF-1α and VEGFA by silencing miR20b in BC cells. (A-B) Luciferase reporter plasmids containing the wild-type or mutant miR20b binding sequence in the 3′-UTRs of HIF-1α and VEGFA mRNA. The luciferase plasmids and a control vector or a miR20b agomir were cotransfected into 4T1 cells and then the relative luciferase activity was analyzed in the cells. (C) The untransfected 4T1 cells or 4T1 cells transfected with a miR20b agomir were cocultured with ASCs and chromatin immunoprecipitation assays were performed using anti-HIF-1α and RNAPII; the amplified products of the VEGFA promoter were analyzed by agarose gel electrophoresis. IgG was used as a negative control. (D) The location of HIF-1α (green fluorescence) in 4T1 cells was assessed by confocal microscopy. (E-F) 4T1 cells were transfected with miR20b agomir, miR20b agomir plus HIF-1α, HIF-1α vector or control constructs for 24 h and then cocultured with ASCs for 24 h. The migration (E) and invasion (F) of these cells were assessed using a transwell assay and western blot. *P < .05, **P < .01, ***P < .001, NS, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

anti-SCF antibody (isotypic IgG was used as a control at 10 μg/ml) resulted in the inhibition of ASC-induced migration and invasion of 4T1 cell, and mouse recombinant SCF (100 ng/ml) induced 4T1 cell migration and invasion (Fig. 1F–G). Moreover, treatment with an anti-SCF antibody resulted in the inhibition of ASC-induced HIF-α and VEGFA expression in 4T1 cells (Fig. 1H, Supplementary Fig. S1A–B). These results indicate that SCF secreted from ASCs predominantly activates HIF-α and VEGFA and mediates the ASC-induced migration and invasion of the BC cells.

3.2. ASC-induced BC cell migration and invasion are mediated by SCF-dependent -induction of miR-20b downregulation in BC cells

Since miR20b is negatively correlated with the advanced invasive stages of breast cancer as an antisense-miR, we examined the contribution of miR20b to the ASC-induced migration and invasion of 4T1 cells by coculture of 4T1 cells with ASCs or by treatment of 4T1 cells with SCF, and analyzed the expression levels of primary/precursor and mature miR20b in 4T1 cells. We observed that the expression levels of miR20b in 4T1 are downregulated after coculture and treatment with SCF (Fig. 2A–B). Furthermore, the overexpression of miR20b inhibited ASC-induced migration and invasion of 4T1 cells (Fig. 2C–D). To further investigate the effects of miR20b on HIF-1α and VEGFA protein expression levels in BC cells, a miR20b agomir or a miR20b inhibitor were used to upregulate or knockdown the expression of miR20b, respectively. We found that transfecting 4T1 cells with a miR20b agomir inhibited ASC-induced HIF-1α and VEGFA expression; however, intracellular HIF-1α and VEGFA protein levels increased after miR20b silencing (Fig. 2E, Supplementary Fig. S1C–D). Pretreatment of 4T1 cells with an anti-SCF antibody resulted in the upregulation of ASC-induced miR20b (Fig. 2F). These results suggest that ASC-induced breast cancer cell migration and invasion are mediated by SCF-dependent induction of miR20b downregulation and subsequent regulation of HIF-1α and VEGFA expression in BC cells.

3.3. The p-c-kit/MAPK-p38/E2F1 cascade is involved in ASC/SCF-induced miR20b downregulation in BC cells

To elucidate the signaling pathways involved in ASC-induced miR20b downregulation in BC cells, we examined whether the MAPK pathway was activated in 4T1 cells treated with SCF and found that the activation of SCF/c-Kit triggers phosphorylation of ERK (p-ERK) and the upregulation of p38, which could inhibit the expression of E2F1 in the nucleus (Fig. 3A). In consideration of the ERK1/2 is the only downstream protein target of MEK1/2, and to further confirm the contribution of the MAPK p38 pathway to E2F1 expression and ASC-induced migration and invasion of the BC cells, we treated 4T1 cells with a MEK-specific siRNA and a p38-specific siRNA and analyzed the changes in E2F1 levels by western blot. We found that the E2F1 expression levels in the nuclei were significantly enhanced by p38-specific siRNA following the phospho-P38 decrease (Fig. 3B, Supplementary Fig. S2A–C). Interestingly, the MEK-specific siRNA did not significantly enhance the expression of E2F1 in the nuclei (Fig. 3B, Supplementary Fig. S2C). In addition, the ASC-induced migration and invasion of cancer cells were abrogated by the p38-specific siRNA (Supplementary Fig. S2D–E).

Considering that the promoter region of miR20b contains E2F1 binding sites according to the MATCH software (Fig. 3C), we investigated whether E2F1 regulates miR20b at the transcriptional level. The results showed that the expression levels of miR106a-363, primary precursors and mature miR20b-5p were significantly changed when 4T1 cells were transfected with an E2F1-specific siRNA (Supplementary Fig. S2F). To further explore whether E2F1 can regulate the expression of miR106a-363 clusters directly, 4T1 cells with stable expression of E2F1 were cotransfected with the miR106a-363-promoter-driven luciferase plasmid. We found that the overexpression of E2F1 markedly increased miR106a-363 promoter activity in 4T1 cells (Fig. 3D). A ChIP assay demonstrated that ASCs induced the binding of E2F1 to the promoter region of miR106a-363 in 4T1 cells through the SCF/MAPK p38 cascade (Fig. 3E–F). These results indicate that ASCs induce miR20b downregulation in BC cells at the transcriptional level through the production of SCF and the subsequent SCF-dependent induction of the MAPK-p38/E2F1 cascade in BC cells.

3.4. ASC induction of miR20b downregulation activates HIF-1α and VEGFA

To further investigate the mechanism of miR20b regulation of the migration and invasion of breast cancer cells, we hypothesized that HIF-1α and VEGFA may be the target genes of miR20b according to the predictions of the PicTar, miRWalk and TargetScan programs. Then, the luciferase reporter plasmids containing HIF-1α-3′UTR or VEGFA-3′UTR with a wild type miR20b-bingding site or a mutated miR20b-bingding site were cotransfected with a miR20b agomir in 4T1 breast cancer cells (Fig. 4A–B). As shown in Fig. 4A and Fig. 4B, the overexpression of miR20b significantly inhibited the luciferase reporter activity of the wild-type HIF-1α-3′UTR and the VEGFA-3′UTR but not the activity of the mutant HIF-1α-3′UTR and the VEGFA-3′UTR constructs. A previous study showed that HIF-1α can bind to the VEGFA promoter and reactivate its activity in BC cells. Therefore, a ChIP assay was performed to detect the proteins bound to the VEGFA promoter in 4T1 cells. We found that the binding of HIF-1α to the VEGFA promoter was significantly decreased in the 4T1 cells transfected with miR20b and cocultured with ASCs compared to that in the untreated cells (Fig. 4C, Supplementary Fig. S3A). In agreement with a report of Sandra Cascio et al. on miR20b [14], our results showed that HIF-1α levels were considerably reduced in the nuclei of 4T1 cells transfected with miR20b and stimulated with ASCs compared to the levels in the 4T1 cells treated with ASCs, but which was higher than the levels in untreated 4T1 cells (Fig. 4D, Supplementary Fig. S3B). In addition, we found that the overexpression of HIF-1α markedly increased the ASC-induced breast cancer cell migration and invasion, whereas miR20b upregulation markedly attenuated HIF-1α promoter activity (Fig. 4E–F). These results indicate that ASCs can induce BC cell migration and invasion through the SCF/miR20b/HIF-1α/VEGFA signaling cascade.

3.5. SCF released from c-kit+ ASCs induces BC cell metastasis through inhibition of miR20b in vivo

To confirm that ASC-stimulated BC cells exhibit the EMT phenotype in vitro, we detected the mRNA levels of the EMT-related genes in untreated 4T1 cells, 4T1 cells overexpressing miR20b, and 4T1 cells

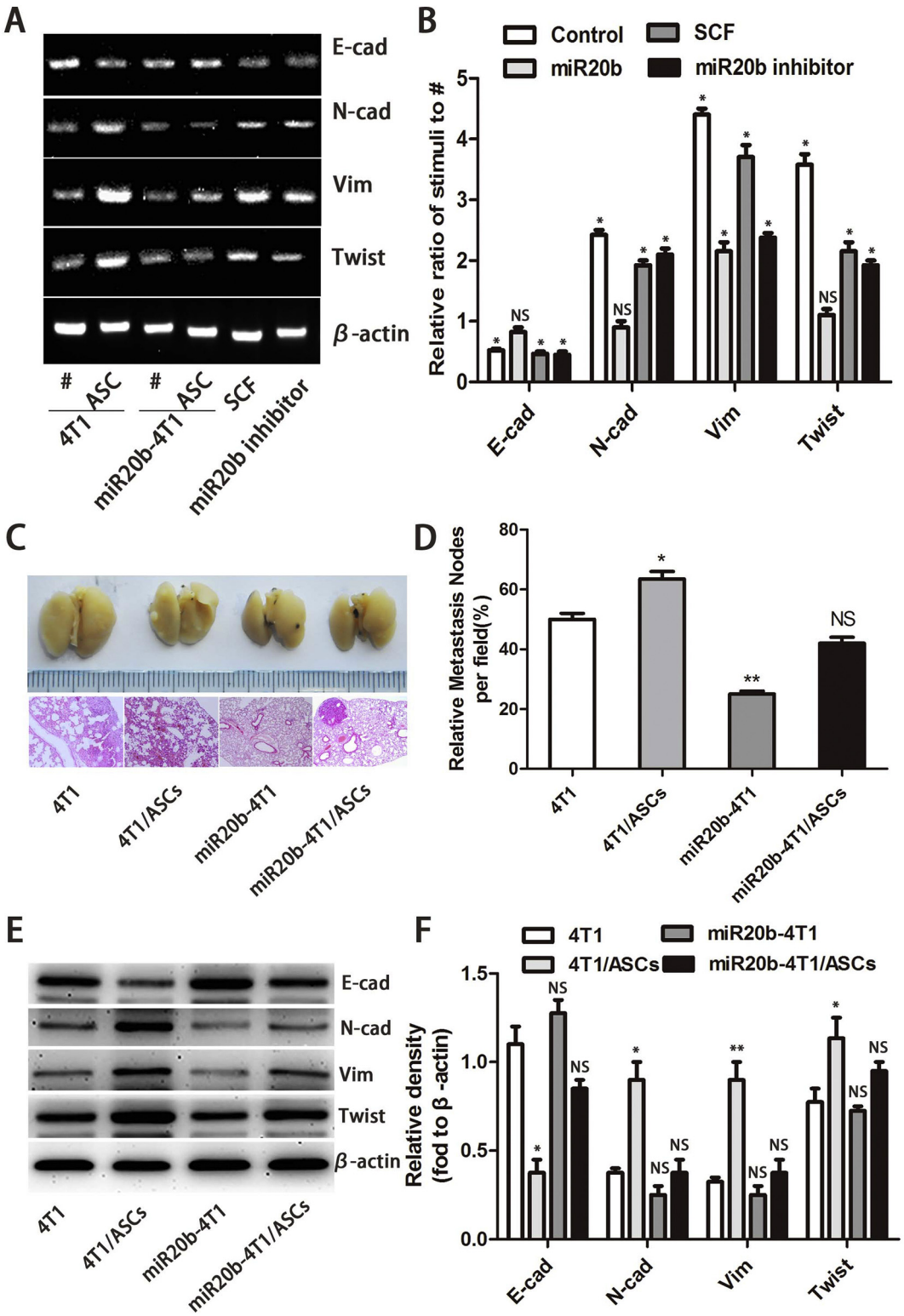

Fig. 5. ASC-stimulated BC cells exhibit the EMT phenotype in vitro and possess the pulmonary metastasis potential in vivo. (A-B) The mRNA levels of the indicated EMT-related genes were examined in monocultured 4T1, miR20b^{up} 4T1 cells, and cells cocultured with ASCs for 3 days were examined. The results were normalized, and the density value of the 4T1(#) or miR20b-4T1(#) was set to 1. The cells were treated with SCF and a miR20b inhibitor was used as a positive control. (C) Tumor metastasis analysis. Representative images of the lung tissue samples and micrographs of the HE staining. (D) Relative metastasis in lung nodules was analyzed in each group of nude mice (n = 5 per group). (E-F) Western blot analysis of the expression levels of the indicated EMT-related proteins in tumor xenografts. *P < .05, **P < .01, ***P < .001.

cocultured with ASCs. The results indicate that coculture of 4T1 cells with ASCs resulted in increased expression levels of N-cadherin, vimentin and Twist and decreased expression levels of E-cadherin (Fig. 5A–B). These changes can be mimicked by treatment with SCF and a miR20b inhibitor, but the expressions levels of the EMT-related genes were reduced in the 4T1 cells overexpressing miR20b that were

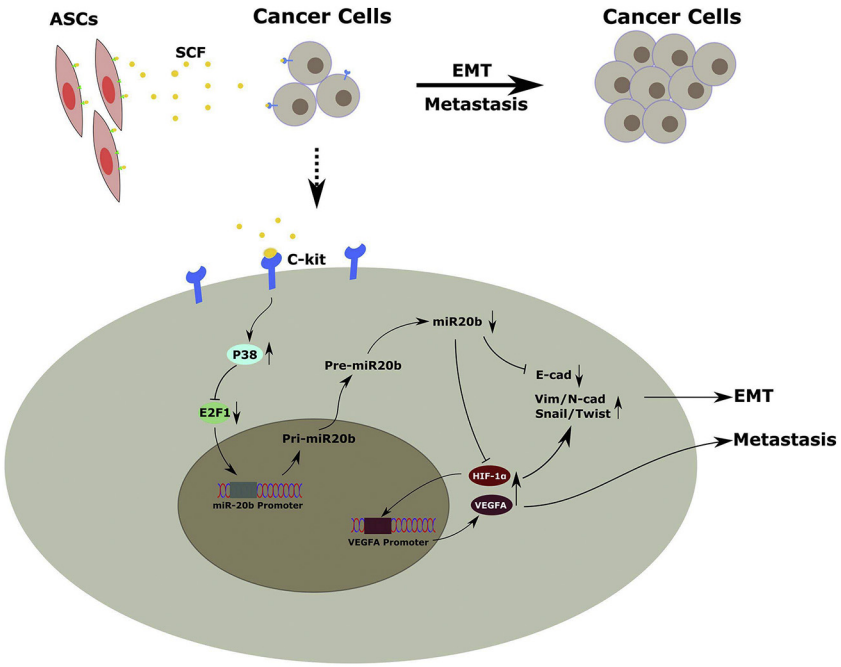

Fig. 6. Overview of the proposed pathways of ASC-induced metastasis of breast cancer cells.

cocultured with ASCs (Fig. 5A–B).

To investigate ASC-educated BC cell metastasis in vivo, we performed subcutaneous coinjection of ASCs with untreated 4T1 cells or with 4T1 cells overexpressing miR20b into nude mice. The results showed that ASC-stimulated BC cells spread out to form more nodules in the lung compared to the untreated BC cells (Fig. 5C–D). In contrast, interfering with miR20b expression in 4T1 cells inhibited pulmonary metastasis (Fig. 5C–D). Similarly, upregulating miR20b in 4T1 cells reduced the numbers of ASC-induced metastasis nodules in the lung (Fig. 5C–D). Furthermore, the results were confirmed by detecting the expressions levels of E-cadherin, N-cadherin, vimentin and Twist using western blot of xenograft tumor tissue samples (Fig. 5E–F). Taken together, our findings demonstrated that ASCs can induce pulmonary metastasis of BC cells in vivo through the inhibition of miR20b.

4. Discussion

Breast cancer metastasis after surgery is associated with the wound healing signaling pathways that can increase local immunosuppression, exert direct effects on the tumor microenvironment and promote distant metastasis [15]. ASCs with autologous fat grafts are used to repair the loss of breast tissue after breast cancer surgery, however, our previous study has shown that ASCs can promote tumorigenesis and metastasis in breast cancer [12]. The precise mechanisms that govern tumor metastasis remain to be elucidated. In this study, we found that c-Kit-positive ASCs can produce high levels of SCF to influence BC cells and promote their EMT and metastasis through the inhibition of miR20b biogenesis (Fig. 6). Mechanistically, we demonstrated that SCF-dependent induction of miR20b downregulation is mediated by the MAPK-p38/E2F1 pathway that can activate HIF-1α/VEGFA and induce EMT and metastasis of BC cells.

SCF plays a critical role in guiding cell migration during development. In addition, SCF activates tissue-resident mast cells to generate a tumor-promoting angiogenic microenvironment [16]. The tumor-promoting effect of SCF are considered to be caused by KitL stimulation of c-Kit+ tumor cells by triggering downstream activation of the MAPK pathway [4], of which the p38 and ERK proteins are generally involved in survival, proliferation, and cell cycle progression [17]. E2F1, as a transcriptional factor, plays a critical role in cell cycle progression [5]. E2F1 activity is mediated by interaction with p38, which modifies its Ser403 and Thr433 residues [8]. Our results clearly confirm that E2F1 is regulated by the p-c-Kit/ MAPK-p38 pathway to mediate the ASC-induced progression of breast cancer cells.

Recent studies have shown the importance of miRNA in regulating cancer progression [10]. miR20b has been reported as a negative regulator of breast cancer angiogenesis and metastasis [18]. However, only a few studies have elucidated the mechanisms by which miR20b interacts with ASCs in the modulation of target gene expression and function in BC cell migration, invasion and metastasis. Previous studies have indicated that posttranscriptional expression of miR20b is triggered by E2F1 during the regulation of myoblast proliferation and differentiation [12]. In addition, several cancer metastasis-associated genes, including HIF-1α and VEGFA, have been reported to be targeted by miR20b in breast cancer cells [14]. In this study, we demonstrated that miR20b biogenesis in BC cells can be inhibited by ASC-released SCF through the downstream c-Kit/MAPK-p38/E2F1 signaling cascade and that miR20b acts as a tumor suppressor miRNA by inhibiting BC cell migration and invasion, particularly in EMT and metastasis. Moreover, bioinformatic approaches suggested that miR20b may target HIF-1α/VEGFA, and our study has shown a positive correlation between ASC-released SCF and HIF-1α/VEGFA; additionally, HIF-1α can be recruited to the VEGFA promoter in BC cells following ASC treatment to inhibit the miR20b-dependent attenuation of the HIF-1α nuclear accumulation during the ASC-induced BC cell migration and invasion. Previous studies have shown hypoxia can induce cytoskeletal remodeling and angiogenesis through HIF-1α/VEGFA to drive EMT and metastasis [19,20], and our results demonstrated that ASC-released SCF induced miR-20b downregulation in BC cells and might induce EMT and lung metastasis through the activation of the HIF-1α/VEGFA to upregulate the N-cadherin, vimentin and Twist transcriptions in vitro and vivo.

Accumulating evidence suggests that breast cancer cells are highly heterogenous, it is worth evaluating whether this is a common mechanism shared by the other breast cancer cell lines. However, tumor microenvironment is complex, non-homologous interference could result in false-negative, therefore, we choose the 4T1 mouse breast cancer cells to perform these experiments. Certainly, human tissue-derived ASCs are needed to prove the mechanism in human breast cancer cell

lines.

In conclusion, we have revealed a new regulatory mechanism by which ASC/SCF downregulation of miR20b activates HIF-1α/VEGFA and induces BC cell EMT and metastasis, and we show that this process is activated by the p-c-Kit/MAPK-p38/E2F1 pathway (Fig. 6). Taken together, our data demonstrate that the p-c-Kit/MAPK-p38/E2F1-miR20b-HIF-1α/VEGFA signaling pathway can serve as a potential therapeutic target in ASC-induced breast cancer cell metastasis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109350.

Conflicts of interests

The authors declare that they have no conflicts of interest.

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