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17β-Estradiol promotes angiogenesis of bone marrow mesenchymal stem cells by upregulating the PI3K-Akt signaling pathway

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ABSTRACT

Objective: Estrogen is an important hormone affecting angiogenesis in women and is important for female physical development. Menopausal women are prone to serious cardiovascular and cerebrovascular diseases when estrogen is significantly reduced. Bone marrow mesenchymal stem cells (BMSC) have potential roles in processes such as angiogenesis and remodeling. This study is to investigate the effect of 17β-estradiol on BMSC angiogenic differentiation and its underlying molecular mechanism, and to provide a basis for the treatment of microvascular diseases.

Methods: Enrichment analysis of apoptosis, migration or angiogenesis processes and molecular mechanisms of BMSC treated with 17β-estradiol was performed to screen core proteins and perform molecular docking validation. Human MSCs were cultured in vitro to examine the effect of 17β-estradiol on BMSC migration or angiogenic differentiation.

Results: 17β-estradiol acted on 48 targets of BMSC and was involved in regulating 52 cell migration processes or 17 angiogenesis processes through 66 KEGG pathways such as PI3K-Akt, MAPK, etc. 17β-estradiol bound tightly to 10 core proteins including APP, NTRK1, EGFR, and HSP90AA1. 17β-estradiol promoted cell scratch area closure rate and CD31 expression in BMSCs, downregulated BMSC apoptosis rate, and promoted Akt and p-Akt protein expression in BMSC.

Conclusion: 17β-estradiol binds to FN1, MCM2, XPO1, NTRK1 and other proteins to initiate PI3K-Akt, MAPK and other signaling pathways, so as to regulate BMSC to promote or remodel angiogenesis, verifying that 17β-estradiol up-regulates PI3K-Akt signaling pathway to promote BMSC angiogenic differentiation.

1. Introduction

Atherosclerosis, diabetes, and other factors can lead to the thickening of the microvascular wall and stenosis of the lumen, which often forms a hypoxic environment and is complicated by serious dysfunction [1]. Vascular endothelial cell injury after childbirth is a high-risk factor for concurrent pregnancy-induced thrombotic microangiopathy (TMA) [2]. Therefore, ensuring good vascular endothelial cell function and promoting angiogenesis are important methods of correcting microcirculation disorders [3].

Bone marrow mesenchymal stem cells (BMSC) are important types of mesenchymal stem cells(MSC)[4], which have multiple differentiation potentials and are considered to be the most potential seed cells for tissue repair[5]. Under certain induction conditions, osteoblasts, chondrocytes, vascular endothelial cells, and neurons are directionally differentiated[6,7], and therefore stem cells have been intensively explored as alternative sources of mature endothelial cells in order to improve vascular tissue engineering [8]. MSC are recruited by inflammatory factors, chemokines, and other targeted chemokines to damaged tissues [9], differentiate into vascular endothelial cells, fibroblasts and other cells, and participate in tissue damage repair. BMSCs homing to the ischemic site of myocardial infarction reduce the apoptosis rate of vascular endothelial cells through the Fas pathway, significantly promote angiogenesis and improve cardiac function [10]. In addition, MSCs can also secrete VEGF, EGF and other cytokines to promote angiogenesis, promote the healing of ischemic endometrium, and maintain uterine physiological function [11]. The periodic generation of blood vessels is an important physiological process of endometrial regeneration and remodeling. 17β-estradiol promotes stromal cell angiogenesis, regulates angiogenesis, or remodels vascular development and plays a role in regulating endometrial

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angiogenesis in the menstrual cycle [12,13]. The expression of 17β-estradiol can lead to myocardial hypertrophy or a progressive increase in the left ventricular loading response in female rats [14,15]. The use of 17β-estradiol and MSCs to promote angiogenesis can accelerate angiogenesis in female reproductive organs, promote the repair of endometrial damage and reverse microvascular disorders. Some studies believe that human umbilical cord mesenchymal stem cells (hUC-MSCs) can home to the endometrium or myometrium of the rat endometrial adhesion animal model, reducing the degree of intrauterine adhesion and restoring periodic endometrial hyperplasia [16]. In addition, it was found that 17β-estradiol significantly promoted the secretion of stromal cell-derived factor-1α (SDF-1α), a CXC-type chemokine-12 (CXCL-12), by BMSCs, promoting BMSC migration [17]. This shows that 17β-estradiol can recruit MSCs to homing, it positively affects MSCs in regeneration and tissue damage repair. This study aimed to investigate how 17β-estradiol regulates BMSC angiogenesis and remodeling and its potential molecular mechanism to build a research foundation for the treatment of female microcirculatory disorders.

2. Materials and methods

2.1. Target analysis of 17β-estradiol in BMSCs

The ChEMBL database (https://www.ebi.ac.uk) was used to analyze potential targets of 17β-estradiol. The BMSC differential gene expression profile was obtained from the GSE9520 dataset in the Gene Expression Omnibus (GEO) database after adjusting to $P < 0.05$ and $|logFC| \geq 1$. The 17β-estradiol targets on BMSCs were screened using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html). The expression heatmap of 17β-estradiol and BMSCs was prepared by using TBtools v1.046 [18].

2.2. Gene Ontology (GO) and KEGG pathway enrichment analysis

The effects of 17β-estradiol on BMSC apoptosis, migration, angiogenesis remodeling, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway were analyzed by Gene Ontology (GO) enrichment analysis by using Metascape (https://metascape.org) [19]. The median target expression was used as the standard to screen highly expressed targets.

2.3. Core proteins and molecular docking

The BisoGenet 3.0.0 plug-in of Cytoscape 3.8 was used to predict the protein–protein interaction (PPI) network between 17β-estradiol and BMSCs [20]. Cytoscape3.8′s cytoHubba 0.1 plug-in was used to screen the top 10 core proteins with Degree as the topology method. The core protein 3D structure was downloaded using the PDB database (https://www.rcsb.org). The molecular binding energy of 17β-estradiol to the core protein was calculated using AutoDock Vina 1.1.2 [21]. The visualization model diagram of molecular docking interactions was constructed using PyMOL 1.8. With a binding energy < -5.0 kJ/mol, the core protein that better binds to 17β-estradiol was screened [22].

2.4. BMSC culture and experimental grouping

α-Minimum Essential Medium (α-MEM) that contains 10 % fetal bovine serum (cat. no. 16140071; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mmol/l glutamine (cat. no. 1294808; Sigma–Aldrich, St. Louis, MO, USA) (cat. no. 12571063; Thermo, Waltham, MA, USA) was used to cultivate BMSCs (cat. no. HUXMA-01001; Cyagen Biosciences, Guangzhou, Guangdong, China). After resuscitation, 3- to 4-generation cells were used for the experiment, and none of the cells died or senesced during the experiment. The control group consisted of BMSCs cultured without any stimulation; the 17β-estradiol group consisted of BMSCs stimulated with 10^{-4}μmol/L 17β-estradiol (cat. no. E8875, Sigma–Aldrich, MO, USA)[17]; the PI3K inhibitor group consisted of BMSCs stimulated with 50 μmol/L LY294002 (cat. no. S1105; Selleck Chemicals, Shanghai, China) and 10^{-4}μmol/L 17β-estradiol.

2.5. 5Angiogenic endothelial cell differentiation

α-MEM medium (cat. no. 12571063; Thermo, USA) that contained 10 % fetal bovine serum (cat. no. 16140071; Thermo, USA), 2 mmol/l glutamine (cat. no. 1294808; Sigma–Aldrich, USA), 10 ng/mL VEGF (cat. no. 293-VE-010/CF; R&D, Minneapolis, MN, USA), 5 ng/mL bFGF (cat. no. 233-FB-010/CF; R&D, MN, USA) served as the vascular differentiation-inducing solution. BMSC cultured in vascular endothelial cell induction medium were used as the negative control group, BMSC cultured in vascular endothelial cell induction medium containing 10^{-4}μmol/L 17β-estradiol were used as the 17β-estradiol group, and BMSC cultured in vascular endothelial cell induction medium containing 50 μmol/L LY294002 and 10^{-4}μmol/L 17β-estradiol were used as the PI3K inhibitor group. BMSC in each group were cultured in vascular endothelial cell differentiation induction medium at 37 °C and 5 % CO_2 for 14 days.

2.6. Flow cytometry cell apoptosis experiment

An Annexin V-FITC Cell Apoptosis Detection Kit (cat. no. C1062S, Beyotime Biotechnology, Shanghai, China) was used to perform Annexin V-FITC/PI double labeling flow cytometry cell apoptosis experiments. Briefly, BMSCs from each 4.5×10^5 group were resuspended in 12.5 ml/L Annexin V-FITC for 15 min; centrifugation at 4 °C and 1000 r/min for 5 min; 20 g/L propidium iodide (PI) was added and incubated for 3 min; centrifugation at 4 °C and 1000 r/min for 5 min; and 0.01 mmol/L phosphate-buffered saline (PBS; cat. no. ZLI-9061, Zhongshan Golden Bridge Technology Co. ltd., Beijing, China) to suspend the cells. A FACSAria Ⅱ flow cytometer (BD Biosciences, San Jose, CA, USA) was used to detect the apoptosis rate.

2.7. Cell scratch experiments

2.0×10^5 BMSCs were cultured in 6-well flat-bottom cell plates (Corning Co. ltd., Corning, NY, USA) at 37 °C for 12 h in 5 % CO_2. A scratch was created by scraping the cells away with a p1000 pipette tip. Serum-free α-MEM medium containing relevant reagents was added according to the experimental groups and cultured for 24 h at 37 °C, 5 % CO_2. The cells were fixed with 4 % paraformaldehyde (cat. no. P0099, Beyotime Biotechnology, Shanghai, China) for 4 h. An IX53-type inverted fluorescence microscope (Olympus, Tokyo, Japan) was used to record the cell scratch area. The closure rate of the BMSC scratch was calculated at 24 h [23].

2.8. Western blot

Cell lysate (cat. no. P0013J, Beyotime, Shanghai, China) lysed 8.5×10^7 BMSCs in each group and centrifuged at 4 °C and 12000 rpm for 10 min. Protein concentrations were measured by a BCA protein concentration detection kit (cat. no. P0011, Beyotime, China), 55 μg of BMSC lysate samples from each group were subjected to SDS–PAGE on a 0.45 μm PVDF membrane (cat. no. FFP36, Beyotime, China). After the skimmed milk powder was sealed for 60 min, rabbit anti-human phospho Akt antibody (P-Akt, cat. no. ab8933, 1:250, Abcam, Cambridge, USA) and rabbit

anti-human Akt antibody (cat. no. ab18785, 1:200, Abcam, USA) and incubated at 4 °C for 18 h; horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (cat. no. ab6721, 1:500, Abcam, USA) was incubated for 240 min at room temperature. β-actin (cat. no. ab5964, 1:400, Abcam, USA) was the internal reference control. An ultrasensitive ECL chemiluminescence kit (cat. no. P0018AS, Beyotime, Shanghai, China) and a Tanon 1600 gel image analysis system (Tanon. ltd., Shanghai, China) were used to detect protein expression. Image-Pro Plus 6.0 (Mediacy Cybernetics, Inc. Bethesda, MD, USA) analyzes the expression of each protein band.

2.9. Statistical analysis

All experiments were repeated 3 times. Data were analyzed by using GraphPad Prism 8.0.2.263 software (GraphPad Software, San Diego, CA, USA). One-way analysis of variance was used to compare multiple groups, and the Q test was used for comparisons between two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. 17β-estradiol targets on BMSCs

A total of 246 potential 17β-estradiol targets were predicted by using the ChEMBL platform, and a total of 2830 BMSC gene expression profiles were detected by the GSE9520 dataset, including 250 upregulated genes and 406 downregulated genes. The number of 17β-estradiol targets on BMSCs was 48. Fig. 1, Table 1.

3.2. Cellular component (CC) analysis of 17β-estradiol acts on BMSCs

To reveal that 17β-estradiol acts on the cellular components (CCs) of BMSC targets. The Metascape database found 47 cellular components of 17β-estradiol in BMSCs ($P < 0.05$) (Fig. 2A). Among them, 25 cellular components distributed in membrane structures, such as cell membranes and lysosomes, included membrane rafts, membrane microdomains, receptor complexes, nuclear envelopes, basal plasma membranes, basal parts of cells, plasma membrane protein complexes, plasma membrane signaling receptor complexes, etc. It was also found that 22 organelles are neurons and synaptic structures. These organelles include neuronal cell bodies, presynapses, myelin sheaths, distant axons, glutamatergic synapses, and synaptic vesicles, as shown in Fig. 2B. There were 32 targets related to the above cellular component, of which APP, ADORA1, HTR2A, HSP90AA1, BACE1, ADRA2C, EGFR, APH1A, ITGA4, CAPN1, PTK2, LGMN, MAPK14, and NAAA were the main targets, as shown in Fig. 2C. Cellular component enrichment analysis suggested that 17β-estradiol mainly acts on the BMSC cell membrane or membrane organelle receptors and can affect the biological process of BMSCs by releasing neurotransmitters.

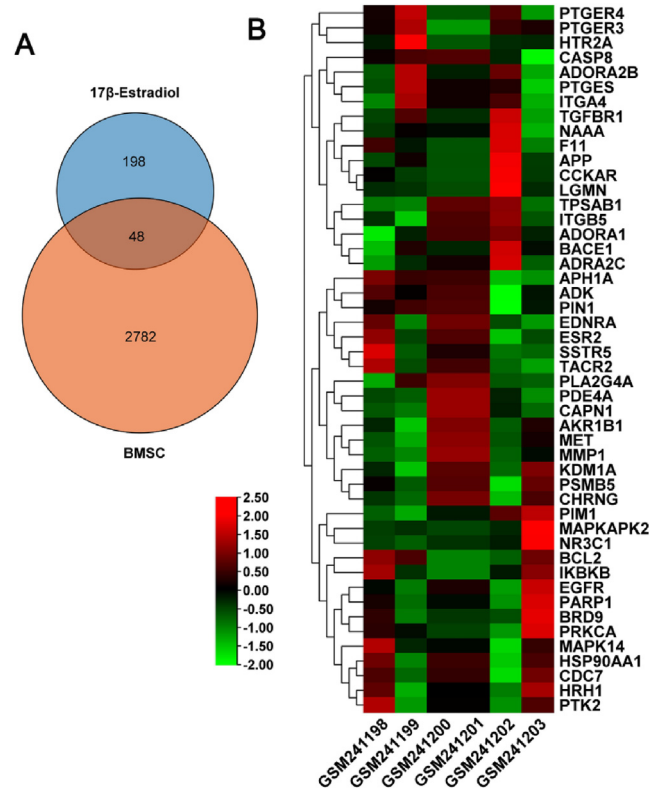

Fig. 1. The 17β-estradiol target on BMSC. A. Veen diagram of the 17β-estradiol target on BMSC. **B.** Heat map of 17β-estradiol on the 48 targets on BMSC. Pearson correlation distance metric and the average link clustering algorithm were used.

3.3. 17β-estradiol regulates the apoptosis process of BMSCs

There are 23 pathways by which 17β-estradiol acts on BMSCs to regulate cell apoptosis, including the apoptotic signaling pathway, extrinsic apoptotic signaling pathway, intrinsic apoptotic signaling pathway, anoikis, apoptotic mitochondrial changes, extrinsic apoptotic signaling pathway via death domain receptors, and intrinsic apoptotic signaling pathway by p53 class mediator (Fig. 3A). The main targets related to apoptosis are BCL2, CASP8, KDM1A, and PARP1 (Fig. 3B). Flow cytometry apoptosis experiments showed that compared with the control group, the BMSC cell apoptosis rate was significantly different ($F = 213.8$, $P < 0.01$) in the 17β-estradiol group and the PI3K inhibitor group. The apoptosis rate of BMSCs was 0.28 % of the control group in the 17β-estradiol group and 0.45 % in the PI3K inhibitor group ($P < 0.01$); compared with the 17β-estradiol group, the BMSC apoptosis rate in the PI3K inhibitor group was significantly increased ($P < 0.01$), Fig. 3C-D. In vitro experiments indicate that 17β-estradiol may inhibit BMSC apoptosis through the PI3K signaling pathway.

3.4. 17β-estradiol regulates BMSC migration and the process of blood vessel development

There are 52 processes by which 17β-estradiol regulates BMSC migration, including cell migration, regulation of cell migration, positive regulation of cell migration, leukocyte migration, ameboidal-type cell migration, endothelial cell migration, epithelial cell migration, epithelium migration, regulation of leukocyte migration, and tissue migration regulation of endothelial cell migration. Fig. 4A. This result suggests that 17β-estradiol regulates BMSC migration through ADORA1, APP, BCL2, CCKAR, EGFR, HRH1, ITGA4, ITGB5 and other targets. PRKCA, PTK2, TGFBR1 and MAPKAPK2 are related to the regulation of BMSC sapoptosis or migration by 17β-estradiol. In addition, there are 17 ways that 17β-estradiol regulates the angiogenesis or remodeling process of BMSCs, including angiogenesis, blood vessel development, blood vessel diameter maintenance, blood vessel morphogenesis, blood vessel endothelial cell migration, tissue remodeling, angiogenesis involved in coronary vascular morphogenesis, positive regulation of blood vessel endothelial cell migration, regulation of blood vessel endothelial cell migration, artery development, blood coagulation, etc. Fig. 4B. In the cell scratch experiment, it was found that the 24-hour cell scratch area closure rate of BMSCs in the control group, 17β-estradiol group and PI3K inhibitor group was significantly different ($F = 5382$, $P < 0.01$). The cell scratch area closure rate of BMSCs in the 17β-estradiol group was 2.20 times higher than that of the control group ($P < 0.01$); in contrast, the cell scratch area closure rates of BMSCs in the PI3K inhibitor group

Table 1
17β-estradiol target information on BMSC.

Target	Protein	Gene	Activity Threshold	Target	Protein	Gene	Activity Threshold
CHEMBL203	P00533	EGFR	7.5	CHEMBL278	P13612	ITGA4	6.5
CHEMBL4439	P36897	TGFBR1	7.5	CHEMBL3816	P47712	PLA2G4A	6
CHEMBL260	Q16539	MAPK14	7.5	CHEMBL3108640	Q9H8M2	BRD9	6
CHEMBL299	P17252	PRKCA	7.5	CHEMBL4822	P56817	BACE1	6
CHEMBL3717	P08581	MET	7.5	CHEMBL4662	P28074	PSMB5	6
CHEMBL2147	P11309	PIM1	7.5	CHEMBL2617	Q15661	TPSAB1	6
CHEMBL2208	P49137	MAPKAPK2	7.5	CHEMBL1900	P15121	AKR1B1	6
CHEMBL1991	O14920	IKBKB	7.5	CHEMBL2820	P03951	F11	6
CHEMBL2695	Q05397	PTK2	7.5	CHEMBL254	P27815	PDE4A	6
CHEMBL5443	O00311	CDC7	7.5	CHEMBL3105	P09874	PARP1	6
CHEMBL1792	P35346	SSTR5	7	CHEMBL3891	P07384	CAPN1	6
CHEMBL1901	P32238	CCKAR	7	CHEMBL6136	O60341	KDM1A	6
CHEMBL1916	P18825	ADRA2C	7	CHEMBL3880	P07900	HSP90AA1	6
CHEMBL3710	P43115	PTGER3	7	CHEMBL332	P03956	MMP1	6
CHEMBL252	P25101	EDNRA	7	CHEMBL3776	Q14790	CASP8	6
CHEMBL2034	P04150	NR3C1	7	CHEMBL5658	O14684	PTGES	6
CHEMBL1836	P35408	PTGER4	7	CHEMBL3589	P55263	ADK	6
CHEMBL2327	P21452	TACR2	7	CHEMBL2487	P05067	APP	6
CHEMBL224	P28223	HTR2A	7	CHEMBL4349	Q02083	NAAA	6
CHEMBL231	P35367	HRH1	7	CHEMBL2288	Q13526	PIN1	6
CHEMBL226	P30542	ADORA1	7	CHEMBL2096675	P18084	ITGB5	6
CHEMBL255	P29275	ADORA2B	7	CHEMBL2094135	Q8WW43	APH1A	5
CHEMBL242	Q92731	ESR2	7	CHEMBL4860	P10415	BCL2	5
CHEMBL4244	Q99538	LGMN	6.5	CHEMBL1907588	P07510	CHRNG	5

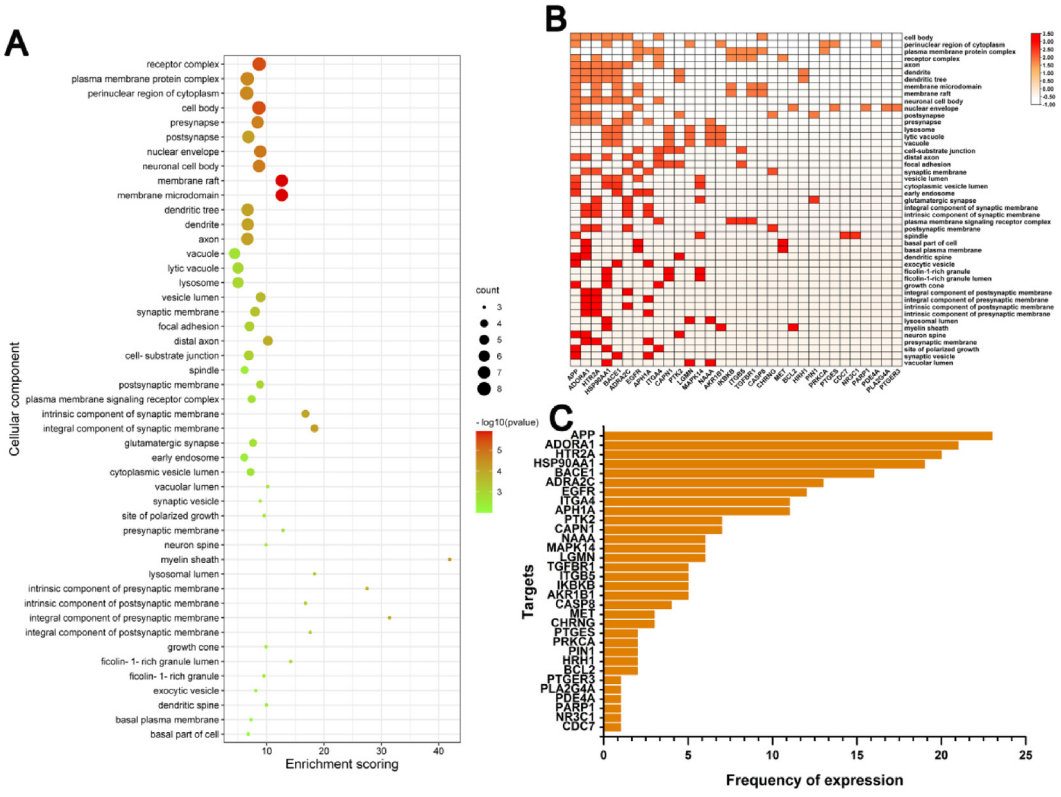

Fig. 2. Cellular component analysis of 17β-estradiol on BMSC. A. Bubble plot showing the effects of 17β-estradiol targets on BMSC and cellular components. The x-axis is the enrichment score, and the y-axis is the term Cellular component. The area of the node is consistent with the number of targets. **B.** Heatmap of 17β-estradiol target on BMSC and cellular component. **C.** Histogram of the expression frequency of 17β-estradiol on BMSC targets. The median frequency of target expression is 5.

were 0.55 % of that of the 17β-estradiol group (P < 0.01) (Fig. 4C-D). In addition, the OD value of CD31 fluorescence of BMSCs in the 17β-estradiol group after differentiation induced by vascular endothelial cells was 9.43 times higher than that of the control group (P < 0.01), but the OD value of CD31 in the PI3K inhibitor group was 0.38 % of the 17β-estradiol group (P < 0.01), Fig. 4E-F. In vitro experiments showed that 17β-estradiol is able to significantly promote the angiogenic differentiation of BMSCs.

3.5. The molecular mechanism by which 17β-estradiol regulates BMSCs

KEGG pathways with 48 genes common to 17β-estradiol and BMSCs had 66 items (P < 0.05), as shown in Table 2. The top 20 pathways included Neuroactive ligand–receptor interaction, Pathways in cancer, Calcium signaling pathway, PI3K-Akt signaling pathway, inflammatory mediator regulation of trp channels, Focal

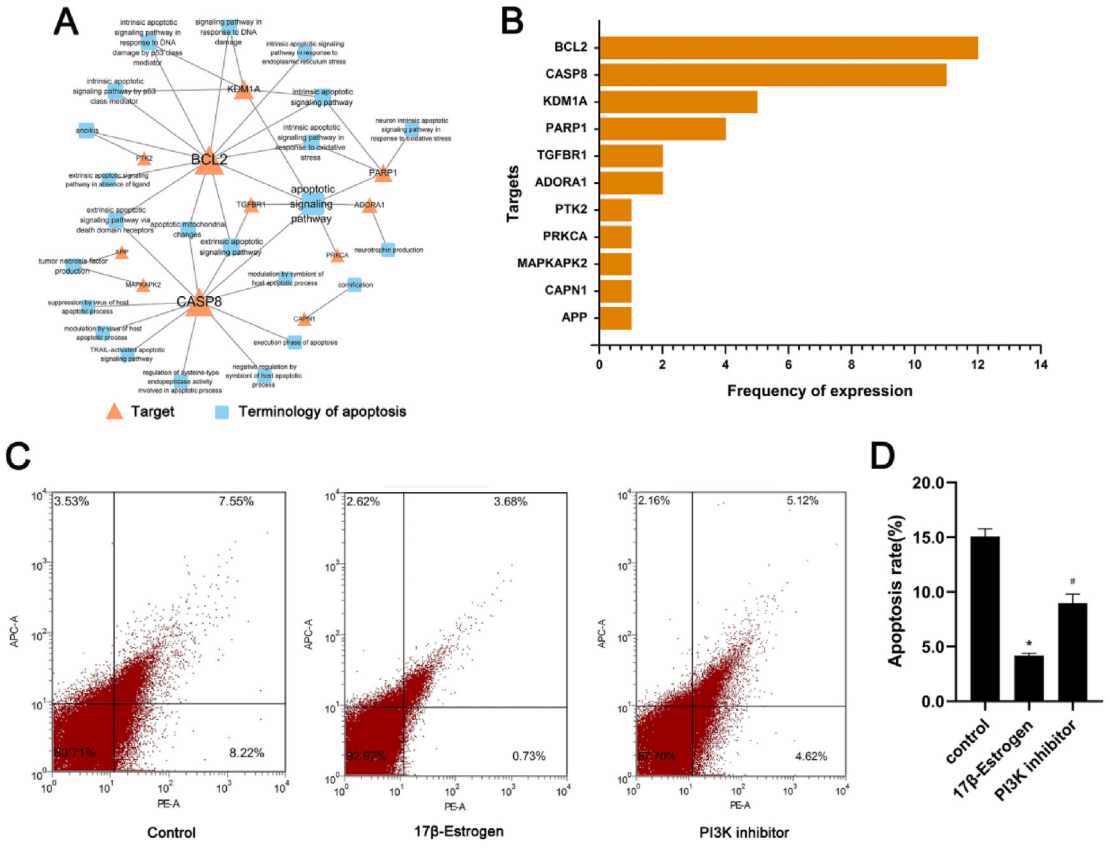

Fig. 3. 17β-estradiol regulates the apoptosis process of BMSC. A. Network diagram of 17β-estradiol regulating the apoptosis process of BMSC.The node area or font size is positively correlated with the degree value of the node. **B.** The histogram of the target expression frequency of 17β-estradiol in the apoptosis process of BMSC. The median frequency of target expression is 2. **C.** Typical pictures of BMSC cell apoptosis detected by flow cytometry. **D.** Comparison of the apoptosis rate of BMSC cells in each group. $^{*}P < 0.01(n = 3)$, compare with the control group; $^{\#}P < 0.01(n = 3)$, compared with the 17β-estradiol group.

adhesion, VEGF signaling pathway, MAPK signaling pathway, IL-17 signaling pathway and other KEGG pathways (Fig. 5A). PRKCA, MAPK14, BCL2, EGFR, IKBKB, PTK2, CASP8, EDNRA, HSP90AA1, MET, PTGER3, PTGER4, TGFBR1 and other targets are related to KEGG pathways (Fig. 5A). The results suggest that the Calcium signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, and cAMP signaling pathway are the main KEGG pathways by which 17β-estradiol promotes BSMC angiogenesis. In vitro experiments verified that the Akt and P-Akt protein contents of BMSCs in the control group, 17β-estradiol group and PI3K inhibitor group were significantly different ($F = 79.9$, $P < 0.01$). The relative protein expression levels of Akt and p-Akt in the 17β-estradiol group were 0.99 times higher and 1.01 times higher than those in the control group ($P < 0.01$), respectively. while the relative protein expression levels of Akt and P-Akt in the PI3K inhibitor group were 0.75 % and 0.70 % of those in the 17β-estradiol group ($P < 0.01$), respectively (Fig. 5B-C). Experiments have confirmed that 17β-estradiol can activate the PI3K-Akt signaling pathway and promote BMSC differentiation in vitro.

3.6. 17β-estradiol acts on the core protein of BMSCs and molecular docking verification

To verify the interaction between 17β-estradiol and the proteins encoded by the BMSC target, BisoGenet 3.0.0 detected 5238 nodes and 11,057 connections in the PPI network of common genes between 17β-estradiol and BMSCs (Fig. 6A). CytoHubba 0.1 screened the top 10 core proteins, including APP, NTRK1, EGFR, HSP90AA1, CUL3, XPO1, HSP90AB1, TP53, MCM2, and FN1 (Fig. 6B, Table 3). The binding energy of these core proteins with 17β-estradiol is less than − 5.0 kJ/mol (Fig. 6C-D, Table 3). The results verified that the core protein of BSMCs activated by 17β-estradiol is consistent with the main KEGG pathway target induced by 17β-estradiol. It is suggested that 17β-estradiol may initiate PI3K-Akt, MAPK and other signaling pathways through core proteins such as FN1, MCM2, XPO1, NTRK1, HSP90AB1, HSP90AA1, TP53, EGFR, APP, and CUL3 and play a role in regulating BMSC migration and promoting angiogenesis and other biological processes.

4. Discussion

Estrogen affects the periodic formation of blood vessels in female reproductive organs. After menopause, the level of estrogen in women is significantly reduced; therefore, elderly women are prone to coronary heart disease, atherosclerosis, stroke and other cardiovascular and cerebrovascular diseases[24]. MSCs can not only differentiate into vascular endothelial cells but also secrete proangiogenic factors such as VEGF and SDF-1α, which can positively affect vascular remodeling and development. BMSCs circulate in blood vessels and are recruited by local tissue inflammatory factors and chemokines, making them easy to home to diseased tissues. Paracrine cytokines such as VEGF and bFGF promote the self-differentiation of MSCs into vascular endothelial cells, which improves blood vessel density in ischemic heart disease, increasing the left ventricular ejection index [25]. Therefore, estrogen has a potential role in angiogenesis, remodeling and other processes. Combined with 17β-estradiol and BMSCs, it can improve

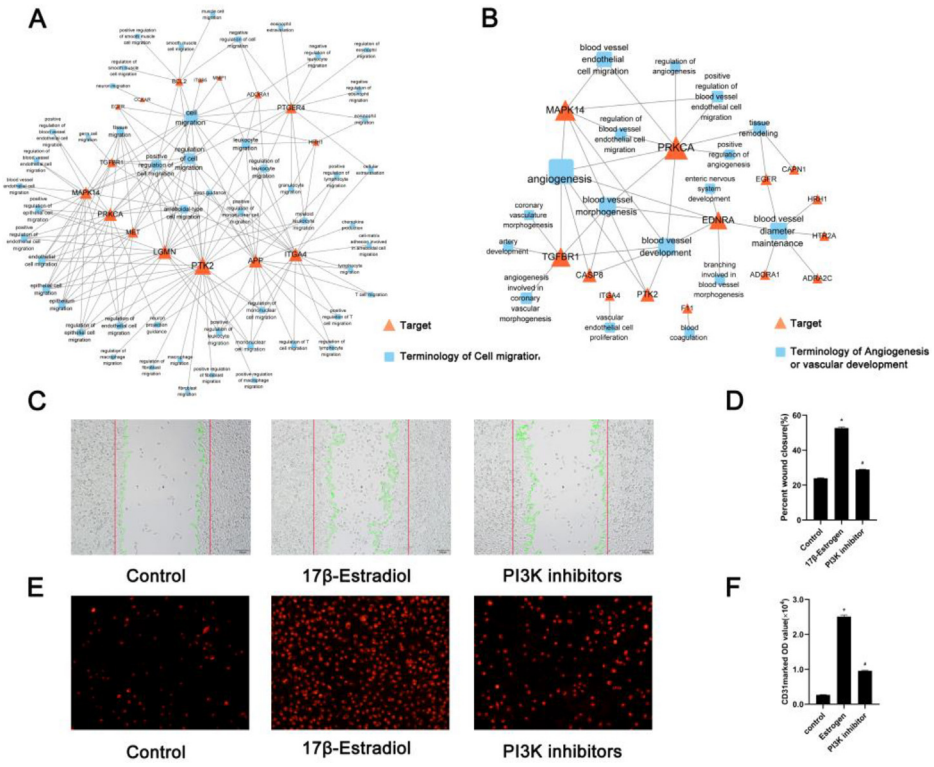

Fig. 4. 17β-estradiol regulates BMSC migration and vascular development. A. Network diagram of 17β-estradiol regulating the migration process of BMSC. The size of the node area and font size are positively related to the Degree value of the node. **B.** The network diagram of 17β-estradiol regulating the angiogenesis process of BMSC. The size of the node area and font size are positively related to the Degree value of the node. **C.** A typical picture of 24 h of BMSC cell scratches in each group. The area between the red lines represents the scratch area of the 0 h cell, and the area between the green lines represents the scratch area of the 24 h cell. Ruler = 100 μm. **D.** Comparison of scratch rate of BMSC cells in each group in 24 h. $^{*}P < 0.01(n = 3)$, $^{\#}P < 0.01(n = 3)$. **E.** The typical photos of CD31-labeled BMSCs in each group detected by immunofluorescence experiment. Ruler = 100 μm. **F.** Comparison of CD31-labeled optical density values of MSCs in each group. $^{*}P < 0.01(n = 3)$, compare with the control group; $^{\#}P < 0.01(n = 3)$, compared with the 17β-estradiol group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the biological activity of vascular endothelial cells, promote the development and regeneration of blood vessels, and relieve the problems of microangiopathy or microcirculation disorders.

We investigated the effect and the mechanism of 17β-estradiol on MSCs to promote angiogenesis in detail. This experiment first used bioinformatics technology to detect the 17β-estradiol targets and BMSC differential genes with the ChEMBL platform and GSE9520 dataset, respectively. Since only the two databases were analyzed by bioinformatics technology, the screening results of 17β-estradiol and BMSC genes may not be comprehensive, but 48 targets of 17β-estradiol on BMSCs have been obtained and analyzed and verified. This experiment found that 32 targets of 17β-estradiol on BMSCs, such as APP, ADORA1, HTR2A, HSP90AA1, EGFR and their encoded proteins, are distributed in cell membranes, endocytic membranes, mitochondrial membranes and other membranous organelles. This shows that 17β-estradiol acts on the receptors of the BMSC cell membrane and key proteins. It has also been found that 17β-estradiol can cause the release of neurotransmitters and cause changes in the biological process of BMSCs. To clarify the effect of 17β-estradiol on the angiogenesis of BMSCs, we first observed the effect of 17β-estradiol on the migration of BSMCs and found that 17β-estradiol regulates 52 BMSC migration processes through ADORA1, APP, BCL2, CCKAR, EGFR, HRH1, ITGA4 and ITGB5. Zhang et al found that 17β-estradiol can promote BMSC chemotaxis and migration[26]. We used biological information technology analysis to find that 17β-estradiol promotes BMSC migration through the above targets, more clearly identifying the site of action of 17β-estradiol. Adenosine can increase the proliferation of BMSCs and promote the paracrine secretion of VEGF and other vasoactive factors in BMSCs. BMSCs activated by adenosine can promote the angiogenesis of skin ulcers in diabetic mice and accelerate the healing of diabetic ischemic and hypoxic skin ulcers [27]. ADORA1, ADORA2B, ADRA2C, and ADRA3 are different subtypes of adenosine receptors, which are G protein-coupled receptors [28]. In ischemia or hypoxia, vascular endothelial cells and cardiomyocytes release a large amount of adenosine and regulate cardiovascular system angiogenesis and improve blood supply through ADORA1, ADORA2B, and ADRA2C [29]. In this study, we found that there was a significant difference in the 24-h cell scratch area closure rate among BMSCs from the control, 17β-estradiol, and PI3K inhibitor groups in the cell scratch assay. The OD value of CD31 fluorescence was increased in BMSCs induced to differentiate into vascular endothelial cells in the 17β-estradiol group, but decreased in the PI3K inhibitor group. In vitro experiments demonstrated that 17β-estradiol could significantly promote BMSC angiogenic differentiation. This result is consistent with previous studies that 17β-estradiol upregulates the expression of stromal cell-derived factor-1α (SDF-1α) and promotes the migration of BMSCs[16], and also suggests that Akt signaling pathway plays an important role in 17β-estradiol-induced BMSC migration or angiogenic differentiation. The migration of vascular endothelial cells is an important process of angiogenesis. Cell scratch assay showed that 17β-estradiol promoted cell migration and recruited cell homing in BMSC, and was important to reveal that 17β-estradiol promoted BMSC angiogenic differentiation.

In addition, Feng et al found that E2 induced endothelial cell migration and proliferation through formation of VEGF and pro-

Table 2
KEGG Pathway enrichment analysis of BMSC regulated by 17-estradiol.

GO	Description	P	GO	Description	P
hsa04080	Neuroactive ligand-receptor interaction	8.63195E-16	hsa04270	Vascular smooth muscle contraction	5.45078E-05
hsa05200	Pathways in cancer	8.26812E-14	hsa04071	Sphingolipid signaling pathway	6.78524E-05
hsa04020	Calcium signaling pathway	2.17583E-11	hsa04014	Ras signaling pathway	7.84699E-05
hsa04151	PI3K-Akt signaling pathway	1.42238E-08	hsa04068	foxo signaling pathway	8.83273E-05
hsa04750	inflammatory mediator regulation of trp channels	2.37199E-08	hsa04923	Regulation of lipolysis in adipocytes	0.000134241
hsa04510	Focal adhesion	4.5597E-08	hsa05212	Pancreatic cancer	0.000179967
hsa04370	VEGF signaling pathway	5.4233E-08	hsa05160	Hepatitis C	0.000198196
hsa04010	MAPK signaling pathway	2.46563E-07	hsa04664	Fc epsilon RI signaling pathway	0.000215443
hsa05161	Hepatitis B	5.58149E-07	hsa04520	Adherens junction	0.000255162
hsa04657	IL-17 signaling pathway	5.99544E-07	hsa05222	Small cell lung cancer	0.000401859
hsa01522	Endocrine resistance	6.31682E-07	hsa04012	ErbB signaling pathway	0.000430588
hsa04933	AGE-RAGE signaling pathway in diabetic complications	1.08336E-06	hsa04540	Gap junction	0.000460605
hsa05205	Proteoglycans in cancer	1.87892E-06	hsa04810	Regulation of actin cytoskeleton	0.000468604
hsa04024	cAMP signaling pathway	2.31444E-06	hsa05032	Morphine addiction	0.000524597
hsa04210	Apoptosis	3.79727E-06	hsa04620	Toll-like receptor signaling pathway	0.000749905
hsa04915	Estrogen signaling pathway	5.00244E-06	hsa04064	NF-kappa B signaling pathway	0.000814442
hsa05418	Fluid shear stress and atherosclerosis	5.34603E-06	hsa05231	Choline metabolism in cancer	0.000814442
hsa05120	Epithelial cell signaling in Helicobacter pylori infection	5.58258E-06	hsa04668	TNF signaling pathway	0.001003506
hsa04622	RIG-I-like receptor signaling pathway	6.2696E-06	hsa04066	HIF-1 signaling pathway	0.0011076
hsa04924	Renin secretion	7.4147E-06	hsa04611	Platelet activation	0.001428298
hsa01521	EGFR tyrosine kinase inhibitor resistance	1.01591E-05	hsa05169	Epstein-Barr virus infection	0.001678008
hsa04621	NOD-like receptor signaling pathway	1.04878E-05	hsa04380	Osteoclast differentiation	0.001694638
hsa05010	Alzheimer's disease	1.079E-05	hsa04261	Adrenergic signaling in cardiomyocytes	0.002029196
hsa05206	MicroRNAs in cancer	1.33443E-05	hsa05162	Measles	0.002490486
hsa05215	Prostate cancer	1.49007E-05	hsa04921	Oxytocin signaling pathway	0.002626615
hsa04912	GnRH signaling pathway	1.85884E-05	hsa04072	Phospholipase D signaling pathway	0.002767265
hsa05142	Chagas disease (American trypanosomiasis)	3.36947E-05	hsa04141	Protein processing in endoplasmic reticulum	0.003011865
hsa04659	Th17 cell differentiation	4.02995E-05	hsa04022	cGMP-PKG signaling pathway	0.003216839
hsa04015	Rap1 signaling pathway	4.29223E-05	hsa04360	Axon guidance	0.003322462
hsa04670	Leukocyte transendothelial migration	4.31897E-05	hsa05164	Influenza A	0.003708839
hsa05145	Toxoplasmosis	4.62286E-05	hsa05152	Tuberculosis	0.004306728
hsa04722	Neurotrophin signaling pathway	5.10763E-05	hsa05203	Viral carcinogenesis	0.005596527
hsa04726	Serotonergic synapse	5.10763E-05	hsa04144	Endocytosis	0.009907629

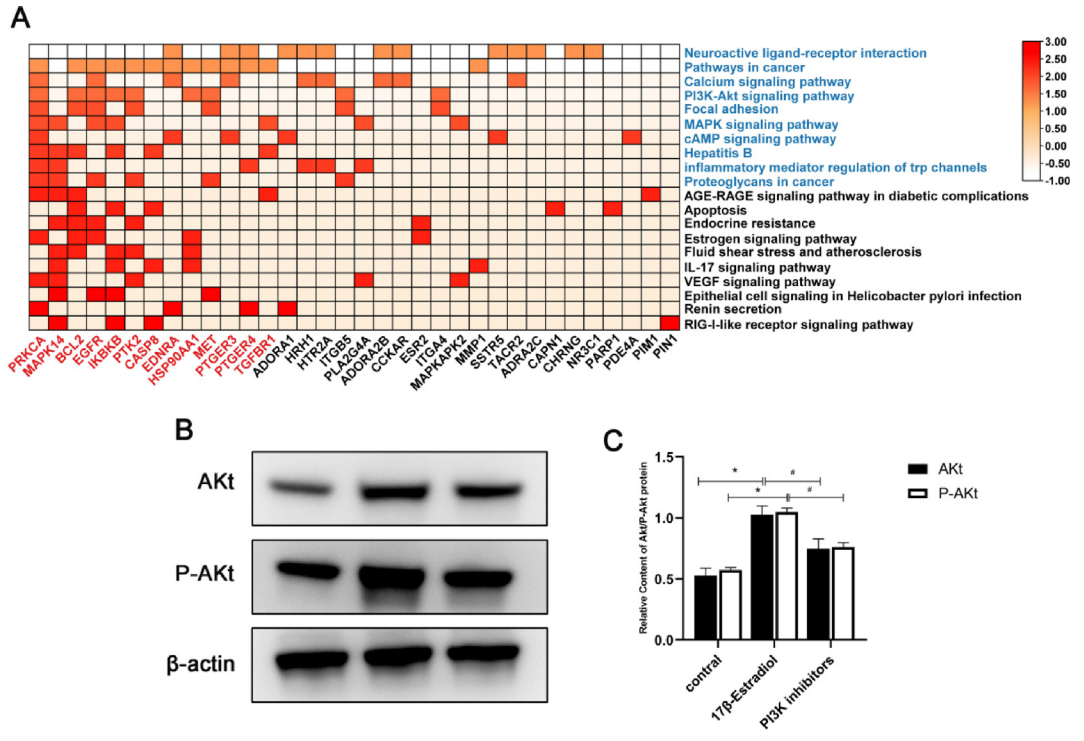

Fig. 5. The molecular mechanism of 17β-estradiol regulating BMSC. A. Top 20 KEGG Pathway heatmap of 17β-estradiol acting on BMSC.The x-axis is the target of the KEGG Pathway that 17β-estradiol regulating BMSC. The median frequency of target expression is 3.0, and the red font is the target that expression frequency greater than 3.0. The y-axis is the KEGG Pathway term. The median frequency of KEGG Pathway expression is 5.5, and the blue font is KEGG Pathway with an expression frequency greater than 5.5. **B.** Typical expression bands of Akt and P-Akt protein of BMSCs in each group detected by Western blot. **C.** Comparison of the relative expression of Akt and P-Akt proteins. $^{*}P < 0.01 (n = 3)$, compare with the control group; $^{\#}P < 0.01 (n = 3)$, compared with the 17β-estradiol group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

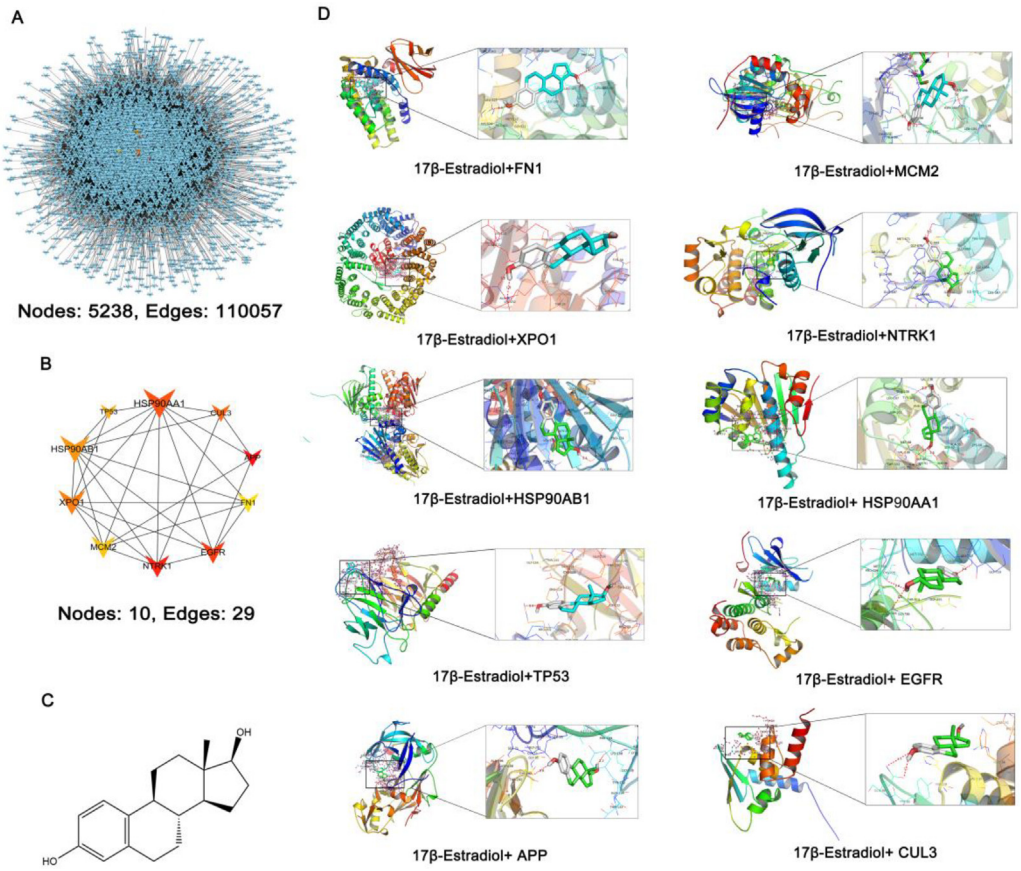

Fig. 6. 17β-estradiol acts on the core protein of BMSC. A. BisoGenet 3.0.0 wused to search the protein interaction network of 17β-estradiol acting on BMSC. Node area was positively correlated with protein Degrrre values.**B.**cytoHubba 0.1 was used to screen 17β-estradiol acting on core proteins of BMSC. Node area was positively correlated with core protein Degrrre values.**C.** The 2D chemical structure of 17β-estradiol. **D.** The molecular docking diagram of 17β-estradiol and BMSC core protein. The red dotted line represents the hydrogen bond. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Top 10 core proteins and their docking energy with 17β-estradiol (KJ/mol).

Protein	Gene	PDB	Ligand	Binding energy	Degree	Protein	Gene	PDB	Ligand	Binding energy	Degree
P07900	HSP90AA1	6GPR	CMP	−31.401	9	P04629	NTRK1	6NSS	LOM	−36.007	6
P08238	HSP90AB1	5UCJ	KU3	−32.239	8	P02751	FN1	2OCF	EST	−44.799	5
O14980	XPO1	6TVO	GTP	−37.263	7	P05067	APP	4I12	1BC	−29.726	4
P49736	MCM2	6YA7	ADP	−39.775	6	Q13618	CUL3	4CXT	SXJ	−27.214	4
P00533	EGFR	5XGM	85X	−30.145	6	P04637	TP53	5G4O	O80	−30.564	3

moted angiogenesis through PI3K/Akt pathway enhanced by estrogen receptor (ER)[30]. To further explore the biological process and molecular mechanism of 17β-estradiol on BMSC angiogenic differentiation, we found that 17β-estradiol could significantly regulate 17 important processes such as BMSC promoting angiogenesis and remodeling vascular structure. PRKCA, MAPK14, TGFBR1, EDNRA, CASP8, PTK2, and EGFR are important targets for promoting angiogenesis. These targets are basically consistent with the targets of 17β-estradiol in regulating BMSC apoptosis or migration. In particular, it should be noted that EGFR and MAPK14 are also common targets for 17β-estradiol to regulate BMSC migration and angiogenesis. EGFR and MAPK14 are the key targets of 17β-estradiol in regulating BMSC cell migration and angiogenesis. Studies have found that activation of the epidermal growth factor receptor (EGFR) of vascular endothelial cells will activate its downstream VEGFR2/NF κB signaling pathway, upregulate the expression of vascular endothelial growth factor (VEGF)-A and VEGF-C, and promote the migration and angiogenesis of vascular endothelial cells in bladder cancer tissue, changing the structure of the tumor microenvironment and accelerating the metastasis of bladder cancer cells through blood vessels [31].p38 MAPK regulates the production of inflammatory mediators and controls cell proliferation, differentiation, migration, and survival, and activation in endothelial cells leads to actin remodeling, angiogenesis, DNA damage responses, which have a major impact on cardiovascular homeostasis and cancer progression[32].17β-estradiol activates the distribution of synaptic vesicles and the presynapse of BMSCs and exerts the PI3K-Akt signaling pathway, MAPK signaling pathway, Calcium signaling pathway, Neuroactive ligand–receptor interaction and other signaling pathways related to vascular endothelial cell activation, vascular survival or remodeling. Studies have found that the growth of nerves and blood vessels follow the same pathway and promote each other[33]. BMSC-conditioned medium activated by neurotrophic factor-3 (NT-3) can promote the proliferation and migration of human umbilical vein endothelial cells (HUVECs) [23]. Brain-derived neurotrophic factor (BDNF), NT-3 and other neu-

rotrophic factors bind to Trk B or Trk C receptors on the surface of BMSCs, activate BMSC paracrine VEGF, FGF and other cytokines, and promote the increase in vascular endothelium, blood vessel density of diabetic skin tissue in C57BL/6J mice cells, and ulcer healing is accelerated [34]. Estrogen stimulates BCPAP papillary thyroid cancer cell lines and ML-1 follicular thyroid cancer cell lines, and it was found that both BCPAP and ML-1 thyroid cancer cells express ER-α and ER-β subtypes of estrogen. Estrogen promotes the secretion of VEGF by ML-1 thyroid cancer cells by upregulating the expression of PI3K protein and activating the Akt pathway, leading to the migration of vascular endothelial cells in thyroid cancer tissue. This phenomenon indicated that estrogen induces a proangiogenic endothelial cell phenotype and angiogenesis in the thyroid tumor microenvironment[35]. Calcium signals induce vascular endothelial cells or smooth muscle cells to contract microfilaments and microtubules, causing cell migration, sprouting, and growth of blood vessels, which effectively regulates blood vessel diameter and contributes to the remodeling of heart function[36]. Inflammatory factors such as IL-1 and IL-8 cause migration of vascular endothelial cells, angiogenesis, and typical inflammatory reactions[37,38]. VEGF is a very clear cytokine that promotes blood vessel growth. It can promote blood vessel growth through PI3K/Akt, MAPK and other signaling pathways and protect the heart from ischemia–reperfusion injury[39]. In addition, Hirata T exposed human pulmonary artery endothelial cells (HPAECs) to laminar flow shear stress for 24 h to perform an overall analysis of cell lipids. It was found that 198 kinds of intracellular lipids were significantly expressed with shear stress stimulation[40]. This shows that the laminar shear stress generated by blood flow stimulates vascular endothelial cells and activates the signal transduction process, which plays an essential role in vascular homeostasis. EGFR can activate the PI3K-Akt signaling pathway and promote the proliferation and migration of seminoma cells[41]. Blocking the PI3K-Akt-BCL-2 signaling pathway can reduce the occurrence and development of colorectal cancer in SD rats[42].

The above studies suggest that EGFR, MAPK14, ADORA1 and PRKCA are not only key targets for 17β-estradiol to promote BMSC to exert angiogenesis or development, but also targets of concern for exerting BMSC to promote angiogenesis. In vitro experiments verified that there were significant differences in Akt and P-Akt protein contents in BMSC among the control, 17β-estradiol, and PI3K inhibitor groups. Akt and p-Akt protein expression was enhanced in the 17β-estradiol group, while Akt and P-Akt protein expression was decreased in the PI3K inhibitor group. It was confirmed that 17β-estradiol could activate PI3K-Akt signaling pathway and promote BMSC vascular differentiation in vitro.Although in vitro experiments found that 17β-estradiol can promote MSC differentiation, further verifying the results of bioinformatics predictions, we cannot rule out that 17β-estradiol also exerts its effect on MSCs through other pathways. These hypotheses need to be further verified using high-throughput gene sequencing and other technologies.

Cytoscape3.8′s cytoHubba 0.1 plug-in predicts the core proteins that 17β-estradiol on BMSCs. Genes related to these proteins are basically high expression targets for 17β-estradiol to act on the migration of vascular cells in BMSCs. In this study, we used a binding energy less than − 5.0 kJ/mol as the criterion to screen the top 10 core proteins with high binding energy with 17β-estradiol. The interaction between 17β-estradiol and core proteins such as FN1, MCM2, XPO1, NTRK1, HSP90AB1, HSP90AA1, TP53, EGFR, APP, and CUL3 needs to be studied. However, it has been suggested that 17β-estradiol can initiate PI3K-Akt[43], Wnt[44], MAPK[45]and other signaling pathways to regulate cell biological processes. The results of this experiment paved the way for research on the effect of 17β-estradiol on BMSCs to improve microcirculation disorders and reduce the incidence of microcirculation diseases.

5. Conclusion

Combined with FN1, MCM2, XPO1, NTRK1 and other proteins, 17β-estradiol is able to activate PI3K-Akt, MAPK and other signaling pathways to regulate BMSCs to promote or remodel angiogenesis, and 17β-estradiol upregulates the PI3K-Akt signaling pathway to promote the BMSC angiogenesis process of differentiation.

CRediT authorship contribution statement

Xiaodong Zhang: Conceptualization, Methodology, Software, Validation, Investigation, Resources, Writing – original draft, Writing – review & editing. **Ligang Liu:** Conceptualization, Software, Writing – original draft, Writing – review & editing. **Danyang Liu:** Conceptualization. **Yongtao Li:** Conceptualization. **Jun He:** Conceptualization. **Lei Shen:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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