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

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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.

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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 GSE95250 dataset in the Gene Expression Omnibus (GEO) database after adjusting to P < 0.05 and |logFC| > 1. The 17β-estradiol targets on BMSCs were screened using Venny 2.1.0 (https://bioinfoogg.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-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, MO, USA), 5 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 containing 10-5μmol/L 17β-estradiol were used as the negative control group, BMSC cultured in vascular endothelial cell induction medium containing 50 μmol/L LY294002 and 10-5μmol/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 % CO2 for 14 days.

2.5. Angiogenic 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 10-5μmol/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 10-5μmol/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 % CO2 for 14 days.

2.6. Flow cytometry cell apoptosis experiment

An Annexin V-FITC Cell Apoptosis Detection Kit (cat. no. C10625, 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 × 105 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 % CO2. 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% CO2. 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 5 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). 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-β-actin (cat. no. A5441, 1:500, Sigma-Aldrich, USA) were used as primary antibodies.
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, EGF, 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.

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, EGF, HRH1, ITGA4, ITGB5 and other targets. PRKCA, PTK2, TGFBRI and MAPKAPK2 are related to the regulation of BMSC apoptosis 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.
were 0.55% of that of the 17\(\beta\)-estradiol group (P < 0.01) (Fig. 4C-D). In addition, the OD value of CD31 fluorescence of BMSCs in the 17\(\beta\)-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 17\(\beta\)-estradiol group (P < 0.01), Fig. 4E-F.

In vitro experiments showed that 17\(\beta\)-estradiol is able to significantly promote the angiogenic differentiation of BMSCs.

### 3.5. The molecular mechanism by which 17\(\beta\)-estradiol regulates BMSCs

KEGG pathways with 48 genes common to 17\(\beta\)-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...
adhesion, VEGF signaling pathway, MAPK signaling pathway, IL-17 signaling pathway and other KEGG pathways (Fig. 5A). PRKCA, MAPK14, BCL2, EGFR, IKBBK, 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 17\beta-estradiol promotes BSMC angiogenesis. In vitro experiments verified that the Akt and P-Akt protein contents of BMSCs in the control group, 17\beta-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 17\beta-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 17\beta-estradiol group (P < 0.01), respectively (Fig. 5B-C). Experiments have confirmed that 17\beta-estradiol can activate the PI3K-Akt signaling pathway and promote BMSC differentiation in vitro.

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

To verify the interaction between 17\beta-estradiol and the proteins encoded by the BMSC target, BioGenet 3.0.0 detected 5238 nodes and 11,057 connections in the PPI network of common genes between 17\beta-estradiol and BMSCs (Fig. 6A). CytoHubba 0.1 screened the top 10 core proteins, including APP, NTRK1, EGFR, HSP90AA1, CUL3, XP01, HSP90AB1, TP53, MCM2, and FN1 (Fig. 6B, Table 3). The binding energy of these core proteins with 17\beta-estradiol is less than – 5.0 kJ/mol (Fig. 6C-D, Table 3). The results verified that the core protein of BMSCs activated by 17\beta-estradiol is consistent with the main KEGG pathway target induced by 17\beta-estradiol. It is suggested that 17\beta-estradiol may initiate PI3K-Akt, MAPK and other signaling pathways through core proteins such as FN1, MCM2, XP01, 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-1\alpha, 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 17\beta-estradiol and BMSCs, it can improve...
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, HTRA2, 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 BMSCs and found that 17β-estradiol regulates 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 up-regulates 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                             | 8.3273E-05 |
| hsa04750    | inflammatory mediator regulation of trp channels  | 2.37199E-08 | hsa04923    | Regulation of lipolysis in adipocytes             | 0.000134241 |
| hsa04510    | Focal adhesion                                    | 4.5979E-08  | hsa05212    | Pancreatic cancer                                  | 0.00017967 |
| 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                            | 0.00041859 |
| 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               | 0.000749505 |
| hsa04913    | 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    | TGF-beta signaling pathway                        | 0.001003506 |
| hsa04622    | RIG-I-like receptor signaling pathway             | 6.6966E-06  | hsa04066    | HIF-1 signaling pathway                           | 0.0011076 |
| hsa04924    | Renin secretion                                   | 7.4147E-06  | hsa04611    | Platelet activation                               | 0.001428298 |
| hsa0521    | 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 |
| hsa05100    | Alzheimer's disease                               | 1.079E-05   | hsa04261    | Adrenergic signaling in cardiomyocytes            | 0.002029196 |
| hsa05206    | MicroRNAs in cancer                               | 1.3344E-05  | hsa05162    | Measles                                           | 0.002940486 |
| hsa04215    | Prostate cancer                                   | 1.49005E-05 | hsa04921    | Oxytocin signaling pathway                        | 0.002626615 |
| hsa04912    | GmRH 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                         | 3.02995E-05 | hsa04022    | cGMP-PKG signaling pathway                       | 0.003216839 |
| hsa04015    | Rap1 signaling pathway                            | 4.2923E-05  | hsa04360    | Axon guidance                                     | 0.003322462 |
| hsa04670    | Leukocyte transendothelial migration              | 4.31895E-05 | hsa05164    | Influenza A                                       | 0.003798839 |
| hsa05145    | Toxoplasmosis                                     | 4.62288E-05 | hsa05152    | Tuberculosis                                      | 0.004346728 |
| hsa04722    | Neurotrophin signaling pathway                    | 5.10763E-05 | hsa05203    | Viral carcinogenesis                              | 0.005596527 |
| hsa04726    | Serotonergic synapse                              | 5.10763E-05 | hsa04144    | Endothcys                                        | 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.)
promoted 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 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-

| Protein Gene | PDB | Ligand | Binding energy | Degree | Protein Gene | PDB | Ligand | Binding energy | Degree |
|--------------|-----|--------|----------------|--------|--------------|-----|--------|----------------|--------|
| P07900 HSP90AA1 | 6GPR | CMP/C0 | -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 | 5X | -27.214 | 4 |
| P00533 EGFR | 5XGM | 85X | -30.145 | 6 | P04637 TP53 | 5G40 | O80 | -30.564 | 3 |

**Fig. 6.** 17β-estradiol acts on the core protein of BMSC. A. BisConet 3.0.0 was used to search the protein interaction network of 17β-estradiol acting on BMSC. Node area was positively correlated with protein Degree 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 Degree 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.)
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 cell 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 P13K 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 P-Akt 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. 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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References

[1] George JN, Nester CM. Syndromes of thrombotic microangiopathy. N Engl J Med 2014;371(7):654–66.
[2] George JN, Nester CM, McIntosh JJ. Syndromes of thrombotic microangiopathy associated with pregnancy. Hematology Am Soc Hematol Educ Program 2015;2015:644–8.
[3] Fakhouri F, Scully M, Provôt F, Blasco M, Coppo P, Noris M, et al. Management of thrombotic microangiopathy in pregnancy and postpartum: from an international working group. Blood 2020;136(19):2105–17.
[4] Phelps J, Sanati-Nezhad A, Ungrin M, Duncan NA, Sen A. Bioprocessing of Mesenchymal Stem Cells and Their Derivatives: Toward Cell-Free Therapeutics. Stem Cells Int 2018 Sep 12;2018:9415367.
[5] Jimenez-Puerta C, Marchal JA, Lopez-Ruiz E, Gálvez-Martín P. Role of Mesenchymal Stromal Cells as Therapeutic Agents: Potential Mechanisms of Action and Implications in Their Clinical Use. J Clin Med 2020 Feb 6;9(2):445.
[6] Zhang Y, Chen W, Feng B, et al. The Clinical Efficacy and Safety of Stem Cell Therapy for Diabetes Mellitus: A Systematic Review and Meta-Analysis. Aging Dis 2020;11(1):141–53.
[7] Budgude P, Kale V, Vaidya A. Mesenchymal stromal cell-derived extracellular vesicles as cell-free biologics for the ex vivo expansion of hematopoietic stem cells. Cell Biol Int 2020;44(5):1078–102.
[8] Ferreira LS, Gerecht S, Shieh HF, et al. Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle-like cells and form vascular networks in vivo. Circ Res 2007;101(3):286–94.
[9] Wang L, Li H, Lin J, He R, Chen M, Zhang Y, et al. CCR2 improves homing and engraftment of adipose-derived stem cells in dystrophic mice. Stem Cell Res Ther 2021;12(1):12.
[10] Chen L, Guo L, Chen F, Xie Y, Zhang H, Quan P, et al. Transplantation of menstrual blood-derived mesenchymal stem cells (MMMSCs) promotes the regeneration of mechanical injured endometrium. Am J Transl Res 2020;12(9):4941–54.
[11] He G, Li HR, Li BR, Xie QL, Yan D, Wang XJ. Bone marrow mesenchymal stem cells overexpressing GATA-4 improve cardiac function following myocardial infarction. Perfusion 2019;34(8):696–704.
[12] Losordo DW, Isner JM. Estrogen and angiogenesis: A review. Arterioscler Thromb Vasc Biol 2001;21(1):6–12.
Qi QR, Lechuga TJ, Patel B, Nguyen NA, Yang YH, Li Y, et al. Enhanced Stromal Cell CBS-H2S Production Promotes Estrogen-Stimulated Human Endometrial Angiogenesis. Endocrinology 2020;161(1): bgaa176.

Walsh-Wilkinson E, Beaumont C, Drolet MC, Roy EM, Le Houillier C, Beaudoin J, et al. Effects of the loss of estrogen on the heart’s hypertrophic response to chronic left ventricle volume overload in rats. Peef 2019;7:e7924.

Gianfrilli D, Pofi R, Feola T, Lenzi A, Giannetta E. The Woman’s Heart: Insights into New Potential Targeted Therapy. Curr Med Chem 2017;24(24):2650–60.

Zhou YH, Han QF, Gao L, Sun Y, Tang ZW, Wang M, et al. HMGB1 Protects the Vydra N, Janus P, Toma-Jonik A, Stokowy T, Mrowiec K, Korfanty J, et al. 17β Estradiol promotes bone marrow mesenchymal stem cell migration mediated by chemokine upregulation. Biochem Biophys Res Comm 2020;530(2):381–8.

Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, et al. tBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. Mol Plant 2020;13(8):1194–202.

Zhou Y, Zhou B, Fache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. MetaScape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun 2019;10(1):1523.

Martin A, Ochagavia ME, Rabasa LC, Miranda J, Fernandez-de-Cossio J, Bringas R, BosoGenet: a new tool for gene network building, visualization and analysis. BMC Biol 2010;11(1):.

Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 2010;31(2):455–61.

Wu WH, Wu F, Gao WY, Wu ZG, Chao YC, Liang C. Network pharmacology for the identification of phytochemicals in traditional Chinese medicine for COVID-19 that may regulate interleukin-6. Biosci Rep 2021;41(11).

Shen L, Zhang Z, Zhang Z, Xiao Y, Yang Y, Wang W, et al. C-X-C motif chemokine ligand 8 promotes endothelial cell homing via the Akt-signal transducer and activator of transcription pathway to accelerate healing of ischemic and hypoxic skin ulcers. Exp Ther Med 2017;13(6):3021–31.

Kamat A, Rajoria S, George A, Suriano R, Shannamugam A, Mewgula U, et al. Estrogen-mediated angiogenesis in thyroid microenvironment is mediated through VEGF signaling pathways. Arch Otolaryngol Head Neck Surg 2011;137(11):1146–53.

Scheppelmann M, Varoja PL, Lopez-Fernandez I, Davies TS, Brennan SC, Edwards PJ, et al. The vascular Ca2+-sensing receptor regulates blood vessel tone and blood pressure. Am J Physiol Cell Physiol 2016;310(3):c193–204.

Weber A, Wasiliw P, Kuchar M. Interleukin-1 (IL-1) signaling pathway. Sci Signal 2010;3(105). cm1.

Shen L, Zhang P, Zhang Z, Xie Y, Xie Y, Yang Y, et al. C-X-C motif chemokine ligand 8 promotes endothelial cell homing via the Akt-signal transducer and activator of transcription pathway to accelerate healing of ischemic and hypoxic skin ulcers. Exp Ther Med 2017;13(6):3021–31.

Zhou YH, Han QF, Gao L, Sun Y, Yang Y, Wang W, et al. HMGBl Protects the Heart Against Ischemia-Reperfusion Injury via PI3K/AKT Pathway-Mediated Upregulation of VEGF Expression. Front Physiol 2019;10(1):.

Hirata T, Yamamoto K, Ikeda K, Arita M. Functional lipidomics of vascular progenitor cells grown on a biomaterial. Sci Rep 2015;5:11594.

He S, Shen L, Wu Y, Li L, Chen W, Hou C, et al. Effect of brain-derived neurotrophic factor on mesenchymal stem cell-seeded electrospinning biomaterial for treating ischemic diabetic ulcers via mi-31:1255–62.

Liu Z, Mikrani R, Zubair HM, Taleb A, Naveed M, Baig M, et al. Local delivery of mesenchymal stem cells for heart renovation: Challenges and innovations. Eur J Pharmacol 2020;876:173049.

Zhang W, Li X, Li H, Lu X, Chen J, Li L, et al. 17β estradiol promotes bone marrow mesenchymal stem cell migration mediated by chemokine upregulation. Biochem Biophys Res Comm 2020 Sep 17;530(2):381–8.

Chen W, Wu Y, Li L, Yang M, Shen L, Liu G, et al. Adenosine accelerates the healing of ischemic diabetic ulcers by improving autophagy of endothelial progenitor cells grown on a biomaterial. Sci Rep 2015;5:11594.

Xiao C, Liu N, Jacobson KA, Gavrilova O, Reitman ML. Physiology and effects of nucleosides in mice lacking all four adenosine receptors. PLoS Biol 2019;17(3): e3000161.

Gaudry M, Vairo D, Marlinge M, Gauthier M, Gruell C, Mottola C, et al. Adenosine and Its Receptors: An Expected Tool for the Diagnosis and Treatment of Coronary Artery and Ischemic Heart Diseases. Int J Mol Sci 2020;21(15):5321.

Feng ZY, Huang TL, Li XR, Chen L, Deng S, Xu SR, et al. 17β Estradiol promotes angiogenesis of stria vascularis in cochlea of C57BL/6j mice. Eur J Pharmacol 2021 Dec;15(913):174642.

Huang Z, Zhang M, Chen G, Wang W, Zhang P, Yue Y, et al. Bladder cancer cells interact with vascular endothelial cells triggering EGFR signals to promote tumor progression. Int J Oncol 2019;54(5):1555–66.

Corre I, Paris F, Huot J. The p38 pathway, a major pleiotropic cascade that transduces stress and metatarsic signals in endothelial cells. Oncotarget 2017;8(33):55684–714.

Weinstein BM. Vessels and nerves: marching to the same tune. Cell 2005;120(3):299–302.

He S, Shen L, Wu Y, Li L, Chen W, Hou C, et al. Effect of brain-derived neurotrophic factor on mesenchymal stem cell-seeded electrospinning biomaterial for treating ischemic diabetic ulcers via milieu-dependent differentiation mechanism. Tissue Eng Part A 2015;21(5–6):928–38.

Kamat A, Rajoria S, George A, Suriano R, Shannamugam A, Megwala U, et al. Estrogen-mediated angiogenesis in thyroid microenvironment is mediated through VEGF signaling pathways. Arch Otolaryngol Head Neck Surg 2011;137(11):1146–53.

Scheppelmann M, Varoja PL, Lopez-Fernandez I, Davies TS, Brennan SC, Edwards PJ, et al. The vascular Ca2+-sensing receptor regulates blood vessel tone and blood pressure. Am J Physiol Cell Physiol 2016;310(3):c193–204.

Weber A, Wasiliw P, Kuchar M. Interleukin-1 (IL-1) signaling pathway. Sci Signal 2010;3(105). cm1.

Shen L, Zhang P, Zhang Z, Xie L, Yao L, Lang W, et al. C-X-C motif chemokine ligand 8 promotes endothelial cell homing via the Akt-signal transducer and activator of transcription pathway to accelerate healing of ischemic and hypoxic skin ulcers. Exp Ther Med 2017;13(6):3021–31.

Zhou YH, Han QF, Gao L, Sun Y, Yang Y, Wang W, et al. HMGBl Protects the Heart Against Ischemia-Reperfusion Injury via PI3K/AKT Pathway-Mediated Upregulation of VEGF Expression. Front Physiol 2019;10(1):.

Hirata T, Yamamoto K, Ikeda K, Arita M. Functional lipidomics of vascular endothelial cells in response to laminar shear stress. FASEB J 2021;35(3): e21301.

Guerra F, Quintana S, Giustina S, Mendeluk G, Jufe L, Avagnina MA, et al. Investigation of EGFR/p38/Akt signaling pathway in seminomas. Biochimie 2021;96(2):125–37.

Huang Z, Liu CA, Cai PZ, Xu FP, Zhu WJ, Wang WW, et al. Omega-3PUFA Attenuates MNU-Induced Colorectal Cancer in Rats by Blocking PI3K/AKT/Bcl-2 Signaling. Onco Targets Ther 2020;13:1953–65.

Meng Q, Li J, Chao Y, Bi Y, Zhang W, Zhang Y, et al. 17β-estradiol adjusts intestinal function via ERβ and GPR30 mediated PI3K/AKT signaling activation to alleviate postmenopausal dyslipidemia. Biochem Pharmacol 2020;180:114134.

Tian L, Shao W, Ip W, Song Z, Badakhshi J, Yin T. The developmental Wnt signaling pathway effector β-catenin/TCF mediates hepatic functions of the sex hormone estradiol in regulating lipid metabolism. PLoS Biol 2019;17(10): e3000444.

Vydra N, Janus P, Toma-Jonik A, Stokowy T, Mrowiec K, Korfanty J, et al. 17β-Estradiol Activates HSF1 via MAPK Signaling in ERα-Positive Breast Cancer Cells. Cancers (Basel) 2019;11(10):1533.