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Title

Over-expression of EBP50 suppresses the invasion and migration of CIA-FLS in vitro by downregulating MMP-9

Full names

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Abstract

Studies had confirmed that the abnormal proliferation and activation of FLS play a key role in the process of arthropathy. Inhibiting the abnormal proliferation and activation of FLS may be an effective method to control the development of RA. Many studies showed that EBP50 was a powerful factor in inhibiting the abnormal tumor cell proliferation and activation, but the expression characteristics and function of EBP50 in FLS had not yet been reported. In this study we showed that overexpression of EBP50 in CIA-FLS can inhibit the proliferation, which had no effect on the autophagy, and promoted the cell apoptosis by activating caspase-3. At the same time, relative results revealed that EBP50 overexpression markedly down-regulated the expression of MMP-9, but had no effect on MMP-2, E-cadherin and β -catenin, suggesting that one of the mechanisms for EBP50-regulated aggressive behavior of FLS is reducing MMP-9 production.

Keywords: EBP50, FLS, Rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic of unknown etiology, progressive autoimmune disease characterized by aggressive and bone damage^[1,2]. The main pathological changes of rheumatoid arthritis include persistent synovial inflammation, abnormal proliferation of synovial cells, extensive infiltration of inflammatory cells, formation of synovial membrane pannus, gradually eroded cartilage and subchondral bone, then resulting in destruction of joint tissue structure and loss of function^[3,4]. RA has been reported to occurred in people aged 40 to 60, while showing predominance in women more than men. Although current clinical treatment can alleviate some symptoms, it cannot reverse the damage caused to cartilage and related bone tissue, and the high disability rate of RA seriously affects the quality of patients life. The latest research have shown that the occurrence and development of RA is due to a combination of genetic, environmental and immune factors, but the specific mechanism is still unclear^[5].

Fibroblast-like synoviocytes (FLS) are predominant component of joint synovial membrane^[6], and plays an pivotal physiological role in controlling the volume of synovial fluid and maintaining the stability of joint cavity^[7]. At the same time, FLS are also important factor leading to joint pathological damage^[8]. During the occurrence and development of RA, FLS can occur three major abnormal changes: such as releasing large amounts of proinflammatory cytokines^[9], FLS can promote the occurrence and maintenance of inflammatory response by involving in the activation of macrophages, T lymphocytes and NK cells^[10]; such as dysplasia and apoptosis defect, both proliferation and invasion of FLS were significantly enhanced^[11], exhibiting tumor-like behavior, accompanied by significant inhibition of apoptosis^[12]; such as secreting matrix degrading enzymes, including matrix apoptosis-deficient metalloproteinases and cathepsin, leading to destruction of bone and cartilage^[13,14]. A large number of studies have confirmed that abnormal proliferation and activation of FLS play a key role in the process of joint lesions in RA patients^[15,16]. Therefore, inhibiting abnormal proliferation and activation of FLS may be a potentially therapeutic strategy to block or delay the progression of RA.

EBP50, also known as Na⁺/H⁺ exchange regulatory factor (NHERF1), is widely distributed in

tissues and could bind to a variety of cellular proteins through two tandem PDZ and ERM binding domain, and is involved in the transport and targeted expression of a variety of intracellular proteins. As a novel tumor suppressor gene. Previous studies have demonstrated that EBP50 expression was down-regulated in breast cancer^[17], gastric cancer^[18] and prostate cancer^[19], and overexpression of EBP50 could effectively inhibit the proliferation of tumor cells and promote the apoptosis of tumor cells, at the same time, Related researchs also show that EBP50 can inhibit the migration and invasion of breast cancer cells by down-regulating MMPs and interacting with β -catenin^[20,21].

However, most studies of EBP50 have focused on tumor cells, and it is not clear whether EBP50 can perform the same function in other cells. In this study, we explored the influences and mechanisms of EBP50 overexpression on FLS in vitro.

Materials and Methods

Materials. Antibodies specific for EBP50 (ab3452), MMP-2 (ab37150), MMP-9 (ab38898) were purchased from Abcam (San Francisco, CA); Caspase-3 (9662), LC-3 (4108), β -catenin (8480) and E-cadherin(14472) were purchased from CST (Cell Signaling Technology); β -actin mAb (AC062), HRP Goat Anti-Rabbit IgG (AS014) and HRP Goat Anti-Mouse IgG (AS003) was purchased from ABclonal Technology; The CIA-FLS were kindly gifted by professor Yingyuan Fu from the Department of Immunology, Medical College of Nanchang University, Nanchang, China, The lentiviral expression plasmid pLVX-IRES-ZsGreen1 was kindly gifted by ZhangLin Zhang from the Department of Transfusion, First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China.

Cell culture. The CIA-FLS were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS). 293T cells (ATCC, TIB-202) were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% FBS. The cells were cultured at 37°C with an atmosphere of 5% CO₂ and 95% air.

Lentivirus vector construction. EBP50 encoding gene was amplified from RAW264.7 cells. The forward and reverse primer are 5'-AGAGAATTC-ATGAGCGCGGACG-3' and 5'-CGCGGATCC-TCAGAGGTTGCTGAAGA-3' respectively. The recombinant lentiviral expression vector Plvx-EBP50 is formed by relevant Enzyme digestion and ligation.

Lentiviral vectors package. The recombinant lentivirus vector Plvx-EBP50 and the corresponding lentivirus frame expression vector Plvx-Ires-ZsGreen1 were co-transfected with lentivirus packaging vector(pMD2.G and psPAX2) in 293T cells, and the virus supernatant was collected for 72h to obtain LV-EBP50 and LV-Plvx. The ultrafiltration centrifuge tube was used for concentration and packaging, and stored at -80°C for use.

Real-time PCR. We extracted total RNA by TRIzol method, real-time PCR was used to detect the expression of EBP50 gene, The GAPDH forward and reverse primer are 5-

GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAGTGGA-3' respectively. The EBP50 forward and reverse primer are 5'-AGGCCAGTTCATCCGAGCAG-3' and 5'-CAGCAGCTTGGCCTCATCAC-3' respectively. The Relative expression content was calculated by $2^{-\Delta\Delta CT}$.

Western blotting. Proteins are collected by RIPA lysate, The collected proteins were sampled in equal amounts to SDS-PAGE, then transferred to NC membranes for 1.5 h at 4°C, Membranes were incubated with primary antibodies against EBP50, LC3, Caspase-3, β -catenin, E-cadherin, Akt, MMP-2 and MMP-9 at 4°C overnight. Membranes were visualized using enhanced chemiluminescence after incubating the corresponding secondary antibody for 1 h. Gray value analysis By the corresponding instrument software.

MTT cytotoxicity assay. The FLS were maintained in DMEM supplemented with 10% FBS, 5×10^3 cells/well were resuspended into a 96-well plate. The MTT reagent (0.5 mg/mL) was supplemented into the medium after 12 h, The culture medium was discarded and added 150 μ L DMSO after 4 h. The absorbance was measured at 490 nm.

Phagocytosis assay by flow cytometry (FCM). FLS transduced with or without recombinant lentivirus for 48 h, 72 h, 96 h. Then cells were washed thoroughly with D-Hanks buffer to remove extracellular recombinant lentivirus and depleted media, at the same time, fls were transferred seeded in 96-well plates 5×10^3 /cells with annexin V binding buffer resuspended. Then were incubated at 2-8°C for 15 min with 5 μ L Annexin V-FITC, and were incubated at 2-8°C for 5 min with 5 μ L PI, finally cells were collected and analyzed using Cytomics FC500 flow cytometer (Beckman Coulter Inc., Brea, CA, USA).

In Vitro Migration and Invasion Assay of FLS. Transwell assay was performed using Transwell chamber with 8- μ m pore size to evaluate the ability of FLS migration and invasion. For cell invasion, transwell chambers was pre-coated with Matrigel. However, Transwell chambers without Matrigel were used for cell migration. When cells reached subconfluency (75%–80%), cells were starved in serum-free medium for 24 hours. After detachment with trypsin, cells were washed with PBS and resuspended by serum-free medium. The cell density was adjusted to 2×10^5 cells/mL. Then, cell suspension with 100 μ L was added to the apical chamber of transwell while complete medium was added to the transwell basolateral chamber. 24 h later, the transwell chamber was taken out, cells that had not migrated were wiped off from the lateral membrane of the apical chamber using cotton swabs. The migrated and invading cells on the bottom side were fixed with 4% paraformaldehyde solution for 30 min after washing with PBS, followed by crystal violet staining for 15 min, and then the numbers of the stained cells were measured using an inverted microscope. The mean of triplicate assays for each experimental condition was used.

Statistical analysis. All experiments were repeated three times independently to confirm the presented data and results. Data were analyzed using unpaired Student's t test or one-way analysis.

P<0.05 was considered to statistically significant.

Results

Overexpression of EBP50 Suppresses the proliferation of FLS

Inhibiting abnormal proliferation and activation of FLS may be a potentially therapeutic strategy to block or delay the progression of RA. To investigate the potential effects of EBP50 on the proliferation of FLS, fls were transfected with LV-EBP50 or LV-Plvx for 72 h, then analyzed for the EBP50 expression and the proliferation of FLS. The results showed that LV-EBP50 transfection significantly increased the expression of EBP50 in both mRNA levels (Fig. 1A) and protein levels (Fig. 1B-1C), and MTT assay demonstrated that overexpression of EBP50 can suppress the proliferation of FLS significantly, when compared with untransfected cells and LV-Plvx transfected cells (Fig. 1D). It has been reported that EBP50 can inhibit the proliferation of breast cancer cells by stimulating the autophagic lysosomal degradation, so we speculate whether EBP50 inhibits FLS cell proliferation by inhibiting autophagy. Then, the expression of LC3 was measured by western blotting in LV-EBP50 and LV-plvx transfected cells and untreated cells. As shown in (Fig. 1E-1F), the expressions of LC3 in FLS remain unchanged after transfected with LV-EBP50. Collectively, these results suggest that overexpression of EBP50 suppresses the proliferation of FLS and has no influence on autophagy.

Overexpression of EBP50 promote apoptosis of FLS By activating caspase-3

FLS can exhibit tumor-like behavior, such as significant inhibition of apoptosis. As the major tumor suppressor genes, related literature reports have indicated that overexpression of EBP50 promoted significantly the tumor cells apoptosis, and our previous results have showed that the overexpression of EBP50 upregulates the apoptosis ratio of macrophage by increasing the expression of Bax and caspase-3 in an NO-dependent mechanism. To investigated whether EBP50 can perform the same function in FLS, FLS were transfected with LV-EBP50 and LV-Plvx at the MOIs of 10 for 48 h, 72 h, 96 h, and the cell apoptosis of FLS was analyzed by FCM. Results showed that EBP50 overexpression increased distinctly the apoptosis of FLS when compared with LV-Plvx transduced FLS ($P < 0.001$) (Fig. 2A); The expression of apoptosis-related protein caspase-3 in FLS were measured by western blotting. As shown in (Fig. 2B), when FLS were transduced with LV-EBP50 and LV-Plvx for 96h, the protein expressions level of cleaved caspase-3 were significantly increased.

overexpression of EBP50 Suppresses the Migration and Invasion of FLS By downregulating MMP-9

Related researchs have showed that EBP50 could inhibit the migration and invasion of breast cancer cells by down-regulating MMPs and interacting with β -catenin(30,33). Since FLS migration and invasion are essential steps leading to joint destruction of RA, we evaluated the effect of EBP50 on these aggressive phenotypes of FLS in vitro. The primary FLS were left untreated (CON) or transfected with LV-EBP50 or LV-Plvx for 72 h, then the migration and invasion of FLS were evaluated using the transwell assay. FLS overexpressing EBP50 displayed

dramatic reduction in the number of migrating and invading cells across transwell assay (Fig. 3A-3B). As we known, the FLS could express MMPs and a variety of surface adhesion molecules to influence the migration and invasion. To study the potential mechanisms underlying the biological effect of EBP50 in FLS, the expression of β -catenin, E-cadherin, MMP2 and MMP9 were analyzed in LV-EBP50 and LV-Plvx transfected cells and untreated cells. The results have showed that protein levels of β -catenin were unchanged and E-cadherin were not expressed (Fig. 3C-3D), and the expression of MMP-9 in the EBP50-overexpressing cells were significantly decreased, whereas MMP-2 remained the same (Fig. 3E-3F-3G). These results have indicated that overexpression of EBP50 impaired both the migratory and invasive ability of FLS by downregulating MMP-9.

DISCUSSION

So far, the pathogenesis of RA has not been completely clarified, therefore no therapeutic specific has been found clinically. The current treatment mainly focuses on drug therapy and surgical treatment. Current clinically used drugs include nonsteroidal anti-inflammatory drugs, glucocorticoid, disease modifying antirheumatic drugs, biologics, traditional Chinese medicine, etc. However, various therapeutic methods have their own limitations. Recent studies have indicated that genetic approach might be a new therapeutic methods, Several genes have been confirmed to be genetic factors of RA or to play a potential regulatory role in the pathogenesis of RA. such as microRNAs^[22,23], lncRNAs^[24], etc. There is growing evidence that FLS of active RA play a major role in the onset and progression of RA. In addition, FLS in synovial tissues from patients with RA present tumour-like behaviours, including aggressive proliferation, increased migration, invasion, and decreased apoptosis. Emerging evidence indicate that inhibition of RA-FLS effector molecules might be beneficial for RA therapy. In this study, we explored the role of overexpression of EBP50 in FLS proliferation, migration/invasion, and cell apoptosis.

There have many studies reported that EBP50 expression was down-regulated in breast cancer, gastric cancer and prostate cancer, and overexpression of EBP50 could effectively inhibit the proliferation of tumor cells and promote their apoptosis. Here in our study, to explore the potential effects of EBP50 on the proliferation of FLS, FLS were left untreated (CON) or transfected with control lentivirus (LV-Plvx) or LV-EBP50 for 72 h, the expression of EBP50 were detected by WB and qRT-PCR firstly. The results indicated that the expression of EBP50 was significantly up-regulated in LV-EBP50 transfected cells compared with untransfected cells and LV-Plvx transfected cells. Then, we found that EBP50 overexpression in FLS significantly inhibited cell proliferation by MTT assay, consistent with previous reports showing EBP50 as an inhibitor against cell proliferation in certain proliferative diseases such as cancer^[25-27]. At the same time, EBP50 can stimulate autophagy and silencing EBP50 restrains it in cytoplasm^[28]. EBP50 is the activator of autophagy, inhibiting the growth of McF-7 breast cancer cells overexpressing by lysosome degradation of C-MYC. Recent studies also have shown that in RA-FLS from RA patients or RA animal models, autophagy the pathway is continuously stimulated and apoptosis is down-regulated, leading to the proliferation and activation of synovial fibroblasts. So, we investigated the effect of EBP50 overexpression on autophagy in FLS. We observed that the autophagy-related proteins LC3 remain unchanged. Our results thus suggest that EBP50 overexpression had no effect on the autophagy of FLS, which might inhibit FLS proliferation

through other pathways.

Apoptosis is another important target for anticancer therapy. Related literature reports that overexpression of EBP50 significantly promoted the tumor cells apoptosis, and we found that overexpression of EBP50 upregulates the apoptosis ratio of macrophage by increasing the expression of Bax and caspase-3 in an NO-dependent mechanism in a previous study[30]. Thus, we investigated whether EBP50 can perform the same function in FLS. The Bcl-2 and Caspase families are known to play crucial roles in cell survival and death. The Bcl-2 family including Bcl-2, Bcl-XL, Bcl-w, and CED 9 were belonged to the antiapoptotic members, and Bax, Bad, and Bid were proapoptotic members^[31,32]. The Caspase-3, -6, -7 are the part of members of the caspase family, which are the effector caspases involved in the execution of apoptosis stimulation, among them, Caspase-3 is the most important end-effector molecule in the process of apoptosis, and activation will lead to irreversible apoptosis^[33]. In this study, after transduced with LV-EBP50, the apoptosis of FLS were increased when compared with LV-Plvx transduced FLS (Fig. 4B). Furthermore, we evaluated the expression of the apoptosis-related proteins Caspase3 (Fig. 4B). The results showed that cleaved caspase-3 were significantly increased in LV-EBP50-transduced FLS, which revealed that EBP50 overexpression promote apoptosis of FLS through activating caspase-3. Our results demonstrated EBP50 transient overexpression significantly increased RA-FLS apoptosis, and therefore, may offer novel approaches against synovial hyperplasia.

FLS is the main cell group in synovial tissues and participated in the development of RA . Prior studies have demonstrated that the suppression of FLS migration and invasion might protect against joint destruction in RA . There is a new therapeutic strategy that regulating the FLS migration and invasion might be used to prevent the destructive progress of RA. Therefore, we determined the effect of overexpression of EBP50 on the migration and invasion of FLS in this study. As a result, transfection of FLS with the LV-EBP50 over-expression vector had a profound inhibitory effect on their migration and invasion. It is well documented that membrane-associated proteins formed by the combination of E-cadherin and β -catenin participate in the regulation of cell adhesion, and cell movement, including metastasis and invasion. EBP50 stabilizes β -catenin on the cell surface by interacting with β -catenin and promotes the binding of β -catenin to E-cadherin complex, inhibiting cell migration and thus tumor cell metastasis. In addition, The main protease that causes invasion and degradation of basement membrane and extracellular matrix is matrix metalloproteinases, which are the main proteases. In RA patients ,MMP-1, MMP-3, MMP-9, and MMP-13 are markedly elevated in synovial fluid, contributing to bone erosion and disease progression^[34]. Among these proteins, endogenous MMP2 and MMP9 contribute to synovial fibroblast survival, proliferation, migration, and invasion in RA^[35]. Studies have shown that EBP50 can down-regulate the expression of MMPs in breast cancer cells, especially MMP-2 and MMP-9, thus inhibiting the invasion ability of breast cancer cells, indicating that EBP50 can inhibit the invasion of tumor cells by inhibiting the expression of MMPs. To determine the effect of EBP50 on E-cadherin, β -catenin, MMP2 and MMP9 expression of FLS, we used western blot to measure the expression of E-cadherin, β -catenin, MMP2 and MMP9. In the present study, we observed that EBP50 overexpression markedly down-regulated the expression of MMP-9, but had no effect on MMP-2, E-cadherin and β -catenin, suggesting that one of the mechanisms for EBP50-regulated aggressive behavior of FLS is reducing MMP-9 production.

Conclusions

To summarize, EBP50 suppressed FLS proliferation, migration and invasion, and induced apoptosis. These findings have therapeutic significance and indicate the potential of EBP50 as a therapeutic target for RA, because the EBP50-mediated effects on FLS involve targeting synovial hyperplasia. Further research is needed to investigate the pathways involved in these protective effects and clarify if EBP50 could reduce proinflammatory cytokine secretion from RA-FLS.

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Disclosure

The authors declare no conflicts of interest.

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Figure legends

Figure 1. Overexpression of EBP50 Suppresses the proliferation of FLS.

FLS were left untreated (CON) or transfected with control lentivirus (LV-Plvx) or LV-EBP50 for 72 h. The mRNA levels of EBP50 were measured by real-time RT-PCR (A). The protein levels of EBP50 were detected by Western blotting (B). Band density of the specific protein was analyzed with Quantity One image software, and the results were expressed as average density to β -actin (C). FLS were left untreated (CON) or transfected with control lentivirus (LV-Plvx) or LV-EBP50 for 48 h, Then were transferred seed in 96-well plates 5×10^3 /cells, after night, 20 μ L MTT solution (0.5 mg/mL) was added in FLS culture medium and incubated for 4 h in 5% CO₂ atmosphere at 37°C. The culture medium in each well was discarded and added 150 μ L DMSO. The absorption of each well was measured at 490 nm. (D). The protein levels of LC3 were detected by Western blotting (E). Band density of the specific protein was analyzed with Quantity One image software, and the results were expressed as average density to β -actin (F). Data are represented as mean \pm SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the uninfected group.

Figure 2. Overexpression of EBP50 promote apoptosis of FLS By activating caspase-3

FLS were left untreated (CON) or transfected with control lentivirus (LV-Plvx) or LV-EBP50 for 48 h, 72 h, 96 h. the apoptosis incidence was measured by FCM (A). The expression levels of caspase-3 were determined by Western blotting (B). Data are represented as mean \pm SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the uninfected group.

Figure 3. overexpression of EBP50 Suppresses the Migration and Invasion of FLS By downregulating MMP-9

FLS were left untreated (CON) or transfected with control lentivirus (LV-Plvx) or LV-EBP50 for 72 h. Then the effect of EBP50 on migration was detected with transwell Boyden chamber. The images are representative of migration through the membrane after staining. Original magnification 200 \times (A, C). The effect of EBP50 on migration was detected with transwell Boyden chamber. The images are representative of invasion through the membrane after staining.

Original magnification 200×(B,D).The expression levels of β -catenin and E-cadherin were determined by Western blotting(E). Band density of the specific protein was analyzed with Quantity One image software (F). The expression levels of MMP-2 and MMP-9 were determined by Western blotting(G). Band density of the specific protein was analyzed with Quantity One image software (H-I). Data are represented as mean \pm SEM of at least three independent experiments.*P < 0.05, **P < 0.01,***P < 0.001 compared with the uninfected group.

Figure 1. Overexpression of EBP50 Suppresses the proliferation of FLS.

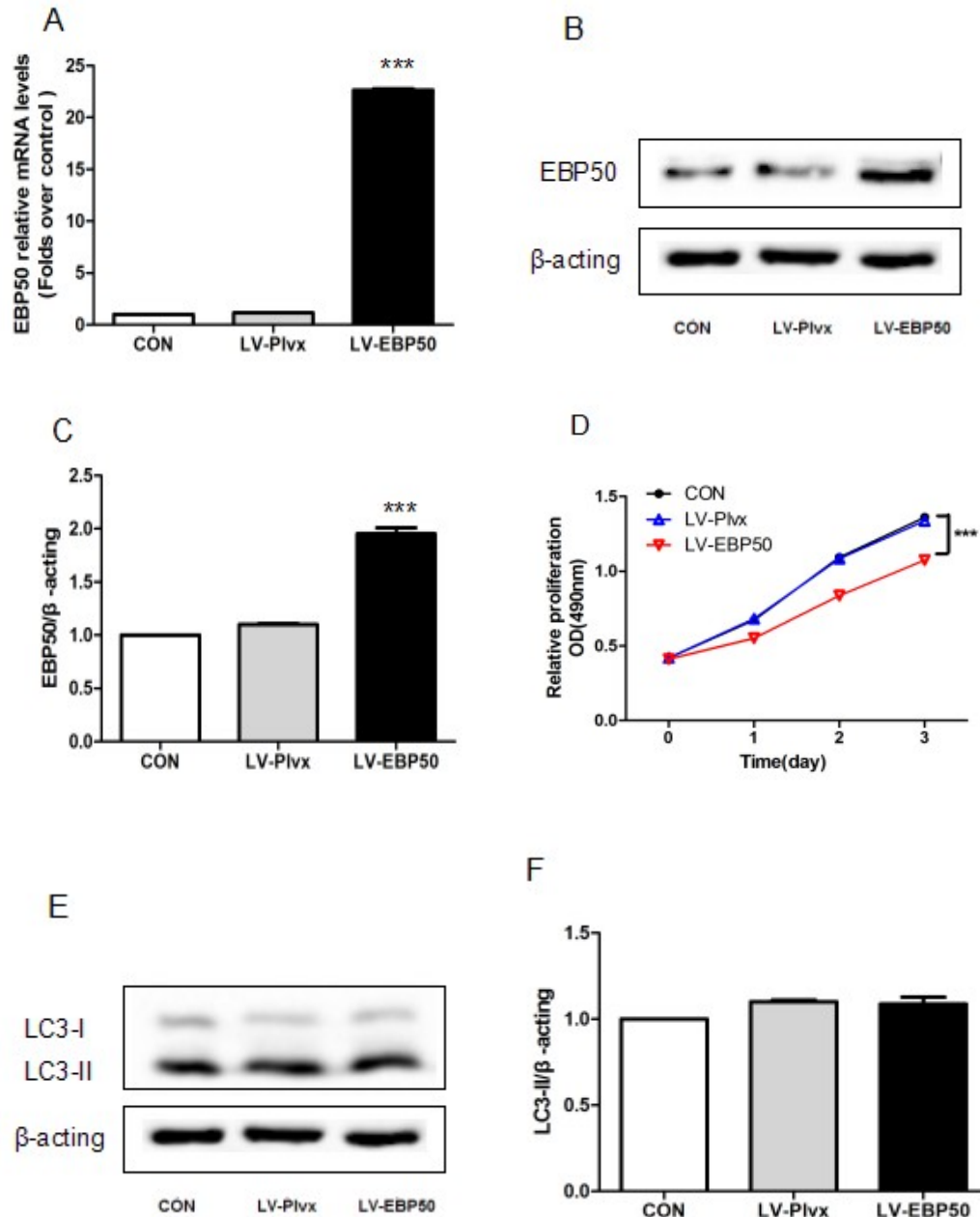


Figure 2. Overexpression of EBP50 promote apoptosis of FLS By activating caspase-3

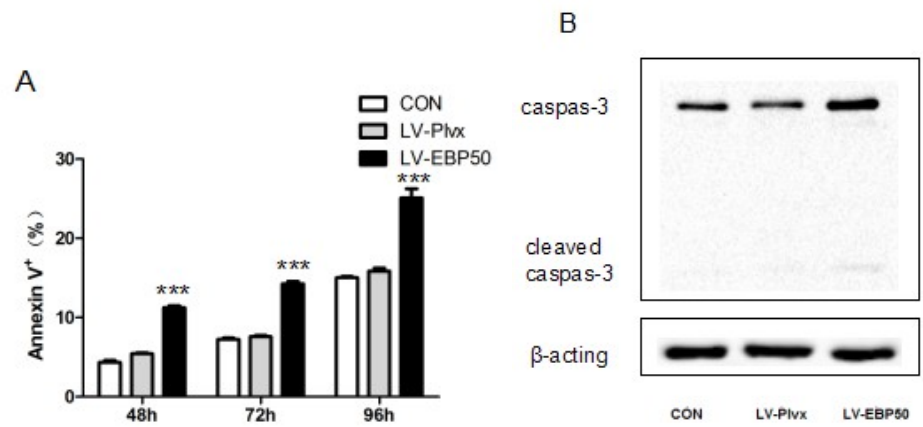


Figure 3. overexpression of EBP50 Suppresses the Migration and Invasion of FLS By downregulating MMP-9

