β-arrestin1 promotes epithelial-to-mesenchymal transition in colorectal cancer through regulating GSK-3β/β-catenin signaling pathway

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Abstract

Recurrence and metastasis seriously affects the prognosis of tumor patients, and Epithelial-mesenchymal transition (EMT) plays a key role in promoting tumor invasion and metastasis. Previous studies have showed that β-arrestin1 acted as a tumor promoting factor in various tumors. However, the exact role and mechanism of β-arrestin1 in colorectal cancer (CRC) progression still have not been well elucidated. In this study, by data mining using GSE41258 dataset, we firstly found that CRC patients with liver/lung metastasis had a higher expression of β-arrestin1, and the expression of β-arrestin1 was reversely correlated with the prognosis of CRC patients. Further mechanism study demonstrated that β-arrestin1 could promote the migration of CRC cells through regulating the EMT process by activating Wnt/β-catenin signaling pathways. Blocking Wnt/β-catenin signaling with inhibitor ICG001 would decrease the promoting effect of β-arrestin1 on EMT in CRC. *In vivo* imaging experiment further demonstrated the promoting effect of β-arrestin1 on the lung metastasis of CRC cells by tail vein injection in mice. In summary, our results reveal a novel mechanism of β-arrestin1 in regulating EMT via Wnt/β-catenin signaling pathway in CRC metastasis, providing a potential therapeutic target for CRC progression.

Keywords: β-arrestin1, Colorectal cancer, Epithelial-to-mesenchymal transition, Wnt/β-catenin signaling pathway, Metastasis

Introduction

Colorectal cancer (CRC) is the second most frequent malignant cause of cancer-related death worldwide that leading to 0.8 million new deaths per year approximately[1]. Metastasis is the main cause of treatment failure and poor prognosis[2]. Multiple signaling pathways and key factors are involved in the process of CRC cells from the primary sites into the distal locations/organs[3].

Studies have shown that Epithelial-mesenchymal transition (EMT) plays a key role in promoting tumor invasion and metastasis[4]. During EMT, loss of a pical-basal polarity, reorganization of the cytoskeletal architecture and increased cell motility contribute to cancer cells acquire the ability of invasion and metastasis[5]. Various important genes or proteins are involved in regulating EMT, such as E-cadherin, N-cadherin, Vimentin, etc[6]. Multiple signal pathways such as TGF-β/SMAD, PI3K, MAPK were reported to regulate the EMT process[7-9]. Recently, several studies have demonstrated that the Wnt signaling is closely associated with EMT, however, the detailed mechanism remains unclarified[10-13].

β-arrestins, including β-arrestin1 and β-arrestin2, are important intracellular regulatory proteins. They were initially found to act as scaffolds to regulate signal transduction mediated by G protein coupled receptor (GPCR)[14]. β-arrestin1 (53 kDa), located on chromosomes 7, were comprised of two major domains, the N-domain and C┐ domain, which basically consist of β-sheets and connecting loops with one short a-helix[3]. β-arrestin1 serves as ligand-regulated scaffolds for GPCR-mediated signaling. Evidence showed that blocking β-arrestin-dependent GPCR desensitization could reduce the occurrence of drug tachyphylaxis in chronic pain, asthma, and psychiatric illness. As signaling scaffolds, β-arrestin1 was also participated in regulating cell growth, migration, and survival in inflammatory diseases, fibrosis, and cancer[15]. Especially β-arrestin1 could regulate cell proliferation, promote cell invasion and migration, transmit anti-apoptotic survival signals and affect other characteristics of tumors, such as tumor growth rate, angiogenesis, drug resistance, invasion and metastatic potential in a variety of tumors, including lung cancer, breast cancer, colorectal cancer, prostate cancer, ovarian cancer, et al[16-19]. Research discoveries that β-arrestin1 plays an important

role in the development and progression of colorectal cancer. An early study found that PGE2-induced transactivation of the EGFR and downstream Akt signaling through β-arrestin1 act as mediator in GPCR activation of c-Src, thereby promoting colorectal cancer cell migration and metastasis[20]. Recent research indicated that miR-296 inhibits colorectal cancer cell growth and enhances apoptosis by activation of Akt mediated by β-arrestin1[21]. Another study revealed that β-arrestin1 could reduce E-cadherin expression and increase Vimentin expression in tumor epithelial-mesenchymal transformation[12]. Although previous studies reveal that β-arrestin1 can promote EMT in CRC, the underlying molecular mechanisms of β-arrestin1 regulating EMT is largely unknown.

In this study, we investigated the biological function and molecular mechanism of β-arrestin1 in EMT and metastasis of colorectal cancer, providing a potential therapeutic target for CRC progression.

Results

High β-arrestin1 expression in CRC predict poor prognosis. The expression of β-arrestin1 in CRC tissues was analyzed using GSE41258 dataset. There were 206 samples in the dataset, including 155 primary tumor, 32 liver metastasis and 19 lung metastasis. By data analysis, we found that β-arrestin1 was obviously overexpressed in CRC metastasis tissues in compared with CRC primary tissues (P=0.0005; Fig. 1A). Especially, liver metastasis tissues had a higher level of β-arrestin1 than primary CRC tissues (P=0.05; Fig. 1B). Compared with CRC primary tissues, the expression of β-arrestin1 significantly also increased in lung metastasis tissues (P=0.0001; Fig. 1C). Accordingly, CRC patients with high expression of β-arrestin1 in CRC primary tissues had a poorer prognosis, in compared with those with low expression of β-arrestin1 (P=0.041; Fig. 1D). We used Pathway Commons, KEGG, GO and dbemt to analyze the genes co-expressed with β-arrestin1. The genes with degree ⩾10 in the network are shown in the figure. The results found that β-arrestin1 was closely related to the genes of EMT, including Twist, Snail, CDH1, Vimentin, MMP2 and MMP9, and GSK-3β, NF-κB, PI3K/Akts, MAPK signaling pathways were involved in regulating this process (Fig. 1E).

β-arrestin1 regulates EMT in CRC cells. Since the bioinformation analysis implied the close

association between β-arrestin1 and EMT related signaling pathway, and EMT is a key step in the invasion and metastasis of CRC, we next observed the function of β-arrestin1 in EMT. β-arrestin1 expression was characterized in seven CRC cells. It was found that β-arrestin1 had a quite higher level in HCT-116 and LoVo cells, compared to HCT-8, Caco-2, HT-29, SW620 and RKO cells (Fig. 2A, 2B). To investigate the function of β-arrestin1, we firstly knocked down and overexpressed β-arrestin1 gene in CRC cells. By real time PCR, we confirmed that the expression of β-arrestin1 was obviously down-regulated in shRNA/β-arrestin1 groups, and significantly up-regulated in the over/β-arresin1 group, in compared with the control group (Fig. 2C). The protein expressions of β-arrestin1 in above groups were also detected by western blot, and the results were consistent with the real time PCR results (Fig. 2D, 2E).

EMT is mainly featured by the destruction of adherens junctions (AJs), which is due to cells losing epithelial characteristics[22-23.] In the process, the conversion from E-cadherin to N-cadherin results in decreasing the function of AJs[24]. The change of cytoskeletal and polarity complex proteins leads to EMT[25]. The activation of Vimentin could regulate organelles and membrane-associated proteins, and promote cell motility[26]. Therefore, E-cadherin, N-cadherin, Vimentin, Snail are recognized broadly as the characteristic proteins of EMT. Our data indicated that, in compared with the control groups, the expression of E-cadherin increased remarkably, and the expression of Snail, N-cadherin and Vimentin decreased obviously in shRNA/β-arrestin1 groups, while the over/β-arrestin1 groups had an opposite effect in HCT-116 or LoVo cells (Fig. 2F-2I).

Since E-cadherin and Vimentin are the most characteristic proteins of EMT, we further detected their expression by immunofluorescence. The result demonstrated that, compared with the control groups, the expression of E-cadherin was increased in shRNA/β-arrestin1 group, but decreased in over/β-arrestin1 group. However, compared with the control groups, the expression of E-cadherin was increased in shRNA/β-arrestin1 group, but decreased in over/β-arrestin1 group (Fig. 3A-3D).

β-arrestin1 promotes the migration of CRC cells. EMT is the initial step of invasion and metastasis of cancer cells. Above results showed that β-arrestin1 could regulated Epithelial mesenchymal transformation in CRC cells, further we investigated the effect of β-arrestin1 on invasion and metastasis. By transwell and wound healing assays, we observe the effect of β-arrestin1 on the migration of CRC cells. According to the data of transwell analysis, we found that

shRNA/β-arrestin1 can suppress the migration of HCT-116 or LoVo cells obviously, but over/β-arrestin1 has the opposite effect (Fig. 4A). The result of wound healing assays was similar with transwell migration assay (Fig. 4B). Furthermore, we detected the expression of MMP2 and MMP9, which are associated closely with invasion and metastasis. In compared with the control group, the expressions of MMP2 and MMP9 were down-regulated in shRNA/β-arrestin1 group, while over/β-arrestin1 could upregulate the expression of MMP2 and MMP9 in HCT-116 or LoVo cells (Fig. 4C, 4D).

β-arrestin1 activates the Wnt/β-catenin signaling pathway in CRC. A series of studies indicated that Wnt/β-catenin was involved in tumor invasion and metastasis[12]. When Wnt/β-catenin signaling is activated, the translocation of β-catenin into the nucleus from the cytoplasm is increased[27]. To investigate the effect of β-arrestin1 on the expression and location of β-catenin, firstly, we applied immunofluorescence assays to examine the expression of β-arrestin1 in shRNA/β-arrestin1 and over/β-arrestin1 groups. As we expected, β-arrestin1 was lowly expressed in shRNA/β-arrestin1 group, and highly expressed in over/β-arrestin1 group (Fig. 5A, 5B). Then we discovered that shRNA/β-arrestin1 decreased the nucleus translocation of β-catenin in colorectal cancer cells, but over/β-arrestin1 increase the expression of β-catenin (Fig. 5C, 5D).

To understand the mechanism of β-arrestin1 on EMT in detail, we examined the function of β-arrestin1 in modulating the expressions of GSK-3β, p-GSK-3β, Axin1, CK1, β-catenin, c-Myc, and CyclinD1, which are associated with Wnt signaling and the downstream proteins of β-catenin. From our data, we found that shRNA/β-arrestin1 promoted the protein expression of GSK-3β, while inhibit the protein expression of p-GSK-3β, c-Myc, and CyclinD1, yet over/β-arrestin1 had a promoting effect in HCT-116 or LoVo cells (Fig. 6A, 6B). What's more, β-arrestin1 had no obviously change in the whole β-catenin expression. Then we further detected the expression of cytoplasm and nucleus β-catenin. As shown, cytoplasm β-catenin expression increased and nucleus β-catenin expression decreased in shRNA/β-arrestin1 group, while over/β-arrestin1 had the opposite result (Fig. 6C, 6D). Next, β-arrestin1 overexpressing HCT-116 or LoVo cells were treated with or without ICG001 for 24h, and the protein levels of Wnt/β-catenin signaling including GSK-3β, p-GSK-3β, β-catenin, c-Myc and CyclinD1 were detected by western blotting. The result indicated that the expression levels of p-GSK-3β, β-catenin, c-Myc and CyclinD1 were all increased in over/β-arrestin1 group

except GSK-3β expression, but this effect was reversed by blocking Wnt/β-catenin signaling with ICG001 (Fig. 6E, 6F). The expression of E-cadherin was upregulated, but the expressions of N-cadherin, Snail and Vimentin were downregulated by using ICG001 (Fig. 6G, 6H). Taken together, these data indicated that β-arrestin1 taken effect as a result of activating Wnt/β-catenin signaling in CRC cells.

β-arrestin1 regulates CRC EMT and metastasis *in vivo*. To detect the function of β-arrestin1 in tumor metastasis *in vivo*, we used a lung metastatic tumor model of fluorescence-labeled colorectal cancer cell line, including HCT-116/luc, shRNA/β-arrestin1-HCT-116/luc and over/β-arrestin1-HCT-116/luc cells. We observed the lung metastasis on day 28, 33, 38, and 43 respectively. As shown, the shRNA/β-arrestin1 group showed smaller lung metastasis than the controls, while over/β-arrestin1 group showed the biggest lung metastasis in three groups (Fig. 7A). Then, we examined the lung metastasis numbers of shRNA/β-arrestin1 and over/β-arrestin1 HCT-116 cells in metastatic tumor model, and found fewer lung metastatic sites in lungs of the shRNA/β-arrestin1 group. Among the three groups, shRNA/β-arrestin1 group showed the best results to extend the overall survival times. After that, the metastatic lesions were excised and examined by HE staining. According to the imaging results we found that more metastatic lesions were seen in the lung organs with over/β-arrestin1, and the metastatic lesions reduced obviously in shRNA/β-arrestin1 group (Fig. 7B-7E). Immunohistochemistry was applied to test the expression levels of E-cadherin and Vimentin. Compared with controls, shRNA/β-arrestin1 group had a higher expression of E-cadherin and lower expression of Vimentin, however, over/β-arrestin1 group had an opposite result (Fig. 7F). The results of western blot were basically consistent with immunohistochemistry (Fig. 7G). In summary, our data showed that β-arrestin1could promote the lung metastasis of CRC cells through regulating EMT process *in vivo*.

Discussion

In recent years, accumulating evidences have revealed that β-arrestin1 play important roles in CRC progression, but its detailed mechanism in CRC is unclear. In this study, our data showed that β-arrestin1 facilitated the metastasis of CRC cells through activating Wnt/β-catenin signal pathway and initiating EMT process. In detail, β-arrestin1 could promote β-catenin translocating into nucleus,

activating the β-catenin signaling pathway, further trigger the proceeding of EMT, leading to CRC progression.

β-arrestins, as ubiquitously scaffolding proteins, are involving in regulating cancer cell phenotypes including cell proliferation, migration, invasion and metastasis[20,28-30]. Recently, β-arrestin1 is reported to be responsible for EMT in tumor cells through a variety of signaling molecules, however, its role and mechanism in CRC still have not been well elucidated[12, 31].

EMT is one of the most key steps for tumor cells to acquire invasive ability, which is significant in promoting tumor invasion and metastasis[32]. Activation of the mesenchymal phenotype and suppression of the epithelial phenotype are the results of changes in genes expression, such as Snail, Twist and zinc-finger E-box-binding (ZEB) transcription factors. When these transcription factors are abnormally expressed, EMT will be triggered[33]. In order to observe the role of β-arrestin1 in CRC, we firstly observed the effect of β-arrestin1 on EMT in CRC cells. Our data showed that silencing β-arrestin1 inhibited EMT in CRC cells, while overexpression of β-arrestin1 significantly promoted EMT in CRC cells, which were visualized by the down-regulation of E-cadherin and up-regulation of snail, N-cadherin and Vimentin in CRC cells. The results demonstrated β-arrestin1 probably exerted a vital role in promoting EMT and facilitating CRC metastasis.

Subsequently, we investigated the effect of β-arrestin1 on CRC cell migration, and the data indicated that silencing of β-arrestin1 expression in CRC cells significantly inhibited cell migration, while overexpression of β-arrestin1 had an opposite effect. Therefore, above results showed *in vitro* that β-arrestin1 can promote CRC cells migration partly through initiating EMT, which are similar to previous researches about the important roles of β-arrestin1 in prostate, ovarian and non-small cell lung cancer [31, 33-35].

Next, we further consider the possible mechanism how β-arrestin1 regulate the EMT and metastasis in CRC. As we know, Wnt/β-catenin signaling promotes carcinogenesis and progression in multiple cancers via activating β-catenin to trigger the transcription of its downstream target genes[36-38]. The interaction between β-arrestin1 and β-catenin increases β-catenin target gene expression, which is responsible for cell migration, invasion and epithelial-to-mesenchymal transition[32]. Therefore, we further investigated the effect of β-arrestin1 on β-catenin signaling pathway. The data demonstrated that overexpression of β-arrestin1 could increase the accumulation of β-catenin in the nucleus and reduction in the cytoplasm, but silencing of β-arrestin1 had the

opposite effect[39-40]. Blocking β-catenin signaling pathway would greatly shrink the promoting effect of β-arrestin1 on EMT in CRC, implying the important roles of β-arrestin1 that promoted EMT partly through β-catenin signaling pathway in CRC progression.

In summary, our results discovered the significant role of β-arrestin1 in the regulation of EMT in CRC cells, and β-arrestin1-mediated Wnt/β-catenin signal pathway may be account for its potential mechanism for CRC metastasis.

Methods

Ethics Statement. All the experimental procedures were performed in strict accordance with the guidelines for the care and use of animals in Shanghai University of Traditional Chinese Medicine. All the animal experimental procedures were approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (No. 20140516). Every effort was made to minimize animal pain, suffering, and distress.

Materials. Three shRNAs sequences for β-arrestin1 gene were as follow: shRNA1: 5′-GAACTGCCCTTCACCCTAATG-3′, shRNA2: 5′-TCTGGATAAGGAGATCTATTA-3′, shRNA3: 5′-GGATCATTGTTTCCTACAAAG-3′, and the control shRNA sequence was as follow: 5′-TTCTCCGAACGTGTCACGT-3′. Human β-arrestin1 (Gene ID: 6421) gene was chemically synthesized. Both the shRNA/β-arrestin1 sequence and the over/β-arrestin1 sequence were subcloned into lentiviral expression vector (PGMLV-CMV-MCS-EF1-ZsGreen1-T2A-Puro) by Genomeditech (Shanghai, China). The shRNA lentivirus, the control lentrvirus and the overexpression lentivirus were transfected into the relative high expressing β-arrestin1 cell lines HCT-116 and LoVo. Rabbit monoclonal antibodies against human E-cadherin, N-cadherin, Vimentin, Snail, β-arrestin1, GSK-3β, p-GSK-3β, Axin1, CK1, β-catenin, c-Myc, CyclinD1, PCNA were purchased from Cell Signaling Technology. Rabbit monoclonal antibodies against human MMP2 and MMP9 were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies against human GAPDH were purchased from Proteintech. β-catenin inhibitor ICG001 was purchased from APExBIO.

Cell culture. Human CRC cell line HCT-116 (ATCC, USA) was cultured in RPMI 1640 medium and LoVo (ATCC, USA) was cultured in Ham's F-12K medium, containing 10% fetal bovine serum (FBS), 100U/mL penicillin, 100mg/mL streptomycin, and incubated in a humidified, 5% CO_2

atmosphere, at 37°C. HCT-116 or LoVo monoclonal cells stably expressing shRNA/β-arrestin1, empty vector or over/β-arrestin1 was generated by screening with purinomycin (2μg/mL). Other five human CRC cells including HCT-8, Caco-2, HT-29, and RKO cells were cultured as HCT116 cells, and SW620 cells were cultured in Leibovitz's L-15 medium supplemented with 10% FBS, 100U/mL penicillin, 100mg/mL streptomycin.

Lentivirus transfection. Before transfection, HCT-116 and LoVo cells (4×10^5) were seed on 6-well plates in incubator at 37°C overnight. Then, the shRNA lentivirus, the control lentrvirus and the overexpression lentivirus were added into the plates (Lentivirus volume = (MOI×number of cells)/lentivirus titer), and cultured in suitable medium. After 48 hours of transfection, the medium containing lentivirus was removed and the fresh medium was added.

Western blot. In brief, all cell proteins were prepared according to the instructions of ProteoJET Cytoplasmic Kit (Fermentas, USA). The extracted protein was quantified by BCA protein assay. Proteins were separated onto the 10% SDS-PAGE gels for electrophoresis, then transferred to PVDF membranes, blocked in 5% milk, and incubated with the primary antibodies following by the HRP-conjugated secondary antibodies. All the resulting immunocomplexes were visualized by enhanced chemiluminescence. Each experiment was repeated independently three times.

Immunofluorescence microscopy. After transfection, HCT-116 and LoVo cells (3×10^4) that adhered to the coverslips were fixed for 30 minutes with 4% paraformaldehyde at room temperature, permeabilized with Triton X-100 (0.5%) for 15 min, then blocked with 5% BSA solution. The cells were first stained with E-cadherin rabbit antibody followed by Cy3-conjugated goat anti-rabbit IgG or first stained with the Vimentin rabbit antibody followed by FITC-conjugated goat anti-rabbit IgG. After incubating with first and secondary antibodies, DAPI was applied for nuclear staining with 30 minutes. Finally, cells were tested with a DMI3000B inverted microscope (Leica, Germany). All the experiments were repeated three times.

Transwell assay for migration. After transfection, HCT-116 (5×10^4, in RPMI 1640 medium with 0.5% FBS) and LoVo cells (5×10^4, in Ham's F-12K medium with 0.5% FBS) were seeded into the upper part of a transwell chamber. 600 μl RPMI 1640 or Ham's F-12K medium with 10 μg/ml fibronectin and 15% FBS was added in the lower part of the chamber. The migration cells were analyzed after co-culture for 48 hours. Migrated cells were analyzed by crystal violet staining, followed by observing under a DMI3000B inverted microscope (Leica, Germany). Five random

views were selected to count the migrated cells. Each experiment was repeated independently three times.

Wound healing assay. After transfection, HCT-116 or LoVo cells were seed on 6-well plates, then were created an artificial scratch wound using a 20μl pipette tip and detached cells were removed by washing with PBS three times. After 48h or 72h incubation, cell migration was photographed using inverted microscope and evaluated by measuring the difference in wound width.

Real time PCR. For real time PCR of β-arrestin1, the primers were designed as follows: Forward primer: 5-ACCAGGCAGTTCCTCATGTC-3, Reverse primer: 5-CGTCTTGTTGGTGTTGTTGG-3, with control primers for GAPDH: Forward primer: 5-GAAGGCTGGGGCTCATTTG-3, Reverse primer: 5-GGGCCATCCACAGTCTTC-3. Real-time PCR was performed by using SYBR® 2×Taq Master Mix (Vazyme, Nanjing, China) with an iCycler® thermal cycler (Thermofisher 7500, USA). All assays were performed in triplicate and independently repeated three times.

Data analysis using GSE41258 datasets. The β-arrestin1 expression and clinical data from GSE41258 datasets were collected, including CRC primary tissues, lung metastasis tissues and liver metastasis tissues. β-arrestin1 expression was measured by IlluminaHiSeq platform. There were 206 samples, including 155 primary tumor, 32 liver metastasis and 19 lung metastasis in GSE41258 datasets. The influence of β-arrestin1 expression on survival was assessed by the Cox proportional hazards model. The genes interacting with β-arrestin1 were screened by Pathway Commons, KEGG, GO and dbemt.

Animal model. Male BALB/c nude mice (5 weeks old) were purchased from Department of Experimental Animals of Shanghai University of Traditional Chinese Medicine and fed in specific pathogen-free conditions for 1 week. The mice were injected with 2×10^6 HCT-116/luc cells, or shRNA/β-arrestin1-HCT-116/luc cells, or over/β-arrestin1-HCT-116/luc cells through the tail vein. For *in vivo* imaging, mice were anesthetized and injected intraperitoneally with D-luciferin (15mg/ml in 200μl PBS), and observed by using an IVISLumina system (Caliper, USA). After that, the lung organs were excised, fixed in 4% paraformaldehyde, and paraffin-embedded. The lung sections were fully cut, and each section was set to 6 μm. All the lung sections were stained with hemaoxylin-eosin (HE), and valued for E-cadherin and Vimentin by immunohistochemistry.

Statistical analysis. All the data were presented as the means ± SD of at least three independent experiments and analyzed with SPSS22 Software. The mean values of two groups were compared by

Student's t test. $P<0.05$ was considered as statistically significant.

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Author Contributions

QL and QJ designed the research and prepared the manuscript. QS, ZFH, XNW, LHZ and LY performed the experiments. QJ, QS, QL, NNL and HS analyzed the data. QL, QJ, JFC and YW conceived and supervised the project.

Competing interests

The authors declare no competing interests.

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Figures legends

Figure 1 | Expression of β-arrestin1 in CRC tissues and its association with the prognosis of CRC patients. (A-C) Analysis of β-arrestin1 expression profile using the data from GSE41258 dataset. (D) Association between β-arrestin1 expression and the prognosis of CRC patients. (E) Network representation of β-arrestin1 interactions with multiple genes, including Twist, Snail, CDH1, Vimentin, MMP2, MMP9, GSK-3β, MAPK, etc.

Figure 2 | β-arrestin1 regulates EMT in colorectal cancer cells. (A-B) The expression of β-arrestin1 in seven CRC cells. β-arrestin1 had a higher expression level in LoVo and HCT-116 cells, $*P<0.05$, compared with other five established human CRC cell lines HCT-8, Caco-2, HT-29, SW620, and RKO. (C) The mRNA expression of β-arrestin1 in shRNA/β-arrestin1 and over/β-arrestin1 groups was detected by RT-PCR. $*P < 0.05$; $**P < 0.01$, compared with HCT-116-vector or LoVo-vector cells. (D-E) Western blot detected the protein expression of

β-arrestin1 in shRNA/β-arrestin1 and over/β-arrestin1 groups in HCT-116 or LoVo cells. ***$P<0.001$, compared with HCT-116-vector or LoVo-vector cells. (F-I) Expressions of epithelial or mesenchymal markers in HCT-116 or LoVo cells were analyzed by western blotting. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared with HCT-116-vector or LoVo-vector cells.

Figure 3 | β-arrestin1 regulates the protein expression associated with EMT. (A-D) Immunofluorescence staining of E-cadherin and Vimentin in HCT-116 or LoVo cells transfected with shRAN/β-arrestin1 or over/β-arrestin1 lentivirus. *$P < 0.05$; **$P < 0.01$, compared with HCT-116-vector or LoVo-vector groups.

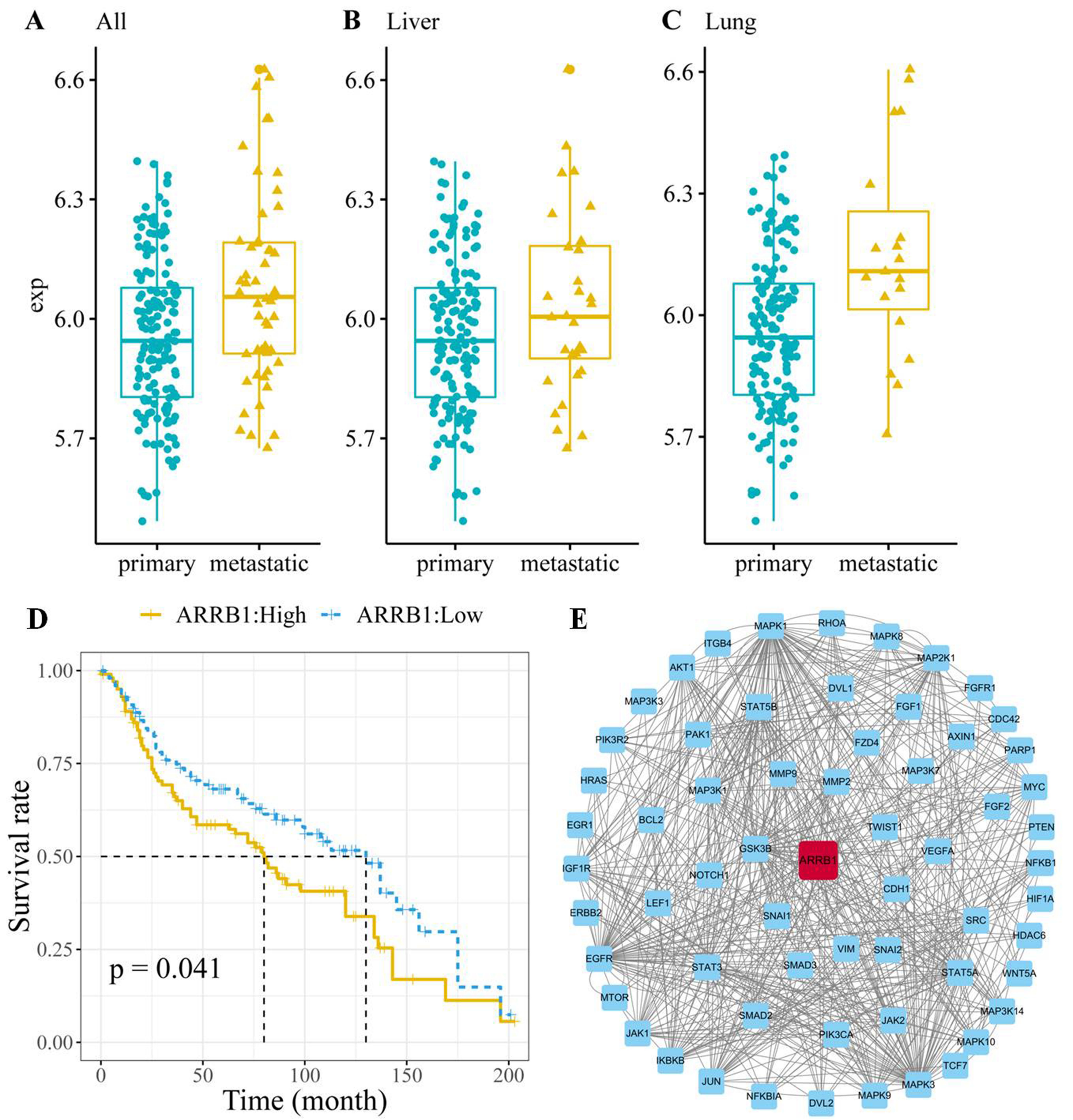
Figure 4 | β-arrestin1 promotes the migration of CRC cells. (A) HCT-116 or LoVo cells transfected with shRNA/β-arrestin1 or over/β-arrestin1 lentivirus were applied for the transwell experiment. Typical images of cells migrating in a transwell migration chamber were captured and quantitative measured. *$P<0.05$; **$P<0.01$, compared with HCT-116-vector or LoVo-vector cells. (B) shRNA/β-arrestin1 or over/β-arrestin1 cells were cultured to confluence on 6-well plates. Representative pictures and a bar graph of the wound-healing assay are shown. The black line was used to mark the ranges of the scratches. *$P<0.05$, compared with HCT-116-vector or LoVo-vector cells. (C-D) MMP2 and MMP9 were examined by western blot. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared with HCT-116-vector or LoVo-vector cells.

Figure 5 | β-arrestin1 promotes nucleus translocation of β-catenin. (A-B) Immunofluorescence staining of β-arrestin1 in shRNA/β-arrestin1 and over/β-arrrestin1 groups. **$P<0.01$, compared with HCT-116-vector or LoVo-vector groups. (C-D) Immunofluorescence staining for β-catenin (green), nucleus (blue) and membrane (red) through using an inverted fluorescence microscope (magnification: 400x). **$P<0.01$; ***$P<0.001$, compared with HCT-116-vector or LoVo-vector groups.

Figure 6 | β-arrestin1 regulates Wnt/β-catenin signal pathway. (A-B) Western blot detected the proteins of GSK-3β, p-GSK-3β, Axin1, CK1, β-catenin, c-Myc, CyclinD1 in shRNA/β-arrestin1 and

over/β-arrestin1 groups. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared with HCT-116-vector or LoVo-vector cells. (C-D) The protein expression of β-catenin in whole or cell extracts of different cellular compartments from HCT-116 or LoVo cells. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared with HCT-116-vector or LoVo-vector cells. (E-F) β-catenin inhibitor ICG001 (10μM) was added into over/β-arrestin1 group, and then cultured for 24h. The related proteins of Wnt signaling pathway were examined by western blot. *$P<0.05$; **$P<0.01$, compared with HCT-116-vector or LoVo-vector cells. (G-H) Western blot detected the proteins expression of epithelial or mesenchymal markers after using ICG001. *$P<0.05$; **$P<0.01$; ***$P<0.001$, compared with HCT-116-vector or LoVo-vector cells.

Figure 7 | β-arrestin1 regulates CRC EMT and metastasis *in vivo*. (A) Fluorescence intensity was evaluated in 8 mice of each group at day 28, 33, 38 and 43 after tail vein injection. *$P<0.05$, compared with HCT-116-vector group. (B-E) The lung metastasis were excised after day 43, the numbers of metastatic lesions were counted. *$P<0.05$, compared with HCT-116-vector group. The survival curve of animals injected with HCT-116/luc or shRNA/β-arrestin1-HCT-116/luc or over/β-arrestin1-HCT-116/luc cells. *$P<0.05$; **$P<0.01$, compared with HCT-116-vector group. Representative hemaoxylin-eosin (H&E) staining of tumour cytostructure and cytomorphology analysis in tumors. (F) Immunohistochemistry detected the expression of E-cadherin and Vimentin in three groups. (G) The protein expression of E-cadherin, N-cadherin, Snail and Vimentin were examined by western blot. *$P<0.05$; **$P<0.01$, ***$P<0.001$, compared with HCT-116-vector group.


A
All
B
Liver
C
Lung
exp
6.6
6.3
6.0
5.7
primary
metastatic
D
ARRB1:High
ARRB1:Low
Survival rate
1.00
0.75
0.50
0.25
0.00
p = 0.041
0
50
100
150
200
Time (month)
E
ARRB1
MAPK1
RHOA
MAPK8
ITGB4
MAP2K1
AKT1
DVL1
FGFR1
MAP3K3
STAT5B
FGF1
CDC42
PIK3R2
PAK1
FZD4
AXIN1
PARP1
HRAS
MMP9
MMP2
MAP3K7
MAP3K1
MYC
FGF2
EGR1
BCL2
TWIST1
PTEN
GSK3B
VEGFA
IGF1R
NOTCH1
NFKB1
CDH1
HIF1A
ERBB2
LEF1
SNAI1
SRC
HDAC6
VIM
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EGFR
STAT3
SMAD3
STAT5A
WNT5A
MTOR
SMAD2
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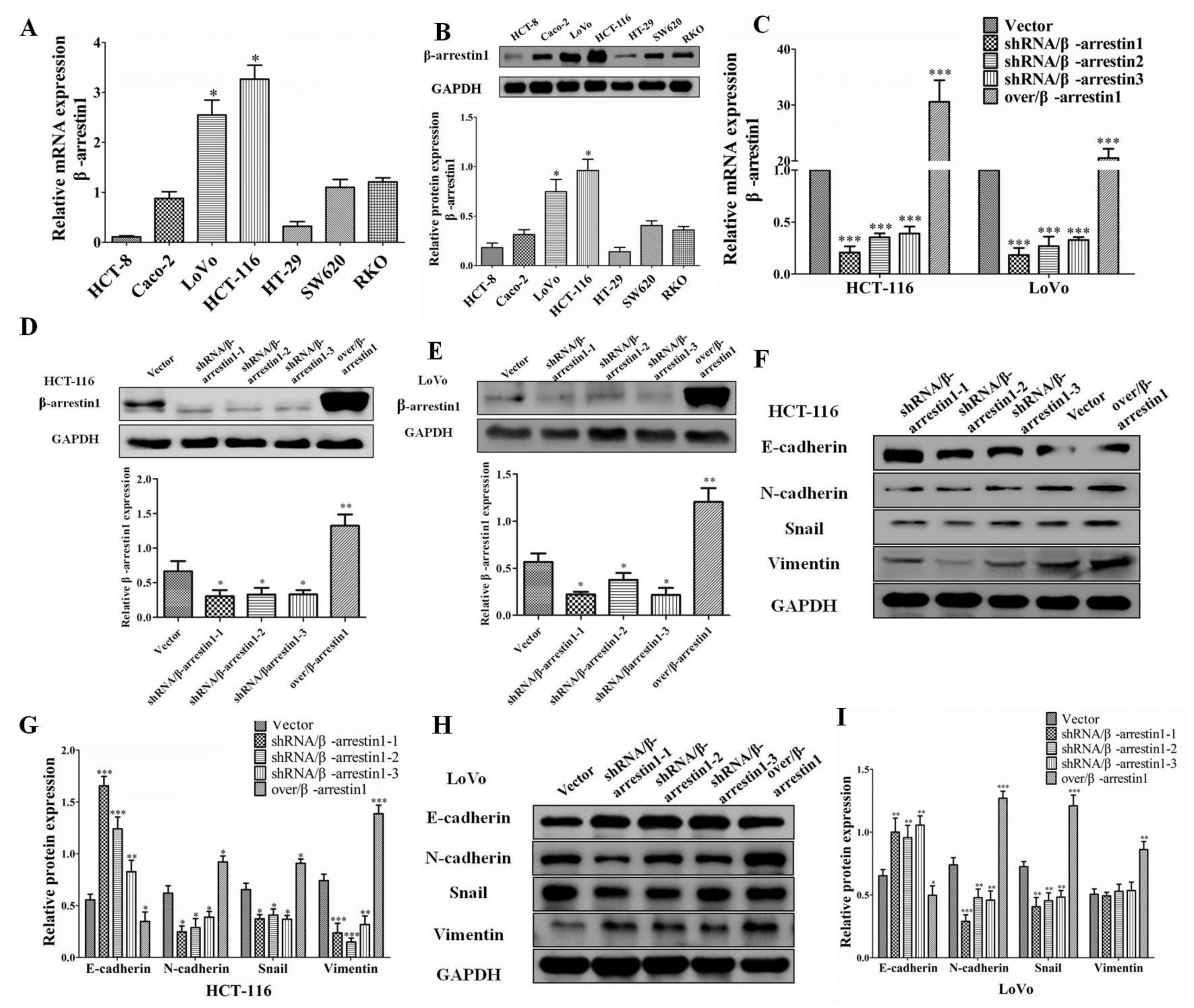
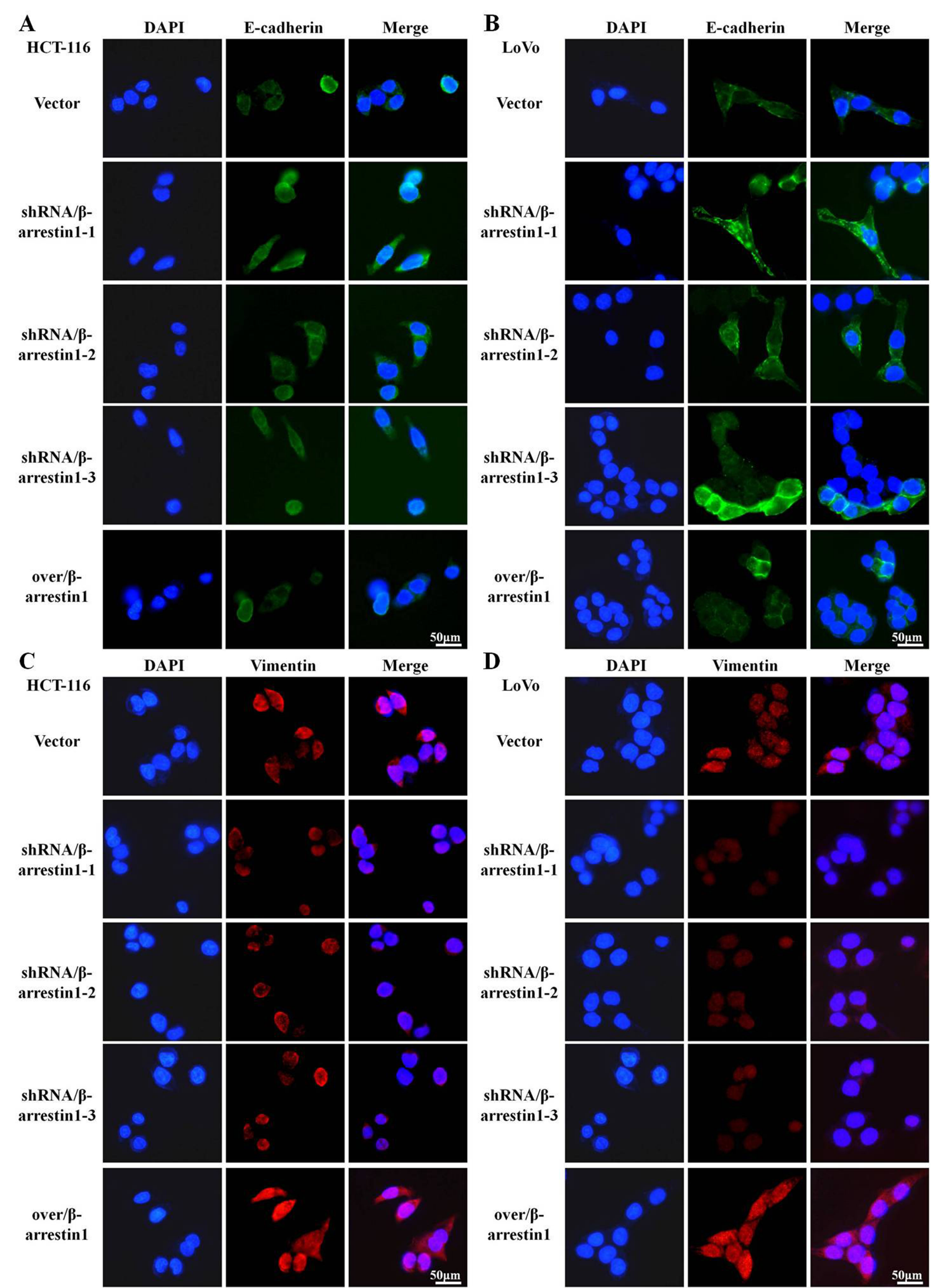
DVL2


A
Relative mRNA expression
β -arrestin1
HCT-8
Caco-2
LoVo
HCT-116
HT-29
SW620
RKO
B
β-arrestin1
GAPDH
Relative protein expression
β -arrestin1
C
Vector
shRNA/β -arrestin1
shRNA/β -arrestin2
shRNA/β -arrestin3
over/β -arrestin1
Relative mRNA expression
β -arrestin1
HCT-116
LoVo
D
HCT-116
β-arrestin1
GAPDH
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
Relative β -arrestin1 expression
E
LoVo
β-arrestin1
GAPDH
Relative β -arrestin1 expression
F
HCT-116
E-cadherin
N-cadherin
Snail
Vimentin
GAPDH
G
Vector
shRNA/β -arrestin1-1
shRNA/β -arrestin1-2
shRNA/β -arrestin1-3
over/β -arrestin1
Relative protein expression
E-cadherin
N-cadherin
Snail
Vimentin
HCT-116
H
LoVo
E-cadherin
N-cadherin
Snail
Vimentin
GAPDH
I
Vector
shRNA/β -arrestin1-1
shRNA/β -arrestin1-2
shRNA/β -arrestin1-3
over/β -arrestin1
Relative protein expression
E-cadherin
N-cadherin
Snail
Vimentin
LoVo


A
DAPI
E-cadherin
Merge
HCT-116
Vector
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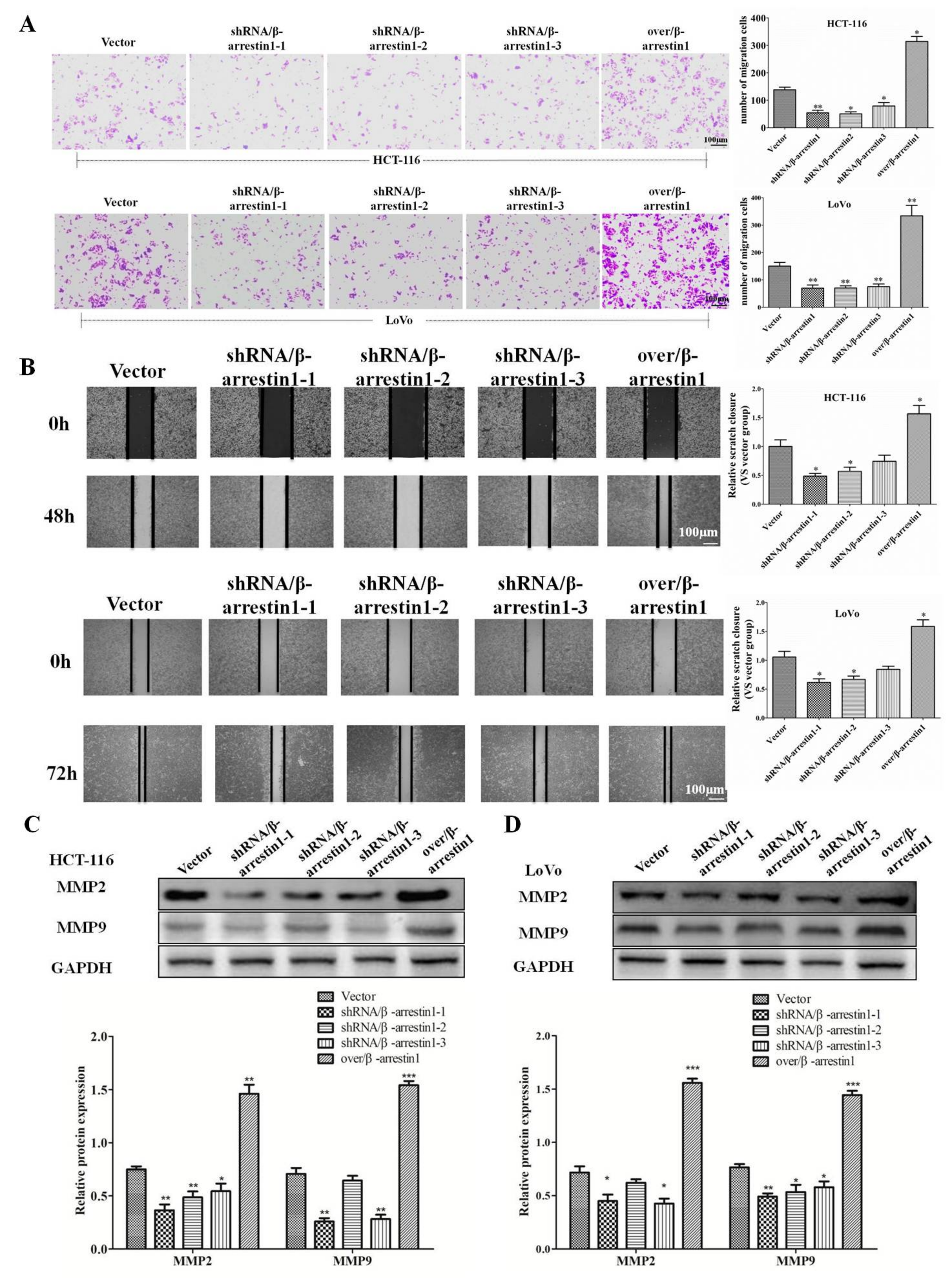
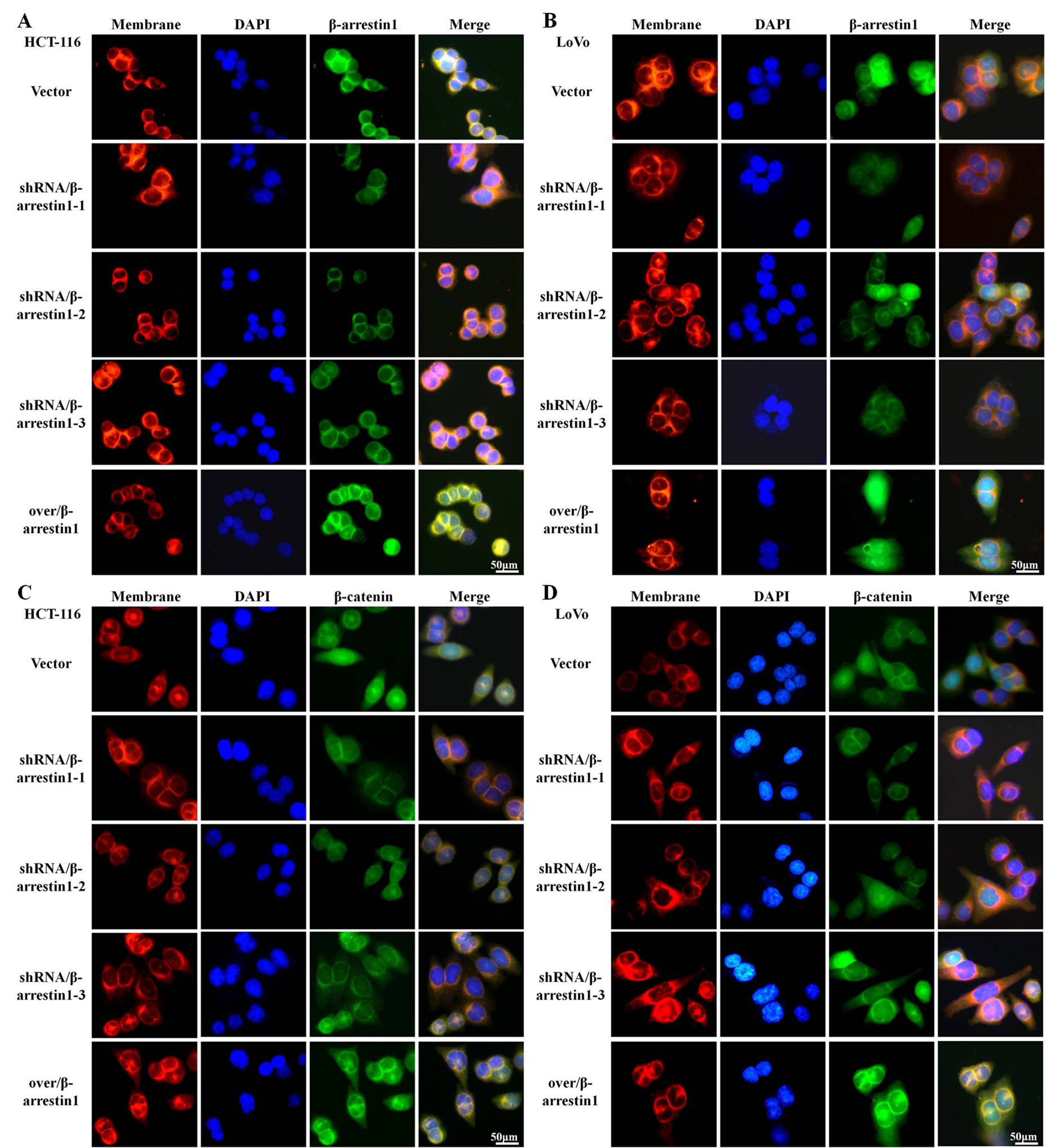
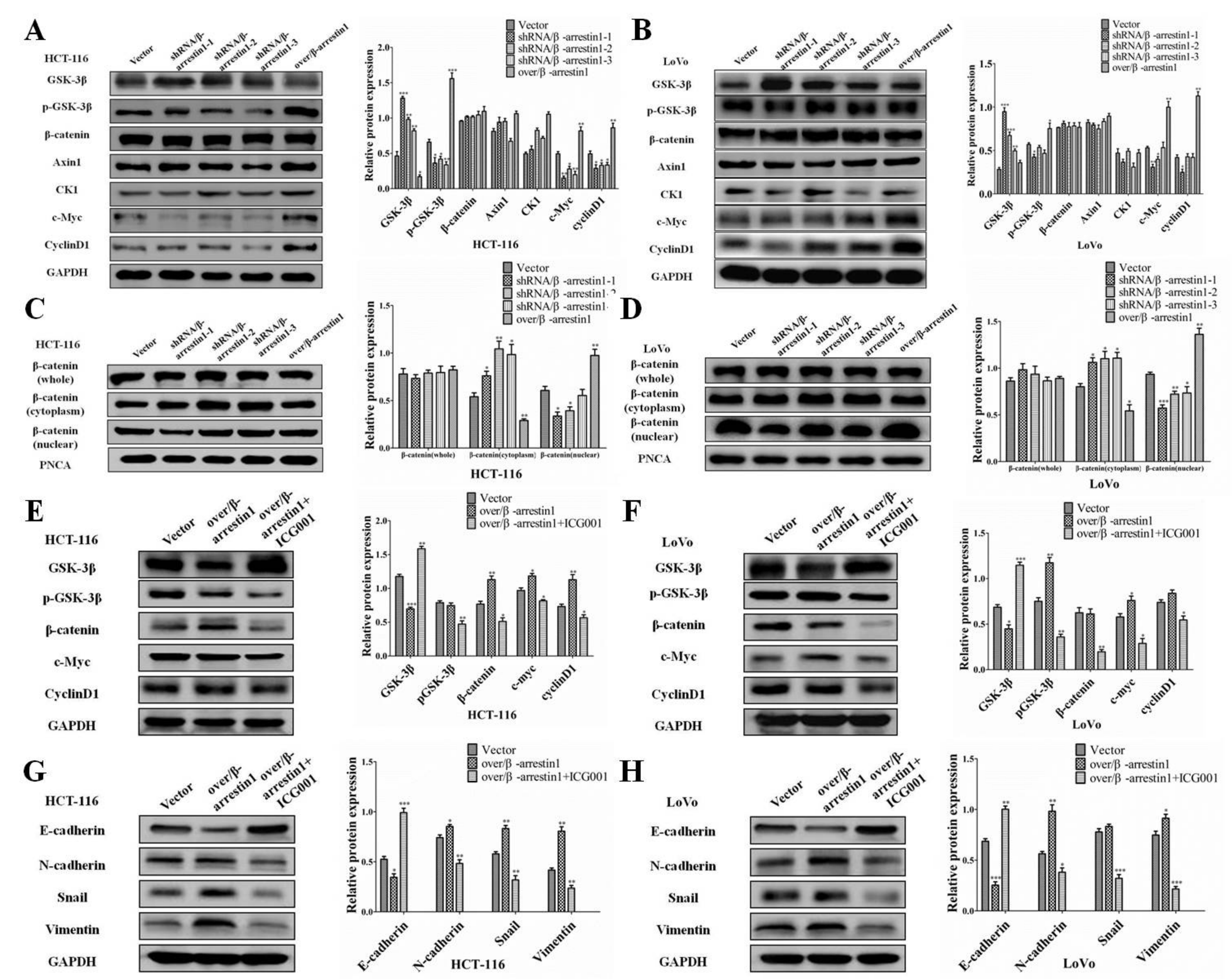
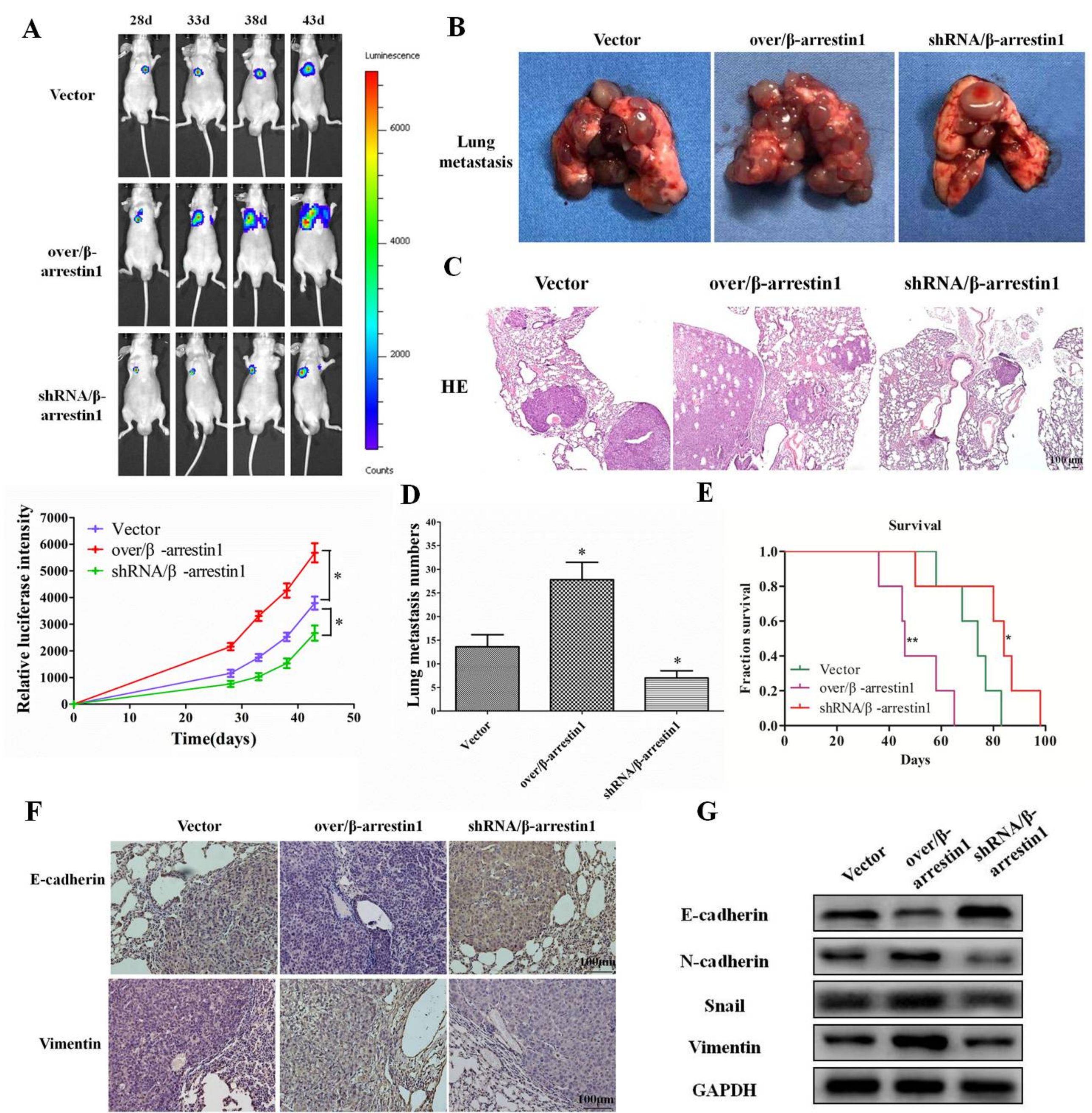
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm
B
DAPI
E-cadherin
Merge
LoVo
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm
C
DAPI
Vimentin
Merge
HCT-116
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm
D
DAPI
Vimentin
Merge
LoVo
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm


A
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
100μm
HCT-116
LoVo
number of migration cells
B
0h
48h
72h
Relative scratch closure (VS vector group)
C
D
MMP2
MMP9
GAPDH
shRNA/β -arrestin1-1
shRNA/β -arrestin1-2
shRNA/β -arrestin1-3
over/β -arrestin1
Relative protein expression


A
Membrane
DAPI
β-arrestin1
Merge
HCT-116
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm
B
Membrane
DAPI
β-arrestin1
Merge
LoVo
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm
C
Membrane
DAPI
β-catenin
Merge
HCT-116
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm
D
Membrane
DAPI
β-catenin
Merge
LoVo
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
50μm


A
HCT-116
Vector
shRNA/β-arrestin1-1
shRNA/β-arrestin1-2
shRNA/β-arrestin1-3
over/β-arrestin1
GSK-3β
p-GSK-3β
β-catenin
Axin1
CK1
c-Myc
CyclinD1
GAPDH
Relative protein expression
GSK-3β
p-GSK-3β
β-catenin
Axin1
CK1
c-Myc
cyclinD1
HCT-116
B
LoVo
LoVo
C
HCT-116
β-catenin (whole)
β-catenin (cytoplasm)
β-catenin (nuclear)
PNCA
β-catenin(whole)
β-catenin(cytoplasm)
β-catenin(nuclear)
HCT-116
D
LoVo
LoVo
E
HCT-116
over/β-arrestin1
over/β-arrestin1+ICG001
pGSK-3β
c-myc
HCT-116
F
LoVo
LoVo
G
HCT-116
E-cadherin
N-cadherin
Snail
Vimentin
HCT-116
H
LoVo
LoVo


A
28d
33d
38d
43d
Vector
over/β-arrestin1
shRNA/β-arrestin1
Luminescence
6000
4000
2000
Counts
B
Vector
over/β-arrestin1
shRNA/β-arrestin1
Lung metastasis
C
Vector
over/β-arrestin1
shRNA/β-arrestin1
HE
100 μm
D
Relative luciferase intensity
Vector
over/β -arrestin1
shRNA/β -arrestin1
Time(days)
E
Lung metastasis numbers
Vector
over/β-arrestin1
shRNA/β-arrestin1
Survival
Fraction survival
Days
F
Vector
over/β-arrestin1
shRNA/β-arrestin1
E-cadherin
Vimentin
100μm
G
Vector
over/β-arrestin1
shRNA/β-arrestin1
E-cadherin
N-cadherin
Snail
Vimentin
GAPDH