IGF- I induced growth and metatasis suppressed by calycosin via STAT3/ BATF2/ NF-κB /FOXM1 in colorectal cancer cells

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Abstract

To determine whether up-regulation of BATF2 by calycosin suppresses IGF- I induced growth and metastasis in human CRC,Cells were cultured and treated with calycosin and IGF-I. Protein and mRNA levels were determined by western-blot and real-time PCR respectively. Cell migration and invasion were assessed by transwell experiments. Apoptosis was measured by Flow cytometry. Cell proliferation was evaluated by the MTT assay.Co-immunoprecipitation and luciferase assays were used to analyze the interaction between BATF2 and MAT2Aand FOXM1.Cytoimmunofluorescence staining was applied for β-catenin and FOXM1 cellular localization.As results,Calycosin induced the up-regulation of BATF2 antagonized by IGF- I via STAT3, STAT1and NF-κB pathways resulting in cell apoptosis promotion via increasing BAX/Bcl-2 ratio subsequent contribute to caspase-3 and caspase-9 release. IGF- I induced cell migration,invasion and EMT suppressed by calycosin via BATF2 to block FOXM1 mediated β-catenin nuclear accumulation in three CRC cells. IGF- I induced cell proliferation was inhibited by calycosin down-regulating MAT2A and FOXM1. IGF-I and calycosin impacted MAT2A on transcriptional level. BATF2 interated with MAT2A and FOXM1 directly.We believed up-regulation of BATF2 may be a practicable treatment strategy for suppression IGF-1 induced growth and metatasis in CRC.

Introduction

Calycosin (C16H12O5) inhibited IGF- IR resulting in apoptosis in CRC[1],but IGF- IR blockade was also discovered to stimulate tumor angiogenesis and promote metastasis [2]. IGF /IGF-IR signaling activiated IGF-I by is highly active in CRC, contributing to the activation of multiple pathways that promote the aggressiveness of tumor phenotype.The effect of calycosin mading on IGF-I induced malignant phenotypes needs to be clarified .Our current data firstly demonstrated that calycosin blocked IGF-I induced migration ,invasion and EMT via BATF2 to repress FOXM1 mediated β-catenin nuclear translocation. BATF2 induced by calycosin via STAT3 pathway overcame IGF- I induced cell growth promotion and apoptosis inhibition through targeting on FOXM1 and increased BAX/Bcl-2 ratio in three CRC cells. Our previous study uncovered an unreported role of calycosin in repressing β-catenin nuclear accumulation as a result of cell migration and EMT suppression in CRC[3], but the mechanism underlying remained to be revealed. High expression of FOXM1 was proved to facilitate to metastasis of CRC via interacting with β-catenin nuclear accumulation in CRC[4]. FOXM1 was previously confirmed a downstream target of STAT signaling[5]. IGF-1is a known activator of the STAT3 pathway and promotes liver metastasis of CRC [6]. Over-expression of IGF- I leads to EMT, increases migration,by activting β-catenin, the mechanism was not documented completely. IGF- I induced invasion could be blocked by knockdowning FOXM1 [7],which carries NF-κB and AP-1sites in the promoter [8][9] can be targeted by BATF2 [10] .We have firstly induced BATF2 up-regulation with calycosin successfully in CRC [3].Therefore, we speculated that up-regulation BATF2 induced by calycosin could suppress IGF- I induced invasion, migration and EMT by suppression FOXM1 interacting with β-catenin nuclear accumulation via JAK/STAT and NF-κB sailings.

IGF-I was also reported to up-regulae MAT2A ,an essential regulators of growth in CRC via binding promoter NF-κB and AP-1 sites [11]resulting in cell proliferation promotion and apoptosis inhibition [12].but BATF2 was confirmed to inhibit AP-1-mediated gene expression effectively [13],As a result, a controversial regulation existed between BATF2 and MAT2A.IGF-I should up-regulate NF-κB subsequently to promote MAT2A expression ,conversely,BATF2 up-regulation inhibits AP-1 activity subsequent contributes to MAT2A repression. We wander which is dominant between NF-κB and AP-1 sites in the MAT2A promoter under the condition of calycosin and IGF-I.

In order to investigate the effect of BATF2 made on CRC cell growth, apoptosis, migration,invasion and EMT in the tumor environment of calycosin and IGF-I. we determined expressions and their interaction between FOXM1 and BATF2 as well as STAT3, STAT1 and NF-κB sailings.

Results

Calycosin suppressed IGF-I induced cell proliferation promotion in three CRC cells

To determine cell proliferation under IGF-I and calycosin, human colorectal cancer cells SW480,HCT116 and LoVo were incubated with 100μM calycosin and 100ng/ml IGF-I alone and together. Cell proliferation was assayed by MTT and growth curves were generated,Cell proliferation suppressed by calycosin and promoted by IGF-I and calycosin reversed IGF-I induced proliferation promotion successfully over 12 hour compared to control group ($p < 0.05$)(Fig. 1)

Calycosin reversed IGF-I induced apoptosis suppression via increasing BAX/Bcl-2 ratio in three CRC cells

To assess the effect on IGF-I induced cell apoptosis made by calycosin in three CRC cells.After HCT116, LoVo and SW480 cells were cultured with 100μM calycosin and100ng/ml IGF-I respectively and simultaneously, Flow cytometry stained with annexin V and PI was performed to determined cell apoptosis rate. In all three CRC cells ,apoptosis was promoted by calycosin and inhibited by IGF-I (Fig. 2), IGF-I induced cell apoptosis suppression was reversed by calycosin which was invovled in BAX and Bcl-2 expressions (Fig. 3, Fig. 7).Expressions of apoptosis related proteins BAX,Bcl-2,caspase-3 and caspase-9 were determined by western blot analysis.The levels of BAX, caspase-3 and caspase-9 were increased by calycosin and decreased by IGF-I, in contrast, the expression of Bcl-2 was just the opposite ,as a result, BAX/Bcl-2 ratio was enhanced (Fig. 3, Fig. 7). siBATF2 was found to increase BAX exprression. And siFOXM1 can also reverse IGF-I induced changes of Bcl-2,caspase-3 and caspase-9 expression consisitant with calycosin (Fig. 7)

MAT2A was regulated by calycosin, IGF-1and BATF2 at different level

To figure out interation between BATF2 and MAT2A, we determined mRNA and protein levels of BATF2 and MAT2A whose expressions were confirmed associated with cell proliferation and apoptosis in human CRC cells. After HCT116, LoVo and SW480 cells were treated as mentioned before, it was shown in Fig. 3 that mRNA of BATF2 was increased by calycosin and decreased by IGF-I significantly ($p < 0.05$), conversely, mRNA of MAT2A was increased significantly by IGF-I and decreased by calycosin in all three CRC cells, Up-regulation of MAT2A mRNA induced by IGF-1 was blocked by calycosin significantly ($p < 0.05$).Down-regulation BATF2 mRNA induced by IGF-1 was also rescued by calycosin significantly ($p < 0.05$) (Fig. 3). Co-IP assay was performed to determine the interation between BATF2 and MAT2A under the condition of IGF-I and calycosin,as a result,BATF2 interates with MAT2A directly. Although there was no difference found in MAT2A protein after treatment with IGF-I and calycosin ($p > 0.05$), siBATF2 up-regulated MAT2A protein in all three CRC cells(Fig. 4).

Calycosin reversed IGF-1-induced protein changes related to BATF2

To clarify the effects of IGF-I and calycosin on BATF2 and genes related to BATF2 expressions which also play important roles in signal pathway of cell proliferation and apoptosis. JAK/ STAT signaling and NF-κB(p65) was confirmed to be upstream and downstream signal pathway of BATF2 respectively, therefore, phosphorylated STAT3 (p-STAT3), phosphorylated STAT1 (p-STAT1), phosphorylated p65 (p-p65) were determined by western-blot under the condition of 100μM calycosin and100ng/ml IGF-I respectively and simultanously. MAT2A,BAX and FOXM1 which may be target downstream of BATF2 and related to cell proliferation and apoptosis were also determined.As results,expressions of p-STAT3,p-STAT1, p-p65 and FOXM1 were increased by IGF-I and decreased by calycosin($p < 0.05$) (Fig. 4), conversely, expressions of BATF2 and BAX were down-regulated by IGF-I and up-regulated by calycosin($p < 0.05$). Calycosin reversed IGF-I 's effect on expressions of p-STAT3,p-STAT1, p-p65, FOXM1, BATF2 and BAX ($p < 0.05$) (Fig. 4); siBATF2 successfully suppressed BAX and significantly promoted p-STAT3,p-STAT1, p-p65, FOXM1,and MAT2A expressions ($p < 0.05$) (Fig. 4).

FOXM1 was regulated by calycosin and IGF-1 mediated by BATF2 in an antagonistic manner

To demonstrate the regulation between BATF2 and FOXM1 at different level, we performed luciferase assays to determine wild-type and mutant FOXM1 promoter activity excited by pmirGLO-BATF2 in LoVo and HCT116 cells. After treatment with calycosin, IGF-1 and both of them, three CRC cells were collected and transfected with the plasmid constructs, and no significant fluorescence was observed in the blank control (pmirGLO-Basic) and mutant control plasmid (pmirGLO-FOXM1-MT). Compared to pGL3-Basic and pmirGLO-FOXM1-MT plasmids, significant fluorescence in the pmirGLO-FOXM1-WT plasmid was observed ($p < 0.05$) after the pmirGLO-BATF2 reporter construct was added to the cells. It is revealed by Luciferase assays in control group that direct effect made by BATF2 on FOXM1 transcriptionally. Wild-type FOXM1 promoter activity was significantly down-regulated by calycosin ($p < 0.05$) and up-regulated by IGF- I via BATF2 ($p < 0.05$) (Fig. 5C and D). It was demonstrated that promotion of IGF- I induced

FOXM1 promoter activity was blocked by calycosin mediated by BATF2 (Fig. 5C and D); ($p < 0.05$) The interaction of BATF2 and FOXM1 was identified by Co-IP. IGF-I and calycosin enhanced the effect BATF2 made on FOXM1 alone (Fig. 5A and B).But the interaction was attenuated after calycosin and IGF- I were added together (Fig. 5A and B).

FOXM1 mediated IGF-I induced cell migration and invasion in three CRC cells and reversed by calycosin

To explore the impact on IGF-I induced-cell migration and invasion made by calycosin and FOXM1, cell migration and invasion were assayed with transwell experiments in human invasive CRC cells HCT116 and LoVo, which were transfected with siFOXM1. SW480, which was low invasive ,was transfected with pFOXM1 to enhance migration and invasion ability, Three cells were incubetd with 100μM calycosin and100ng/ml IGF-I for 48hour.Transferred cell was counted. Migrations and invasion of Three cells were promoted by IGF-I and inhibited by calycosin ($p < 0.05$). Calycosin reversed IGF-I induced cell migration and invasion in three CRC cells($p < 0.05$) (Fig. 6A,B and C). siFOXM1 decreased migrated and invaded cell count and reversed IGF-I induced-cell migration and invasion in HCT116 and LoVo ($p < 0.05$). Calycosin hightened the inhibition effect of siFOXM1 on migration and invasion. IGF-I alleviated inhibition effect of siFOXM1 and calycosin in HCT116 and LoVo (Fig. 6A).The migration and invasion of SW480 were promoted by pFOXM1 and IGF-I. IGF-I enhanced the promotion effect of pFOXM1 in SW480. Calycosin alleviated promotion of IGF-I combination with pFOXM1 in SW480 (Fig. 6B and C).

Calycosin blocked IGF-1 induced EMT mediated by FOXM1

To investigate the role of calycosin in IGF-1-induced EMT and the underlying mechanisms, western blot was used to detect Vimentin, SNAIL and N-Cadherin, which were regarded as promoting regulators of the EMT process in CRC. The human highly invasive CRC cells LoVo and HCT116,as well as low invasive CRC cell SW480,were collected and cultured with PBS (control), 100μM calycosin, 100ng/ml IGF-1, calycosin combination with IGF-I, siFOM1combination with IGF-I for 48h. The expressions of Vimentin, SNAIL, N-Cadherin were significantly promoted by IGF-1($p < 0.05$) and suppressed by calycosin ($p < 0.05$) in three cells(Fig. 6). Up-regulation of Vimentin, SNAIL and N-Cadherin induced by IGF-1 was blocked by calycosin and siFOXM1 in three cells (Fig. 6). E-Cadherin ,a suppressing regulator of EMT, was decreased by IGF- I and increased by calycosin. calycosin and siFOXM1 reversed down-regulating of E-Cadherin due to IGF- I in all three CRC cells($p < 0.05$).

IGF-1 induced β-catenin and FOXM1 nuclear localization was impeded by calycosin

To confirm the roles played by β-catenin and FOXM1 in human highly invasive CRC cells LoVo and HCT116. Cytoimmunofluorescence staining and western blot were performed to demonstrate FOXM1 subcelluar localization and distribution. β-catenin and FOXM1 expressions in cytoblast, cytoplasm and cytomembrane were determined by western blot.Two CRC cells were collected and cultured with PBS (control), 100μM calycosin, 100ng/ml IGF- I, calycosin combination with IGF- I for 48h,then stained with Anti- FOXM1. Compared to control,IGF- I promoted FOXM1 nuclear accumulation and calycosin reduced it

significantly, calycosin impeded IGF- I induced FOXM1 nuclear translocation (Fig. 8A). IGF- I promoted distributions of β-catenin and FOXM1 from cytoplasm to cytoblast (Fig. 8B and C). Calycosin reversed IGF- I induced nuclear importing of β-catenin and FOXM1 simultaneously (Fig. 8B and C).No significant difference was found in Cytomembrane expression between groups ($p > 0.05$) (Fig. 8B and C).

Calycosin blunted IGF-1 induced β-catenin nucleus importing via FOXM1

To identify the relationship between β-catenin and FOXM1 in CRC cells, we investigated the effect of altered FOXM1 expression on β-catenin subcelluar localization and distribution.HCT116 and LoVo cells were heavily seeded, FOXM1 and β-catenin subcelluar localization were analyzed by Cytoimmuno-fluorescence staining with different FOXM1 expression. Subcelluar distribution of β-catenin was determined by western-blot in the cytoplasm and cyteblast respectively. Interestingly, β-catenin subcelluar localization was synchronous with FOXM1, β-catenin and FOXM1nuclear localization was clearly repressed by siFOXM1(Fig. 9A and B), IGF- I alleviated the repression due to siFOXM1, calycosin suppressed nucleus accumulation of β-catenin caused by pFOXM1.Calycosin reversed IGF- I induced nucleus importing of β-catenin via FOXM1 in both HCT116 and LoVo (Fig. 9A and B). Subcelluar distributions of β-catenin between cyteblast and cytoplasm were determined by western-blot. Cyteblast β-catenin level were decreased and cytoplasm level increased by siFOXM1 correspondingly ($p < 0.05$) (Fig. 9C) and siFOXM1 transferred β-catenin from cyteblast to cytoplasm (Fig. 9C), IGF-1 relieved β-catenin cytoplasm accumulation due to siFOXM1, calycosin blunted nuclear translocation of β-catenin via FOXM1.

Discussions

In our previous study, BATF2 induced by calycosin played an important role in TGF-β induced- migration and EMT suppression.BATF2 was reported to regulate numerous cellular processes [14] including growth inhibition and [15] apoptosis promotion [16][17]. However, its role in EMT of CRC is unclear.Here,for the first time, we discovered that calycosin induced BATF2 up-expression and inhibited STAT3, STAT1 and NF-κB pathways, conversely,siBATF2 promoted their up-expressions,then, negative feedback regulations were found between BATF2 and the sailings in all three CRC cells. Calycosin reversed IGF-I induced cell proliferation and apoptosis inhibition via FOXM1 and increased BAX/Bcl-2 ratio subsequent contribute to caspase-3 and caspase-9 release and The present research demonstrated that IGF- I induced cell migration, invasion and EMT were inhibited by BATF2 interating with FOXM1 to meditate subcelluar translocation of β-catenin.

MAT2A was believed to be an essential regulators of growth in CRC[13]. In present study, IGF-I and calycosin regulated MAT2A expression at the transcriptional level and calycosin reversed IGF-I induced cell proliferation promotion and apoptosis inhibition in HCT116, LoVo and SW480. Our results of IGF-I activiating MAT2A were in line with with previous findings [5][13]. But MAT2A was not regulated by IGF-I and calycosin at protein level.The reason need further investigation. We have a conclusion that the

MAT2A protein regulation by IGF-I and calycosin was more than dependent on BATF2.Our conclusion based on the fact that we confirmed BATF2 interation with MAT2A under the conditions of IGF-I and calycosin by Co-IP assay ,furthermore, MAT2A protein was up-regulated by siBATF2 signifcantly in all CRC cells.NF-κB(p65) and AP-1 site in MAT2A promotor can be suppressed by BATF2 in CRC,as a result, BATF2 inhibition activiates NF-κB(p65) and AP-1subsequent contributes to MAT2A promotion.

Just recently, the joint action of NF-κB, STAT3, and AP-1 factors involved in many human cancers except for CRC was confirmed to form complexes of STAT3 and NF-κB interaction with AP-1 factors that binding to target gene[18] .But the mechanism underlying remained clarified,we speculated BATF2, a negative regulator of AP-1 activity and AP-1-mediated gene in tumor [11] [13], may be a potential target genes

of the joint action of NF-κB, STAT3, and AP-1 factors in CRC cells. The NF-κB family of transcription factors, consisting of RelA, RelB, c-Rel, p50, and p52, is fundamentally involved in inflammation and interaction of STAT3 and RelB was found active in colon cancer [19],but the p-RelA (p-p65) can physiologically interact with p-STAT3 and their association can modify their transcriptional activity with the most common mechanism of activating cytokines and chemokines and growth factors, which in turn activate STAT3 in stromal cells [20].We demonstrated growth factor,IGF-1 activated NF-κB (p65), STAT3 besides STAT1 signalings resulted in down-regulating in BATF2 and reversed by calycosin. And it was clarified in present data that BATF2 was down- and up-regulated by IGF-1 and calycosin corresponding to NF-κB (p65), STAT3 and STAT1sailings activated and inactivated. Decreased BATF2 by us with small RNA interference also activated the three signaling pathway molecules of p-STAT3, p-STAT1 and p-p65. BATF2 was confirmed to be a direct downstream target of STAT3 [21] and upstream of NF-κB (p65) [11] [22]and was also discovered to promote STAT1 instead of STAT3 and NF-κB (p65) degradation [23]. Thereore, negative feedback regulations were demonstrated between BATF2 and the sailings, a STAT3/ BATF2/ STAT1(NF-κB) axis was found in all three CRC cells. In addition, it has been believed that simultaneous rather than single inhibition of STAT3 and NF-κB (p65) activiation was effective strategy for combat CRC[24]. BATF2 may be a feasible candidate target of the joint action to repress STAT3 and NF-κB (p65) simultaneously .

FOXM1 is a common forkhead box transcription factor and master regulator of cancer tumorigenesis and metastasis [25]. Indeed, FOXM1is only expression in proliferating cells,over-expression of FOXM1 is believed to be associated with worse survival of patients[26]. IGF-I inhibited cell apoptosis by decreasing BAX/Bcl-2 ratio and caspase-3 and caspase-9 expressions ,but IGF-I induced apoptosis inhibition was bolcked by calycosin and siFOXM1which both contributed to FOXM1 repression. We confirmed FOXM1 interated with BATF2 directly by Co-IP and Luciferase assays, in addition, siBATF2 up-regulated FOXM1 significantly in all three CRC cells. As a result, we identified that IGF-I induced cell proliferation and apoptosis inhibition were reversed by calycosin via up-regulating BATF2 to down-regulate FOXM1 and increase BAX/Bcl-2 ratio subsequent contribute to caspase-3 and caspase-9 release in three CRC cells.

FOXM1 regarded as an oncogene could be activated by oncogenic Sonic Hedgehog[27], STAT1[28],Ras-MAPK, NF-κB and EGFR pathways [29],especially, NF-κB(p65) was demonstrated to regulate FOXM1 at three layers[30]. As mentioned earlier, the joint action of NF-κB, STAT3, and AP-1 factors takes active in tumor cells ,it is not surprise for us to discovered that FOXM1 was regulated by calycosin and IGF-1via STAT3 and NF-κB sailings mediated by BATF2. In the current study, p-p65 was promoted by siBATF2 accompanied with FOXM1 upregulation in three CRC cells and the regulation of FOXM1 resulted from BATF2 was itdentified by us, we conclude that up-regulation of BATF2 by calycosin via STAT3 subsequent contributes to FOXM1 suppression via NF-κB and STAT1 pathways in three CRC cells, IGF-I is antagonistic to calycosin in STAT3/ BATF2/ NF-κB (STAT1)/FOXM1 axis. FOXM1 has been confirmed to be involved in malignant phenotypes in CRC [8] and it mediated cell migration, invasion and EMT by interacting with β-catenin via Wnt pathway [32][33]; IGF- I mediated invasion could be blocked by knockdowning FOXM1 in breast cancer cells [7] In our research, IGF- I induced-cell migration, invasion and EMT was mediated by FOXM1 and could be reversed by calycosin and siFOXM1. Correspondingly pFOXM1 promoted cell migration, and invasion and hightened IGF- I induced-cell migration, and invasion which were blocked by calycosin. Besides,IGF-I induced nuclear accumulations of FOXM1 and β-catenin were also blunted by calycosin and we further to ascertained that it was FOXM1 mediated subcelluar localization and distribution of β-catenin ,FOXM1 was regulated by IGF-I and calycosin via BATF2 in CRC cells. It is worth noting that both MAT2A and FOXM1 carrying NF-κB and AP-1 sites in the promoter were both inhibited by BATF2,which was inactivaited by STAT3, consequently, BATF2 was identified to candidate target of joint action of NF-κB, STAT3, and AP-1 factors in CRC cells.

Methods

Cell culture

SW480,HCT116 and LoVo human CRC cell lines were obtained from Wuhan Health Care Biotechnology Company (Wuhan, China).The cells were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS)and incubated at 37°C with 5% CO_2 in a humidified atmosphere.

MTT cell viability assay

The effects of calycosin and IGF- I on CRC cells proliferation were evaluated by the MTT cell viability assay. Briefly, cells were plated into 96-well plates (5×10^3 cells) for 12 h, and then cultrred with 100 μM calycosin and 100ng/ml IGF-1alone and together,PBS was used as control. After 6, 12, 24,and 48h, 20 UL MTT solution (5 mg / ml) was added for 4hour,cell viability was analyzed at 490 nm by ELISA. The cells were counted and a growth curve was generated.

Flow cytometry

The cells were collected, washed with cold PBS, and suspended in 500 μl binding buffer. Then 5 μl annexin V-FITC and 5 μl propidium iodide (Keygen, Nanjing, China) were added and mixed with the cells. After the incubation at room temperature for 15 min in the dark, the cells were subjected to flow cytometry

analysis. The percentage of apoptotic cells was calculated as the sum of cells stained with both annexin Vand PI

Quantitative PCR

Total RNA was extracted with Trizol reagent (Ambion, USA), and first-strand cDNA was synthesized referring to manufacturer's protocol (TAKARA,Japanse). Quantitative PCR was performed in triplicate with SYBR Green PCR(KAPABiosystems, USA) according to the manufacturer's protocol, using GAPDH as a control. The relative mRNA levels of the target genes were calculated with the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Standard western blot was performed using whole-cell protein lysates, and primary antibodies BATF2, MAT2A, FOXM1(Abcam),phosphorylated STAT3(p-STAT3), phosphorylated STAT1(p-STAT1), phosphorylated p65 (p-p65),Bcl-2, BAX, caspase-3, caspase-9 and GAPDH (bioswamp, China), a secondary antibody (anti-rabbit IgG ;bioswamp, China). Equal protein-sample loading was monitored using an anti-GAPDH antibody.

Cell migration and invaison assays

For migration and invasion assays, 80μl Matrigel glue was laid before invasion assay ,cells were seeded into the upper chamber of a 8μM pore transwell, 1×10^5 cells of different treatment with 100ng/ml IGF-1 and 100 μM calycosin respectively and simultaneously ,HCT116 and LoVo cells were transfected with siFOXM1,SW480 was transfected with pFOXM1 then added with IGF-1 and calycosin respectively and simultaneously, and 0.75 ml of culture medium containing 10% FBS was added into the lower chamber for 48 hour,Migrated cells were fixed, stained, and counted from six random fields and averaged. The experiments were repeated three times.

Co-immunoprecipitation

For Co-immunoprecipitation (Co-IP) assay, HCT116 and LoVo cells were treated with phosphate-buffered saline (PBS) control, 100 μM calycosin, 100ng/ml IGF-1 and a mixture of 100ng/ml IGF-1 and 100 μM calycosin for 48 h, then following experimental procedure referred to our previous study[3]

Plasmids and small interfering RNAs

The pmirGLO and pmirGL3-Basic plasmids were obtained from bioswamp company,China. The reporter gene construct (pmirGLO-BATF2), mutant control constructs (pmirGLO-FOXM1-MT), wild control constructs (pmirGLO-FOXM1-WT) and pmirGL3-Basic plasmid encoding Renilla luciferase were constructed referred to earlier research [3]; The plasmids pcDNA-FOXM1 (p-FOXM1); Small interfering RNA (siRNA) plasmids: siRNA BATF2(siBATF2), siRNA FOXM1(siFOXM1) and pcDNA-FOXM1 (pFOXM1)were comstructed and identified by PCR and western blot. siRNA targeting sequences were as follows: FOXM1-F CTTCTGGACCATTCACCC ,FOXM1-R CTTCATCTTCCGCCGT;

BATF2-F GCACGAGTCTCTGGAAAA, BATF2-R TGAGCAGGAGGCACAAT.

Promoter reporter and dual luciferase assay

1×10^5 cells of HCT116 and LoVo per well in 6-well culture plates and transfected with Lipofectamine 2000 transfection reagent (Invitrogen) for 24 h. For luciferase assays, cells were transiently co-transfected with the reporter gene construct (pmirGLO-BATF2), mutant control constructs (pmirGLO-FOXM1-MT), wild control constructs (pmirGLO-FOXM1-WT) and pmirGL3-Basic plasmid encoding Renilla luciferase. Cells were incubated for 24 h and then mixed with PBS (control), 100Mm calycosin, 100mg/ml IGF-1, and calycosin mixed with IGF-1for 48 h. Then firefly andrenilla luciferase activities were assayed using the dual-luciferase reporter assay system (Promega).

Cytoimmunofluorescence staining

HCT116 and LoVo cells were cultured with phosphate-buffered saline PBS (control), 100 μM calycosin, 100ng/ml IGF- I, both 100ng/ml IGF-1 and 100 μM calycosin,transfected with siFOXM1, transfected with siFOXM1 then added IGF- I, transfected with pFOXM1 then added calycosin for 48 h. Cytoimmuno-fluorescence staining of β-catenin and FOXM1referred to previous research[3]

Conclusions

In total ,our study revealed for the first time that up-regulation of BATF2 by calycosin via STAT3 suppressed FOXM1 through STAT1 and NF-κB pathways in HCT116, LoVo and SW480 cells.BATF2 induced by calycosin overcomed IGF- I induced cell proliferation promotion and apoptosis inhibition through targeting on FOXM1 and increased BAX/Bcl-2 ratio subsequent contribute to caspase-3 and caspase-9 release, calycosin blocked IGF-I-induced migration, invasion and EMT via FOXM1 mediated β-catenin nuclear translocation suppression in LoVo and HCT116 cells. The results of this study suggest that up-regulation of BATF2 may be a therapeutic option for CRC.

Abbreviations

BATF2: Basic leucine zipper ATF-like transcription factor 2 ;GAPDH: Glyceraldehyde-3-phosphate dehydrogenase ;

MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide ;CRC: Colorectal cancer

PBS: Phosphate buffer saline;SD: Standard deviation ;Annexin V-FITC: Annexin V-Fluorescein isothiocyanate

PI: Propidium Iodide ;ANOVA: One-way analysis of variance ;EMT: epithelial-mesenchymal transition ;STAT3: signal transducer and activator of transcription 3 ;p-STAT3: phosphorylation signal transducer and activator of transcription 3;STAT1: signal transducer and activator of transcription 1;p-STAT1: phosphorylation signal transducer and activator of transcription1;p-p65: phosphorylation p65;BAX: Bcl-2-

Associated X ;FOXM1: Forkhead box protein M1;MAT2A: Methionineadenosyltransferase2A;IGF-1:insulin growth factor-1;NF-κB : nuclear factor kappa-B;AP-1: activator protein-1;IP: Co- immunoprecipitation

Declarations

Availability of data and materials

All data and materials are contained and described in the main paper.

Authors' contributions

QW and SZW conceived and designed the study.QW ,GLP and Lu Li carried out the experiment. QW, WJL , DDW analyzed the data. QW and SZW drafted and revised the manuscript.All authors read and approved the final manuscript.

Informed Consent Statement

Not applicable.

Consent for publication

All of the authors consent for publication in *International Journal of Molecular Sciences*

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Conflict of Interest Disclosure Statement

The authors have no conflicts of interest to declare.

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Figures

Figure1

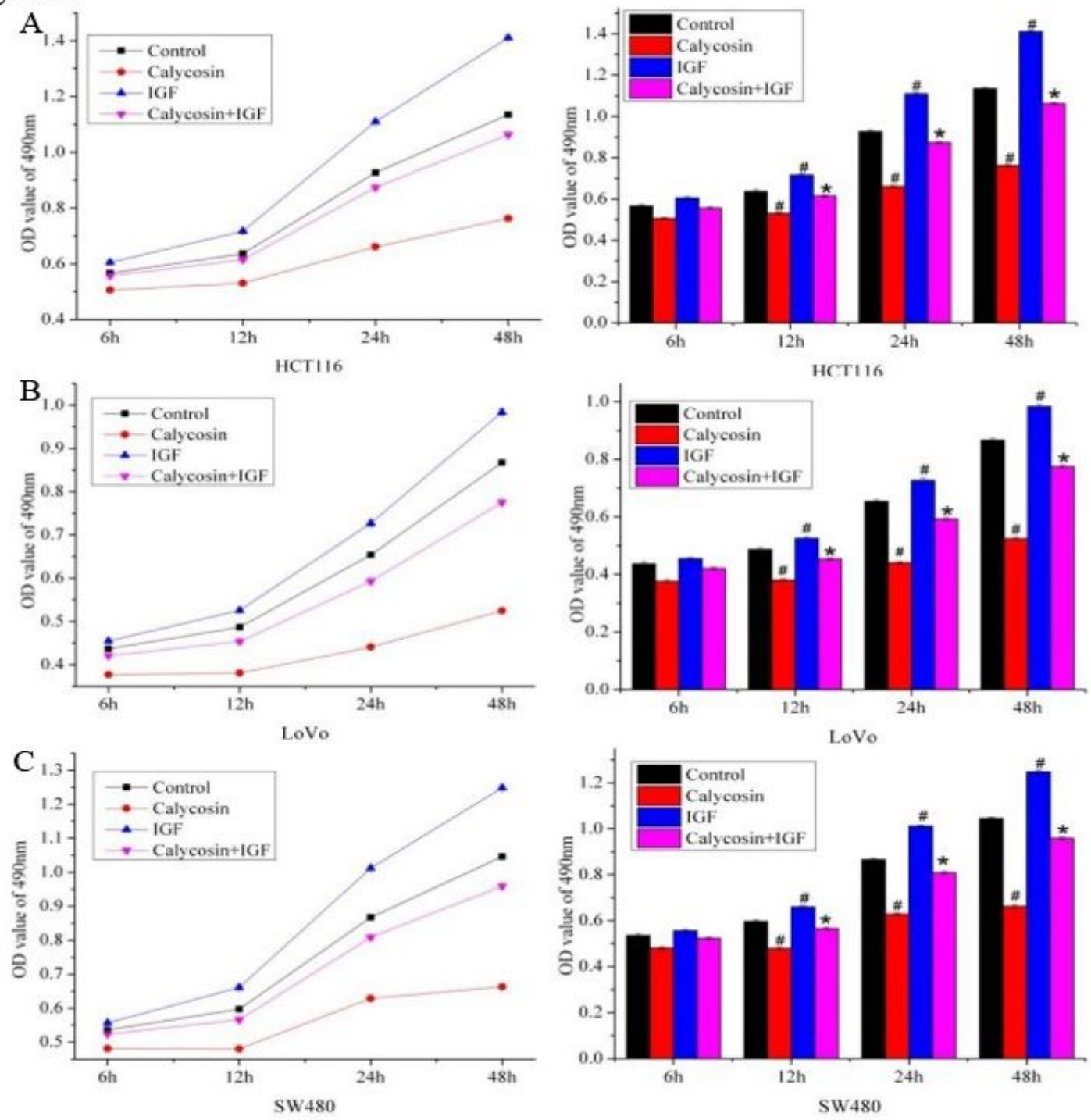

Figure 1

MTT was used to evaluate cell promotion, A,B and C demonstrate that IGF-1 induces proliferation of CRC HCT116, LoVo and SW480 cells,and cell proliferation promotion of HCT116,LoVo and SW480 cells were reversed by calycosin. (# mens VS control group $p<0.05$; * mens VS IGF group $p<0.05$;)

Figure2

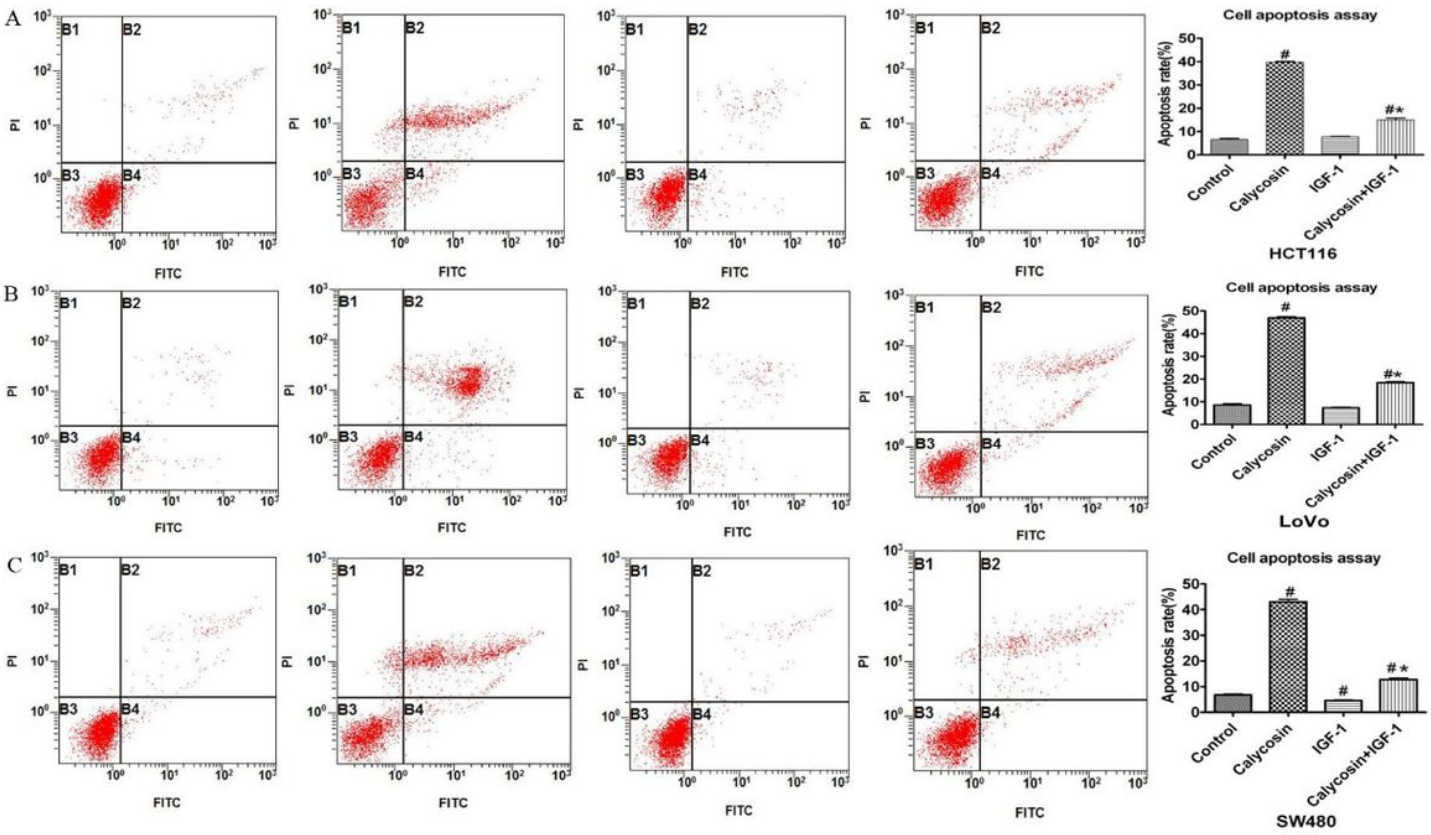

Figure 2

After HCT116, LoVo and SW480 cells were treated with PBS, 100 μM calycosin, 100ng/ml IGF-I and a mixture of 100ng/ml IGF-I and 100 μM calycosin for 48 h, stained with both annexin Vand PI,then,cell apoptosis were determined by Flow cytometry,the apoptosis rates were shown in A,B and C respectively. (# mens VS control group $p<0.05$; * mens VS IGF group $p<0.05$;)

Figure3

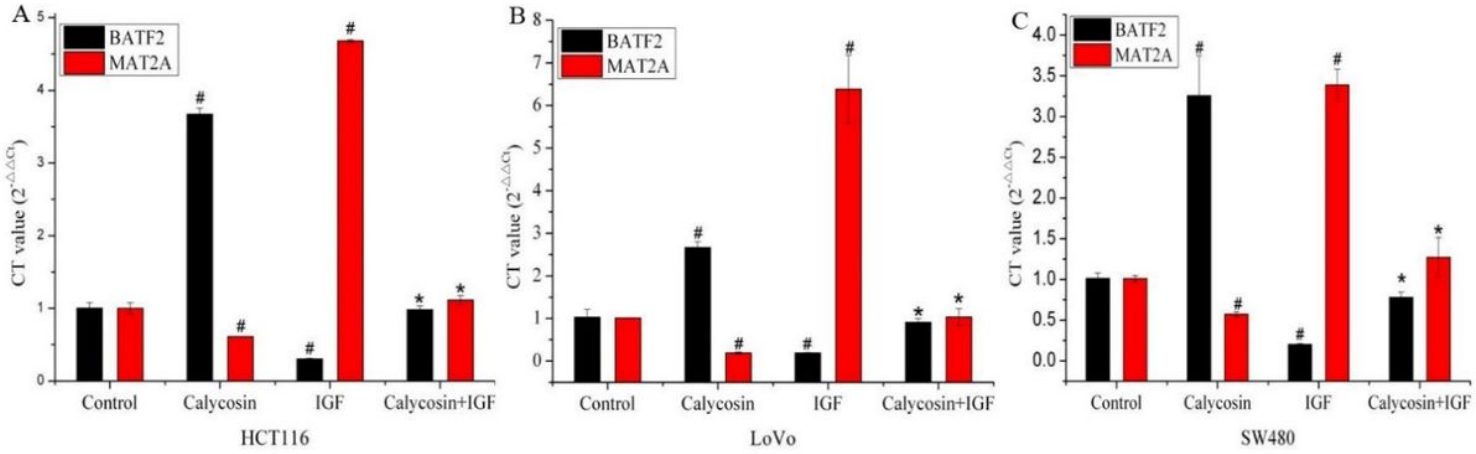

Figure 3

Real-time PCR was performed to determine mRNA of BATF2 and MAT2A under the contition of PBS, 100 μM calycosin, 100ng/ml IGF-I and a mixture of 100ng/ml IGF-1 and 100 μM calycosin for 48 h,the relative values of HCT116, LoVo and SW480 were calculated and demonstrated in A,B and C respectively.(# mens VS control group $p<0.05$; * mens VS IGF group $p<0.05$;)

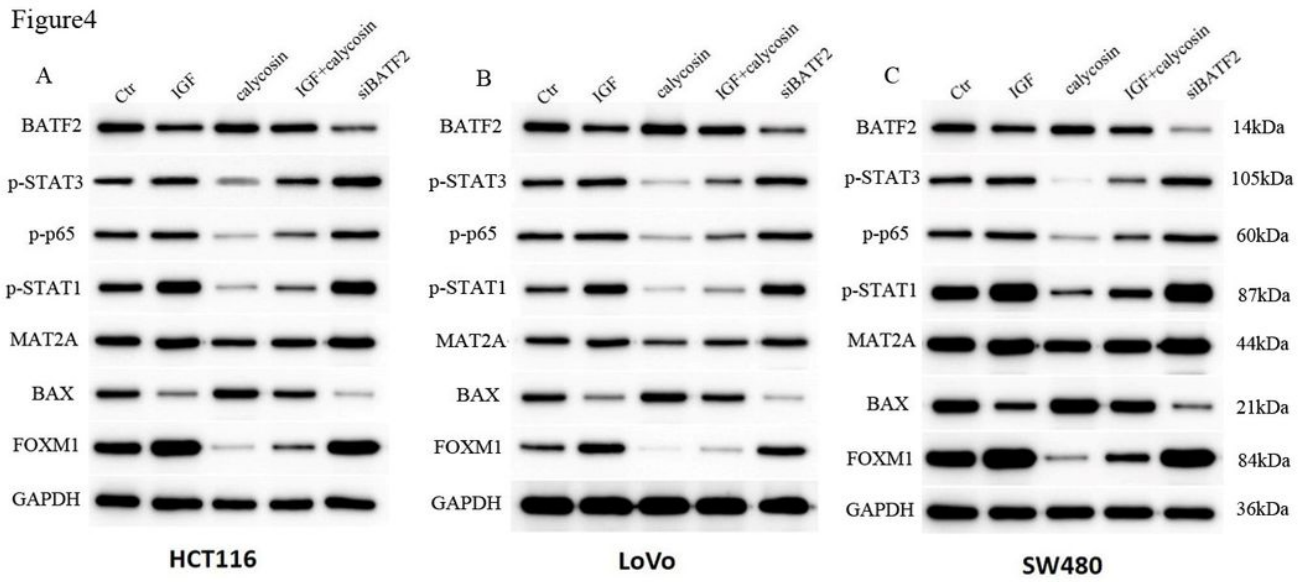

Figure 4

Proteins of BATF2, p-STAT3, p-p65,p-STAT1,MAT2A,BAX and FOXM1 were determined by Western-blot after HCT116, LoVo and SW480 cells treated with PBS, 100 μM calycosin, 100ng/ml IGF-I , mixture of calycosin and IGF-1 and siBATF2;The picture A,B and C display results of HCT116, LoVo and SW480 protein expression.

Figure5

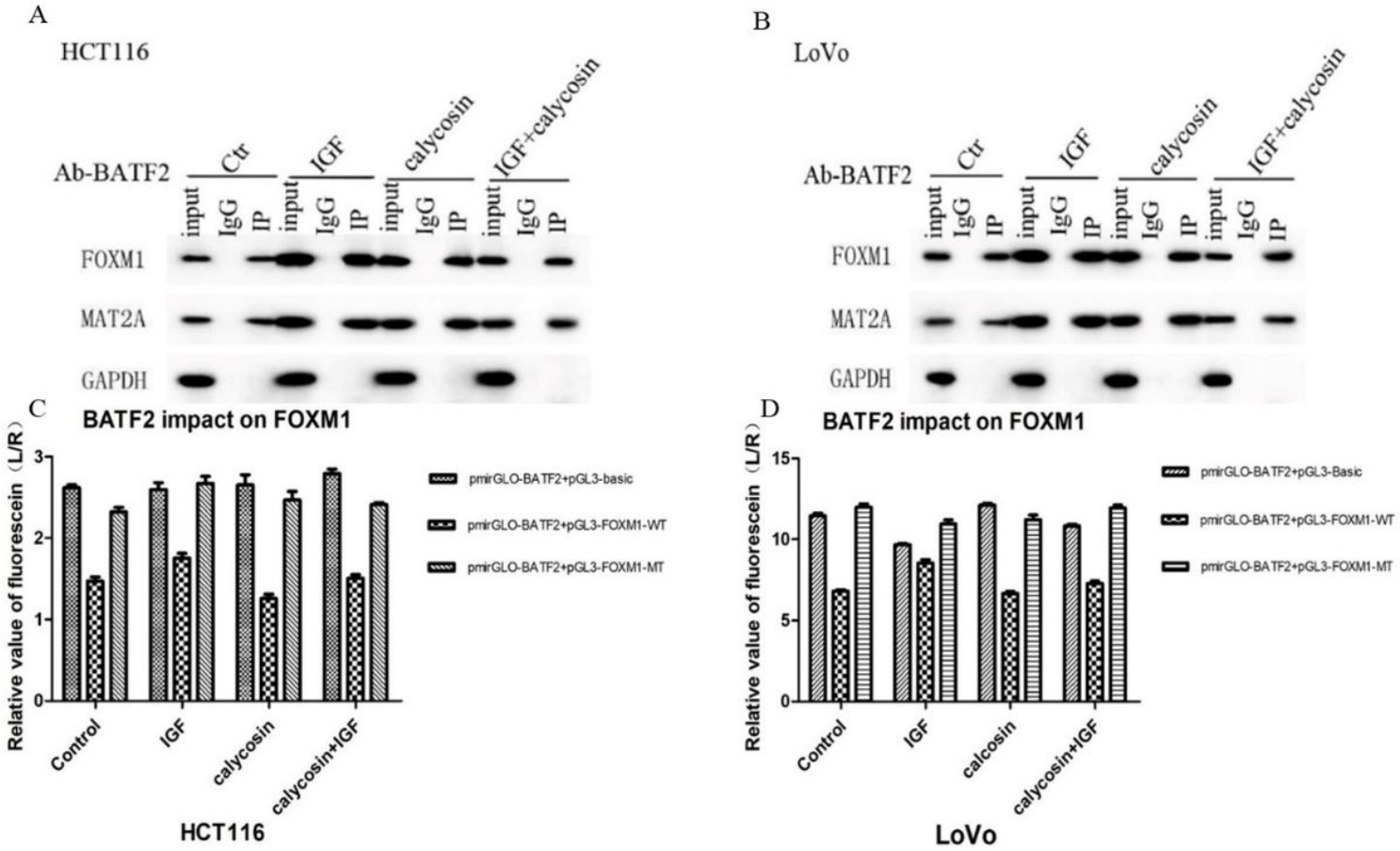

Figure 5

The interations of BATF2 and FOXM1, BATF2 and MAT2A were determined by assays Co-IP and dual-luciferase reporter systems in HCT116 and LoVo cells the circumstances of PBS, 100 μM calycosin, 100ng/ml IGF-I and a mixture of 100ng/ml IGF-I and 100 μM calycosin for 48 h. A and B show BATF2 interated with FOXM1 and MAT2A with and without IGF-I and calycosin ;C and D show pmirGLO-BATF2 impacted on Wild-type rather than mutant or null FOXM1 promoter significantly in HCT116 and LoVo cells with and without IGF-I and calycosin .

Figure6

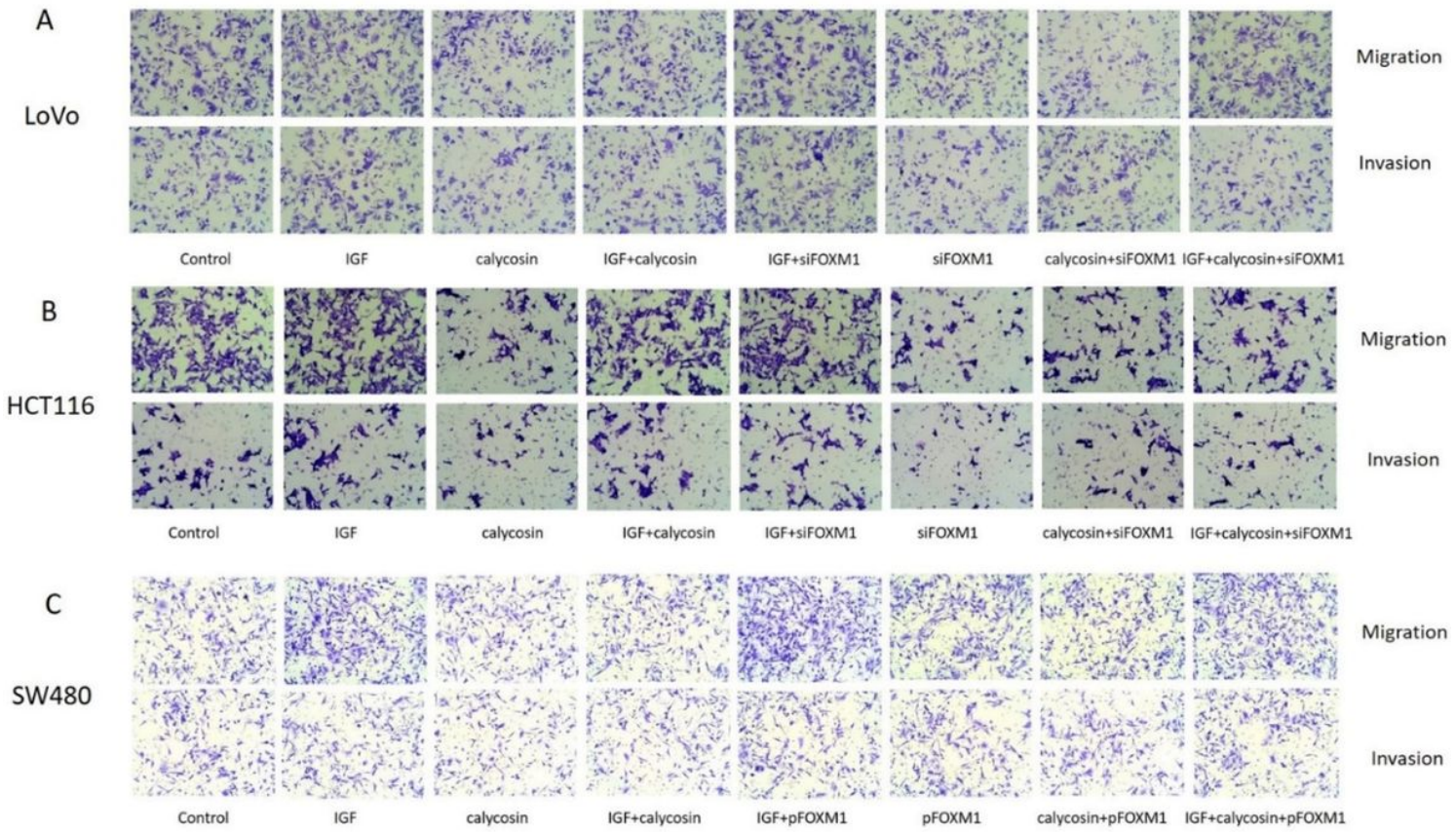

Figure 6

Cell migration and invasion were investigated by transwell experiments, A and B demonstrate HCT116 and LoVo cells were treated with PBS, 100 μM calycosin, 100ng/ml IGF-I , calycosin and IGF-I,siFOXM1 and IGF-I, siFOXM1, calycosin and siFOXM1, calycosin and siFOXM1 and IGF-I;C demonstrate SW480 was treated with PBS, 100 μM calycosin, 100ng/ml IGF-I , calycosin and IGF-I,pFOXM1 and IGF-I, pFOXM1, calycosin and pFOXM1, calycosin and pFOXM1and IGF-I. Migrated cells were fixed, stained, and counted from six random fields and averaged.

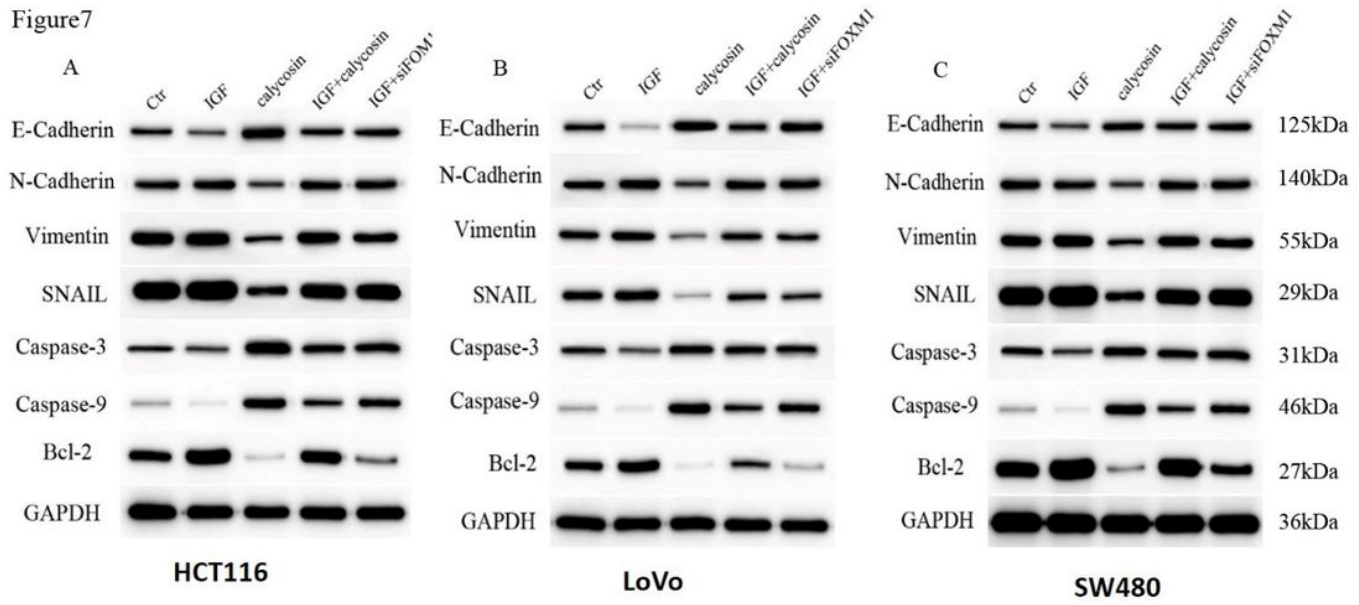

Figure 7

A,B and C display that EMT and cell apoptosis related proteins: E-Cadherin, N-Cadherin, Vimentin, SNAIL, caspase-3 caspase-9 and Bcl-2 were determined by Western-blot after HCT116, LoVo and SW480 cells treated with PBS, 100 μM calycosin, 100ng/ml IGF-I , calycosin and IGF-I, siFOXM1 and IGF-I.

Figure8

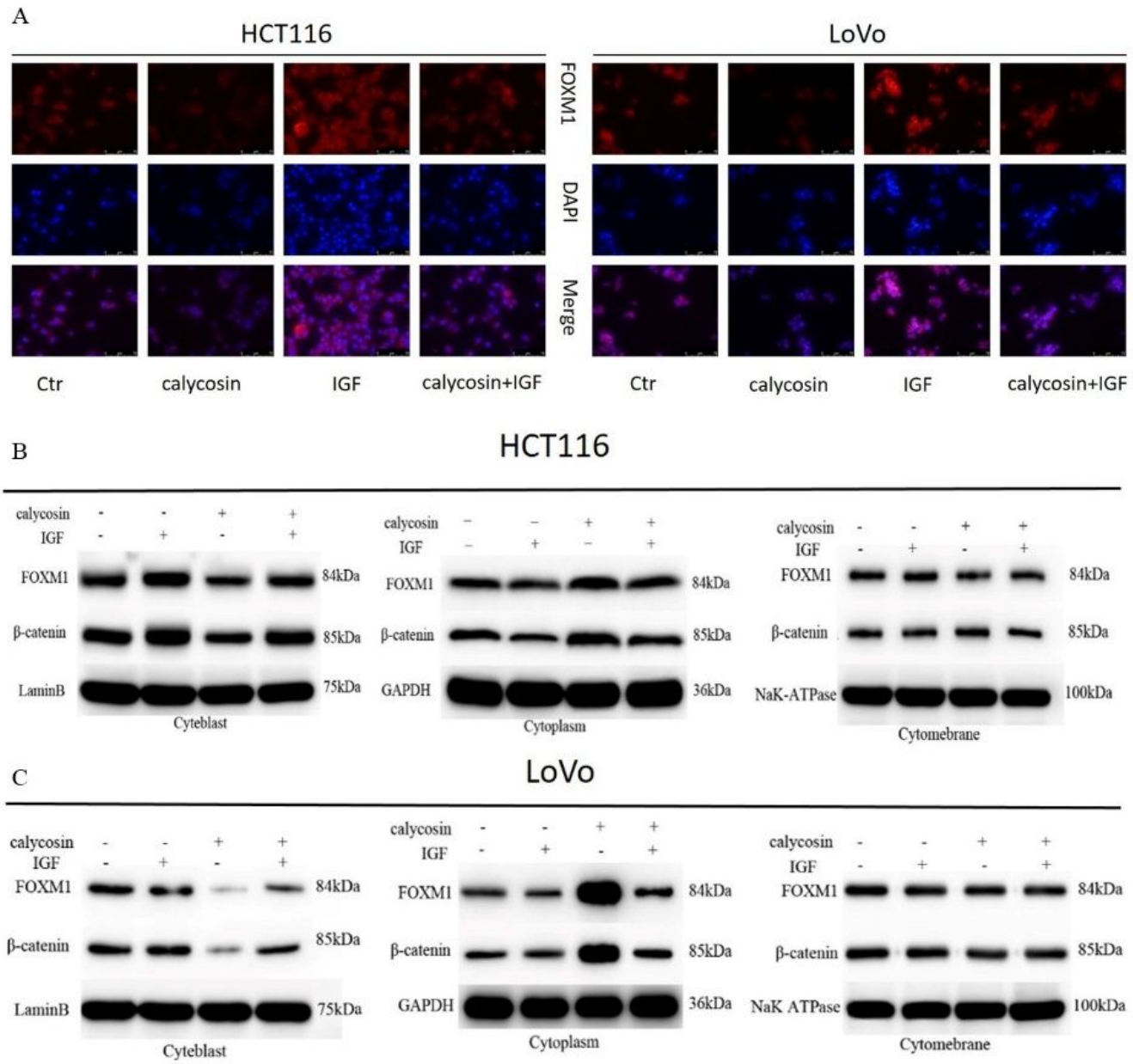

Figure 8

A show subcelluar localization of FOXM1 was demonstrated by cytoimmunofluorescence stainings ;B and C dislpay respectively cytoblast, cytoplasm and cytomembrane distribution of β-catenin and FOXM1 protein that were assessed by Western-blotting after HCT116 and LoVo cells were treated with PBS, 100 μM calycosin, 100ng/ml IGF-I , calycosin and IGF-I simultaneously.

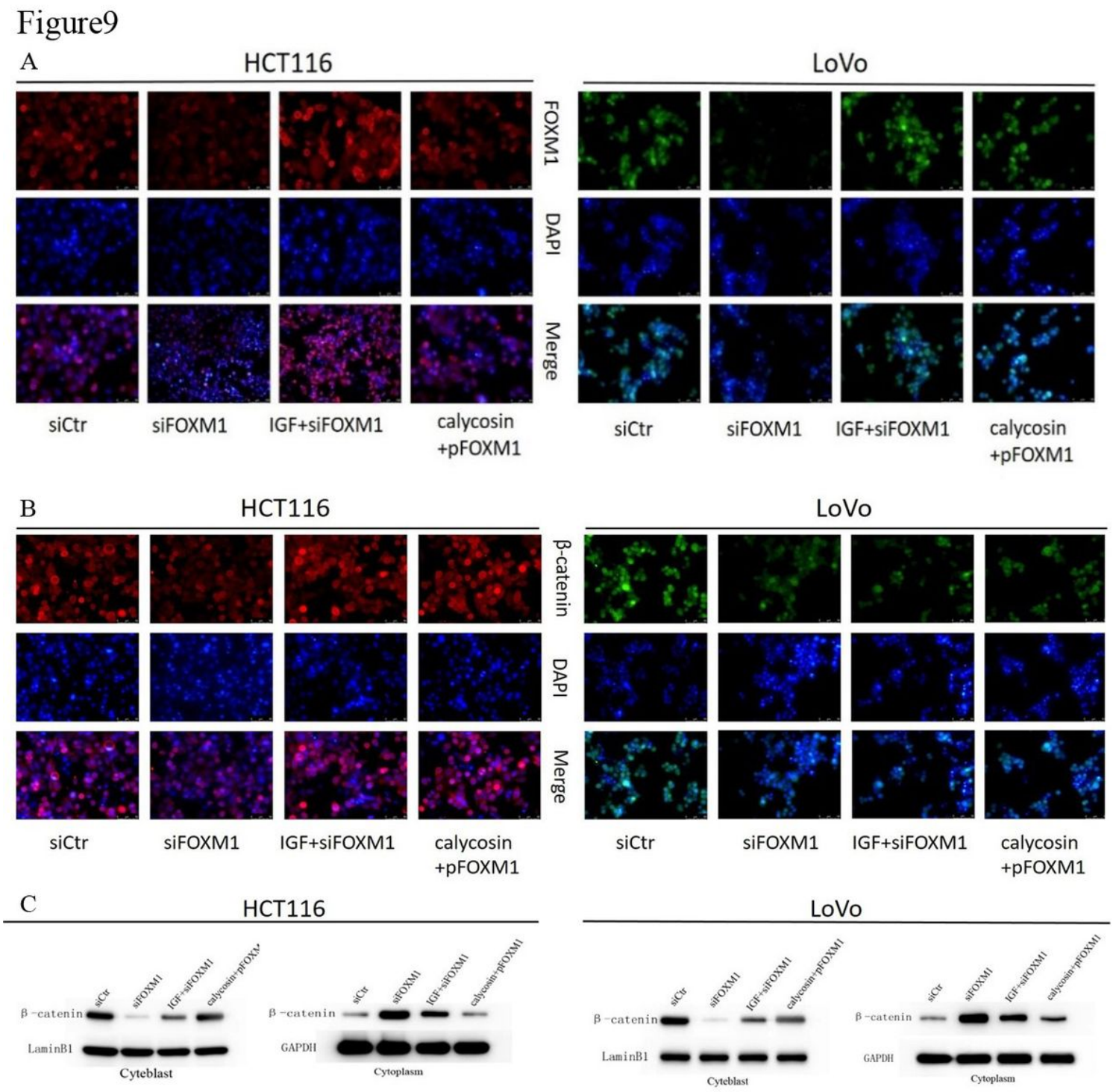

Figure 9

A dislpays cytoblast and cytoplasm localization of FOXM1 with cytoimmunofluorescence stainings in HCT116 and LoVo cells treated with siControl, siFOXM1, IGF-I mixtured with siFOXM1, calycosin together with pFOXM1. B and C show subcelluar localization and distribution of β-catenin in cytoblast and cytoplasm after HCT116 and LoVo cells treated with siControl, si FOXM1, IGF-I mixtured with si FOXM1, calycosin together with pFOXM1.

Supplementary Files

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- Graphicalabstract.jpg