Integrin β4-mediated metabolic reprogramming of cancer-associated fibroblasts in triple negative breast cancer

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The Graduate School, Yonsei University

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Directed by Professor Nam Hoon Cho

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Jin Sol Sung

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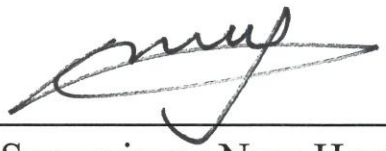
This certifies that the Doctoral Dissertation of
Jin Sol Sung is approved.



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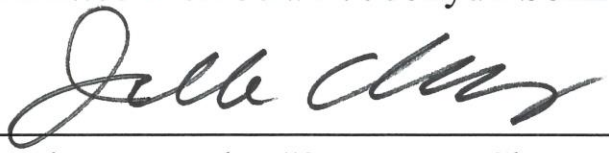

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ABSTRACT

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New therapeutic strategies are urgently required for triple-negative breast cancer (TNBC), as few targeted therapies are currently available. Targeting the tumor microenvironment is a novel approach that may prove beneficial for TNBC patients. In this study, we found that TNBC-specific integrin β4 expression and its extracellular release via exosomes led to its uptake by cancer-associated fibroblasts (CAFs). Blockade of exosomal integrin β4 secretion by MDA-MB-231 cells lowered its uptake by CAFs. Exosomal integrin β4 uptake by CAFs induced lactate production and glycolysis-related gene expression. Moreover, integrin β4-induced glycolytic stress elevated the rates of mitochondrial fission, which in turn stimulated mitophagy to preserve mitochondrial quality. BCL2 interacting protein 3 like (BNIP3L) expression was upregulated by integrin β4-mediated c-Jun activation.

Integrin β4-overexpression in CAFs increased microtubule associated protein 1 light chain 3 (LC3) levels and stimulated autophagosome formation. Excess lactate production by integrin β4-overexpressing CAFs induced TNBC proliferation and invasion by upregulating the expression of monocarboxylate transporter 1 (MCT1). In a xenograft experiment, exosomal integrin β4 promoted tumor growth and angiogenesis. Collectively, these results demonstrate that integrin β4 uptake in CAFs via exosomal transfer induces aerobic glycolysis, which results in excess lactate production and promotes TNBC cell proliferation and invasion as well as tumor growth and angiogenesis.

Key words : integrin β4, mitophagy, reverse Warburg effect

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I. INTRODUCTION

There is a lack of promising therapeutic strategies for triple-negative breast cancers (TNBCs), as they lack the expression of validated breast cancer therapeutic targets like estrogen (ER), progesterone (PR), and herceptin 2 receptor (Her2). Therefore, targeting stromal cells, such as fibroblasts, is being considered as a potential therapeutic strategy against TNBC[1-3]. The importance of stromal cells within the tumor microenvironment is well accepted, as these cells play an important role in determining cancer cell behavior and clinical outcomes[4,5]. Cancer-associated fibroblasts (CAFs), the major population of tumor

stromal cells, have been identified by expression profiling to predict clinical prognosis in breast cancer patients, independent of other factors[6,7]. Therefore, understanding the biological role of CAFs is required for further insight into TNBC progression.

Integrin α6β4 is a heterodimeric transmembrane receptor that binds to laminin-332 in the extracellular matrix (ECM). As integrin β4 only pairs with integrin α6, integrin β4 expression can be used as a proxy to measure integrin α6β4 expression[8]. The latter complex plays an important role in signal transduction by relaying signals from the extracellular matrix to the cell interior. Emerging studies suggest that integrin β4 contributes to tumor progression in various cancers such as lung, ovarian, bladder, and breast cancer[9-11]. Indeed, high levels of integrin β4 expression are associated with poor prognosis and facilitate the migration, invasion, and survival of TNBC cells[12-14].

Exosomes are small vesicles that play important roles in cancer progression. These vesicles are secreted by various cells and contain functional biomolecules such as proteins, lipids, RNA, and DNA. With respect to cancer, cell-derived exosomal proteins are transferred to recipient cells within the tumor microenvironment establishing a malignant phenotype and promoting cancer cell invasion and migration. Interestingly, a recent study indicated that exosomal integrin β4 derived from breast cancer cells is involved in organ-specific metastasis[15,16].

CAFs affect adjacent epithelial tumor cells by partaking in growth factor secretion, ECM remodeling and tumor immunity; all of which promote cancer progression[4,17-19]. Moreover, work by Pavlides has indicated that CAFs play a key role in cancer cell metabolism, resulting higher proliferative capacity of cancer cells. CAFs rely on the reverse Warburg effect, whereby these cells utilize aerobic glycolysis for energy, resulting in increased secretion of lactate that is exported by monocarboxylated transporter 4 (MCT4) present on the CAF membranes. This lactate can then be taken up by the lactate importer MCT1 present in cancer cells and used as fuel. Several studies have suggested that CAFs with altered metabolism induce breast tumorigenesis[20-22]. Increased metabolic stress in CAFs triggers mitochondrial fragmentation and degradation[23]. Depending on the severity, this increased metabolic stress may even lead to apoptosis[23,24]. Under conditions where the mitochondria become dysfunctional, AMP-activated protein kinase (AMPK) is activated that restores energy homeostasis, to maintain mitochondrial function and eliminate damaged mitochondria via mitophagy[25-28].

The aim of this study is to investigate the contribution of integrin β4 overexpression in CAFs to TNBC progression. Integrin β4 may be acquired by CAFs from TNBC cell-derived exosomes. Our study implicates the potential use of exosomal integrin β4 as a therapeutic target in TNBC.

II. MATERIALS AND METHODS

1. Human fibroblast isolation and cell cultures

Cancer associated fibroblasts (CAFs) were isolated from the breast cancer patients undergoing surgery at Severance Hospital of the Yonsei University Health System, South Korea. The research protocol was approved by the Severance Hospital Ethics Committee (IRB number 4-2008-0383). All patients were informed of tissue use of comprehensive experiments and signed consent forms. To isolate CAFs, tissue was cut into small pieces, placed in a digestion solution of enzyme cocktail (ISU ABXIS, Seoul, South Korea), and incubated in a humidified incubator at 37°C and 5% CO_2 overnight. The digested cell mixture was filtered through a 70 μm cell strainer (BD Bioscience, Franklin Lakes, NJ) and then centrifuged at 485 x *g* for 5 min. The resulting pellet was resuspended with medium, added into the Ficoll (Histopaque®-1077, sigma, 1.077 g/ml), and centrifuged at 90 x *g* for 2 min. The supernatant containing fibroblasts was further centrifuged at 485 x *g* for 9 min. The final pellet was resuspended with the Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco BRL, Grand island, NY) containing 20% fetal bovine serum (FBS; Gibco BRL, Grand island, NY) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Grand island, NY), and then placed in a humidified incubator containing 5% CO_2 at 37°C. The fibroblastic characteristics of the isolated cells were determined by both microscopic morphology and immunostaining with antibodies

against vimentin (Abcam, Cambridge, UK), cytokeratin (Dako, Glostrup, Denmark) and cytoketarin 5 (Novocastra Newcastle upon Tyne, UK). Breast cancer cell lines (MDA-MB-231, BT-20, MDA-MB-453, MCF7, BT-474, and SK-BR-3 cells) were purchased from the Korean Cell Line Bank (authenticated using morphology and STR profiling) and cultured with the DMEM (Gibco BRL, Grand island, NY) containing 10% FBS (Gibco BRL, Grand island, NY) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Grand island, NY) under the same experimental conditions as the fibroblasts. For co-culture, CAFs were incubated with the serum-free DMEM/F12 containing 5 μM CellTracker Green CMFDA (5-chloromethylfluroecein diacetate; Invitrogen, Carlsbad, CA) dye at 37°C for 30 min, washed with phosphate buffered saline (PBS, Gibco BRL, Grand island, NY), and added with a fresh medium. Unstained cancer cells were seeded onto the CMFDA-stained CAFs, and then cultured with serum-reduced DMEM/F12 (0.5% FBS) for 1 day.

2. Real-time PCR analysis

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and quantitated by NanoDrop Spectrophotometer (ThermoFisher Scientific, Waltham, MA). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using Hyperscript™ First strand synthesis kit (Geneall, Seoul, Korea) in a PTC-200 Thermal Cycler (MJ Research, Reno, NV, USA). For quantitative analysis, 25 ng of the resulting cDNA was amplified in a

CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories; Hercules, CA, USA) using LaboPass™ SYBR Green Q Master (Cosmogenetech, Seoul, South Korea). The PCR experiments were performed in triplicated, and relative expression values were calculated according to the ΔΔCt method. All primer sequences were provided in Supplemental table S1.

3. Genetic manipulation

For a transient gene overexpression, CAFs were seeded into 6-well plate to reach 80% confluence. Plasmid vectors (empty, integrin α6, integrin β4, or pQCXI Puro DsRed-LC3-GFP) were transfected into the CAFs using Lipofectamine® LTX with Plus™ and then incubated for 48 hr. pQCXI Puro DsRed-LC3-GFP was a gift from David Sabatini (Addgene plasmid #31182 ; http://n2t.net/addgene:31182 ; RRID:Addgene_31182) [21]. To establish an integrin β4 knockdown MDA-MB-231 cells, MDA-MB-231 cells were transduced with lentivius containing integrin β4 shRNA.

4. Western blot analysis

Protein extracts were prepared by cell lysis with a PRO-PREP™ kit (iNtRON biotechnology, Seongnam-si, South Korea) and centrifuged at 15,000 × *g* for 15 min using Centrifuge 5810R (Eppendorf, Hamburg, Germany). The protein concentration of the

resulting supernatant was measured using the Bradford protein assay kit (Bio-Rad, Hercules, CA). 20 μg protein was electrophoresed on 10% polyacrylamide gels in Tris/glycine (Invitrogen®, Carlsbad, CA), transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA), and then probed with primary antibodies against LAMC2, Integrin α6, Integrin β4, GAPDH, ACTB, CD63, PINK1, PRKN, BNIP3, BNIP3L, JNK, p-JNK, c-Jun, p-c-Jun, AMPK, FIS1 (Santa Cruz Biotechnology, Dallas, TX), OPA1, MFN1, DRP1, DRP1(Ser616), DRP1(Ser637) (Cell signaling technology, Danvers, MA), ULK1 (Invitrogen, Carlsbad, CA), LDHA, LDHB, MCT4, p-AMPK, and LC3 (Abcam; Cambridge, UK). The blotted membrane was blocked with 0.5% BSA at RT for 1 h and then followed by incubation with HRP-tagged secondary antibodies against mouse, rabbit (GenDEPOT, Barker, TX) or goat (Invitrogen, Carlsbad, CA). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection kit (GenDEPOT, Barker, TX).

5. Lactate assay

The amount of lacatate in cell lysate and supernatant was measured using Lactate assay kit (Biovision, Milpitas, CA) according to manufacturer's instructions. Briefly, 50 μl of sample was mixed with 50 μl of the master reaction mix containing 46 μl of lactate assay buffer, 2 μl of lactate enzyme mix, and 2 ul of lactate probe in a well of a 96-well plate. 30 min after incubation, absorbance was measured in at 570 nm using Spectramax plus 96/384 (MTX

Lab Systems, Bradenton, FL, USA). Lactate amount was measured in triplicate, calculated using the standard curve prepared with known concentration of lactate, and then normalized with protein concentration.

6. Migration matrigel assay

CAFs and MDA-MB-231 cells were seeded to lower chamber and matrigel-coated transwell insert respectively, and then placed in a humidified incubator at 37°C and 5% CO2 for 24 hr. After incubation, non-invading cells were removed from the upper surface of the matrigel by scrubbing. The invaded cells to the lower surface of the matrigel was stained with 0.1% crystal violet, and rinsed with distilled water. The dried matrigel was placed on a slide, added with a drop of immersion oil, and then covered with a coverslip.

7. Exosome isolation

CMs were collected from cancer cells and centrifuged at 3000 x *g* for 15 min. The resulting supernatant was transferred to a new tube, mixed with the appropriate volume of ExoQuick-TC (SBI, Palo Alto, CA), incubated at 4°C for 24hr, and then was centrifuged at 1500 x g for 30 min. Supernatant was aspirated and then the mixture was centrifuged at 1500 x *g* for 5 min to remove residual supernatant. The exosome pellet was resuspended to use or stored at -20°C.

8. Apoptosis detection assay

Apoptosis was analyzed using Annexin V-FITC detection kit (BD Bioscience, Franklin Lakes, NJ) according to the manufacturer's instructions. Briefly, CAFs were washed twice with cold PBS, centrifuged, and then resuspended with binding buffer at a concentration of 1×10^6 cells/ml. 100 μl of the cell suspension was mixed with 5 μl of Annexin V-FITC and PI. 15 min after incubation in the dark at room temperature, the mixture was added with 400 μl of binding buffer and then analyzed using FACSVerse (BD Bioscience, Franklin Lakes, NJ).

9. Glucose uptake assay

Glucose uptake was measured using Glucose Uptake Glo kit (Promega, Madison, WI) according to the manufacturer's instructions. CAFs (1.5×10^4) were seeded in a well of 96-well plate and placed for 24 hr. The CAFs were washed with glucose-free PBS and incubated with 2DG (1 mM) for 10 min. Stop and Neutralization buffer were added to the well and mixed one by one. 2DG6P detection reagent was added to the CAFs and then incubated at RT for 30 min. Glucose uptake was measured using luminometer (EG & G Berthold, Bad Wildbad, Germany) in triplicate.

10. Flow cytometry analysis

Cells (0.5×10^6) was washed with 1 ml of the PBS containing 2% FBS (2% FBS-PBS) and

then centrifuged at 150 x *g* for 3min. After removing supernatant, the resulting cell pellet was resuspended with 50 μl of the 2% FBS-PBS containing primary antibody and incubated at 4°C for 1 hr. After resuspending and centrifugation, cell pellet was incubated in the dark with 100 μl of FITC or PE-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX) in the same way as the primary antibody. After washing, the cell pellet was resuspended with 500 μl of 2% FBS-PBS, and then analyzed by FACSVerse (BD Bioscience, Franklin Lakes, NJ).

11. Xenograft mouse model

GFP-overexpressing CAFs (3×10^6) were subcutaneously co-transplanted to six-week-old BALB/c Nude mice with RFP-overexpressing MDA-MB-231 or RFP-overexpressing integrin β4 knockdown MDA-MB-231 cells (1×10^6) using Matrigel (Invitrogen, Carlsbad, CA). Tumor size (length and width) was measured every 3 days using calipers for one month. The volume of tumor mass was calculated using the formula: V = (W x W x L), where W is the width and L is the length.

12. Transmission electron microscopic analysis

CAFs transfected with control or integrin β4 plasmid were harvested with trypsin-EDTA (Gibco BRL, Grand island, NY), placed to 1.5 ml tube, and immediately fixed with 4%

paraformaldehyde at RT for 30 min. After washing three times with PBS by centrifugation at 300 x *g* for 5 min, the CAFs were embedded in 100% Eponate resin (Ted Pella Inc, Redding, CA) at 60°C for 24 hr. 1-μm-thick sections were cut using an ultramicrotome (Leica, Wetzlar, Germany), stained with 2% aqueous uranyl acetate for 15 min, rinsed with distilled water, and then stained with lead citrate (Leica, Wetzlar, Germany). The stained sections were examined on JEM1011 transmission electron microscope (JEOL, Tokyo, Japan).

13. Confocal imaging

CAFs were fixed with 4% paraformaldehyde at 4˚C for 15 min and then permeabilized with 1% Triton X-100 (Sigma-Aldrich, St. Louis. Mo) at RT for 10 min. After being washed twice with PBST, the CAFs were blocked with 1% BSA at RT for 1hr and then incubated with primary antibodies against integrin β4 and CD63 (Santa Cruz Biotechnology, Dallas, TX) at 4˚C overnight. Following to washing, fluorescence-conjugated secondary antibodies against mouse (Santa Cruz Biotechnology, Dallas, TX) were added to the CAFs and incubated at RT for 1hr. The CAFs were washed, added with a drop of mounting solution (Abcam, Cambridge, UK), covered with a coverslip, and then analyzed with with LSM700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

14. Luciferase assay

The putative c-Jun binding sites were analyzed in the 2kb upstream sequences of human *BNIP3L* gene using PROMO[38]. Three putative binding sites on *BNIP3L* were amplified from the genomic DNA of CAFs using conventional PCR. Deletion mutant were constructed from the PCR products by an overlap extension PCR method [39]. The wild-type and deletion mutant constructs were cloned into pGL3 vector. For dual luciferase assays, each cloned plasmid was co-transfected with pRL-TK in HEK293T (2×10^5) cells using Lipofectamine® LTX with Plus™ Reagent (Invitrogen®, Carlsbad, CA, USA). 48 hr after transfection, luciferase activity was measured from the cell lysates using a dual-luciferase reporter assay system (Promega; Madison, WI, USA).

15. Statistical analysis

Statistical significance was determined using t-test and anova (two-tailed). The results were considered to be significant at $p < 0.05$. All statistical analyses were performed using Prism 6 for Windows (GraphPad Software, Inc.; La Jolla, CA, USA). Asterisks indicate p values: one for $p < 0.05$, two for $p < 0.01$, and three for $p < 0.001$.

III. RESULTS

Immunohistochemical staining was performed to assess the expression of integrin β4 in each breast cancer subtype. Integrin β4 expression was significantly higher in TNBC patients than non-TNBC patients (Figure 1A). In addition, integrin β4 was specifically expressed in the CAFs of TNBC patients. Consistent with this result, integrin β4 expression in TNBC cells, and in CAFs co-cultured with TNBC cells was also upregulated *in vitro* (Figure 1B and C). Since integrin β4 expression can be induced by the soluble factors secreted from MDA-MB-231 and BT-20, CAFs were stimulated with the conditioned medium (CM) from MDA-MB-231, BT-20, and Hs578T (negative control) cells. However, integrin β4 expression was not induced by the CM from any of TNBC cells (Figure 1D).

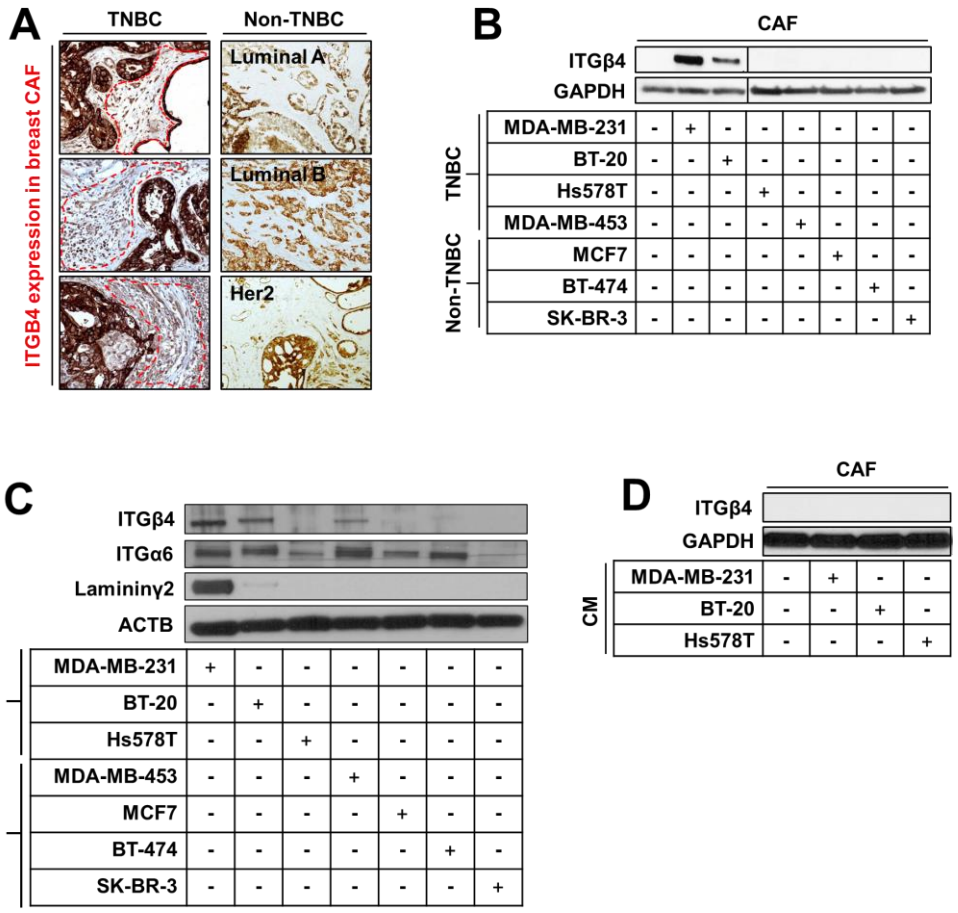


Figure 1. TNBC-specific integrin β4 expression in CAFs. Integrin β4 overexpression in (A) TNBC stroma using the patient tumor tissues and (B) CAFs co-cultured with TNBC cells. Integrin β4 expression in (C) breast cancer cell lines and (D) CAFs stimulated with CMs from breast cancer cells.

We next investigated the mechanism of expression of integrin β4 in CAFs. Previous studies have indicated that tumor-derived exosomal integrin β4 uptake in distant CAFs contributes to metastasis[15]. As shown in Figure 2A, integrin β4 was present abundantly in exosomes derived from the TNBC cell line MDA-MB-231, compared to those derived from other TNBC cell lines, BT-20 and Hs578T. Integrin β4 and CD63 (an exosome marker) were weakly co-localized in the CAFs cultured with MDA-MB-231 cells (control) in the transwell co-culture system, however they were not co-localized in the CAFs cultured with integrin β4 knockdown MDA-MB-231 cells (Figure 2B). Although CD63 was detected in the CAFs co-cultured with integrin β4 knockdown MDA-MB-231 cells, integrin β4 expression was not detected. Similarly, in the transwell co-culture system, integrin β4 and CD63 were strongly co-localized in the CAFs cultured with MDA-MB-231 cells (control), however they were not co-localized in the CAFs cultured with integrin β4 knockdown MDA-MB-231 cells (Figure 2C). To further evaluate exosomal integrin β4 transfer, MDA-MB-231 cells were pretreated with GW4869 (10 μM, an exosome inhibitor) for 2 days, and then cultured with CAFs in a direct co-culture system. As shown in Figure 2D, the co-localization of integrin β4 and CD63 was dramatically reduced in the CAFs cultured with GW4869-treated MDA-MB-231 cells. Integrin β4 protein expression was strongly detected in the CAFs co-cultured with MDA-MB-231 cells, but not in those co-cultured with integrin β4 knockdown MDA-MB-231 cells (Figure 2E) and GW4869-treated MDA-MB-231 cells (Figure 2F).

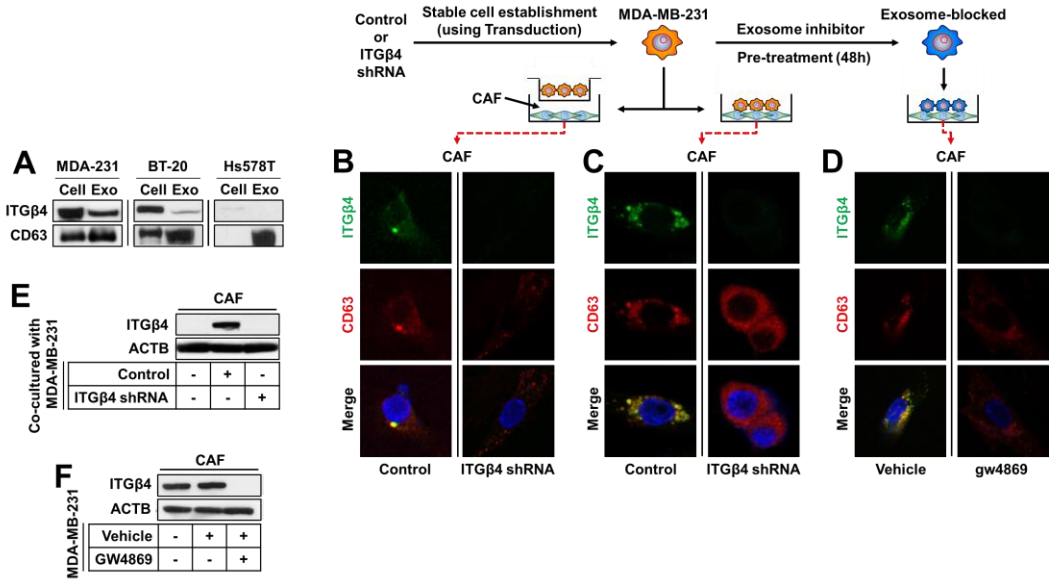


Figure 2. TNBC cells transfer integrin β4 protein to CAFs via exosome. (A) Integrin β4 expression in TNBC cells and -derived exosomes. TNBC-derived integrin β4 transfer to CAFs in (B) a transwell co-culture and (C) a direct co-culture. (D) Exosome-mediated integrin β4 transfer in the direct co-culture. MDA-MB-231 cells were pretreated with 10 μM of GW4869 for 48h. Integrin β4 expression in (E) the direct co-culture and (F) the direct co-culture with or without GW4869. To establish stable integrin β4 knockdown cancer cells, MDA-MB-231 cells were transduced with the lentivirus containing control (empty) or integrin β4 shRNA. To block the exosomal transfer of integrin β4, MDA-MB-231 cells were pretreated with GW4869 for 1day.

To investigate the role of integrin β4 expression on metabolic shift in CAFs (from oxidative phosphorylation to glycolysis), we measured lactate production[29]. Uptake of exosomal integrin β4 by CAFs elevated their lactate production (Figure 3A). A similar response was also observed in whole cell lysate of CAFs that overexpressed integrin β4 (Figure 3B) and its CM (Figure 3C), however the effect was inhibited by integrin β4 knockdown (Figure 3D) and pre-treatment with GW4869, an exosome inhibitor (Figure 3E). Further, glucose uptake also increased in CAFs that overexpressed integrin β4 (Figure 3F).

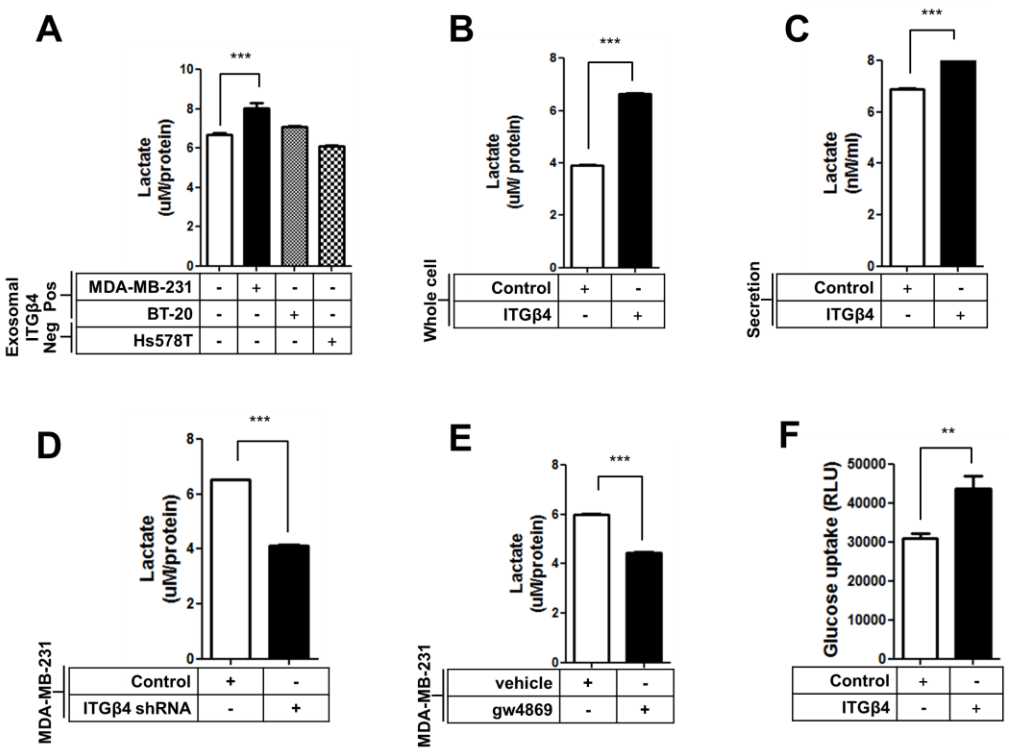


Figure 3. Exosomal integrin β4 induces glycolysis in CAFs. Lactate production in (A) CAFs co-cultured with TNBC cells, (B) whole lysate of CAFs transfected with integrin β4 plasmids, (C) CM of CAFs transfected with integrin β4 plasmids, (D) CAFs co-cultured with integrin β4 knockdown MDA-MB-231 cells, and (E) CAFs co-cultured with MDA-MB-231 pretreated with GW4869 for 1 day. (F) Glucose uptake in integrin β4-overexpressing CAFs. Co-culture was performed in the transwell co-culture. The membrane pore size of transwell insert was 0.4 μm.

Consistent with these results, an increased expression of lactate dehydrogenase A (LDHA, which converts pyruvate to lactate) was observed upon the uptake of integrin β4 by CAFs from MDA-MB-231-derived exosomes and also upon the overexpression of integrin β4 in CAFs (Figure 4). In contrast, the expression of lactate dehydrogenase B (LDHB, showing antagonistic effect to LDHA) remained unchanged in integrin β4-overexpressing CAFs. MCT4 expression was downregulated in CAFs upon the uptake of integrin β4 from MDA-MB-231-derived exosomes but was upregulated in integrin β4-overexpressing CAFs (Figure 4). These observations suggest that integrin β4 expression in CAFs is involved in aerobic glycolysis, even in the presence of oxygen.

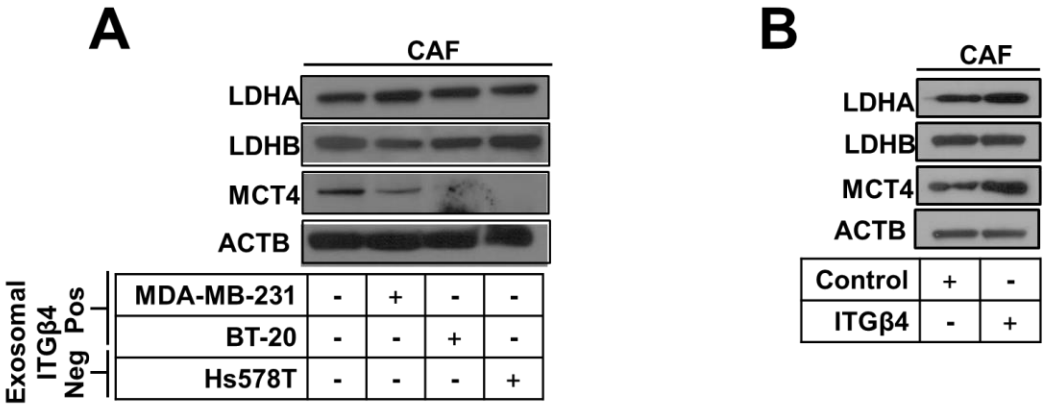


Figure 4. Exosomal integrin β4 regulates glycolysis-related gene expression in CAFs. The expression of glycolysis-related genes in (A) CAFs co-cultured with TNBC cells and (B) integrin β4-overexpressing CAFs.

Mitochondrial fission significantly increases in response to high levels of glycolytic stress, while mitochondrial fusion concomitantly decreases[30-32]. Mitochondrial dynamin like ATPase (Opa1) and mitofusin1 (Mfn1) proteins, essential for mitochondrial fusion, were downregulated upon integrin β4-overexpression (Figure 5A). In contrast, expressions of mitochondrial fission 1 protein (Fis1), dynamin-1-like protein (Drp1), and phosphorylation of Drp1(Ser616), which have critical roles in mitochondrial fission, were upregulated in integrin β4-overexpressing CAFs, suggesting increased mitochondrial fission (Figure 5B). Transmission electron microscopy (TEM) was performed to visualize mitochondrial morphology. Consistent with the expression levels of mitochondrial proteins, we observed a higher rate of mitochondrial fission in integrin β4-overexpressing CAFs than in control CAFs (Figure 5C).

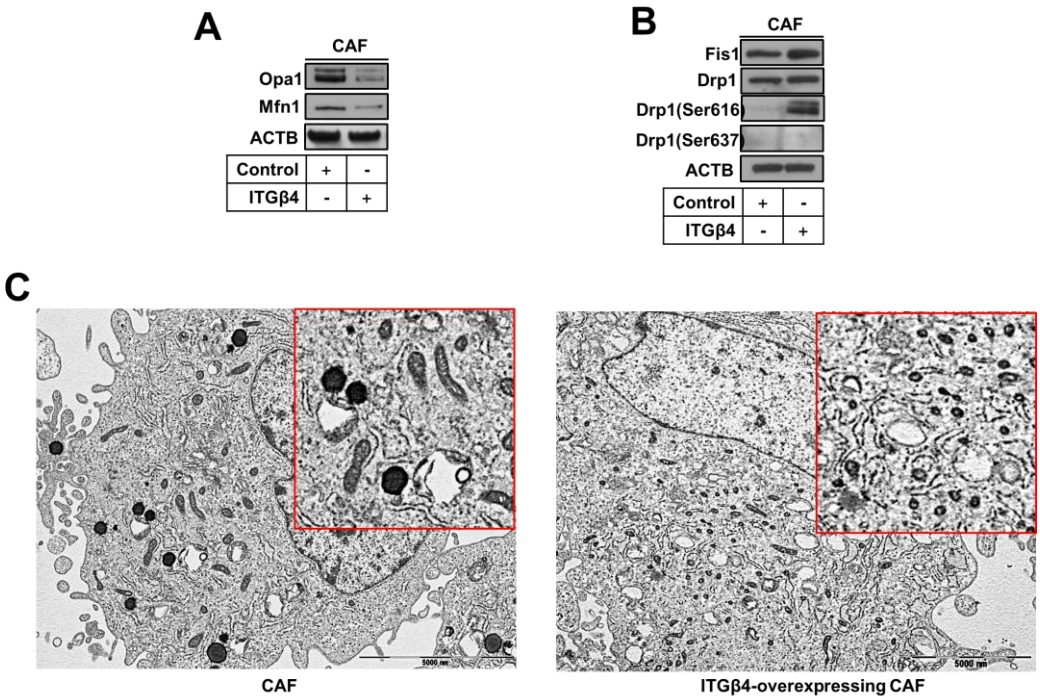


Figure 5. Glycolytic CAFs promote mitochondria fragmentation. The expression of mitochondrial (A) fusion and (B) fission-related genes. (C) The transmission electron microscopic image of integrin β4-induced mitochondrial fission.

Mitochondrial fission facilitates mitophagy and promotes the recruitment of BCL2 interacting protein 3 like (BNIP3L) to that is essential for mitochondrial clearance[33-35]. Here, we found that BNIP3L was upregulated in integrin β4-overexpressing CAFs, reflected by an increase in mitophagy (Figure 6A). However, no change in expression was detected in PTEN-induced kinase1/parkin (PINK/PARKIN), suggesting that integrin β4-mediated mitophagy in CAFs is related to BNIP3L. Indeed, immunofluorescence analyses indicated that integrin β4-positive CAFs co-expressed BNIP3L (Figure 6B).

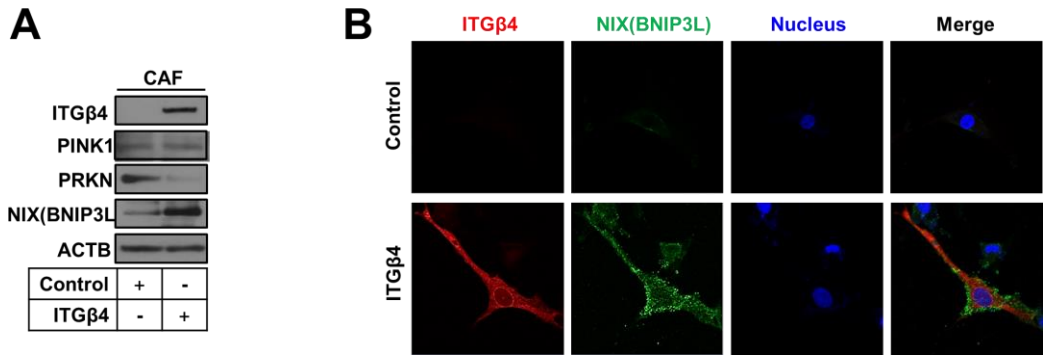


Figure 6. Integrin β4-induced mitochondrial fission leads to mitophagy in CAFs. (A) The expression of mitophagy-related factors in the integrin β4-overepxressing CAFs. (B) The fluorescent microscopic image of integrin β4-induced BNIP3L overexpression in CAFs.

We further confirmed the occurrence of mitophagy using TEM. Strikingly, a large population of mitochondria was observed enclosed within double-membraned autophagosomes, in integrin β4-overexpressing CAFs compared to control CAFs (Figure 7A). To test the prevalence of mitophagy upon integrin β4 overexpression, annexin V staining was performed. However, no significant change in cell death was observed between control cells and CAFs overexpressing integrin β4 (Figure 7B).

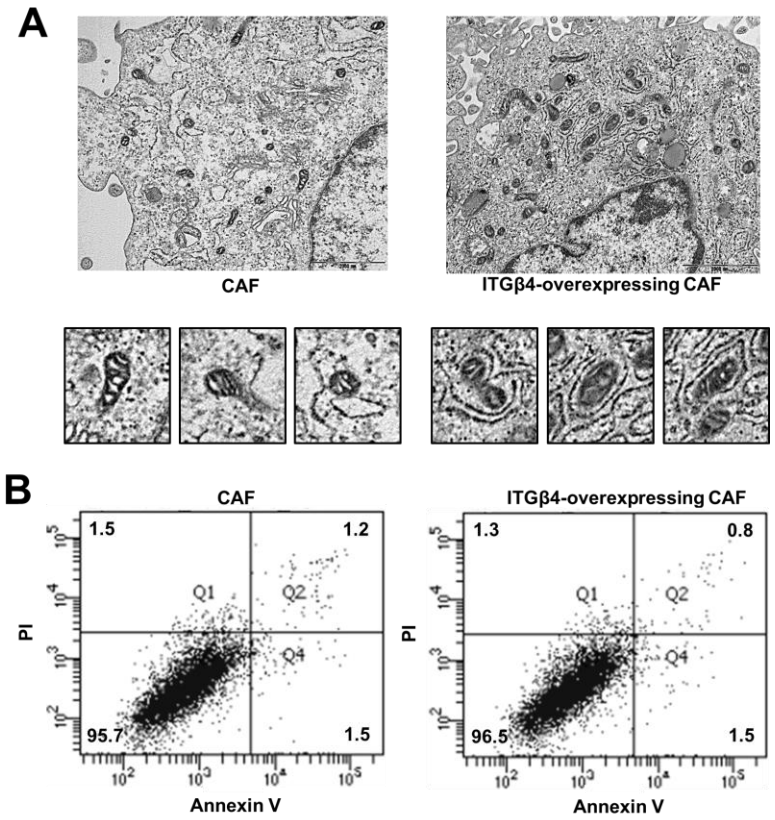


Figure 7. Integrin β4-overexpressing CAFs facilitate damaged mitochondria clearance. (A) The transmission electron microscopic image of integrin β4-induced mitochondrial clearance. (B) Cell viability comparison between wild-type and integrin β4-overexpressing CAFs.

As BNIP3L was strongly upregulated upon integrin β4 overexpression in CAFs, we sought to examine the direct role of integrin β4 in BNIP3L regulation. To this end, the upstream 2 kb sequence of the BNIP3L promoter region was analyzed using a transcription prediction program (TFSEARCH). Through this program, we identified that the putative transcription factor c-Jun was capable of binding the queried 2 kb promoter sequence of BNIP3L. We generated three c-Jun deletion mutants within specific binding sites; and assessed the binding affinities of wild-type c-Jun and the three mutants to the 2 kb promoter sequence, using a dual luciferase assay (Figure 8A, left). The luciferase activity was significantly higher in the assay with wild-type c-Jun as compared to c-Jun that contained a mutation in the first binding site (-13 bp to -7 bp, TCAGTC) (Figure 8A, right). No significant difference was observed between wild-type and deletion mutants for the other binding sites. Thus, integrin β4 could potentially activate BNIP3L transcription via the transcription factor c-Jun. To further confirm if integrin β4 directly regulates BNIP3L through c-Jun activation, we determined the levels of phosphorylated (activated) c-Jun and its upstream kinase – c-Jun N-terminal kinase (JNK). Phosphorylated c-Jun and JNK were both upregulated in integrin β4-overexpressing CAFs (Figure 8B). Interestingly, BNIP3L expression was suppressed in integrin β4-overexpressing CAFs in the presence of sp600124, an inhibitor of c-Jun (Figure 8C).

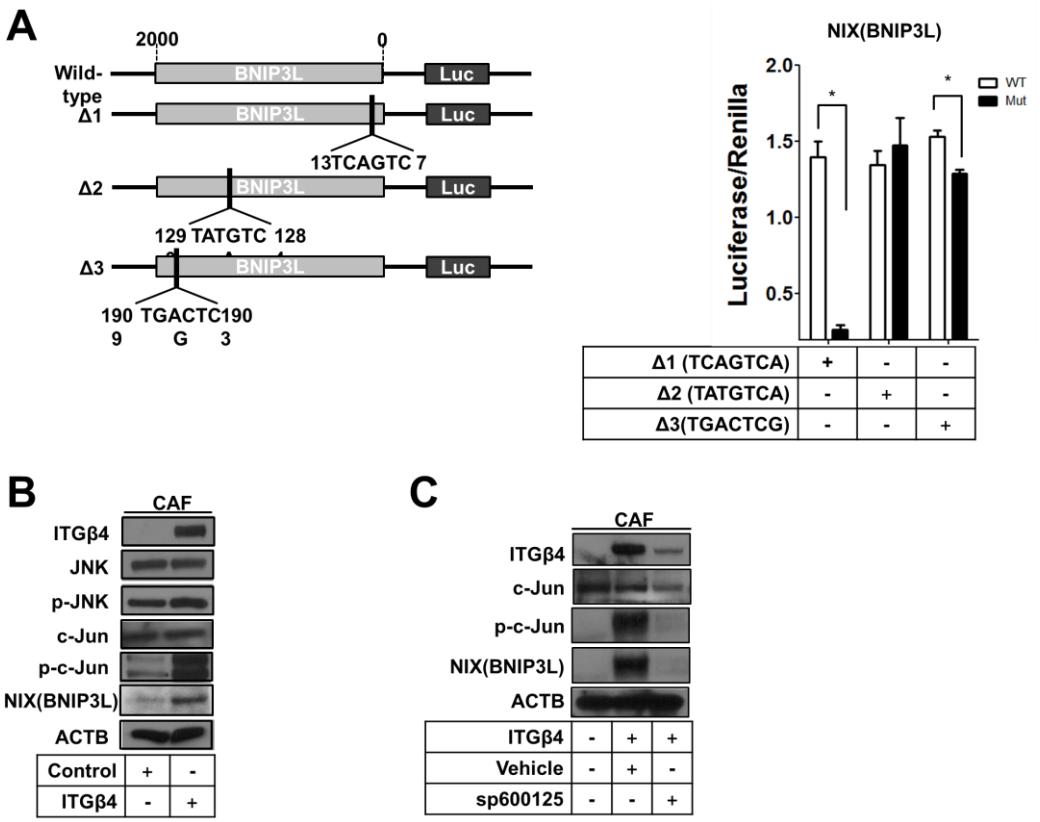


Figure 8. Integrin β4 directly regulates BNIP3L by c-Jun activation in CAFs. (A) Dual luciferase reporter assay. The wild-type or mutant sequences of putative c-Jun binding sites on BNIP3L were cloned into pGL3 vector. c-Jun binding to BNIP3L was examined by dual luciferase assay. (B) Integrin β4-dependent mitophagy signaling pathway in CAFs. (C) The suppression of integrin β4-dependent mitophagy signaling in CAFs by JNK inhibitor.

Emerging evidences suggest that BNIP3L-dependent mitophagy is capable of recruiting the autophagy machinery to the mitochondria. Further, BNIP3L directly interacts with the microtubule associated protein 1 light chain 3 (LC3)[36-40]. Autophagy activation is regulated by the AMP-activated protein kinase (AMPK). Phosphorylation of AMPK promotes autophagy and activates unc-51 like autophagy activating kinase 1 (ULK1), which inhibits the autophagic cascade[41,42]. In this study, AMPK phosphorylation was upregulated in integrin β4-overexpressing CAFs (Figure 9A). Further, ULK1 and LC3-II expression levels exhibited similar trends of upregulation (Figure 9A). To visualize autophagosome formation, we used GFP-LC3B-DsRed whose GFP tag is degraded during autophagosome formation. The number of DsRed-LC3 puncta increased in integrin β4-overexpressing CAFs suggesting an increase in autophagosome formation (Figure 9B). Since BNIP3L is known to directly bind LC3, we next visualized the co-localization of BNIP3L and LC3, marked by GFP and RFP, respectively. Compared to the control CAFs, co-localization between BNIP3L and LC3 was higher in integrin β4-overexpressing CAFs (Figure 9C). Taken together, these data imply that integrin β4-induced mitophagy facilitates damaged mitochondrial clearance by entering the autophagic pathway, but acts independently of autophagic cell death.

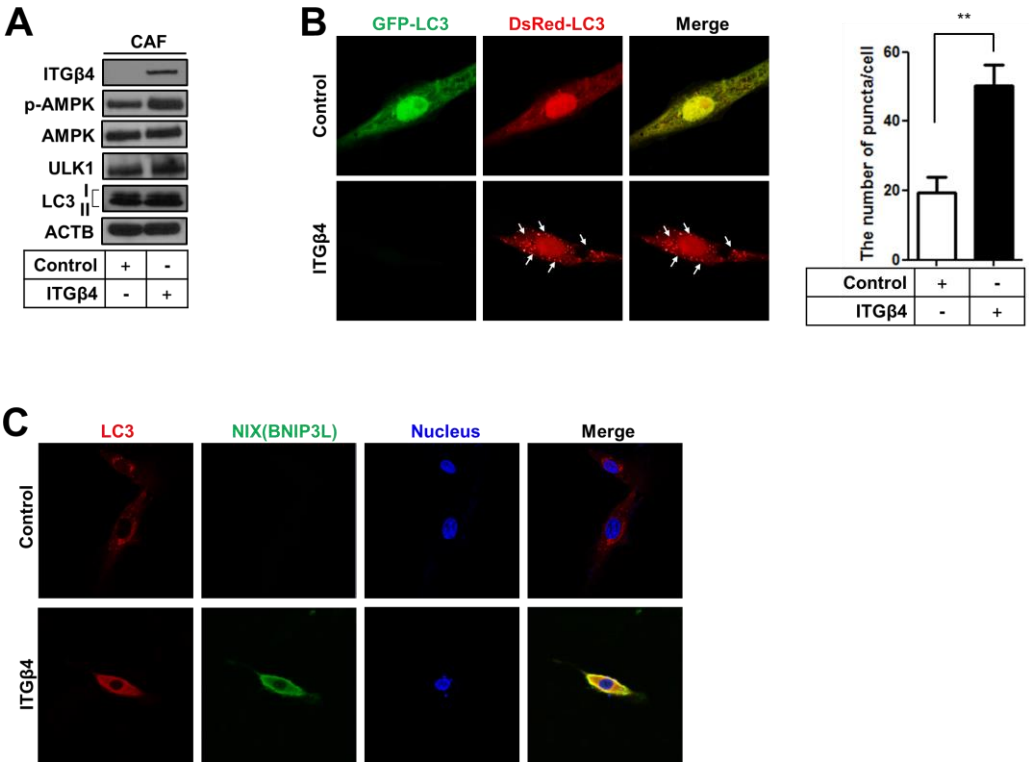


Figure 9. Integrin β4-overexpressing CAFs undergo autophagy through AMPK activation. (A) Integrin β4-induced autophagy signaling in CAFs. (B) Puncta formation in integrin β4-overexpressing CAFs. For a puncta formation assay, pOCXI puro DsRed-LC3-GFP was transfected into CAFs. (C) Co-localization between LC3 and BNIP3L in integrin β4-overexpressing CAFs.

Since integrin β4 plays a role in regulation of glycolysis in CAFs, we next set out to determine the effect of integrin β4 overexpressing CAFs on TNBC cells. We first investigated the level of membrane localized MCT1 in MDA-MB-231 cells to test whether MDA-MB-231 cells uptake lactate as an energy source through MCT1. Interestingly, MDA-MB-231 incubated with conditioned medium (CM) from integrin β4-overexpressing CAFs demonstrated significantly higher levels of MCT1, compared to those grown in CM from control CAFs (Figure 10A). Next, a proliferation assay was used to assess MDA-MB-231 cell proliferation. CM from integrin β4-overexpressing CAFs strikingly accelerated the growth of MDA-MB-231 cells. However, CM from integrin β4-overexpressing CAFs treated with the c-Jun inhibitor, sp600125, or the AMPK inhibitor, compound C, suppressed MDA-MB-231 cell growth (Figure 10B). These data demonstrate that integrin β4 expression in CAFs potentially plays a critical role in cancer cell proliferation by creating a tumor microenvironment replete with the high-energy metabolite – lactate, upon which cancer cells preferentially feed. We further examined MDA-MB-231 cell invasiveness, a commonly described histological characteristic of TNBC[44]. Our results indicate that cell invasiveness was significantly higher in MDA-MB-231 cells incubated with CM from integrin β4-overexpressing CAFs in trans-well migration assays (Figure 10C). In contrast, CM from integrin β4-overexpressing CAFs treated with sp600125 or compound C decreased the invasiveness of MDA-MB-231 cells (Figure 10C). Concomitantly, mRNA levels of epithelial-mesenchymal transition (EMT) inducer genes were also higher in MDA-MB-231 cells

stimulated with CM from integrin β4-overexpressing CAFs, compared to both control MDA-MB-231 cells, and MDA-MB-231 cells stimulated with CM from integrin β4-overexpressing CAFs that were treated with sp600125 or compound C (Figure 10D).

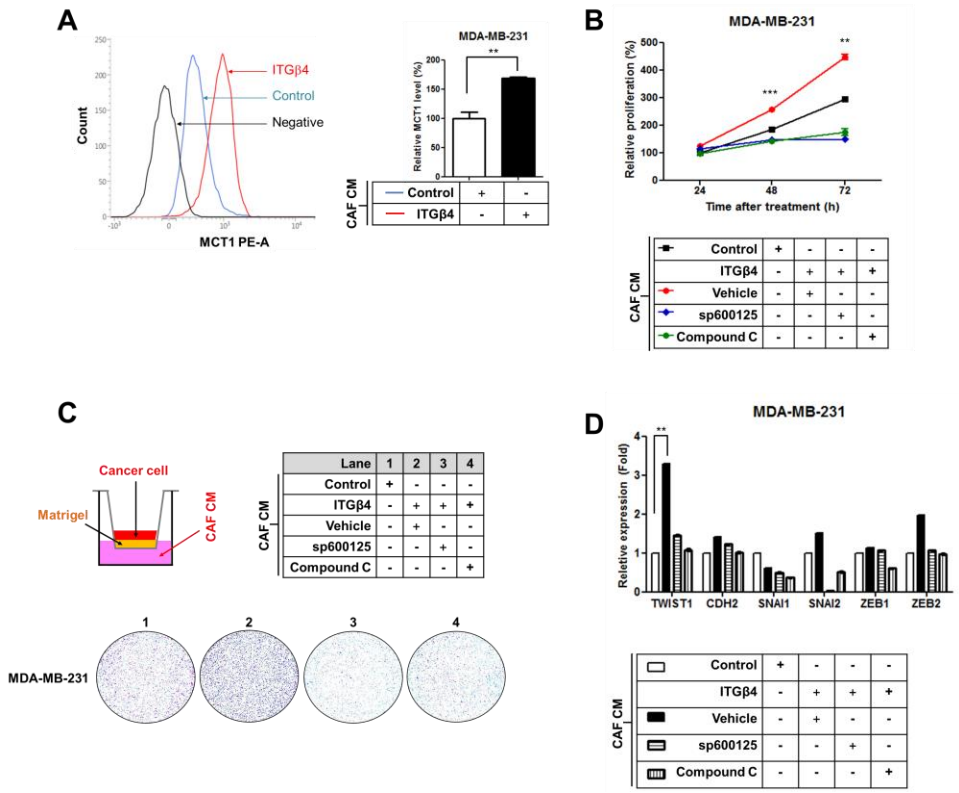


Figure 10. Integrin β4-dependent reverse Warburg effect contributes to breast cancer progression. (A) The surface MCT1 level of TNBC cells stimulated by CM from integrin β4-overexpressing CAFs. (B) The proliferation of TNBC cells stimulated by CM from integrin β4-overexpressing CAFs and integrin β4-overexpressing CAFs pretreated with sp600125 and Compound C. (C) The invasiveness of MDA-MB-231 cells was measured using matrigel invasion chamber. (D) The upregulation of EMT inducer expression was analyzed using real-time PCR. MDA-MB-231 cells were stimulated by CM from integrin β4-overexpressing CAFs and integrin β4-overexpressing CAFs pretreated with sp600125 and Compound C.

Immunohistochemical staining was performed to evaluate integrin β4 expression in CAFs in the xenograft. As expected, increased expression of integrin β4was observed in CAFs injected with MDA-MB-231 (CAFs+MDA-MB-231), compared to CAFs injected with integrin β4 knockdown MDA-MB-231 cells (CAFs+shITGβ4_MDA-MB-231) (Figure 11A). In addition, CAFs+MDA-MB-231 tumors were significantly larger compared to CAFs+shITGβ4_MDA-MB-231 tumors (Figure 11B). Interestingly, we also found that CAFs+MDA-MB-231 tumors developed blood vessels (Figure 11C) and further investigated angiogenesis in the tumors using immunohistochemical staining of CD31 and vascular endothelial growth factor (VEGF). Angiogenesis was induced in CAFs+MDA-MB-231 tumors as demonstrated by increased staining of CD31 and VEGF (Figure 11C). The viability of immortalized CAFs in vivo was confirmed by fluorescence microscopiy after scarifying mice (Supplementary Figure S2). Together, these data demonstrate that functional exosomal integrin β4 uptake by CAFs promote tumor growth and angiogenesis in TNBC.

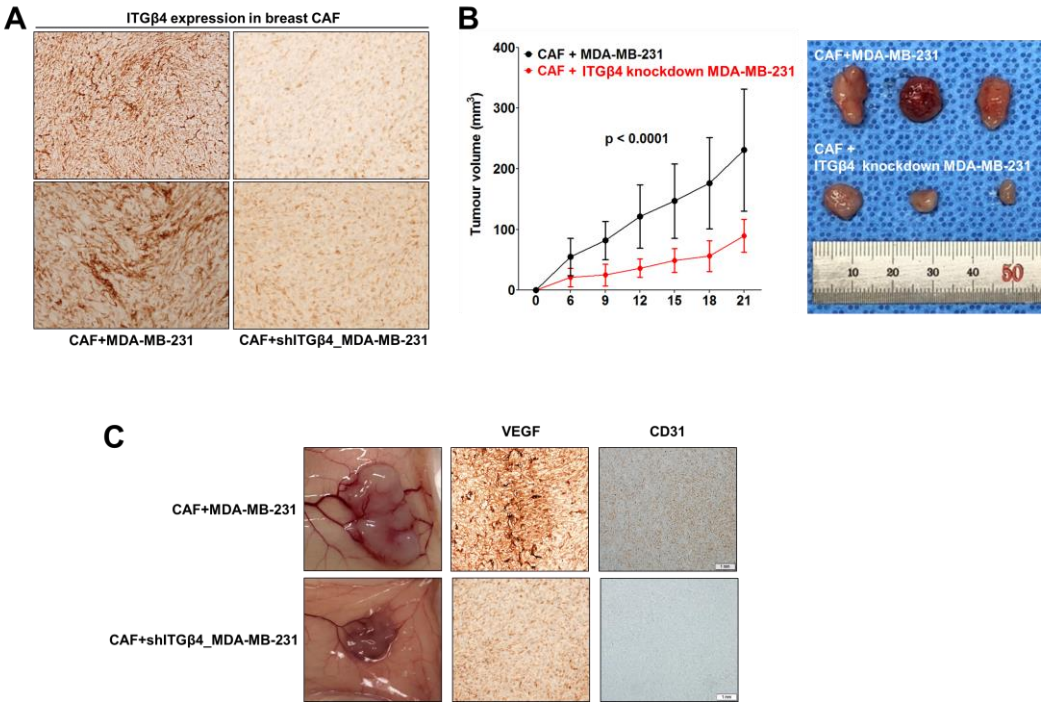


Figure 11. Exosomal integrin β4 promotes tumor growth and angiogenesis *in vivo.* (A) Integrin β4 overexpression in TNBC stroma using the mouse tumor tissues. Increased (C) tumor growth and (D) angiogenesis by integrin β4-overexpressing CAFs in a mouse xenograft model. MDA-MB-231 or integrin β4 knockdown MDA-MB-231 cells were subcutaneously co-transplanted with CAFs.

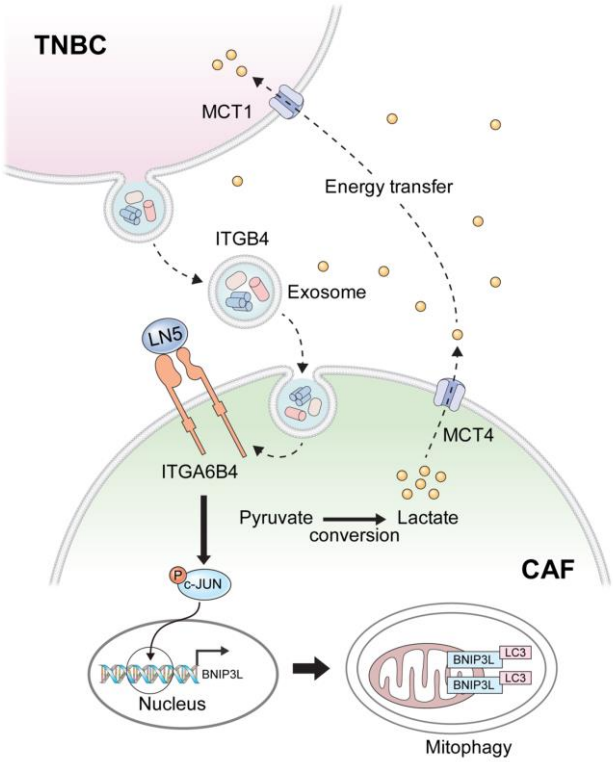
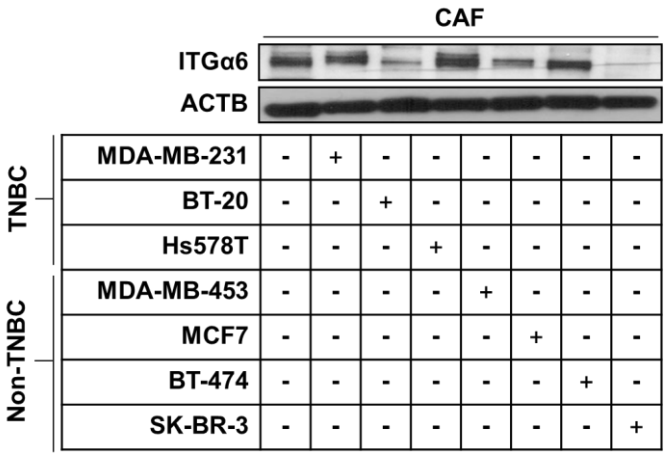
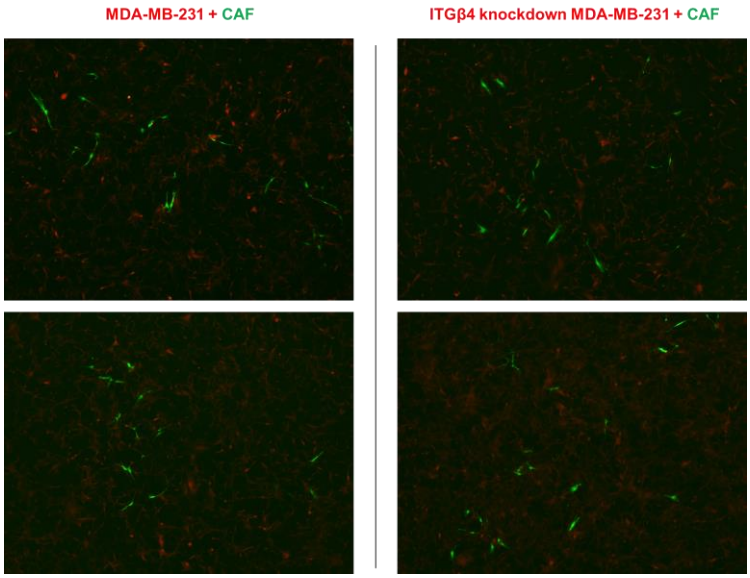


Figure 12. Integrin β4-mediated metabolic reprogramming of cancer-associated fibroblasts in triple negative breast cancer. Integrin β4-overexpressing TNBC cells provide CAFs with integrin β4 via exosomes. Once integrin β4 is transferred, integrin α6β4 is displayed on the surface of CAFs. LN5 (laminin-332) in extracellular microenvironment binds to the integrin α6β4, and then initiates the mitochondrial expression of BNIP3L in CAFs via c-Jun phosphorylation. BNIP3L triggers mitophagy by binding with LC3, which results in the conversion of pyruvate to lactate in the CAFs. The produced lactates are exported from CAFs to extracellular microenvironment through MCT4, and then TNBC cells uptake them through MCT1.



Supplementary Figure S1. The expression of ITGα6 expression in the CAFs co-cultured with or without breast cancer cell lines.



Supplementary Figure S2. The fluorescence microscopic images of the mouse tumor tissues.

Supplementary Table S1. Primers for real-time PCR analysis.

Gene name	Strand	Sequence (5' to 3')	Annealing Tm (°C)
TWIST1	Forward	GTC CGC AGT CTT ACG AGG AG	53
	Reverse	CCA GCT TGA GGG TCT GAA TC	
SNAI1	Forward	TTT ACC TTC CAG CAG CCC TA	53
	Reverse	CCC ACT GTC CTC ATC TGA CA	
SNAI2	Forward	AAGCATTTCAACGCCTCC AAA	54
	Reverse	GGATCTCTGGTTGTGGTATGACA	
ZEB1	Forward	TGC ACT GAG TGT GGA AAA GC	53
	Reverse	TGG TGA TGC TGA AAG AGA CG	
ZEB2	Forward	CGC TTG ACA TCA CTG AAG GA	53
	Reverse	CTT GCC ACA CTC TGT GCA TT	
CDH2	Forward	CTCCTATGAGTGGAACAGGAACG	53
	Reverse	TTGGATCAATGTCATAATCAAGTGCTGTA	
GAPDH	Forward	GTC AGT GGT GGA CCT GAC CT	52
	Reverse	TGC TGT AGC CAA ATT CGT TG	

IV. DISCUSSION

Patients with TNBC have better response to chemotherapy than patients with other breast cancer subtypes[45]. This could be explained by the lack of therapeutic targets, leading to cause a high rate of fatality[46]. Personalized cancer therapy has been developed in the field of TNBC therapeutics for those who do not respond to chemotherapy. However, due to the heterogeneity of TNBC, recent studies propose the identification of a single targetable molecule in tumor microenvironment, imposing challenges for its treatment[47-49].

Our study identified a novel function of integrin β4 in CAFs. Although integrin β4 is highly expressed in TNBC cell lines, only specific TNBC CAFs show its expression. Elevated integrin β4 expression accompanied with high levels of integrin α6 and laminin-332, is associated with TNBC-specific integrin β4 expression in CAFs via exosomes. Considering that a laminin-332-rich tumor microenvironment is naturally established around TNBC and that integrin α6 (the only pair partner) is intrinsically expressed at a high level in CAFs (Supplementary Figure S1). The role of integrin β4 is well-known to facilitate migration, invasion and survival of TNBC cells[9-11]. A recent study indicated that exosomal integrin β4 derived from breast cancer cells is involved in organtropic metastasis[15]. However, there has been little knowledge about the function of integrin β4 in CAFs. We showed that Increase in integrin β4 expression in CAFs up-regulated glycolysis and lactate production. These CAFs showed enhanced mitochondrial fission, possibly as a response to glycolytic stress.

Emerging studies have described that cleavage of Opa1 preventing further re-fusion of mitochondria facilitates the elimination of damaged mitochondria by mitophagy[23-28,30-33].

In this study, induction BNIP3L suggested a significant increase in mitophagy upon integrin β4 overexpression. c-Jun was identified as a transcription factor regulating BNIP3L gene expression activated by integrin β4 in CAFs. BNIP3L is a key molecule that recruits the autophagic machinery[35-39]. Autophagy is promoted by AMPK followed by the overexpression of ULK1[41-42]. Integrin β4-induced BNIP3L expression enhanced AMPK phosphorylation, and upregulated ULK1 and LC3 expression. Although integrin β4 overexpressing CAFs showed an increase in autophagosome formation by entering autophagic pathway, they were not relevant for autophagic cell death. These observations suggest the potential role of integrin β4 acquired by CAFs from TNBC-derived exosomes, leading to the production of excess lactate by controlling mitochondrial quality via mitophagy.

Lactate secreted by CAFs serves as a high-energy metabolite for adjacent TNBC cells. According to reverse Warburg effect, lactate is exported by MCT4 and imported by MCT1 in cancer cells, to be used as a fuel. In this study, MCT1 levels were elevated in TNBC cells grown in CM from integrin β4-overexpressing CAFs, thus favoring TNBC cell proliferation and invasion. However, the detailed mechanism of this process remains unknown at this time. Our *in vivo* studies suggested the critical role of exosomal integrin

β4 in tumor growth and angiogenesis, as integrin β4 knockdown in TNBC cells led to tumor suppression. Notably, the expressions of VEGF and CD31 were significantly upregulated in the presence of exosomal integrin β4 in the xenograft mouse model. We speculate that rapid tumor growth and angiogenesis of TNBC is mediated by exosomal integrin β4-induced glycolysis in CAFs. Further studies are required to uncover the mechanisms by which TNBC cells are regulated by glycolytic CAFs. We predict that exosomal integrin β4 will emerge as an effective therapeutic target against TNBC.

V. CONCLUSION

Here, we provide a novel function for the TNBC-specific expression of integrin β4 in CAFs. Integrin β4 is transferred to CAFs via exosomes derived from TNBC cells and promotes aerobic glycolysis. These glycolytic CAFs facilitate mitochondrial fission and mitophagy to maintain mitochondrial homeostasis. As CAFs elevate their lactate production, adjacent TNBC cells import lactate via MCT1 and utilize it as an energy source for cell proliferation, invasion, and angiogenesis. Together, these results represent a basis for TNBC development and further suggest that exosomal integrin β4 may be a potent therapeutic target against TNBC.

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ABSTRACT (IN KOREAN)

삼중 음성 유방암 내 섬유아세포의 integrin β4 매개 신진대사 재구성

< 지도교수 조남훈 >

연세대학교 대학원 의과학과

성 진 솔

삼중 음성 유방암은 (TNBC)은 ER/PR/HER2가 모두 음성인 유방암으로 공격적인 특성을 보이며 재발률이 높고 유방암 중 가장 예후가 나쁜 것으로 알려져 있다. 현재까지 TNBC에 대해 효과가 입증된 표적 치료가 없어 새로운 치료 전략이 필요한 상황이다. 최근에는 종양 미세 환경을 표적으로 하는 새로운 치료전략이 주목을 받고 있다. 본 연구에서는 암세포의 주변 섬유아세포 (Cancer-associated fibroblast, CAF)에서 TNBC 특이적인 integrin β4의 발현이 엑소좀 (exosome)을 통해 CAF로 이동하는 것을 확인하였다. MDA-MB-231 세포에 exosome 분비를 억제 시켰을 때 CAF에서 integrin β4의 발현이 감소되었다. CAF에서의 integrin β4 발현은 젖산 생산과 해당 과정에 관여하는 유전자들의 발현을 증가시켰고, 이로 인한 당 분해 스트레스는 미토콘드리아의 분열과 미토콘드리아 기능유지를 위한 미토파지(mitophagy)를 증가시켰다. 또한 integrin β4

과발현된 CAF는 c-Jun의 활성화를 통해 미토파지에 관여하는 BCL2 interacting protein 3 like (BNIP3L) 발현을 증가시키는 것을 확인하였다. CAF에서의 integrin β4 과발현은 microtubule associated protein 1 light chain (LC3)의 발현과 자가포식소체(autophagosome) 형성이 증가되었다. 또한 과량으로 생성된 젖산 증가는 monocarboxylate transporter 1 (MCT1)을 통해 TNBC세포의 성장(proliferation)과 침투(invasion)를 촉진시켰다. 이종이식 모델에서 exosome을 통해 CAF로 이동한 integrin β4가 종양의 성장과 혈관생성을 증가시켰다. 종합적으로 본 연구에서는, exosome을 통한 CAF의 integrin β4 발현이 과도한 젖산 생산을 증가시키고, TNBC 세포의 증식과 침투뿐만 아니라 종양의 성장과 혈관 생성을 촉진시키는 호기성 해당작용을 유도함을 확인하였다.

Key words : integrin β4, 미토파지, 반대 월버그 효과

PUBLICATION LIST

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