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이학석사학위논문

Molecular transformation of non-tumorigenic MCF10A cells caused by direct contacts with breast cancer cells.

유방암세포와의 직접접촉을 통해 유도되는

MCF10A 세포주의 분자생물학적 변화

2017년 2월

서울대학교 대학원

의과대학 협동과정 종양생물학 전공

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지도교수 노 동 영

이 논문을 이학석사학위논문으로 제출함

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조 설 화

조설화의 이학석사학위논문을 인준함

20 년 월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

Abstract

Molecular transformation of non-tumorigenic MCF10A cells caused by direct contacts with breast cancer cells.

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College of Medicine

The effect of epithelial cancer cells on the adjacent normal epithelial cells has not been investigated clearly. In this study, we hypothesized that the normal mammary epithelial cells can undergo molecular transformation due to the paracrine effects exerted by the

adjacent cancer cells. To test the hypothesis, we used both direct and indirect co-culture methods for human mammary epithelial cells and breast cancer cells. MCF10A normal mammary epithelial cells and MDA-MB-231 breast cancer cells were used. MDA-MB-231 cells had minimal effects on MCF10A cells in the indirect co-culture systems. However, when MCF10A cells were grown in direct co-culture system, where the direct contacts between the cancer cells and normal cells were allowed, the cells underwent substantial phenotypic changes. Direct co-culture with MDA-MB-231 cells enhanced MCF10A cell's capacity in cell proliferation, colony formation, and migration. Also, cells showed increased expression of various EMT-related markers. The dynamic interactions between the cancer cells and normal cells in the direct co-culture system via cell projections and extracellular vesicles were observed in the live cell

imaging assays. To find specific genes and pathways involved in this phenomenon, we performed a phosphorylation assay and RNA sequencing analysis. In phosphorylation assay, phospho-p53 (S392, S46, S15 residue) level was reduced in direct co-cultured MCF10A. Global gene expression profiles of mono-cultured MCF10A cells versus co-cultured MCF10A cells showed downregulation of some keratin genes and upregulation of S100A8 and S100A9. Our findings suggest that MDA-MB-231 cells may disrupt the cell cycle regulation and suppress the expression of E-cadherin in MCF10A cells through upregulation of S100A8/A9 and downregulation of p53 by physical contact and vesicular transport. Future study is needed to find specific pathways which is involved in S100A8/9 and p53.

Keywords: breast cancer; normal mammary epithelial cell; S100A8/A9; p53

Student number: 2015-22063

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List of abbreviation

ECM	Extracellular matrix
EGF	Epidermal growth factor
FACs	Fluorescence-activated cell sorting
qRT-PCR	Quantitative real time polymerase chain reaction

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Introduction

Breast cancer is the most common disease which occurs usually in women. WHO (World Health Organization) reported that breast cancer ranked second in a cancer incidence rate in both sexes worldwide in 2012. In the human body, cancer cells do not develop alone. They interact with many cells, such as fibroblasts, immune cells, and extracellular matrix. We called this complex is the tumor microenvironment. Weaver et al. reported the importance of interaction between cells and their microenvironment. In this study, phenotype of cells could be reverted through interactions with their microenvironment although their genetic backgrounds were same (7, 8). Therefore, tumor microenvironment has critical role to promote cancer cell growth and metastasis (1). Many studies have shown that fibroblast, immune cells and endothelial cells greatly influence on

breast cancer progression (2, 4, 5, 6). However, studies on interaction between normal epithelial cells and breast cancer cells were not clear (3, 5, 9). In this study, we hypothesized that normal mammary epithelial cells can undergo molecular transformation due to the paracrine effects exerted by the adjacent cancer cells. We investigate interactions between breast cancer cells and normal mammary epithelial cells.

Materials and Methods

Cell culture

MCF10A cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium with 5% horse serum, 20 ng ml^{-1} epidermal growth factor (EGF), 100 ng ml^{-1} cholera toxin, 10 μg ml^{-1} insulin and 500 ng ml^{-} hydrocortisone. MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium with 10% FBS and 1% penicillin and 1% streptomycin. For fluorescence tagged cells, puromycin was added.

Flow Cytometry

MCF10A cells and MDA-MB-231 cells were transfected with lentivirus GFP and RFP construct respectively. Cells were co-cultured for 10days and sorted by FACs Aria Ⅱ.

Co-culture of MCF10A cells and MDA-MB-231 cells

Firstly, to find an appropriate medium for co-culture, two mediums were mixed in varying ratios, after checking the proliferation and morphological change, we selected an appropriate condition. All experiments were performed with this co-culture medium. GFP-labeled MCF10A cells and RFP-labeled MDA-MB-231 cells were mixed 3:7 and 7:3 ratio. Mixed cells were seeded in 6 well plate and maintained for 10days. During co-culture, cells were sub-cultured two times.

Indirect co-culture was performed by using transwell insert (pore size 0.4um). Cancer cells (MDA-MB-231 and MCF7) were seeded on the membrane of the insert (pore size 0.4um) MCF10A cells were seeded in the lower six well plates. Mono- cultures were also performed. In that case, same cells were seeded in the lower well and

upper chamber. Cells were cultured in co-culture medium (DMEM:DMEM/F12=7:3) for 10days.

Cell proliferation assay

Cells were seeded in triplicate into 96-well plates at a density of 2,000 cells per well. Proliferation assays were conducted using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, USA) solution following the manufacturer's protocol.

Quantitative real-time PCR

Total cells were extracted from isolated cells with TRIzol (Favorgen, Taiwan). PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan) were used for reverse transcription of RNA, and resulting cDNA was amplified using Power SYBR® Green PCR Master Mix (Applied Biosystems). The primer sequences used were as a Table 1.

Colony-formation assay

2,000 cells per well were seeded in triplicate into 6-well plates. After 2 weeks, colonies were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet.

Immunofluorescence

Cells were seeded in Cell imaging coverglass with 8 chambers (Ependorf, Hamburg, Germany). Cells were fixed with 4%paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum. Samples were incubated with primary antibodies overnight at 4℃. TRITC conjugated secondary antibodies or Alexa conjugated secondary antibodies were incubated 1hr at RT. In case of phalloidin, fluorescence was already conjugated so, it do not need two steps.

CytoPainter Phalloidin-iFluor 488 Reagent (Abcam, Cambridge, MA, USA) and CytoPainter Phalloidin-iFluor 647 Reagent (Abcam, Cambridge, MA, USA) were used.

Western blot

MDA-MB-231 cells and MCF10A cells were co-cultured for 10days and then two cell-lines were isolated by FACs. Isolated cells were seeded and proteins were harvested after 2 days with RIPA buffer (Thermo scientific, Palm Springs, CA, USA), protease&phosphatase inhibitor and 0.5M EDTA solution. Protein concentration was measured by BCA assay kit (Thermo scientific, Palm Springs, CA, USA). Cell lysates were loaded onto 15% gels and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk and incubated with primary antibody overnight at 4℃. The peroxidase-

conjugated secondary antibody was used for detection. Bands were detected by LAS and ECL system.

Live cell imaging

Cells were seeded in 8well chambers and cultured for 48hrs. Images were taken every two minutes with fixed position for 16hrs.

Migration assay

2×10^4 cells were seeded in an insert(8 μm pore size) with serum free media. Media with 10% FBS was added in lower chambers. Cells were incubated for 20hrs and fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Experiments were duplicated.

Table 1.

Name		Sequences
S100A8	F	ATGCCGTCTACAGGGATGAC
	R	CCACGCCCATCTTTATCACC
S100A9	F	GGGAATTCAAAGAGCTGGTGC
	R	AGCTGCTTGTCTGCATTTGTG
KRT17	F	CAGTCCCAGCTCAGCATGAA
	R	CCACAATGGTACGCACCTGA
KRT16P3	F	TCAGACCGGTGGAGAAGTGA
	R	TGTGCCGGGTCCTTCATACT
KRT16	F	AGTCCCAGCTCAGCATGAAA
	R	GCGGGAAGAATAGGATTGGC
KRT10	F	CAACTCACATCAGGGGGAGC
	R	CAGCTCATCCAGCACCCTAC
CD24	F	GCTCCTACCCACGCAGATTT
	R	GAGACCACGAAGAGACTGGC
ZEB1	F	GTGACGCAGTCTGGGTGTAA
	R	TGAGTCCTGTTCTTGGTCGC
Snail	F	GAGGACAGTGGGAAAGGCTC
	R	TGGTTCGGATGTGCATCTT
ZO-1	F	GACAGCAGACCACGTTACGA
	R	TGAAGGTATCAGCGGAGGGA
β-actin	F	CACTGTGTTGGCGTACAGGT
	R	TCATCACCATTGGCAATGAG
E-cadherin	F	CCCTCGACACCCGATTCAAA
	R	TGGATTCCAGAAACGGAGGC

N-cadherin	F	ATGTGCCGGATAGCGG
	R	ACATTGAGAAGAGGCTGTCC

Results

Ⅰ. Indirect co-culture with MDA-MB-231 cells changed the mRNA expression of EMT markers but it did not affect morphology and proliferation of MCF10A cells.

MCF10A cells and breast cancer cells (MCF7 or MDA-MB-231) were indirectly co-cultured with a transwell culture system. To find an appropriated media for co-culture, two types of cell culture media were mixed with varying ratio, after checking the proliferation and morphological changes. All experiments were performed with this co-culture media. Breast cancer cells were seeded in the upper and MCF10A cells were seeded in lower wells. In this system, breast cancer cells exchange their secreted factors with MCF10A cells through the pore of the insert (pore size 0.4 μm). Co-culture was maintained for 10 days. Morphological changes were not observed in indirect co-cultured MCF10A (Figure 1). Indirect co-culture with MDA-MB-231 cells changed the mRNA expression of E-cadherin, Zo-1 and N-cadherin (Figure 2). However, proliferation changes did not show a consistent pattern in indirect co-cultured MCF10A (Figure

3). Indirect co-culture did not affect MCF10A cell growth and morphology in 3D matrigel culture (Figure 4).

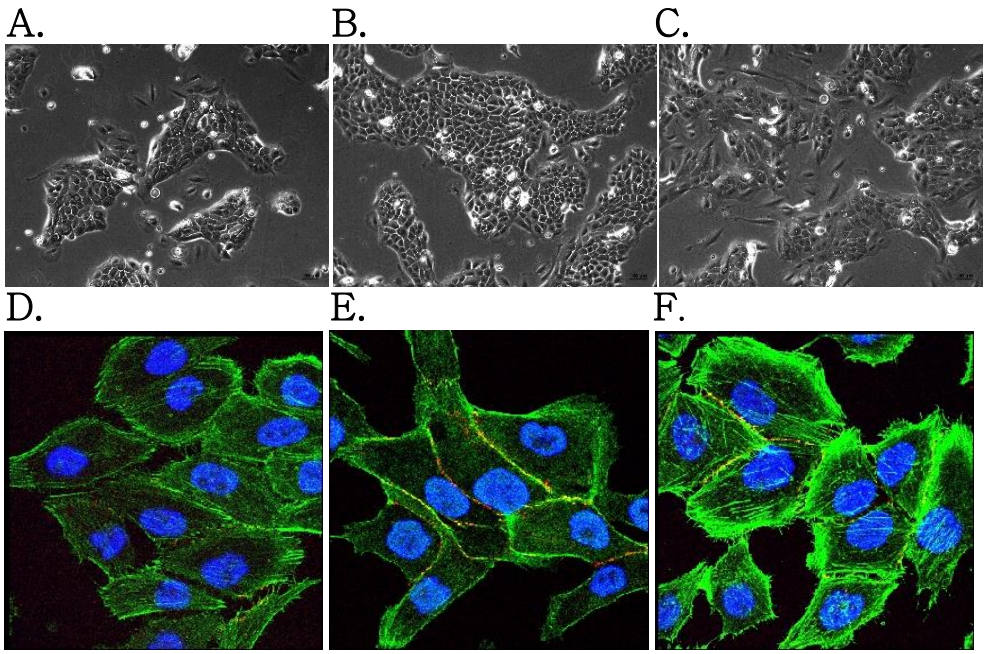


Figure 1. Effect of indirect co-culture on the morphology of MCF10A.

Indirect co-culture with MCF10A cells and breast cancer cells (MCF7 or MDA-MB-231) were maintained for 10days at a ratio 3:7. Cells were observed after 10days by microscopy (A-C). Cells were reseeded in a confocal dish after indirect co-culture and stained with F-actin (Green) and DAPI (Blue). Localization of F-actin in MCF10A cells were examined by a confocal microscopy (D-F). Mono-cultured MCF10A (A, D), co-cultured MCF10A with MDA-MB-231 (B, E),

co-cultured MCF10A with MCF7 (C, F).

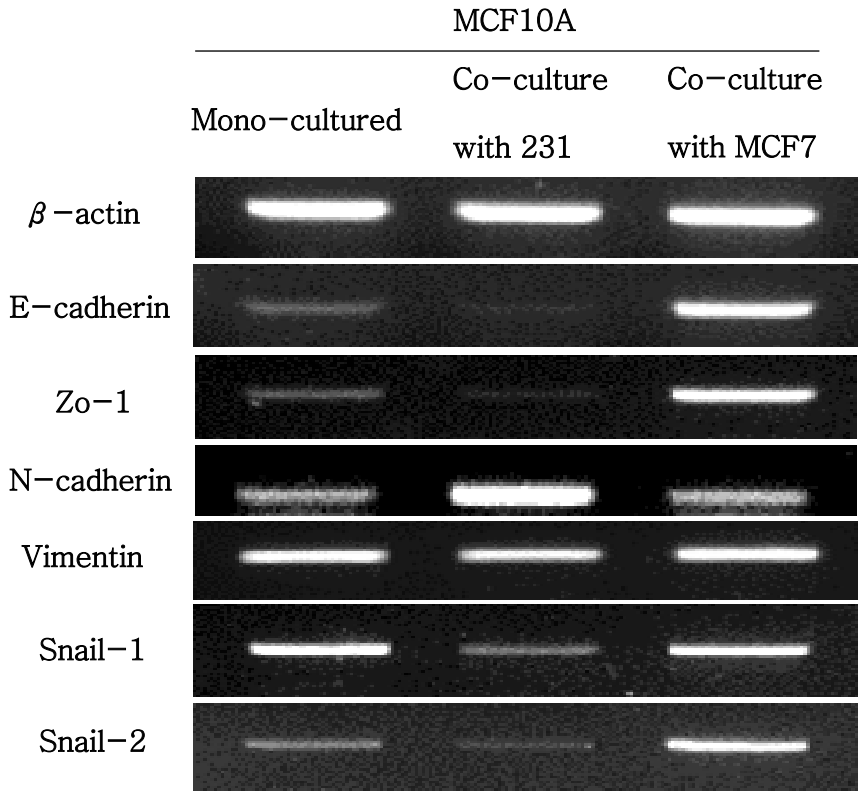


Figure 2. EMT related mRNA expression.

Total RNA was extracted from indirect co-cultured MCF10A or mono-cultured MCF10A with TRIzol reagent. Expression of EMT related mRNA was analyzed by RT-PCR. Primer information was shown in Table 1.

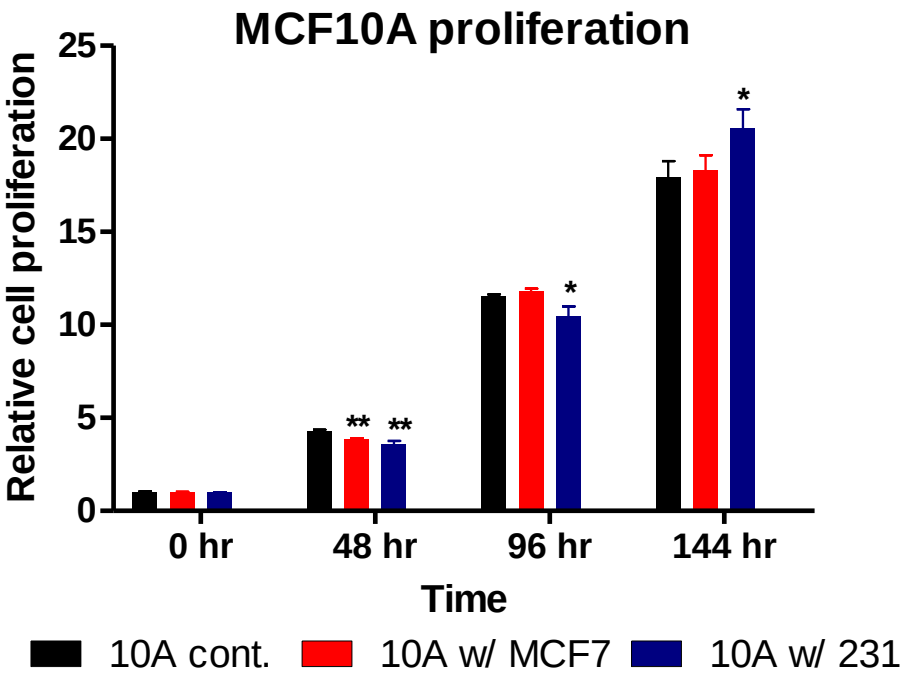


Figure 3. Effect of indirect co-culture on cell proliferation in MCF10A cells.

2,000 cells per well were seeded in a 96well plate in triplicated. Cell proliferation was measured using a CellTiter-Glo Luminescent Cell Viability Assay kit. (*p<0.05, **p<0.01)

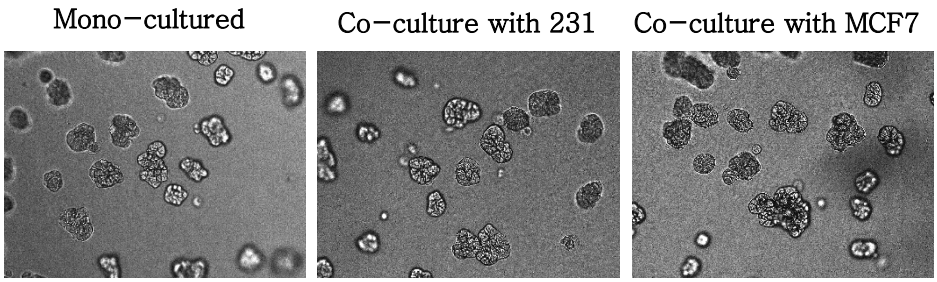


Figure 4. Morphology and growth of MCF10A cells in 3D matrigel culture.

Indirect co-cultured and mono-cultured MCF10A cells were embedded in matrigel (3mg/ml) for 3D culture. 5,000 cells per well were seeded in a 96 well plate. Media was changed every two days. Culture was maintained for 2 weeks. Images were taken on day 7 by a bright field microscopy (200x).

.

Ⅱ. Direct co-culture with MDA-MB-231 cells suppressed the expression of E-cadherin and enhanced proliferation, migration and colony formation ability.

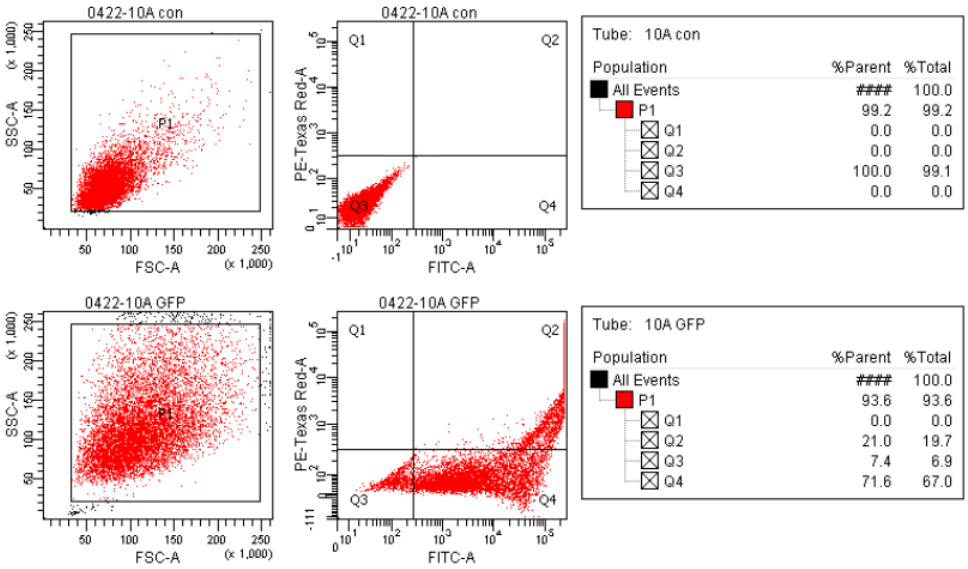
We did direct co-culture using two cell-lines. Before direct co-culture we tagged fluorescent proteins to each cell-line for distinction.

The number of MCF10A-GFP cells was fixed 120,000 cells. MCF10A cells were mixed with MDA-MB-231 cells at a ratio 3:7 and 7:3. Each mixture was seeded in a 60mm cell culture dish and 6well plate. After 10 days, cells were sorted with FACs AriaⅡ (Figure 5). Co-cultured MCF10A cells showed decreased cell-cell adhesion and grew dispersed (Figure 6). Direct co-culture with MDA-MB-231 cells suppressed the expression of E-cadherin in mRNA level (Figure7, 8) and protein level (Figure9). We did immunocytochemistry without FACs sorting. Two cell-lines were existed in the same chamber. When two cell-lines contact each other, the protein expression of E-cadherin in MCF10A cells was diminished (Figure 10). Direct co-cultured MCF10A cells were increased cell proliferation significantly in 2D and 3D matrigel culture (Figure 11 and Figure 12). In 3D matrigel culture, spheroids were larger and more irregular in direct co-cultured MCF10A, compared with mono-cultured MCF10A (Figure 12). Direct

co-culture with MDA-MB-231 cells also increased MCF10A cell's ability to form a colony from a single cell (Figure 13) and migration (Figure 14). We did direct co-culture with MDA-MB-231 cells and MCF10A cells for 40 days. MCF10A cells were directly co-cultured with MDA-MB-231 cells and were FACs sorted after 10 days. Isolated MCF 10A cells were reseeded with new MDA-MB-231 cells.

This step was repeated for 40 days. Direct co-culture with MDA-MB-231 cells increased the proportion of MCF10A cells at the end of co-culture (Figure 15).

A.


0422-10A con
SSC-A
FSC-A
(x 1,000)
P1
PE-Texas Red-A
FITC-A
Q1
Q2
Q3
Q4
Tube: 10A con
Population %Parent %Total
All Events #### 100.0
P1 99.2 99.2
Q1 0.0 0.0
Q2 0.0 0.0
Q3 100.0 99.1
Q4 0.0 0.0
0422-10A GFP
Tube: 10A GFP
Population %Parent %Total
All Events #### 100.0
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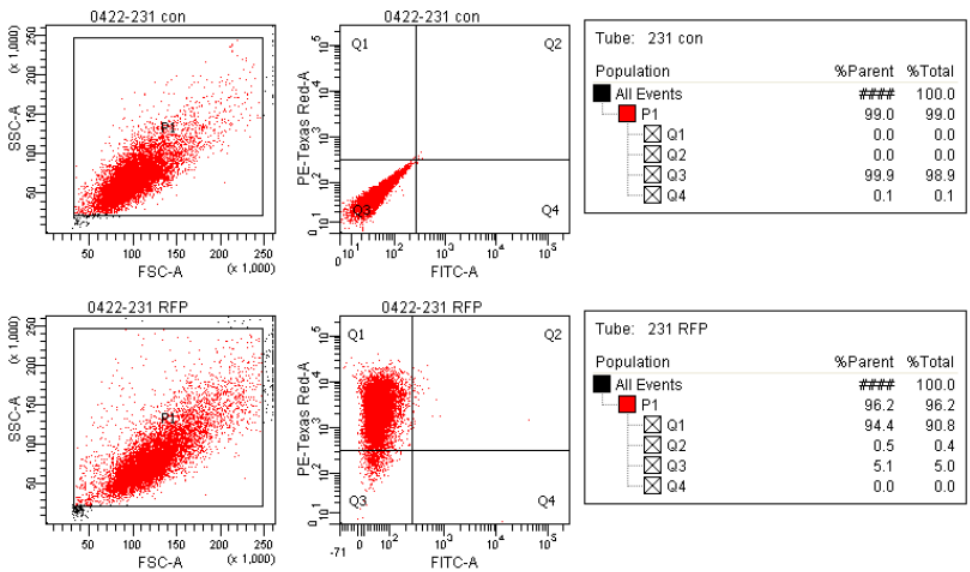
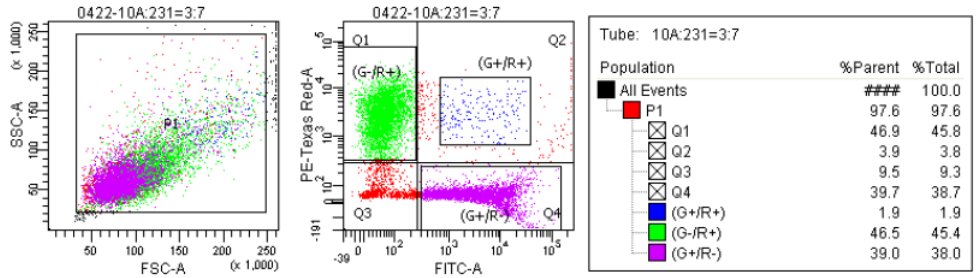
Q1 0.0 0.0
Q2 21.0 19.7
Q3 7.4 6.9
Q4 71.6 67.0

B.


0422-231 con
SSC-A
FSC-A
(x 1,000)
P1
PE-Texas Red-A
FITC-A
Q1
Q2
Q3
Q4
Tube: 231 con
Population %Parent %Total
All Events #### 100.0
P1 99.0 99.0
Q1 0.0 0.0
Q2 0.0 0.0
Q3 99.9 98.9
Q4 0.1 0.1
0422-231 RFP
Tube: 231 RFP
Population %Parent %Total
All Events #### 100.0
P1 96.2 96.2
Q1 94.4 90.8
Q2 0.5 0.4
Q3 5.1 5.0
Q4 0.0 0.0

C.



D.

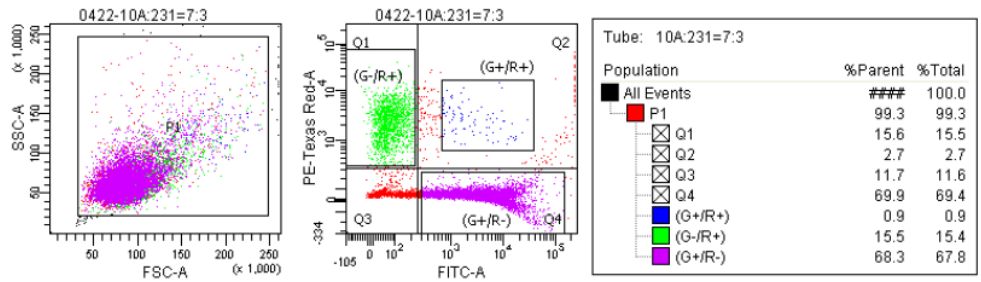


Figure 5. Direct co-culture with MCF10A cells and MDA-MB-231 cells were isolated by FACs.

The number of MCF10A-GFP cells was fixed 120,000 cells. MCF10A cells were mixed with MDA-MB-231 cells at a ratio 3:7 and 7:3. Each mixture was seeded in a 60mm cell culture dish and 6well plate. After 10 days, cells were sorted with FACs Aria Ⅱ. Mono-cultured MCF10A (A), Mono-cultured MDA-MB-231(B), Co-culture at a ratio 3:7(C), at a ratio 7:3(D).

A. B. C.

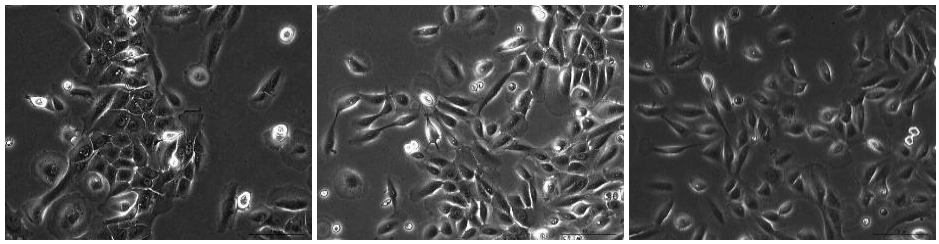


Figure 6. Effect of direct co-culture on the morphology of MCF10A.

Direct co-cultured MCF10A cells were reseeded after isolation by FACs sorter. Mono-cultured MCF10A (A), Co-cultured MCF10A with MDA-MB-231 at a ratio 7:3(B) and 3:7(C) (200x).

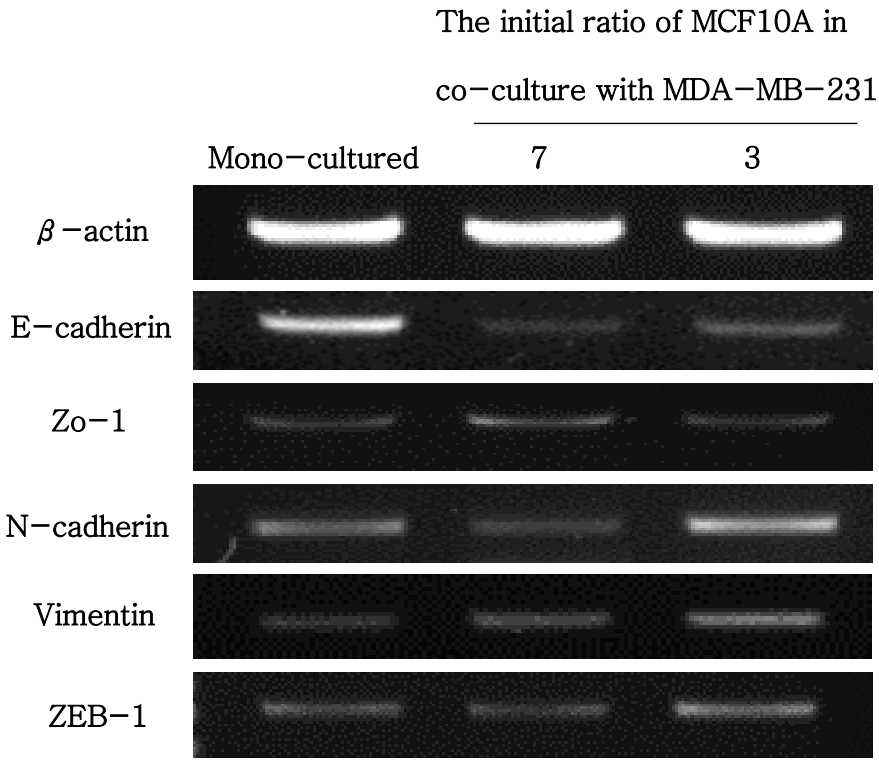


Figure 7. mRNA expression of EMT-related markers in direct co-cultured MCF10A.

Expression of EMT-related genes in MCF10A after direct co-culture with MDA-MB-231. The mRNA level was measured by RT- PCR.

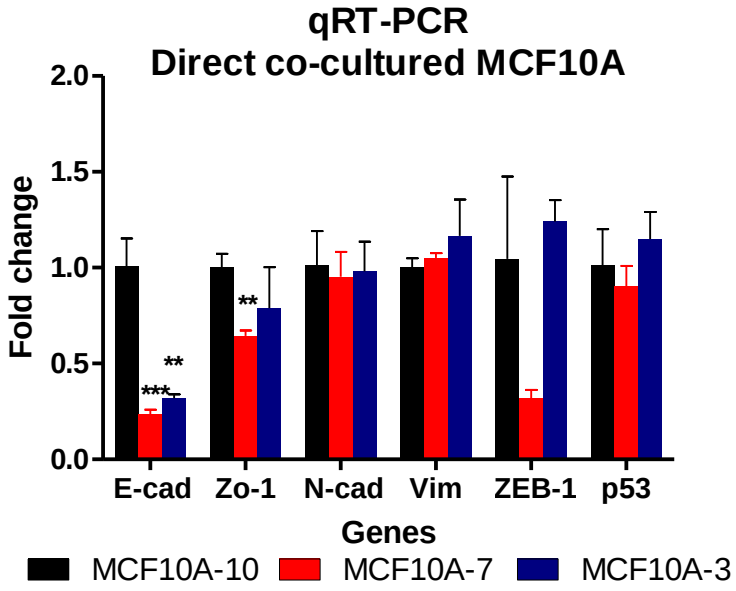
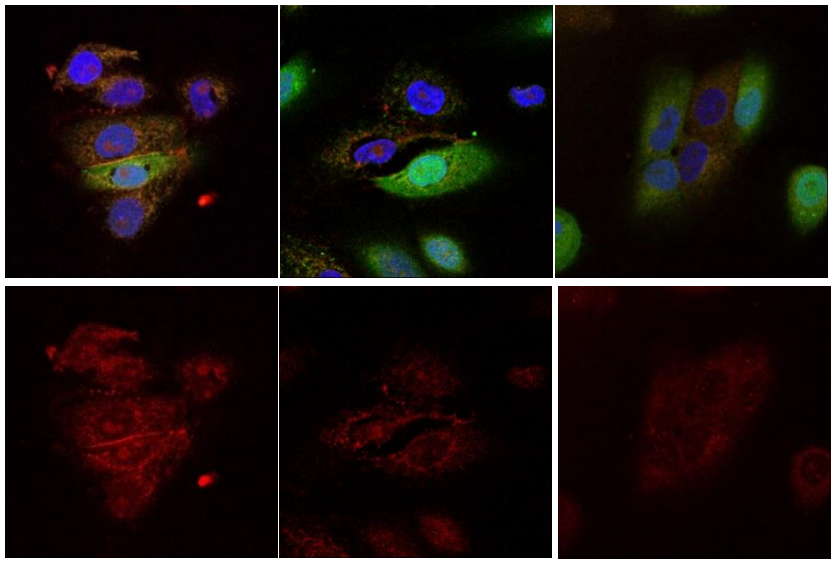


Figure 8. The expression of mRNA was measured by qRT-PCR.

Quantitative real-time PCR was also performed in triplicated. Each data was normalized to the expression of β-actin (B) (*P<0.05, **P<0.01, ***P<0.0001).

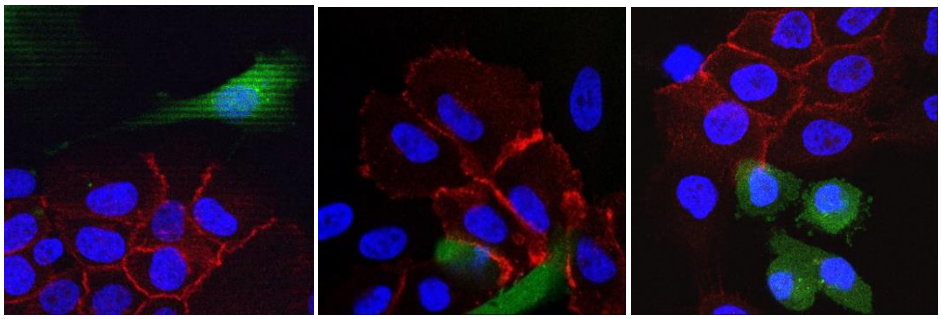
A. B. C.



MCF10A, E-cadherin, Nucleus

Figure 9. The protein level of E-cadherin was measured by immunocytochemistry assay in direct co-cultured MCF10A.

Immunocytochemistry of MCF10A-GFP (green), E-cadherin (red) and nucleus (blue) were shown. Co-cultured MCF10A cells at a ratio 7(B), 3(C) showed reduced E-cadherin expression comparing with mono-cultured MCF10A (A). Leica confocal microscopy was used with 40X*2.5.



MDA-MB-231, E-cadherin, Nucleus

Figure 10. E-cadherin expression was measured by immunocytochemistry when MCF10A-GFP and MDA-MB-231-RFP were co-cultured directly.

MCF10A cells and MDA-MB-231-GFP cells were seeded in 8 well confocal chambers. Cells were fixed and stained at day 2. Images were taken with 40x*2.5.

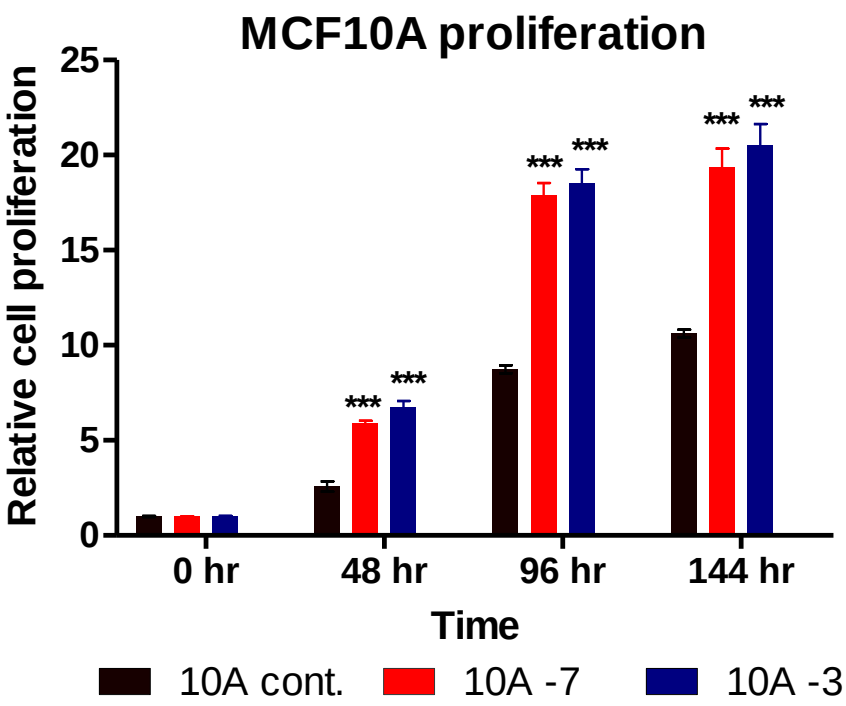
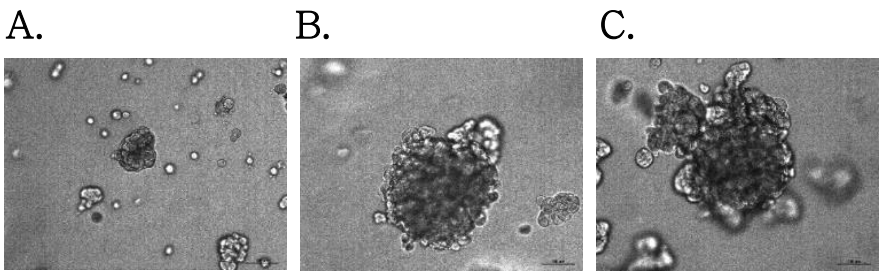


Figure 11. Direct co-culture effects on MCF10A proliferation.

MCF10A cells were directly co-cultured with MDA-MB-231 cells for 10days at a ratio 3:7 and 7:3. Cells were isolated using fluorescent protein by FACs. Isolated cells were seeded in a 96well plate. Proliferation was measured using a CellTiter-Glo Luminescent Cell Viability Assay kit (*P<0.05, **P<0.01, ***P<0.0001).



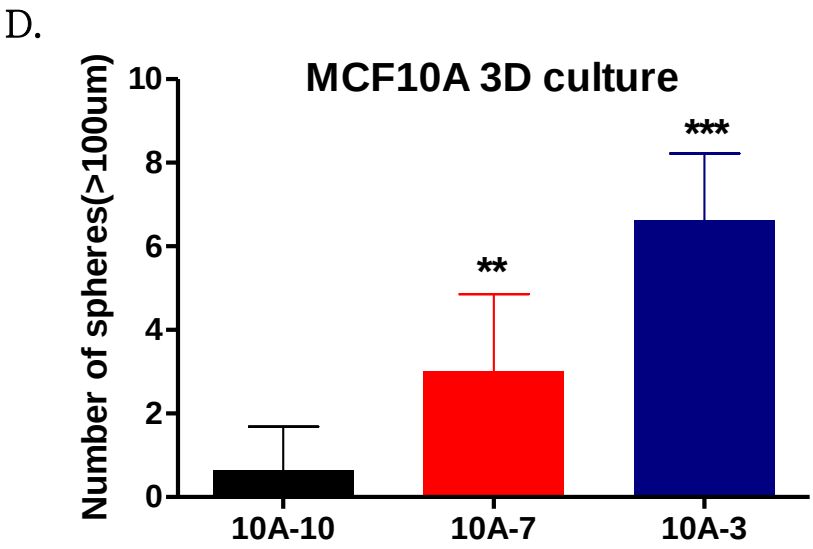


Figure 12. 3D matrigel culture of direct co-cultured MCF10A.

Mono-cultured and co-cultured cells were embedded in matrigel (3mg/ml). 3D matrigel culture was maintained for 14 days. Media was changed every 2 days. Mono-cultured MCF10A (A), Co-cultured MCF10A with MDA-MB-231 at a ratio 7:3(B) and 3:7 (C). Spheres with more than 100 μm diameter were counted (D).

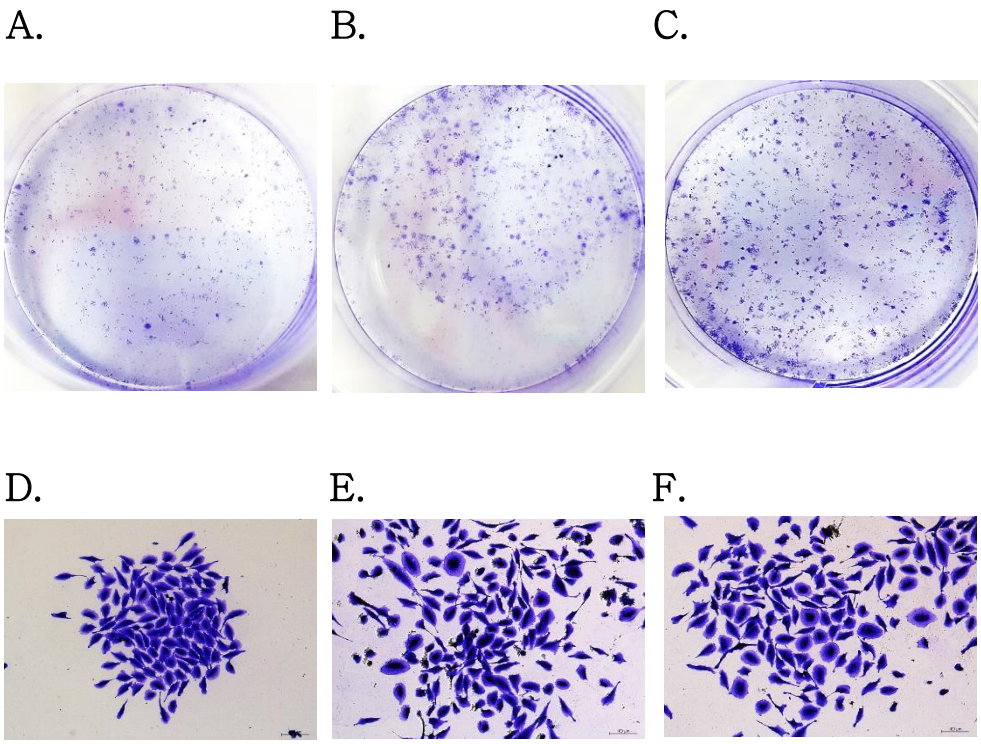


Figure 13. Colony formation assay for mono-cultured MCF10A and co-cultured MCF10A.

Sorted MCF10A cells (2,000 cells) were seeded in 6well plates in triplicated. Cells were cultured for 14 days and fixed with paraformaldehyde. 0.1% crystal violet was used for staining. Mono-cultured MCF10A (A, D), Co-cultured MCF10A with MDA-MB-231 at a ratio 7:3(B, E) and 3:7(C, F). The image for colony formation was

taken by a bright-field microscopy (200x) (D-F).

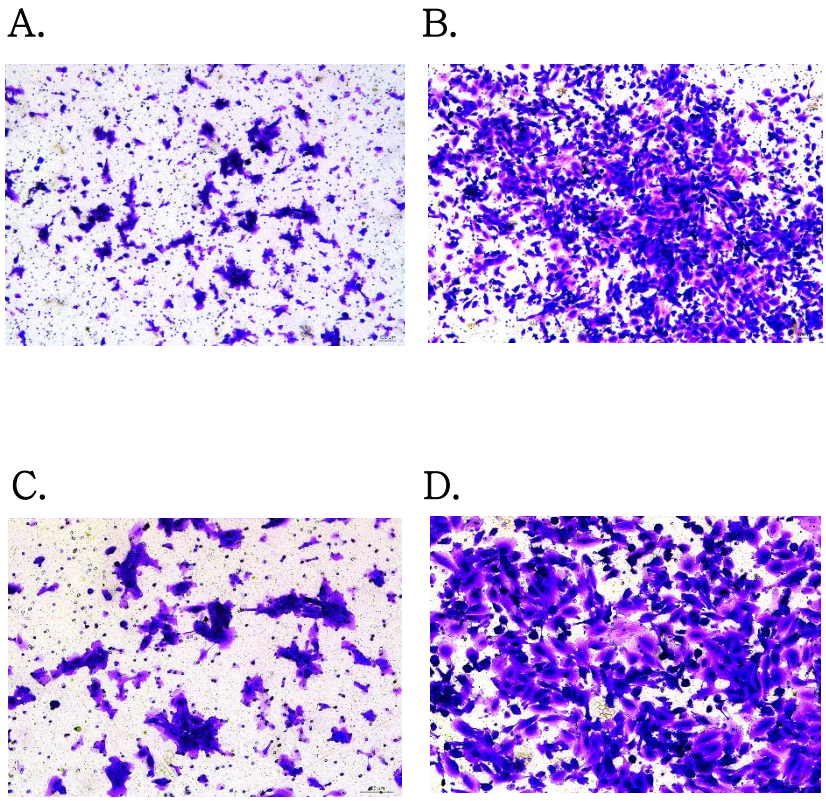
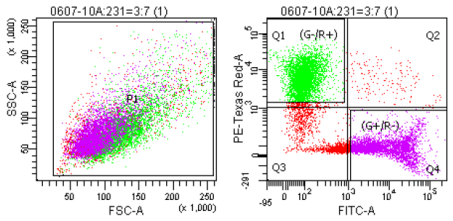


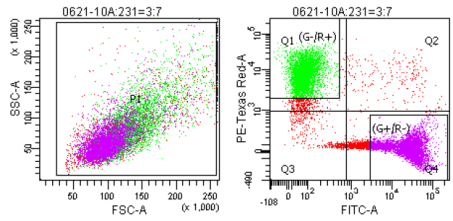
Figure 14. Migration assay about mono-cultured MCF10A and direct co-cultured MCF10A.

2 x 10^4 cells were seeded in an insert(8.0 μm pore size). Cells were fixed and stained after 20hrs. Mono-cultured MCF10A cells (A, C), Co-cultured MCF10A cells (with MDA-MB-231 cells at a ratio 3:7) (B,D). Upper images (50x), lower images (100x).

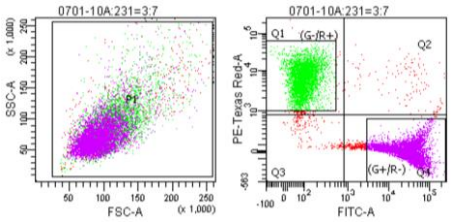
A.



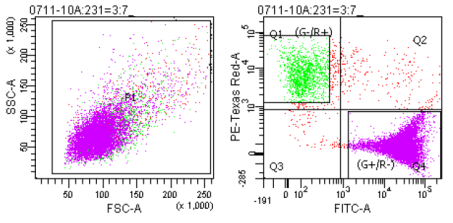
B.



C.



D.



E.

		1st	2nd	3rd	4th
MCF10A	Ratio	38%	40%	54.4%	81.3%
	Cell number	$1x10^6$	$1.9X10^6$	$4x10^6$	$5.7x10^6$
MDA-MB-231	Ratio	41.9%	40.7%	38.9%	13.5%
	Cell number	$2x10^6$	$2x10^6$	$3x10^6$	$1.8x10^6$

F.

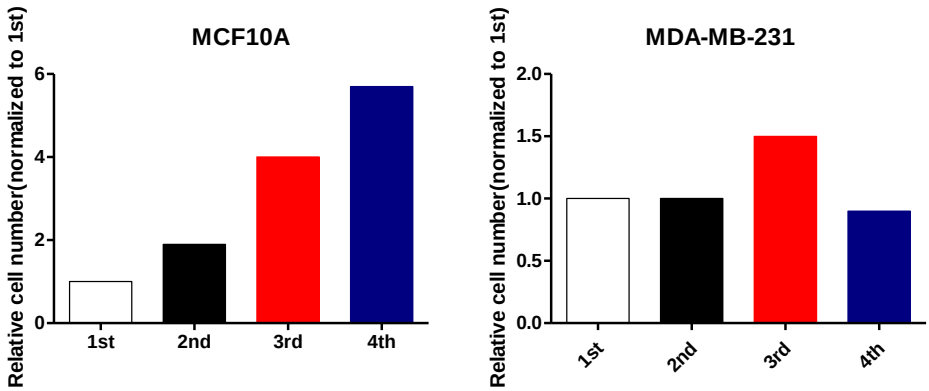


Figure 15. FACs data for series of direct co-cultures.

Direct co-culture with MCF10A and MDA-MB-231 cells at a ratio 3:7 were maintained for 10 days and sorted by FACs (MCF10A-GFP, MDA-MB-231-RFP). Isolated MCF10A cells and new MDA-MB-231 cells were co-cultured again as a same condition for a first time. This step was repeated four times. Co-culture period: 10days (A), 20days (B), 30days(C), 40days (D). FACs data were shown in a table (E) and graphs(F).

Ⅲ. Breast cancer cells cause phenotypical changes in MCF10A cells by vesicle transfer and direct contact.

During direct co-culture with MCF10A and MDA-MB-231 cells, MCF10A cells grew as a cluster and surrounded by MDA-MB-231 cells (Figure 16). To know, how two different cells communicate each other, we did live cell imaging using a confocal microscopy. MDA-MB-231 cells moved quickly while MCF10A cells were stationary. MDA-MB-231 cells stretched membrane nanotubes. Using nanotubes they touched other cells and transferred some molecules which look like micro vesicles to adjacent cells. Vesicular transport was more active between MDA-MB-231 cells than MDA-MB-231 cells and MCF10A cells. Vesicular transport between MDA-MB-231 cells were achieved quite long distance. There were three patterns when MCF10A cells and MDA-MB-231 cells communicate with each other. First, MDA-MB-

231 cells touched MCF10A cells and transferred molecules by using nanotubes (Figure 17A). Second, vesicles from MDA-MB-231 cells to MCF10A cells transferred when they were close each other (Figure 17B). Third, MCF10A cells moved to uptake particles from MDA-MB-231 cells. A part of MDA-MB-231 cells was extended and then cut. The detached one was engulfed by MCF10A cells (Figure 17C). We took z-stack images to make sure these molecules from MDA-MB-231 cells were existed in inside MCF10A cells (Figure 18).

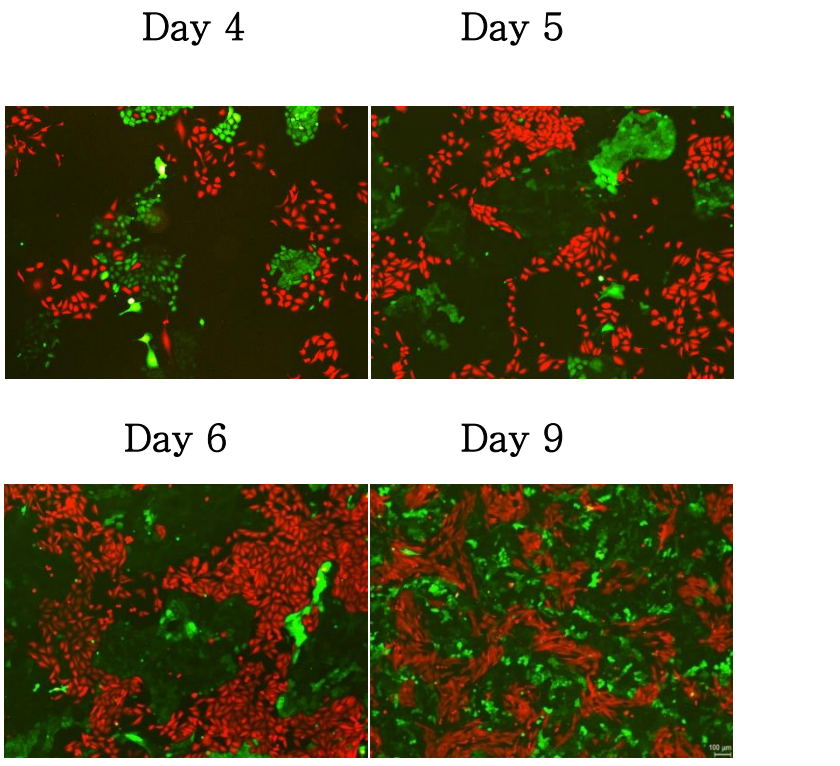
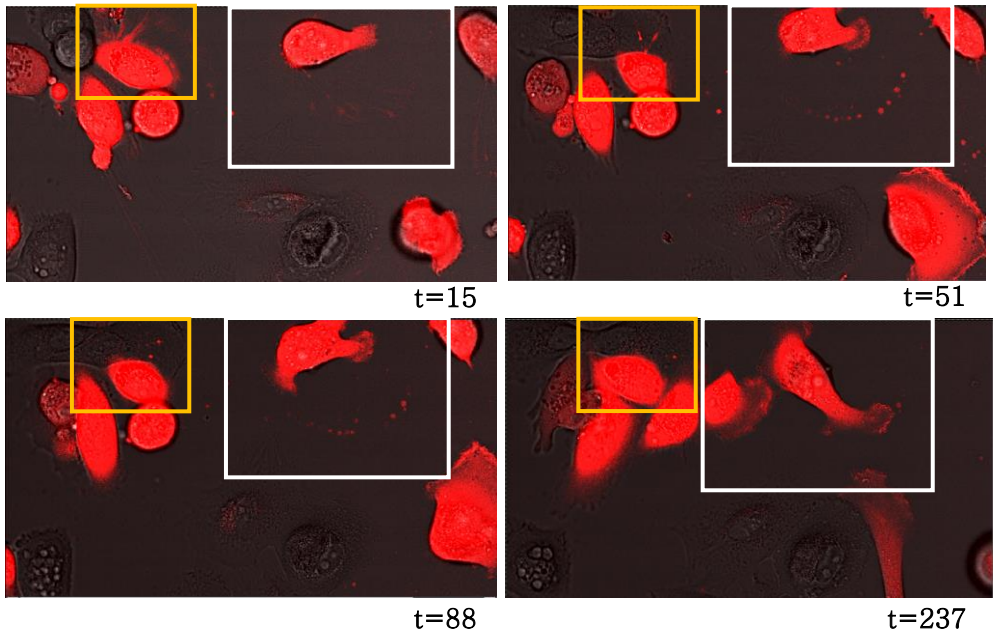


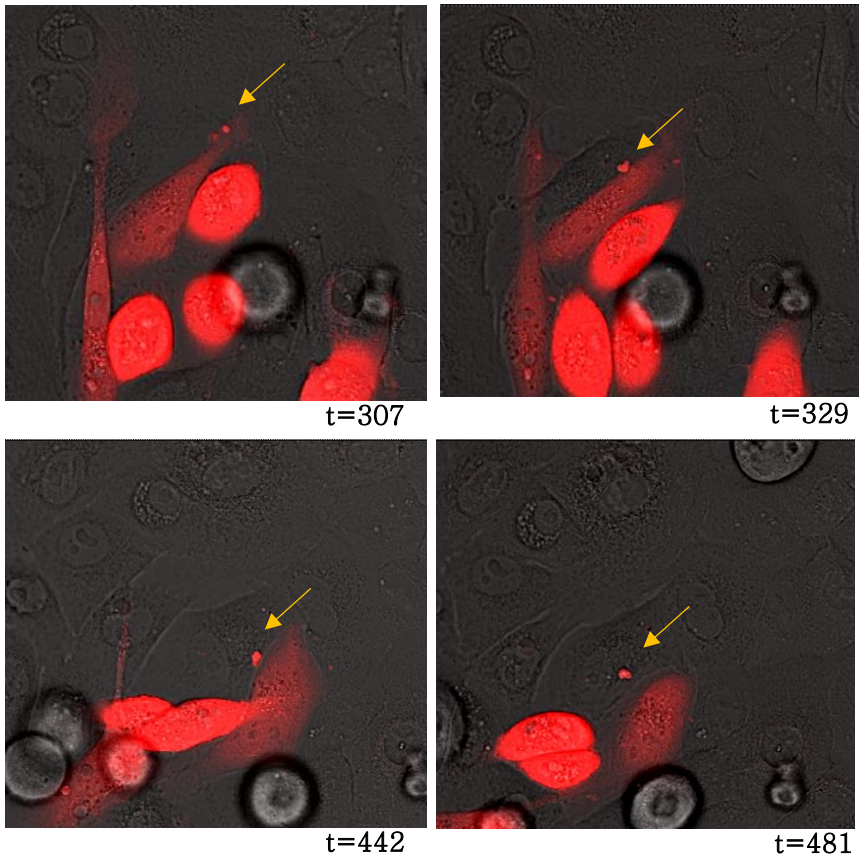
Figure 16. Cells were observed by fluorescent microscopy, during direct co-culture with MCF10A cells and MDA-MB-231 cells.

MCF10A-GFP cells and MDA-MB-231 cells were seeded in 6well plates at a ratio 3:7. Cells were observed by fluorescent microscopy. Representative images were shown (40x).

A.



B.



C.

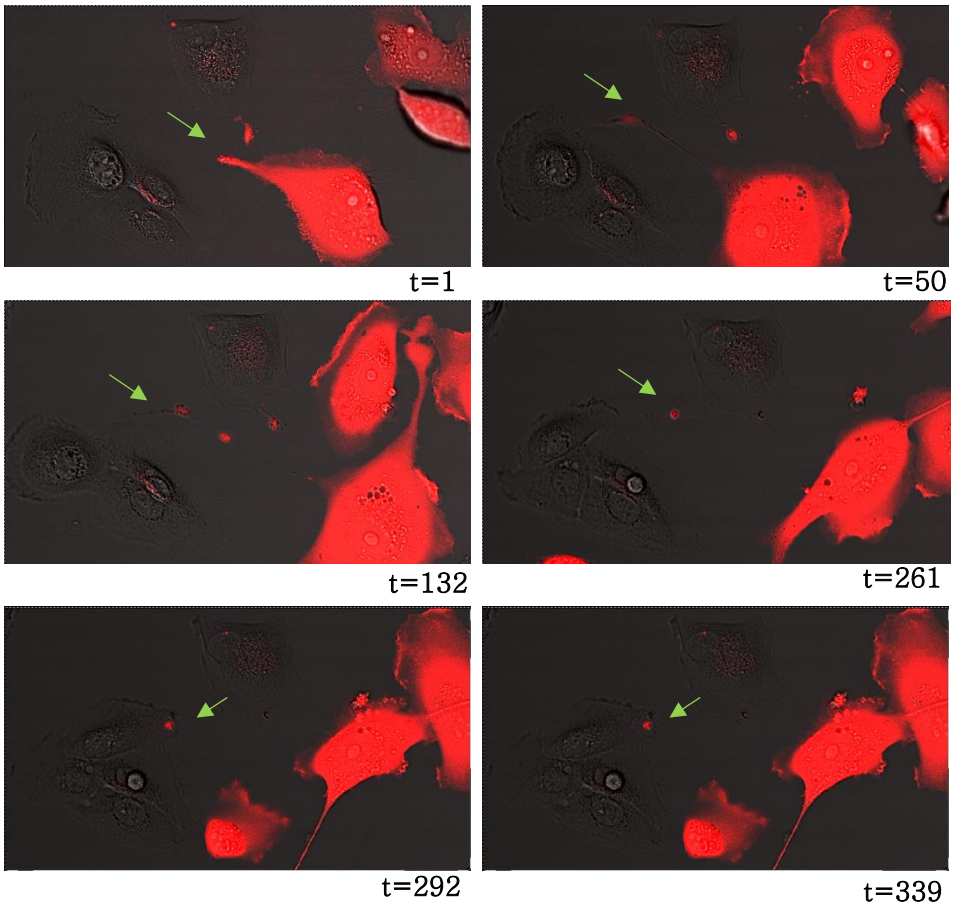
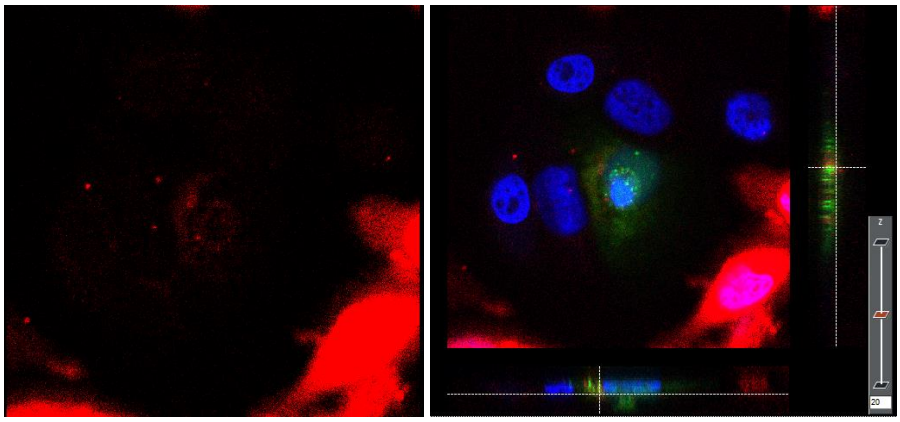


Figure 17. Cell movement was detected by a confocal microscopy. The position was fixed at a point.

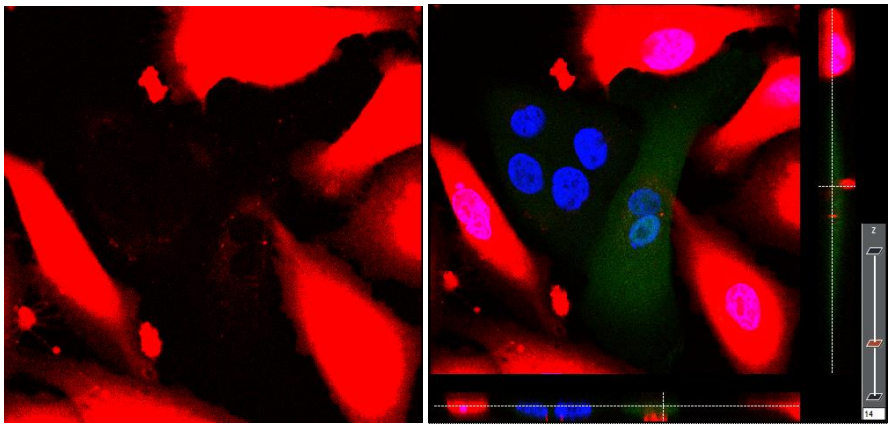
MCF10A-GFP and MDA-MB-231-RFP cells were seeded in a confocal chamber at a different ratio. Live cell imaging was taken every 2miniutes for 16hrs using a time lapse confocal microscopy

(1t=2min) (40x). To see the movement of vesicles more clear, green fluorescence was excluded in these images. Vesicles were transferred from MDA-MB-231 cells to MDA-MB-231 cells (white box) and from MDA-MB-231 cells to MCF10A cells (yellow box, yellow arrow) (A), (B). The part of MDA-MB-231 cells were engulfed by MCF10A cells(C).

A.



B.



MCF10A, MDA-MB-231

Figure 18. Z-stack images were taken by confocal microscopy.

(A) Total depth =16.60 μm, The depth of this image is 0.415 μm from bottom to top(400x*3). (B). Total depth =15.32 μm. The depth of this image is 0.414 μm from bottom to top (400x*2).

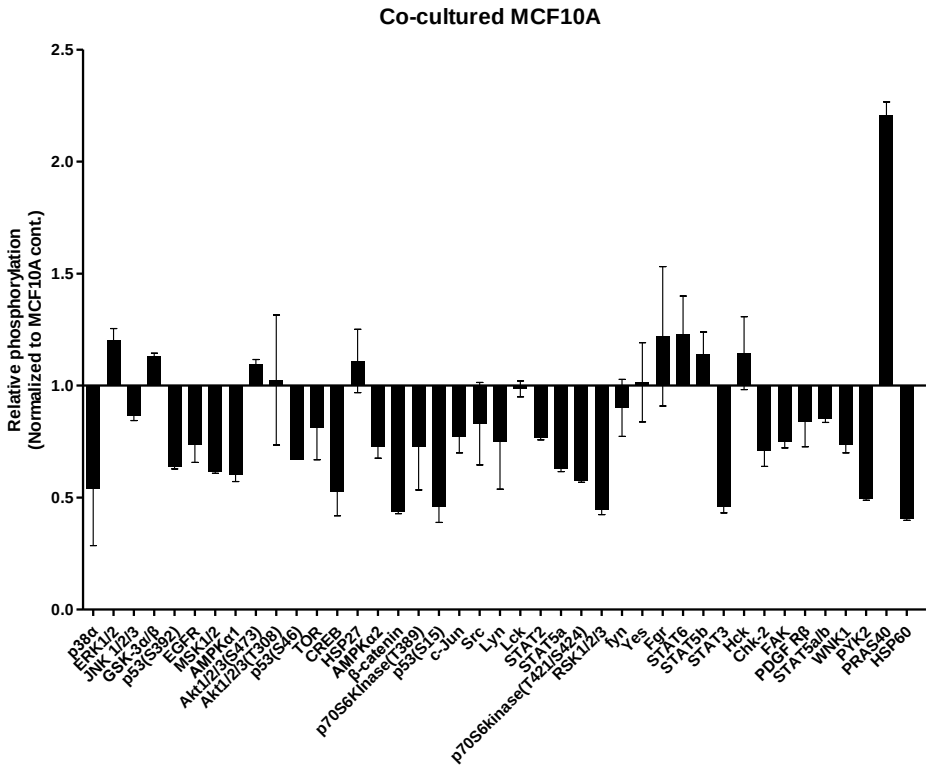
Ⅳ. AMPKα/p53/Akt/mTOR pathway may enhance direct co-cultured MCF10A proliferation.

To investigate proteome changes in direct co-cultured MCF10A, we performed phosphorylation assay (Figure19A, B). These data provide the levels of phosphorylated proteins. Many cellular signaling pathways were modulated in direct co-cultured MCF10A. Especially the protein levels of phospho-p53 (S392, S46, S15 residue) was downregulated in direct co-cultured MCF10A. p53 is activated and it arrests cell cycle in G1 check point, when cells were damaged. In co-cultured MCF10A, p-p53 was reduced so, it could not regulate cell cycle. It might promote abnormally cell growth in direct co-cultured MCF10A. We drew the expected pathway by putting data together (Figure 19C). PI3K/Akt pathway is known for having important role in tumor growth especially for proliferation. mTOR, the downstream of

Akt, is consisted of mTORC1 and mTORC2. PRAS40 (proline-rich Akt substrate 40) is one of the mTORC1 complex (10). In our data, p-AMPK α and p-p53, the downstream of AMPK α, was downregulated. p-Akt, which is inhibited by p-p53 was upregulated. Phosphorylated PRAS40 was upregulated more than two fold in direct co-cultured MCF10A (Figure 19C). This data suggests MDA-MB-231 cells promote the proliferation of MCF10A through AMPK α/p53/Akt/mTOR pathway.

A. Mono-cultured MCF10A Co-cultured MCF10A

B.



C.

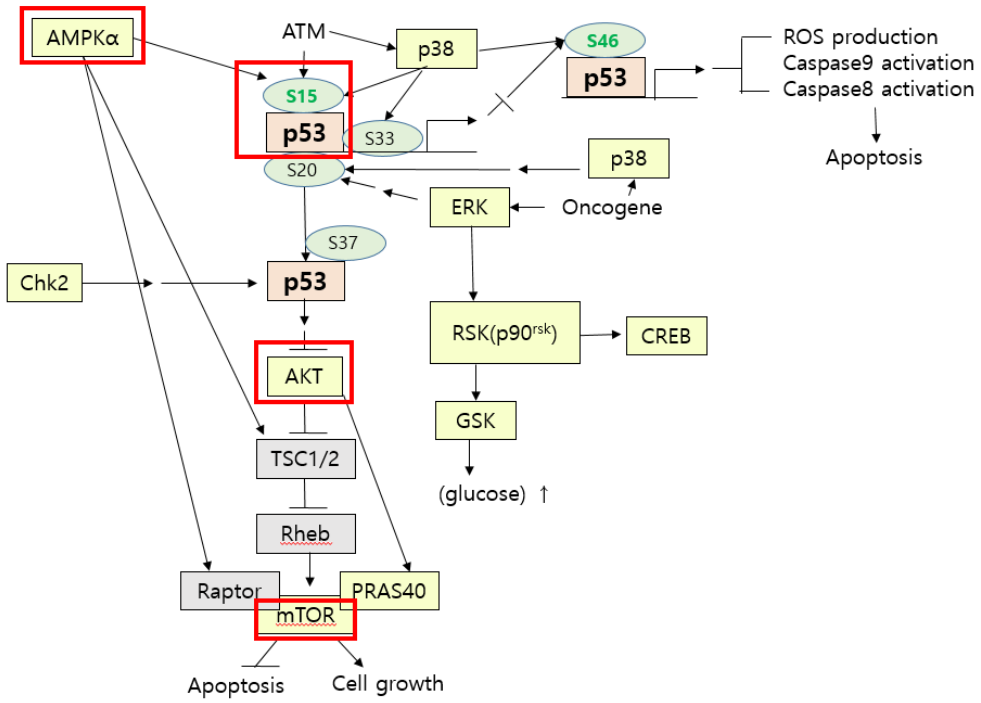


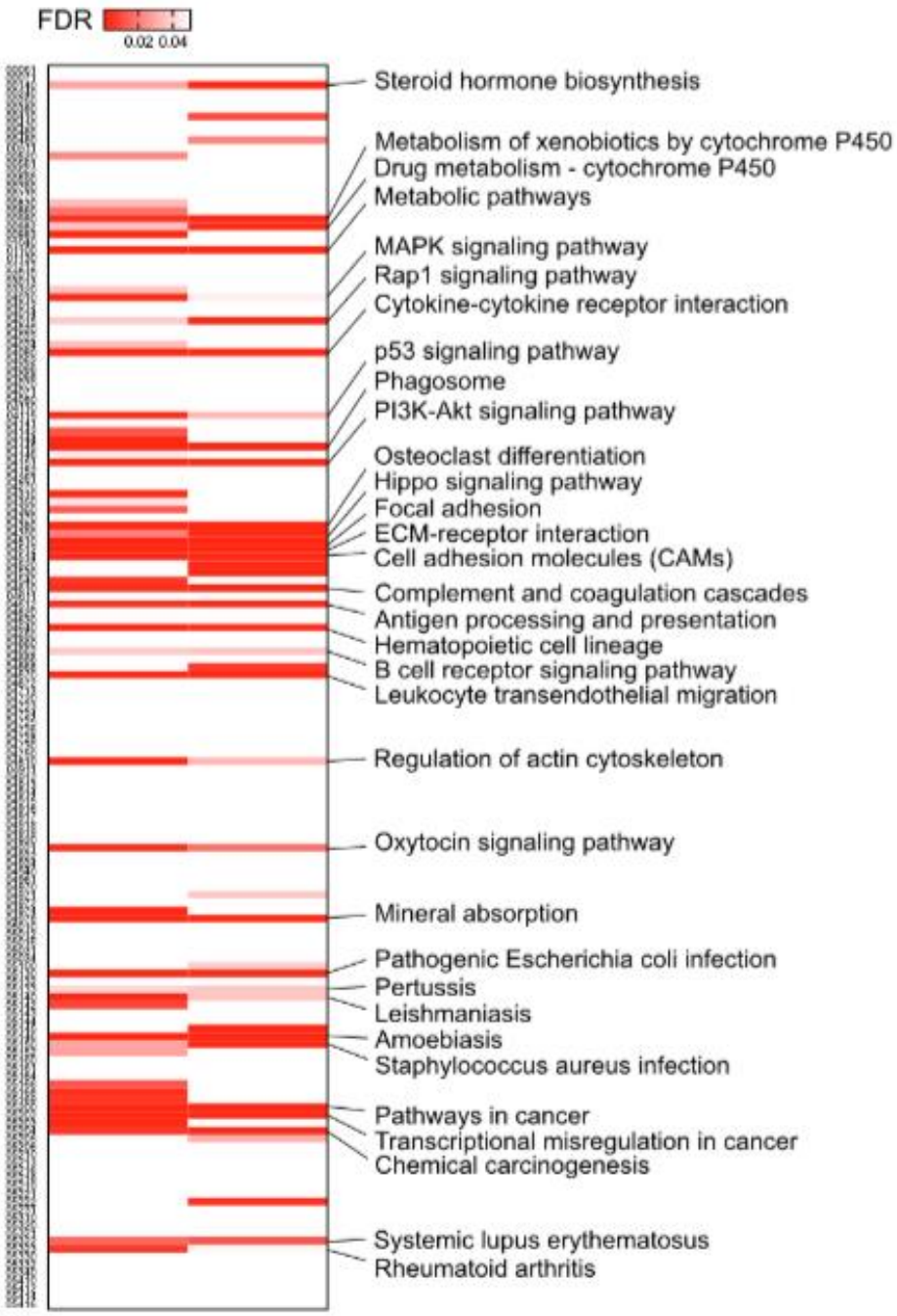
Figure 19. Proteome changes in direct co-cultured MCF10A.

$1x10^7$ cells were harvested with lysis buffer and cell lysates were measured using BCA assay kit. Spots on the membrane were visualized by an autoradiography with multiple exposure times (A). Pixel densities in each spot were measured by image J and normalized to reference spot and mono-cultured MCF10A (B). Expected pathway was drawn(C).

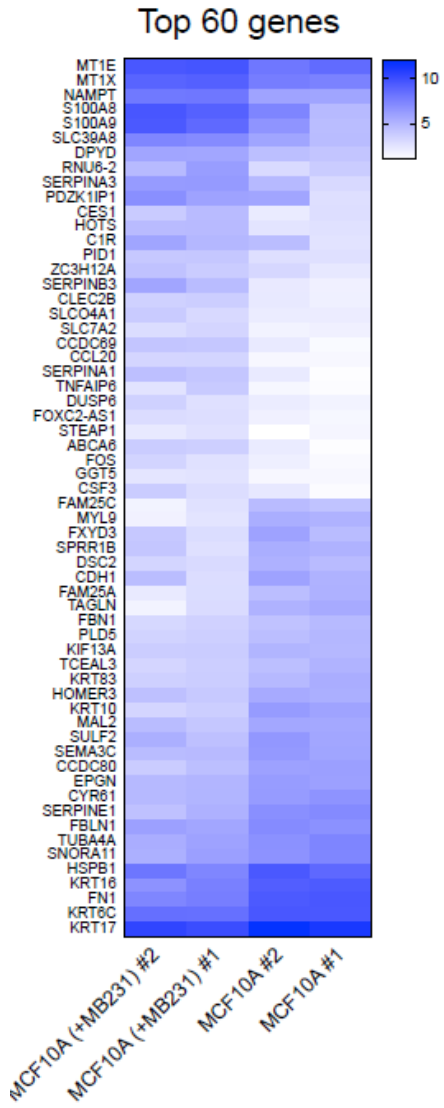
V. Breast cancer cells induced upregulation of S100A8/9 in direct co-cultured MCF10A cells.

We did global gene expression profiling to identify differentially expressed genes between mono-cultured MCF10A and direct co-cultured MCF10A. The KEGG pathway, which is based on mRNA sequencing, PI3K-Akt and p53 signaling pathways were associated with direct co-cultured MCF10A. It could be matched with phosphorylation array data. Cancer associated pathways were also increased in direct co-cultured MCF10A (Figure 19C, Figure 20A). Highly upregulated genes in co-cultured MCF10A with considering gene volume and fold changes, S100A8 and S100A9 were in the upper ranks of top 60 genes (Figure 20B). The mRNA and protein expression of S100A8 and S100A9 was also confirmed (Figure 20E).

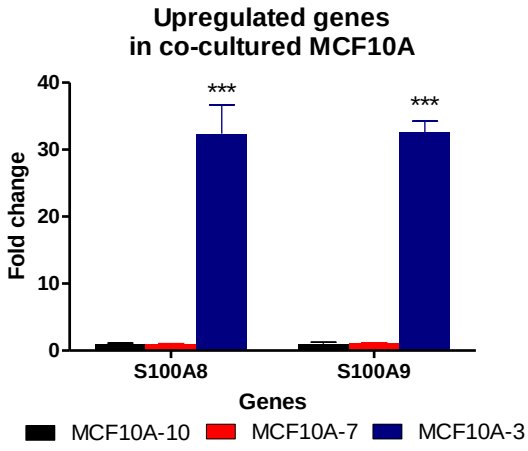
A.


FDR
0.02 0.04
Steroid hormone biosynthesis
Metabolism of xenobiotics by cytochrome P450
Drug metabolism - cytochrome P450
Metabolic pathways
MAPK signaling pathway
Rap1 signaling pathway
Cytokine-cytokine receptor interaction
p53 signaling pathway
Phagosome
PI3K-Akt signaling pathway
Osteoclast differentiation
Hippo signaling pathway
Focal adhesion
ECM-receptor interaction
Cell adhesion molecules (CAMs)
Complement and coagulation cascades
Antigen processing and presentation
Hematopoietic cell lineage
B cell receptor signaling pathway
Leukocyte transendothelial migration
Regulation of actin cytoskeleton
Oxytocin signaling pathway
Mineral absorption
Pathogenic Escherichia coli infection
Pertussis
Leishmaniasis
Amoebiasis
Staphylococcus aureus infection
Pathways in cancer
Transcriptional misregulation in cancer
Chemical carcinogenesis
Systemic lupus erythematosus
Rheumatoid arthritis

B.


Top 60 genes
MT1E
MT1X
NAMPT
S100A8
S100A9
SLC39A8
DPYD
RNU6-2
SERPINA3
PDZK1IP1
CES1
HOTS
C1R
PID1
ZC3H12A
SERPINB3
CLEC2B
SLCO4A1
SLC7A2
CCDC69
CCL20
SERPINA1
TNFAIP6
DUSP6
FOXC2-AS1
STEAP1
ABCA6
FOS
GGT5
CSF3
FAM25C
MYL9
FXYD3
SPRR1B
DSC2
CDH1
FAM25A
TAGLN
FBN1
PLD5
KIF13A
TCEAL3
KRT83
HOMER3
KRT10
MAL2
SULF2
SEMA3C
CCDC80
EPGN
CYR61
SERPINE1
FBLN1
TUBA4A
SNORA11
HSPB1
KRT16
FN1
KRT6C
KRT17
10
5
MCF10A (+MB231) #2
MCF10A (+MB231) #1
MCF10A #2
MCF10A #1

C.


Upregulated genes
in co-cultured MCF10A
Fold change
40
30
20
10
0
***
***
S100A8
S100A9
Genes
MCF10A-10
MCF10A-7
MCF10A-3

D.

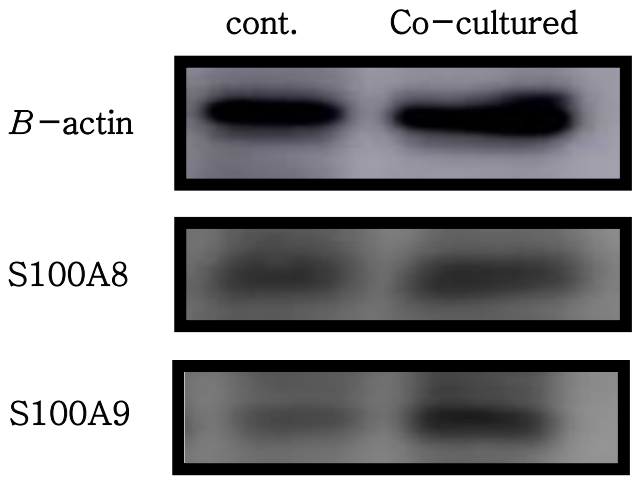


Figure 20. mRNA expression was measured in mono-cultured MCF10A and co-cultured MCF10A by mRNA sequencing and qRT-PCR. The protein level of S100A8 and S100A9 were detected by western blotting.

Total RNA was extracted from MCF10A cells after direct co-culture with MDA-MB-231 cells. We performed global gene expression profiling on both mono-cultured and direct co-cultured MCF10A. KEGG pathway which is based on mRNA sequencing for direct co-cultured MCF10A (A). Top 60genes were shown (B). qRT-PCR data

(C). Total cell lysates were measured by western blotting. The size of protein about S100A8 and S100A9 is 11kDa and 14kDa respectively. β-actin was used for control(D).

Discussion

Normal breast is comprised of epithelial cells and stromal cells (11). The interaction between two types of cells via ECM is important to progress or suppress of breast cancer (12). It is well known that tumor microenvironment has critical role in development of cancer. Many studies were shown cancer associated fibroblasts, cancer associated adipocytes or immune cells promote breast cancer progression (12-15). There was a study about physical contact with HER-2 positive breast cancer cell and mesenchymal stem cell activate tumor growth and resistance of trastuzumab by src pathway. Their method was similar with us (16).

To study how cancer cells affect adjacent normal cells, we used direct co-culture and indirect co-culture methods with two cell-lines. We found that MDA-MB-231 breast cancer cells touch and transfer vesicles to MCF10A normal mammary epithelial cells. So, we guessed molecular changes in MCF10A cells were associated with such a course of action. From the protein expression and mRNA expression data, we knew that S100A8 and S100A9 expression were upregulated and p-p53 protein level was downregulated in direct co-cultured MCF10A cells.

S100A8 and S100A9 are calcium-binding proteins and form a homodimer or heterodimer. Heterodimer of S100A8 and S100A9 called calprotectin. There was a report about calprotectin in breast cancers. In this report, S100A8 and S100A9 had a critical role to promote oncogenesis in HER2 transformed MCF10A cells (17). MBweiss et al (2010) reported p53 knock down in MCF10A cells induce enhanced proliferation and chromosome instability but do not undergo transformation (18).

We are studying the function of S100A8/A9, but we do not know the relationship between S100A8/A9 and p53 yet. Therefore, further study is needed that if S100A8/9 and p53 expression were changed, they could induce same phenotypical changes as direct co-cultured MCF10A.

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국문초록

상피 암이 주변 정상상피세포에 미치는 영향에 대해서 명확히 연구가 되어있지 않다. 본 연구에서는 주변 암세포에서 분비되는 인자들에 의해 정상 유선 상피세포가 molecular transformation을 일으킬 수 있다고 가정하였다. MCF10A 정상유선세포와 MDA-MB-231 유방암 세포를 직접적, 간접적 방법으로 공생배양 하였다. 간접적 공생배양에서는 MDA-MB-231 세포가 MCF10A 세포에 미미한 영향만 주었다. 하지만, 암세포와 정상세포 사이에 물리적 접촉이 일어날 수 있도록 직접적인 공생배양을 하였을 때, MCF10A 세포의 세포증식, 콜로니 형성, migration 능력이 증가하는 등, 상당한 표현형 변화가 일어났다. 또한, 다양한 EMT 관련 marker들의 발현이 증가되는 것도 확인되었다. Live cell imaging 을 이용해 직접적 공생 배양 시, 암세포와 정상세포들이 Cell projection 과 extracellular vesicle 들을 통해 다양한 상호작용을 하는 것을 관측했다. 이러한 현상과 관련된 특정 유전자와 경로를 찾기 위해서

phosphorylation assay 와 RNA sequencing analysis 를 하였다.

Phosphorylation assay 를 통해 p-p53(S392, S46, S15 residue) 발현이 직접적 공생 배양한 MCF10A 세포에서 감소되어 있는 것을 확인하였고, global gene expression profile을 통해 몇몇 keratin 유전자의 발현이 감소되어 있고, S100A8과 S100A9의 발현이 증가되어있음을 확인하였다. 실험결과를 통해, MDA-MB-231 세포가 MCF10A 세포에 물리적 접촉과 vesicle 전달을 하여 S100A8/A9 발현 증가와 p53 발현 감소를 일으키고, 이로 인해 MCF10A 세포의 세포주기가 파괴되고 proliferation을 촉진한다는 것을 알 수 있었다. S100A8/9와 p53이 포함된 특정 경로를 찾는 연구가 추가적으로 필요하다.

주요 단어: 유방암; 정상유선상피세포; S100A8/A9; p53

학번: 2015-22063