Study on the Effect of Obesity on Epithelial Defense against Cancer (EDAC)

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Abbreviations

Acetyl-CoA	acetyl coenzyme A
BSA	bovine serum albumin
CK19	cytokeratin-19
CRE	cyclization recombination
DMEM	Dulbecco's modified Eagle's medium
DNMT1	DNA (cytosine-5)-methyltransferase 1
DMSO	dimethyl sulfoxide
EDAC	epithelial defense against cancer
EPLIN	epithelial protein lost in neoplasm
ER	estrogen receptor
F-actin	filamentous actin
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
HFD	high-fat diet
HRAS	Harvey sarcoma virus-associated oncogene
IRES	internal ribosome entry site
KRAS	Kirsten sarcoma virus-associated oncogene
LA	linoleic acid
LoxP	locus of X-ing over of P1
LSL	LoxP-STOP-LoxP
MDCK	Madin-Darby canine kidney
ND	normal diet
NRAS	neuroblastoma RAS viral oncogene
PA	palmitic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDK4	pyruvate dehydrogenase kinase 4
PDH	pyruvate dehydrogenase
Ras	rat sarcoma
SA	stearic acid
SD	standard deiation
Tam	tamoxife
TMRM	tetramethylrhodamine methyl ester
TMZ	trimetazidine
Tet	tetracycline
WT	wild type
αLA	alpha-linolenic acid

Abstract

Recent studies have revealed that newly emerging transformed cells are often eliminated from epithelial tissues via cell competition with the surrounding normal epithelial cells. This cancer preventive phenomenon is termed Epithelial Defense Against Cancer (EDAC). However, it remains largely unknown whether and how EDAC is diminished during carcinogenesis.

In this study, using a newly established cell competition mouse model, I show that high-hat diet (HFD)-feeding substantially attenuates the frequency of apical elimination of RasV12-transformed cells from intestinal and pancreatic epithelia, leading to the formation of precancerous tumors. This process involves both lipid metabolism and chronic inflammation. Furthermore, aspirin treatment significantly facilitates eradication of transformed cells from the epithelial tissues in HFD-fed mice.

This is the first report demonstrating that obesity can profoundly influence competitive interaction between normal and transformed cells, providing new insights into cell competition and cancer preventive medicine.

Chapter 1:

GENERAL INTRODUCTION

1. General Introduction

1.1. The Idea Behind This Study

Cancer is one of the leading cause of death globally, and was responsible for 8.8 million deaths in 2015. Globally, nearly one in 6 deaths is due to cancer. In the case of Japan, one in 5 deaths is due to cancer and one in two people get a cancer. Recently, there are various treatment methods and therapeutic drugs for cancer thanks to a lot of findings and theories from many studies for cancer. Because of that, some types of cancer can be diagnosed at early stage and can be cured. However, cancer has been overcome only a little, and it has been required that the more development of new therapeutic methods or drugs. To get a breakthrough cures for cancer, it is important to know how cancer is made, what molecules involved in the becoming malignancy and why our body loses to cancers.

Recently, one interesting findings about defense mechanism against cancer at early stage of carcinogenesis were reported. These reports showed the newly emerging oncogenic transformed cells are eliminated from the epithelia by a competitive behavior with surrounding normal epithelial cells, and this phenomenon is considered as a new defense mechanism against cancer equipped in the epithelia. And, the findings have been expected to give a new strategy for cancer treatment and/or cancer prevention and deeper understandings about carcinogenesis.

At the same time, such a competitive behavior between transformed cells

and surrounding normal epithelial cells is generally classified into a “cell competition”. Cell competition is defined as a competitive behavior between different types of cells to induce cell death or elimination of loser cells and expansion of winner cells (Figure 1.1).

Cell competition was firstly discovered in *Drosophila Melanogaster* in 1975 (Morata and Ripoll, 1975). So far, it is known that cell competition is related in various biological events including development, aging and cancer prevention (Maruyama and Fujita, 2017; Moreno, 2008; Vincent et al., 2013). Summary about cell competition are given below, briefly.

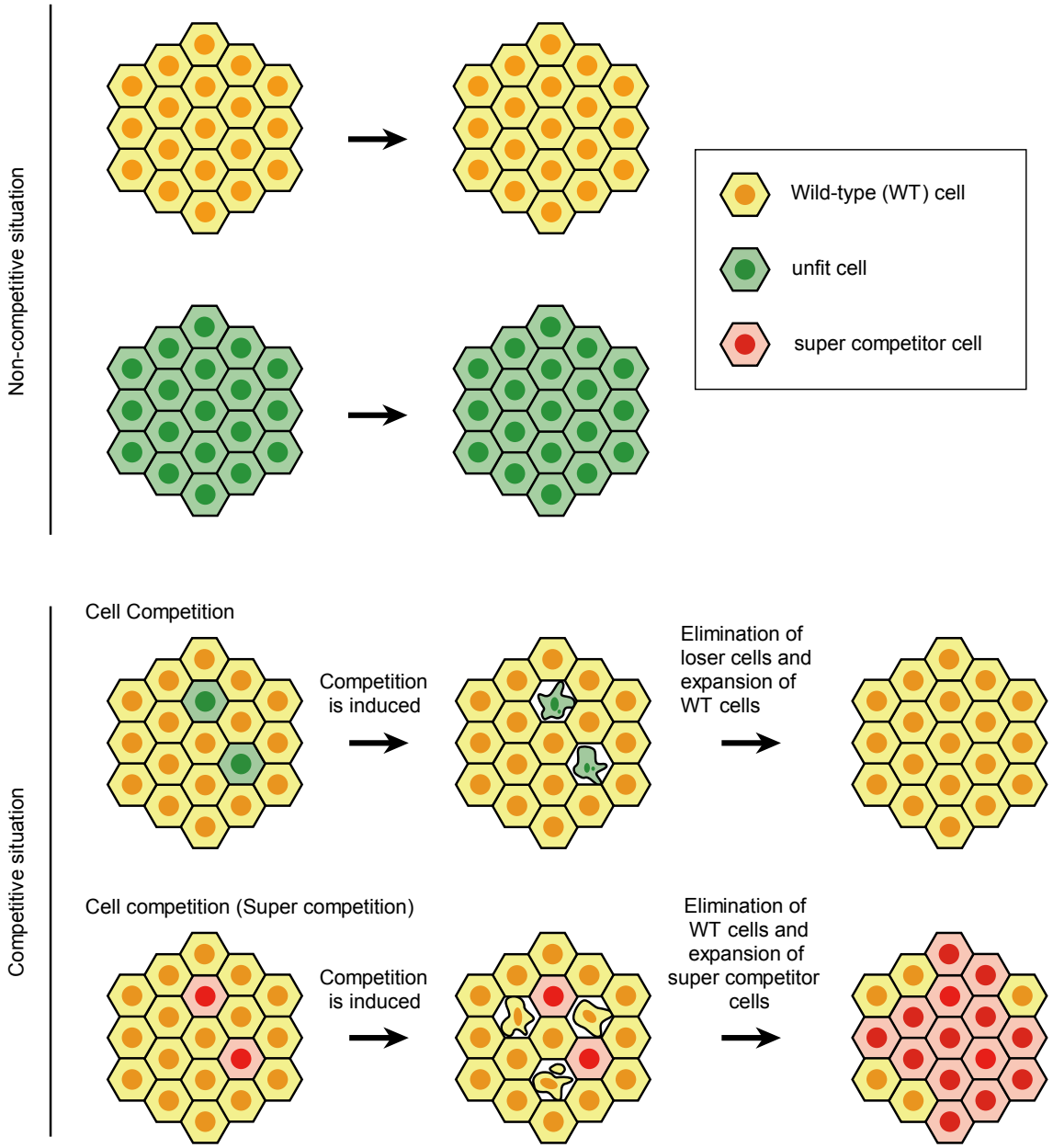


Figure 1.1 Conceptual model of “Cell Competition”

1.2. Cell Competition

1.2.1. Cell competition in *Drosophila*

1.2.1.1. Minute

Cell competition was discovered in the imaginal discs of *Drosophila Melanogaster* in 1975 (Morata and Ripoll, 1975). According to the paper, in the situation of co-existence of wild-type epithelial cells and mutated cells which have a mutation in minute gene, which encodes a ribosomal protein, mutated cells which contacted to neighbor wild-type cells is died, and surrounding normal cells compensatory proliferate (Figure 1.1). After that, the adult is made from only wild-type cells despite there were two types of cells at early stage of development. However, minute mutated cells can generate adult without visible abnormalities under the existence of mutated cells alone (Figure 1.1). In other words, the results indicate that the existence of interaction and competitive phenotype between wild-type cells and minute mutated cells. The fact showed the sociality in the living cells, like as a human society.

It is known that in the case of the cell competition derived from minute mutation, the competitive behavior was caused by the difference about the speed of cell division.

1.2.1.2. dMyc

dMyc, known as an oncogenic gene, also involved in cell competition. It was reported by two independent groups in 2004 (de la Cova et al., 2004;

Moreno and Basler, 2004). The difference of the dMyc expression levels induces cell competition. Cells with higher level of dMyc expression became "winner", and cells which express dMyc in the lesser level are eliminated by neighboring winner cells and become "loser" (de la Cova et al., 2004; Moreno and Basler, 2004). And a big difference between minute-derived cell competition and dMyc-derived cell competition is whether the cells can be a super competitor or not. Even if the number of dMyc high-expressing cells surrounded by wild-type cells are very few, the dMyc cells can kill the neighboring wild-type cells and can compensatory growth (figure 1.1). On the other hand, minute mutated cells cannot kill the surrounding cells and are died.

So far, some molecular mechanisms about the cell competition mediated by dMyc is revealed (Vincent et al., 2013). One is the recognition mechanism between winner and loser cells. To be sensed the dMyc lower expressing cells as loser cells, a protein, named Flower, is used like "loser marker". Flower has some isoforms. When the cells which must be loser were genarated, the specific Flower isoforms, fwe^{LoseA} and fwe^{LoseB}, are expressed at plasma membranes of loser cells. And the loser cells are sensed by winner cells and eliminated by apoptosis (Rhiner et al., 2010).

1.2.1.3. Other genes

In *Drosophila Melanogaster*, other genes which induce cell competition are identified (Table 1.1). Not only mutations in genes related to cell growth, but also mutations in genes involved in cell polarity (Scribble, Lgl) and

endocytosis (Vps25) leads to cell death.

Table 1.1 Triggers of cell competition in *Drosophila Melanogaster*.

Mutation	Phenotype	References
Minute	Apostosis of Minute$^{+/-}$ cells	(Morata and Ripoll, 1975)
Scribble	Apoptosis of Scribble-knockdown cells	(Brumby and Richardson, 2003)
dMyc	Apoptosis of dMyc-lower expressing cells	(Moreno and Basler, 2004) (de la Cova et al., 2004)
Vps25	Apoptosis of Vps25 mutant cells	(Thompson et al., 2005)
Csk	Apoptosis and basal exclusion of Csk-deficient boundary cells	(Vidal et al., 2006)
Ras	Apoptosis of Ras^{N17} cells	(Prober and Edgar, 2000)
	Apical or basal extrusion of Ras-transformed cells	(Hogan et al., 2009)
Lgl/Mahjong	Apoptosis of Lgl/Mahjong-knockdown cels	(Grzeschik et al., 2010) (Tamori et al., 2010)

1.2.2. Cell competition in mammal

1.2.2.1. Cell competition at the initial stage of carcinogenesis

Cell competition is also observed in mammals. Mammalian cell competition was firstly reported under the concept, what happens in the initial stage of carcinogenesis (Hogan et al., 2009). Previous cancer studies using cultured cells had been performed about cancer cells alone or normal cells alone. However, newly emerging transformed epithelial cells are surrounded by normal epithelial cells at the initial stage of carcinogenesis. The investigation of interactions between transformed cells and surrounding normal cells was the purpose of the research.

In the study, they used special culture method. When two different types of cells are cultured together, cell sorting occurs and the same type of cells form colonies (Steinberg and Takeichi, 1994) (Foty and Steinberg, 2005) (Krieg et al., 2008). Under such a cell sorted condition, it is difficult to make mosaic patterns like the situation of the most initial stage of carcinogenesis. To avoid such sorting phenotype, they used a genetic technique in Mardin-Darby canine kidney (MDCK) cell lines, and they established a new MDCK cell line, MDCK-pTR-GFP-RasV12 cells, which expressing oncogenic Ras in a tetracycline inducible manner (Figure 1.2). This cell line and parental MDCK cell line can be mixed in a mosaic manner in absence of tetracycline. By the treatment of tetracycline after the formation of a monolayer, the situation of initial stage of carcinogenesis can be mimicked.

Using this culture system, it was revealed that the newly emerging RasV12-

transformed cells are apically extruded from the monolayer. Because such apically elimination was not observed in a condition cultured Ras-transformed cells singly, it was revealed that the apical extrusion was caused by the interaction between Ras-transformed cells and surrounding normal MDCK cells. This competitive manner belongs to cell competition, and it is considered a new defense mechanism against carcinogenesis within normal epithelial tissues because the eliminated cell is newly generated transformed-epithelial cells.

So far, about other genes involved in carcinogenesis including other oncogene genes (Src and ErbB2 etc.) and tumor suppressor genes (Scribble, Mahjong etc.), the cell lines were also established by almost same strategy, and competitive behavior was observed, respectively (Table 1.2).

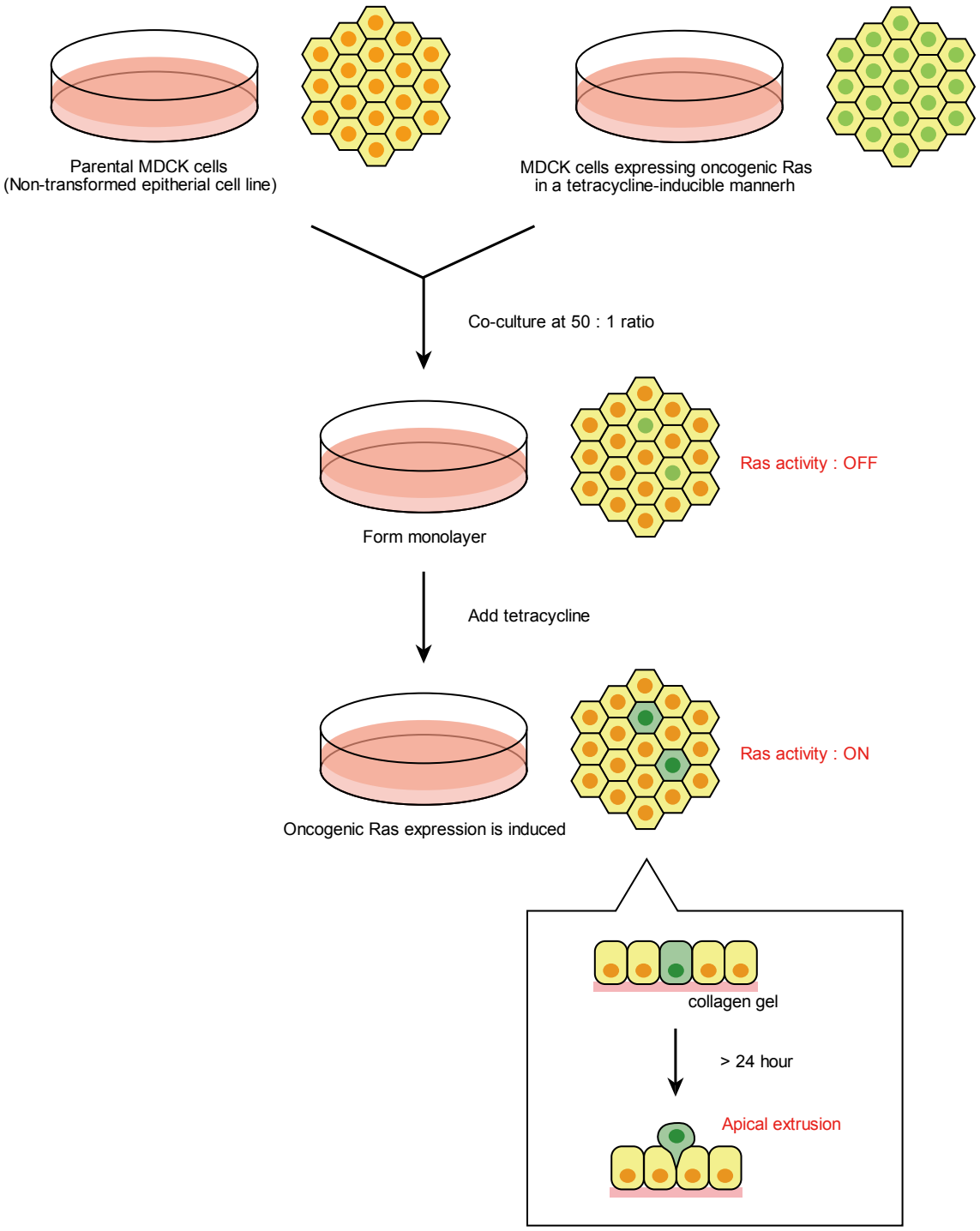


Figure 1.2. A strategy for the study of mammalian cell competition

Table 1.2. Cell competition in mammalian cell culture systems

Mutation	Phenotype	References
Ras	Apical extrusion or basal protrusion of Ras-transformed cells	(Hogan et al., 2009)
Src	Apical extrusion of Src-transformed cells	(Kajita et al., 2010)
Mahjong	Apoptosis of Mahjong-knockdown cells	(Tamori et al., 2010)
Scribble	Apoptosis of Scribble-knockdown cells	(Norman et al., 2012)
ErbB2	Translocation and clonal expansion of ErbB2-overexpressing cells	(Leung and Brugge, 2012)
Cdc42	Apical extrusion by expression of the constitutively active cells	(Grieve and Rabouille, 2014)
Yap	Apical extrusion by expression of the constitutively active form	(Chiba et al., 2016)

1.2.2.2. Ras gene

In this thesis, I focused on cell competition derived from oncogenic Ras expression. Ras family proteins are classified into a small GTPase superfamily that regulates multiple cellular processes including cell proliferation, differentiation, and motility. In humans, three isoforms about *RAS* genes have been identified, and these three *RAS* are known as famous oncogenic genes. *HRAS* is Harvey sarcoma virus-associated oncogene (Harvey, 1964), *KRAS* is Kirsten sarcoma virus-associated oncogene (Kirsten and Mayer, 1967), and *NRAS* was identified from neuro blastoma and leukaemia cell lines. Point mutations in these three genes are often observed in various cancers including large intestinal cancer, biliary tract cancer, and pancreatic cancer (Table 1.3). Mutations in Ras protein mainly occurred in fixed positions as follows: glycine 12 (G12), glycine 13 (G13), and glutamine 61 (Q61) (Cox and Der, 2010).

Ras is a member of small GTPase superfamily which regulated by the state of GTP/GDP switches (Figure 1.3). Its GDP form which is inactive form turn into GTP form which is active form by guanine nucleotide exchange factors (GEFs). Conversely, GTP form is changed to GDP form by GTPase activating proteins (GAPs). In means the activity of Ras is regulated by GEFs and GAPs. On the other hand, Ras proteins having a point mutation including G12, G13 and Q61 is out of control by GEFs and GAPs, and are constitutively activated. Activated Ras induces a lot of downstream pathways such MAPK pathway, PI3K pathway etc. (Figure 1.3).

Table 1.3. Frequency of RAS isoform mutations in human cancers. Data from the COSMIC database from 2017/12/02. Yellow cells mean > 5% mutation is found.

Tissue	Point Mutation								
	HRAS			KRAS			NRAS		
	Tested	Mutated	%	Tested	Mutated	%	Tested	Mutated	%
Adrenal gland	44	1257	4%	1	1288	0%	10	1231	1%
Autonomic ganglia	5	1134	0%	1	1094	0%	11	1212	1%
Biliary tract	2	709	0%	710	3179	22%	28	926	3%
Bone	7	897	1%	11	1001	1%	13	1039	1%
Breast	30	4519	1%	86	6215	1%	11	4185	0%
Central nervous system	8	3153	0%	32	3711	1%	25	3642	1%
Cervix	24	603	4%	72	1157	6%	4	533	1%
Endometrium	6	2027	0%	602	4043	15%	26	1180	2%
Eye	0	284	0%	4	405	1%	27	736	4%
Fallopian tube	0	2	0%	0	7	0%	0	4	0%
Female genital tract (site indeterminate)				0	2	0%			
Gastrointestinal tract (site indeterminate)	0	1	0%	70	1083	6%	0	477	0%
Genital tract	2	227	1%	23	293	8%	8	283	3%
Haematopoietic and lymphoid	65	10206	1%	938	18080	5%	2154	22090	10%
Kidney	4	2183	0%	25	2651	1%	9	2372	0%
Large intestine	20	3881	1%	24207	73532	33%	457	12239	4%
Liver	2	2514	0%	67	3079	2%	16	2764	1%
Lung	22	5010	0%	6051	37681	16%	100	13869	1%
Meninges	0	169	0%	0	203	0%	16	230	7%
NS (not specified)	7	969	1%	68	1519	4%	434	2357	18%
Oesphagus	5	2261	0%	36	2656	1%	1	1735	0%
Ovary	2	1241	0%	800	6273	13%	19	1433	1%
Pancreas	1	2408	0%	6194	10869	57%	15	2764	1%
Paratesticular tissues				0	1	0%			
Parathyroid	1	135	1%	0	136	0%	0	135	0%
Penis	2	28	7%	1	29	3%	0	28	0%
Peericardium				0	2	0%	0	2	0%
Perineum	0	1	0%	0	1	0%	0	1	0%
Peritoneum	0	93	0%	168	340	49%	1	120	1%

Pituitary	12	361	3%	0	357	0%	0	357	0%
Placenta	0	3	0%	0	11	0%	0	3	0%
Pleura	0	199	0%	5	325	2%	3	233	1%
Prostate	39	2434	2%	97	3186	3%	9	2464	0%
Salivary gland	83	647	13%	10	526	2%	4	400	1%
Skin	542	5185	10%	120	4809	2%	2047	13470	15%
Small intestine	0	64	0%	238	930	26%	2	151	1%
Soft tissue	64	1742	4%	99	3067	3%	54	1688	3%
Stomach	15	2467	1%	302	5452	6%	11	1638	1%
Testis	5	360	1%	25	677	4%	9	571	2%
Thymus	7	296	2%	9	552	2%	3	372	1%
Thyroid	325	7454	4%	198	9050	2%	614	8372	7%
Upper aerodigestive tract	163	2984	5%	84	4218	2%	40	2627	2%
Urinary tract	270	3112	9%	100	2201	5%	25	1914	1%
Vagina	0	1	0%	0	4	0%	0	2	0%
Vulva	16	162	10%	2	186	1%	0	162	0%

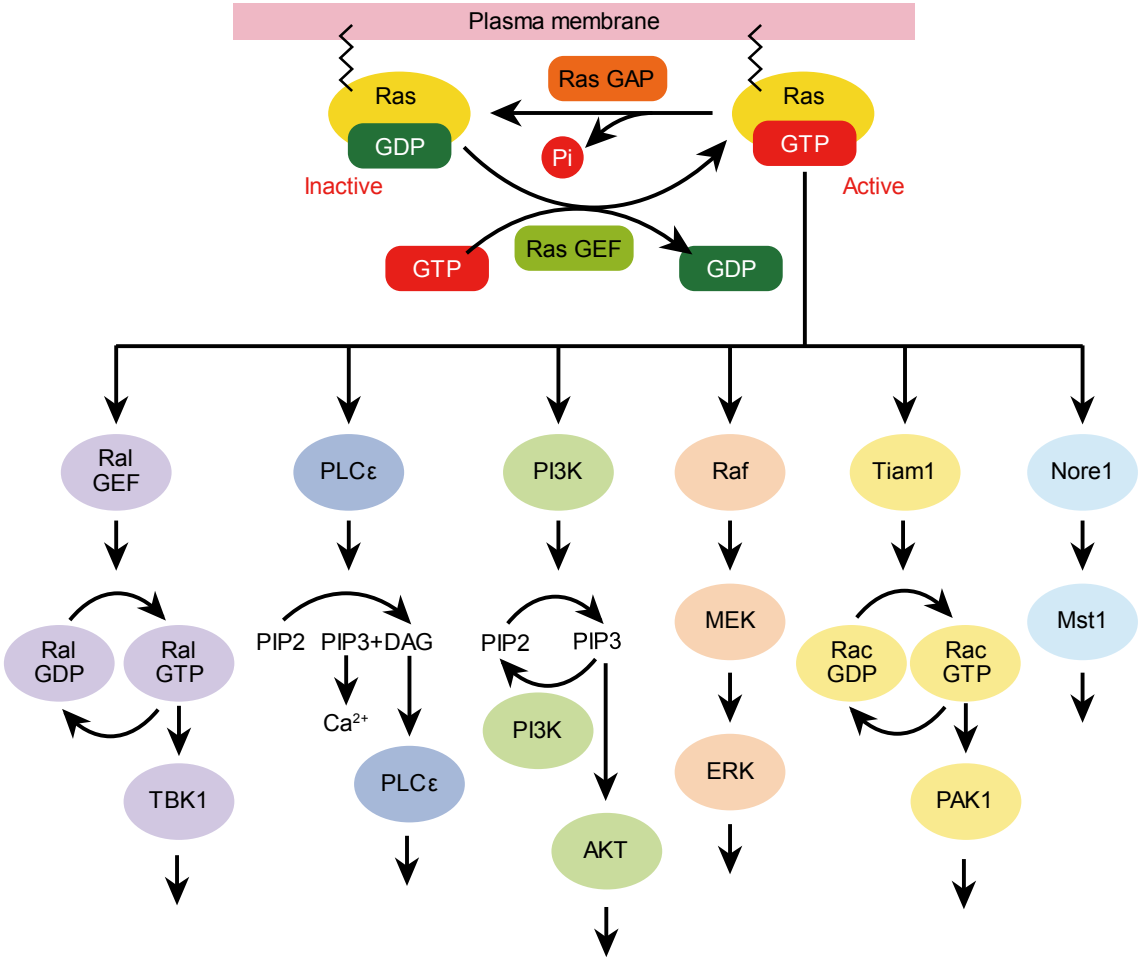


Figure 1.3. Ras activation and downstream signaling

1.2.2.3. Epithelial Defense against Cancer (EDAC)

In the case of the cell competition in mammals, the elimination occurs in transformed cells under the mixed culture condition, while the elimination is not observed when transformed cells cultured alone. It suggests that the normal epithelial tissues have a role for the defense mechanism against cancer. However, it is important that whether the elimination of transformed cells occur in active or passive manner to know the ability of defense mechanism. That is to say, it should be reveal whether the surrounding normal epithelial cells actively eliminate transformed cells or not.

So far, some molecular mechanisms which can be evidences for the involvement of surrounding normal cells in apical extrusion were reported as follows (Kajita et al., 2010)(Kon et al., 2017)(Ohoka et al., 2015)(Yamamoto et al., 2016).

1.2.2.3.1. Cytoskeletal organization

To reveal the changes in surrounding normal cells, biochemical screening was performed, and filamin, which is actin binding protein, and vimentin, which is an intermediate filament, was identified (Kajita et al., 2014)(Kajita and Fujita, 2015). This study revealed that filamin and vimentin are strongly accumulated at the boundary between transformed cells and normal cells, and the accumulation occurred within surrounding normal cells. And it also showed that filamin regulate vimentin reorganization as a downstream, and vimentin generate force to push transformed cells.

The active role of surrounding normal epithelial cells in the elimination of transformed cells was named “epithelial defense against cancer (EDAC)”.

1.2.2.3.2. Mitochondrial dysfunction

Recently, it was also reported that, glycolysis is elevated, but the mitochondrial activity decreased in the Ras-transformed cells surrounded by normal cells, and the metabolic changes promote apical extrusion of transformed cells (Figure 1.4) (Kon et al., 2017). The mitochondrial dysfunction is caused by the inactivation of pyruvate dehydrogenase (PDH). PDH inactivation is caused by the accumulation of phosphorylated pyruvate dehydrogenase kinase 4 (PDK4). The phosphorylation of PDK4 is induced by the accumulation of epithelial protein lost in neoplasm (EPLIN) in Ras-transformed cells. EPLIN is accumulate in the RasV12-transformed cells by the filamin within neighboring normal cells (Figure 1.4) (Ohoka et al., 2015). In other words, the filamin accumulation in surrounding normal cells which is caused by the interaction between RasV12-transformed cells and surrounding normal epithelial cells also induces the mitochondrial dysfunction, other than reorganization of vimentin, and promotes apical extrusion (Figure 1.4).

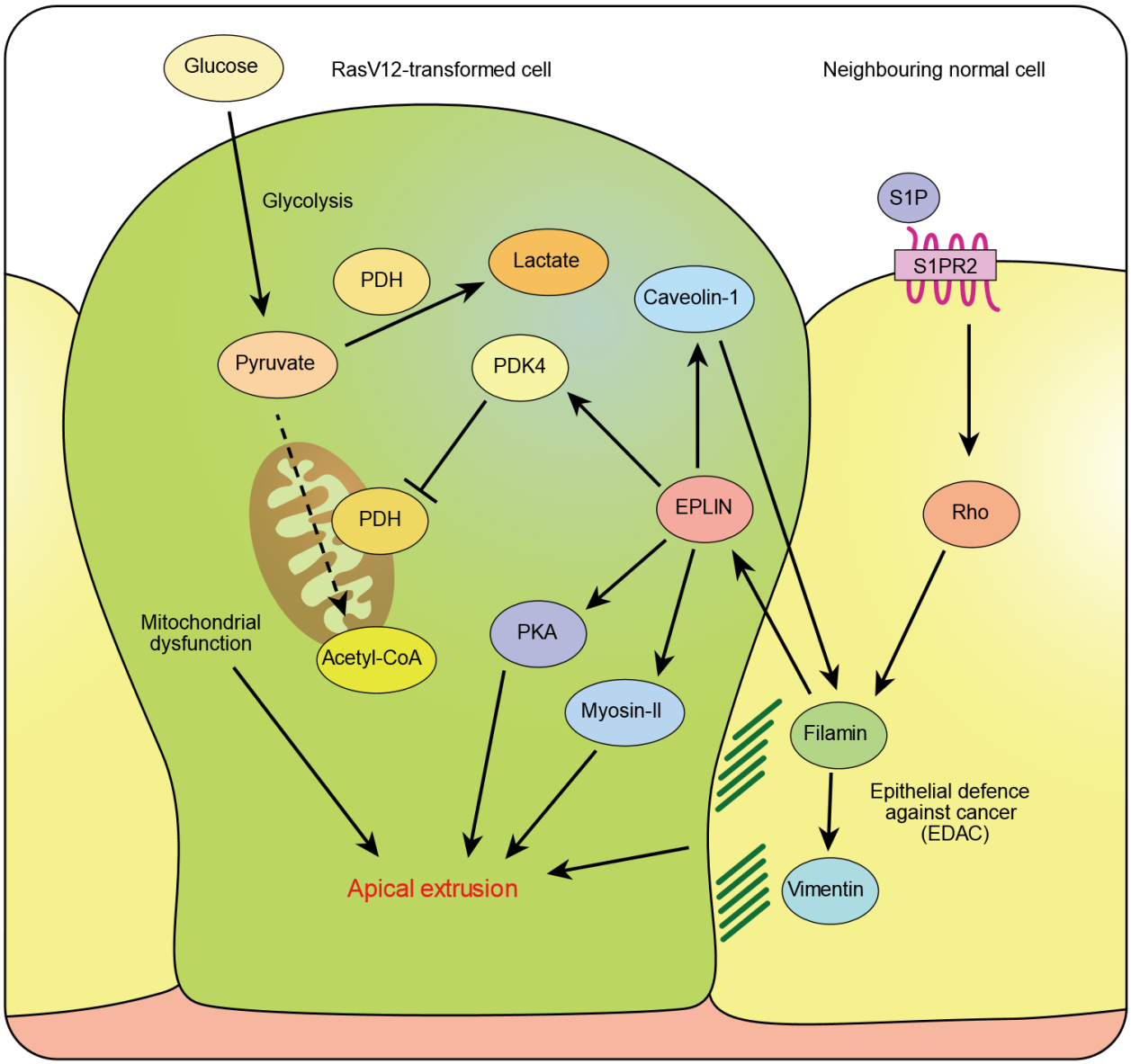


Figure 1.4 Schematic model of molecular mechanism of apical extrusion of RasV12-transformed cells surrounded by normal cells.

1.2.3. Cell competition in mouse model

Cell competition is also observed *in vivo* mouse models (Table 1.3).

1.2.3.1. Ras-transformed Cells

Recently, we reported apical extrusion of RasV12-transformed cells using mouse model system (Kon et al., 2017). In this model, Cre-Lox recombination system has been applied to generate RasV12-transformed cells in a mosaic manner (Figure 1.5). CreERT2 is an advanced Cre which can be activated by tamoxifen, and activated CreERT2 can recognize the DNA sequence of "loxP" and induce recombination at that site. We used offspring of Villin-CreERT2 mice and CAG-LSL-RasV12-IRES-eGFP mice. In this mouse, CreERT2 is expressed in only intestinal epithelia because "Villin" is a specific promotor of intestinal epithelial tissue. CreERT2 can kick out the STOP codon sequence in front of RasV12 stochastically in presence of tamoxifen, thereby RasV12-transformed cells are generated in a mosaic manner at the intestinal tissue. Using this mouse model, the apical extrusion of RasV12-transformed cells also observed in mouse small intestine (Kon et al., 2017).

1.2.3.2. The Others

Other types of cell competition in mouse models also have been reported (Table 1.3). For example, induction of Myc-overexpression in a mosaic manner in the cardiomyocytes of the myocardium in embryo or adult mice cause cell competition. Myc-overexpressing cells dominates the myocardial tissue, which is accompanied by the elimination of wild-type cells.

Table 1.3 Cell competition in mouse model system

Mutation	Phenotype	References
Minute	Elimination of Minute-knockout cells in the livier	(Oliver et al., 2004)
p53	Loss of wild-type cells by senescence-like phenotype in the hematopoietic system	(Bondar and Medzhitov, 2010) (Marusyk et al., 2010)
Myc	Cell death of low myc-expressing cells in the epiblast and myocardium	(Claveria et al., 2013) (Sancho et al., 2013) (Villa del Campo et al., 2014)
Ras	Apical extrusion of Ras-transformed cells	(Kon et al., 2017)

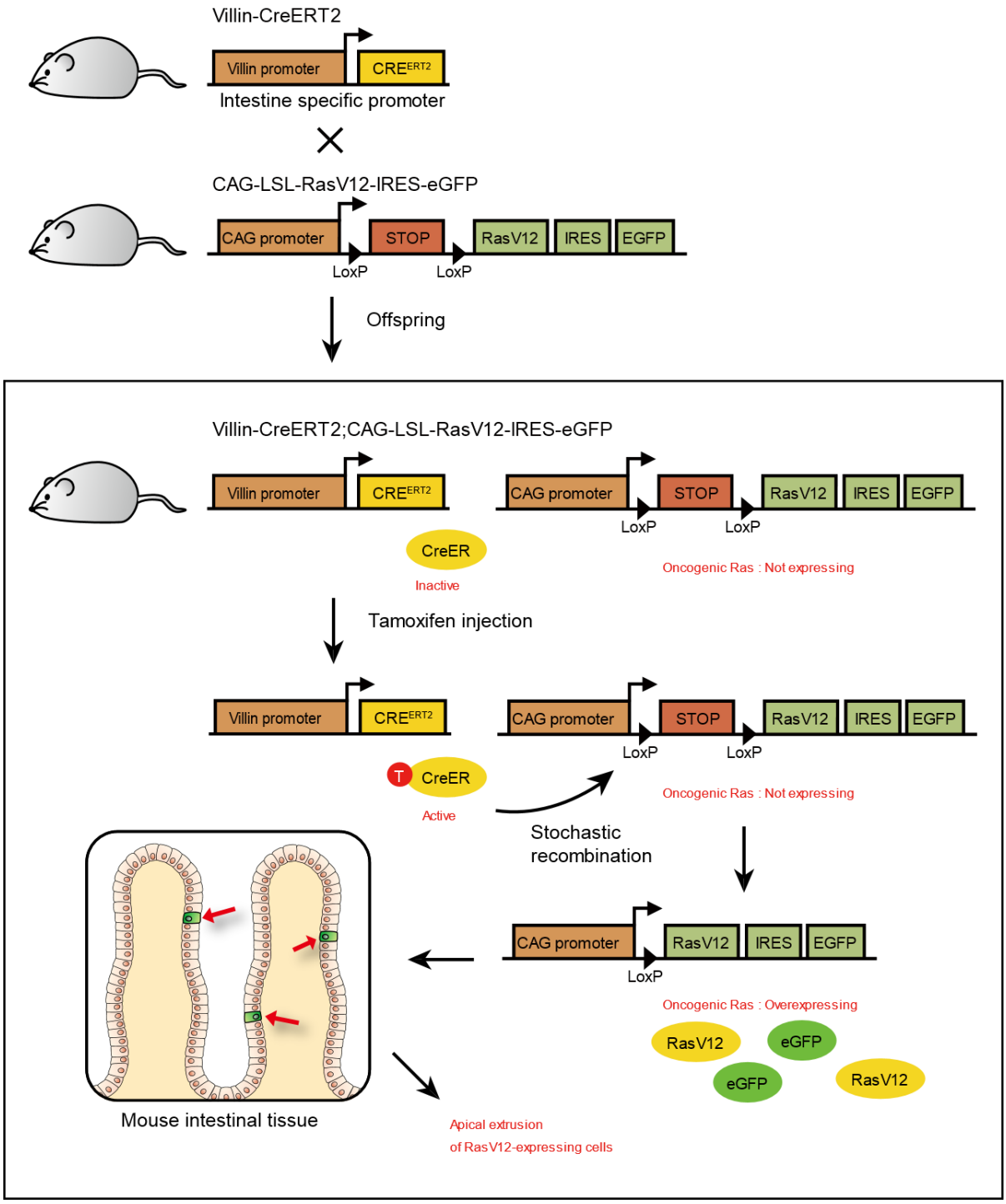


Figure 1.5 Schematic model of cell competition *in vivo* mouse system

1.3. Obesity and Cancer

1.3.1. Obesity in the world

Obesity is one of the major risk factors in metabolic syndromes, and the number of obese individuals has been increasing worldwide (Collaboration, 2016)(Collaborators et al., 2017).

1.3.2. Changes in obesity

Obesity can induce various systemic disorders such as altered lipid metabolism, dysregulated hormone secretion, dysbiosis and chronic inflammation (Gonzalez-Muniesa et al., 2017)(Heymsfield and Wadden, 2017)(Kopelman, 2000)(Rosenbaum et al., 1997).

1.3.3. Obesity and cancer risk

It has also become evident that obese individuals have higher incidents of certain types of malignancies, including colon, pancreatic and breast cancer (Lauby-Secretan et al., 2016). Previous studies have revealed molecular mechanisms of how ovesity promotes tumor growth and malignant progression: e.g. oxidative stress, chronic inflammation, dysbiosis and hormonal alterations (Bianchini et al., 2002)(Donohoe et al., 2017)(Font-Burgada et al., 2016)(Hopkins et al., 2016)(Khandekar et al., 2011)(Lauby-Secretan et al., 2016)(Poloz and Stambolic, 2015)(Renehan et al., 2015).

However, it remains elusive whether and how obesity is also involved in tumor iniciation.

1.3.4. Obesity mouse models

There are a lot of obese mouse models used in research, and they could be classified into two groups. One is an obese mouse model caused by genetic mutation in genes which involved in leptin that regulate the amount of food intake and body weight. Ob/ob mouse that has mutations in the gene responsible to produce leptin is most famous.

The other is diet-induced obesity mouse model without genetic mutations. The representative one is high-fat diet (HFD) induced obesity mouse model. (Nilsson et al., 2012) In this thesis, I used HFD-induced obesity mouse model using a diet containing 60% kcal fat.

1.4. The Aim of This Study

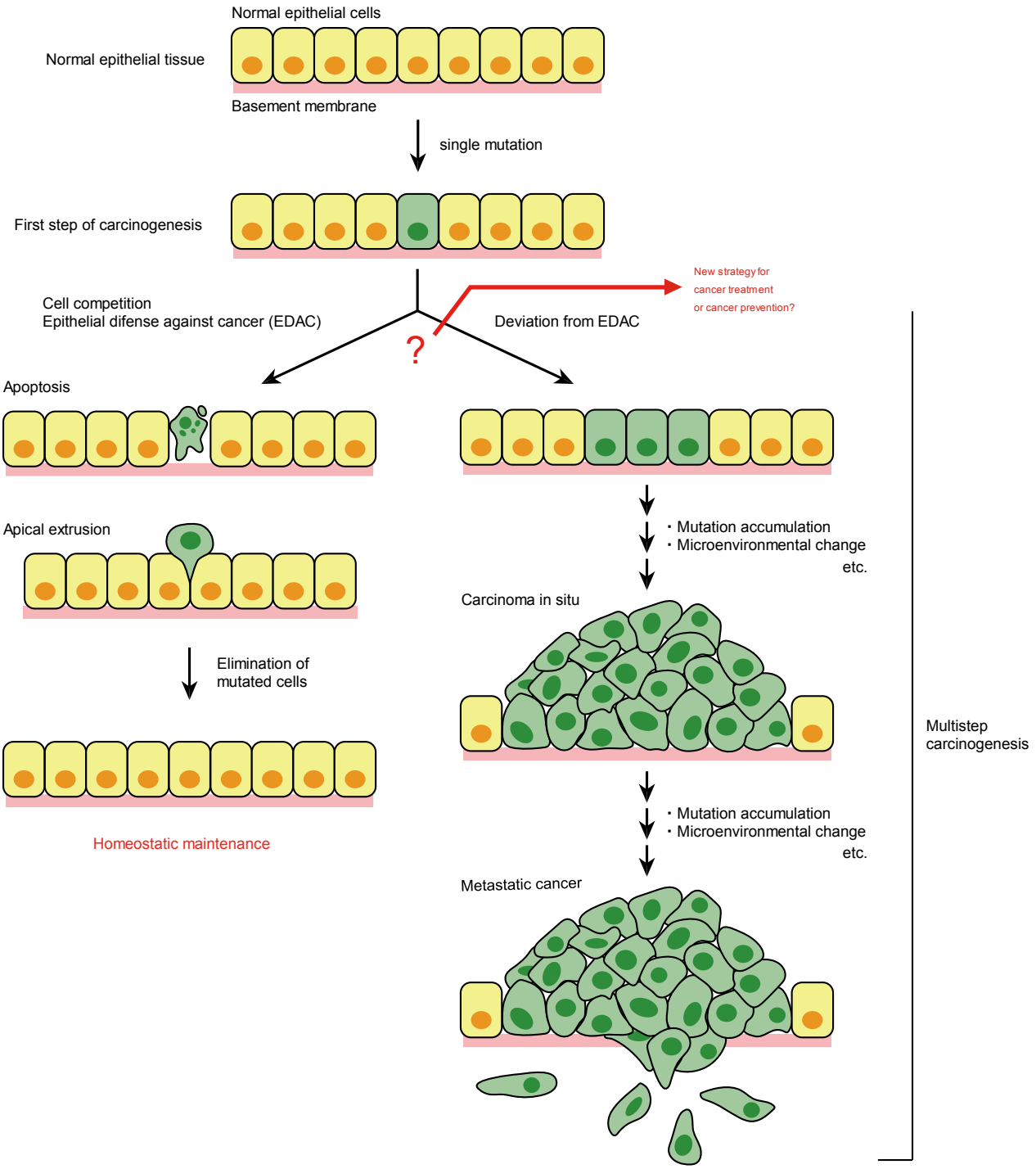
When RasV12-transformed cells are surrounded by normal epithelial cells, RasV12 cells are apically eliminated from epithelia *in vitro* culture system and *in vivo* mouse model system (Hogan et al., 2009)(Kon et al., 2017). During the apical extrusion of transformed cells, various non-cell-autonomous changes occur in both normal and transformed cells at their boundary. That data imply a notion that normal epithelia have anti-tumour activity that does not involve immune cells: the process is called EDAC.

However, cancer cause a lot of people to die all over the world despite the presence of such defence mechanism. The understanding why such defence mechanism is not perfect might be able to bring a new insight and strategy for cancer treatment and prevention (Figure 1.6). To investigate the relationship between EDAC and cancer risk factors, I focused on obesity as a cancer risk factor.

It is known that obese individuals have higher incidents of certain types of malignancies (Lauby-Secretan et al., 2016). Previous studies have revealed molecular mechanisms of how obesity promotes tumour growth and malignant progression: e.g. oxidative stress, chronic inflammation, dysbiosis

and hormonal alterations (Bianchini et al., 2002; Donohoe et al., 2017; Font-Burgada et al., 2016; Hopkins et al., 2016; Khandekar et al., 2011; Lauby-Secretan et al., 2016; Poloz and Stambolic, 2015; Renehan et al., 2015). However, it remains elusive whether and how obesity is also involved in tumour initiation.

To reveal the relationship between cell competition and obesity, and the mechanism of cancer initiation in obese individuals, I performed experiments using mouse cell competition model with obesity.



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1.5. References

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Chapter 2:

EFFECTS OF OBESITY ON EDAC IN MOUSE TISSUES

1. Effects of obesity on EDAC in mouse tissues

2.1. Introduction

EDAC is expected to act as a defense mechanism against cancer. However the role of EDAC in carcinogenesis have still been required to be made clear. The understanding how cell competition contributes to the suppression of carcinogenesis and whether the inhibition of cell competition induces carcinogenesis may provide deeper insight for the meaning of cell competition. On the other hand, obesity is known as a risk factor of cancers. And the mechanism of carcinogenesis derived by obesity have not been clearly known. I then made a hypothesis that cancers led by obesity is caused by the inhibition of cell competition, and I investigated the relationship between cell competition and obesity.

Previously, our group reported about a cell competition mouse model system that can observe apical extrusion of RasV12-transformed cells in the small intestinal epithelia. In this thesis, I used a new cell competition mouse model system, to monitor the fate of newly emerging RasV12-transformed cells in various epithelial tissues. Then, the frequencies of apical extrusion of RasV12-transformed cells were tested in the small intestine, lung and pancreatic tissues in lean mouse and high-fat diet induced obese mouse.

2.2. Experimental procedures

2.2.1. Antibodies and materials

Chicken anti-GFP (ab13970) antibody was purchased from Abcam. Rat anti-E-cadherin (131900) antibody was from Life Technologies. Alexa-Fluor-488-conjugated secondary antibody was from Abcam, and Alexa-647-conjugated secondary antibody was from Life Technologies. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000.

2.2.2. Mice

All animal experiments were conducted under the guidelines by the Animal Care Committee of Hokkaido University. The animal protocols were reviewed and approved by the Hokkaido University Animal Care Committee (approval number 12-0116). We used 6-10 weeks old C57BL/6 mice for mating. *Cytokeratin19* (CK19)-Cre^{ERT2} mice (Means et al., 2008) were crossed with *DNMT1*-CAG-*loxP*-STOP-*loxP*-HRas^{V12}-*IRES-eGFP* mice to create CK19-*RasV12*-GFP mice. Mice heterozygous for each transgene were used for experiments. For PCR genotyping of mice, the following primers were used: 5'-AATCGCCAGGAATTGACCAATGGGG-3', 5'-CGGCAAACGGACAGAAGCATTTTCC-3' and 5'-CGCCCGTACCCCCAAAGGAAGACAT-3' for the CK19-Cre^{ERT2} mice, 5'-CACTGTGGAATCTCGGCAGG-3' and 5'-GCAATATGGTGGAAAATAAC-3' for the *DNMT1*-CAG-*loxP*-STOP-*loxP*-HRas^{V12}-IRES-eGFP mice. The expected sizes of PCR products were

265 bp, 369 bp and 403 bp for CK19-Cre^{ERT2}, *DNMT1*-CAG-*loxP*-STOP-*loxP*-HRas^{V12}-*IRES*-*eGFP*, respectively.

HFD treatment was achieved by feeding female CK19-*RasV12*-GFP mice a dietary chow consisting of 60% kcal fat (Research Diets D12492). The HFD feeding began at the age of 6-10 weeks and were extended for a period of 3 months. Control mice were age-matched and fed with normal diet (NOSAN). The mice were given a single intraperitoneal injection of 1 mg of tamoxifen in corn oil (Sigma) per 20 g of body weight, and were then sacrificed at 3 days after Cre activation.

2.2.3. Immunofluorescence

For immunohistochemical examinations of the small intestine, pancreas and lung, the mice were perfused with 1% paraformaldehyde (PFA, Sigma-Aldrich), and the isolated tissues were fixed with 4% PFA in PBS for 24 h and embedded in FSC 22 Clear Frozen Section Compound (Leica). Then, 10-μm-thick frozen sections were cut on a cryostat. The sections were blocked with Block-Ace (DS Pharma Biomedical) and 0.1% Triton X-100 in PBS. Primary or secondary antibodies were incubated for 2 h or 1 h respectively at ambient temperature. All primary antibodies were used at 1:1000, and all secondary antibodies were at 1:500. Immunofluorescence images of mouse tissues were acquired using the Olympus FV1000 system and Olympus FV10-ASW software.

3.1.5 Statistics and reproducibility

For data analyses, Chi-squared test (Figures 2.3C, 2.4B, 2.5B) and unpaired two-tailed Student *t*-tests (Figures 2.2B) were used to determine *P*-values using GraphPad Prism7 and Microsoft Excel, respectively. *P*-values less than 0.05 were considered to be statistically significant. For animal studies, the experiments were not randomized, and the investigators were not blinded to allocation during experiments. All results were reproduced in at least three mice for each experimental condition.

2.3. Results

2.3.1. Cell competition also observed in the small intestine, lung and pancreatic tissues.

To monitor the fate of newly emerging RasV12-transformed cells in various epithelial tissues, I have established a novel cell competition mouse model system (Figure2.2). To this end, I used an LSL-RasV12-IRES-eGFP mouse whereby RasV12 expression is induced in a Cre-dependent fashion and traced by simultaneous expression of eGFP (Figure 2.2)(Kon et al., 2017). I then crossed LSL-RasV12-IRES-eGFP mice with cytokeratin 19 (CK19: epithelial specific marker)-Cre-ERT2 mice (Figure 2.2). In the RasV12; CK19-Cre mice, administration of a low dose of tamoxifen induced recombination events less frequently, resulting in mosaic expression of RasV12 within various epithelial tissues (Figures 2.3A, 2.4A, 2.5A). In a previous study, using villin (intestinal specific marker)-Cre-ERT mice, I have shown that newly emerging RasV12-transformed cells are eliminated into the apical lumen of the intestinal epithelium(Kon et al., 2017). Similarly, using the new mouse model, I found that after three days of tamoxifen treatment, more than 65-90% of RasV12-expressing cells underwent apical extrusion from small intestine, pancreas and lung epithelia (Figures 2.3, 2.4, 2.5).

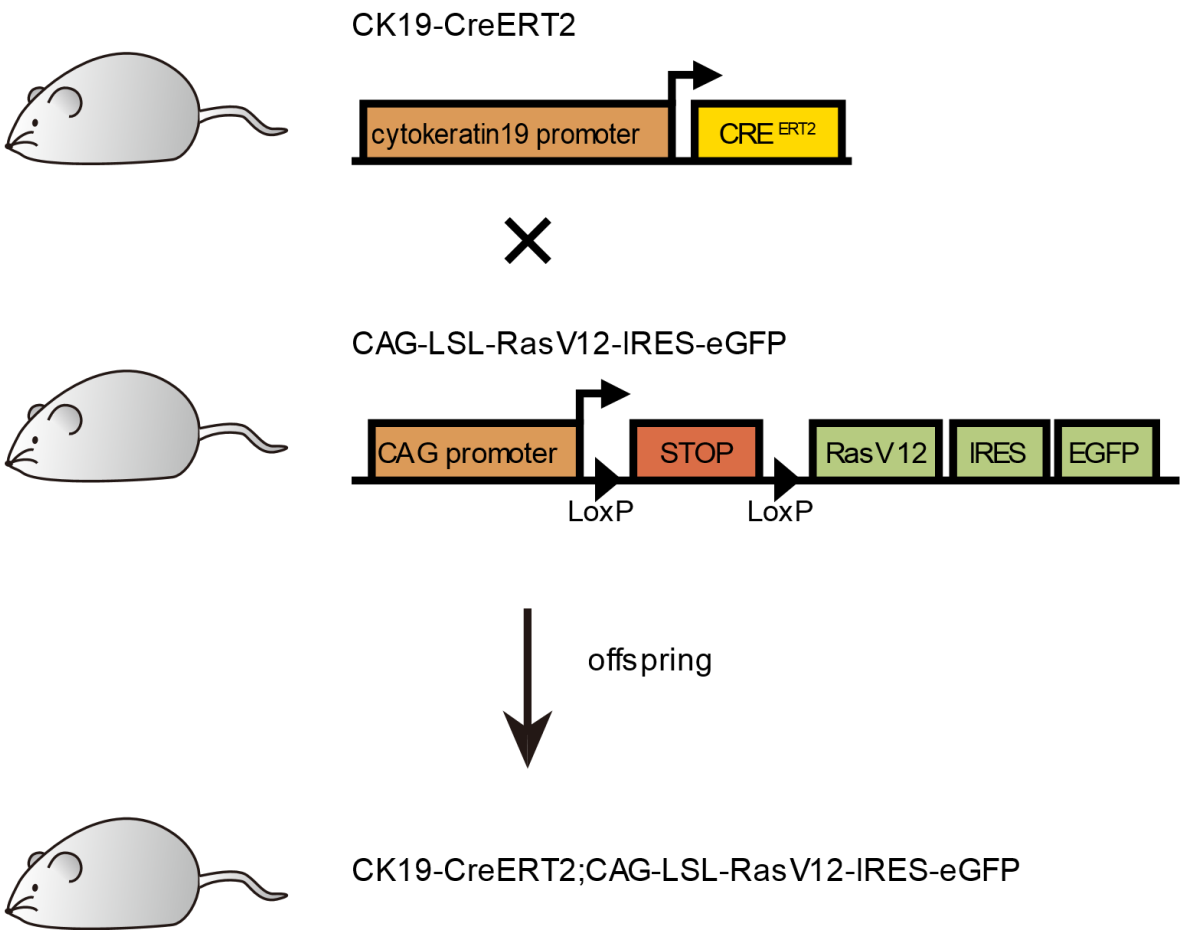

Figure 2.1. Strategy of the establishment of the establishment of the new cell competition mouse model.

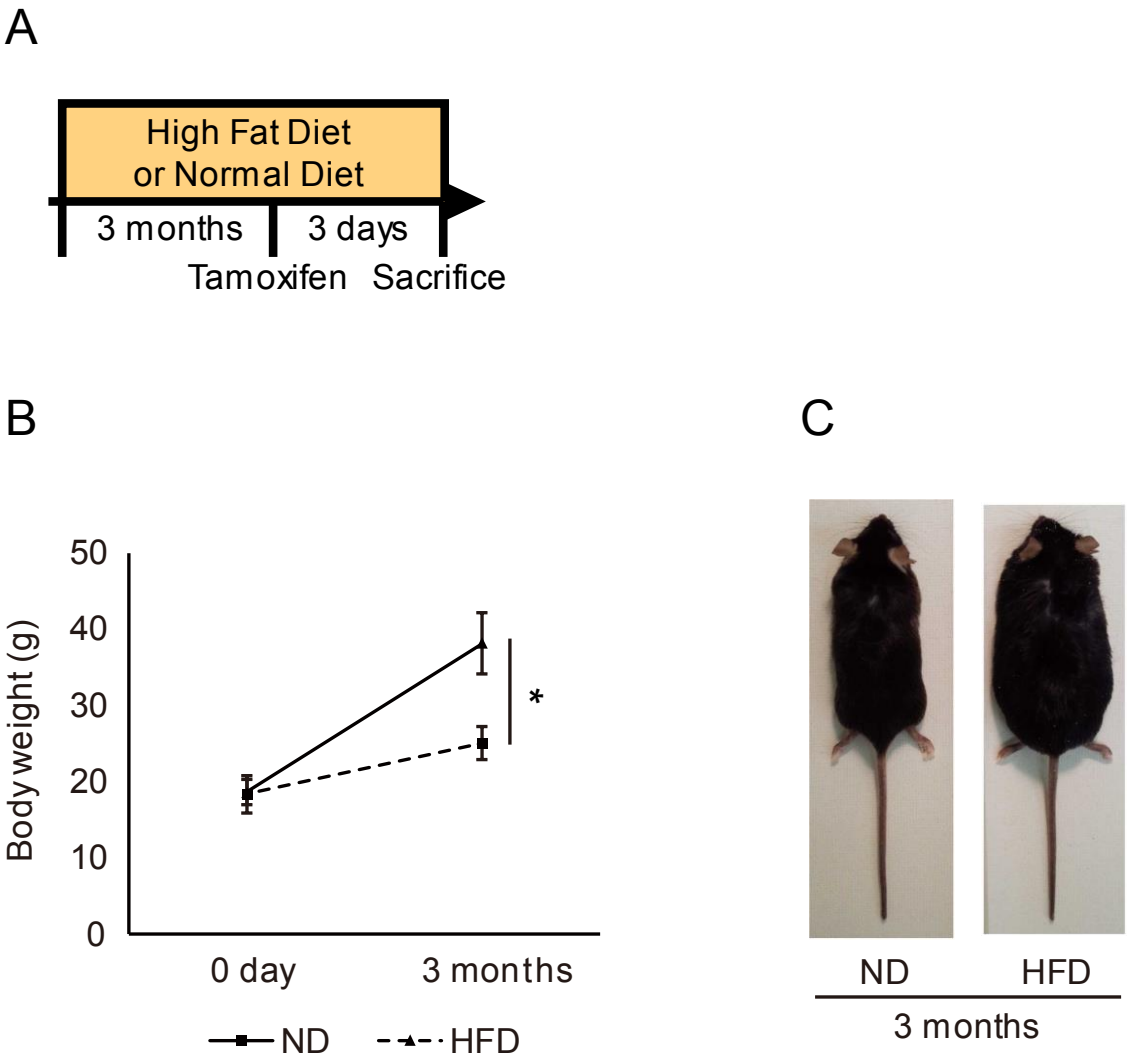

Figure 2.2. Experimental design of HFD treatment. (A) Experimental design for feeding and tamoxifen administration. (B) Effect of ND or HFD on body weight. (C) Representative image of mice treated with ND or HFD. Data represent mean ± SD from three independent experiments. *$P < 0.001$ (Student *t*-test)

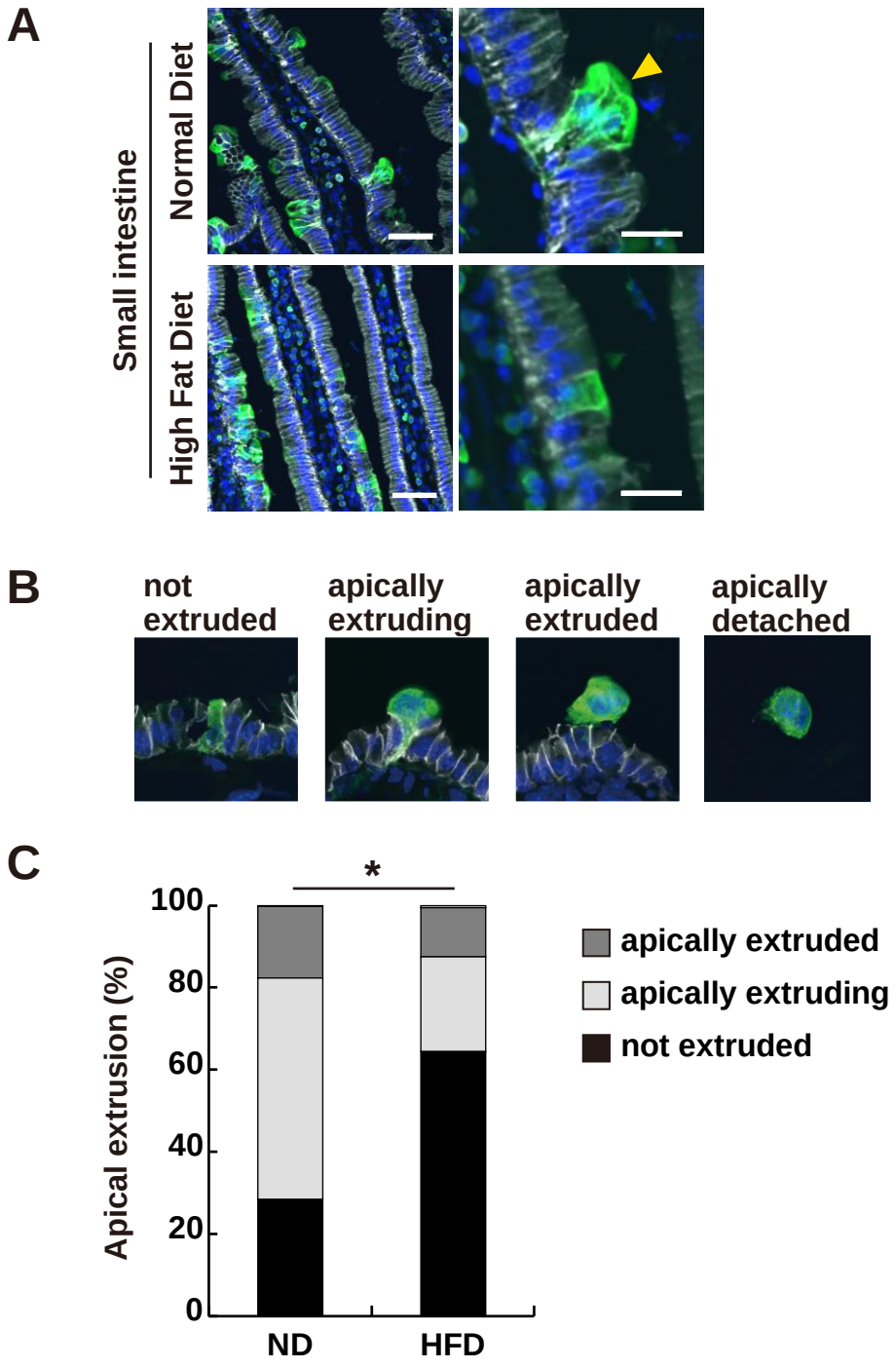

Figure 2.3. Effect of HFD treatment on apical elimination of RasV12-transformed cells from the small intestinal epithelial tissue. (A) Immunofluorescence images of RasV12-transformed cells in the epithelium of the small intestine. The tissue samples were stained

with anti-GFP (green) and anti-E-cadherin (white) antibodies and Hoechst (blue). The yellow arrow and arrow heads indicate apically extruded and extruding cells, respectively. Scale bars, 50 um (left panel) and 20 um (right panel). (B) Representative images of RasV12-transformed cells. ‘Not extruded’: remaining within the epithelium. ‘Apically extruding’: with their nucleus apically shifted, but still attached to the basement membrane. ‘Apically extruded’: completely detached from the basement membrane and translocated into the apical lumen. ‘Apically detached’: completely detached from the epithelium. (C) Quantification of apical extrusion of RasV12 cells for B. ND 2,063 cells from 8 mice, HFD 1,117 cells from 3 mice. *P < 0.0001 (chi-square test).

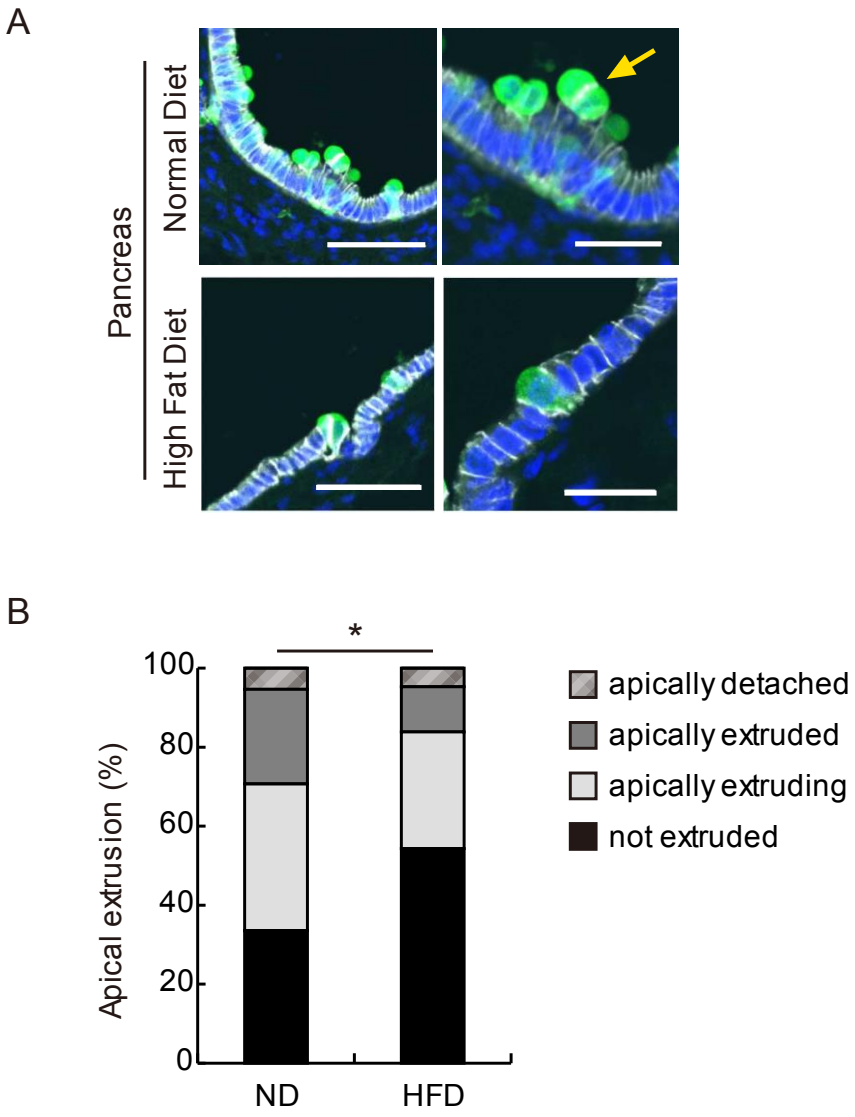

Figure 2.4. Effect of HFD treatment on apical elimination of RasV12-transformed cells from the pancreatic epithelial tissue. (A) Immunofluorescence images of RasV12-transformed cells in the epithelium of the pancreas. The tissues samples were stained as previously described in Figure 2.3. Scale bars, 50 um (left panel) and 20 um (right panel). (B) Quantification of apical extrusion of RasV12 cells for B. ND 560 cells from 9 mice, HFD 298 cells from 4 mice. *$P < 0.0001$ (chi-square test).

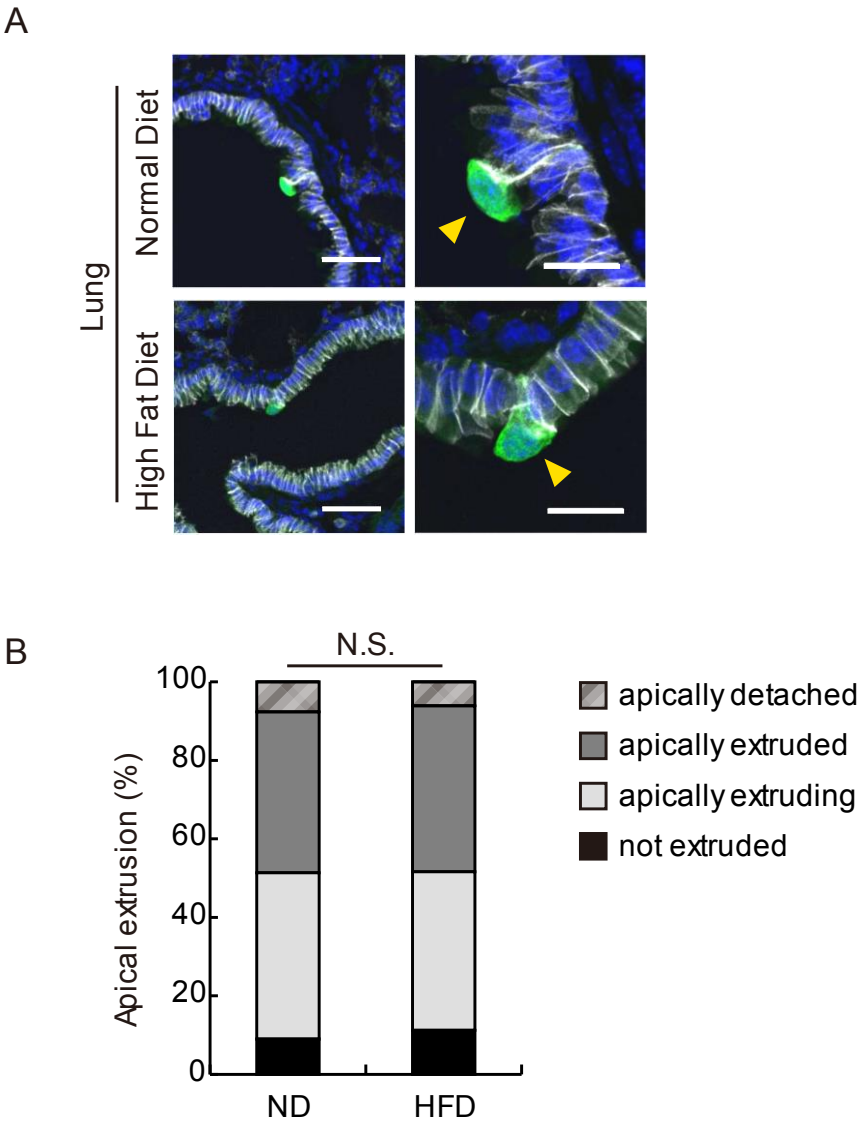

Figure 2.5. Effect of HFD treatment on apical elimination of RasV12-transformed cells from the lung epithelial tissue. (A) Immunofluorescence images of RasV12-transformed cells in the epithelium of the lung. The tissues samples were stained as previously described in Figure 2.3. Scale bars, 50 μm (left panel) and 20 um (right panel). (B) Quantification of apical extrusion of RasV12 cells for B. ND 144 cells from 4 mice, HFD 213 cells from 4 mice. N.S.: not significant.

2.3.2. HFD-Induced Obesity Suppresses EDAC in the Small Intestine and Pancreas.

Then, we examined whether obesity affects EDAC by analysing the effect of high-fat diet (HFD) treatment on the fate of RasV12 cells. Mice were fed with normal diet (ND) or HFD for three months prior to tamoxifen administration (Figure 2.2A). Compared with ND-fed mice, HFD-fed mice profoundly gained body weight and became severely obese (Figure 2.2B and C). In the small intestine and pancreas, compared with ND, HFD treatment significantly suppressed the frequency of apical extrusion, and consequently RasV12 cells more frequently remained within the epithelium (Figures 2.3 and 2.4). Compared with the small intestine and pancreas, in the lung, most of RasV12 cells underwent apical extrusion in ND-fed mice, and HFD treatment did not significantly affect the frequency of the elimination of RasV12 cells (Figure 2.4). These data indicate that HFD treatment could suppress the elimination of transformed cells in certain epithelial tissues.

Discussion

Although previously we have reported that cell competition of RasV12-transformed cells occurred in the mouse small intestinal tissue, in this thesis, I newly showed cell competition of RasV12 cells occur in also the mouse pancreatic and lung epithelial tissues. While the elimination occurred in these three tissues, the frequency of apical extrusion was different between these tissues. It is interesting that how the difference of elimination frequency is generated, and it is required to be revealed. But, in this thesis, I couldn't reveal the mechanism.

In addition, the frequency of apical extrusion was decreased in the small intestinal and pancreatic tissues in obese mice compared with the tissues in lean mice. The results newly indicate that individual environmental conditions like obesity can affect cell competition. And, the results support the hypothesis that the cancers led by obesity is caused by the inhibition of cell competition.

On the other hand, the suppression of apical extrusion in obese mice was not observed in the lung tissue. This result corresponds with epidemiologic studies which reported about the correlation between cancer risk and BMI (Bianchini et al., 2002; Collaborators et al., 2017; Lauby-Secretan et al., 2016). Why the frequency of apical extrusion in the lung tissue was not affected by the high-fat diet treatment is still unknown. But, one possibility is that because the lung epithelial tissue has high ability to eliminate transformed cells compared with the small intestine and pancreas, obesity could not inhibit the apical extrusion efficiency.

2.4. References

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Chapter 3:

LIPID METABOLISM AND EDAC

3. Lipid metabolism and EDAC

3.1. Introduction

To investigate the mechanism of the inhibition of apical extrusion by HFD, I focused on the changes in the mouse under the HFD treatment. It is known that HFD treatment increases the concentration of circulating and intracellular fatty acids and induces various systemic disorders including altered lipid metabolism and chronic inflammation (Font-Burgada et al., 2016; Hotamisligil, 2006; Khandekar et al., 2011; Newgard, 2017). First, I decided to investigate the effect of fatty acids on apical extrusion using *in vitro* cell competition model with MDCK epithelial cells.

HFD consist of higher amounts of fatty acids containing palmitic acid, stearic acid, linoleic acid and α-linolenic acid. Treatment of cultured cells with fatty acids can affect intracellular lipid metabolisms and signalling pathways (Beyaz et al., 2016; Laugerette et al., 2012). Therefore, I examined whether and how alteration of lipid metabolism influences the behaviour and fate of RasV12-transformed cells within the epithelium using our *in vitro* cell competition model with Madin-Darby canine kidney (MDCK) epithelial cells (Hogan et al., 2009).

3.2. Experimental Procedures

3.2.1. Antibodies and materials

Chicken anti-GFP (ab13970) antibody was purchased from Abcam. Rat anti-E-cadherin (131900) antibody was from Life Technologies. Alexa-Fluor-568-conjugated phalloidin from Life Technologies was used at 1.0 U/ml. Alexa-Fluor-488-conjugated secondary antibody was from Abcam, and Alexa-Fluor-647-conjugated secondary antibody was from Life Technologies. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000. TMRM (tetramethylrhodamine methyl ester) was obtained from Molecular Probes. Palmitic acid, stearic acid, linoleic acid and α-linolenic acid were from Wako. Trimetazidine (Abcam) was used at 10 μM.

3.1.2. Cell culture

MDCK and MDCK-pTR GFP-RasV12 cells were cultured as previously described (Hogan et al., 2009; Kon et al., 2017). To induce the expression of GFP-RasV12 in MDCD-pTR GFP-RasV12 cells, 2 μg/ml of tetracycline (Sigma-Aldrich) was added. For immunofluorescence, cells were plated onto collagen gel-coated coverslips (Hogan et al., 2009). To quantify the frequency of apical extrusion, the indicated fatty acids and/or trimetazidin were added together with tetracycline, and then cells were further cultured for 24 h. To monitor the mitochondria activity, cells were cultured for 15.5 h after tetracycline addition and then loaded with 50 nM TMRM for 30 min and subjected to microscopic observation as previously described (Kon et al.,

2017).

3.2.3. Mice

HFD treatment was achieved by feeding female CK19-*RasV12*-GFP mice a dietary chow consisting of 60% kcal fat (Research Diets D12492). The long- and short-term HFD feeding began at the age of 6-10 weeks and were extended for a period of 5 days. Control mice were age-matched and fed with normal diet (NOSAN). The mice were given a single intraperitoneal injection of 1 mg of tamoxifen in corn oil (Sigma) per 20 g of body weight, and were then sacrificed at 3 after Cre activation.

3.1.4. Immunofluorescence

For immunohistochemical examinations of the small intestine, pancreas and lung, the mice were perfused with 1% paraformaldehyde (PFA, Sigma-Aldrich), and the isolated tissues were fixed with 4% PFA in PBS for 24 h and embedded in FSC 22 Clear Frozen Section Compound (Leica). Then, 10-μm-thick frozen sections were cut on a cryostat. The sections were blocked with Block-Ace (DS Pharma Biomedical) and 0.1% Triton X-100 in PBS. Primary or secondary antibodies were incubated for 2 h or 1 h respectively at ambient temperature. All primary antibodies were used at 1:1000, and all secondary antibodies were at 1:500. For immunofluorescence of cultured cells, MDCK-pTR GFP-RasV12 cells were mixed with MDCK cells at a ratio of 1:50 and cultured on the collagen matrix as previously described (Hogan et al., 2009). The mixture of cells was incubated for 8 h

until they formed a monolayer, followed by tetracycline treatment for 24 h. Cells were fixed with 4% PFA in PBS and permeabilized with 0.5% Triton X-100 in PBS, followed by blocking with 1% BSA in PBS. Alexa-Fluor-568-conjugated phalloidin was incubated for 1 h at ambient temperature. Immunofluorescence images of mouse tissues and cultured cells were acquired using the Olympus FV1000 system and Olympus FV10-ASW software. Images of TMRM were quantified with the MetaMorph software (Molecular Devices).

3.1.5 Statistics and reproducibility

For data analyses, Chi-squared test (Figures 3.7B and 3.8B) and unpaired two-tailed Student *t*-tests (Figures 3.1B, 3.2B, 3.5B and 3.6B) were used to determine *P*-values using GraphPad Prism7 and Microsoft Excel, respectively. *P*-values less than 0.05 were considered to be statistically significant. For animal studies, the experiments were not randomized, and the investigators were not blinded to allocation during experiments. All results were reproduced in at least three mice for each experimental condition.

3.3. Results

3.3.1. Fatty acids treatment inhibit apical elimination of RasV12-transformed cells.

In the MDCK model system, when RasV12-transformed cells are surrounded by normal cells, the transformed cells are often apically extruded from the monolayer of normal epithelial cells (Hogan et al., 2009). I found that addition of either fatty acid in condition media significantly suppressed frequency of apical extrusion of RasV12 cells from the epithelial monolayer (Figures 3.1).

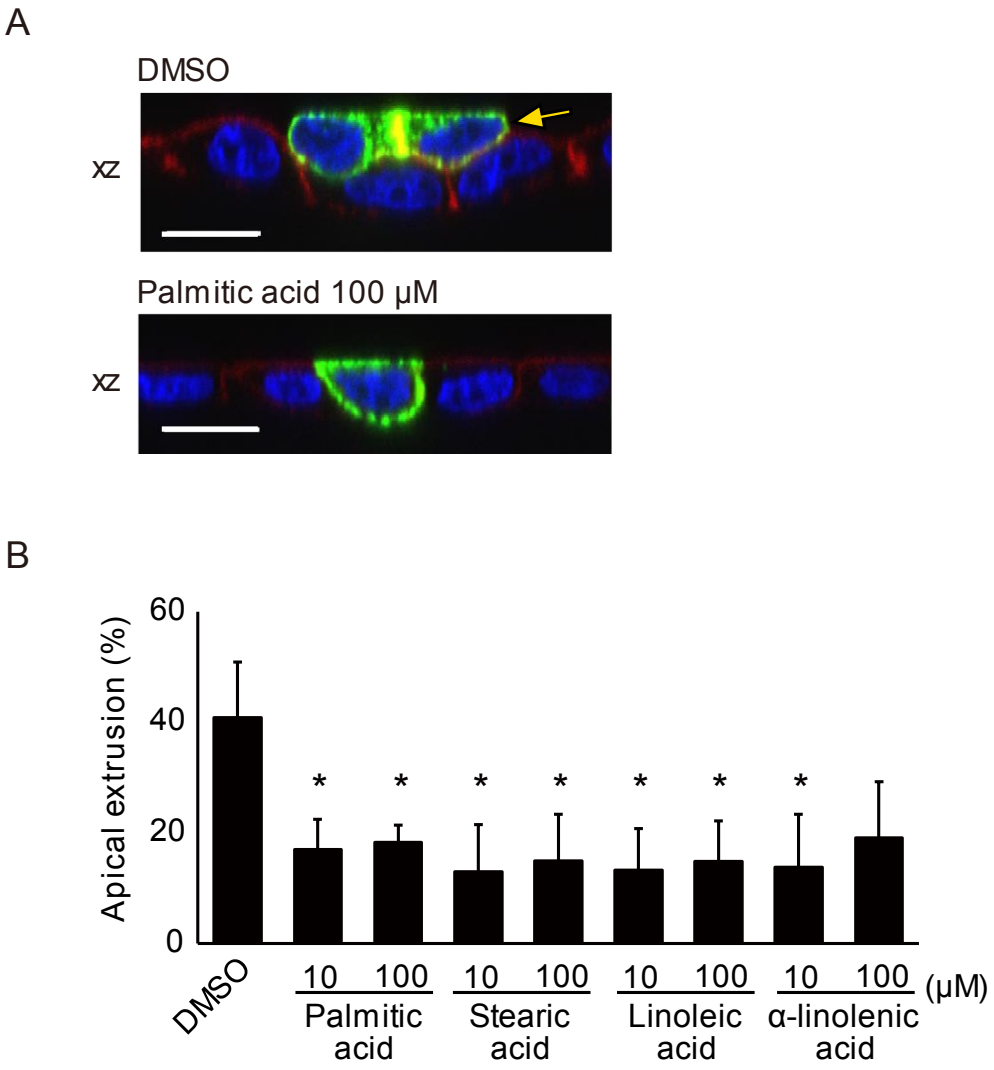

Figure 3.1. Effect of various fatty acids on apical extrusion of RasV12-transformed cells. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells on collagen gels. (A) Representative images of cells were cultured with the indicated concentration of fatty acids and fixed after 24 h incubation with tetracycline and stained with Alexa-Fluor-568-phalloidin (red) and Hoechst (blue). Confocal microscopy images of xz sections. Arrows indicate the apically extruded cells.

Scale bars, 10 μm. (B) Quantification of apical extrusion. n≧100 cells for each experimental condition. Data are mean ± SD from three independent experiments. *P<0.05 (Student t-tests).

3.3.2. Alteration of lipid metabolism Influences apical elimination of RasV12-transformed cell from epithelia.

When RasV12 cells are surrounded by normal epithelial cells, mitochondrial membrane potential is diminished via increased expression of pyruvate dehydrogenase kinase (PDK4) (Kon et al., 2017). Accumulated PDK4 phosphorylates and inactivates pyruvate dehydrogenase (PDH) that catalyses the conversion of pyruvate to acetyl-CoA, thereby blocking the entry into tricarboxylic acid (TCA) cycle (Figure 1.4 and 3.3) (Kon et al., 2017). Consequently, mitochondrial membrane potential is decreased in RasV12 cells that are surrounded by normal cells, which positively regulates apical extrusion of RasV12 cells. TMRM (tetramethylrhodamine methyl ester) is a positively charged red fluorescent dye that accumulates in active mitochondria according to the negative membrane potential gradient across their inner membranes. Using TMRM, I observed that mitochondrial membrane potential was profoundly decreased in RasV12 cells when they were surrounded by normal cells as previously reported (Figures 3.2 and 3.4) (Kon et al., 2017). Incubation with the excessive amount of palmitic acid, stearic acid or linoleic acid significantly restored the mitochondrial membrane potential (Figure 3.2). Next, I examined the effect of the fatty acid oxidation inhibitor Trimetazidine (TMZ), which blocks the conversion from fatty acids to acetyl-CoA (Figure 3.3). When TMZ was added together with palmitic acid or linoleic acid, incorporation of TMRM was substantially diminished (Figure 3.4). Furthermore, TMZ treatment suppressed the inhibitory effect of palmitic acid or linoleic acid on apical extrusion (Figure

3.5). Collectively, these data suggest that the excess fatty acids are converted into acetyl-CoA and thus restore mitochondrial membrane potential in RasV12 cells surrounded by normal cells, thereby inhibiting the eradication of transformed cells from the epithelium.

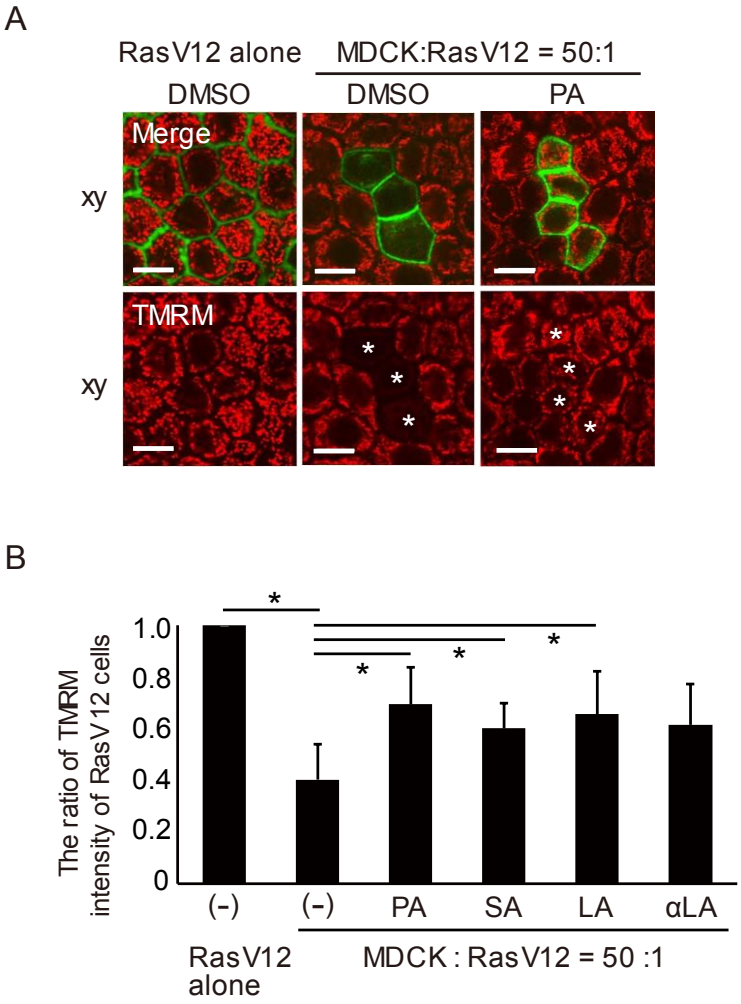

Figure 3.2. Effect of various fatty acids on TMRM incorporation of RasV12-transformed cells. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells on collagen gels. Cells were cultured with the indicated fatty acid (100 μM), and TMRM incorporation was examined after 16 h incubation with tetracycline. (A) Confocal microscopy images of xy sections. Asterisks indicate RasV12 cells surrounded by normal cells. Scale bars, 10 μm. (B) Quantification of the fluorescence intensity of TMRM. Data are mean ± SD from three independent experiments. *P<0.05 (Student t-tests).

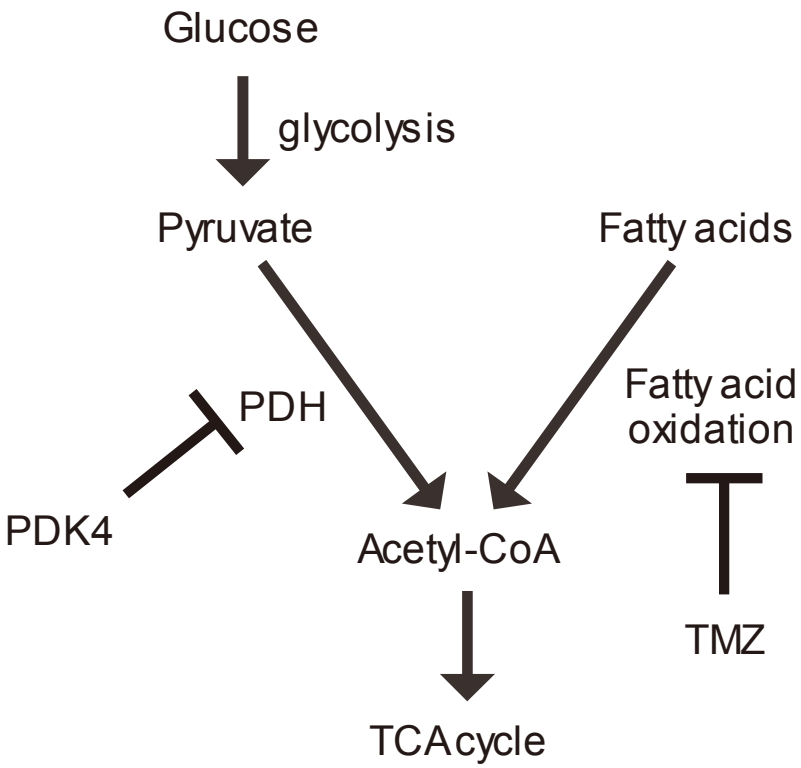

Figure 3.3. Schematic diagram for glycolysis and fatty acid oxidation.

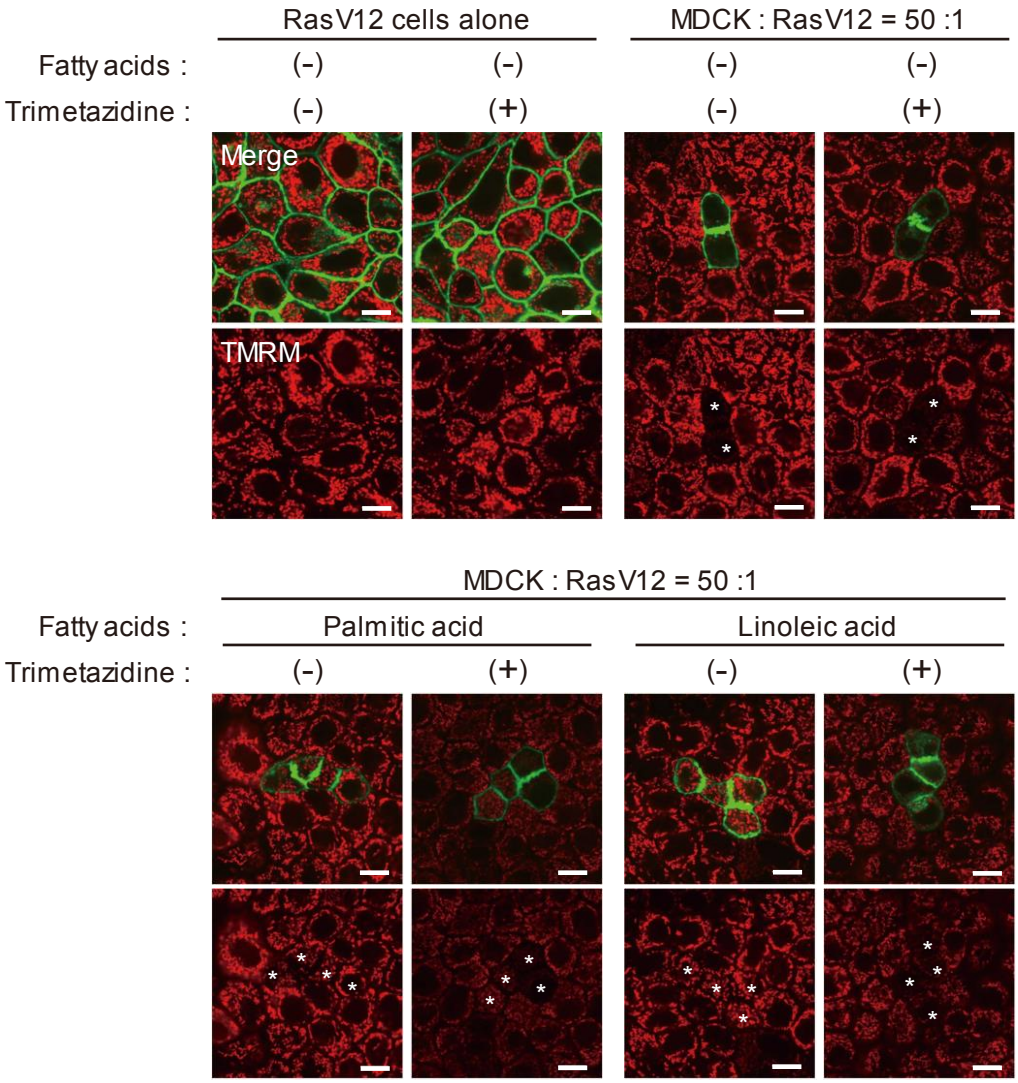

Figure 3.4. Effect of Trimetazidine on TMRM Incorporation in RasV12-transformed cells surrounded by normal cells. Representative images of TMRM incorporation in RasV12-transformed cells with Trimetazidine (TMZ) treatment. MDCK-pTR GFP-RasV12 cells were mixed with normal MDCK cells on collagen gels. Cells were cultured with the indicated fatty acid (100 μM) in the presence or absence of TMZ, and TMRM incorporation was examined after 16 h incubation with tetracycline. Asterisks indicate RasV12 cells surrounded by normal cells. Scale bars, 10 μm.

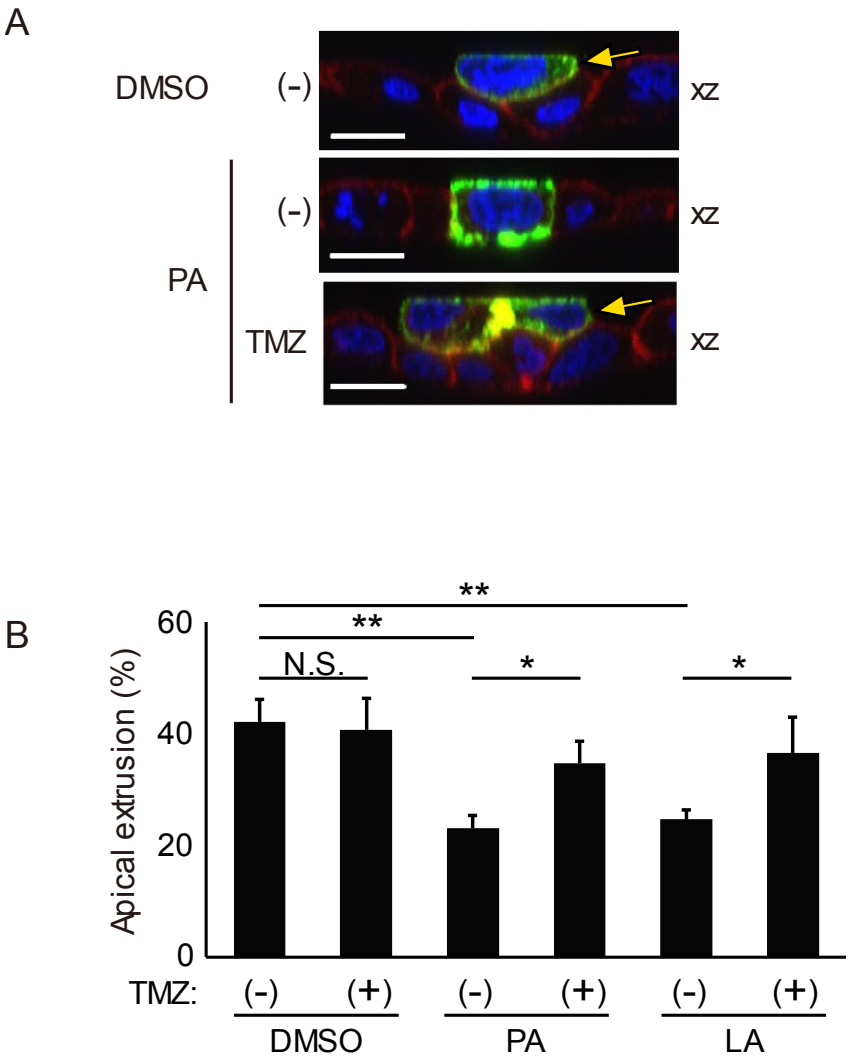

Figure 3.5. Effect of the fatty acid oxidation inhibitor Trimetazidine (TMZ) on apical extrusion of RasV12 cells. TMZ was added together with tetracycline and the fatty acid (100 μM) where indicated. (A) Representative images. Confocal microscopy images of xz sections. Arrows indicate the apically extruded cells. Scale bars, 10 μm. (B) Quantification of apical extrusion. n≧100 cells for each experimental condition. **P*<0.05, ***P*<0.01 (Student *t*-tests).

3.3.3. Alteration of lipid metabolism influences apical elimination of RasV12-tansformed cells from epithelia.

To further explore the involvement of lipid metabolism in apical extrusion of RasV12-transformed cells, I examined the effect of short-term HFD-feeding in which HFD were fed for only 4 days prior to tamoxifen administration (Figure 3.6). Under this condition, adipose tissue mass and adipocyte size are increased, and plasma free fatty acids are elevated, while chronic inflammation is not yet induced (Hernandez Vallejo et al., 2009; Lee et al., 2011). The short-term HFD-feeding did not substantially affect body weight (Figure 3.6). In the small intestine and pancreas, short-term HFD significantly suppressed apical extrusion, and RasV12 cells remained within the epithelium more frequently, compared with ND-feeding (Figures 3.7 and 3.8).

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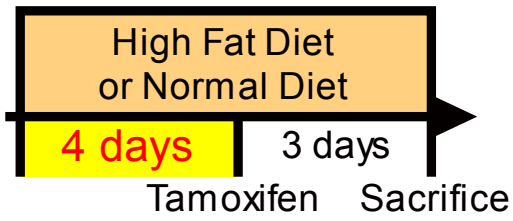

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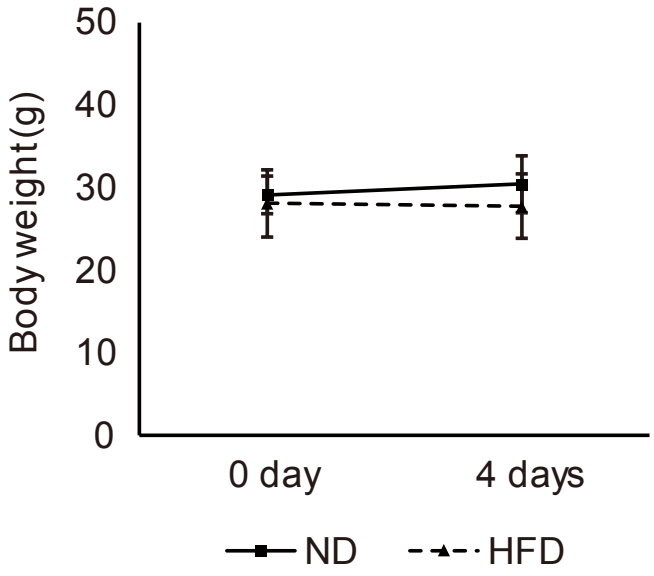

Figure 3.6. Experimental design of short-term HFD treatment.

(A) Experimental design for feeding and tamoxifen administration. (B) Effect of ND or HFD on body weight. Data represent mean ± SD from three independent experiments. *P < 0.001 (Student t-test)

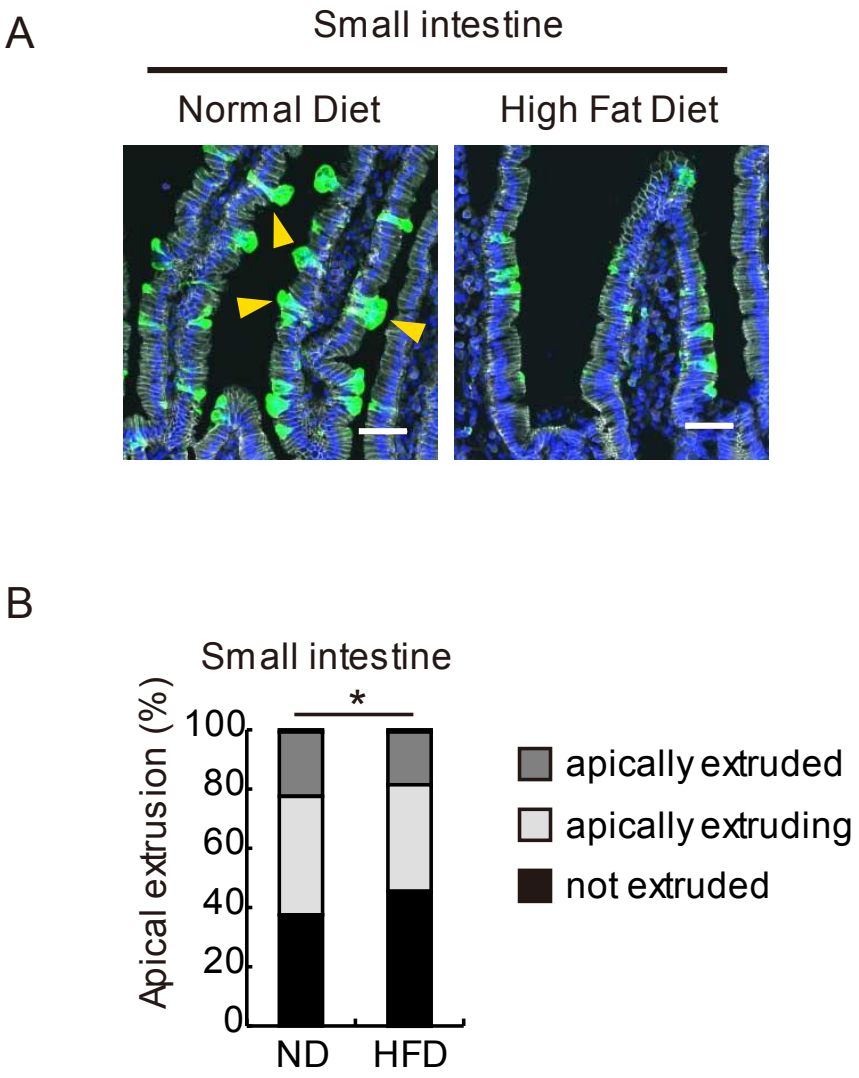

Figure 3.7. Effect of short-term HFD treatment on apical elimination of RasV12-transformed cells from the mouse small intestinal epithelial tissue. (A) Immunofluorescence images of RasV12-transformed cells in the epithelium of the small intestine. The tissue samples were stained with anti-GFP (green) and anti-E-cadherin (white) antibodies and Hoechst (blue). The arrowheads indicate extruding cells. Scale bars, 50 μm. (B) Quantification of apical extrusion of RasV12 cells in the small intestine. ND 940 cells from 3 mice, HFD 749 cells from 4 mice. *$P < 0.05$ (chi-square test).

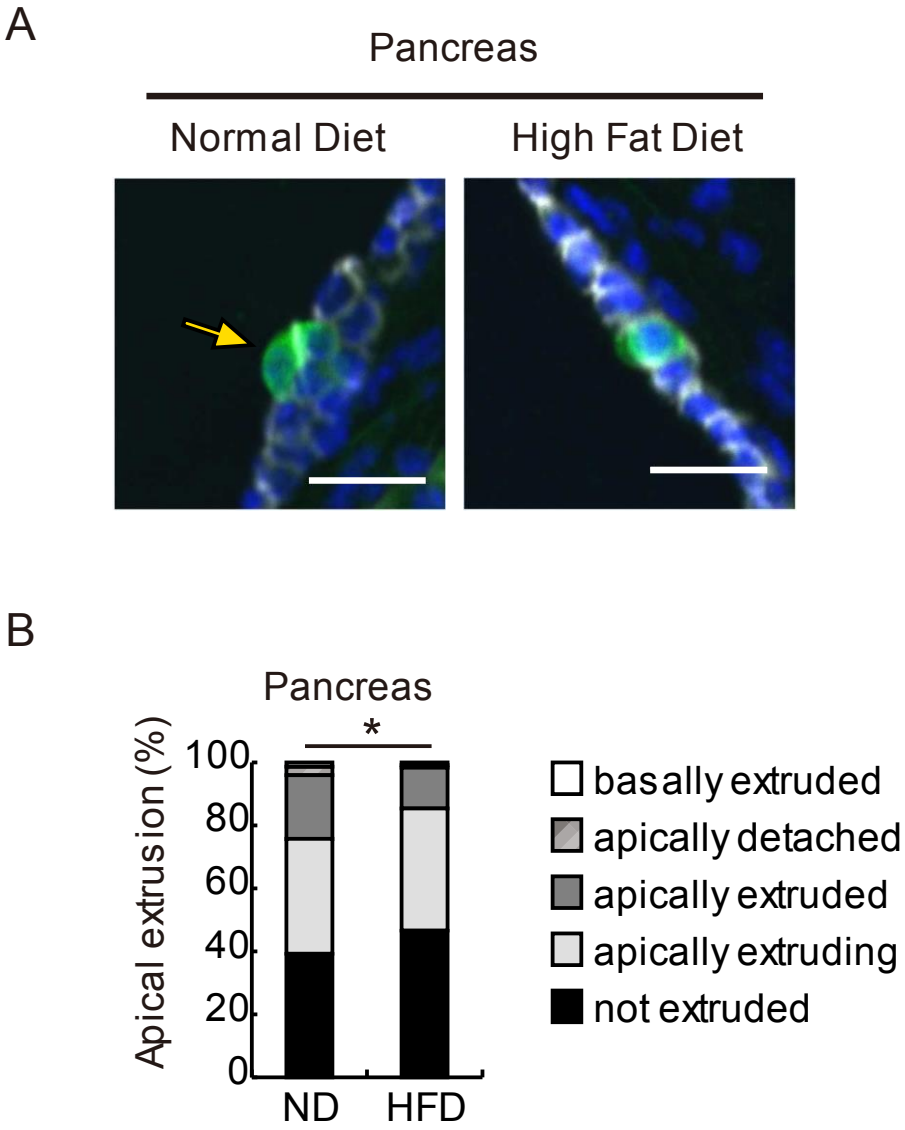

Figure 3.8. Effect of short-term HFD treatment on apical elimination of RasV12-transformed cells from the mouse pancreatic epithelial tissue. (A) Immunofluorescence images of RasV12-transformed cells in the epithelium of the pancreas. The tissue samples were stained as previously described in Figure 3.7. The arrow indicates apically extruded cell. Scale bars, 50 μm. (B) Quantification of apical extrusion of RasV12 cells in the pancreas. ND 222 cells from 3 mice, HFD 348 cells from 4 mice. *$P < 0.05$ (chi-square test).

3.4. Discussion

To reveal the mechanisms of the inhibition of apical elimination by HFD treatment, I examined the involvement of the change of lipid metabolism in the cell competition. As a result, it has revealed that the addition of fatty acids rescued the decreased mitochondrial activity by increase of fatty acids β-oxidation, and the metabolic change inhibits the elimination of mutated cells. Short-term HFD also significantly suppressed apical extrusion in the small intestine and pancreatic tissues. But the inhibitory effect of short-term HFD was smaller than that of long-term HFD. These results suggest that the change of intracellular metabolism is partially involved in HFD-mediated suppression of EDAC. On the other hand, these results also indicate that the possibility that the additional factors such chronic inflammation is involved in the inhibition of elimination of RasV12-transformed cells in the obese mice.

3.5. References

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Chapter 4:

CHRONIC INFLAMMATION AND EDAC

4. Chronic inflammation and EDAC

4.1. Introduction

Next, I examined whether chronic inflammation is involved in the extrusion of transformed cells as well. For mammals, essential fatty acids mainly consist of unsaturated ω6 and ω3 fatty acids (Smith, 2008). As energy source, both ω6 and ω3 fatty acids can be metabolized into acetyl-CoA through oxidation. In another metabolic pathway, ω6 fatty acids are metabolized to arachidonic acid, which can cause chronic inflammation in various tissues including the intestine and pancreas. In contrast, ω3 fatty acids are converted into eicosapentaenoic acid and thus have the anti-inflammatory effect (Miyata and Arita, 2015; Serhan, 2014). Indeed, it has been previously demonstrated that ω3 fatty acid-enriched linseed oil presents anti-inflammatory effect *in vivo* (Kunisawa et al., 2015; Wahli and Michalik, 2012). I then examined the effect of linseed oil or ω6 fatty acid-enriched soy bean oil on apical elimination of RasV12-transformed cells in the cell competition mouse model.

4.2. Experimental procedures

4.2.1. Antibodies and materials

Chicken anti-GFP (ab13970) antibody was purchased from Abcam. Rat anti-E-cadherin (131900) antibody was from Life Technologies. Alexa-Fluor-488-conjugated secondary antibody was from Abcam, and Alexa-Fluor-647-conjugated secondary antibodies were from Life Technologies. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000. Acetylsalicylic acid (aspirin) was from SIGMA.

4.2.2. Mice

HFD treatment was achieved by feeding female CK19-*RasV12*-GFP mice a dietary chow consisting of 60% kcal fat (Research Diets D12492). The HFD feeding began at the age of 6-10 weeks and were extended for a period of 3 months. For the experiment of the soy bean oil- or linseed oil-enriched diet, mice were maintained for 3 months on diets composed of chemically defined materials with 10% each dietary oil (Oriental Yeast). Control mice were age-matched and fed with normal diet (NOSAN). The mice were given a single intraperitoneal injection of 1 mg of tamoxifen in corn oil (Sigma) per 20 g of body weight, and were then sacrificed at 3 or 30 days after Cre activation. To examine the effect of aspirin, the ND- or HFD-fed mice were pretreated with 30 mg/L in their drinking water for 4 days. Subsequently, the mice were injected intraperitoneally with tamoxifen and sacrificed 3 days later; aspirin was continuously administered during this period.

4.2.4. Immunofluorescence

For immunohistochemical examinations of the small intestine, pancreas and lung, the mice were perfused with 1% paraformaldehyde (PFA, Sigma-Aldrich), and the isolated tissues were fixed with 4% PFA in PBS for 24 h and embedded in FSC 22 Clear Frozen Section Compound (Leica). Then, 10-μm-thick frozen sections were cut on a cryostat. The sections were blocked with Block-Ace (DS Pharma Biomedical) and 0.1% Triton X-100 in PBS. Primary or secondary antibodies were incubated for 2 h or 1 h respectively at ambient temperature. All primary antibodies were used at 1:1000, and all secondary antibodies were at 1:500. Immunofluorescence images of mouse tissues were acquired using the Olympus FV1000 system and Olympus FV10-ASW software.

4.2.4. Statistics and reproducibility

For data analyses, Chi-squared test (Figures 4.2B, 4.3B, 4.4B and 4.5B) and unpaired two-tailed Student *t*-tests (Figure 4.1B) were used to determine *P*-values using GraphPad Prism7 and Microsoft Excel, respectively. *P*-values less than 0.05 were considered to be statistically significant. For animal studies, the experiments were not randomized, and the investigators were not blinded to allocation during experiments. All results were reproduced in at least three mice for each experimental condition.

4.3. Results

4.3.1. Soy bean oil-enriched diet inhibited apical elimination of RasV12-transformed cells than linseed oil-enriched diet.

To investigate the relationship between chronic inflammation driven by obesity and cell competition, I examined the effect of linseed oil or ω6 fatty acid-enriched soy bean oil on apical elimination of RasV12-transformed cells in the cell competition mouse model (Figure 4.1A). Soy bean oil- and linseed oil-fed mice gained weight to a comparable extent (Figure 4.1B). Soy bean oil-feeding profoundly diminished apical extrusion of RasV12-expressing cells in the small intestine and pancreas (Figures 4.2 and 4.3). Linseed oil also suppressed apical extrusion, but to a lesser extent than soy bean oil (Figures 4.2 and 4.3). These data suggest that chronic inflammation may have an inhibitory role in the elimination of transformed cells.

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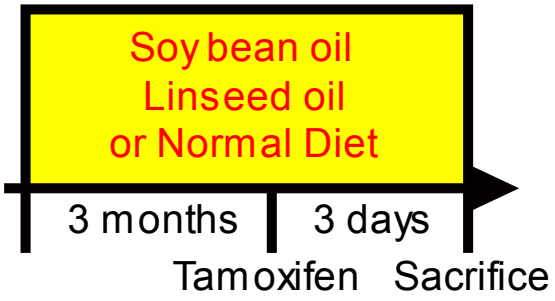

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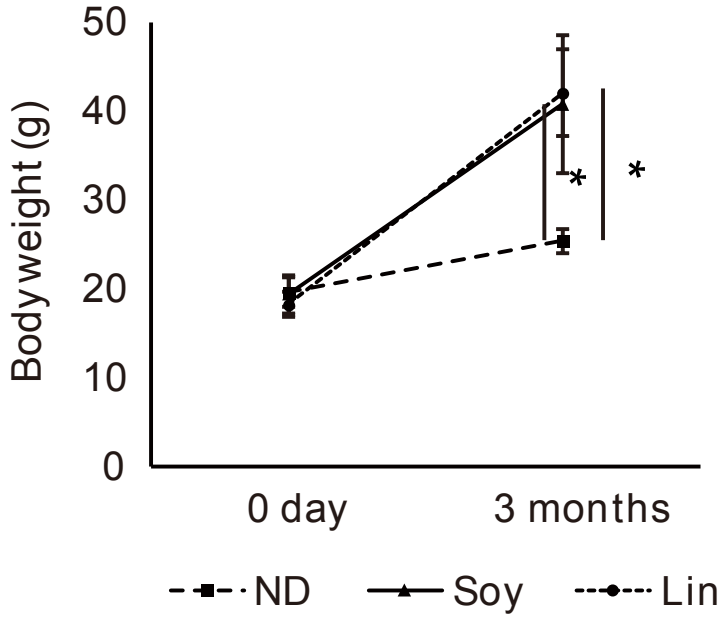

Figure 4.1. Experimental design of soy bean oil- or linseed oil-enriched diet treatment. (A) Experimental design for feeding and tamoxifen administration. (B) Effect of ND or soy bean oil- or linseed oil-enriched diet on body weight. Data represent mean ± SD from three independent experiments. *P < 0.001 (Student t-test).

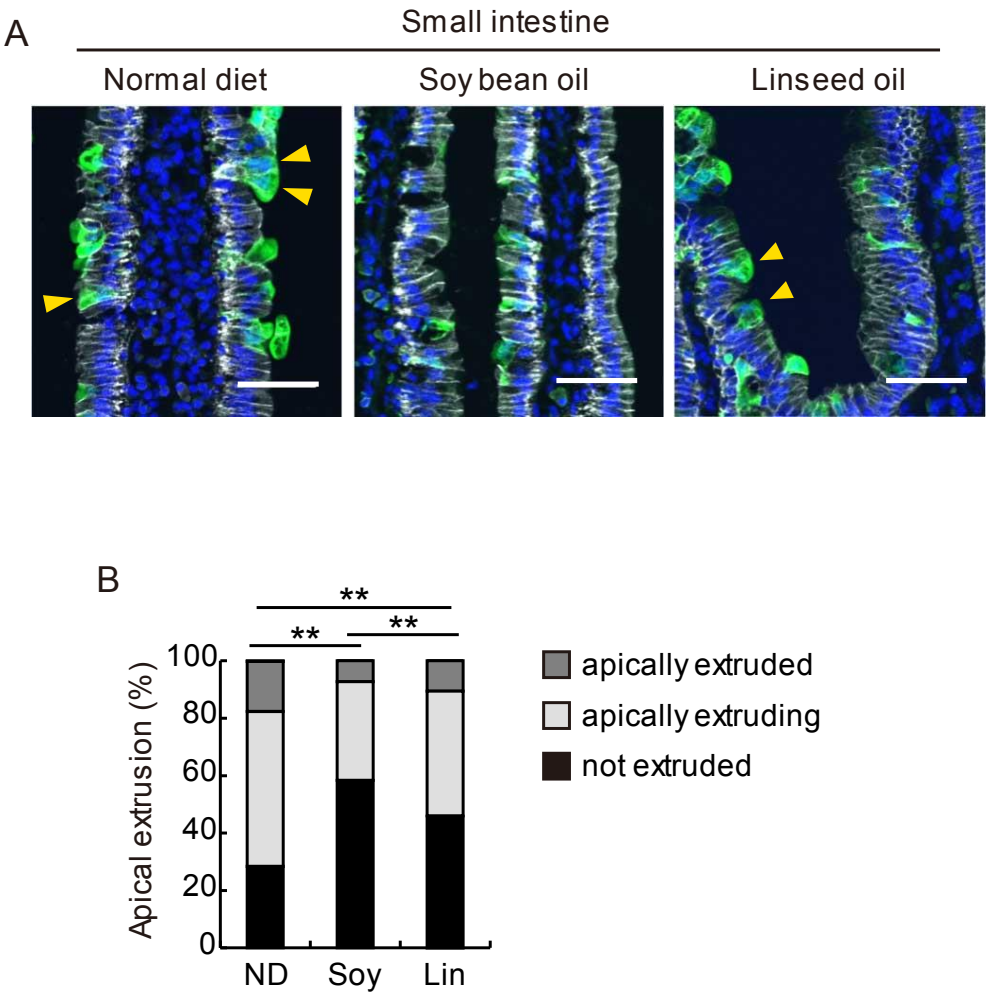

Figure 4.2. Effect of the soy bean oil- or linseed oil-enriched diet treatment on apical elimination of RasV12-transformed cells from the small intestinal epithelial tissue. (A) Immunofluorescence images of RasV12-transformed cells in the epithelium of the small intestine. The tissue samples were stained with anti-GFP (green) and anti-E-cadherin (white) antibodies and Hoechst (blue). Arrowheads indicate apically extruding cells. Scale bars, 50 μm. (B) Quantification of apical extrusion of RasV12 cells in the small intestine. ND 2,863 cells from 8 mice, Soy 1,584 cells from 4 mice, Lin 1,215 cells from 4 mice. ***P* < 0.0001 (chi-square test).

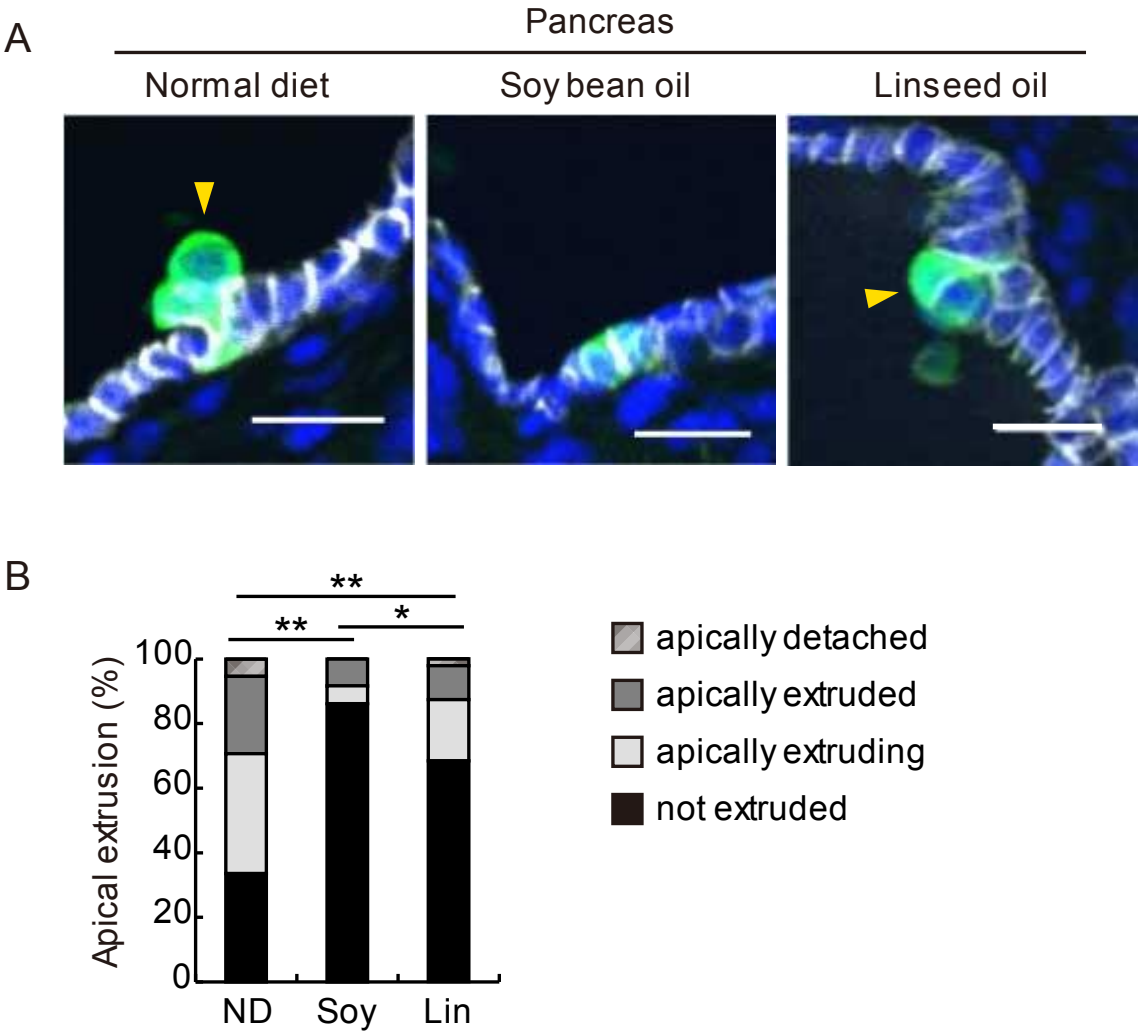

Figure 4.3. Effect of the soy bean oil- or linseed oil-enriched diet treatment on apical elimination of RasV12-transformed cells from the pancreatic epithelial tissue. (A) Immunofluorescence images of RasV12-transformed cells in the epithelium of the pancreas. The tissue samples were stained as previously described in Figure 4.2. Arrowheads indicate apically extruding cells. Scale bars, 20 μm. (B) Quantification of apical extrusion of RasV12 cells in the pancreas. ND 560 cells from 9 mice, Soy 72 cells from 4 mice, Lin 190 cells from 4 mice. *$P < 0.05$, **$P < 0.0001$ (chi-square test).

4.3.2. Aspirin rescued suppression of apical extrusion of RasV12-transformed cells from epithelial tissues.

To further clarify the involvement of chronic inflammation, we examined the effect of Non-Steroidal Anti-Inflammatory Drug (NSAID) (Figure 4.4A). In ND-fed mice, treatment of aspirin, one of the most commonly used NSAIDs, did not significantly affect the frequency of apical extrusion in both the small intestine and pancreas (Figures 4.4 and 4.5). In contrast, in HFD-fed mice, aspirin treatment substantially facilitated apical extrusion, and the lesser number of RasV12-transformed cells remained within intestinal and pancreatic epithelia (Figures 3G-J), demonstrating that suppression of chronic inflammation can facilitate the elimination of transformed cells from epithelial tissues.

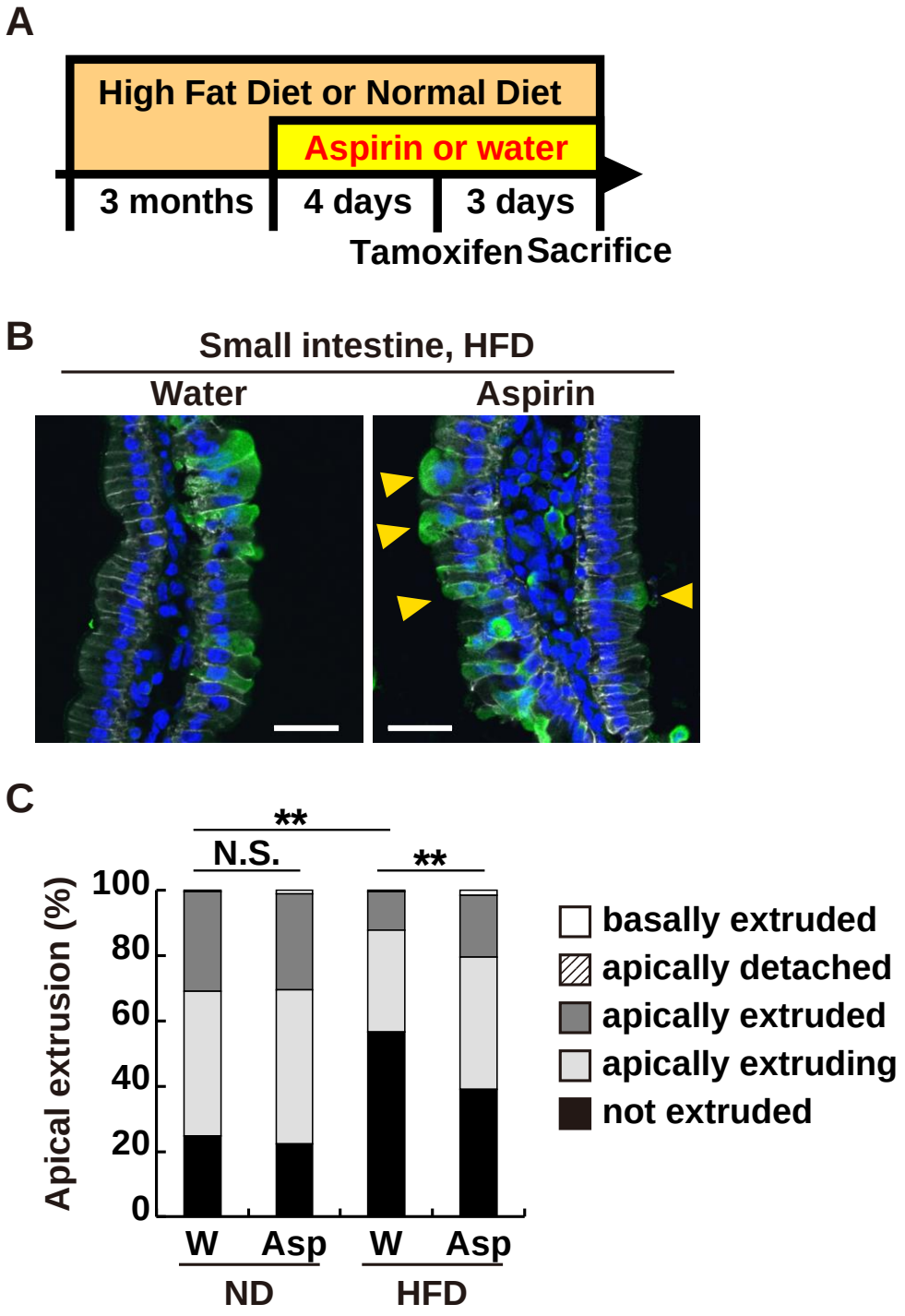

Figure 4.4. Effect of Aspirin on apical elimination of RasV12-transformed cells from the small intestinal epithelial tissue in ND- or HFD-fed mice (A) Experimental design for diet feeding, aspirin

treatment and tamoxifen administration. (B) Immunofluorescence images of RasV12 cells in the epithelium of the small intestine. The arrowheads indicate apically extruded cells. Scale bars, 30 μm. (C) Quantification of apical extrusion of RasV12 cells in the small intestine. ND (W: water) 481 cells, ND (Asp: aspirin) 517 cells, HFD (W) 600 cells, HFD (Asp) 372 cells. ND (W: water) 481 cells, ND (Asp: aspirin) 517 cells, HFD (W) 600 cells, HFD (Asp) 372 cells. For each condition, cells were collected from 3 mice. $^{**}P < 0.0001$ (chi-square test).

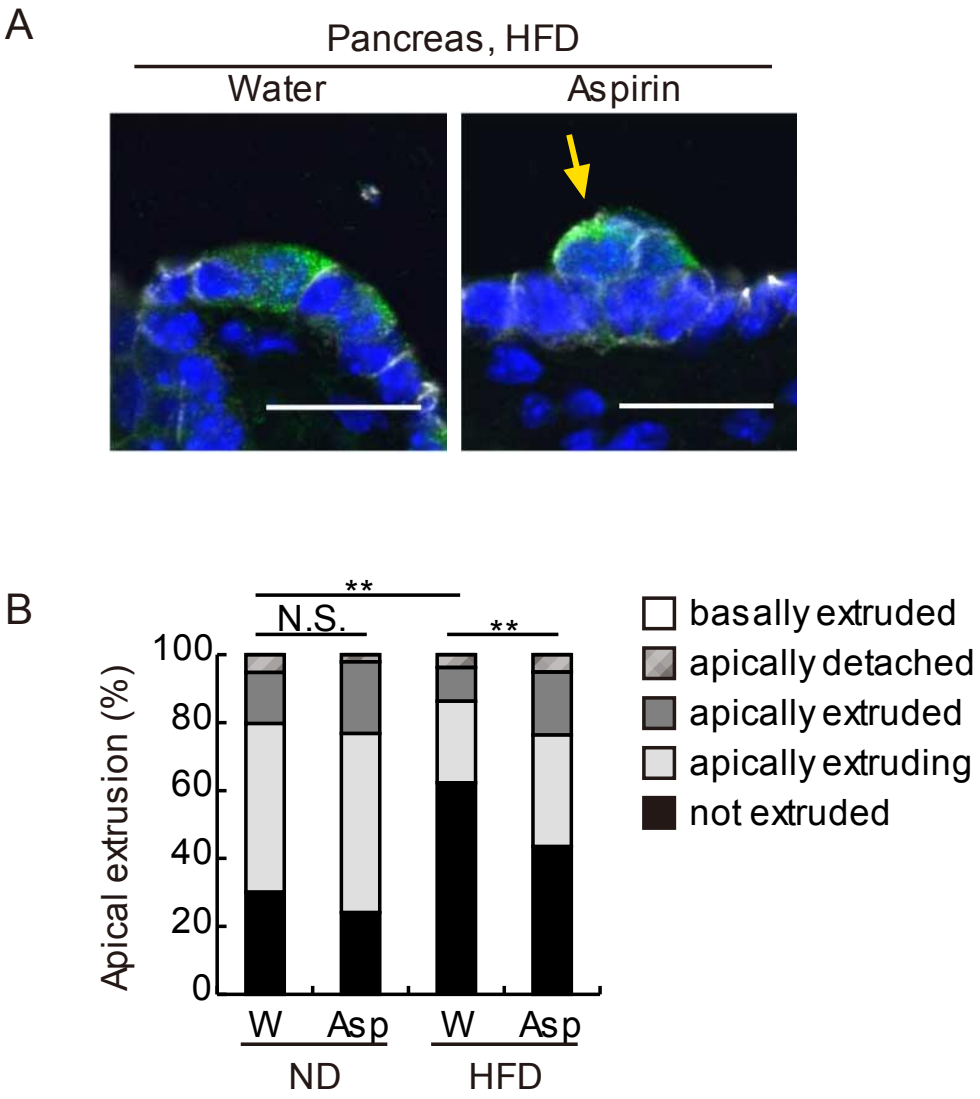

Figure 4.5. Effect of Aspirin on apical elimination of RasV12-transformed cells from the pancreatic epithelial tissue in ND- or HFD-fed mice (A) Immunofluorescence images of RasV12 cells in the epithelium of the small intestine. The arrowheads indicate apically extruded cells. Scale bars, 30 μm. (B) Quantification of apical extrusion of RasV12 cells in the small intestine. ND (W) 153 cells, ND (Asp) 237 cells, HFD (W) 212 cells, HFD (Asp) 216 cells. For each condition, cells were collected from 3 mice. $^{**}P < 0.0001$ (chi-square test).

4.4. Discussion

To investigate the mechanism of inhibition of apical extrusion by HFD treatment, I examined the involvement of chronic inflammation as a candidate approach. To assess the effect of chronic inflammation, soy bean oil- and linseed oil-enriched diet were used as inflammatory diet and anti-inflammatory diet, respectively.

The suppression of apical extrusion of RasV12-transformed cells in linseed oil-fed mice was lower than that in soybean oil-fed mice. And, aspirin rescued the inhibition of apical elimination of RasV12-transformed cells. These results suggest that chronic inflammation induced by the HFD negatively affect EDAC. And, these results suggest the potential that aspirin would diminish the risk of cancer of obesity by promoting apical extrusion of transformed cells in obese mouse.

Previous studies have revealed that NSAIDs can suppress the frequency of tumour formation in various tissues including intestine, pancreas and breast (Chubak et al., 2016; Drew et al., 2016; Giovannucci et al., 1995; Giovannucci et al., 1994; Kune et al., 1988; Streicher et al., 2014; Zhang et al., 2015); the underlying molecular mechanisms, however, remain obscure. In this study, I have demonstrated that aspirin promotes elimination of RasV12-transformed cells in HFD-fed mice, implying that reinforcement of EDAC can be one of the potential targets of NSAIDs in cancer prevention.

4.5. References

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Chapter 5:

OBESITY INCREASES THE FORMATION OF TUMOR-LIKE LEGIONS

5. Obesity increases the formation of tumor-like regions

5.1. Introduction

At three days after tamoxifen injection, in both ND- and HFD-fed mice, a substantial number of RasV12-expressing cells remained within the intestinal and pancreatic epithelium (Figure 2.3 and 2.4).

Although the apical elimination of transformed cells has been considered epithelial defence mechanism against cancer as EDAC (Kajita et al., 2010; Kon et al., 2017), it has not been reported whether cell competition actually inhibits carcinogenesis and whether the collapse of cell competition triggers carcinogenesis. To approach that is important for the evaluation of the ability of cell competition.

To investigate whether the rupture of cell competition lead to cause carcinogenesis or not, I analysed the fate of the remaining transformed cells after one month of induction of RasV12 expression in the pancreas.

5.2. Experimental Procedures

5.2.1. Antibodies and materials

Chicken anti-GFP (ab13970) antibody was purchased from Abcam. Rat anti-E-cadherin (131900) antibody was from Life Technologies. Alexa-Fluor-488-conjugated secondary antibody was from Abcam, and Alexa-Fluor-647-conjugated secondary antibody was from Life Technologies. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000.

5.2.2. Mice

HFD treatment was achieved by feeding female CK19-*RasV12*-GFP mice a dietary chow consisting of 60% kcal fat (Research Diets D12492). The long- and short-term HFD feeding began at the age of 6-10 weeks and were extended for a period of 3 months. Control mice were age-matched and fed with normal diet (NOSAN). The mice were given a single intraperitoneal injection of 1 mg of tamoxifen in corn oil (Sigma) per 20 g of body weight, and were then sacrificed at 30 days after Cre activation.

5.2.3. Immunofluorescence

For immunohistochemical examinations of the small intestine, pancreas and lung, the mice were perfused with 1% paraformaldehyde (PFA, Sigma-Aldrich), and the isolated tissues were fixed with 4% PFA in PBS for 24 h and embedded in FSC 22 Clear Frozen Section Compound (Leica). Then, 10-μm-thick frozen sections were cut on a cryostat. The sections were blocked with Block-Ace (DS Pharma Biomedical) and 0.1% Triton X-100

in PBS. Primary or secondary antibodies were incubated for 2 h or 1 h respectively at ambient temperature. All primary antibodies were used at 1:1000, and all secondary antibodies were at 1:500. Immunofluorescence images of mouse tissues were acquired using the Olympus FV1000 system and Olympus FV10-ASW software.

Statistics and reproducibility

For data analyses, unpaired two-tailed Student *t*-tests (Figure 5.2) was used to determine *P*-values using Microsoft Excel. *P*-values less than 0.05 were considered to be statistically significant. For animal studies, the experiments were not randomized, and the investigators were not blinded to allocation during experiments. All results were reproduced in at least three mice for each experimental condition.

5.3. Results

5.3.1. HFD treatment induces tumorous lesions in the pancreas.

At three days after tamoxifen injection, in both ND- and HFD-fed mice, a substantial number of RasV12-expressing cells remained within the intestinal and pancreatic epithelium (Figures 2.3 and 2.4). I then analysed the fate of remaining transformed cells after one month of induction of RasV12 expression in the pancreas (Figure 5.1). In ND-fed mice, the ratio of the epithelial ducts harbouring RasV12-expressing cells was profoundly reduced during the one month-period (Figures 5.1 and 5.2). In the majority of the pancreatic ducts, RasV12 cells were not observed, and remaining RasV12 cells were often found in a small cell cluster (Figures 5.1 and 5.2). In contrast, in HFD-fed mice, RasV12-expressing cells continued to remain within the ducts after one month and frequently formed a tumorous mass into the apical lumen of the epithelium (Figures 5.1 and 5.2). In addition, a small number of RasV12 cells were basally delaminated (Figure 5.1, arrows). These data indicate that HFD treatment can reduce Epithelial Defence Against Cancer (EDAC) and induce potentially precancerous lesions.

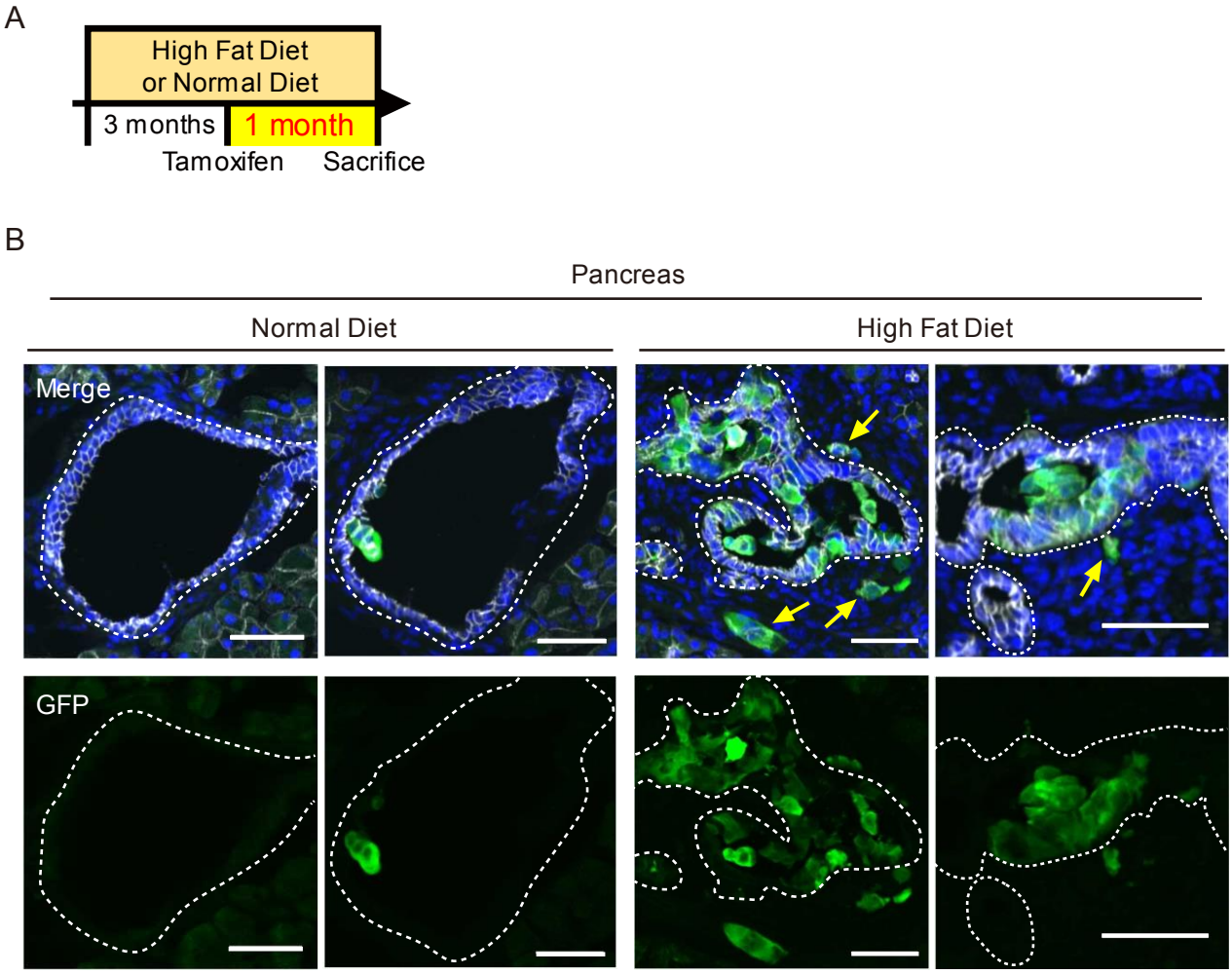

Figure 5.1. The fate of RasV12-transformed cells after one month of tamoxifen administration in the pancreas in ND- or HFD-fed mice (A) Experimental design for diet feeding and tamoxifen administration. (B) Immunofluorescence images of RasV12-transformed cells in the pancreas. The tissue samples were stained with anti-GFP (green) and anti-E-cadherin (white) antibodies and Hoechst (blue). The dotted lines delineate the basement membrane of the pancreatic epithelium. Arrows indicate basally extruded cells. Scale bars, 50 μm.

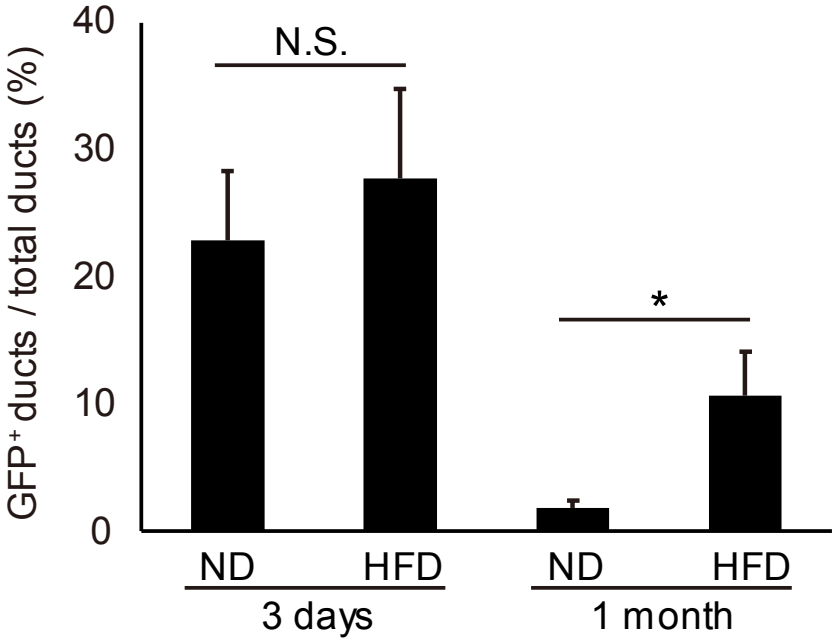

Figure 5.2. Quantification of pancreatic epithelial ducts harbouring RasV12-expressing cells. The percentage of ducts containing GFP-RasV12 cells relative to total ducts is shown. The total number of analysed ducts: 198 (ND 3 d), 139 (HFD 3 d), 428 (ND 1 m), 354 (HFD 1 m). Data are mean ± SD from three mice. *$P < 0.05$ (Student *t*-tests). N.S.: not significant.

5.4. Discussion

The cell competition of transformed cells in mammals has been considered epithelial defense mechanism against cancer as EDAC. But, it has not been examined how cell competition actually important for suppression of carcinogenesis. To approach this, I analyzed the fate of RasV12-transformed cells which escaped from the elimination mechanism. At 1 month after tamoxifen administration, the RasV12-transformed cells were remained in the pancreatic duct of obese mice compared with that of lean mice. And the RasV12-transformed cells harbored in the pancreatic ducts formed tumor-like legions.

Of course, the cell cluster was not visible size and it is not sure whether it will become to cancer after that. However, such accumulation of abnormal cells means breakdown of homeostasis of epithelial tissues, and these results suggest the possibility that cancers made by such accumulation of abnormal cells which evade from EDAC. And, such a model in which cell competition has inhibited is considered to be useful in application of cell competition in the future.

5.5. References

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Chapter 6:

CONCLUSION

6. CONCLUSION

In this study, I reveal that HFD treatment suppresses the eradication of newly emerging transformed cells from the intestinal and pancreatic epithelia, but not from the lung.

I also show that chronic inflammation is, at least partly, involved in HFD-mediated suppression of EDAC. Chronic inflammation involves recruitment and activation of various cells including immune cells and fibroblasts. Soluble factors secreted from these cells may affect competitive interaction between normal and transformed epithelial cells. The molecular mechanism of how chronic inflammation influences cell competition needs to be elucidated in future studies.

In addition, I demonstrate that in HFD-fed mice, tumorous lesions are formed in the pancreas after one month of induction of RasV12 expression. It is currently uncertain whether these potentially precancerous lesions can develop into malignant cancer. This issue also needs to be clarified in future by analysing the fate of RasV12-expressing cells after the longer period of time, probably in combination with certain carcinogen treatment.

Several lines of studies have suggested molecular mechanisms whereby obesity leads to higher incidence of cancer: oxidative stress, hormonal disorder, dysbiosis and chronic inflammation (Bianchini et al., 2002; Donohoe et al., 2017; Font-Burgada et al., 2016; Hopkins et al., 2016; Khandekar et al., 2011; Lauby-Secretan et al., 2016; Poloz and Stambolic, 2015; Renehan et al., 2015). These obesity-mediated processes are thought

to facilitate tumour growth and malignant transformation at the mid or later stage of tumorigenesis. But, my data indicate that obesity can also promote tumour initiation by influencing cell competition between normal and newly emerging transformed cells at the initial stage of carcinogenesis.

This is the first report demonstrating that environmental factors such as obesity and chronic inflammation influence cell competition within the epithelium. Previous studies have shown that at the boundary between normal and transformed epithelial cells, various non-cell-autonomous changes occur in both cells, which induces the competitive interaction between them (Maruyama and Fujita, 2017). But, this study implies that not only epithelial intrinsic factors, but also extrinsic environmental factors from the outside of epithelia could profoundly influence the outcome of cell competition. It is plausible that other environmental factors such as infection, smoking, sleep and ageing also affect cell competition. Indeed, a previous *Drosophila* study has demonstrated that suboptimal cells accumulate in the aged flies and that intensification of cell competition reduces the number of suboptimal cells, leading to extended lifespan (Merino et al., 2015); however it remains unclear whether ageing itself diminishes cell competition. Further development of these studies would lead to comprehensive understanding of cell competition in physiology and pathology.

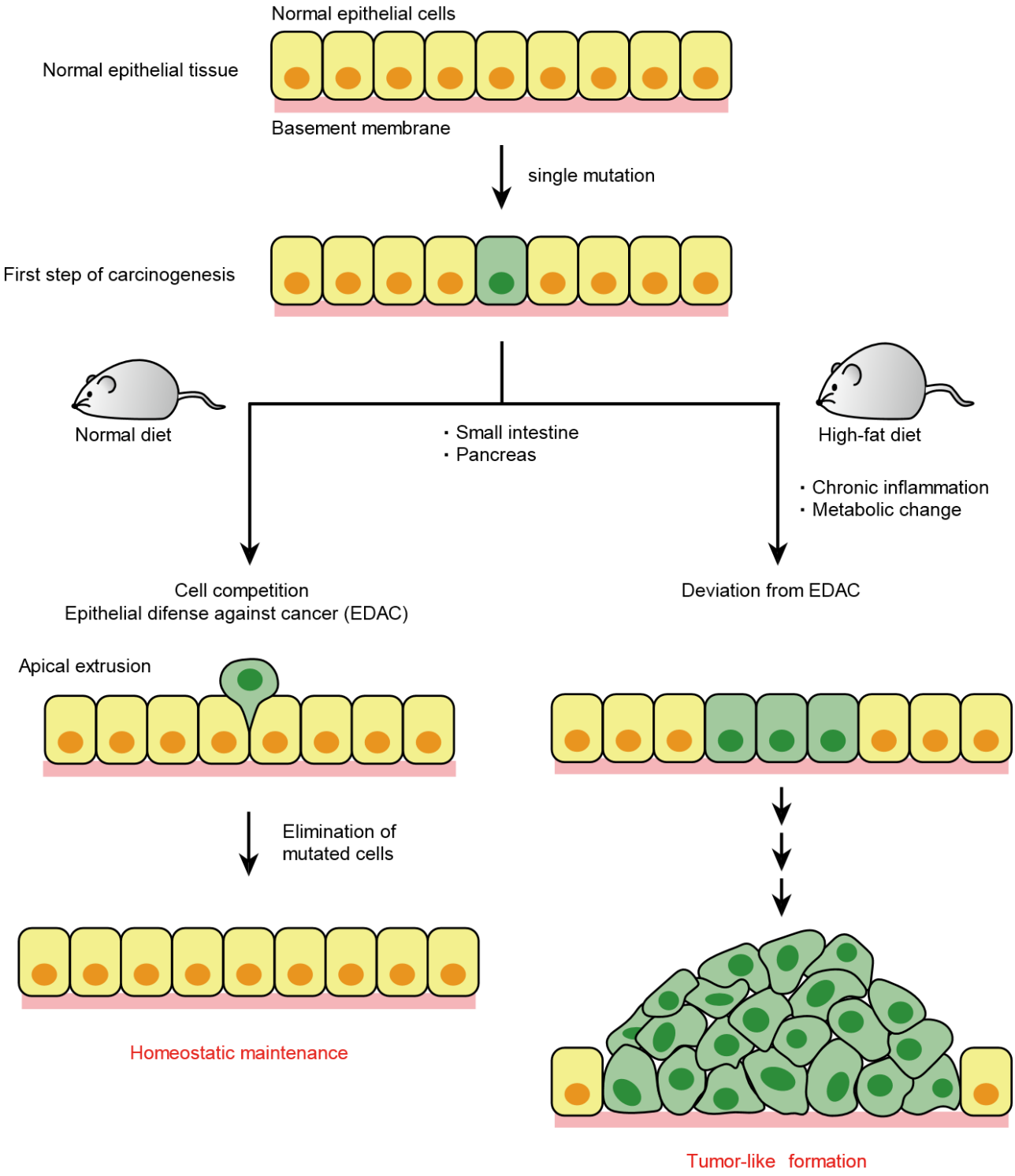

Figure 6.1. Schematic model of this study

6.2. References

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