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Study on the COX-2/PGE₂ Pathway on Apical Elimination of RasV12- transformed Cells from Epithelia

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Abstract

At the initial stage of carcinogenesis, when RasV12-transformed cells are surrounded by normal epithelial cells, RasV12 cells are often extruded from the apical lumen of the epithelial layer through cell competition with the surrounding normal cells. However, the underlying molecular mechanisms of this cancer preventive process are not fully understood. In this study, we first demonstrate that expression of cyclooxygenase (COX)-2 is transcriptionally upregulated in normal epithelial cells that surround RasV12-transformed cells in a non-cell-autonomous manner. Addition of COX inhibitor or COX-2-knockout in normal cells promotes apical extrusion of RasV12 cells. Furthermore, production of Prostaglandin (PG) E₂, a downstream prostanoid of COX-2, is elevated in normal cells surrounding RasV12 cells. Addition of PGE₂ suppresses apical extrusion of RasV12 cells, while PGE₂ synthase inhibitor enhances it. In a cell competition mouse model, expression of COX-2 is elevated in pancreatic epithelia harbouring RasV12-expressing cells, and administration of the COX inhibitor ibuprofen promotes apical extrusion of RasV12 cells. Moreover, caerulein-induced chronic inflammation substantially suppresses apical elimination of RasV12 cells. These results indicate that intrinsically or extrinsically mediated inflammation can promote tumour initiation by diminishing cell competition between normal and transformed cells.

Abbreviations

COX-2	Cyclooxygenase-2
PG	Prostaglandin
PGE ₂	Prostaglandin E ₂
EDAC	Epithelial defense against cancer
MDCK	Mardin-Darby canine kidney
CK19	Cytokeratin-19
GFP	Green fluorescent protein
YFP	Yellow fluorescent protein
FACS	Fluorescence-activated cell sorting
qPCR	quantitative polymerase chain reaction
PKC	Protein kinase C
BIM	Bisindolylmaleimide
ELISA	Enzyme-linked immunosorbent assay
NSAID	Non-steroidal anti-inflammatory drug

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CHAPTER 1:

General Introduction

1. General Introduction

1.1. The concept behind this study

Cancer is the one of leading cause of death globally and is estimated to account for 9.6 million death in 2018. Especially in Japan, more than 300,000 people die from cancer every year which means one in five deaths is due to cancer. Early detection is the key to complete cure in cancer, but it is often too late for refractory cancers such as pancreatic cancer, as it has already metastasized when detected by clinical diagnosis. One promising measure is to detect and remove cancer mutant cells at an initial stage prior to the current clinically diagnosable stage (Fig.1).

Generation of an abnormal cell in the epithelial cell layer is considered to be the first step to cancer development. When the first oncogenic mutation occurs in epithelia, then what happens? Recently, it has been reported that newly emerging mutant cells are eliminated from epithelial monolayer by cell competition with surrounding normal epithelial cell (see section 1.3). And, the findings have been expected to give a new strategy for cancer treatment and/or cancer prevention and deeper understandings about carcinogenesis. However, despite this anti-cancer activity, cancer often arises from epithelial tissues, suggesting that there may be molecular mechanisms that diminish it. Therefore, it is necessary to understand the mechanism that controls cell competition positively or negatively in more detail.

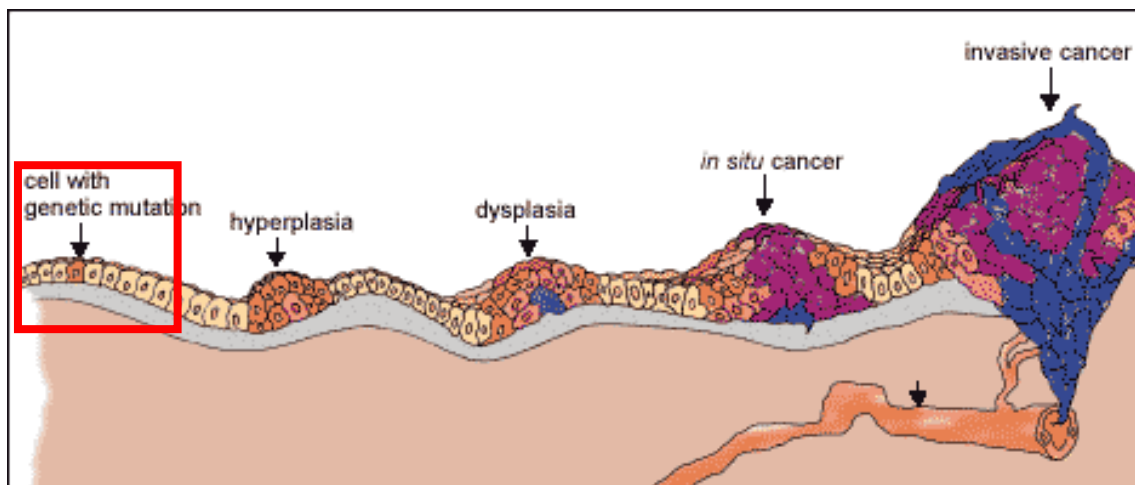


Figure 1-1. Stage of cancer development adopted from Understanding Cancer, teacher's guide NIH 2017

Tumor development begins when a cell sustains an oncogenic mutation. We focus on this stage: the cells that have just one or two mutation that is difficult to detect in current clinical diagnosis as shown in red circle.

1.2. Oncogene dealt with in this study

1.2.1. Ras

In this study, I focused on cell competition caused by Ras mutant overexpression. A summary of the importance of Ras mutations in cancer is given below briefly.

1.2.1.1. Ras gene mutation in human cancer

Ras genes were originally identified as a retroviral oncogene from the Harvey and Kirsten rat sarcoma virus genomes in the 1960/70s. Three human *Ras* genes are known: H-RAS (Harvey), K-RAS (Kirsten) and N-RAS, which share approximately 90% amino acid identity. These three RAS genes are the most frequently mutated oncogene family in human cancers. Most common mutation in *Ras* is single missense mutation typically at codons 12, 13 or 61. But the missense mutation frequency at each position varies widely between isoforms, with G12 mutations the most common in *K-Ras* and *H-Ras*, while Q61 mutations are the most common in *N-Ras* (Malumbres and Barbacid, 2003). In human cancers, *K-Ras* mutations are far more frequently observed in the three genes, especially most commonly in malignancies of lung, intestines and pancreas (Table 1-1). *H-Ras* mutations are found in cancers arising from the skin, brain, and reproductive organs, and *N-Ras* is found in cancers originating from the skin, thyroid, and blood (Table 1-1).

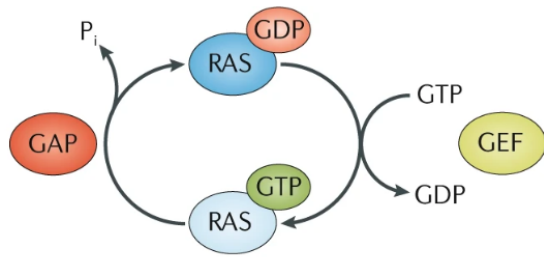
Cancer	% KRAS	% NRAS	% HRAS	% All RAS
Pancreatic ductal adenocarcinoma	97.7	0.0	0.0	97.7
Colorectal adenocarcinoma	44.7	7.5	0.0	52.2
Multiple myeloma	22.8	19.9	0.0	42.6
Lung adenocarcinoma	30.9	0.9	0.3	32.2
Skin cutaneous melanoma	0.8	27.6	1.0	29.1
Uterine corpus endometrioid carcinoma	21.4	3.6	0.4	24.6
Uterine carcinosarcoma	12.3	1.8	0.0	14.3
Thyroid carcinoma	1.0	8.5	3.5	12.5
Acute myeloid leukaemia	3.1	6.7	1.6	11.4
Bladder urothelial carcinoma	3.1	1.4	5.9	10.6
Gastric adenocarcinoma	11.4	0.9	0.0	10.0
Cervical adenocarcinoma	8.3	0.0	0.0	8.3
Head and neck squamous cell carcinoma	0.5	0.3	4.7	5.5
Diffuse large B cell lymphoma	5.2	0.0	0.0	5.2
Oesophageal adenocarcinoma	3.8	0.0	0.6	4.4
Chronic lymphocytic leukaemia	1.9	2.5	0.0	4.4
Lung squamous cell carcinoma	2.2	0.0	2.2	4.4
Small cell lung carcinoma	1.4	0.0	1.4	2.8
Renal papillary cell carcinoma	1.2	0.6	0.6	2.4
Adenoid cystic carcinoma	0.0	0.0	1.7	1.7
Chromophobe renal cell carcinoma	0.0	1.5	0.0	1.5
Hepatocellular carcinoma	0.8	0.4	0.0	1.4
Breast invasive carcinoma	0.7	0.4	0.3	1.4
Cervical squamous cell carcinoma	1.3	0.0	0.0	1.3
Ovarian serous adenocarcinoma	0.6	0.6	0.0	1.3
Adrenocortical carcinoma	1.1	0.0	0.0	1.1
Prostate adenocarcinoma	0.3	0.0	0.3	0.9
Neuroblastoma	0.0	0.8	0.0	0.8
Brain lower grade glioma	0.5	0.5	0.0	0.8
Glioblastoma	0.7	0.0	0.0	0.7
Medulloblastoma	0.0	0.4	0.0	0.4
Kidney renal clear cell carcinoma	0.2	0.0	0.2	0.4
Oesophageal squamous cell carcinoma	0.0	0.0	0.0	0.0
Osteosarcoma (paediatric)	0.0	0.0	0.0	0.0
Ovarian small cell carcinoma	0.0	0.0	0.0	0.0
Rhabdoid tumours	0.0	0.0	0.0	0.0
Sarcoma	0.0	0.0	0.0	0.0
Small intestine neuroendocrine tumours	0.0	0.0	0.0	0.0
T cell prolymphocytic leukaemia	0.0	0.0	0.0	0.0

Table 1-1. Frequency of *RAS* mutations in human cancers, adopted from (Cox et al., 2014)

1.2.1.2. Function and regulation of Ras protein

All Ras proteins belong to small GTPase family that function as GDP/GTP-regulated molecular switches. Ras GTPases cycles between an active and inactive GDP binding state. They are inactivated by GTPase activating protein (GAP) and activated by guanine nucleotide exchange factor (GEF) (Figure 1-2A). Ras GTPase plays an important role in the regulation of cell growth, cell differentiation, cell migration, and lipid vesicle trafficking. (Figure 1-2B). Oncogenic potential of Ras is enhanced by point mutations (see 1.2.1.1), which not only impair the intrinsic GTPase activity but also render Ras insensitive to the GAP action, resulting in the constitutive activation of the downstream effectors (Figure 1-2B).

A



B

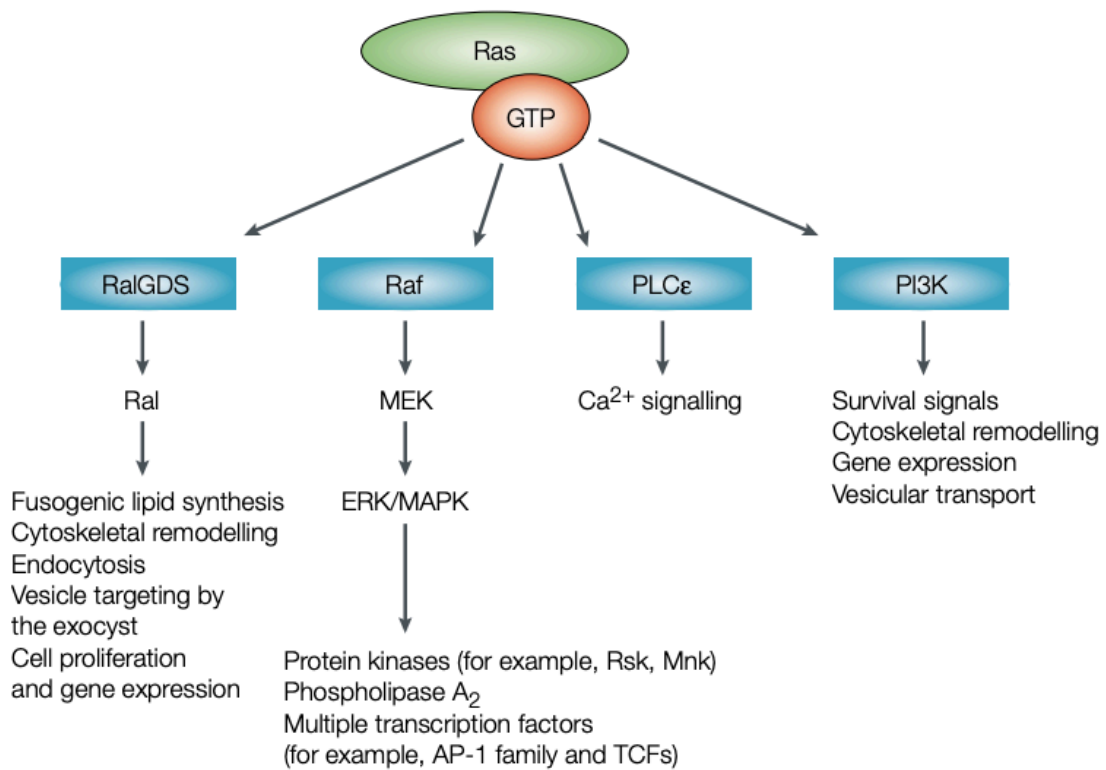


Figure 1-2. Ras activation and downstream effectors

(A) Ras proteins function as molecular switches, cycling between the GDP-bound ('off') and the GTP-bound ('on') states and exist predominantly in the GDP-bound state, adopted from (Ryan and Corcoran, 2018).

(B) A key pathway that signals downstream from activated Ras, adopted from (Cullen and Lockyer, 2002).

1.3. Cell competition

Cell competition is defined as the elimination or exclusion of cells caused by the short-range cell-cell interaction between two different types of cells. For example, in growing tissue, cells with lower fitness are eliminated as a “loser” by neighboring cells with higher fitness, and then cell with high fitness grows as a “winner” (Figure1-2B).

Importantly, cells with low fitness are viable when situated among cells from the same genotype (Figure 1-2A). Initially, cell competition was discovered as a phenomenon in which mutant cells are eliminated from the tissue however it was also found that some mutant cells became “super-competitors” and eliminated surrounding wild-type cells (Figure1-2B). Recently, it has revealed that various oncogenic mutations also induce cell competition.

1.3.1. Cell competition in *Drosophila*

Cell competition was first described four decades ago in developing epithelia of *Drosophila* (Morata and Ripoll, 1975). Since then, a number of *Drosophila* studies have revealed that cell competition can occur between normal and various types of transformed cells in epithelial tissues.

1.3.1.1. Minute

Cell competition was first reported in 1975 through the analysis of *Drosophila Minute* mutants by Morata and Ripoll. *Minute* mutants are a class of *Drosophila* mutants which have mutations in the ribosomal protein genes. Homozygous *Minute* flies (*Minute*^{-/-}) are lethal. Although

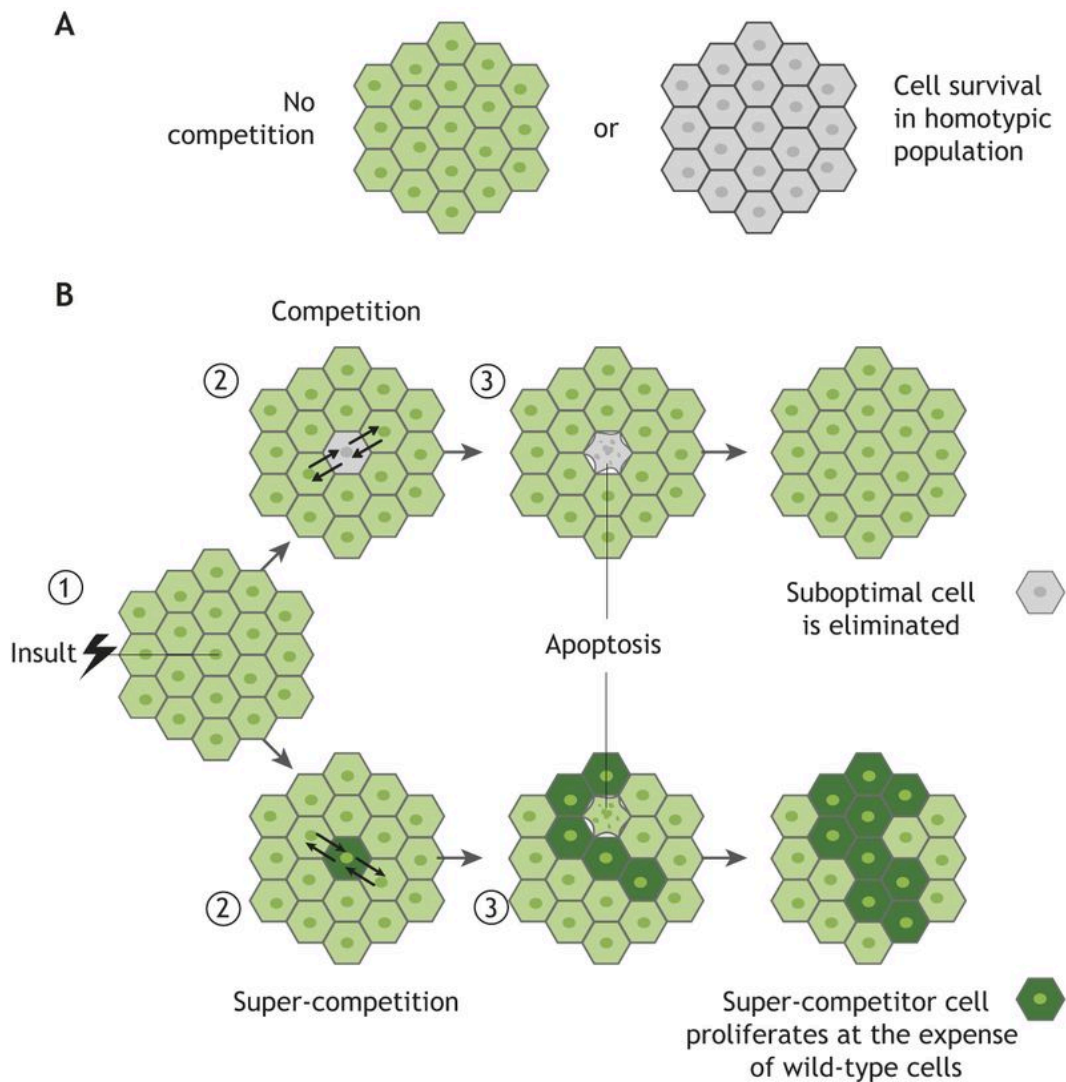


Figure 1-3. Conceptual model of “Cell Competition”, adopted from (Coelho and Moreno, 2019)

(A) In homotypic cell populations composed of winner cells (light green) or loser cells (gray), there are no relative fitness differences between cells. Competition is not initiated, and all cells survive.

(B) An initial insult or mutation can induce competition. In classical cell competition, suboptimal cells (gray) are eliminated from the tissue by wild-type cells (light green), which proliferate to maintain organ size. In super-competition, mutant cells (dark green) become super-competitors.

heterozygous *Minute* (*Minute*^{+/-}) are viable develop into essentially normal flies with relatively thinner bristles, interestingly, a small population of *Minute*^{+/-} clones induced in wild-type *Drosophila* wing discs disappeared during development. These observations suggest that *Minute*^{+/-} cells were eliminated via competitive interactions with neighboring wild-type cells and thus the phenomenon was called “cell competition”. Further study shows that *Minute*^{+/-} cells that directly contact with wild-type cells seemed to be eliminated, suggesting that cell competition occurs through local cell-cell interaction (Simpson and Morata, 1981). In this paper, they also reported the growth difference in wild-type cells and *Minute*^{+/-} cells; the winner cells growing faster than the loser ones. It has been hypothesized 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. However, recent studies neglected this hypothesis by showing that elimination of *Minute*^{+/-} cells was apoptosis-dependent, and the apoptosis in loser cell is induced by lower ability to capture the secreted survival factor than the surrounding wild-type cells (Moreno et al., 2002). The mechanisms of cell competition triggered by *Minute* mutation is still controversy (Li and Baker, 2007; Lolo et al., 2012). However, at least, it has become evident that cell growth speed is not the absolute determinant for the consequence of cell competition; rather, the interaction between winner and loser cells induces non-cell-autonomous changes that influence the behavior of both cells.

1.3.1.2. dMyc

It took another 30 years until cell competition became a subject of interest again, when two independent group reported that cell competition was not limited to *Minute*

mutation, but also caused by different expression level of *dMyc* (*Drosophila myc*), the homolog of the mammalian proto-oncogene c-Myc, (De La Cova et al., 2004; Moreno and Basler, 2004). Cells with higher level of dMyc expression became “winner”, while cells with lesser level of dMyc are eliminated by neighboring winner cells and become “loser”. Moreover, Myc-overexpressing cells eliminate their wild-type neighbors, leading them to be considered as supercompetitors. Since cells that highly expression of onco-protein Myc kills neighbors and become winner, it has attracted attention that the role of cell competition not only in development but also in the context of cancer progression. To support this, it has been shown that abnormalities in various signaling pathways related to cancer induce cell competition.

1.3.1.3. Other genes

In *Drosophila Melanogaster*, other genes which induce cell competition are identified (Table 1-2). Not only mutations in genes related to cell growth, but also mutations in tumor suppressor genes involved in cell polarity (Scribble, Lgl) or endocytosis (Vps25) leads to cell death.

Table 1-2. Triggers of cell competition in *Drosophila Melanogaster*.

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

1.3.2. Cell competition in vertebrates

Recent studies have revealed that cell competition also occurs in vertebrates (Table 1-3 and 1-4) under various physiological and pathological conditions such as embryonic and organ development, tissue homeostasis, delay of ageing and various disease models. In this thesis, I examined the mechanism of cell competition using the mammalian cell culture system (see section 1.3.2.1) and cell competition model mouse (see section 1.3.2.2) in the context of cancer development (see section 1.3.2.3).

1.3.2.1. *In vitro* cell competition model

To observe the cell competition in cell culture system, one of the technical problems was making mosaic pattern; both two type of cells should interact each other at short distance. However, in cell culture system, it is widely known that when two type of cells are exist, cell sorting occurs and the same type of cells form colonies (Foty and Steinberg, 2005; Krieg et al., 2008; Steinberg and Takeichit, 1994). Therefore, it is difficult to form mosaic pattern in co-cultured system. To solve this problem, our group has established a mammalian epithelial cell culture system, where transformed cells emerges from a monolayer of normal epithelial cells in a tetracycline inducible manner (Hogan et al. 2009; Figure1-4). In this system, we use normal Mardin-Darby canine kidney (MDCK) cell line and tetracycline-inducible transforming MDCK cell line. When these two types of cells are mixed, they can make the mosaic pattern in absence of tetracycline. By the treatment of tetracycline after the formation of a monolayer, therefore

Table 1-3. 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)
p53	Necroptosis of constitutively active p53 expressing cells	(Watanabe et al., 2018)
Cdc42	Apical extrusion of constitutively active Cdc42-expressing cells	(Grieve and Rabouille, 2014)
Yap	Apical extrusion of constitutively active YAP expressing cells	(Chiba et al., 2016)

Table 1-4. Cell competition *in vivo*

Mutation	Phenotype	References
Mouse		
Minute	Elimination of Minute-knockout cells in the liver	(Oliver et al., 2004)
p53	Loss of wild-type cells by senescence-like phenotype in the hematopoietic system	(Bondar and Medzhitov, 2010)
Myc	Cell death of low myc-expressing cells in the epiblast and myocardium	(Clavería et al., 2013) (Villa et al., 2014) (Sancho et al., 2013)
Ras	Apical extrusion of Ras-transformed cells	(Kon et al., 2017) (Sasaki et al., 2018)
Zebrafish		
Src	Apical extrusion of Src-transformed cells	(Kajita et al., 2010) (Kajita et al., 2014)

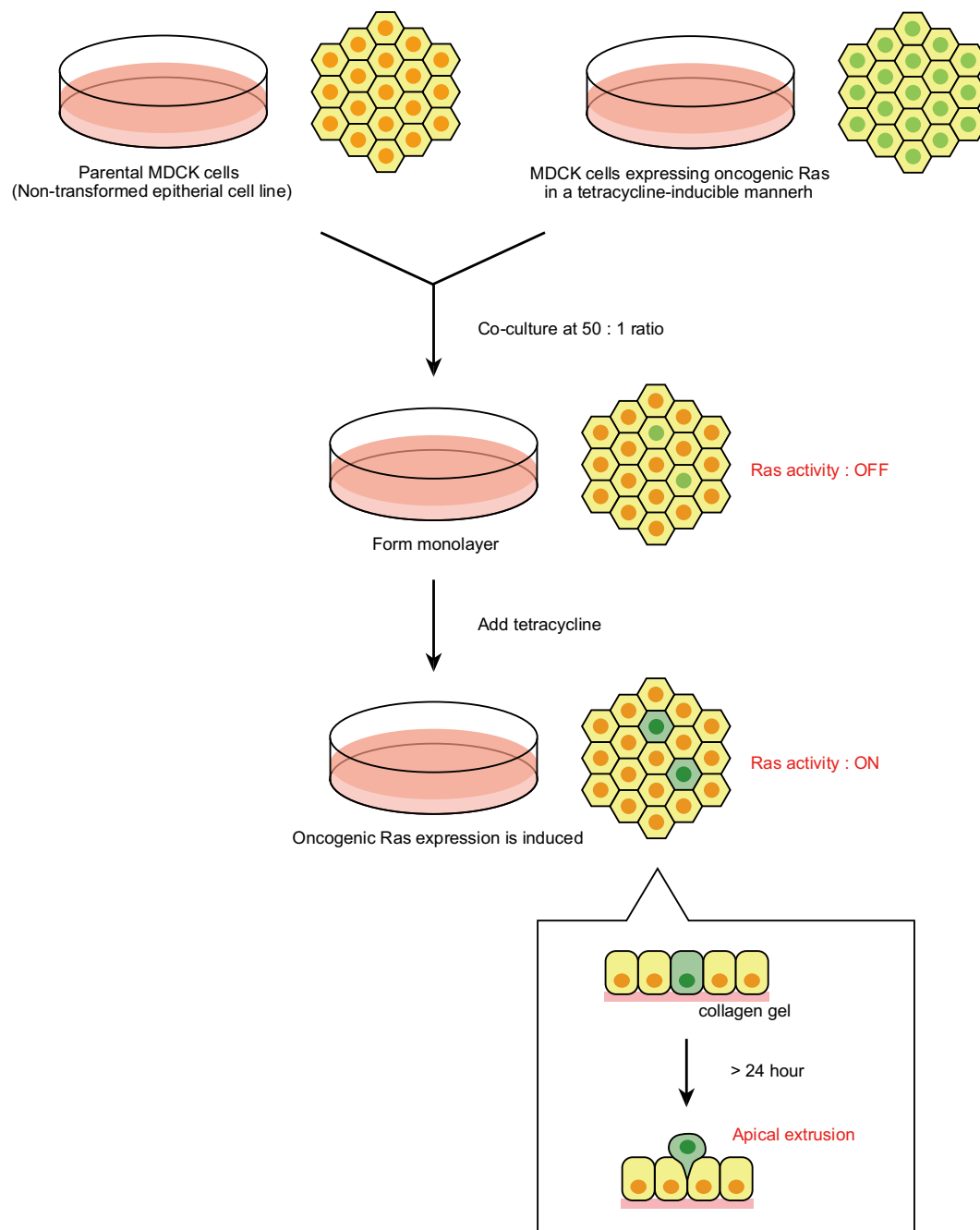


Figure 1-4. Experimental design for the study of mammalian cell competition adopted from Dr. Ayana Sasaki (<http://doi.org/10.14943/doctoral.k13277>)

potentially transformed cells are surrounded by normal cells. Also, by controlling the concentration of mutant cells, the situation of initial stage of carcinogenesis can be mimicked (Figure 1-1). Using this system, our group and other group demonstrated that a variety of newly emerging transformed cells are eliminated from normal epithelia as a loser (Table 1-3). In this thesis, I describe about Ras- and Src-transformed cells in more depth.

1.3.2.2. Cell competition model mouse

Recently, we reported apical extrusion of RasV12-transformed cells using mouse model system (Kon et al., 2017; Sasaki et al., 2018). 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 CK19-CreERT2 mice and CAG-LSL-RasV12-IRES-eYFP mice. In this mouse, CreERT2 is expressed in only intestinal epithelia because “CK19” is a specific promotor of 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 various epithelial tissue. Administration of a low dose of tamoxifen induced recombination events less frequently, resulting in mosaic expression of RasV12 within various epithelial tissues. The apical extrusion of RasV12-transformed cells also

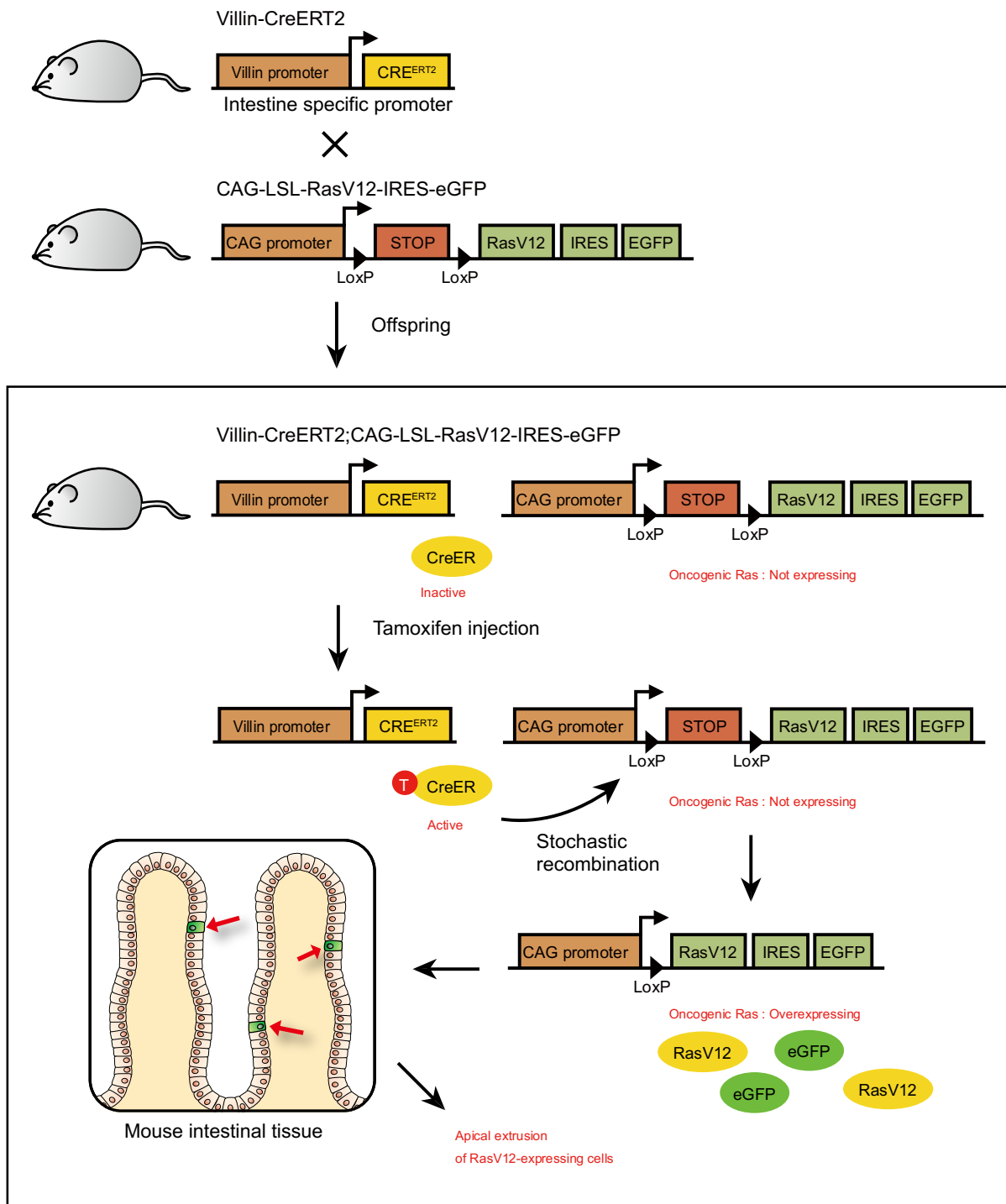


Figure 1-5. Schematic model of cell competition in vivo mouse system adopted from Dr. Ayana Sasaki (<http://doi.org/10.14943/doctoral.k13277>)

observed in mouse small intestine, pancreas and lung in this model mouse (Sasaki et al., 2018).

1.3.2.3. Cell competition at the initial stage of carcinogenesis

At the very initial stage of carcinogenesis, a single transformed cell is emerged within the epithelial layer. And in this point, the newly emerging transformed cells are supposed to interact with the neighboring normal cells. Recently, several studies have revealed that interactions between transformed cells and the neighboring normal cells induce cell competition (Table 1-3 and 1-4). Of importance, the elimination is not observed when transformed cells cultured alone. It implies that the normal tissue has ability to eliminate or exclude the pre-cancerous cells. Now, cell competition-mediated anti-tumor mechanism is spotlighted.

1.3.2.4. RasV12- or Src-transformed cells in cell competition in Mammal

Using a newly established cell culture system, our group reported that when RasV12-transformed cells are surrounded by normal epithelial cells, the majority of RasV12 cells are apically extruded from a monolayer of normal epithelial cells (Hogan et al., 2009). Importantly, when RasV12 cells alone are present, apical extrusion does not occur, indicating that activation of the cell-autonomous RasV12 signaling pathway itself is not sufficient to induce apical extrusion, but that the interaction with surrounding normal cells is also required.

Similar to RasV12 cells, when oncoprotein v-Src-transformed cells are surrounded by normal epithelial cells, v-Src cells are apically extruded from a monolayer of normal epithelial cells (Kajita et al., 2010). Apical extrusions of RasV12 or v-Src cells share several common features. First, apical extrusion is induced only when the transformed cells are surrounded by normal epithelial cells. Second, the transformed cells are apically extruded in an apoptosis-independent manner. Third, the height of the transformed cells is increased when they are surrounded by normal cells. Moreover, recent studies have shown that other types of transformed cells are also apically extruded upon interaction with normal epithelial cells (Table 1-3). These results indicate that apical extrusion is a one of the general mechanisms of the elimination of transformed cells from epithelia.

1.3.2.5. Epithelial defense against cancer (EDAC)

Recent studies have revealed that various molecules and signaling pathways are non-cell autonomously modulated in both normal and transformed cells during apical extrusion (Anton et al. 2014; Yamamoto et al. 2016; Ohoka et al. 2015; Kon et al. 2017; Figure 1-6). However, it was not known whether and how the neighboring normal cells play an active role in this process. By a biochemical screening, our previous report shows that Filamin is specifically modulated under the mix culture condition of normal and Ras- and Src-transformed epithelial cells. Filamin acts upstream of vimentin, and the accumulated vimentin filaments somehow generate tensile forces that promote apical extrusion of transformed cells. These results suggest that normal epithelial cells are able to sense the presence of the neighboring transformed cells and actively eliminate them by dynamically modulating those cytoskeletal proteins. Thus, at the

initial stage of carcinogenesis, normal epithelia are equipped with anti-tumor activity that does not involve immune systems; this process is termed ‘epithelial defense against cancer (EDAC)’.

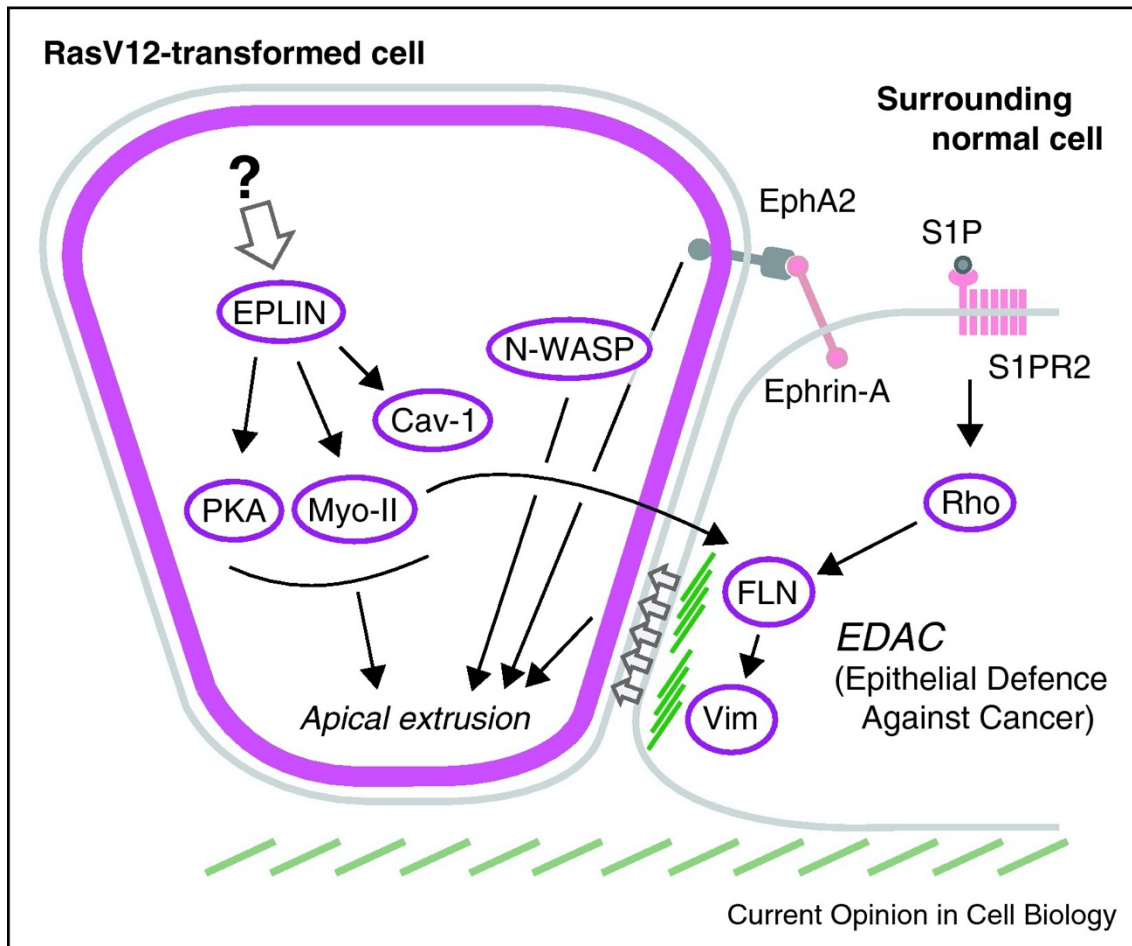


Figure 1-6. Molecular mechanisms of cell competition between normal and RasV12-transformed cells, adopted to (Maruyama and Fujita, 2017).

At the interface between normal and RasV12-transformed cells, a variety of non-cell-autonomous changes occur in both cells.

1.4. Inflammation and Cancer

Accumulating evidence shows that chronic inflammation such as pancreatitis and is associated to increased risk of cancer. Pathways linking inflammation and cancer have been identified: an intrinsic pathway (driven by genetic alterations) and an extrinsic pathway (driven by inflammatory conditions which predispose to cancer).

It is also widely known by epidemiological study that suppression of inflammation reduces the cancer risk. Non-steroidal anti-inflammatory drugs (NSAIDs) which target COX enzyme reduces the certain type of cancers such as colon and breast cancer.

1.5. Molecules involved in this study

1.5.1. COX-2

Cyclooxygenase (COX) is one of the key mediators in inflammation. It catalyses the conversion from arachidonic acid to prostaglandin (PG) H_2 , which is further converted to various PGs or thromboxane (Figure 1-6). COX family is comprised of three members, and COX-1 and COX-2 play major physiological and pathological roles (Simmons et al., 2004; Turini and DuBois, 2002). Constitutively expressed COX-1 is involved in tissue homeostasis. In contrast, expression of COX-2 is stimulated by various proinflammatory cytokines, leading to synthesis of PGs.

NSAIDs inhibit COX catalysed prostaglandin production. Among the various NSAIDs, non-selective NSAIDs such as Ibuprofen and Aspirin inhibit both COX-1 and COX-2, on the other hand, selective NSAIDs such as lumiracoxib and celecoxib selectively suppress COX-2.

1.5.2. PGE₂

Among the COX-2-mediated PGs, PGE₂ is one of the most abundant PGs produced in the body (Serhan and Levy, 2003), and is most widely characterized in animal species, and exhibits versatile biological activities. Under physiological conditions, PGE₂ is an important mediator of many biological functions, such as regulation of immune responses, blood pressure, gastrointestinal integrity, and fertility. PGE₂ is synthesized from PGH₂ by PGE₂ synthases (Samuelsson et al., 2007). In inflammation, PGE₂ is of particular interest because it is involved in all processes leading to the classic signs of inflammation: redness, swelling and pain (Foty and Steinberg, 2005). PGE₂ can bind G protein-coupled receptors named EP₁-4 and activates downstream signaling. Activation of EP₁ is known to increase intracellular Ca²⁺ via PLC. Activation of EP₂ or EP₄ stimulates cAMP production, on the other hand, EP₃ upregulates or inhibits cAMP production (Figure 1-6).

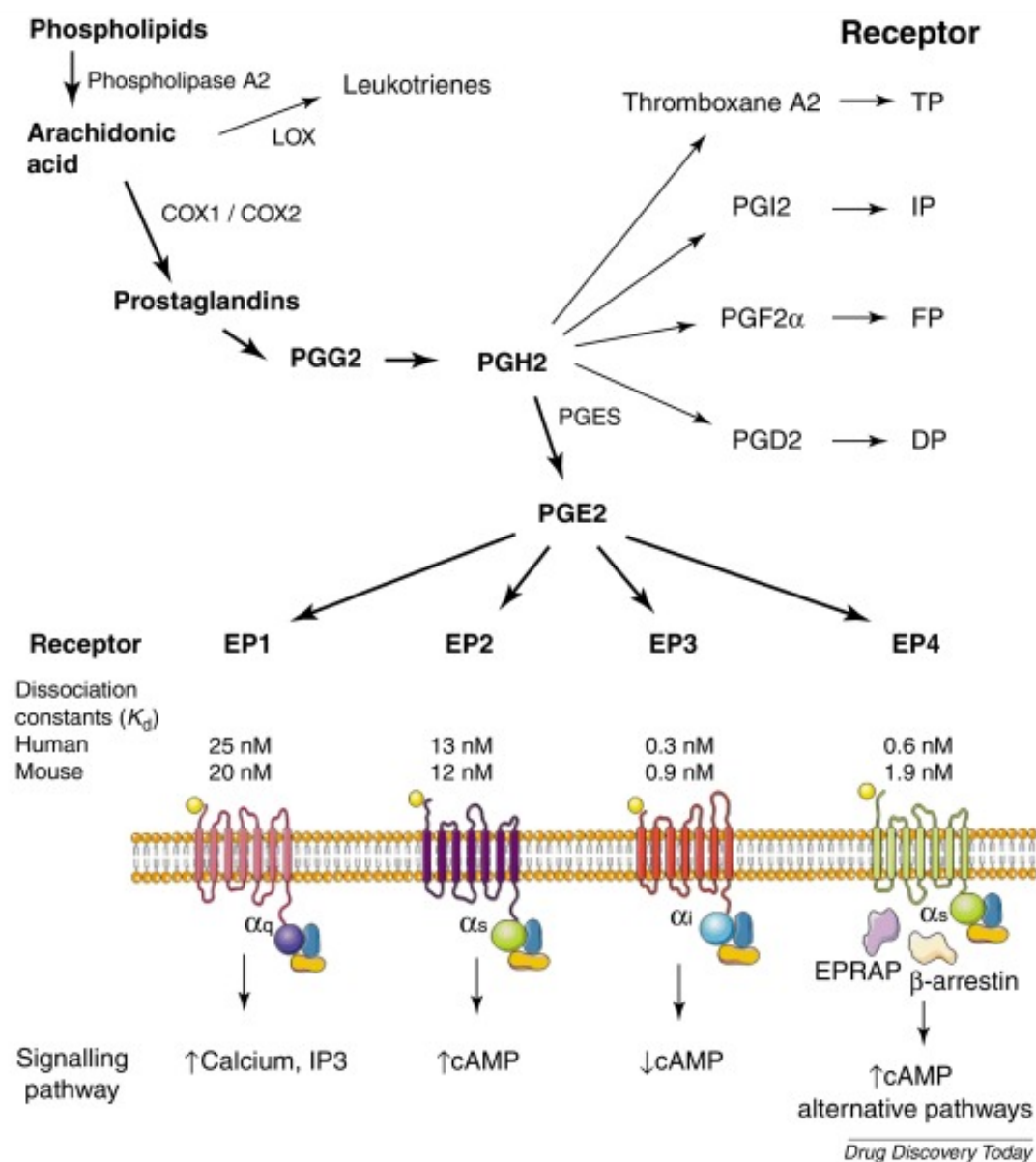


Figure 1-6. Arachidonic acid cascades adopted to (Markovič et al., 2017)

1.6. Aim of this study

During cell competition, normal cells actively eliminate transformed cells by dynamically accumulating cytoskeletal proteins, e.g. Filamin, at the boundary with transformed cells, implying that normal epithelial cells have anti-cancer activity termed EDAC (Kajita et al., 2014). However, the molecular mechanisms in normal cells still remain elusive. In this study, I investigated how the elimination process in normal cells is regulated at a gene expression level.

1.7. References

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Thesis

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

上皮細胞に備わる発がん防御機構[EDAC]における肥満の影響に関する研究

<http://doi.org/10.14943/doctoral.k13277>

Web site

Understanding Cancer, teacher's guide NIH 2017

(<https://science.education.nih.gov/supplements/webversions/cellBiology/guide/understanding1.html>) 2020/3/2

CHAPTER 2:
**Identification of COX-2/PGE₂ Pathway
that Negatively Regulates the Apical
Elimination of RasV12-transformed Cells**

2. Identification of COX-2/PGE₂ pathway that negatively regulates the apical elimination of RasV12 transformed cells

2.1. Introduction

Our previous report indicates that normal epithelial cells have an ability to sense and eliminate neighboring transformed cells, termed EDAC (Kajita et al., 2014). Furthermore, vasodilator-stimulated phosphoprotein (VASP), a member of the Ena/VASP family of actin cytoskeletal regulators, generally works as a negative regulator for elimination of RasV12-transformed cells, but when VASP is phosphorylated via PKA in RasV12-transformed cells mixed with normal MDCK cells, it works as a positive regulator for cell competition (Anton et al., 2014). These reports suggest that apical extrusion of transformed cells is regulated various positive and negative regulators in both normal cells and transformed cells. However, negative regulators in normal cells surrounding transformed cells now still remain unclear.

In this thesis, I focused on how EDAC process is regulated in normal cells in more depth. To identify the molecules whose expression level changes during cell competition in normal cells, I performed gene expression profiling and identified that COX-2 is upregulated in normal cells. Furthermore, I demonstrate that COX-2/PGE₂ pathway is upregulated in normal cells neighboring RasV12-transformed cells in a non-cell-autonomous fashion, which plays an inhibitory role in apical extrusion of transformed cells from epithelia.

2.2. Experimental procedures

2.1.1. Antibodies, Materials and Plasmids

The following primary antibodies were used in this study: mouse anti- β -actin (MAB1501R clone C4) and mouse anti-GAPDH (MAB374) antibodies from Merck Millipore, mouse anti-filamin (F6682) and mouse anti-vinculin (V44505) antibodies from Sigma-Aldrich, rabbit anti-COX-2 (ab15191) and chicken anti-GFP (ab13970) antibodies from Abcam, rabbit anti-Phospho-(Ser) PKC Substrate (#2261) antibody from Cell Signaling Technology. Peroxidase-conjugated AffiniPure anti-mouse and anti-rabbit IgG were from Jackson ImmunoResearch. Alexa-Fluor-568- and -647-conjugated phalloidin (Life Technologies) were used at 1.0 U ml⁻¹. Alexa-Fluor-568- and -647-conjugated secondary antibodies were from Life Technologies. Alexa-Fluor-488-conjugated secondary antibody was from Abcam. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000 for immunofluorescence. Type-I collagen (Cellmatrix Type I-A) was obtained from Nitta Gelatin and was neutralized on ice to a final concentration of 2 mg ml⁻¹ according to the manufacturer's instructions. To fluorescently stain living MDCK-pTR GFP or MDCK-pTR GFP-RasV12 cells, CMFDA (green dye) (Life Technologies) was used according to the manufacturer's instructions. The following reagents were obtained from Cayman Chemical: PGE₂, agonists selective for each EP subtype (Iloprost, Butaprost (free acid), Sulprostone and L-902,688 for EP1, EP2, EP3 and EP4, respectively) and antagonists selective for each EP receptor (ONO-8711 and PF-04418948 for EP1 and EP2, respectively). L-798106 (EP3 antagonist) was obtained from Sigma-Aldrich. ONO-AE3-208 (EP4 antagonist) was obtained from ChemScene. The following COX inhibitors, ibuprofen, ibuprofen sodium salt and lumiracoxib were from Sigma-Aldrich. Ibuprofen and ibuprofen sodium salt were used for cell culture and mice,

respectively. PKC ζ pseudosubstrate-derived ζ -inhibitory peptide (ZIP) was from abcam. MF63 was from ChemScene. Bisindolylmaleimide (BIM)-I and Go6976 were from Calbiochem. SB203580 (10 μ M) and SR11302 (5 μ M) were from TOCRIS. BAY117082 (1 μ M) and C646 (1 μ M) were from Santa Cruz Biotechnology.

2.1.2. Cell culture

MDCK cell lines were used in this study. The parental MDCK cells were a gift from W. Birchmeier (Fujita et al., 2002). Mycoplasma contamination was regularly tested for all cell lines in use using a commercially available kit (MycoAlert, Lonza). MDCK cells stably expressing GFP (MDCK-pTR GFP), GFP-RasV12 (MDCK-pTR GFP-RasV12) or GFP-cSrcY527F (MDCK-pTR GFP-cSrcY527F) in a tetracycline-inducible manner were established and cultured as previously described (Hogan et al., 2009; Ohoka et al., 2015). To establish CRISPR/Cas9-mediated COX-2- or EP2-knockout cells, guide sequences of COX-2 or EP2 single-guide RNA (sgRNA) targeting *Canis* COX-2 or EP2 were designed on exons 1 as described previously (Hsu et al., 2013). COX-2 sgRNA sequence (COX-2 sgRNA, 5'-CGCCCTGGTGCTCTGCGCC-3') or EP2 sgRNA sequence (EP2 sgRNA1, 5'-CATCGCGCTGGCGCTGCTG-3' or EP2 sgRNA2, 5'-GCTGCTGGCGCGCCGCTGG-3') was introduced into the pCDH-QC-sgRNA control (sgControl) vector (Maruyama et al., 2015) using primers listed in Supplementary Table 1. First, MDCK cells were infected with lentivirus carrying pCW-Cas9 as described (Maruyama et al., 2015) and were cultured in the 500 ng ml⁻¹ puromycin-containing medium. The tetracycline-inducible MDCK-Cas9 cells were transfected with pCDH-EF1-COX-2 sgRNA, pCDH-EF1-EP2 sgRNA1 or 2 by nucleofection, followed by selection in the medium containing 200 μ g ml⁻¹ of hygromycin, and subjected to limiting

dilution. Indels on the COX-2 or EP2 exons in each monoclonal were analysed by direct sequencing using primers listed in Supplementary Table 1. To generate MDCK-pTRE3G GFP-RasV12-COX-2-KO or -EP2-KO cells, complementary DNA of GFP-H-RasV12 was cloned into BamHI/EcoRI sites of pPB-TRE3G-MCS-CEH-rtTA3-IP (Kon et al., 2017). pPB-TRE3G GFP-H-RasV12 was introduced into the COX-2- or EP2-deleted cells by nucleofection and antibiotic selection (blasticidin, 5 $\mu\text{g ml}^{-1}$).

2.1.3. Microarray Analysis

9.0×10^6 of 1:1 mix culture of MDCK and MDCK-pTR GFP cells or 1:1 mix culture of MDCK and MDCK-pTR GFP-RasV12 cells were cultured in non-coat 10-cm plastic dishes. After incubation with tetracycline for 16 h, following trypsin treatment, GFP-negative cells were collected by an analytical flow cytometer FACS AriaTM II. Total RNA was extracted from the isolated cells using TRIzol (Thermo Fisher Scientific) and RNeasy Mini Kit (QIAGEN). The gene expression profiling was performed using Canine (V2) Gene Expression Microarray Kit (Agilent Technologies) by Oncomics Co., Ltd. For analysis of the microarray data, the canine gene database canFam2:V2.0:May2005 was used.

2.1.4. Quantitative Real-Time PCR

For cultured cells, the purified total RNA was obtained from GFP-negative cells as described above. For RNA isolation from murine pancreas, pancreas was cut into small pieces using scissors and incubated in 2 mg ml⁻¹ collagenase (Wako) in the RPMI1640 medium (Sigma-Aldrich) containing 2% (vol/vol) new-born calf serum (Equitech-Bio)

for 30 min at 37°C with stirring. The cell suspensions were filtered through cell strainers (pore size, 70 µm; BD Biosciences) and used for gene expression analysis. The total RNA was extracted from the isolated cells using TRIzol and an RNeasy Mini Kit (QIAGEN) and reverse-transcribed using a QuantiTect Reverse Transcription Kit (QIAGEN). GeneAmp SYBR qPCR Mix (NIPPON GENE) was used to perform qPCR using the StepOne system (Thermo Fisher Scientific). The primer sequences used are listed in Supplementary Table 1. We used *GAPDH* or *RPL13A* as a reference gene to normalize data.

2.1.5. Immunofluorescence

For immunofluorescence of cultured cells, MDCK-pTR GFP-RasV12 cells were mixed with MDCK cells at a ratio of 1:50, 1:10 or 1:1 and cultured on the collagen matrix as previously described (Hogan et al., 2009). The mixture of cells was incubated for 8-12 h until they formed a monolayer, followed by tetracycline treatment for 6 h (for immunofluorescence analyses of p-PKC substrate), 16 h (for immunofluorescence analyses of COX-2 or filamin) or 24 h (for apical extrusion analyses). Cells were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS and permeabilized with 0.5% Triton X-100 in PBS, except for filamin immunofluorescence where cells were fixed in methanol at -20°C for 2.5 min, followed by blocking with 1% BSA in PBS. Primary or secondary antibodies were incubated for 2 h or 1 h respectively at ambient temperature. Alexa-Fluor-568- or -647-conjugated phalloidin was incubated for 1 h at ambient temperature. Immunofluorescence images of cultured cells and mouse tissues were acquired using the Olympus FV1000 system and Olympus FV10-ASW software. All primary antibodies were used at 1:100, except for mouse tissues: anti-GFP (1:1,000), anti-

E-cadherin (1:2,000) and anti-CD45.2 (1:1,000) antibodies. All secondary antibodies were used at 1:200 for cultured cells.

2.1.6. Western Blotting

Western blotting was performed as previously described (Hogan et al., 2004). Primary antibodies were used at 1:1,000 except anti-GAPDH at 1:2,000. Western blotting data were analysed using ImageQuant™ LAS4010 (GE healthcare).

2.1.7. Lipidomics Analysis

1.0 x 10⁶ MDCK cells, MDCK-pTR GFP-RasV12 cells, or 1:1 mix culture of MDCK and MDCK-pTR GFP-RasV12 cells were plated into non-coat plastic dishes (6-well, greiner) (triplicate under each condition). After incubation for 12 h, tetracycline was added to induce GFP-RasV12 expression, followed by incubation for 16 h. The culture media were then changed to FCS-free DMEM media with tetracycline, which were collected after 6 h and stored at -80°C. After thawing, the samples were centrifuged for 10 min at 10,000 x g and were purified with solid phase extraction with an Oasis HLB cartridges (Milford, MA, USA) as previously described (Kita et al., 2005). Liquid chromatography-selected reaction monitoring-mass spectrometry (LC-SRM-MS) analysis was performed using a Nexera UHPLC system and a triple quadrupole mass spectrometer LCMS-8040 (Shimadzu, Kyoto, Japan) with a reversed-phase column (Kinetex C8, 2.1 x 150 mm, 2.6 µm; Phenomenex, Torrance, CA) as described previously (Yamada et al., 2015). LabSolutions software (Shimadzu) was used for peak detection and integration. Chromatographic peak area for the corresponding SRM transitions were used for

comparison of lipid mediators.

2.1.8. Measurement of PGE₂ Concentration

For measurement of PGE₂ concentration, 3.25×10^5 MDCK cells, MDCK-pTR GFP-RasV12 cells, or 1:1 mix culture of MDCK and MDCK-pTR GFP-RasV12 cells were cultured into non-coat plastic dishes (24-well, greiner). After 16 h tetracycline incubation with or without the indicated COX inhibitor, culture media were changed to FCS-free DMEM with tetracycline and incubated for 2 h. Culture media were then collected, and PGE₂ concentration was measured using the PGE₂ high sensitivity ELISA kit (ENZO Life Sciences) according to the manufacturer's instructions.

2.1.9. Data Analyses

For data analyses, Mann-Whitney test, Chi-square test, unpaired *t*-test or two-tailed Student's *t*-test was used to determine *p* values as indicated in the figure legends. *p* values less than 0.05 were considered to be statistically significant. For quantification of apical extrusion of RasV12-transformed cells, 2-12 RasV12-transformed cells that were surrounded by the indicated cells were analysed. Filamin accumulation in the surrounding cells at the interface with RasV12-transformed cells was quantified for transformed cells of which more than 75% of the perimeter was surrounded by normal cells. When filamin accumulation was observed at more than one-third of the perimeter of the transformed cell, it was counted as filamin accumulation-positive. More than 100 cells were analysed for apical extrusion, and more than 30 cells were analysed for filamin accumulation in each experiment. All immunofluorescence images were analysed by confocal microscopy. Immunofluorescence intensity of COX-2 was quantitatively analysed with a Cell Insight

image cytometer (Thermo Fisher Scientific).

2.1.10. Oligonucleotides

Primers used in this thesis is described in (Table 2-1).

Table 2-1. The sequences of primers for site-directed mutagenesis, direct sequencing, genotyping and qPCR.

	Name	5' to 3' sequence
Site-directed mutagenesis primer	canis sgCOX-2 QC primer Fw	GGAAAGGACGAAACACCGTAGGATTAACAGGCTTTAACGTTTTAGAGCTAGAAATAGC
	canis sgCOX-2 QC primer Rv	GCTATTTCTAGCTCTAAACGTTAAAGCCTGTTAATCCTACGGTGTTCGTCCTTTCC
	canis sgEP2#1 QC primer Fw	GGAAAGGACGAAACACCGCAGCAGCGCCAGCGCATGGTTTTAGAGCTAGAAATAGC
	canis sgEP2#1 QC primer Rv	GCTATTTCTAGCTCTAAACCATCGCGCTGGCGCTGCTGCGGTGTTCGTCCTTTCC
	canis sgEP2#2 QC primer Fw	GGAAAGGACGAAACACCGGCTGCTGGCGCGCCGCTGGGTTTTAGAGCTAGAAATAGC
	canis sgEP2#2 QC primer Rv	GCTATTTCTAGCTCTAAACCCAGCGGCGCGCCAGCAGCCGGTGTTCGTCCTTTCC
Primers used for direct sequencing	canis COX-2 KO ex1 Fw	GGAAGGTTCCGTCCTTAG
	canis COX-2 KO ex1 Rv	ATGACAAGGGAGACCCACAC
	canis EP2 KO ex1 Fw	CCTGCTCCCAGACTCTCCT
	canis EP2 KO ex1 Rv	GAGGCTGAAGAAGGTCATGG
genotyping primer	CK19-CreERT2 Fw	AATCGCCAGGAATTGACCAATGGGG
	CK19-CreERT2 Rv	CGCCCGTACCCCAAAGGAAGACAT
	DNMT1-CAG-loxP-STOP-loxP-HRasV12-IRES-eGFP Fw	CACTGTGGAATCTCGGCAGG
	DNMT1-CAG-loxP-STOP-loxP-HRasV12-IRES-eGFP Rv	GCAATATGGTGAAAAATAAC
	LSL-YFP Fw	AAAGTCGCTCTGAGTTGTTAT
	LSL-YFP mut Rv	GGAGCGGGAGAAATGGATATG
	LSL-YFP wt Rv	GGAGCGGGAGAAATGGATATG
qPCR primer	canis PTGS2 Fw	CGAGGACCAGCTTTCACCA
	canis PTGS2 Rv	GACCTGAGTATCTTTGACGGTAGGA
	canis GAPDH Fw	ATTCTATCCACGGCAAATCC
	canis GAPDH Rv	GGACTCCACAACATACTAG
	mouse TNF alpha Fw	CTGTAGCCCACGTCGTAGC
	mouse TNF alpha Rv	TTGAGATCCATGCCGTTG
	mouse IL-1 beta Fw	TTGACGGACCCCAAAAGAT
	mouse IL-1 beta Rv	GAAGCTGGATGCTCTCATCTG
	mouse CCL2 Fw	CATCCACGTGTTGGCTCA
	mouse CCL2 Rv	GATCATCTTGCTGGTGAATGAGT
	mouse IL-6 Fw	TGCCTTCATTTATCCCTTGAA
	mouse IL-6 Rv	TTACTACATTGACCAAAAAGCAC
	mouse RPL13A Fw	GAGGTCGGGTGGAAGTACCA
	mouse RPL13A Rv	TGCATCTTGCCCTTTTCCTT
	mouse PTGS2 Fw	GATGCTCTCCGAGCTGTG
	mouse PTGS2 Rv	GGATTGGAACAGCAAGGATTT

2.3. Results

2.3.1. In vitro screen to identify the regulator of cell competition in normal cells

To investigate whether and how the presence of RasV12-transformed cells influences the gene expression profile of the neighboring normal epithelial cells, I performed microarray analysis. First, normal Madin-Darby canine kidney (MDCK) cells were co-cultured with MDCK cells expressing GFP or GFP-RasV12 (Fig. 2-1A). GFP-negative normal MDCK cells were then collected by FACS, and expression of various genes was compared between the two conditions (Fig. 2-1A). Expression of a number of genes was upregulated or downregulated in normal cells co-cultured with GFP-RasV12 cells, compared with those co-cultured with GFP cells (Figure. 2-1B). Among them, the expression of the *PTGS2* gene encoding COX-2 was most profoundly upregulated (Figure. 2-1B).

2.3.2. COX-2 is upregulated in normal cells co-cultured with transformed cells

To validate the microarray result, I performed qPCR analysis and western blot analysis (Figure 2-2 A and B). Comparable upregulation of COX-2 expression was also observed in normal cells co-cultured with Src-transformed cells (Figure 2-2C). Furthermore, I showed by western blotting and immunofluorescence that the protein level of COX-2 was also upregulated in normal cells co-cultured with RasV12-transformed cells (Figure 2-2 D and E). Collectively, these data indicate that the presence of RasV12 cells augments the expression of COX-2 in the surrounding normal epithelial cells in a non-cell-autonomous fashion.

2.3.3. Examination of upstream of COX-2

The expression of COX-2 can be regulated by a variety of stimuli or signalling pathways (Kang et al., 2007; Tanabe and Tohnai, 2002). I then explored the upstream signalling pathways/molecules of the COX-2 expression by examining the effect of various inhibitors. Addition of inhibitors for NF- κ B, AP-1, CREB or p38MAPK did not significantly affect the expression level of COX-2 (Figure 2-3 A-D).

By contrast, addition of the PKC inhibitor bisindolylmaleimide (BIM)-I substantially suppressed the COX-2 expression (Figure 2-4 A). Furthermore, to detect PKC activity, I used an antibody against a phosphorylated consensus sequence recognized by PKC. Immunofluorescence analysis showed that activity of PKC was elevated in normal cells co-cultured with RasV12 cells, which was diminished by BIM-I (Figure 2-4 B). It has been reported that Ca²⁺-dependent PKC or PKC- ζ can act upstream of COX-2. To identify the PKC isozyme, I tested Go6976: an inhibitor for Ca²⁺-dependent PKC isoforms and ZIP: PKC zeta specific inhibitor. Go6976 partially affected the expression of COX-2 in normal cells surrounding RasV12 cells and ZIP significantly decrease the expression COX-2 (Figure 2-4 C and D). Thus, it is possible that multiple PKC subtypes are involved in the regulation of COX-2.

2.3.4. COX-2 has a negative role in the elimination of RasV12 transformed cells

Next, I examined a functional role of COX-2 in cell competition. Previous studies showed that RasV12-transformed MDCK cells are apically extruded when they are surrounded by normal MDCK cells (Hogan et al., 2009; Kajita et al., 2010). Ibuprofen suppresses activity of COX-1 and COX-2, whereas lumiracoxib suppresses COX-2

activity. I found that addition of either ibuprofen or lumiracoxib significantly elevated the apical extrusion ratio of RasV12 cells (Figure 2-5 A). I then established COX-2-knockout normal or RasV12-transformed MDCK cell lines (Figure 2-5 B and C). When RasV12 cells were surrounded by COX-2-knockout normal cells, apical extrusion was profoundly enhanced (Figure 2-5 D). In contrast, knockout of COX-2 in RasV12 cells did not affect apical extrusion (Figure 2-5 E). Collectively, these data suggest that COX-2 in the surrounding normal cells negatively regulates apical elimination of RasV12-transformed cells.

2.3.5. PGE₂ plays a negative role in apical extrusion of RasV12-transformed cells

To further understand the involvement of lipid metabolites in cell competition, collaborator performed a comprehensive quantitative-lipidomics analysis using conditioned media from three different culture conditions: normal cells alone, RasV12-transformed cells alone and mix culture of normal and RasV12 cells. Among the detected lipid metabolites, the amount of PGE₂ was most significantly upregulated in the mix culture condition, compared with that in alone culture conditions (Figure 2-6 A and Table 2-2). COX-2 catalyses the conversion from arachidonic acid to PGH₂ (through PGG₂), and PGH₂ is further converted to various prostaglandins and thromboxane, among which the role of PGE₂ in inflammation has been intensively studied (Harizi et al., 2008; Nakanishi and Rosenberg, 2013). Using the high sensitivity ELISA kit, I confirmed that the PGE₂ production was elevated in the mix culture condition (Figure 2-6 B). The level of PGE₂ in the mix culture condition was profoundly diminished by COX-inhibitor or COX-2-knockout in normal cells (Figure 2-

6 B and C), suggesting that the production of PGE₂ is mainly mediated by COX-2 in normal cells under the mix culture condition. In addition, PGE₂ treatment attenuated apical extrusion of RasV12-transformed cells in a dose-dependent manner (Figure 2-6 D and E).

MF63 selectively inhibits microsomal PGE synthase-1, which catalyses the conversion from PGH₂ to PGE₂. Addition of MF63 strongly suppressed the production of PGE₂ (Figure 2-7 A) and significantly promoted apical extrusion of RasV12 cells (Figure 2-7 B). Furthermore, PGE₂ treatment substantially diminished the accumulation of filamin, a positive regulator for apical extrusion, in normal cells that surrounded RasV12 cells (Figure 2-7 C and D). Collectively, these data indicate that PGE₂ production in normal cells negatively regulates apical elimination of RasV12-transformed cells by suppressing EDAC.

2.3.6. Elucidation of the downstream of PGE₂

I further explored the downstream of PGE₂. PGE₂ can bind and activate G-protein-coupled receptors EP (E prostanoid receptor) 1-4, thereby activating various downstream mediators. Agonist for EP2 or EP4 suppressed apical extrusion of RasV12 cells, whereas EP1 or EP3 agonist had no effect (Figure 2-8 A). However, antagonist for the respective receptor did not significantly affect apical extrusion (Figure 2-8 B). In addition, knockout of EP2 in normal or RasV12 cells did not influence the frequency of apical extrusion (Figure 2-8 C-E). These results imply that multiple EP receptors or intracellular PGE₂ might regulate the downstream pathways

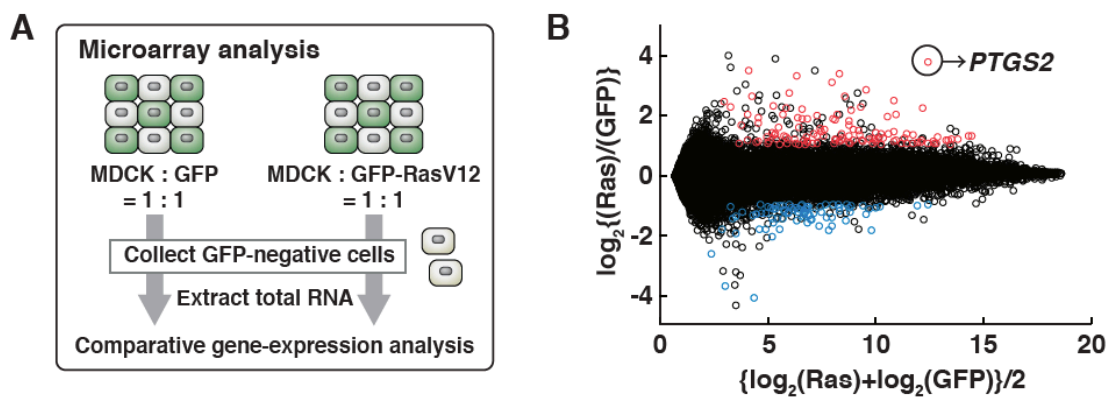


Figure 2-1. *PTGS2* which encodes COX-2 is upregulated in normal cells co-cultured with RasV12-transformed cells.

(A) Schematics for microarray analysis. Normal MDCK cells were co-cultured with GFP- or GFP-RasV12-expressing MDCK cells. GFP-negative normal MDCK cells were then collected by FACS sorting, and the extracted total RNAs were subjected to comparative gene-expression analysis between the two culture conditions.

(B) Graphic display of expression profiling data of the microarray analysis. The vertical axis is the $\log_2$ -ratio (mixed with Ras vs with GFP), while the horizontal axis represents the average log values. Red or blue dots indicate genes of which expression is significantly and more than two-fold upregulated or downregulated respectively in the mix culture with Ras cells. The *P* value for *PTGS2* is 1.8×10^{-4} (Student's *t*-test).

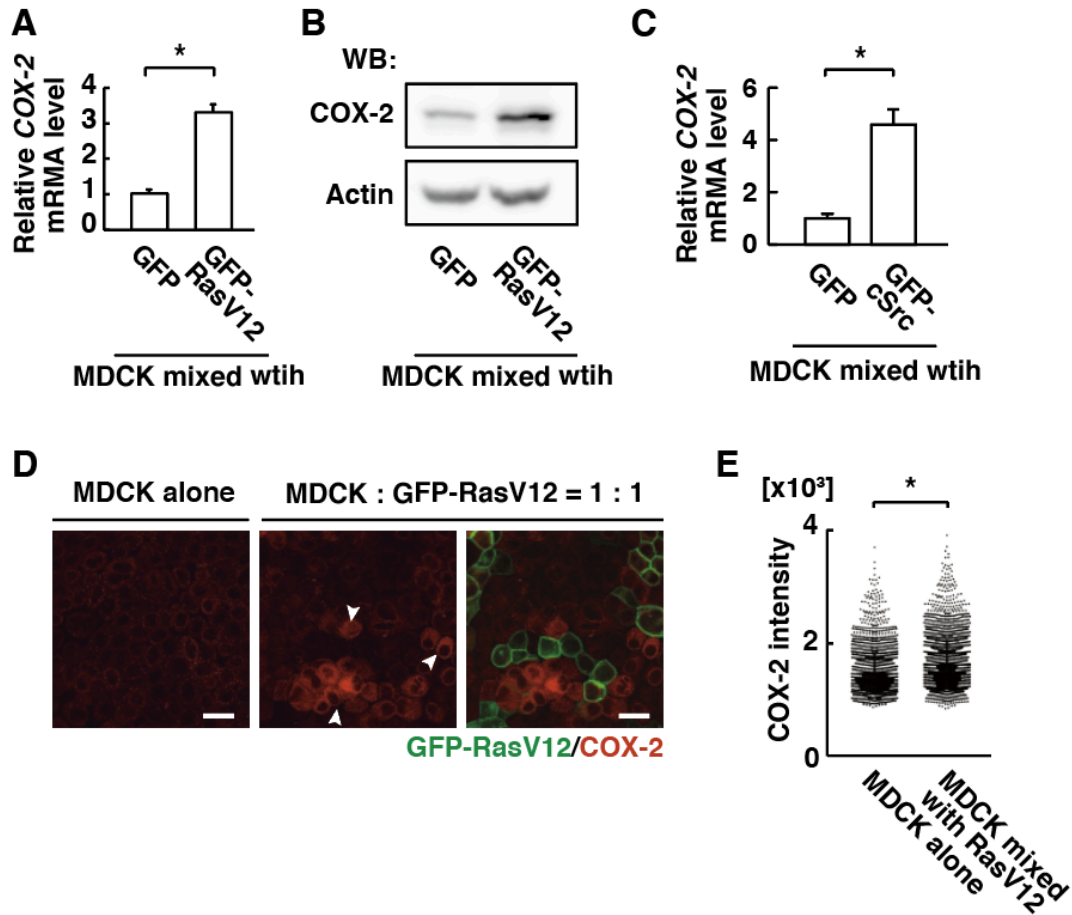


Figure 2-2. COX-2 is upregulated in normal cells co-cultured with transformed cells at a mRNA level and protein level.

(A and C) Quantitative RT-PCR analysis of COX-2 expression. MDCK cells are mixed with MDCK-GFP cells or MDCK GFP-RasV12 cells (A), or MDCK cells mixed with MDCK-GFP cells or MDCK cSrcY527F-GFP cells (C). (A) Data are mean $\pm$ s.d. from three independent experiments. Values are expressed as a ratio relative to GFP. $*P=0.0019$ (Student's *t*-test). (C) Data are mean $\pm$ s.d. from three independent experiments. Values are expressed as a ratio relative to GFP. $*P=0.0088$ (Student's *t*-test). **(B)** Western blotting analysis of COX-2 expression after sorting. Cell lysates from FACS-sorted GFP-negative normal cells were examined. **(D)** Immunofluorescence analysis of COX-2 expression. MDCK cells were cultured alone or co-cultured with MDCK GFP-RasV12 cells at a ratio of 1:1, followed by immunofluorescence analysis with anti-COX-2 antibody. Arrowheads indicate COX-2-positive cells. Scale bars, 20 μ m. **(E)** $n=2,152$ cells (alone) or 2,321 cells (with RasV12) from two independent experiments. $*P<1.0 \times 10^{-4}$ (Mann-Whitney test).

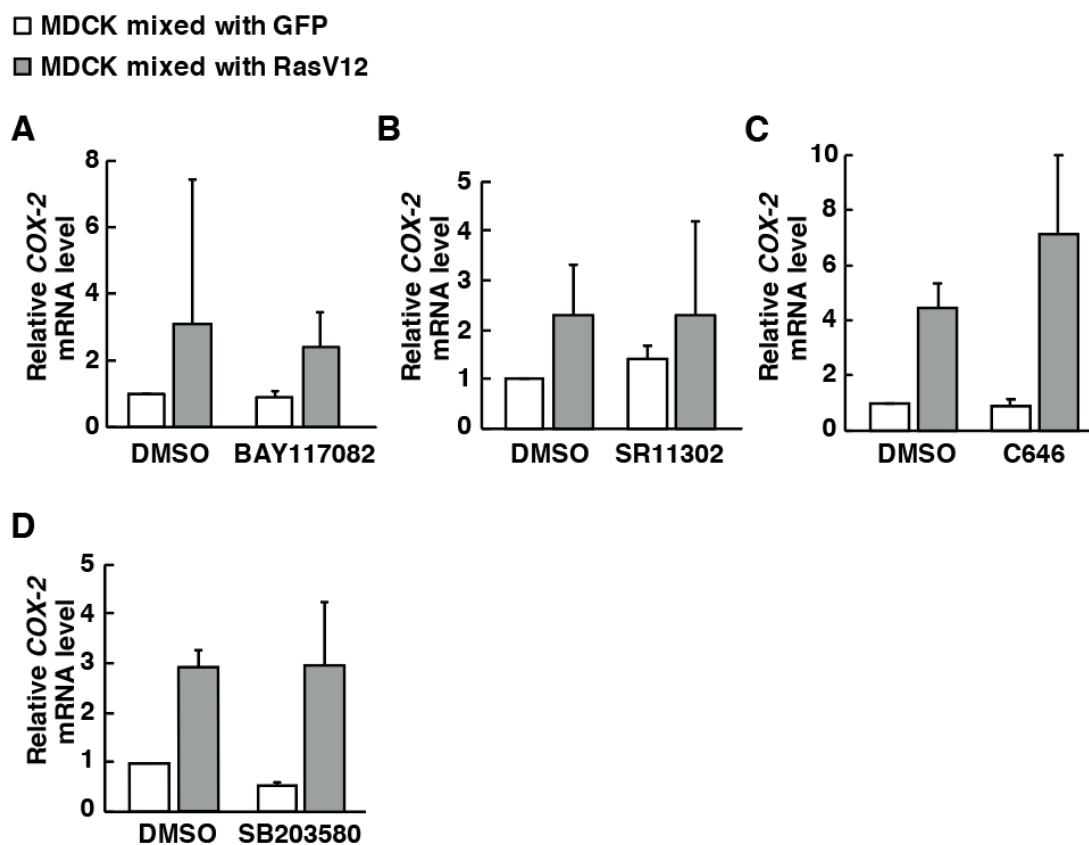


Figure 2-3. Effect of various inhibitors on the *COX-2* mRNA level in normal MDCK cells co-cultured with GFP-expressing MDCK cells (white) or GFP-RasV12-expressing MDCK cells (grey). Data are mean $\pm$ s.d. from four (**A**) or two (**B-D**) independent experiments. Values are expressed as a ratio relative to DMSO (MDCK mixed with GFP). (**A**) BAY117082: NF- κ B inhibitor, (**B**) SR11302: AP-1 inhibitor, (**C**) C646: CREB inhibitor, (**D**) SB203580: p38MAPK inhibitor.

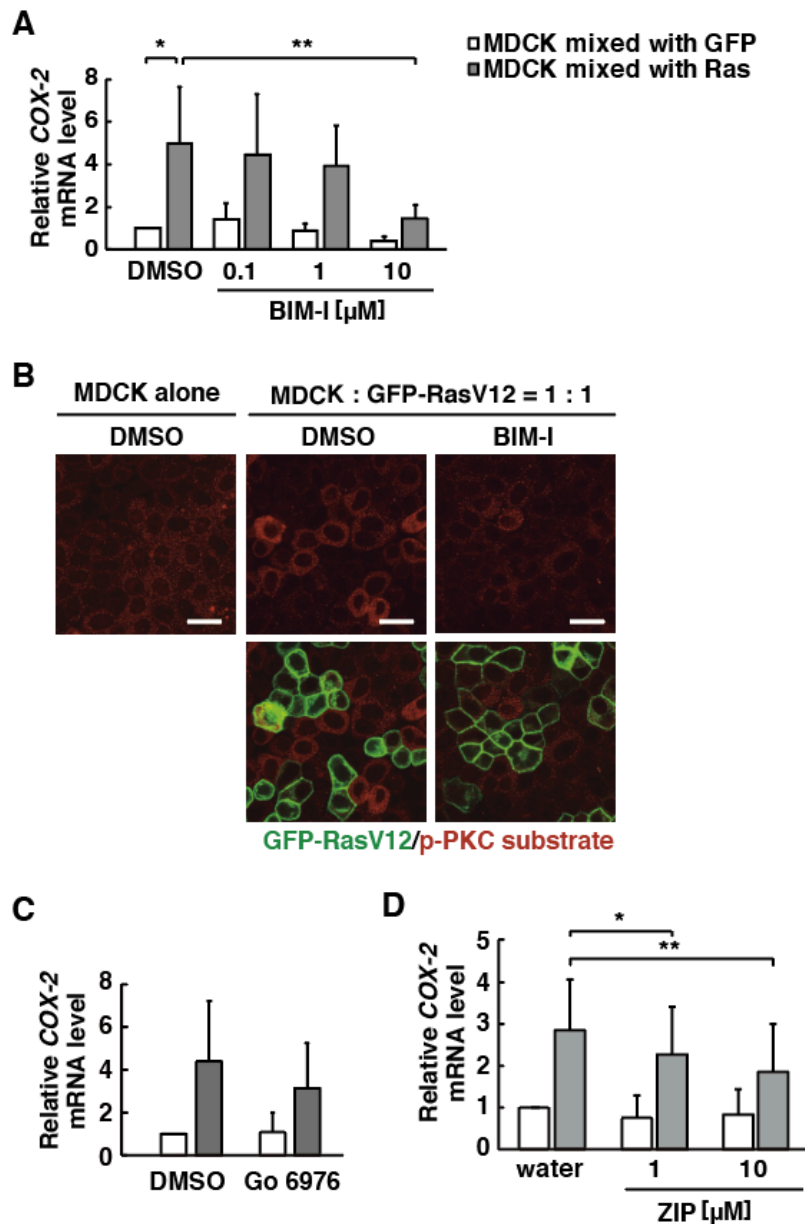


Figure 2-4. The PKC inhibitor BIM-I suppresses PKC activation and COX-2 expression in normal cells co-cultured with RasV12-transformed cells.

(A) Effect of BIM-I on the *COX-2* mRNA level in normal MDCK cells co-cultured with GFP-expressing MDCK cells (white) or GFP-RasV12-expressing MDCK cells (grey). Data are mean $\pm$ s.d. from six (DMSO, 10 μ M BIM-I), five (1 μ M BIM-I) or three (0.1 μ M BIM-I) independent experiments. Values are expressed as a ratio relative to DMSO (MDCK mixed with GFP). * $P=0.014$, ** $P=0.021$ (unpaired t -test). **(B)** Effect of BIM-I on PKC activation in normal cells co-cultured with RasV12 cells. MDCK cells were cultured alone or co-cultured with MDCK GFP-RasV12 cells at a ratio of 1:1 in the presence or absence of BIM-I (10 μ M), followed by immunofluorescence analysis with anti-p-PKC substrate antibody. Scale bars, 20

μm . **(C and D)** Effect of the PKC subtype specific inhibitors on *COX-2* mRNA expression. Data are mean $\pm$ s.d. from five (C) or four (D) independent experiments. * $P=0.022$, ** $P=0.017$ (student *t*-test). Values are expressed as a ratio relative to water or DMSO (MDCK mixed with GFP). (C) Go6976 (10 μM): Ca^{2+} -dependent PKC inhibitor, (D) ZIP: PKC ζ pseudosubstrate-derived ζ -inhibitory peptide

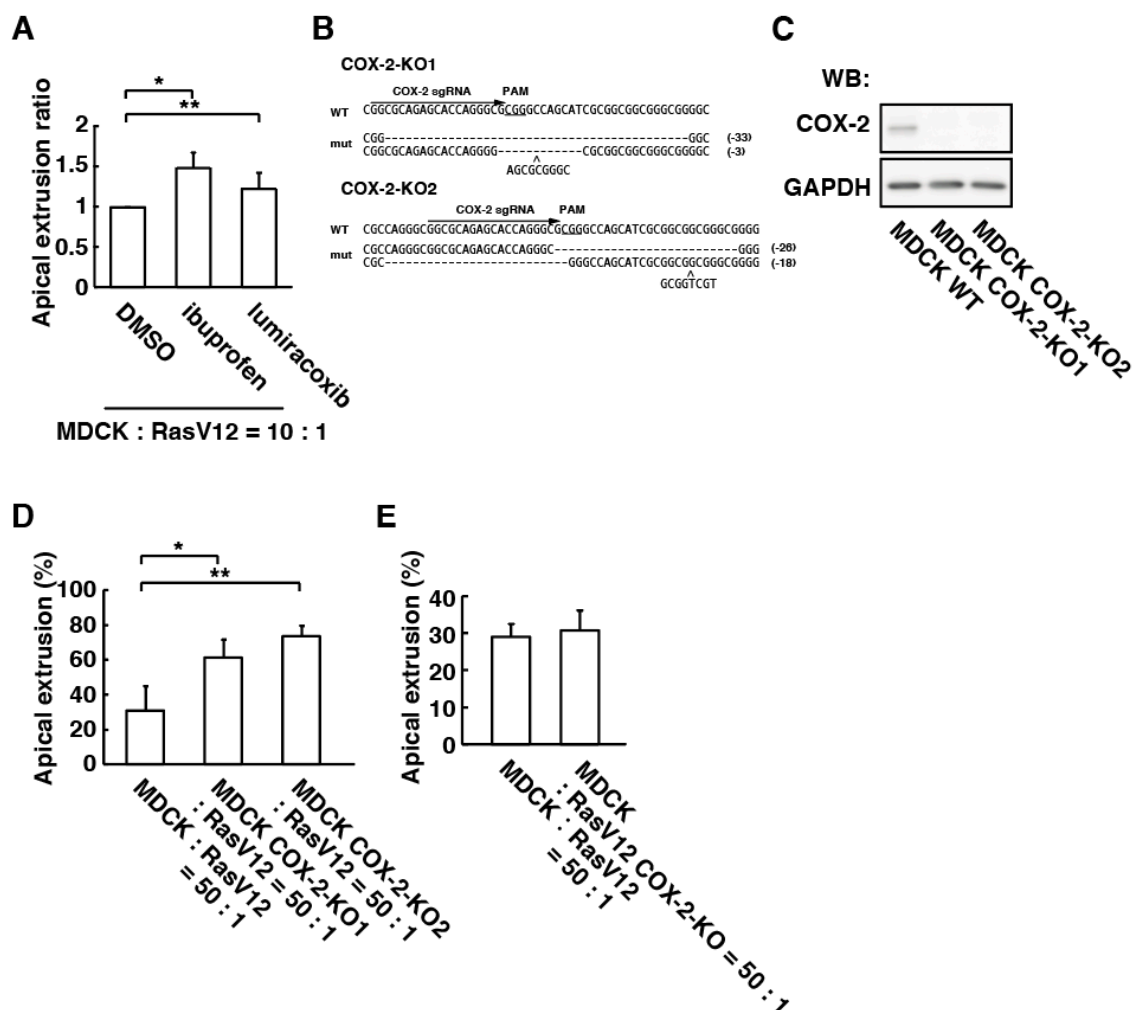


Figure 2-5. COX-inhibitor or COX-2-knockout in normal cells promotes apical extrusion of RasV12-transformed cells.

(A) Effect of the COX-inhibitor ibuprofen (10 μ M) or lumiracoxib (10 μ M) on apical extrusion of RasV12 cells. Data are mean $\pm$ s.d. from seven (DMSO), five (ibuprofen) or six (lumiracoxib) independent experiments. Values are expressed as a ratio relative to DMSO. * $P=0.0054$, ** $P=0.0036$ (unpaired t -test). **(B)** A targeting scheme and DNA sequences of the wild type and COX-2-null MDCK cell lines. PAM motifs are underlined. The spacing indicates a deleted nucleotide. **(C)** Knockout of COX-2 was confirmed by western blotting. Note that RasV12 was stably expressed in MDCK COX-2-KO1 cells to establish the RasV12 COX-2-knockout cell line. **(D and E)** Effect of COX-2-knockout in normal cells (D) or RasV12 cells (E) on apical extrusion of RasV12 cells. (D) Data are mean $\pm$ s.d. from four independent experiments. * $P=0.015$, ** $P=0.0041$ (Student's t -test). (E) Data are mean $\pm$ s.d. from three independent experiments.

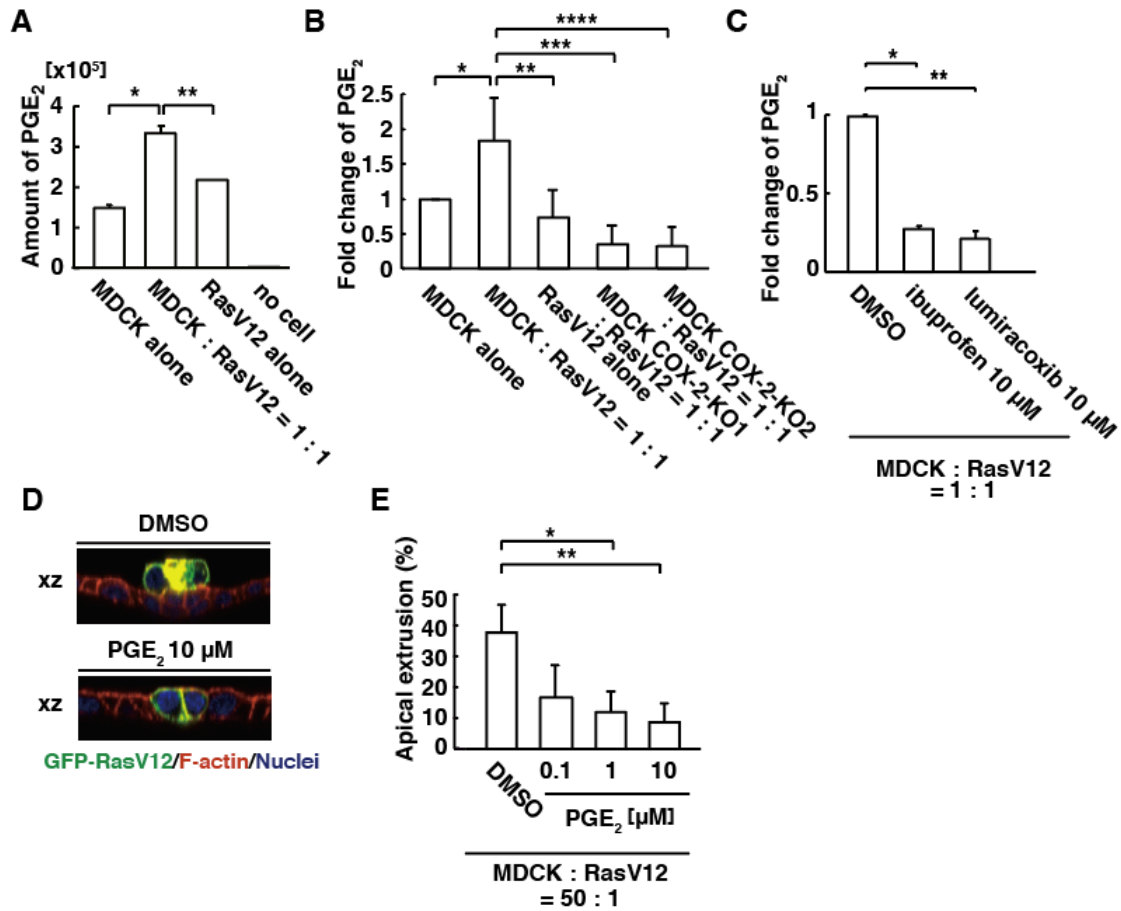


Figure 2-6. PGE₂ negatively regulates apical extrusion of RasV12-transformed cells that are surrounded by normal cells.

(A and B) Measurement of PGE₂ in conditioned media under the indicated culture conditions by comprehensive quantitative-lipidomics analysis (A) or using the high sensitivity ELISA kit (B). (A) The values in the vertical axis indicate the chromatographic peak area of the mass spectrometric analysis. Data are mean $\pm$ s.d. from triplicate samples. * $P=0.0095$, ** $P=0.0013$ (Student's t -test). (B) Data are mean $\pm$ s.d. from six (left three) and four (right two) independent experiments. Values are expressed as a ratio relative to MDCK alone. * $P=0.020$, ** $P=0.0056$ (Student's t -test), *** $P=0.0011$, **** $P=0.017$ (unpaired t -test). **(C)** Effect of the COX inhibitor on the PGE₂ level in the conditioned medium of the mix culture of normal and RasV12-transformed cells. Data are mean $\pm$ s.d. from three independent experiments. Values are expressed as a ratio relative to DMSO. * $P=2.5 \times 10^{-5}$, ** $P=4.1 \times 10^{-5}$ (Student's t -test). **(D and E)** Effect of PGE₂ on apical extrusion of RasV12 cells. **(C)** The xz-immunofluorescence images of GFP-RasV12-expressing cells surrounded by normal cells in the presence or absence of PGE₂. Scale bar, 20 μ m.

Table 2-2. Comprehensive quantitative-lipidomics analysis using liquid chromatography-selected reaction monitoring-mass spectrometry (LC-SRM-MS).

The values for MDCK alone, RasV12 alone or Mix culture indicate the mean of chromatographic peak area for the corresponding SRM transitions from the triplicate samples. The *P* values between ‘Average of alone cultures’ and ‘Mix culture’ were calculated using unpaired *t*-test.

	MDCK alone	RasV12 alone	Average of alone cultures	Mix culture	<i>P</i> value
Prostaglandin E ₂	149032.0	218274.7	183653.3	333123.3	0.00011
Prostaglandin A ₂	6590.7	8940.3	7765.5	13786.3	0.00034
17-hydroxy Docosahexaenoic Acid	1098.0	2104.7	1601.3	3390.7	0.013
11-HETE	1476.7	5309.0	3392.8	6840.3	0.013
8-iso Prostaglandin E ₂	5696.7	6452.7	6074.7	11690.7	0.043
6-keto-Prostaglandin F _{1α}	5651.0	11411.3	8531.2	12791.0	0.048
Prostaglandin F _{2α}	7913.0	22378.7	15145.8	22196.0	0.087
Oleoyl Ethanolamide	1979.3	1353.7	1666.5	4346.7	0.132
Prostaglandin D ₂	1260.3	6405.3	3832.8	4835.0	0.474

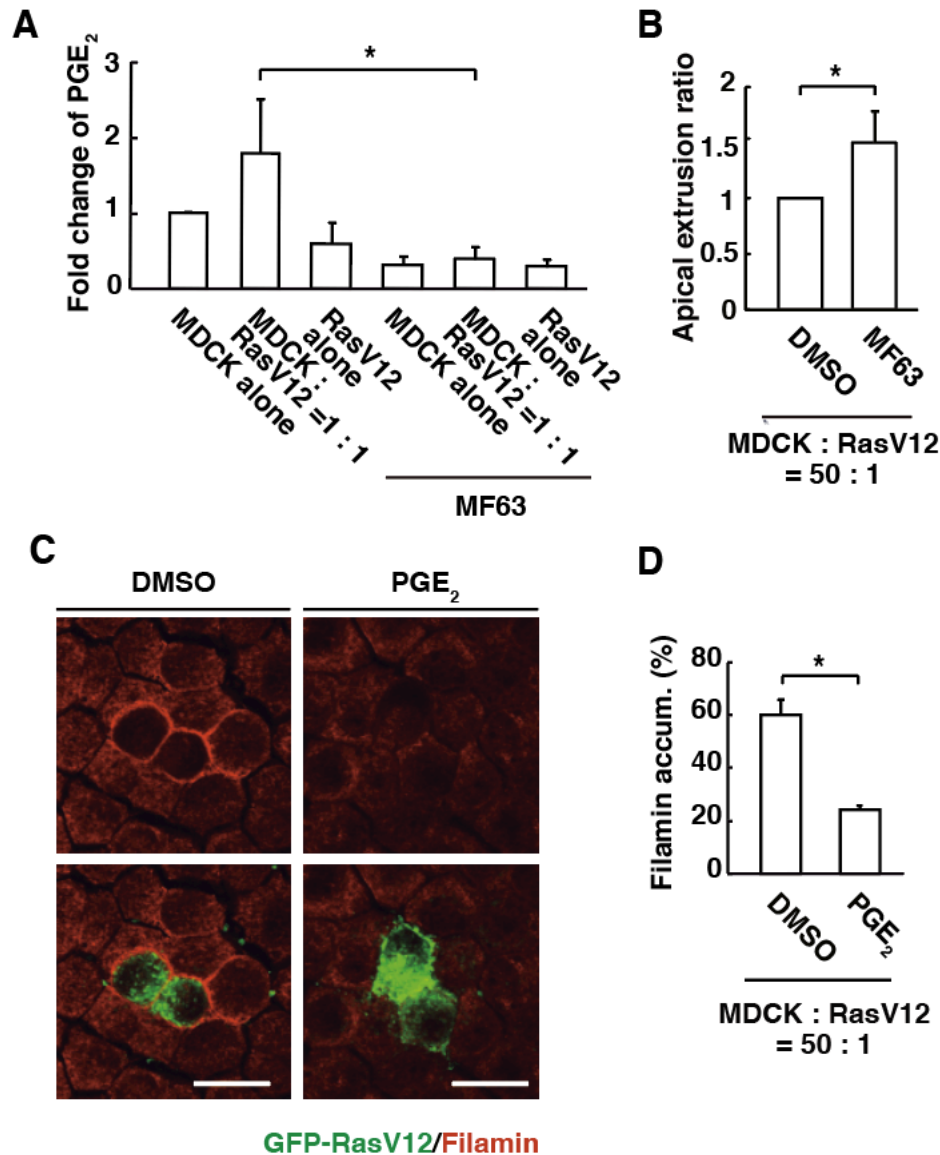


Figure 2-7. Significance of PGE₂ production in normal cells during cell competition.

(A) Effect of the PGE synthase-I inhibitor MF63 (0.1 μ M) on the production of PGE₂. Data are mean $\pm$ s.d. from three independent experiments. Values are expressed as a ratio relative to MDCK alone. * $P=0.021$ (Student's t -test). (B) Effect of MF63 (0.1 μ M) on apical extrusion of RasV12 cells. Data are mean $\pm$ s.d. from three independent experiments. Values are expressed as a ratio relative to DMSO. * $P=0.035$ (Student's t -test). (C and D) Effect of PGE₂ (10 μ M) on filamin accumulation in normal cells at the interface with the neighbouring RasV12 cells. (C) Immunofluorescence images of filamin in the presence or absence of PGE₂. Scale bars, 20 μ m. (D) Data are mean $\pm$ s.d. from three independent experiments. * $P=0.011$ (Student's t -test).

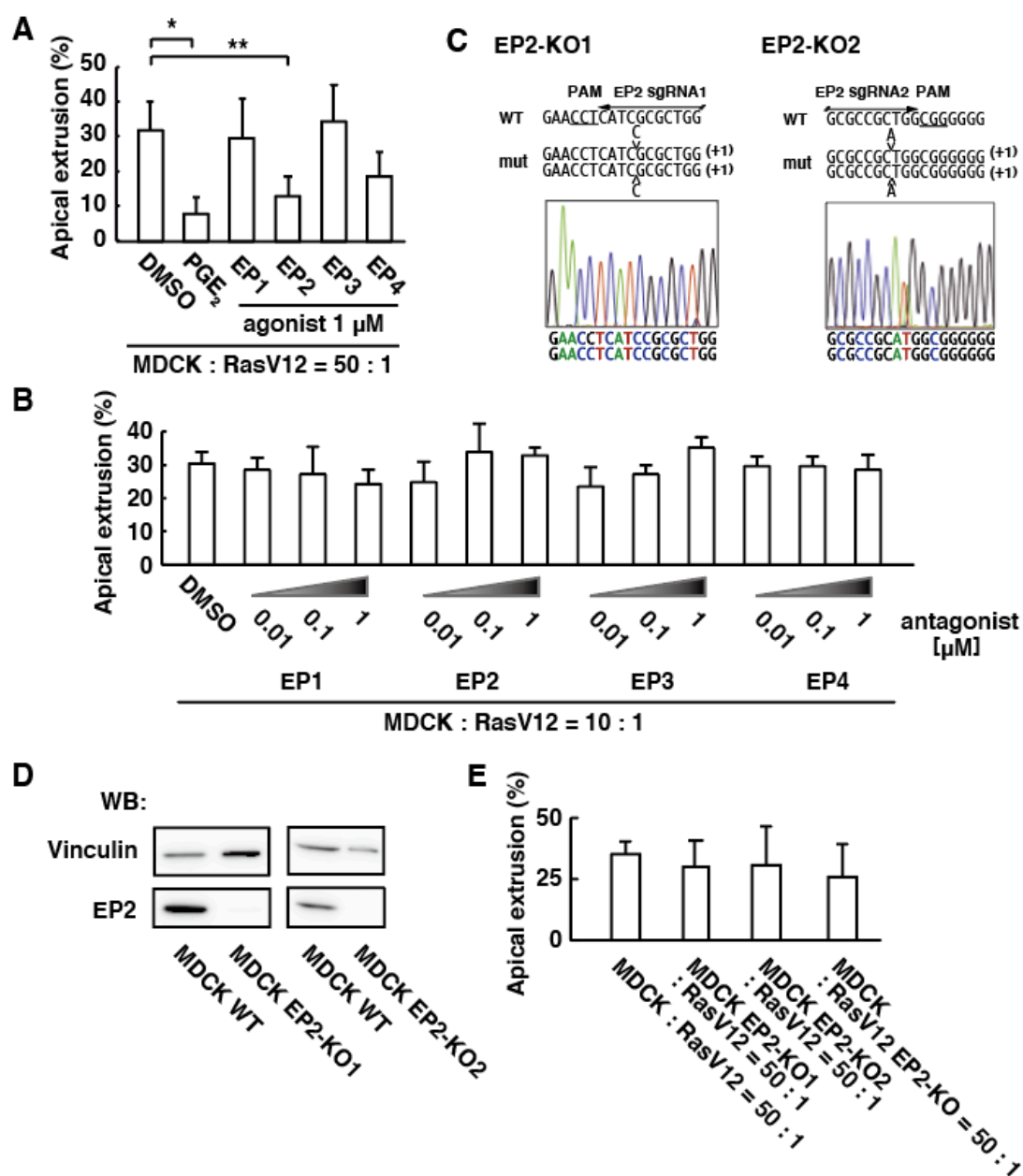


Figure 2-8. Effect of EP receptor agonists, EP receptor antagonists or EP2-knockout on apical extrusion of RasV12-transformed cells.

(A) Effect of PGE₂ (0.1 μM) or EP receptor agonists on apical extrusion of RasV12 cells. Data are mean ± s.d. from three independent experiments. * $P=0.0011$, ** $P=0.0091$ (unpaired t -test).

(B) Effect of EP receptor antagonists on apical extrusion of RasV12 cells. Data are mean ± s.d. from six (DMSO) or three (EP1-EP4) independent experiments. (C) A targeting scheme and DNA sequences of the wild type and EP2-null MDCK cell lines. PAM motifs are underlined.

(D) Knockout of EP2 was confirmed by western blotting. Note that RasV12 was stably expressed in MDCK EP2-KO2 cells to establish the RasV12 EP2-knockout cell line. (E) Effect

of EP2-knockout in normal cells or RasV12 cells on apical extrusion of RasV12 cells. Data are mean $\pm$ s.d. from three (left three) or two (right) independent experiments.

2.4. Discussion

2.3.1. COX-2 as a negative regulator of cell competition

Previous studies in *Drosophila* and mammals have revealed that during the event of cell competition, expression and activity of a variety of molecules/signalling pathways are modulated in both normal and transformed cells in a non-cell-autonomous fashion. Those changes often positively regulate the elimination of transformed cells. For instance, various positive regulators of cell competition have been found in *Drosophila*, including Flower, Spätzle/Toll signalling and Sas/PTP10D (Rhiner et al., 2010; Shin et al., 2011; Yamamoto et al., 2017). A role for Flower as positive regulator is conserved in mammals as well (Fujita, 2019; Madan et al., 2019). In mammals, Warburg-effect-like metabolic changes occur in transformed cells surrounded by normal cells, thereby promoting apical extrusion of transformed cells (Kon et al., 2017). In normal cells, cytoskeletal proteins filamin and vimentin accumulate at the interface with transformed cells, which generate physical forces that are required for apical extrusion (Kajita et al., 2014). On the other hand, it remains elusive whether and how cell competition is also governed by negative regulators. In *Drosophila*, expression of a soluble factor Sparc is upregulated in loser cells, which inhibits caspase activation of loser cells in a self-protective manner (Portela et al., 2010). In this study, I demonstrate, for the first time, that the COX-2-PGE₂ pathway acts as a negative regulator of cell competition in winner cells. The expression level of COX-2 can be influenced by a number of upstream signalling pathways; thus, the inhibitory role of COX-2 would be up- or down-regulated depending on external stimuli or environments. Hence, under various conditions, the consequence of cell competition might be controlled by the power-balance between positive and negative regulators (Table 2-3).

Table 2-3 The summary of major positive/negative regulators for cell competition.

	In mutant cells	In normal cells
Positive regulators	Drosophila	
	Flower, Spätzle/Toll signalling, Sas/PTP10D	?
	Mammal	
	Flower, Mitochondrial disfunction, p-VASP, Myosin-II, EPLIN	Filamin, Vimentin
Negative regulators	Drosophila	
	Sparc	?
	Mammal	
	VSAP	COX-2/PGE₂ pathway

Elimination of unfit cells or pre-cancerous cells from the epithelia should be desirable for the tissue. Why negative regulator exists in normal cells? In this discussion, I summarize the possibilities below.

(i) Mutant cells affect the neighboring cells and make the environment advantage for own survival within epithelia.

(ii) In context of maintaining the epithelial homeostasis, cell extrusion is somehow risky for it because cell extrusion can disturb the cytoskeletal organization and need to change the contractile force to squeeze unfit cells, and after that, have to proliferate to fill the space. Therefore, epithelial tissue originally has mechanisms against cell extrusion.

(iii) This inflammation-like pathway in normal cells is triggered after recognition of mutant cells like innate immune system.

2.3.2. What is the cause of COX-2 induction?

Expression of COX-2 is transcriptionally upregulated in normal epithelial cells that surround RasV12-transformed cells. Among the tested inhibitors, the pan-PKC inhibitor BIM-I suppresses the non-cell-autonomous upregulation of COX-2. Go6976: an inhibitor for Ca^{2+} -dependent PKC isoforms and ZIP: PKC ζ pseudosubstrate-derived ζ -inhibitory peptide affects the expression of COX-2 in normal cells surrounding RasV12 cells. Currently, our group have been reported that Ca^{2+} wave occurs in surrounding normal cells during cell extrusion (Takeuchi et al., 2020). Thus, Ca^{2+} signal can be upstream of COX-2 expression in normal cells via Ca^{2+} -dependent PKC. Some reports also show the relationships between the COX-2 induction and other PKC isoforms. Ca^{2+} -independent PKC isoform(s) positively regulate COX-2 expression. Indeed, previous studies have

shown that PKC- ζ or PKC- ϵ can act upstream of COX-2 (Jerde et al., 2008; Xuan et al., 2005). Furthermore, our group have reported that PKC- ϵ is accumulated in normal cells surrounding transformed cells (Kajita et al., 2014). In future studies, the upstream regulators of COX-2 in cell competition need to be further examined.

2.3.3. How PGE₂ suppresses the apical elimination of mutant cells?

COX-2 promotes the production of various prostaglandins including PGE₂. I present several lines of evidence indicating that the COX-2-PGE₂ pathway plays a crucial role in cell competition between normal and transformed cells. First, the amount of PGE₂ is elevated in the mix culture of normal and RasV12-transformed cells. Second, COX-2-knockout in normal cells diminishes the production of PGE₂. Third, addition of PGE₂ profoundly suppresses apical extrusion of RasV12 cells. Fourth, PGE₂ synthase inhibitor treatment promotes apical extrusion of RasV12 cells. PGE₂ can bind and activate EP 1-4. However, addition of antagonists for the respective receptors does not significantly affect apical extrusion. In addition, EP2-knockout in normal or RasV12 cells does not influence the frequency of apical extrusion. It is possible that multiple PGE₂ receptors on normal or RasV12 cells regulate the process of apical extrusion in a concerted manner. Alternatively, not only extracellular PGE₂, but also intracellular PGE₂, might modulate signalling pathways that affect apical extrusion (Bhattacharya et al., 1998; Fernández-Martínez and Lucio Cazaña, 2014; Lalier et al., 2011).

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CHAPTER 3:
**Effect of Intrinsically or Extrinsically
Induced Inflammation on Apical
Elimination of RasV12-transformed Cells
*in Vivo***

3. Effect of intrinsically or extrinsically induced inflammation on apical elimination of RasV12-transformed cells in vivo

3.1. Introduction

In previous chapter, I demonstrate that COX-2/PGE₂ pathway negatively regulates apical elimination of mutant cells. To further understand physiological role of COX-2/PGE₂ pathway in cell competition, I used cell competition model mice. Also, PGE₂ addition suppresses the apical extrusion dose dependent manner, suggesting that extrinsic inflammation might diminish the cell competition. To further elucidate the relationships between inflammation and cell competition, I analysed the chronic inflammation model mice. In this chapter, I demonstrate that either intrinsically or extrinsically induced inflammation suppressed the apical elimination of RasV12 cells *in vivo*.

3.2. Experimental procedures

3.4.1 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)-CreERT2 mice (Means et al., 2008) were crossed with CAG-loxP-STOP-loxP-EYFP (Srinivas et al., 2001) mice or DNMT1-CAG-loxP-STOP-loxP-HRasV12-IRES-eGFP mice (Kon et al.,

2017) to create CK19-YFP mice or CK19-RasV12-GFP mice respectively. The CAG-loxP-STOP-loxP-EYFP mice were obtained from the Jackson Laboratory (JAX stock #006148). For PCR genotyping of mice, primers listed in Supplementary Table 1 were used. Mice were age-matched and given a single intraperitoneal injection of 2 mg of tamoxifen in corn oil (Sigma-Aldrich) per 20 g of body weight for the induction of YFP or RasV12/GFP expression, and then sacrificed at Day 3, Day 7 or Day 14 after Cre activation. To examine the effect of ibuprofen, ibuprofen sodium salt (Sigma) was dissolved at 1 mg ml⁻¹ in drinking water and then administered for three days before sacrifice. Acute pancreatitis was elicited by hourly (6 times) intraperitoneal injections of 50 µg kg⁻¹ body weight caerulein (Sigma-Aldrich, Tokyo, Japan), whereas control animals received a comparable amount of PBS. Mice were submitted to three episodes of caerulein treatment per week for 3 weeks, resulting in chronic pancreatitis.

3.4.2 Antibodies and Materials

The following primary antibodies were used in this study: rabbit anti-COX-2 (ab15191) and chicken anti-GFP (ab13970) antibodies from Abcam, rat anti-E-cadherin (131900) antibody from Life Technologies, APC (Allophycocyanin)-conjugated CD45.2 (20-0454) antibody from TONBO. Alexa-Fluor-568- and -647-conjugated phalloidin (Life Technologies) were used at 1.0 U ml⁻¹. Alexa-Fluor-568- and -647-conjugated secondary antibodies were from Life Technologies. Alexa-Fluor-488-conjugated secondary antibody was from Abcam. Hoechst 33342 (Life Technologies) was used at a dilution of 1:5,000 for immunofluorescence.

3.4.3 Immunofluorescence

For immunohistochemical examinations of the pancreas, the mice were perfused with 1% PFA, and the isolated tissues were fixed with 1% PFA in PBS for 24 h and embedded in FSC 22 Clear Frozen Section Compound (Leica Biosystems). Then, 10-mm-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.

Immunofluorescence images of cultured cells and mouse tissues were acquired using the Olympus FV1000 system and Olympus FV10-ASW software. All primary antibodies were used at 1:100, except for mouse tissues: anti-GFP (1:1,000), anti-E-cadherin (1:2,000) and anti-CD45.2 (1:1,000) antibodies. All secondary antibodies were used at 1:1,000 for mouse tissues.

3.4.4 Oligonucleotides

Primers used in this thesis is described in (Table 2-1, see page 39).

3.3. Results

3.4.1 COX inhibitor treatment promotes apical elimination of RasV12-transformed cells from pancreatic ductal epithelia

To examine the functional involvement of COX-2 in the eradication of transformed cells *in vivo*, I used a cell competition mouse model system. A CK19-Cre-ERT2 mouse was crossed with an LSL-eYFP or LSL-RasV12-IRES-eGFP mouse (Figure 3-1 A). I then administered a low dose of tamoxifen, which induced recombination events less frequently, resulting in expression of RasV12 in a mosaic manner within various epithelial tissues (Kon et al., 2017). In this study, I focused on the pancreas, as Ras mutations are frequently observed in human pancreatic cancer. By immunofluorescence analysis, I found that expression of COX-2 was elevated in pancreatic epithelia harbouring RasV12-expressing cells (Figure 3-1 B). Accordingly, expression levels of a variety of inflammatory marker proteins were elevated in the RasV12-expressing pancreas compared with the YFP only-expressing pancreas, which was markedly reduced by ibuprofen treatment (Figure 3-2 B). RasV12-expressing cells were often apically eliminated from the pancreatic epithelial duct, but ibuprofen treatment further promoted apical extrusion of RasV12 cells (Figure 3-2 D and E). Collectively, these results suggest that increased expression of COX-2 accompanied by RasV12 induction suppresses apical elimination of transformed cells.

3.3.2. Caerulein-induced inflammation retards apical elimination of RasV12-expressing cells from pancreatic ductal epithelia

I further investigated the effect of extrinsically mediated inflammation on apical extrusion of RasV12-transformed cells. Caerulein is a cholecystokinin analogue that induces an infiltration of inflammatory cells into the pancreas leading to pancreatitis (Lerch and Adler, 1994; Willemer et al., 1992). Caerulein was continuously administered for three weeks to induce chronic pancreatitis (Nagashio et al., 2004), and tamoxifen was then injected to induce RasV12 expression, followed by caerulein treatment for two more weeks (Figure 3-3 A). I also confirmed that inflammatory response is successfully induced by caerulein administration (Figure 3-3 B and C). In control mice, the number of YFP only-expressing cells was not affected by caerulein (Figure 3-4 A and C). In contrast, the number of remaining RasV12 cells within pancreatic tissues was substantially increased by caerulein treatment (Figure 3-4 A and B). This result indicates that extrinsically induced inflammation can also suppress elimination of RasV12-transformed cells from epithelial tissues.

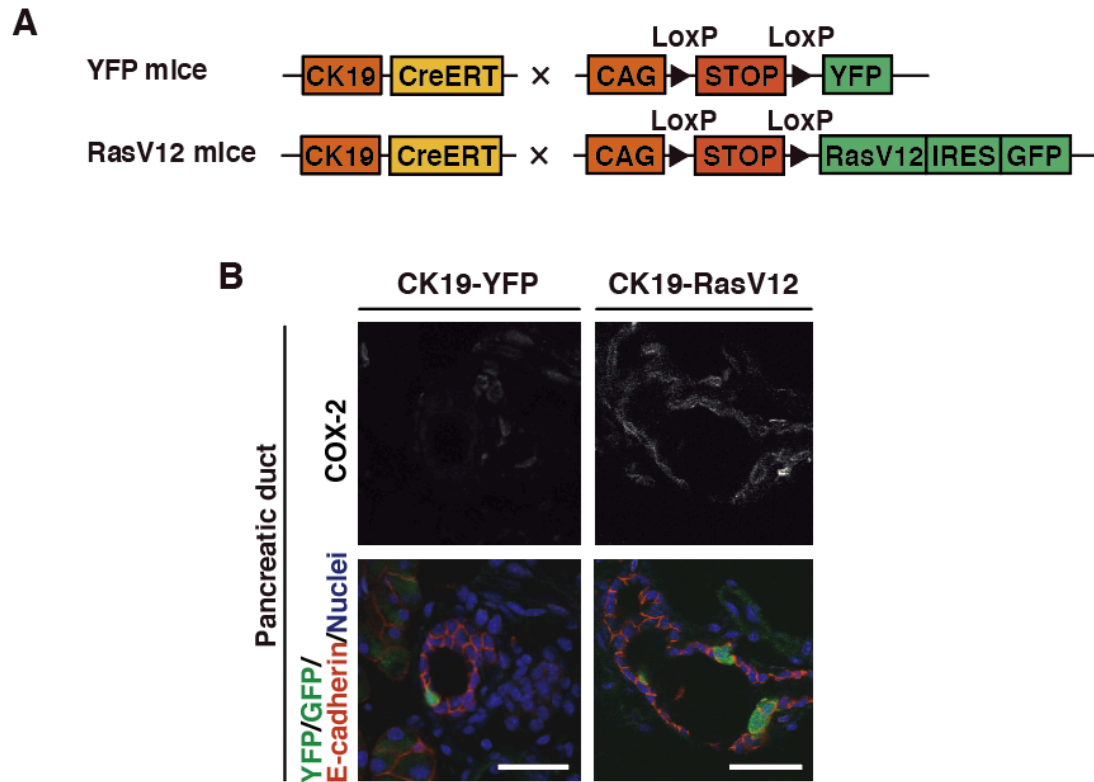


Figure 3-1.

(A) Strategy for the establishment of the cell competition mouse model. (B) Immunofluorescence analysis for COX-2 in the pancreatic ducts harbouring YFP- or RasV12/GFP-expressing cells at three days after tamoxifen administration. Scale bars, 40 μ m.

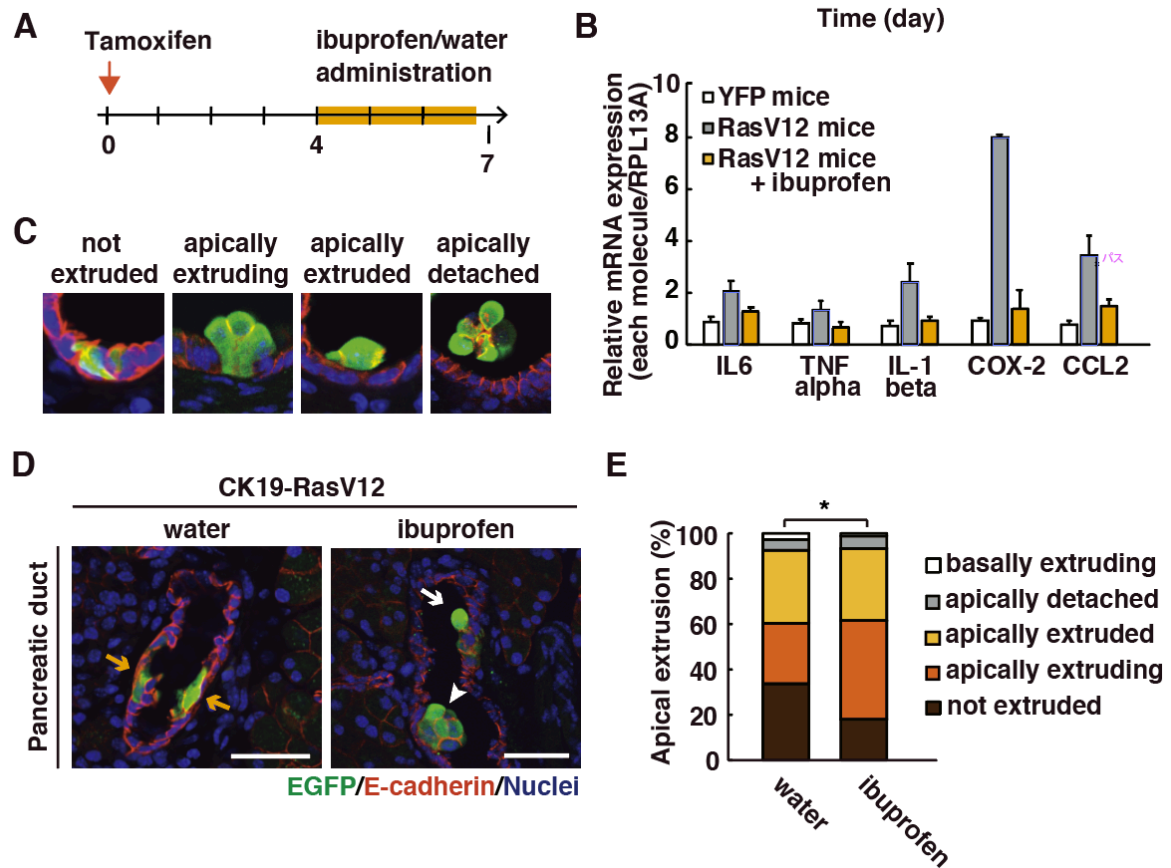


Figure 3-2. Effect of ibuprofen treatment on pancreatic epithelia.

(A) Experimental design for ibuprofen treatment. (B) mRNA expression of inflammatory factors in the RasV12-expressing pancreas with or without ibuprofen treatment. Tissue fractions of the pancreas were examined by quantitative RT-PCR. The mRNA expression level of the indicated inflammatory factors was normalized to that of *RPL13A*. Data are mean $\pm$ s.d. from five (YFP mice or RasV12 mice) or three (RasV12 mice + ibuprofen) independent experiments. (C) Representative images of RasV12-expressing 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 apical side of the epithelial layer. (D) Immunofluorescence images of pancreatic ducts at seven days after tamoxifen administration with or without ibuprofen treatment. Yellow arrows: not extruded; white arrow: apically extruding; white arrowhead: apically extruded. Scale bars, 40 μ m. (E) Quantification of apical extrusion of RasV12 cells from the pancreatic duct. n=174 (water) or 234 (ibuprofen) from three mice. * $P=0.020$ (chi-square test).

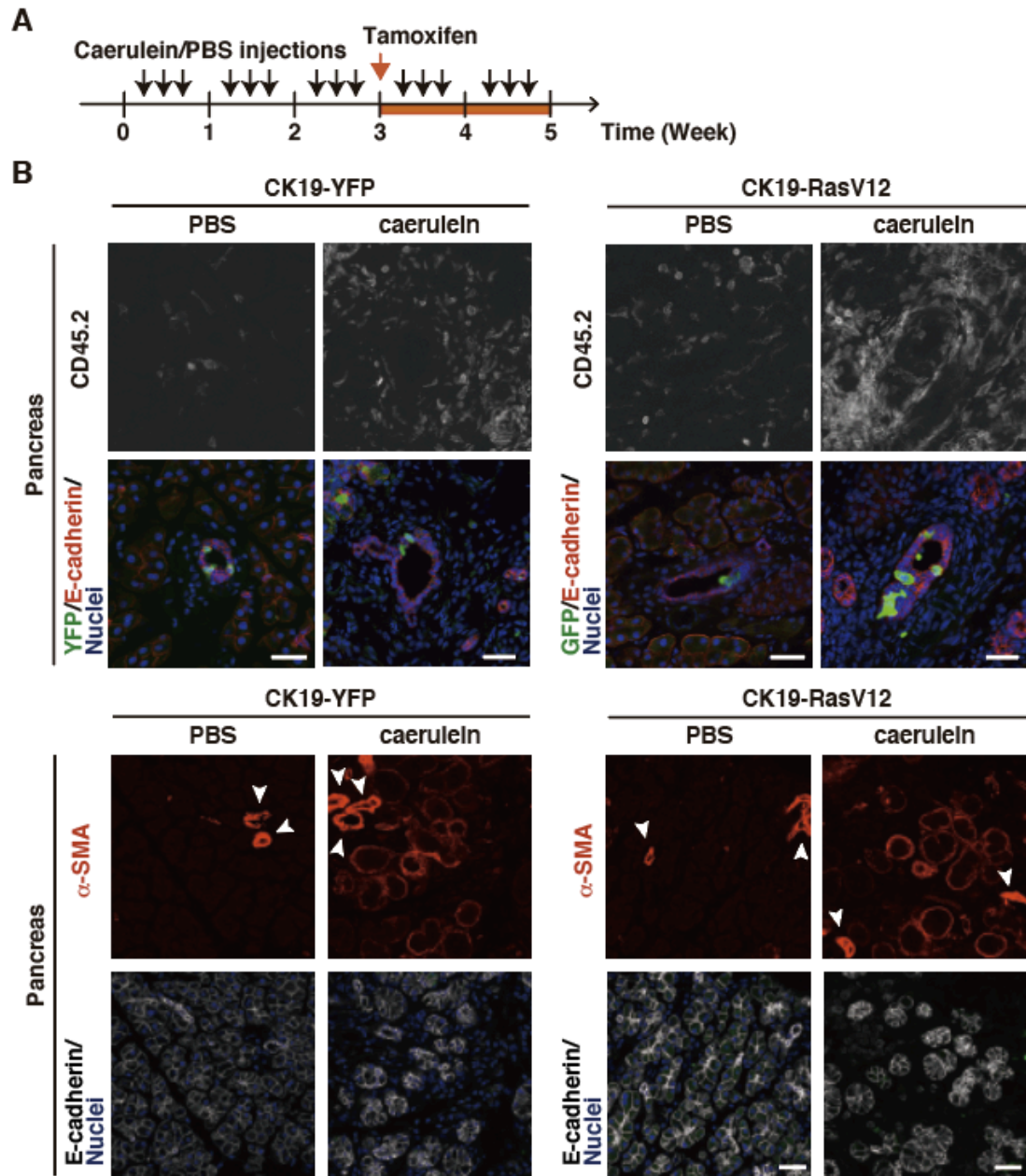


Figure 3-3. Establishment of chronic pancreatitis model mouse.

(A) Experimental design for caerulein treatment. (B and C) Immunofluorescence analysis for the immune cell marker CD45.2 (B) and alpha-SMA (C) in the pancreatic tissues upon caerulein treatment. Scale bars, 40 μ m.

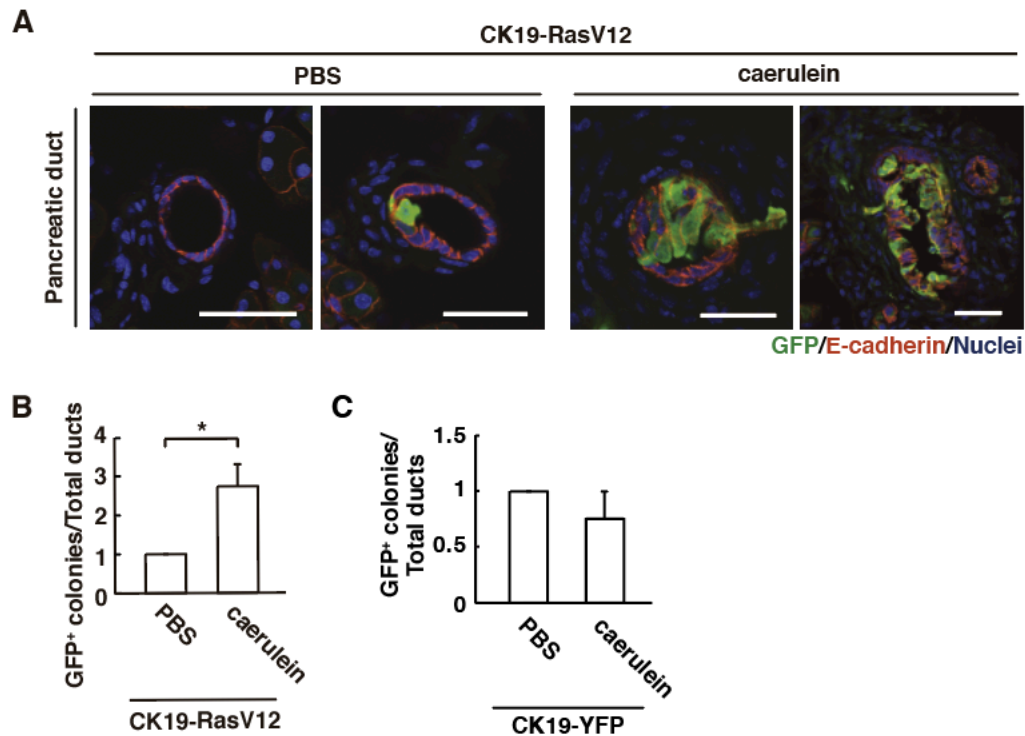


Figure 3-4. Caerulein-induced inflammation retards apical elimination of RasV12-expressing cells from pancreatic ductal epithelia.

(A) Immunofluorescence images of RasV12/GFP-expressing cells with or without caerulein treatment. Scale bars, 40 μ m. (B) Quantification of RasV12-expressing cell groups (colonies) in the pancreatic epithelial ducts. The total number of analysed ducts are 549 (PBS) and 486 (caerulein). Values are expressed as a ratio relative to PBS. Data are mean $\pm$ SD from three mice. * $P=0.033$ (Student's t -test). (C) Quantification of YFP-expressing cell groups (colonies) in the pancreatic epithelial ducts in the presence or absence of caerulein treatment. The total number of analysed ducts are 230 (PBS) and 295 (caerulein). Values are expressed as a ratio relative to PBS. Data are mean $\pm$ s.d. from two mice.

3.4. Discussion

3.4.1 Inflammation at the initial stage of carcinogenesis

At the interface between normal and transformed epithelial cells, the COX-2-PGE₂ pathway is upregulated in normal epithelial cells in a non-cell-autonomous fashion, which suppresses apical extrusion of transformed cells. In addition to this intrinsic inflammatory response, our data suggest that extrinsically mediated inflammation can also suppress cell competition; either addition of PGE₂ or caerulein treatment diminishes the frequency of apical elimination of transformed cells from epithelia. I also demonstrate that administration of NSAID promotes apical extrusion of RasV12-transformed cells from epithelial layers both *in vitro* and *in vivo*. Inflammation is considered to be one of the crucial tumour-promoting factors (Crusz and Balkwill, 2015; Grivennikov et al., 2010; Hanahan and Weinberg, 2011). Chronic inflammatory diseases increase the risk of certain types of cancer such as colon and pancreas. Epidemiological evidence indicates that NSAID treatment significantly decreases the incidence of various types of cancer including colon and pancreatic cancer (Baron et al., 2003; Bradley et al., 2010). It is generally believed that inflammation influences tumour progression at the mid- or late-stage of carcinogenesis. However, the data in this study imply that inflammation can also facilitate tumour initiation by diminishing EDAC at the initial stage of carcinogenesis.

3.5. Reference

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CHAPTER 4: Conclusion

4. Conclusion

Previously, our group reported that normal tissue has the ability to sense the pre-cancerous cells and eliminate them from the epithelia by cell competition. Furthermore, normal epithelial cells actively eliminate mutant cell by generating force to squeeze them, which termed EDAC (Epithelial defence against cancer). However, the mechanisms of EDAC process is still remain elusive.

In this study, I identified that COX-2/PGE₂ pathway is activated in normal epithelial cells that are mix-cultured with RasV12-transformed cells. Then I examined the role of COX-2 in cell competition. COX-inhibitor or COX-2-knockout promotes apical extrusion of RasV12-transformed cells. I also showed that induced inflammation suppresses the apical elimination of RasV12 cells from murine pancreas. Taken together, I demonstrated that either intrinsically or extrinsically induced inflammation suppresses apical elimination of RasV12-transformed cells from tissue (Figure 4-1). This is the first report to reveal the negative regulator in winner cells in cell competition, and this is also the first report to show the possibility that anti-inflammatory drugs could be the medicine for cancer prevention by promoting EDAC.

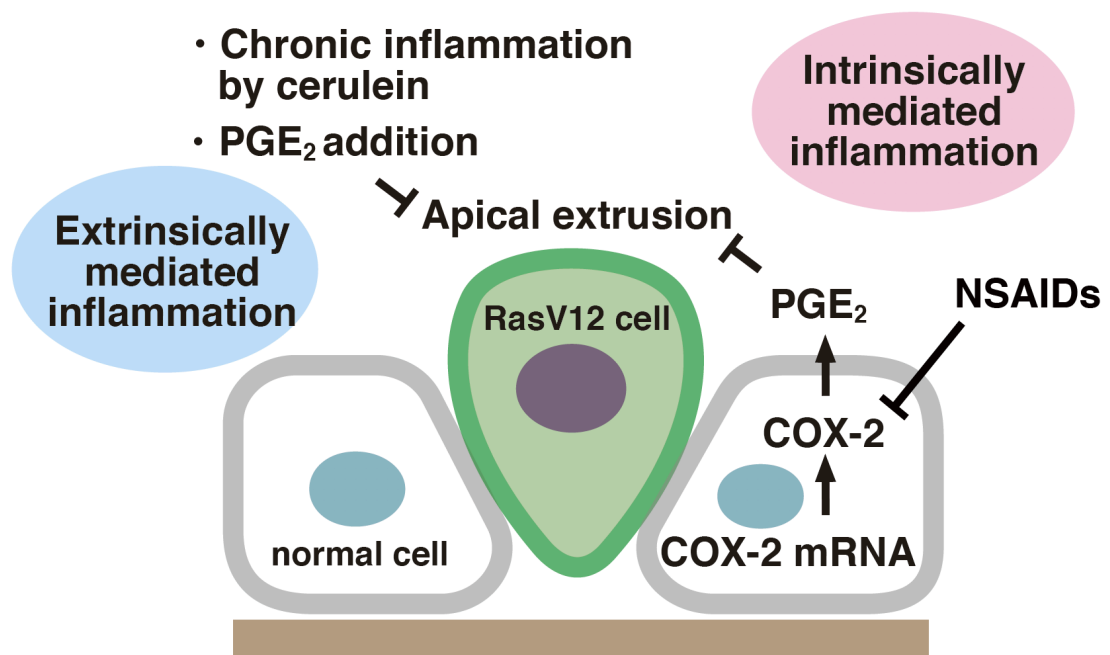


Figure 4-1. Schematics for the inhibitory role of inflammation in apical extrusion of RasV12-transformed cells.

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