Doctoral Thesis

Cell competition between thyroid cancer and normal follicular cells

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1.1 What is cell competition?

In multicellular organism cells consisting tissues/organs always communicate with each other to respond to outside environments as well as to maintain their functions for fulfilling homeostatic condition of organism as a whole. One of the sophisticated mechanisms to keep the homeostasis of their tissues/organs' functions is called cell competition, which has been evolved in living organisms. Cell competition was originally described in the wing imaginal epithelia of *Drosophila melanogaster* in 1975 [1]. In genetically mosaic flies containing clones of so-called *Minute*-mutants, which have mutations in the ribosomal protein genes, and wild-type cells, *Minute*-mutant cells are eliminated from the imaginal disk during development through the competition with more rapidly dividing neighbours. Interestingly, the mutant cells themselves are viable, however, in the presence of neighbouring normal cells, they cannot withstand the competition and subsequently been eliminated from the population [1].

Subsequent researches have shown that cell competition occurs not only in flies, but also in mammals involving embryonic development and adults remodelling belonging to a wide variety of tissues [2−7]. For example, during early embryogenesis in mouse, where various Myc protein expression is observed, cells expressing lower Myc level are eliminated by interaction with neighbors with higher Myc levels [8,9]. Stem cells under normal conditions continuously compete for occupancy of the niche, resulting in dynamic stem cell competitive behavior in the niche [10]. Mutation of the *Belly spot and tail* (*Bst*) gene, the mammalian *Minute* gene homologue that behaves just like the *Drosophila Minute*, has been shown to make the *Bst*/+ cells outcompeted in chimeras [11]. In fact, the *Bst*/+ mice have features remarkably similar to the *Drosophila Minutes*, indicating that cell competition and its mechanism should be uniersal among vertebrates.

Thus, cell competition is taken place when a critical degree of phenotypic heterogeneity is present within a homotypic cell population. More recently cell competition has been shown in a intrinsic condition where the levels of survival factors, such as nutrients or growth factors and space, are limited [2-7]. They might give spatial and size constraint, which is forced on any tissue by homeostatic control mechanisms. Most famous case involves the neurotrophic theory, which describes the phenomenon whereby neurons compete for limited levels of nerve growth factor, resulting in the culling of nearly half the originally produced neurons [12]. Similar mechanisms have been proposed to take place during *Drosophila* development. For example, limited levels of EGFR signalling support the survival of only a subset of cells in larval tissues [13]. Moreover, some studies also indicate that the elimination of less-fit cells in the developing imaginal wing disc results from competition for bone morphogenetic protein [14], although conflicting findings challenge this hypothesis [15, 16]. Competition for interleukin-7 (IL-7) appears to mediate selection of young T cells over old T cells in the thymus [17], which is deeply involved in immunesenescence. Since homeostasis regulated through soluble factors are common, such nutritious cell competition is expected in various

tissues/organs, we should be cautious as it is still possible that fitness-dependent mechanisms should be involved as well.

Within an epithelium, cell crowding is another physiological condition sufficient to induce cell competition. Histolically, mechanical, stress-induced competition was first predicted through the computational modelling [18] and has now been validated in a range of biological contexts, including the human colon, the zebrafish epidermis, MDCK cell sheets and the *Drosophila* pupal notum [19]. For example, in the pupal notum, cells with slower growing are preferentially extruded over fast growing cells [20]. Polarity-deficient MDCK cells are eliminated by extrusion in culture by their wild-type counterparts through mechanical triggers [19]. In fact, the finding that contact-based elimination of MDCK cells is triggered by elevation of p53 alone alludes to the possibility that the activation of cell stress pathways sensitises cells to mechanical stimuli, resulting in the preferential removal of damaged cell types. Thus, it can be concluded that cell competition is an endogenous system, by which an emergence of different types of tissues, cells compete for different factors, and appropriate cell density of tissues/organs are scrutinized.

1.2 The physiological role of cell competition

When cell competition was originally determined in developing *Drosophila melanogaster* tissues, it was considered to play a role of a quality control system in the growing embryo by eradicating undesirable cells during development [1]. Since then cell competition has been confirmed in a variety of physiological processes, including embryogenesis, morphogenesis, and aging [2-7]. In particular, it is now believed that cell competition is a general feature of tissues and organs for eliminating the variants in specified cell lineages, which is critical for tissue homeostasis [14, 16-19]. It also works as a natural selection process between normal and its variant cells [10 –13]. In most cases, cells showing growth advantage predominates their neighbors with growth-disadvantage in the host tissue. Accordingly, the less-fit cells are eliminated by cell death, like apoptosis, while more fit cells proliferate and dominate neighborhood [4,5,7]. Thus, cell competition is recognized as a critical player in coordinating different cell clones consisting tissues/organs. Previous studies have revealed that mechanisms underlying cell competition include the molecular recognition of ‘different’ cells, signalling imbalances between distinct cell populations, and the mechanical consequences of differential growth rates, indicating that these mechanisms may also involve innate immune systems, elimination of clones with oncogenic mutations, and cells with changing translational profiles [21-24].

Homeostasis of tissue physiology might be challenged by several extrinsic insults, such as viral infection, radiation, and chemicals. In such cases, cell competition plays a role in controling tissue quality, otherwise it should be disrupted by dying cells. In particular, in overtly damaged tissues triggered by massive cell death, cell competition eliminates even viable cells in a context-dependent manner, selecting the best available cell lineages in the tissue. During developmental metabolic cell

competition, prospective cells to be eliminated rescued from death can generate normal tissues and organs. Accordingly, cell competition may optimize tissues before cell impairment compromises tissue function to any significant degree, although it should be cautious to conclude as there still be a exceptions, such as mammalian heart development, where cell competition has been induced but not be required for normal development [25].

Recently, one another physiological aspect of cell competition has been described, which is a role in controling tissue/organ size. Most tissues/organs undergoing cell competition, which compensates for the loss of aged or damaged cells, so that final organ size is not affected. In fact, when cell competition through natural apoptosis is blocked in the wing disc in Drosophila, there is an increase in wing size variability [15], suggesting an involvement of cell competition in controling the organ size, although this effect has not been detected in in mammals [26].

It is implicated that cell competition-independent global size regulation mechanisms are more predominant in mammals. In the case of supercompetition driven by Myc-overexpressing cells, flies and mice also show no changes in organ size [4,14,15]. Interestingly, activation of growth pathways that do not induce cell competition produces a mild increase in wing size, and inhibition of loser cell death during supercompetition leads to overgrowth of imaginal disc compartments. Furthermore, when natural apoptosis is blocked in the wing disc, there is an increase in wing size variability. These results suggest that endogenous cell death contributes to the robustness of size control mechanisms, although the competitive nature of this natural cell death has not been determined. Furthermore, these results indicate that elimination of loser cells is important for size control during cell competition, which might be identified in in mammals during embryogenesis or organogenesis.

1.3 Elimination and replacement of competed cells

During cell competition, cells that would be viable in a homogenous environment are eliminated as a result of being surrounded by their competitor cells. Among heterogeneous cell population cell competition is taken place through the process divided into at least three stages. First, cells with heterogeneous population have different fitness levels. In the context of cell competition less-fit cells are often referred to as ‘loser’ cells, whereas more-fit cells can be considered to be ‘winner’ cells. The second step of cell competition involves the elimination of loser cells from the tissue. The mechanisms through which this elimination occurs are diverse. For example, previous studies have shown that elimination is executed through (1) apoptosis/cell death, (2) extrusion from the epithelia, (3) senescence induction, (4) elimination of cells by phagocytosis, or (5) cell differentiation. Among them in particular, senescence induction is worth considering as the ultimate fate of the less-fit epitheelial cells is their death by senescence [27]. Finally, following loser cell elimination, compensation of tissue population occurs through increased proliferation of neighbouring cells or overgrowth of surrounding winner cells. Because a key feature of competition is its ‘silent’ phenotype, despite loser cell elimination, a constant tissue size is maintained, so that the winner cell

compensatory proliferation is a key to maintaining a constant tissue size. Only an exception is when cellular overcrowding triggers competition, which is deeply involved in cancer cell propagation *in situ*.

1.4 The role of cell competition in the pathogenesis of cancer

At earlier stages of carcinogenesis, malignant transformation starts from a single cell that grows within an epithelial monolayer. The initiated cell continues to proliferate and accumulates genetic alterations, which give rise to malignant cancer cells. However, tissue microenvironment, in which multiple types of cells coexist, affects malignant propagation of initiated cells [8,32-34]. In fact, in a tissue microenvironment, the initiated cells are likely to interact with normal counterparts in a dynamic fashion over time. Such interactions may lead to a expansion by promoting cell death or to cell propagation if the balance is impaired [34]. Therefore, gaining a greater understanding of the mechanisms how the initiated and normal cells compete at a cellular level should provide deeper insights into the complexity of cancer development *in vivo*.

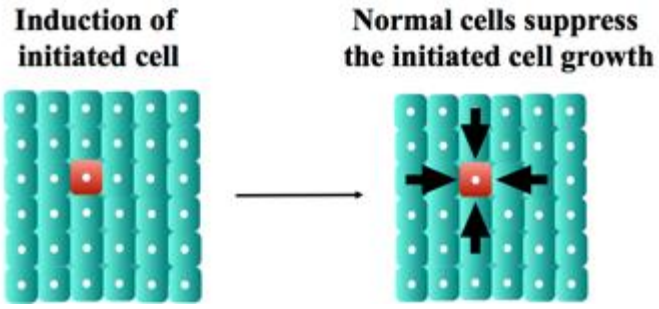

Figure 1. Cell competition between initiated cells and neighbouring normal cells

The initiated cell, induced by a oncogenic mutation, is limited its growth by tissue microenvironment through cell-to-cell competition, otherwise they continue to proliferate and accumulates genetic alterations, which give rise to malignant cancer cells.

Over the last few years, it has been demonstrated that cell competition is a form of cell-cell interaction, which works as a natural selection process between two different types of cells such as mutant and normal cells [1-6]. In most cases, cells showing growth-advantage win out over their neighbours with growth-disadvantage in host tissue. Accordingly, the less-fit cells or namely, ‘losers’ are eliminated, while more fit cells or ‘winners’ proliferate and dominate neighborhood [15], resembling the domination of oncogenic mutated cells in tissue. Thus, cell competition occurs under different physiological conditions naturally, but growing numbers of studies have implicated that cell competition is critically involved in the development of several diseases including cancer [35-40]. In carcinogenesis, Myc-mediated cell competition is the most well-studied example [41]. Originally, cells showing higher expression of Myc were demonstrated to cause competitive cell elimination in

Drosophila wing discs [14,15], whose process was termed super-competition. Subsequently, cell competition between cells with different levels of Myc protein was reported in mouse epiblast, and cells with low Myc-expression were eliminated [42,43]. Since c-Myc protein is involved in many types of cancer cells [44-48], it is likely that cancer cells with excessive oncoprotein expression burden may enable exploiting super-competition to aid their expansion in tissues [9,49]. p53, which is mutated in more than 50% of cancers [50-53], has also been shown to play a role in cell competition, and cells with mutated p53 function outcompeted the wild-type p53 cells in mouse skin tissue [54]. Another study demonstrated that p53 mutations occurred in cells carrying oncogenic Ras supported the competitive growth of RasV12-mutated cells [55]. Thus, accumulating evidences have strongly implied that cell competition potentiated by oncogenic mutations plays a critical role in clonal expansion of the initiated cells within tissue, which gives rise to clinically recognized cancers.

1.5 Thyroid cancers and radiation health effects

Thyroid cancer is the most frequent endocrine malignancy [56-59]. Amongst three major types of thyroid cancers, papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), and anaplastic thyroid cancer (ATC), ATC is a rare but highly aggressive disease [57,60-62]. Using the Surveillance, Epidemiology, and End Results (SEER) database, the median survival was determined to be 3.16 month after the diagnosis among the ATC patients diagnosed in the United States between 1986 and 2015 [60]. While PTCs are well-known to show a good prognosis, ATCs are quite refractory to be cured. In fact, most of the treatment modalities, such as surgical resection, chemotherapy and radiation therapies, have not been effective [57,59,62-64].

Relationship between radiation exposure and thyroid cancers are well described among A-bomb survivors and children affected by the Chernobyl nuclear power plant accident. Enormous amount of childhood thyroid cancers among such children have been induced in a dose-dependent manner, and therefore, after the Fukushima Daiichi nuclear power plant accident, thyroid cancers have also been screened, and they were detected among residents ages 18 years and younger in Fukushima. However, since the estimated radiation doses to thyroid are markedly low, these cancers are not related to radiation exposure. More importantly, considering the frequency, they might not been detected if the ultrasound screening was not carried out. Thus, it is now quite evident that thyroid cancer cases diagnosed in Fukushima are sporadic ones. It has been shown that such thyroid cancers are mostly latent, suggesting that there is a mechanism to limit the propagation of very small thyroid cancers to microcarcinomas [65-69]. If normal cells can suppress tumour growth through cell competition mechanism, and eventually eliminate them, then microcarcinoma remains small and propagation is still slow, so that possible conversion of microcarcinoma to cancer takes a long time, or they even remain in the tissues as subclinical microcarcinomas thoughout the life.

The possibility stimulated me to initiate this thesis project, and I have aimed at proving that normal cells limit propagation of cancer cells. Simply, I have hypothesized that the cell to cell competition could be the critical mechanisms to regulate cancer induction, so that the question must be what if the initiated cells have already had malignant phenotype, normal neighboring cells are able to suppress their growth. To realize my idea it is essential to obtain normal human thyroid follicular epithelial cells and human thyroid cancer cells.

Recently, the molecular abnormalities in ATC have been studied on RNAs, genome and protein levels, which uncovered the most prevalent mutations were those of p53, BRAF and RAS, suggesting that increased cell competitiveness could be involved in the malignant phenotype [70-72]. Considering that cancer cell growth in thyroid follicles is affected by the interaction with the environment and that cell competition could be one of such actual mechanisms, a model employing ATC cell line, ACT1, and normal thyroid follicular epithelial cells (NTECs) was set to evaluate its role. Since ATC is a representative malignant thyroid cancer, I was also hoping to discover any clues for the tratment of ATCs. The study applied co-culture method, which mimicked the small clusters of cancer cells surrounded by normal cells, and the live-cell imaging technique was applied in order to evaluate the dynamics of each cell type.

My second aim of this thesis is to pursue effects of radiation exposure on cell competition, since dysfunction of cell competition is perfectly matched to the idea that explain the biological effects caused by radiation exposure [73]. In fact, cell competition is now a major issue in radiation field. According to the recent ICRP Pub131, studies have shown that damaged stem cells are subject to constant removal competition for residence in the vital niche from undamaged stem cells [10]. But irradiation modifies stem-cell-to-niche interaction by giving selective advantage to stem cells for residence in the bone marrow niche, contributing to the further development of leukemia. It became clear radiation could change cell-to-cell and cell-to-tissue interactions in a tissue's microenvironment through the secretory phenotype of senescence-like dead cells caused by radiation. As a result, tumor suppressing mechanism of cell competition could be disrupted [74-76]. Since cell competition was negated by terminal growth arrest of normal cells, radiation-induced detrimental effects on cells can be hypothesized to be an intrinsic mechanism associated with tumor initiation, propagation, and metastasis, whose possibility has to be determined. To prove this the co-culture system, in which normal cells exposed to lethal dose of radiation were cultured with the clusters of ACT1 cells, was used.

2. Materials and Methods

2.1 Cell culture

Normal human thyroid follicular epithelial cells (NTECs) were spontaneously immortalized cells derived from human thyroid gland [77], and anaplastic thyroid carcinoma cell lines (ACT1) were obtained from Dr. N. Onoda (Osaka City University, Osaka, Japan; originally established by Dr. S. Ohata of Tokushima University [78]. Cells were cultured in DMEM (Wako, Tokyo) supplemented with 10% fetal bovine serum (FBS) (TRACE Bio) under standard conditions in a humidified incubator at 37°C with 5% CO_2. NTECs and ACT1 cells were checked their human origin by chromosome analysis, and mycoplasma contamination was routinely inspected by DAPI staining.

2.2 Irradiation

NTECs cultured in a culture flask were exposed to 10 Gy of γ-rays from a γ-ray irradiator equipped with a ^{137}Cs source (Pony Industry Co., Ltd, Osaka) at a dose rate of 1 Gy/min.

2.3 Cell labelling

ACT1 was labelled with the Qtracker 655 cell labeling reagent (ThermoFisher Scientific, Tokyo, Japan). Cells were cultured onto sterilized 22 x 22 mm glass slips (Matsunami, Tokyo, Japan) placed in the 35-mm culture dishes. For labelling, a 10 nM solution was prepared according to the manufacturer's protocol and added to the dishes with ACT1 cells for 1 hr at 37°C followed by two washes with fresh growth medium.

NTECs were transfected with the pHOS-H2B-GFP plasmid (BD Bioscience, Tokyo) from which the green fluorescent protein (GFP)-tagged histone H2B is produced. Introduction of the plasmid into NTEC was performed by electroporation (Electric Cell Fuser, ECF2001, Wakenyaku, Tokyo). Exponentially growing cells were collected by trypsinization, suspended in PBS, and 200 □l of cell suspension was added to a electroporation cuvette (0.2 cm) with plasmid DNA (1 μg). Three pulses with an intensity of 400V/cm with a constant pulse duration of 1 msec were used. Immediate after the pulse, cell suspension was transfer to 100-mm dishes with 10 ml of DMEM medium and cultured for three days. Cells were then collected by trypsinization, replated onto three 100-mm dishes with 10 ml of DMEM medium containing 400 μg/ml G418 (WAKO Pure Chemicals, Osaka), and cultured for further 7 day before G418-resistant colonies were formed. Colonies were randomly isolated, and independent clones were cultured in T25 flasks. A part of each clone was replated onto glass-bottomed dishes (AGC Techno Glass Co., Ltd, Tokyo), and GFP expression was checked under a fluorescent microscope (BZ-9000, KEYENCE, Osaka) to select GFP-H2B-positive clones. The clone, which expressed the strongest GFP fluorescence, was named GFP-NTECs.

2.4 Co-culture of ACT1 and NTEC cells

Exponentially growing ACT1 cells were plated on glass coverslips at low cell density (400 cells/slip) and incubated for about 5 days until they formed small cell clusters. Then, NTECs were added to the culture (10^5 cells/slip), and incubated for further 3-5 days.

2.5 Time-lapse microscopy

Time-lapse imaging was performed by BioStation-ID (GE Helthcare Bioscience, Tokyo, Japan) with a x10 objective lens. Images were captured in every 5 min for up to 72 h. In each experiment, at least ten fields were imaged in GFP fluorescence channel along with phase contrast.

2.6 Live-cell imaging of apoptosis

For the detection of apoptosis in living cells, CellEvent Caspase-3/7 Green Detection Reagent (ThermoFisher Scientific, Tokyo, Japan) was used. The reagent (2 µl) was diluted in growth medium and added to the dishes containing ATC1 clusters and NTECs. The cultures were incubated for 30 minutes according to the manufacturer's instruction, and apoptotic cells with activated caspase-3/7, which showed bright green fluorescence, were analyzed by time-lapse microscopy using Biostation-ID.

2.7 Immunofluorescence

For immunofluorescence, the cells on glass coverslips were fixed in 4% formaldehyde for 10 minutes on ice, followed by the permeabilization with 0.05% Triton X-100 for 5 min on ice. After extensive wash with PBS, primary antibodies diluted in TBS-DT (20 mM Tris-HCl, pH7.6, 137 mM NaCl, 0.1% Tween 20, 125 µg/ml ampicillin, 5% skim milk) were treated for 2 hr at 37°C, followed by the Alexa Fluor-labeled secondary antibodies for 1 hr at 37 ˚C. After extensive washes with PBS, coverslips were mounted on glass slides with 10% glycerol/PBS containing 1 µg/ml DAPI. The primary antibodies used in the experiments were rabbit anti-phospho ERK1/2 antibody (1:500 dilution, #4370, Cell Signaling Technology Japan, Tokyo), rabbit anti-c-myc antibody (1:500 dilution, ab32071, Abcam Japan, Tokyo), mouse anti-CDH1 antibody (1:500 dilution, 610182, BD Biosciences, San Jose, CA), mouse anti-phospho H2AX antibody (1:500 dilution, 613402, BioLegend, San Diego, USA), rabbit anti-Ki-67 antibody (1:1000 dilution, ab16667, Abcam Japan, Tokyo), chicken anti-Vimentin antibody (1:2000 dilution, ab92547, Abcam Japan, Tokyo), rabbit anti-active YAP1 antibody (1:200 dilution, ab205270, Abcam Japan, Tokyo), and rabbit anti-53BP1 antibody (1:500 dilution, A300-272A, Bethyl Laboratories Inc., Montgomery, TX). The secondary antibodies used were goat Alexa Fluor 488-labeled anti-mouse IgG (1:1000 dilution, A11001, Thermo Fisher Scientific), goat Alexa Fluor 555-labed anti-rabbit IgG (1:1000 dilution, A21428, Thermo Fisher Scientific, Tokyo), and goat Alexa Fluor 647-labed anti-chicken IgY (1:1000 dilution, A150171, Thermo Fisher Scientific, Tokyo). Images were captured by a fluorescence microscope (DM6000B, Leica Japan) and analyzed by image processing system (FW4000, Leica Japan).

2.8 Image analysis

Images were taken under a fluorescent microscope (BZ-9000) and the fluorescence area corresponding to a cell cluster size was determined using Image J software [79]. Briefly, the selection tool was used to set the area of interest, and the image was converted to Grayscale. The pixels were highlighted using the threshold dialog, and the total number of pixels was defined by selecting the menu command "measurements". For analyzing phosphorylation of ERK1/2, the distance from ACT1 cell cluster was marked with the straight selection tool. Next, the colour images were splitted using the menu command "Image-Type-Colour-Split channels", and using the blue channel, which is for the DAPI staining, the total number of NTEC cells per distance was calculated. The number of NTEC cells with phosphorylated ERK1/2 was quantified with the merged image between the blue and red channels using the menu command "Image-Colour-Merge channels", and cells positive for both channels were counted.

2.9 Western blotting

Cells were treated with lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS). The cell lysate was cleared by centrifugation at 15,000 rpm for 10 min at 4°C, and the supernatant was used as the total cellular protein. Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Protein samples (8 or 16 □g) were electrophoresed on a SDS-polyacrylamide gel (ATTO Corporation, Tokyo) and were transferred to a polyvinyl difluoride (PVDF) membrane (Millipore Japan, Tokyo) in a transfer buffer (100 mM Tris, 192 mM glycine). After incubation with blocking solution (10% skim milk in TBS-T buffer (20 mM Tric-HCl, pH7.6, 137 mM NaCl containing 0.1% Tween-20)) for one hour, the membrane was incubated with the primary antibodies for 2 hours followed by the secondary goat anti-mouse IgG or goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (BioRad Laboratories, Inc, Tokyo, Japan). The bands were visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. The primary antibodies used in this study were rabbit anti-ERK1/2 (1:500 dilution, #4695, Cell Signaling Technology Japan, Tokyo), rabbit anti-phosphorylated ERK1/2 (1:500 dilution, #4370, Cell Signaling Technology Japan, Tokyo), rabbit anti-p38 (1:500 dilution, #9212, Cell Signaling Technology Japan, Tokyo), rabbit anti-phosphorylated p38 (1:500 dilution, #4511, Cell Signaling Technology Japan, Tokyo), rabbit anti-phosphorylated RB at S780 (1:500 dilution, #8180, Cell Signaling Technology Japan, Tokyo), rabbit anti-RB (1:500 dilution, #9313, Cell Signaling Technology Japan, Tokyo), mouse anti-$p21^{WAF1/Cip1}$ (1:500 dilution, ab107099, Abcam plc, Cambridge, UK), rabbit anti-$p27^{Kip1}$ (1:500 dilution, ab32034, Abcam plc, Cambridge, UK), mouse anti-$p16^{INK4a}$ (1:500 dilution, ab117443, Abcam plc, Cambridge, UK), rabbit anti-phosphorylated Skp2 at S64 (1:500 dilution, #14865, Cell Signaling Technology Japan, Tokyo), rabbit anti-Akt (1:500 dilution, #4691, Cell Signaling Technology Japan, Tokyo), rabbit anti-phosphorylated Akt at

S473 and T308 (1:500 dilution, #2965 and #4060, Cell Signaling Technology Japan, Tokyo), rabbit anti-c-myc antibody (1:500 dilution, ab32071, Abcam Japan, Tokyo), mouse anti-p53 antibody (1:500, BP53-12, dilution, 628201, BioLegend, San Diego, USA), goat anti-Scrib (1:500 dilution, sc-11049, Santa Cruz Biotechnology Japan, Tokyo), and mouse anti-β-Actin (1:500 dilution, 2F1-1, BioLegend, San Diego, USA).

2.10 Senescence associated β-galactosidase staining

Cells plated on cover glass were washed with PBS and treated with a fixative (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 5 min at room temperature. After a wash with PBS, cells were incubated with staining solution (40 mM citric acid/sodium phosphate, pH 6.0, 5 mM Potassium ferricyanide, 5 mM Potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$ containing 1 mg/ml 5-bromo-4-chloro-3-indolyl □-D-galactoside, WAKO Pure Chemicals, Osaka) for 12-24 hours at 37°C. After extensive washes with PBS, coverslips were mounted on glass slides with 10% glycerol/PBS and stored at 4°C.

2.11 Statistics

For each experiment, at least three independent repeats were carried out. The non-parametric Mann-Whitney test was used for colony size comparison. Weighted linear regression was used to analyze region-specific phosphorylation of ERK1/2. Statistical calculations were performed using JMP 15 Pro software. The *p*-value < 0.05 were considered indicative of statistical significance.

3. Results

3.1 Cell competition between anaplastic thyroid cancer cells and normal thyroid follicular cells

Cell competition was reproduced by establishing a co-culture system between anaplastic thyroid cancer cell line, ACT1, and normal thyroid follicular epithelial cells (NTECs). ACT1 cells plated at a low density grow clonally and form densely packed cell clusters by Day 5 (Fig 2A), and then, NTECs were added to the culture. I observed that all ACT1 clusters were surrounded by NTECs (Fig 2B).

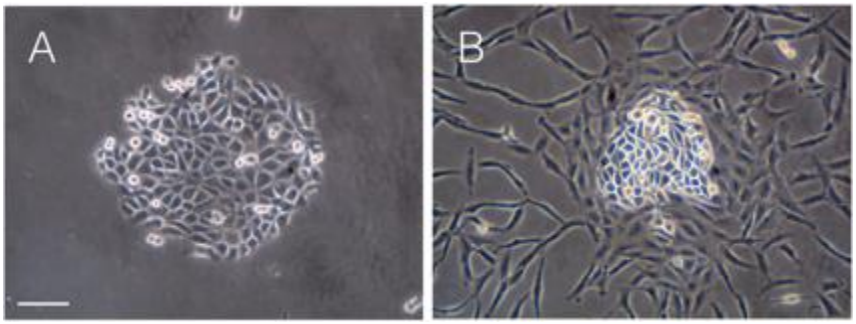

Fig 2. Co-culture of ACT1 cell cluster with NTECs.

ACT1 cells were cultured for 5 days until they formed small cell clusters of approximately 500 mm in diameter. Then, NTECs were added to establish co-cultures. (A) ACT1 cluster in monoculture at Day 7. (B) ACT1 cluster with NTECs two days after co-culture. NTECs were gradually crowded around ACT1 cell clusters. The bar in (A) indicates 100 μm.

The time-lapse analysis demonstrated that NTECs added to the culture are gradually accumulated around ACT1 clusters even at very low cell density, and crowding around ACT1 clusters becomes obvious approximately 6-8 hrs later. After 3-5 days incubation, NTECs fully occupied the space between the ACT1 clusters, and the growth of ACT1 clusters seemed to be suppressed, as I observed significant change in the sizes of the clusters among NTECs compared with ATC1 clusters in monoculture. To confirm the suppressive effects GFP-tagged NTECs, which enable to discriminate normal cells in co-culture, were used (Fig 3).

ACT1 cluster sizes in monocultures (Fig 3A) are obviously larger than those in co-cultures (Fig 3B), and the difference is statistically significant (Fig 3C), demonstrating that cell competition between neighbouring NTECs and ACT1 clusters retarded ACT1 cluster growth. According to the results of immunofluorescence using anti-Ki-67 antibody, a marker for proliferation, ACT1 clusters found to be negative for Ki-67 and lost proliferating potential. Particularly, ACT1 cells in the center of the clusters became completely negative for Ki-67, while all of ACT1 cells are positive for Ki-67 when they were cultured in monoculture. This confirms that cell competition between NTECs and ACT1 clusters retarded proliferative potential of ACT1 cells consisting the ACT1 clusters.

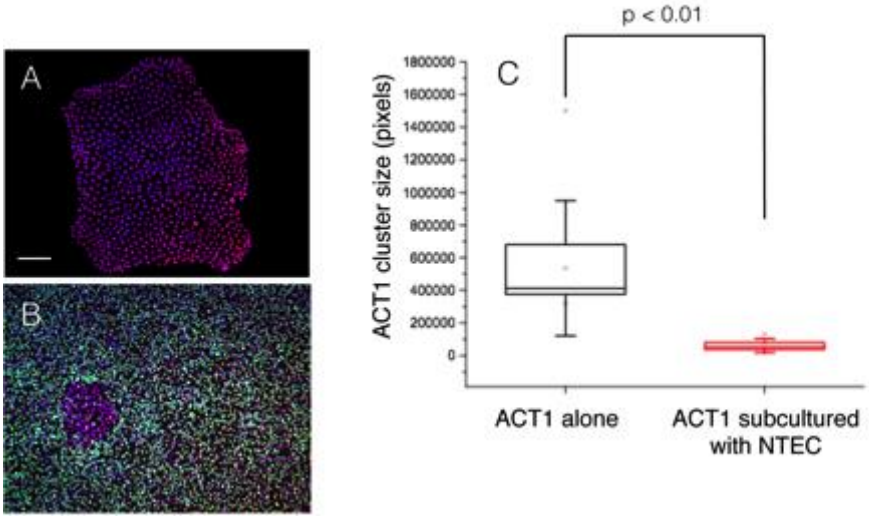

Fig 3. Suppression of ACT1 cell growth by competition with NTECs

ACT1 cells were cultured for 5 days until they formed small cell clusters, and then GFP-NTECs were added and cultured for 3 more days. ACT1 cluster sizes at Day 8 were measured in ATC1 monocultures (A) and in co-cultures (B), and their sizes were compared (C) as described in Materials and Methods. ACT1 clusters and NTECs were fixed and stained with anti-53BP1 antibody (red fluorescence). The bar indicates 100 μm.

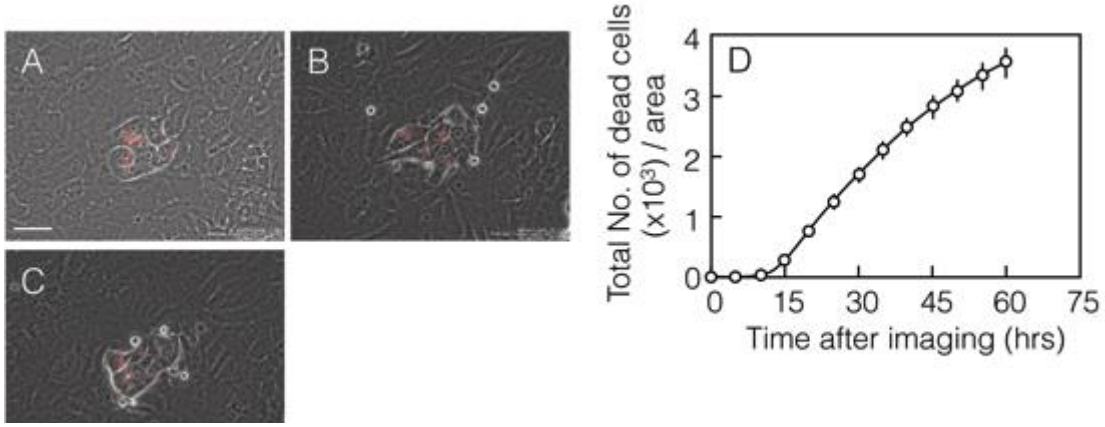

Fig 4. Time-lapse analysis demonstrates NTEC-specific cell death.

ACT1 cells labeled with Qtracker 655 were incubated for 3 days before NTECs were added. Time-lapse imaging was started 48 hours after co-culture (A). Representative images of co-cultures at 18 h (B) and 24 h (C) thereafter. Spatiotemporal occurrence of dead NTECs, which appered as small round-shaped cells within 100 mm distance from the ACT1 clusters, was recorded and summed every 5 hours. Total number of dead cells indicates the summed number of dead cells obtained every 5 hours (D). The bar indicates 40 μm.

In contrast to the growth arrest of ACT1 clusters, the time-lapse analysis also exhibited regional death of NTECs in the areas close to ACT1 clusters (Fig 4). During the first approximately 48 hours after inoculating NTECs, growth of NTECs was more than that of ACT1, so that dividing cells were

observed more in NTECs, and then, morphologically distinct cells, which were small round but not the mitotic cells, were emerging in proximity to ACT1 clusters (Fig 4B and 4C).

Detailed time-lapse analysis revealed that cell blebbing, which is one of the defined features of apoptosis, preceded the cells being round-shaped. It was found that small round-shaped cells were subsequently ruptured. The time-lapse analysis also confirmed that they were derived from Qtracker-negative cells. Small round-shaped cells were also GFP-tagged and positive for vimentin, which is a marker for NTECs, confirming that cell death was induced in NTECs. Temporal analysis of time-lapse images shows that dead cells were appearing continuously over 60 hours (Fig 4D), while none of them was detectable during the first approximately 12 hours. Judging from their morphology, NTECs was likely dead by apoptosis, which was confirmed by live-imaging using cell-permeable fluorescent substrates for Caspase-3 and 7. As shown in Fig 4, NTECs in the areas close to ACT1 clusters are marked by green fluorescence (Fig 5B and 5C), and those cells become round-shaped cells afterwards (Fig 5C and 5D), indicating that Caspase-3/7-mediated apoptosis is involved in NTEC death. Apoptotic cell death is also confirmed by an immunofluorescent analysis using γ-H2AX antibody, which depicts DNA fragmentation.

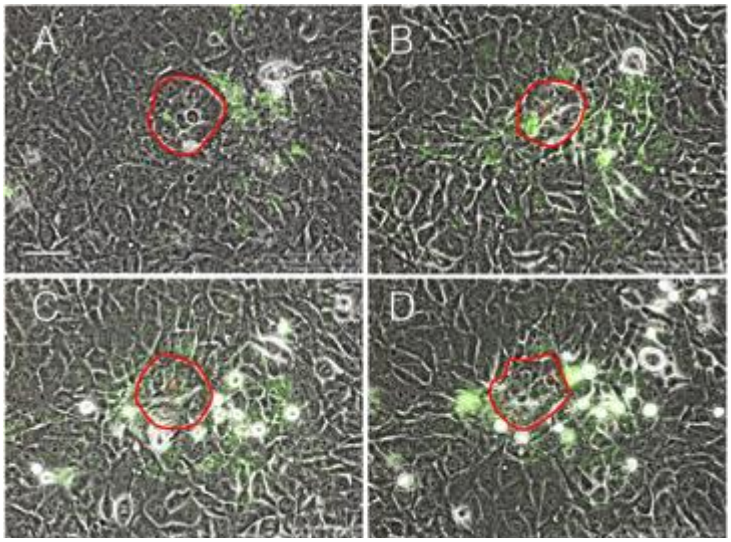

Fig 5. Time-lapse analysis demonstrates NTEC-specific apoptosis.

ACT1 cells labeled with Qtracker 655 (red fluorescence) were incubated for 3 days before NTECs were added. Time-lapse imaging was started 48 hours after co-culture at which time point CellEvent Caspase-3/7 Green detection reagent was added and incubated for 30 min (A). The freeform shape indicated by red lined corresponds to ATC1 cluster borders. Representative images of co-cultures with CellEvent Caspase-3/7 Green detection reagent incubated for further 6 hours (B), 12 hours (C) and 16 hours (D). The bar indicates 50 μm.

To uncover the mechanisms underlying position-specific cell death, I performed an immunofluorescent analysis using antibodies against phosphorylated forms of MAP kinases. Among

ERK1/2, p38 and JNK1/2, only ERK1/2 was found to be phosphorylated in NTECs. whereas augmented phosphorylation is common in ACT1 clusters (Fig 6).

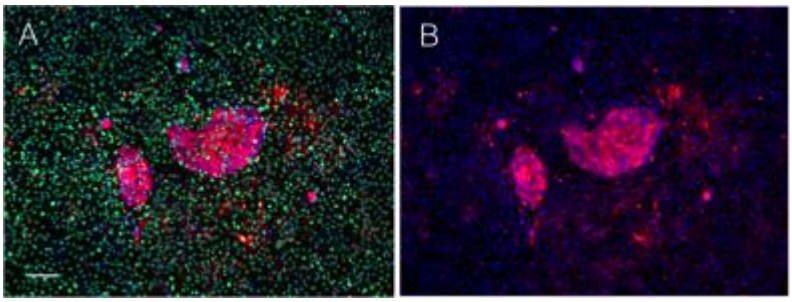

Fig 6. Detection of phosphorylation of ERK1/2.

ACT1 cell clusters were co-cultured with GFP-NTECs for 5 days, fixed with formaldehyde, and stained with anti-phosphorylated ERK1/2 antibody (red) and anti-Ki-67 antibody (green). (A) Merged image. (B) Red fluorescence image showing regional phosphorylation of ERK1/2 in NTECs. The bar indicates 100 μm.

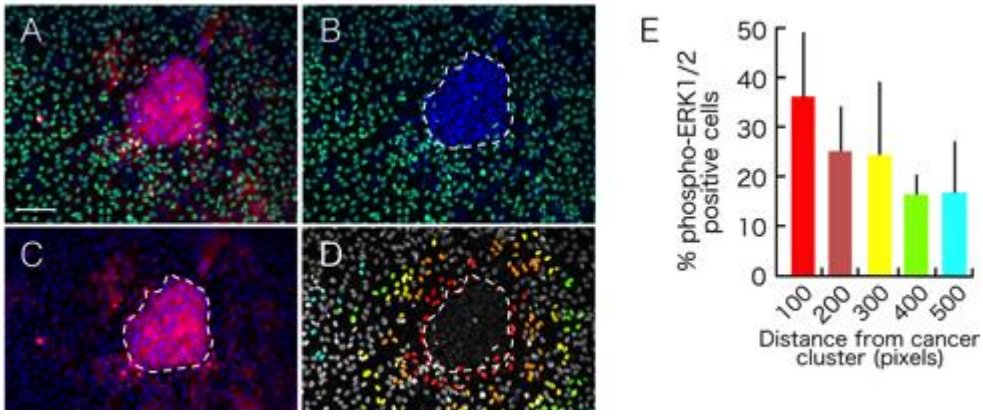

Fig 7. Analysis of region-specific phosphorylation of ERK1/2.

ACT1 cell clusters were co-cultured with GFP-NTECs for 5 days, fixed with formaldehyde, and stained with anti-phosphorylated ERK1/2 antibody. (A) Merged image. (B) Green fluorescence image showing GFP-NTECs. Area designated with a white dashed line indicates ACT1 cell cluster. (C) Red fluorescence image showing phosphorylation of ERK1/2. (D) NTECs with phosphorylated ERK1/2 signals zoned according to their distance from the ACT1 cluster. Red cells are phosphorylated ERK1/2-positive within 100 pixels from the cluster, orange 100 - 200 pixels, yellow 200 - 300 pixels, green 300 - 400 pixels, cyan 400 - 500 pixels. Percentage of NTECs with phosphorylated ERK1/2 is indicated in (E). The bar in (A) indicates 100 μm.

Importantly, NTECs with phosphorylated ERK1/2 are localized to the areas close to ACT clusters, and notably, round-shaped dead NTECs are phosphorylated ERK1/2-positive. As shown in Fig 6, phospho-ERK1/2 positivity was observed more in NTECs closer to the clusters, so that the frequency of phospho-ERK1/2-positive NTECs is determined in the areas every 100 pixels from the ACT1

cluster border (Fig 7). Analysis of distance-dependent ERK1/2 phosphorylation revealed the highest frequency is observed in NTECs closest to the ACT1 clusters (Fig 7E), however, even NTECs within the area more than 500 pixels apart from the ACT1 cluster border, which are equivalent to more than 300 □m apart from the clusters, still show phosphorylation of ERK1/2.

3.2 Molecular pathways involved in cell competition

Since the growth of ACT1 clusters was significantly retarded in co-cultures with NTECs, I further analysed molecular changes in ACT1 cells as well as NTECs using discriminatory cell collection technique. As the substrate attachment of ACT1 cells is much stronger than that of NTECs, I briefly trypsinized co-cultures without PBS wash and collected NTECs. Then, cells were trypsinized again to collect cancer cells remaining in the dish. Purity of the NTEC and ACT1 cell population was checked by using the GFP positivity determined under the fluorescence microscope, and more than 99.9% of the first collected cells and less than 0.5% of the second collected cells were positive for GFP, respectively.

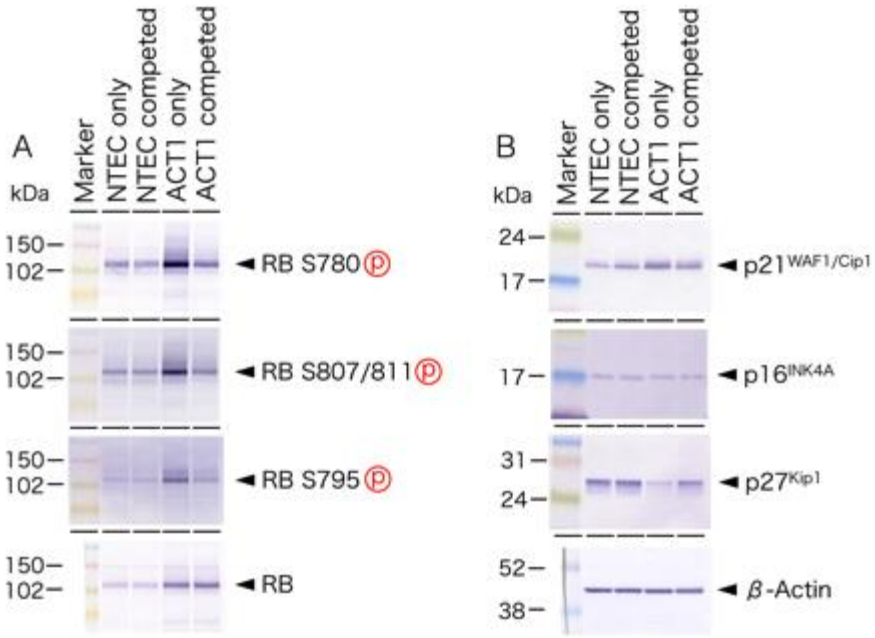

Fig 7. Western blot analysis of RB and CKIs.

Total protein was extracted from both NTECs and ACT1 cells cultured either alone (NTEC or ACT1 only, respectively) or from cell co-cultures (NTEC or ACT1 competed, respectively) and subjected to western blot analysis with indicated antibodies.

Western blot analysis demonstrated that multiple phosphorylation of RB was significantly reduced in ACT1 cells that competed with NTECs, while total RB protein level was not changed (Fig 8A). As RB phosphorylation is targeted by several Cyclin/Cdk kinases, I examined the levels of their

inhibitors. Among $p21^{WAF1/Cip1}$, $p16^{INK4A}$, and $p27^{Kip1}$, the $p27^{Kip1}$ protein, which are expressed in ACT1 cells at lower level as compared with NTECs, was profoundly upregulated in competed ACT1 cells (Fig 8B).

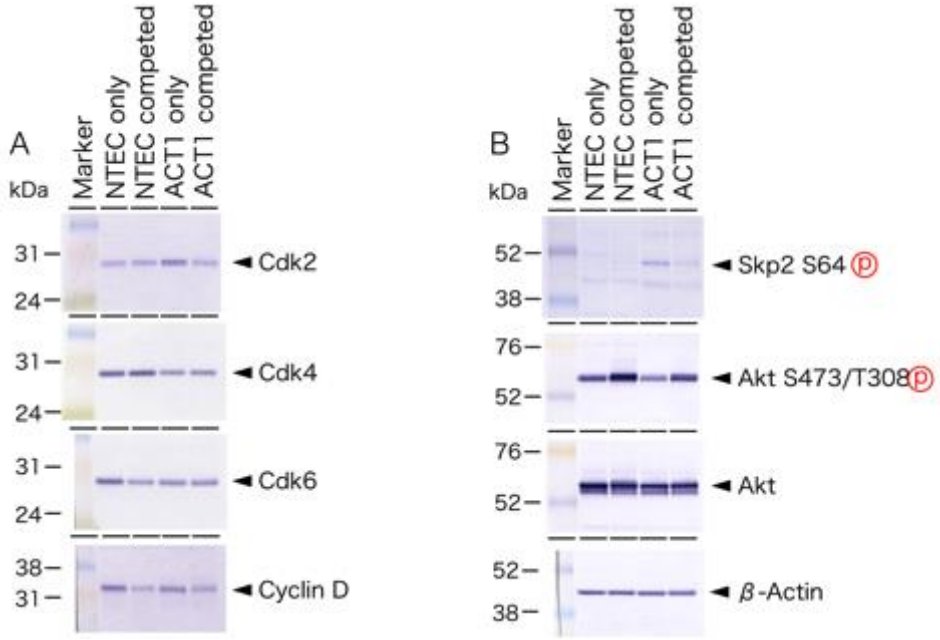

Fig 9. Western blot analysis of cell cycle regulators and Akt.

Total protein was extracted from both NTECs and ACT1 cells cultured either alone (NTEC or ACT1 only, respectively) or from cell co-cultures (NTEC or ACT1 competed, respectively) and subjected to western blot analysis with indicated antibodies.

I also observed the decreased expression of Cdk2 and Cyclin D in competed ACT1 cells (Fig 9A), indicating that upregulation of $p27^{Kip1}$ and downregulation of Cyclin D/Cdk2 could be involved in reduced RB phosphorylation. Competed ACT1 cells also displayed lower level of phosphorylated Skp2 at serine 64 and increased level of phosphorylated forms of Akt (Fig 9B).

In agreement with the immunofluorescence results, phosphorylation of ERK1/2 was higher in ACT1 cells compared with NTECs, however, the level is not changed by cell competition (Fig 10A). As I observed an increased ERK1/2 phosphorylation in NTECs, which were close to ACT1 clusters, I examined phosphorylation of ERK1/2 in NTECs from bulk co-culture. Despite regional phosphorylation was detected on immunofluorescence analysis, I did not detect changes in ERK1/2 phosphorylation under such conditions (Fig 10A). I also observed increased phosphorylation of JNK1/2 in both types of competed cells (Fig 10B).

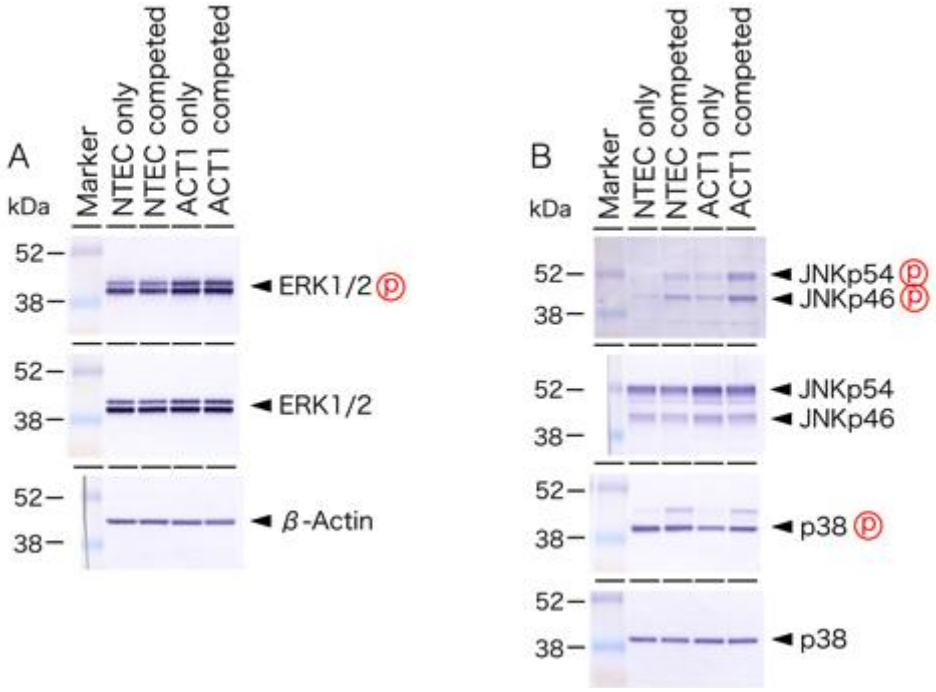

Fig 10. Western blot analysis of MAP-kinase pathway.

Total protein was extracted from both NTECs and ACT1 cells cultured either alone (NTEC or ACT1 only, respectively) or from cell co-cultures (NTEC or ACT1 competed, respectively) and subjected to western blot analysis with indicated antibodies.

3.3 Effect of radiation exposure of NTECs on ACT1 cell growth

To determine whether the proliferation of NTECs is required for cell competition, NTECs were exposed to 10 Gy of γ-rays, which induces senescence-like terminal growth arrest as judged by the expression of senescence associated β-galactosidase activity.

NTECs were exposed to γ-rays and incubated for 3 days before adding to ACT1 clusters. The quantity equivalent to that inducing competition (10^6 cells) were added to ACT1 cluster, and ACT1 cluster sizes were measured 3 days later. It is apparent that the cluster sizes are appeared to be significantly greater than those observed in co-cultures with unexposed NTECs (Fig 11). Immunofluorescent analysis reveals that Ki-67 positivity, which was significantly reduced in competed ACT1 clusters, is remarkably recovered under this condition.

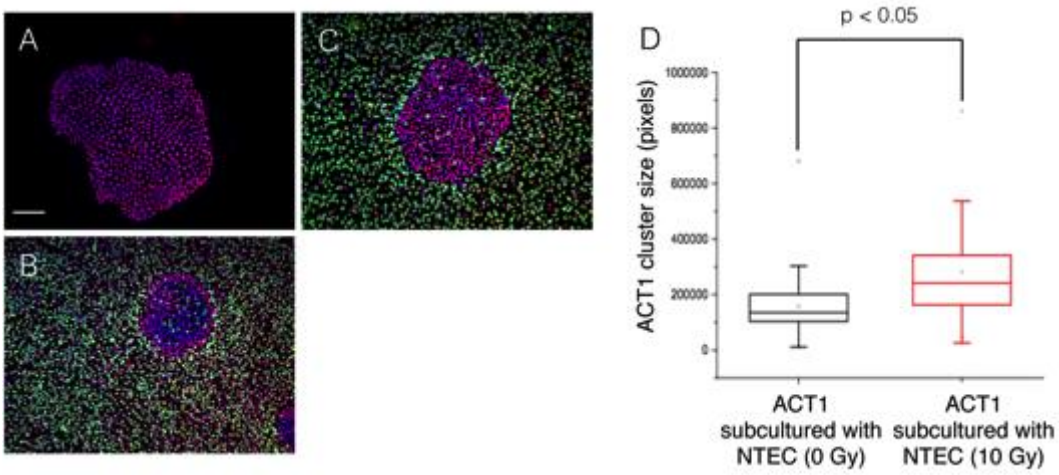

Fig 11. Reduced cell competition between terminally arrested NTECs and ACT1 cell cluster.

ACT1 cells were cultured for 5 days until they formed small cell clusters and then GFP-NTECs irradiated with 10 Gy of γ-rays were added and cultured for 3 more days. ACT1 cluster sizes were measured 3 days later (on Day 8). ACT1 clusters and GFP-NTECs were fixed and stained with anti-53BP1 antibody (red fluorescence). (A) ACT1 cluster in monoculture. (B) ACT1 cluster co-cultured with GFP-NTECs at Day 8. (C) ACT1 cluster with 10 Gy-irradiated GFP-NTECs at Day 8. (D) Comparison of the ACT1 cluster sizes. Statistical difference was evaluated by Mann-Whitney test. The bar indicates 100 μm.

3.4 Radiation exposure and tissue reaction

Tissue reaction is a process, by which tissue/organ injuries are amended. It could also be a fundamental mechanism involved in the manifestation of radiation health effect. Micronuclei are the exo-nuclear DNA, which are created by nondisjunction of un-rejoined chromosome fragments through cell division. It was found that DNA in micronuclei was eliminated through degradation by apoptosis-dependent DNA fragmentation (Fig 12).

Figure 12. Caspase-dependent DNA fragmentation in micronuclei

Exponentially growing normal human diploid cells were exposed to 1 Gy of γ-rays and incubated for further 24 hours before the fixation with methanol. The samples were then incubated with anti-phosphorylated histone H2AX mouse antibody and anti-53BP1 rabbit antibody. The white arrowheads indicate micronuclei. Bar indicates 10 μm.

Apoptotic DNA fragmentation is accompanied by the degradation of nuclear lamina, which results in the release of DNA fragments into the cytoplasm. Such fragmented DNA, particularly in the

cytoplasm, has been proven to execute innate immune response through the activation of the cGAS-STING pathway. Activated cGAS catalyzes the cyclization of ATP and GTP, resulting in the formation of cGAMP. cGAMP then activates STING, which transactivates interferon (IFN) regulatory factor 3 (IRF3) and NF-□B, thereby stimulating transcription and secretion of the type I IFNs and the inflammatory cytokines (Fig 13), which is followed by the secretion of several factors, such as IL-1β, IL-6, IL-8 and TGF-β. These are the same as those secreted from senescent cells, which were involved in senescence-associated phenotypes. Thus, the cGAS-STING pathway is a critical pathway, mediating senescence induction after radiation exposure. It is worthy to note that senescence by itself is also an inducer for cytokines, such as IL−1β, IL-6, and therefore, it further amplifies the effect of cGAS-STING activation, and thereby, tumor-suppressive microenvironment could be enabled.

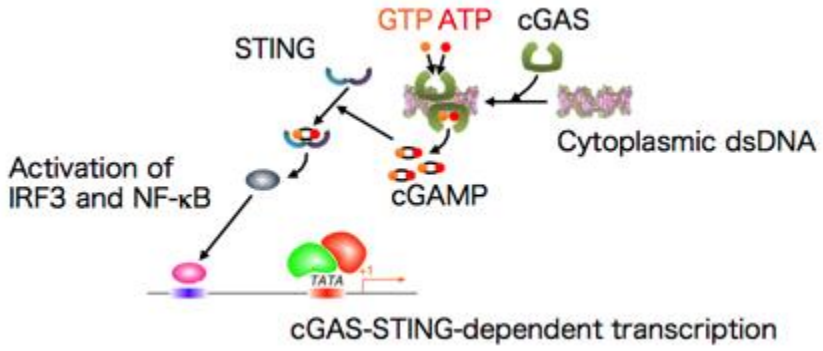

Figure 13. cGAS-STING signaling pathway

Cytoplasmic double-stranded DNA activates the cGAS-STING signaling pathway. cGAS is able to recognize cytoplasmic double-stranded DNA through its unique structure, thereby turning on the enzymatic activity catalyzing the cyclization of AMP and GMP, which results in the formation of cGAMP. cGAMP then binds to STING and activates it as an adaptor, which modulates transcription through IRF3 and NF-□B. Thus, the cGAS-STING signaling pathway plays a critical role in senescence induction after radiation exposure.

Discussion

This study for the first time outlined the crucial role and possible mechanisms of the cell competition between anaplastic thyroid cancer cells and normal thyroid follicular epithelial cells. Comparison of ACT1 cluster size clearly indicated that the growth of ACT1 cells was significantly suppressed by NTECs (Fig 3). In fact, Ki-67 positivity was significantly reduced in ACT1 clusters, confirming that cell competition was able to suppress cancer cell growth. Western blot analysis reveals that RB phosphorylation was significantly repressed accompanied by the up-regulation of $p27^{Kip1}$ (Figs 8 and 9). Since $p27^{Kip1}$ level is regulated through ubiquitin-dependent proteasome activity, I examined phosphorylation of Skp2, an E3 ligase catalyzing $p27^{Kip1}$ ubiquitination [80], and found that it was reduced under competed condition (Fig 9B). I also observed up-regulation of Akt phosphorylation which facilitates Akt-dependent phosphorylation of Cdk2 and sequestering of the latter in the cytoplasm [81,82]. These changes together with down-regulation of Cdk2 and Cyclin D could be involved in the retarded growth of competed ACT1 cells.

Besides ACT1 cell growth suppression, cell competition also affected the fate of NTECs manifested by the elimination of cells with contacting ACT1 clusters or located nearby to those. NTECs elimination was taking place through apoptosis accompanied by the region-specific activation of ERK1/2 (Fig 7), although the overall phosphorylation levels of ERK1/2 showed no change in bulk cultures (Fig 10). While activation of ERK1/2 is generally involved in cell proliferation, ERK activity has been also involved the induction of apoptosis [83]. Considering that no apoptosis in NTECs during the first days before the time-lapse analysis was started, this indicates that physical cell contact is required to induce NTECs elimination. Previously, it was demonstrated that mechanical stress through cell-to-cell contact was involved in cell competition [84,85]. In fact, NTECs neighboring ACT1 cluster show increased cell anisotropy, indicating that an increasing the number of NTECs competed with ACT1 clusters potentiates compression of NTECs at the border. This could cause apoptotic cell death in NTECs.

So far, the molecular nature of cell competition has been discussed extensively in Drosophila [36-40], and several driving factors have been identified including c-myc and p53 [14,15,41,42]. In fact, I confirmed augmented expression of c-myc in ACT1 cells, however, it did not seem to make ACTs cells supercompetitor, since the growth of ACT1 is much slower than NTECs. Also, differential expression of Scrib does not appear to be involved as there was no difference between NTECs and ACT1. The p53 gene is mutated in ACT1 cells, so that the p53 protein level is significantly stabilized. Together with the mutation in the *N-ras* gene p53 deregulation could confer the 'winner' phenotype [55], but ACT1 growth was indeed suppressed by the cell competition with NTECs, suggesting that driver mutations may play a different role in different cell context.

Recently, the suppressive effect of cell competition on liver cancer, which was mediated by the YAP induction in peritumoral hepatocytes, was reported [86]. The YAP as well as TAZ transcriptional coactivators are the downstream effectors of the Hippo signaling pathway, which plays

critical roles in cell-to-cell contact, cell polarity, and fitness to the neighbors [87-89]. While several previous literatures have presented that the YAP1 is overexpressed in tumors including thyroid cancers [90,91], the study clearly demonstrated that the YAP limitedly induced in normal hepatocytes neighboring tumor cells played suppressive role. In fact, the active YAP1 level was augmented in ACT1 cells, which was significantly down-regulated in co-culture (Fig 14). Since high-density culture did not alter the YAP expression in ACT1 cells, cell competition with NTECs might affect the Hippo signaling pathway. Similar observation was reported by other study, confirming that bidirectional cell competition was indeed involved in cancer growth *in vivo* [92]. Thus, the down-regulation of YAP, which might be controlled by the Hippo signaling pathway activated through cell competition with NTECs, should be additional pathway that suppress ACT1 cell growth. The exact mechanism of the Hippo signal activation, and an involvement of Akt activation in this pathway need further investigations to confirm.

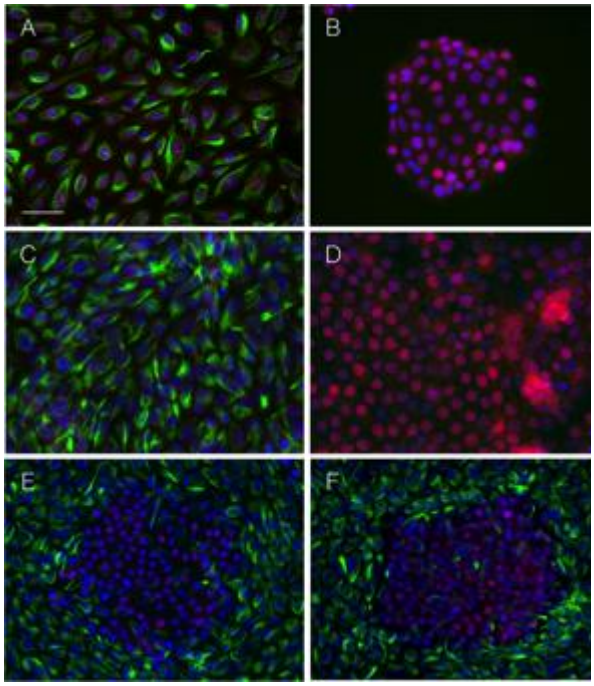

Fig 14. Down-regulation of the YAP expression through cell competition.

ACT1 cells were cultured for 5 days until they formed small clusters before adding NTECs. Then, ACT1 clusters and NTECs were co-cultured for further 3 days, fixed with formaldehyde and stained with anti-vimentin antibody (the secondary antibody is labelled with Alexa647, so that the green pseudo color was applied) and anti-active YAP1 antibody (red fluorescence). Exponentially growing NTECs (A), ACT1 cluster (B), confluent NTECs (C), and confluent ACT1 cells (D) show vimentin (green) and active YAP1 (red) expression. ACT1 cluster co-cultured with NTECs show decreased active YAP1 expression. The bar in (A) indicates 40 μm.

Previously, several reports demonstrated that cell growth rate and fitness *per se* may not drive cell competition as these are not an absolute common quality and does not determine the outcome of competition [93-96].The findings indicated that ATC1 cells have sensitivity to compaction and crowding, and the AKT activation could be a critical event to initiate the downstream cascade lead to RB dephosphorylation (Fig 12). The exact pathway of AKT activation still needs to be determined.

According to the results obtained by live-cell imaging (Fig 4), cell competition was initiated when the numbers of NTECs reached sufficient for cell compression. In order to confirm that continuous cell growth of NTECs is essential for cell competition, NTECs with sufficient cell number but terminated cell growth were examined (Fig 11). As I expected, reduction of ACT1 cluster sizes was significantly negated. I also found no apoptotic cell death in NTECs. Furthermore, it is demonstrated that overall ERK1/2 phosphorylation is up-regulated upon irradiation. While locally activated ERK1/2 seemed to be essential for apoptotic cell death of NTECs, the result suggested that not homogeneous activation but regional and accidental activation of ERK1/2 could be involved in region-specific apoptosis induction. Previously, it was reported that downregulation of ERK was involved in *Drosophila* [97], the results represented that the effect could be cell context-dependent.

It should be emphasized that even if the NTECs close to the ACT1 clusters were forced to die by apoptosis, the presence of NTECs surrounding cancer cells established suppressive cell competition to anaplastic cancer cells. Considering that thyroid follicles are the spheroidal structures formed by a monolayer of follicular cells, it is possible that similar cell competition could be taking place *in vivo*. Recently, accumulating evidences have demonstrated that cell competition is an essential component of tissue microenvironment, which is important not only during tissue development and aging but also in preventing cancer development and invasion [1-8]. Cellular heterogeneity of a tissue plays a key role since cancer cells may have different cell stiffness and different proliferation rates than normal cells. Although many of oncogenic mutations have been though to confer supercompetitor phenotype to cancer cells, normal counterparts exert intrinsic tumor-suppressive effects through cell competition activity, which was also reported by others and termed epithelial defence against cancer (EDAC) [98]. While anaplastic cancer is an aggressive form of thyroid cancer, the results indicated that cell competition therapy could be an option. Of importance, since the study shows that termination of growth of NTECs by radiation exposure abrogates suppressive competition properties of NTECs, it should be important to reduce toxicity of radiotherapy and chemotherapy to normal epithelial cells in order to preserve maximum EDAC.

Recently, an unexpected role of cytoplasmic DSBs enables deciphering mechanisms of radiation effects through exo-nuclear signaling. Micronuclei are the exo-nuclear DNA and created by nondisjunction of un-rejoined chromosome fragments through cell division. DNA in micronuclei was shown to be eliminated through degradation by apoptosis-dependent DNA fragmentation (Fig 12) [99]. Apoptotic DNA fragmentation is accompanied by the degradation of nuclear lamina, which results in the release of DNA fragments into the cytoplasm. Such fragmented DNA, particularly in the

cytoplasm, has been proven to execute innate immune response through the activation of the cGAS-STING pathway [100-103]. cGAS is originally described as a sensor of microbial DNA, and it becomes clear that it is also able to recognize endogenous double-stranded DNA. The cGAS protein has a positively charged surface as well as a zinc ribbon, through which the fragmented DNA duplex is interacted (Fig 13). Activated cGAS catalyzes the cyclization of ATP and GTP, resulting in the formation of cGAMP. cGAMP then activates STING, which transactivates interferon (IFN) regulatory factor 3 (IRF3) and NF-□B, thereby stimulating transcription and secretion of the type I IFNs and the inflammatory cytokines. Subsequently, it was reported that many of the secretory factors, such as IL-1β, IL-6, IL-8 and TGF-β, were the same as those secreted from senescent cells, which were involved in senescence-associated phenotypes [104,105]. Furthermore, since micronuclei are commonly induced by ionizing radiation, cGAS has been shown to be involved in radiation-induced premature senescence [106]. Thus, the cGAS-STING pathway is also a critical pathway, mediating senescence induction after radiation exposure. It is interesting to note that senescence by itself is also an inducer for cytokines, such as IL-1β, IL-6, and therefore, it further amplifies the effect of cGAS-STING activation.

Obviously, ATM-p53 axis-dependent DNA damage response as well as cGAS-STING-dependent innate immunity execute unscheduled senescence [107], predominantly among epithelial tissues exposed to ionizing radiation, causing secretion of soluble factors that mediate non-targeted effects and perpetuate senescence phenotype. This autocrine/paracrine feedback loop promotes modification of tissue microenvironment, which is highly likely to accelerate propagation of pre-cancer cells with spontaneous oncogenic driver mutations. Although the involvement of tissue reaction in late radiation health effects still needs further verification, it should improve our knowledge on the biological mechanisms underlying the dose-response relationship of late radiation health effects, especially dose-dependency of cancer risks from low-dose/low-dose-rate radiation exposure. Since senescent cells have been demonstrated to be eliminated by low-molecular weight chemicals, it might provide a clue to mitigate cancer risk from radiation exposure. Finally, future advances in radiation biology are expected to keep providing novel findings that should shed lights on the better understandings of the mechanisms underlying the radiation-induced carcinogenesis.

In conclusion, the thesis study established *in vitro* cell competition system and demonstrated that the growth of anaplastic thyroid cancer cells was significantly retarded through competition with normal thyroid follicular epithelial cells. Cell competition evoked stress response in cancer cells, which resulted in down-regulation of RB phosphorylation. Reciprocally, it induced stress response in normal cells, which gave rise to position-dependent induction of apoptosis. These results prove that cell competition is obviously a bidirectional phenomenon, in which competed cells are both affected each other. Since ERK1/2 and Akt, well-known pathways involved not only in anaplastic thyroid cancer but also in many other types of tumors, are critical components of cell competition, further

studies on identifying target molecules that govern the struggle between cancer and normal cells could provide opportunities for conditioning the situation in favor of normal cells. Also, since senescent cells have been demonstrated to be eliminated by low-molecular weight chemicals, it might provide a clue to mitigate cancer risk from radiation exposure. Finally, future advances in related radiation biology are expected to keep providing novel findings that should shed lights on the better understandings of the mechanisms underlying the radiation health effects as well as radiation-induced carcinogenesis.

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