Cell competition between thyroid cancer and normal follicular cells
（甲状腺癌と正常甲状腺濾胞細胞との細胞競合）

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［目的］

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. In particular, normal counterparts are likely to interact with the initiated cells in a dynamic fashion over time. Such interactions may lead to a balance to maintain homeostasis at the cellular level through suppression of aberrant cell expansion by promoting cell death or to cell propagation if the balance is impaired. Therefore, gaining understandings of the mechanisms how normal cells and the initiated cells interact at a cellular level should provide deeper insights into the complexity of cancer development *in vivo*.

Cell competition, originally identified more than 40 years ago in developing *Drosophila melanogaster* tissues, plays a critical role of a quality control in the growing embryo by eradicating undesirable cells during development. Since then cell competition has been confirmed in a variety of physiological processes, including embryogenesis, morphogenesis, and aging [10 –15]. Furthermore, it is now generally believed that cell competition is a general feature of tissues and organs for eliminating mutated cells showing growth advantage.

After the TEPCO Fukushima Daiichi nuclear power plant accident, thyroid cancers have 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 also been shown that such thyroid cancers are mostly latent, so that any medical interventions might not be necessary. Since it was suggested that there should be a mechanism to limit the propagation of very small thyroid cancers to microcarcinomas. If normal cells can suppress tumour growth through cell competition mechanism, and eventually eliminate them, then microcarcinoma remains small with less propagation, and they remain in the tissues as subclinical microcarcinomas thoughout the life, which should be proven scientifically.

In my thesis study, I have simply hypothesized that the cell to cell competition could be the mechanism to regulate propagation of initiated cell, so that the question must be what if the growth of initiated cells are suppressed by neighboring normal cells. The second aim of my study is to pursue the 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. Radiation exposure has been suggested to change cell-to-cell and cell-to-tissue interactions through the induction of senescence-like dead cells, which secret soluble factors affecting the tissue’s microenvironments. This should be determined with respect to identifying a role of radiation in cancer development. I set out *in vivo* mimetic unique culture system, in which tiny clusters of anaplastic thyroid cancer cell line, ACT1, were co-cultured with normal thyroid follicular epithelial cells (NTECs). Since cell competition is a dynamic process, a live-cell imaging technique together with the usage of single cell visualization was used.

［結果］

ACT1 cells plated at a low density grow clonally and form densely packed cell clusters by Day 5, and then, NTECs were added to the culture. By five days after incubation, NTECs fully occupied the space between the ACT1 clusters, and the growth of ACT1 clusters seemed to be suppressed, as we

observed significant change in the sizes of the clusters among NTECs compared with ATC1 clusters in monoculture, demonstrating that cell competition between neighbouring NTECs and ACT1 clusters retarded ACT1 cluster growth (**Figure 1**).

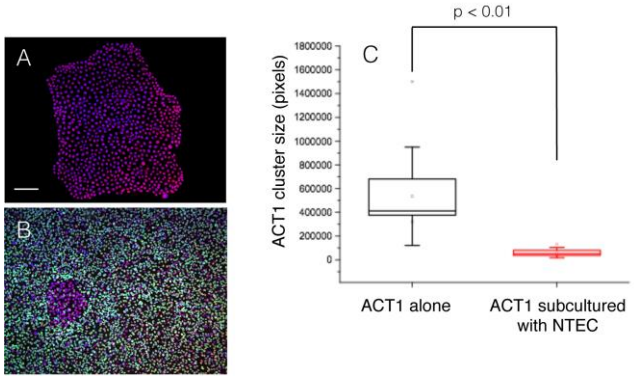

Figure 1. Suppression of ACT1 cell growth by competition with NTECs

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.

In contrast to the growth arrest of ACT1 clusters, the analysis also exhibited regional death of NTECs in the areas close to ACT1 clusters. The time-lapse analysis revealed that NETCs were dead by apoptosis. 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. 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.

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. 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. As RB phosphorylation is targeted by several Cyclin/Cdk kinases, we 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. We also observed the decreased expression of Cdk2 and Cyclin D in competed ACT1 cells, 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.

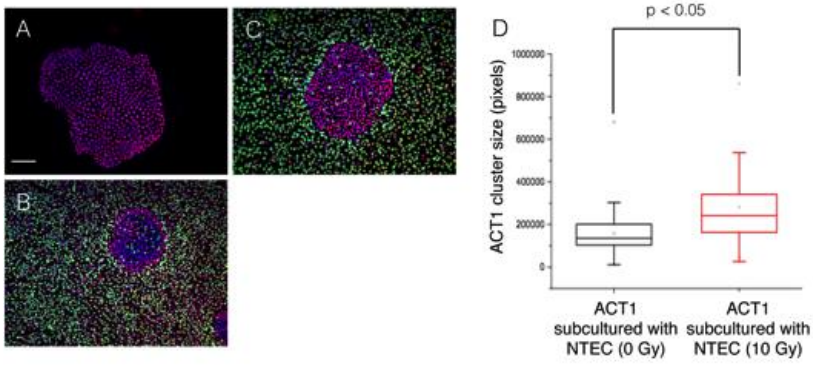

Figure 2. Reduced cell competition between terminally arrested NTECs and ACT1 cell cluster.
ACT1 cells were cultured for 5 days until they formed small cell clusters and then GFP-NTECs irradiated with 10 Gy of γ-rays were added and cultured for 3 more days. ACT1 cluster sizes were measured 3 days later (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.

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 cluster sizes are appeared to be significantly greater than those observed in co-cultures with unexposed NTECs (**Figure 2**), indicating retardation of tumor suppressive effects of NTECs.

［考察］

This thesis study clearly outlined the crucial role and possible mechanisms of the cell competition between anaplastic thyroid cancer cells and normal thyroid follicular epithelial cells. Western blot analysis reveals that RB phosphorylation was significantly repressed accompanied by the up-regulation of $p27^{Kip1}$. Up-regulation of Akt phosphorylation, which facilitates Akt-dependent phosphorylation of Cdk2, 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 occurred through apoptosis induction accompanied by the region-specific activation of ERK1/2. It was demonstrated that mechanical stress through cell-to-cell contact was involved in cell competition. 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. Recently, the suppressive effect of cell competition on liver cancer, which was mediated by the YAP induction in peritumoral hepatocytes, was reported. 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. In fact, the active YAP1 level was augmented in ACT1 cells, whereas it was significantly down-regulated in co-culture. Since high-density culture by itself did not alter the YAP expression in ACT1 cells, cell competition with NTECs might affect the Hippo signaling pathway.

While anaplastic cancer is an aggressive form of thyroid cancer, the results indicated that cell competition therapy could be an option. Of importance, since our study showed that termination of growth of NTECs by radiation exposure abrogates suppressive competition properties of NTECs, it should be critical to reduce toxicity of radiotherapy and chemotherapy to normal epithelial cells in order to preserve maximum suppressive effects of NTECs.

In conclusion, the current study developed an *in vitro* cell competition system, which mimicked in vivo situation, and demonstrated that the growth of anaplastic thyroid cancer cells was significantly retarded by 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. 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.

［基礎となった学術論文］

1. Amrenova A, Suzuki K, Saenko V, Yamashita S, Mitsutake N. Cell competition between anaplastic thyroid cancer and normal thyroid follicular cells exerts reciprocal stress response defining tumor suppressive effects of normal epithelial tissue. *PLoS ONE*, **16**, e0249059, 2021.
2. Suzuki K, Amrenova A, Mitsutake N. Recent advances in radiobiology with respect to pleiotropic aspects of tissue reaction. *J. Radiat. Res.*, 62, i30-i35, 2021.