# Global COE Assistant Professors’ ORAL presentation

13:00 ～

## Effect of Cereblon (CRBN) on vertebrate development

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Ando, Hideki

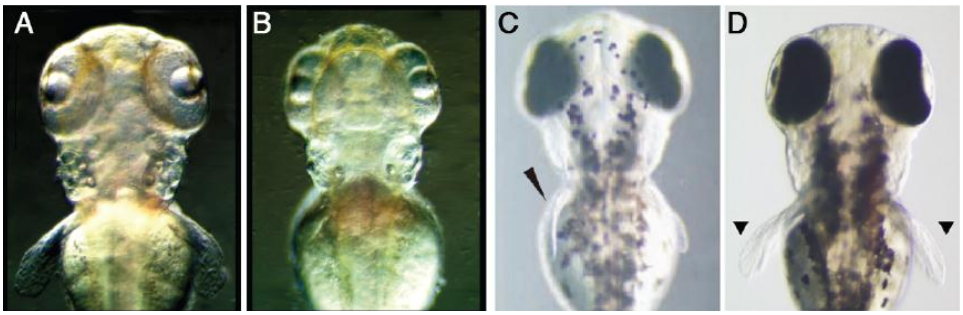
(GCOE Assistant Professor)

Nearly 50 years after thalidomide's teratogenic action to cause limb malformations was firstly reported, we have identified a protein cereblon (CRBN) that is involved in the drug's adverse side-effects (*Science*, 2010, *327*, 1345).
CRBN was identified as a thalidomide-binding protein from cell lysates by using thalidomide-coated microscopic beads. Biochemical analysis appeared CRBN forms an E3 ubiquitin ligase complex with Cullin4a (Cul4a) and Ddb1. When thalidomide binds to CRBN, enzymatic activity of CRBN-containing E3 complex was suppressed *in vitro*.

In order to study the biological function of CRBN, we cloned zebrafish CRBN gene. Thalidomide also binds to zCRBN. Knockdown of CRBN function caused stunted growth in pectoral fins and otic vesicles in zebrafish, those were similar as seen in thalidomide-treated fish and Cul4a-kockdown fish. Fgf8 expressions in the Apical Ectodermal Ridge (AER) were severely diminished in both thalidomide-treated fish and CRBN-knockdown fish.

To confirm the possibility that those defects were induced by loss-of-function of CRBN by functional knockdown and by binding of thalidomide, we overexpressed mutant CRBN protein that cannot bind to thalidomide in zebrafish and chicken. The engineered animals showed much lower sensitivities to thalidomide in development than those seen in untreated animals exposed to the drug, suggesting that CRBN is a primary target of thalidomide teratogenicity (Figure).

This study firstly presents a molecular basis for thalidomide teratogenicity and may provide important information for the development of new thalidomide derivatives without teratogenic activity.




(Figure) Teratogenic effect of thalidomide in pectoral fin development. 3 Days fishes incubated without thalidomide (A), with 200 μM of thalidomide (B), CRBN-knockdown fish without thalidomide (C) and mutant (thalidomide-unbound) CRBN-overexpressed fish with 400 μM of thalidomide (D).

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# Regulation of cell proliferation during ascidian embryogenesis

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(GCOE Assistant Professor)

Kobayashi, Kenji

【Background & Aim】

Urochordate ascidians are marine invertebrate chordates that have a common ancestor shared by the cephalochordate and vertebrates. The organization of ascidian tadpole larvae shows basic features of the chordate body plan. Ascidian tadpole larvae are composed of a remarkably small number of cells (approximately 2,600 cells in Ciona) and tissue types. In addition, the cell division patterns are invariant between individuals, allowing the identification of each cell. During embryogenesis, each individual cell of embryos exits the cell cycle at specific developmental times before their terminal differentiation. Moreover, the compact unduplicated Ciona genome has been sequenced and annotated. Taking advantage of these features, our project focuses on the regulation of cell proliferation during ascidian embryogenesis.

【Results】

(1) Cs-CKI-b is required for the arrest of notochord and muscle cell proliferation

The larvae of Ciona savignyi contain the definite number of notochord and muscle cells, 40 and 36, respectively. During embryogenesis, both of notochord precursors and muscle precursors exits their cell cycle at the early neurula stage. We showed that these cell cycle exits occur at G1 phase by BrdU incorporation experiments. Moreover, we found that there are two Cip/Kip family CDK Inhibitor (CKI) genes and there is no Ink4 family CKI gene in the Ciona genome. Whole-mount in situ hybridization analyses revealed that one of the Cip/Kip family CKI genes, namely Cs-CKI-b, is zygotically expressed in both of notochord precursors and muscle precursors from the gastrula stage onward. Functional knockdown experiments using specific antisense morpholino oligonucleotides showed that Cs-CKI-b is required for the arrest of notochord and muscle cell proliferation. However, functional knockdown of Cs-CKI-b did not affect the notochord and muscle differentiation.

These results suggest that the zygotic expression of Cs-CKI-b in notochord and muscle precursors is responsible for their cell cycle exit.

(2) Cs-CKI-b is required for the arrest of epidermis cell proliferation

Cs-CKI-b is zygotically expressed also in all of epidermis precursors transiently between the late neurula stage and the initial tailbud stage, just before epidermis precursors exit the cell cycle. Approximately 750 monolayer epidermis cells surround the entire surface of the larva. Functional knockdown experiments showed that Cs-CKI-b is required for the proper number of cell divisions of epidermis cells. However, functional knockdown of Cs-CKI-b did not affect the epidermis differentiation. These results suggest that the transient expression of Cs-CKI-b in epidermis precursors is required for the proper timing of cell cycle exit.

(3) Cs-Myc is involved in the continuing proliferation of mesenchyme and endoderm

Although most cells constituting ascidian larva exit cell cycle by the end of tailbud stage, larval mesenchyme and endoderm cells, which give rise to mesodermal and endodermal tissues of adults, respectively, continue their cell division during embryogenesis, larval stage, and metamorphosis. We found that sole C. savignyi ortholog of bHLH-LZ transcription factor Myc, namely Cs-Myc, is zygotically expressed in both of mesenchyme precursors and endoderm precursors from the gastrula stage onward. Functional knockdown experiments showed that Cs-Myc is involved in the continuing proliferation of mesenchyme and endoderm cells of ascidian larva. In contrast, functional knockdown of Cs-Myc did not affect the mesenchyme and endoderm speification.

【Conclusion】

In this study, we identified two specifically expressed and functioned cell cycle regulators, Cs-CKI-b and Cs-Myc. Cs-CKI-b is required for the cell cycle exit of notochord, muscle, and epidermis. Cs-Myc is involved in the continuing proliferation of mesenchyme and endoderm of ascidian tadpole larva.

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# Synthesis of modified ribonucleoside derivatives for useful construction of RNA aptamers

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(GCOE Assistant Professor)

Tsunoda, Hirosuke

Recently molecules binding specifically to target molecules have drawn attention as the sensitive biosensors and efficient therapeutic drugs without side effects. Aptamers are short DNA or RNA folded molecules that can be selected in vitro on the basis of their high affinity for the target molecules. Aptamers are similar to the antibodies in many aspects and also have superior properties, which are low immunogenicity, simple mechanism and brief synthesis process, than the antibodies. Therefore, aptamers can became good tools for developing biosensors and therapeutic drugs instead of such antibodies. On the other hand, there is a weak point that aptamers are decomposed by nucleases in living bodies. To improve such a weak point of aptamers, modified nucleosides have been needed. For example, the above-mentioned weak point is improved by introduction of the modified nucleic acids have resistance against nuclease degradation into aptamer. Moreover, modified nucleosides are able to affect the binding affinity of the aptamers for target molecules and conformation stability. Here are two modification plans (1) to obtain the resistance against nuclease degradation and (2) to enhance the binding affinity of aptamers.

(1) Design aptamers directed toward the resistance against nuclease degradation

It is well known that 2'-O-modified ribonucleoside derivatives have the resistance against nuclease degradation. Herein, we had reported the synthesis of uridine derivatives having cyanoethyl, carbamonyl, and aminobutylcarbamoyl groups at the 2'-hydroxy group site. Therefore, we decided to use these 2'-O-modifications in this study.

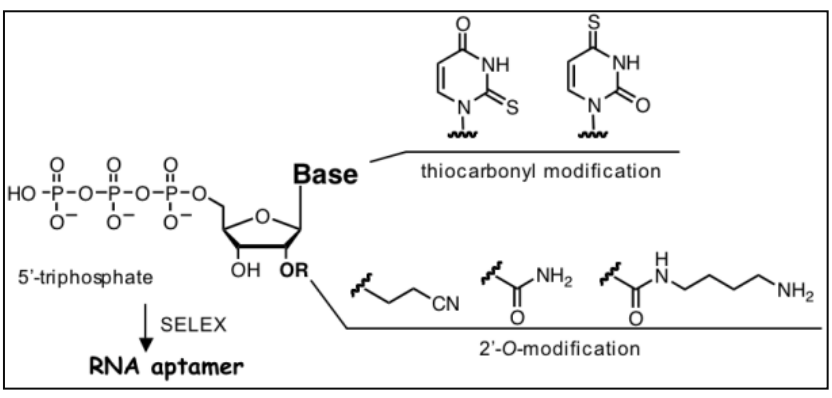
(2) Design aptamers directed toward stability of conformation

To stabilize the aptamer conformation is a good approach to enhance its binding affinity. It is known that the thiocarbonyl group introducted into nucleobase has the stabilizing effect of conformation of oligonucleotides due to interaction with 3'-downstream bases. In this study, we chose thiocarbonylated uridine derivatives as the modification to stabilize the aptamer conformation.

The purpose of this study is the synthesis of these modified ribonucleoside 5'-triphosphate derivatives and evaluation of substrate recognition of their derivatives in RNA polymerase reactions for SELEX system that is to prepare RNA aptamers.

First, we synthesized the 5'-triphosphate derivatives to be used in SELEX system. The synthesis of 5'-triphosphate derivatives was achieved by the reaction of the corresponding 5'-OH derivative and a phosphorouschloride reagent followed by the addition of tributylammonium pyrophosphate. Next, we carried out the evaluation of substrate recognition of the 5'-triphosphate derivatives in T7 RNA polymerase reactions. When 2'-O-cyanoethyl modified derivatives were used in their enzyme reactions, full-length transcription products were observed although the transcription efficiency was lower than that of the reaction using natural 5'-triphosphates. Whereas, the reaction using thiocarbonylated derivatives efficiently gave the full-length products.

It is concluded from these results that 2'-O-cyanoethyl- and thiocarbonyl-modified derivatives can be incorporated into aptamers in the SELEX system.



14:00 ～

# Commonality of speciation by sensory drive revealed by its signatures in Lake Victoria cichlid fishes

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(GCOE Assistant Professor)

Terai, Yohey

Biodiversity has been created through numerous times of speciation events. It is believed that the common mechanism underlying such speciation events may exist, but common mechanism of speciation has not been reported except geographical isolation and polyploidyzation of chromosomes. Recently, we showed clear-cut examples of the mechanism of speciation by sensory drive (adaptation in sensory for signaling systems to different environments cause premating isolation) by showing adaptations of opsins (protein components of visual pigments) to different light environments and divergence in male breeding colorations using rock cichlid fish species in Lake Victoria. Here, we tested the hypothesis of common mechanism of speciation by detecting signatures of sensory drive using ten cichlid species in Lake Victoria from various light environments. We collected species from surface to the deepest bottom, and analyzed light environments, the sequence and function of opsins, and male breeding colorations. The environmental light components were different in various depth and turbidity. The analysis of amino acid sequences of eight opsin genes showed that long-wavelength sensitive opsin (LWS) and rod-opsin (RH1) gene were diversified and specific to species. Reconstitution and measurements of absorption spectra for LWS and RH1 pigments indicated that LWS and RH1 were functionally diversified. The efficiency for absorption of environmental light by opsin pigments showed high light-absorbing efficiencies in their living light environments, suggesting that cichlid species adapted their LWS and RH1 to ambient light environments. The analysis of breeding coloration with environmental light absorbed by LWS pigments showed that the adaptive visual pigments can effectively absorb the light reflected by their breeding colorations.

These results were well match with the process of speciation by sensory drive, suggesting that speciation by sensory drive may be one of the common mechanism of speciation during the adaptive radiation in Lake Victoria cichlids.

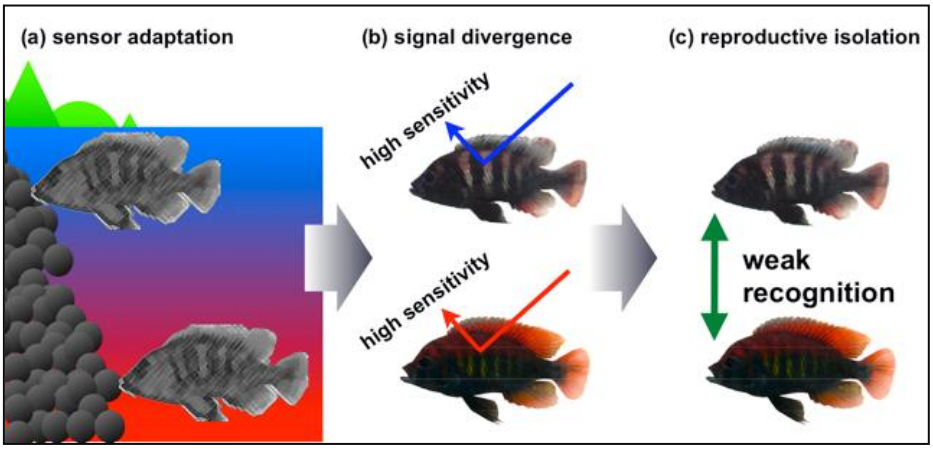


Figure legend. The process of sensory drive speciation
(a) The adaptive divergence of sensors, (b) mating signal divergence, and (c) reproductive isolation by weak recognition between populations

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# The maintenance system of colitogenic memory CD4+ T cells as the target of radical therapy for Inflammatory Bowel Disease

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(GCOE Assistant Professor)

Nemoto, Yasuhiro

**【Background & Aim】**

Inflammatory bowel disease (IBD) is the chronic and persistent disease with the lifelong recurrences. According to the present understanding, this disease occurs as a result of hyper activation of immune system to commensal bacterial antigens with genetic background. We have noticed colitogenic memory $CD4^+$ T cells as the important factor for the perpetuation of IBD. We aimed for the development of a radical therapy to reset "pathogenic memory" of IBD, and analyze the maintenance system of the pathogenic memory $CD4^+$ T cells in chronic colitis.

**【Methods】**

We have conducted a series of *in vivo* experiments using $CD4^+CD45RB^{high}$ T cell transfer IBD mice model with some conditions modified.

**【Results】**

(1) Substantial number of $CD4^+CD44^+CD62L^-IL\text{-}7R\alpha^{high}$ memory T cells proliferated in the colitic SCID mice previously transferred with $CD4^+CD45RB^{high}$ T cells. These cells produced effector cytokine with the stimulation of commensal bacterial antigens. Since re-transfer of these cells to another SCID mice induced colitis, we have defined these cells as colitogenic memory $CD4^+$ T cells.

(2) Although germ free SCID mice transferred with colitogenic memory $CD4^+$ T cells didn't develop colitis, substantial number of memory $CD4^+$ T cells survived in these mice. Furthermore, these mice developed severe colitis when they are moved to SPF condition, which suggest that commensal bacterial antigens are not essential for the maintenance of colitogenic memory $CD4^+$ T cells

(3) Homeostatic cytokine IL-7 is known as an important maintenance factor for $CD4^+$ T cells. We next hypothesized that IL-7 is essential for the maintenance of colitogenic memory $CD4^+$ T cells. IL-$7^{-/-}$ x RAG-$1^{-/-}$ mice transferred with colitogenic memory $CD4^+$ T cells did not develop colitis, and only few memory $CD4^+$ T cells survived in these mice.

(4) IL-7Rα expression on lamina propria (LP) $CD4^+$ T cells from colitic RAG-$2^{-/-}$ mice was significantly higher than that on normal LP $CD4^+$ T cells, whereas that on other colitic LP immune cells, such as NK cells, macrophages, and myeloid dendritic cells was conversely lower than that of paired LP cells in normal mice. RAG-$2^{-/-}$ mice transferred with IL-$7R\alpha^-CD4^+CD25^-$ effector T cells or $CD4^+CD44^-CD62L^+$ naïve T cells didn't develop colitis, while IL-$7R\alpha^{-/-}$ x RAG-$2^{-/-}$ mice transferred with $CD4^+CD25^-$ T cells developed colitis to a similar extent to RAG-$2^{-/-}$ mice transferred with $CD4^+CD25^-$T cells. Together, the present results suggest that IL-7Rα expression on colitogenic $CD4^+$ T cells, but not on other cells, is essential for development of chronic colitis.

(5) On the other hand, we have found that IL-7 level of colitic intestine is less than that of normal one as a result of disappearance of goblet cells. Actually, intestinal IL-7 is not essential for the perpetuation of colitis, because IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice parabiosed with IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice injected with colitic $CD4^+CD45RB^{high}$ T cells develop colitis. Therefore, we hypothesize that colitogenic memory $CD4^+$ T cells are maintained outside the intestine as memory stem cells.

(6) However, it has been believed that intestinal LP $CD4^+$ T cells are gut-tropic and terminally differentiated cells that do not move out of the intestinal LP but undergo apoptosis there. In contrast to this dogma, we recently demonstrated using intrarectal administration (enema) of $CD4^+$ T cells that colitogenic memory $CD4^+$ T cells constantly recirculate from LP to blood.

(7) We next tested the necessity of lymph nodes and spleen, which have been regarded as most important reservoirs so far. So we used LTα$^{-/-}$ mouse, which has no lymph node and payer's patches. We performed that adoptive transfer of colitogenic memory $CD4^+$ T cells into Splenectomized LTα$^{-/-}$ x RAG-$2^{-/-}$ mice or control RAG-$2^{-/-}$ mice. Splenectomized LTα$^{-/-}$ x RAG-$2^{-/-}$ mice developed colitis as same extent as the control mice, which suggest that these organs are not essential reservoir for the colitogenic memory $CD4^+$ T cells.

(8) Finally, we have noticed the bone marrow (BM), the main source of IL-7. $CD4^+CD44^+CD62L^-IL\text{-}7R\alpha^{high}$ memory $CD4^+$ T cells were retained in BM of colitic SCID mice. These BM $CD4^+$ T cells were mostly attached to IL-7-producing BM stromal cells. RAG-$1^{-/-}$ mice transferred with BM $CD4^+$ from colitic RAG-$2^{-/-}$ mice didn't develop colitis, while IL-$7^{+/+}$ x RAG-$1^{-/-}$ mice transferred with colitic BM $CD4^+$ T cells did develop severe colitis. To confirm a role of IL-7-producing BM-derived cells for the perpetuation of IBD, we tried to develop the mice in which IL-7 expression is specific in BM. IL-$7^{-/-}$ x RAG-$1^{-/-}$ mice were transplanted with BM cells of RAG-$1^{-/-}$ (IL-$7^{+/+}$→IL-$7^{-/-}$) or IL-$7^{-/-}$, RAG-$1^{-/-}$ mice (IL-$7^{-/-}$→IL-$7^{-/-}$). IL-7 mRNA and protein were detected in BM, but not in colon, of IL-$7^{+/+}$→IL-$7^{-/-}$ mice. IL-$7^{+/+}$→IL-$7^{-/-}$, but not IL-$7^{-/-}$→IL-$7^{-/-}$ recipients, transferred with $CD4^+CD45RB^{high}$ T cells 4 weeks after transplantation, developed colitis. To identify IL-7-producing cells in BM, we isolated BM mesenchymal stem cells (MSC). MSC isolated from BM of RAG1$^{-/-}$ mice (IL-$7^{+/+}$ MSC), but not from BM of IL-$7^{-/-}$ x RAG1$^{-/-}$ mice (IL-$7^{-/-}$ MSC), expressed higher levels of IL-7 mRNA and protein than those of freshly isolated IL-$7^{+/+}$ BM cells. Co-culture with IL-$7^{+/+}$ MSC, but not IL-$7^{-/-}$ MSC, induced extensive proliferation of CFSE-labeled colitogenic $CD4^+$ T cells *in vitro*. $CD4^+CD45RB^{high}$ T cell-transferred IL-$7^{-/-}$ x RAG1$^{-/-}$ mice previously transplanted with IL-$7^{+/+}$ MSC, but not IL-$7^{-/-}$ MSC, developed colitis.

**【Conclusion】**

Long-lived colitogenic memory $CD4^+$ T cells can be maintained in the absence of commesal bacteria, and participate in the perpetuation of colitis. IL-7-producing BM MSC is the important factor for the maintenance of colitogenic memory $CD4^+$ T cells.

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# Non-stress Homogeneous Culture System for ES/iPS Cells on E-cadherin Based Nano-material

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Hexig, Bayar

Embryonic stem cells (ES cell) and induced pluripotent stem cells (iPS) which have characteristics such as self-renewal and pluripotency, are considered to hold great promise in regenerative medicine and drug design for pharmacological evaluation systems. In the past few years, many types of iPS cell clones have been reprogrammed from different somatic cell sources, by using different transcription factors combination, different selection methods and different carrier system. Recently, the possibility of patient-derived iPS cells for defining the pathogenic mechanism also has been reported. Furthermore, many studies have been reported on differentiation of iPS cells into somatic cells such as hepatic linage, neural cells, cardiomyocyte, and so on. However, most of the studies reported that proliferation of undifferentiated state and induced differentiation of somatic cells from ES and iPS cells have been based on cell-cell aggregated colony culture system. In which, stimulating factors fail to interact with all cells homogeneously and directly in the same time, and leading to generate heterogeneous cell population system. To overcome these problems, we applied a single cell level culture system which established on E-cadherin chimeric (E-cad-Fc) protein, for controlling ES and iPS cells fate. Adsorption behaviors of recombinant E-cad-Fc fusion protein on the polystyrene(PS) surface were studied by quartz-crystal microbalance(QCM) method. The binding interactions of Fc domain of E-cad-Fc on PS surface were investigated by computational method. On E-cad-Fc coated culture system, ES cells and iPS cells showed scattering morphology which offers homogenous microenvironment for all cells and efficient differentiation for hepatocyte-like cells. Furthermore, cultured all cells can be harvested by without enzyme treatment in our culture system. In conclusion, we established a uniform and non-stress single cell level culture system for large scale proliferation of ES and iPS cells. Utilizing this single cell level culture system, comparative investigation of two different iPS cell colons and one ES cell line in undifferentiated state, and the differentiation induction to hepatic lineage was also performed.

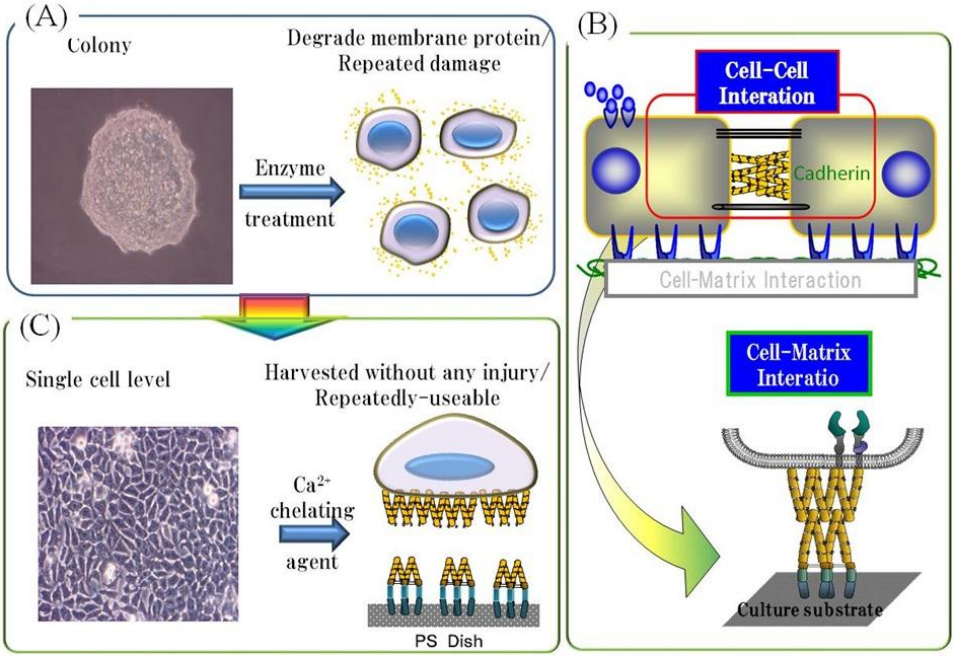


Figure
(A). Cell-Cell aggregated colony culture system.
(B).E-cad-Fc model protein for cell-cell adhesion molecule E-cadherin
(C). Single cell level ES/iPS cells culture system.