Chapter 8

Personal closing remarks on the occasion of retirement: The real course and history of our researches prior to the 4C theory, personal interpretation on life phenomena, and experiences and opinions as a life scientist

by Tatsuo Nakayama

SUMMARY

We advanced the chromatin conformation change code (4C) theory for the bio-system to gain new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications via a lot of generations. The 4C theory had been studied under worst research environments, i.e., without any research grants, in our spare time from main research projects and that by only a womanpower. The 4C theory is the typical fruit of my serendipity and the outcome of some 10 years of my small research group. This article describes real course/history of our researches prior to the 4C theory, my personal interpretation of concept on life phenomena, and experiences and opinions as a life scientist.

In eukaryotes, alterations in chromatin structure are remarkably involved in regulation of gene expression, and replication, repair and recombination of DNA and so on [1-5]. Furthermore, in higher eukaryotes, such alterations participate decisively in development and differentiation of cells, e.g., lymphocytes [6-12], and then determine specificity of diverse cell types. Among several epigenetic modifications of chromatin structure, chemical modification of Lys (K) residues of core histones H2A, H2B, H3 and H4 with acetyl group should be major one [13-26]. Of various chromatin-modifying enzymes participating in alterations of chromatin structure, histone acetyltransferase(s) (HATs) and deacetylase(s) (HDACs) cooperatively control acetylation and deacetylation levels of particular Lys residues of core histones [27-37]. However, my research group had mainly studied on structures (nucleotide sequences, transcriptional elements and/or genome organizations) of core histones and linker histone H1 of chickens until ~20 years ago (~1995) [38-47]. Prior to these researches, during more past ~25 years (started from the master course of graduate school), I had mainly studied on determination of amino acid sequences of protein (cytochrome c of bonito), in vitro and in vivo protein (ovalbumin) acetylation and synthesis in hen's oviduct, RNA synthesis by RNA polymerase of Bacillus subtilis during sporulation, 2D-PAGE analysis of proteins, and structure and function of DNA binding proteins and so on [48-59]. Figuratively speaking, if my whole researches are likened to a game, those must be pre-run of the hop, step and jump.

In the midst of the above-mentioned studies on structures of histone genes, the DT40 cell line, which was derived from chicken pre-B lymphocyte cells [60], was reported to possess an ability of homologous recombination with a very high frequency and to be a powerful system for gene targeting techniques [61, 62]. An approach using the gene targeting techniques in DT40 cells was very advantageous for clarifying not only specific and/or limited functions in the B lymphocyte cell lineage but also fundamental and/or common phenomena in higher eukaryotic cells. I was confident that even my small group could continue any of researches using the ALV-transformed chicken DT40 cell line, although both

of the start and continuance of those using model animals (such as mice and rats) should be very hard for us due to insufficient grants and manpower. In addition, I was fully convinced that peculiar knowledge on basic bio-systems obtained in DT40 cells could be universally expanded for understanding of fundamental and complex bio-systems in higher eukaryotes. Because almost all manners of the most essential life phenomena, including central dogma, which had been elucidated in phage, prokaryote (especially E. coli) and/or yeast systems, could be thoroughly applicable to higher eukaryotes.

Based on the above-mentioned mature consideration, in parallel with studies on in vitro functions of histone chaperones (CAF-1 and HIRA) [63-68], I had immediately changed my main research projects to elucidation of in vivo functions (and structures) of several chromatin-modifying enzymes (HATs and HDACs) and transcription factors as well as histones and histone chaperones (CAF-1, HIRA, ASF1 and others), using the gene targeting techniques in the DT40 cell line. Of course, I had no hesitation in using the DT40 cell line as an experimental system for my research projects at all, because I had become accustomed to the chicken system as mentioned above. First, to assess individual roles of histones and histone chaperones, we had comprehensively generated numerous homozygous DT40 mutant cell lines, lacking the corresponding gene of each of their particular members. The results obtained from resultant mutants, combined, showed that this approach was really useful to know individual functions of these particular genes in vivo [69-79].

In parallel and/or continuously, based on these positive results and my intrinsic interest on universal/global role of histone acetylation, to assess individual roles of HATs and HDACs, we had generated a number of homozygous DT40 mutant cell lines each were devoid of particular member of HATs and HDACs, such as GCN5, PCAF, HAT1, HDAC1, HDAC2, HDAC3 and others [27, 76, 80-95]. Our qualitative analyses of the HDAC2-deficient mutant cell line, HDAC2(-/-), which was initially generated by us [27], revealed not only that HDAC2 regulates the amount of IgM H-chain at the steps of transcription of its gene plus alternative processing of its pre-mRNA [27], but also that it down-regulates IgM L-chain gene promoter activity [81]. The lacking of HDAC2 also represses gene expressions of HDAC7, Pax5, Aiolos, Ikaros and EBF1, elevates gene expressions of HDAC4, HDAC9, PCAF and E2A, and changes bulk acetylation levels of several Lys residues of core histones [85]. Further, we generated Pax5(-), Aiolos(-/-), Ikaros(-/-/+), EBF1(-/-), E2A(-/-), Helios(-/-) and other mutants [85, 96, Chap. 3], and clarified particular functions of these factors in B lymphocytes [85, 93, 96-99]. In consequence, these results, together with others [100], revealed that Pax5, EBF1, Aiolos and Ikaros down-regulate gene expressions of IgM H- and L-chains, and inversely E2A and probably OBF1 up-regulate these two immunoglobulin gene expressions. Therefore, we concluded that HDAC2 regulates indirectly gene expressions of IgM H- and L-chains through opposite transcription regulations of Pax5, EBF1, Aiolos, Ikaros, E2A and also OBF1 genes [85, 88].

However, I had never met my own exciting research that takes me captive and makes my heart beat

fast with joy until then. In addition, I had not been throbbing heavily with almost all of so-called brilliant and/or fashionable achievements in life science field until then, with a few exceptions. I owed such feelings chiefly to the fact that I have been fascinated by the word "life" from boyhood; therefore, I had a great interest in fundamental concept of life phenomena but no interest in any of individual vital substances, reactions and/or functions, even though they were very important. Of course, still now countless important questions remain to be resolved in the life science field.

In the midst of re-constitution (or re-expression) experiments studying detailed participation of HDAC2 in transcription regulations of IgM H- and L-chain genes, we became aware of the surprising phenomena as follows. We first introduced the tetracycline- (tet-) repressive HDAC2 cDNA into HDAC2(-/-) mutants, and isolated transformant cell lines, HDAC2(-/-)tetHDAC2(+), lacking two alleles of the endogenous HDAC2 gene but possessing the artificial tet-repressive HDAC2 cDNA. Contrary to our expectation, surprisingly, IgM H- and L-chains and their mRNAs were very low levels in HDAC2(-/-)tetHDAC2(+) even in the case of non-existence of HDAC2 due to the presence of tet, as well as in the case of existence of HDAC2 due to the absence of the drug and in wild-type DT40 cells having the enzyme (our unpublished data).

Concerning these findings, members of my research group each had different interpretations. One of co-workers intensely asserted not only that the accumulation of IgM H- and L-chains in HDAC2(-/-) was not truly based on the HDAC2-deficiency and resulted from only unknown artificial cause of beneath our notice, but also that the accumulated immunoglobulin proteins were only reduced spontaneously during cultivation for construction of HDAC2(-/-)tetHDAC2(+). Since the co-worker was bigoted toward a vague biased (but ordinary in a sense) view that several properties of higher eukaryotic cells are easily changeable during cultivation. Similarly, another co-worker conservatively insisted that the accumulation and diminution of IgM H- and L-chains in HDAC2(-/-) were not directly due to the HDAC2-lacking but based on only somatic mutations occurred within the genome locus of the IgM H-chain gene during cultivation for construction of the transformants, since the locus was generally considered as a hot spot for the mutations [62]. Thus, they were not interested in these findings.

However, intuitively, I believed that the accumulation of IgM H- and L-chains in HDAC2(-/-) was really caused by the HDAC2-deficiency itself, and their diminution during cultivation for the construction of HDAC2(-/-)tetHDAC2(+) should be attributable to unknown important biological mechanisms. Such my own opinion was supported in part by our following findings. First, several re-experiments (2D-PAGE, Western blotting and/or Northern blotting) showed that protein and mRNA levels of IgM H-chain (and also L-chain) were firstly increased in several independent HDAC2(-/-) mutant clones and thereafter reproducibly reduced, whenever they were cultivated for a long period (our unpublished data). Second, by contrast, insignificant changes (increases and/or decreases) in protein and/or mRNA levels of IgM H- and L-chains were observed in numerous DT40 mutant cell lines (approximately 30), each of

which was devoid of a member of HDACs (except HDAC2), HATs, transcription factors, histones and histone chaperones, etc. [70-75, 85, our unpublished data].

Meanwhile, in the process of the above-mentioned consideration and re-examinations, I noticed some following mistaken speculations due to qualitative results in our previous paper [27]. Re-experiments showed that protein and mRNA levels of HDAC2 in the heterozygous mutants HDAC2(-/+) were decreased to ~60% of those in DT40 cells (our unpublished data), whereas these two levels had been reported to be kept constant (~90%) by means of the compensation mechanism [69-75]. RNase protection assay (and Northern blotting) re-examined showed that total amounts of IgM H-chain protein and ratio of secreted to membrane-bound forms of its mRNA were increased in HDAC2(-/+), compared with those in DT40 cells (our unpublished data), though both of the amounts and ratio had been reported to remain unchanged. Growth rate of initially generated HDAC2(-/-) mutant clones re-tested (our unpublished data), as well as that of most clones of newly generated HDAC2(-/-) mutants [Chap. 4], was slightly different from that of DT40 cells, though growth rate of the initial mutants had been reported to be almost similar to that of DT40 cells. Pulse-labeling/chase experiment and Northern blotting re-examined showed that protein and mRNA of IgM L-chain really existed as two forms (large and small) and their amounts were increased associated with the HDAC2-deficiency (our unpublished data), though the small form of IgM L-chain had been considered to be a specific proteolytic product of the large form at the C-terminal region.

Based on my consideration and enthusiasm as mentioned above, I asked again some members of my research group to study mechanisms of the accumulation and diminution of protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) as a part of their own research works. However, several kinds of approaches gave only following negative (unpublished) data. RT-PCR performed on various transcription factors (including promoter and intron enhancer binding proteins) for the IgM H-chain gene showed only insignificant (or no) changing patterns in their mRNA levels in DT40 cells and HDAC2(-/-) mutants at any cultivation stages. MNase protection assay carried out on several regions within chromatin surrounding the IgM H-chain gene showed insignificant changing patterns in nucleosome ladders in DT40 cells and HDAC2(-/-) mutants at any cultivation stages. In addition, MNase protection assay performed on several genes encoding transcription factors (including Pax5, Aiolos, Ikaros and EBF1) also showed insignificant changing patterns in nucleosome ladders for these genes in both of DT40 and HDAC2(-/-) mutant cells at any cultivation stages.

On the other hand, interestingly, Northern blotting using probes specific for the IgM H-chain gene sometimes showed a mere change in amounts of small positive-like RNA molecules having a possibility of alternative splicing product of IgM H-chain mRNA, between DT40 cells and HDAC2(-/-) mutants at the early and late cultivation stages (our unpublished data). Since small RNA molecules (such as small nuclear RNA, micro RNA, small interference RNA, anti-sense RNA, etc.) were watched as important

players in cell functions at that time, we temporarily focused our attention on these molecules. Unfortunately, these findings were not reproducible; therefore, I decided that in our case the small RNA molecules were not worth studying ever since.

For all that, I believed that these negative results should be caused only by the following facts: 1) IgM H- and L-chain genes were indirect but not direct targets of HDAC2 [85]. 2) MNase protection assay was regrettably carried out only on coding regions but not on 5'-upstream regions of these transcription factor genes. 3) HDAC2(-/-) mutants were really collected at vague early cultivation stage. That is, although HDAC2(-/-) mutants were regarded to be collected at the early stage, those must be actually collected at fairly late/varied cultivation stages, because the mutant cells had been already cultivated for a long time to use for other experiments until then. Therefore, I resolved to analyze again properties of the initially generated HDAC2(-/-) mutants collected at different cultivation periods as correctly as possible. Further, in particular, I should like to emphasize that we newly generated HDAC2(-/-) mutants, collected them at different cultivation periods from virtual early (as quickly as possible) until late stages, and systematically and minutely analyzed their various cellular properties to solve the above problems.

Then, I thought that a full-time researcher, but not a part-time one, must be absorbed in the project to clarify mechanisms for the accumulation and diminution of amounts of proteins and mRNAs of IgM H- and L-chains in HDAC2(-/-) mutants. I asked Dr. Masami Nakayama, my wife, to work with me as a co-worker. She has worked on this project and others full-time, in spite of a part-time contract. Further, another PhD joined in my group as assistant professor for collaboration on the project. This plan, however, came to a deadlock in a short time, because contrary to my expectation the new co-worker also did not contribute towards the achievement of this project at all. Based on the deliberations and results obtained through the above-mentioned processes, we had started and continued the research, which made my heart beat first with joy, even under following worst research environments. Without any research grants, in our spare time from main research projects (supported by some small grants) and that by only a womanpower (of M. N.), we had undertaken the dreamiest study on chromatin conformation change code (4C) theory for gain of new cell functions through irreversible creation of chromatin structure plasticity with epigenetic modifications [Chaps. 2 ~ 7]. At the start of this research, I had firmly resolved that my small research group (of T. N. and M. N.) made some contribution (even if sole and small concept) to recent rapid development of life science field, which had already become a big science like space science, experimental elementary particle physics, geophysics and so on. On the other hand, it was also true that we had to continue principally our ordinary projects (most of which were supported by some small grants) [64-68, 75-78, 80-94, 96-100] and collaborations (with other groups) [79, 95, 101-120], similarly to previous our own plus collaborative researches [27, 38-59, 63, 69-74, 121-144] and some unlisted ones, since minimum research conditions were essential to execute the research on the 4C theory. Throughout

whole research periods, of course, we had referred to numerous papers. Only about forty selected randomly from them were listed as references [1-26, 28-37, 60-62] in this article, although many of these referred papers were cited in our papers [27, 38-59, 63-78, 80-94, 96-100]. If I had not come across this biological phenomenon, besides my failure in formation of excellent research group, my life as a scientist must be ended in imperfect combustion. Anyhow, we had started the exciting research on the 4C theory with sickening anxiety, quiet fighting spirit and a wish that it will be a forerunner in future biology (life science) beyond Biochemistry, Molecular Biology and Cell Biology, all of which have mostly targeted bio-systems already acquired during evolution since the birth of living things, under following mottos:

Exploring only one, pursuing only it, by only one, and Creator of concept, rather than only one, rather than number one

The 4C theory, which is the fruit of my small serendipity and also the outcome of some 10 years of intellectual investigation spirit, intuition plus insight (of T. N.) and enthusiasm, outstanding technique plus diligent work (of M. N.) (Figs. 8-1 ~ 8-14 selected properly from Chaps. 2 ~ 7), is a creative concept on ability of living things and the only visible and biggest evidence for me as a scientist. Outline of the 4C theory is concretely as follows. 1) Somatic cells (and even tumor cells) of higher eukaryotes possess ability to cope with and/or overcome intra- and/or extra-cellular environment change, when they firstly encounter with it. 2) Somatic cells, for gain of new cell function, exhibit pluri-potency, elasticity and flexibility, all of which are originated from those of chromatin structure. 3) New cell function to adapt for and/or eliminate environment change is acquired through irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream chromatin region (named notch and also director) of each of numerous particular genes (principally of chromatin-modifying enzymes, transcription factors and related enzymes/factors). 4) Diversity in chromatin structure plasticity in individuals of the same cell type is triggered by spontaneous unbalanced response to environment change at the time of their first encounter with it and accomplished by successive convergence of response via a lot of generations. 5) Irreversible creation of chromatin structure plasticity occurs inevitably but not incidentally and/or neutrally. 6) Chromatin structure plasticity (from tight to loose forms or vice versa) of proximal 5'-upstream region is created through successive chromatin conformation change by epigenetic modifications with acetyl group etc. via numerous cell divisions. 7) Putative environment change recognition receptor/site (ECRR/ECRS) recognizes environment change and putative chromatin conformation change complex (4C) machinery creates chromatin structure plasticity. 8) Variety in irreversible creation of chromatin structure plasticity fairly depends on antecedents of somatic cells and successive response to environment change, and occurs in descendent cells via a lot of generations but not in the initial cell encountering with it. 9) Chromatin structure of proximal 5'-upstream region(s) of particular gene(s), as just dynamic

three-dimensional conformation, receives signal on environment change. 10) Chromatin structure (loose or tight form) of proximal 5'-upstream region of particular gene directs switch (on or off) for its latent transcription ability. 11) The number of codes in the 4C theory, which may determine complicated/diverse functions and types of higher eukaryotic cells, can be roughly estimated based on combination of the number of candidate genes and the number (probably two, i.e., loose and tight forms of chromatin structure) of codes for each of these genes.

In any case, if we have been able to study this biological subject in favorable research surroundings, some of questions (which were represented in [Chap. 6]) concerning the 4C theory on the exclusion of artificially accumulated IgM H- and L-chains in HDAC2(-/-) mutants during cultivation must have been resolved. Concerning the previously mentioned questions, here, I pick out some momentous ones as follows. 1) Why acetylation levels of particular Lys residues of histone H3 within chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes are reduced at the early stage of cultivation in HDAC2(-/-) mutants, in spite of the deficiency of HDAC2 activity. 2) Why the decreased acetylation levels of the particular Lys residues within proximal 5'-upstream chromatin regions of these genes are increased in HDAC2(-/-) mutants during cultivation. Why the case of the OBF1 gene is opposite. 3) Steric and functional differences between loose and tight forms of chromatin structure surrounding proximal 5'-upstream regions of these genes must be clarified in detail. 4) How putative chromatin conformation change complex (4C) machinery (participating in irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream regions of particular genes) differs from the well-known chromatin modifying machinery. 5) To demonstrate the 4C theory, both of putative environment change recognition receptor/site (ECRR/ECRS) as a first player for recognition of the environment change and putative 4C machinery as a final player for creation of chromatin structure plasticity must be clarified. Anyhow, I earnestly crave that somebody takes over and elucidates the 4C theory including the above-mentioned questions in near future.

In connection with the 4C theory, here, I wish to express briefly my personal interpretation of conception about life phenomena as follows. How higher eukaryotic cells cope with and/or overcome unexpected and disadvantageous environment change, when they firstly encounter it. Probably, there are following four possible countermeasures. 1) In the case of most severe change, cells should die because it is far ahead of their adaptation ability. 2) In the case of considerable severe change, cells should cope with or overcome it by means of alterations in genome information, such as point mutation, insertion, deletion, duplication and multiplication on the DNA molecule via a lot of generations. This mode should be a basis for evolution of species. 3) In the case of moderate change, cells should cope with or overcome it through irreversible creation of chromatin structure plasticity caused by successive

chromatin conformation changes with epigenetic modifications via a lot of generations. This mode should be a basis for differentiation of cells. 4) In the case of minor change, cells should respond to it using only the already acquired regulation mechanism. The exclusion of IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) mutant cells (and also Pax5(-) mutant cells) during cultivation seems to be really the third one [see Chaps. 2 ~ 7].

In addition, I have a following harmless insight about development and differentiation of higher eukaryotes, which should be certainly related to irreversible creation of chromatin structure plasticity. For instance, when ovum and sperm are fertilized, though all components of chromatin (or chromosome) (except DNA) existed in the former one should be generally considered to be evenly and symmetrically divided into two chromatin sets, in fact, asymmetrical allotment of chromatin components must spontaneously become in a few unbalance, i.e., two daughter cells after the first cell division of fertilized ovum must already have quantitative and qualitative differences slightly in constituent elements of their chromatin (chromosome). Successively, the unbalanced distribution of chromatin components should be repeated and converged to create varied chromatin structure plasticity surrounding proximal 5'-upstream regions of different sets of numerous particular genes in individual cells via a lot of cell divisions. Furthermore, such spontaneous and asymmetrical allotment of chromatin components at first cell division (followed by its successive convergence) should be universally occurred in all fertilized ova. Consequently, complexity and diversity of chromatin structure plasticity cause those of cell specificity, i.e., distinct functions of chromatin, nucleus and/or cell, leading to irreversible creation of variety of specific cell (or tissue) types in higher eukaryotes.

Well, genome information, which is inherited to descendant generations via cell divisions (and of course, fertilization or pollination of generative cells), is generally preserved in nucleotide sequences of DNA and translated in amino acid sequences of protein (or polypeptide) through transcription in nucleotide sequences of RNA. In eukaryotes, genome information and nuclear function should be mainly protected by nuclear membrane which acts as cytoplasm-nucleus barrier; naturally, there are two other typical and important barrier systems for conservation (protection) of life, e.g., blood-brain barrier for conservation of individuals in higher animals and insemination (sperm-ovum fertilization or stamen-pistil pollination) barrier for conservation of species in higher animals or plants. Moreover, both of communication and signal transduction between nucleus and cytoplasm, which are essential for normal expressions of cell functions, should be preferentially performed by the going and returning of large and/or small molecules via nuclear pore acting as a guard station at the barrier. By contrast, both of transport of useless molecules and signal transduction on unexpected (and/or unfavorable) change in intra- and/or extra-cellular environment to nucleus must be usually shielded by this barrier system.

Finally, I wished to digress from the subject on researches and to change largely it for my personal

experiences/opinions as follows. I organized "The 9th MBSJ Spring Symposium in Miyazaki" held on 2009 (MBSJ: The Molecular Biology Society of Japan). The theme was "New Quickening of Molecular Biology ~ Its Dawn of Daybreak from Miyazaki (in Japanese) ~". As a matter of fact, I secretly and individually included the meaning of the 4C theory, which was still immature and unpublished at that time, in the theme of the official symposium. Anyhow, I am proud of the 4C theory [Chaps. 2 ~ 7] and also our other researches on histones, histone chaperones, chromatin-modifying enzymes and transcription factors in the DT40 cell line [27, 69-78, 80-94, 96-100], because all of these studies were originally started and achieved by only myself and my small group, except some technical helps, and not directly succeeded to those of any related scientists and/or groups. In particular, I have a credit for the 4C theory, a new fundamental concept on life phenomena, which was created based on only a few and insufficient data in our solitary step, i.e., by very small group (of M. N. and T. N.), in spare time from our other main projects and with no grants.

Incidentally, as easily perceived by the above-mentioned facts, prior to the first paper on the iPS cells, in fact, we started the research on the 4C theory for the exclusion of artificially accumulated IgM H- and L-chains in HDAC2(-/-) mutants during cultivation. However, while I was in active service, all of data for the 4C theory on gain of new cell function through irreversible creation of chromatin structure plasticity could not been published as papers in journals and presentations in meetings. Since I thought it was the minimum requirement that besides all of our results obtained by that time, additional data should be published at the same time, in order to win recognition of scientist community. When I retired, to my regret, nobody took over the research on the 4C theory, and then I had to abandon all of my own old samples, final submitted manuscripts and Experimental Notes (containing raw data, most of which had been already published) kept from graduate school (except some samples and all data of M. N. on the 4C theory), because I lost both academic and private spaces to keep them. It goes without saying that my laboratory members probably keep their own samples and raw data on our other research projects. On the occasion of my thorough retirement, for the first time, I had seriously started writing of rough drafts and manuscripts for our book on the 4C theory in original paper form.

Meanwhile, concerning the acquisition of the pluri-potency of higher eukaryotic cells, following the iPS cells, the analogous cells triggered simply by stress of various reagent-treatments were reported in rapid succession. Nevertheless, to my great surprise, two papers on the STAP cells were reported splendidly and showily toward the end of the writing of these drafts and manuscripts. However, after a little while these two papers were retracted and thereby the existence of the STAP cells came to naught. Regrettably and sorrowfully, the so-called STAP scandal was degraded itself to one of the worst injustice in the whole science world, and the Japanese science world, especially the Japanese life science field, was thereby got a violent blow. Naturally, the scandal must have indirect effects (such as severe criticisms of

reviewers) on our research concerning the 4C theory not a little, although it has not direct effects at all.

Anyhow, regardless of this STAP scandal, I decided to publish at least our book at my own expense as a scientist out of office in the worst case, since I could not obtain additional data any more and feared that these papers should be rejected thereby in the peer review by severe referees, when those are submitted. Based on these my own experiences, concerning a means for further efficient development of science, I should like to express my brief opinion as follows. First, when scientists retire thoroughly, I guess that most of them should have a lot of certain but immature/unpublished results, which are taken over by nobody. To prevent the huge unquestionable data from being left over as unpublished and come to naught secretly, it should be necessary for scientist community to make suitable resorts of publishing these unpublished data (of course, their reliabilities are essential) as gemstone with hidden potential, besides the so-called formal journals in which mature results are published as papers/reports in complete forms. However, though this idea may be contrary to the ordinary opinion of the community and also the general public now, especially after the historic STAP scandal. Because, in future, someone can initiate and develop new research projects using some of these authentic data just as the start line without waste of time, effort and grant, even though they are incompletely/immaturely published.

Next, I wished to change the subject to life scientist's types/styles having no direct connection with the 4C theory. Since old times, roughly speaking, there are three typical researcher's types/styles based on means of taking influential data in life science field as follows. The first type is a category of researchers who find new biological concepts and/or phenomena based on morphological nature observation like C. R. Darwin. Most of primate biologists (in fieldwork) should be classified as this category. The second type is a category of researchers who find new biological concepts, phenomena, functions and/or molecules based on their own experimental results like G. J. Mendel. Most of experimental biologists (in wet laboratory) should be classified as this category. The third type is a category of researchers who find new biological concepts, phenomena, functions and/or molecules based on results/data of other scientist(s) like J. D. Watson and F. H. C. Crick. Most of molecular evolutionists and informatics biologists (in dry/silico laboratory) should be classified as this category. Of course, I belonged thoroughly to the second category; therefore, we could not add any more data to the 4C theory after my thorough retirement. To return from the digression, I should like to emphasize once again that these facts were just the reason/excuse for the above-mentioned decision, i.e., in the worst case I must publish at least our actual book at my own expense as an amateur or an old fossil-like/analog-type scientist out of office.

I should like to just mention a following word in conclusion. Future Biology that I mean must preferentially focus on bio-system (we named the 4C theory) for gain of new cell functions through irreversible creation of chromatin structure plasticity (which should inevitably occur) rather than

alterations in genome information (which should incidentally and/or neutrally occur), in order to adapt, cope with and/or overcome unexpected, disadvantageous and/or advantageous environment changes, when higher living things firstly encounter such changes. As concrete approaches to generalize the 4C theory, for instance, influences of changes in temperature, atmosphere and/or nutrition on ability to gain new cell functions throughout numerous generations (cell divisions) of established cell lines and development and differentiation of model animals (e.g., mice and rats) may be relatively easy to be examined. Consequently, the 4C theory should open the door for acquisition of adaptation ability of higher eukaryotes to the environment changes and thereby innovate the common concept on somatic cells. It is because, the bio-system (the 4C theory) to gain new cell functions through irreversible creation of chromatin structure plasticity with epigenetic modifications should be one of the most fundamental phenomena for the life conservation and cell-type determination of higher eukaryotes.

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FIGURE LEGENDS

Figure 8-1. Alterations in amounts of IgM H- and L-chains in HDAC2(-/-) DT40 mutant cells during cultivation

2D-PAGE was performed on total cellular proteins prepared from HDAC2(-/-) mutant cells at the early (E), middle (M) and late (L) cultivation stages and DT40 cells (W). The figure is equal to Fig. 2-1 in [Chap. 2].

Figure 8-2. Alterations in amounts of IgM H-chain proteins in HDAC2(-/-) DT40 mutant cells during cultivation

Immuno-electron microscopy was performed on HDAC2(-/-) mutant cells at the early (E) and late (L) cultivation stages and DT40 cells (W), using anti-chicken IgM H-chain antiserum. The figure is equal to Fig. 2-3 in [Chap. 2].

Figure 8-3. Alterations in amounts of IgM H- and L-chains in individual HDAC2(-/-) DT40 mutant clones during cultivation

Western blotting was performed on total cellular proteins prepared from six HDAC2(-/-) mutant clones at indicated cultivation periods, using anti-chicken IgM L- and H-chain antisera. The figure is equal to Fig. 4-2 in [Chap. 4].

Figure 8-4. Alterations in gene expressions of IgM H- and L-chains, members of HATs, HDACs and transcription factors in individual HDAC2(-/-) DT40 mutant clones during cultivation

RT-PCR was performed on total RNAs prepared from four HDAC2(-/-) mutant clones at indicated periods of cultivation using appropriate primers. The figure is equal to Fig. 4-6 in [Chap. 4] and shown with slight modifications.

Figure 8-5. Manners for control of gene expressions of IgM H- and L-chains through control of gene expressions of particular transcription factors in the presence or absence of HDAC2 in wild-type DT40 cells (W) or all and individual clones of HDAC2(-/-) mutant cells at early (E) and late (L) cultivation stages

The figure is equal to Suppl. Fig. 6-S1 in [Chap. 6].

Figure 8-6. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of the Pax5 gene in individual clones of HDAC2(-/-) mutants during cultivation

The figure is a set of Figs. 5-1 ~ 5-4 in [Chap. 5].

Figure 8-7. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of the Aiolos gene in individual clones of HDAC2(-/-) mutants during cultivation

The figure is a set of Figs. 5-5 ~ 5-8 in [Chap. 5].

Figure 8-8. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of the EBF1 gene in individual clones of HDAC2(-/-) mutants during cultivation

The figure is a set of Figs. 5-9 ~ 5-12 in [Chap. 5].

Figure 8-9. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of the OBF1 gene in individual clones of HDAC2(-/-) mutants during cultivation

The figure is a set of Figs. 5-13 ~ 5-16 in [Chap. 5].

Figure 8-10. Summary on alterations in mRNA levels of IgM H- and L-chains, Pax5, Aiolos, EBF1 and OBF1 genes, and acetylation levels of particular Lys residues of histone H3 and chromatin structure of their proximal 5'-upstream regions in individual HDAC2(-/-) DT40 mutant clones during cultivation

The 4C machinery should contain particular member of each of HATs and HDACs and others. The TFC machinery should contain RNA polymerase, particular transcription factors and others. The figure is equal to Fig. 6-5 in [Chap. 6].

Figure 8-11. Localization of IgM H-chain proteins at peri-nuclear space, endoplasmic reticulum and cell surface of HDAC2(-/-) DT40 mutant cells

Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was performed on HDAC2(-/-) mutant cells. Arrows P, E and S indicate IgM H-chain proteins localized at peri-nuclear space, endoplasmic reticulum and surface. The figure is equal to Fig. 6-1 in [Chap. 6].

Figure 8-12. A model for signal transduction on IgM H- and L-chains accumulated in peri-nuclear space to chromatin structure in HDAC2(-/-) DT40 mutant cells

Left panel: Accumulated IgM H-chain proteins were localized in peri-nuclear space of HDAC2(-/-) mutant cells. Right panel: Signal on accumulated IgM H- and L-chains in peri-nuclear space of HDAC2(-/-) mutant cells was transmitted to chromatin, followed by unbalanced correspondence and

convergence for the signal to particular genes (Pax5, Aiolos, EBF1, OBF1, etc.) in individual clones. ECRR/ECRS: environment change recognition receptor/site. 4C machinery: chromatin conformation change complex (4C) machinery. The figure is equal to Fig. 6-4 in [Chap. 6].

Figure 8-13. Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region in descendent cells

Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region of particular gene occurs in descendent cells (upper panel). By contrast, reversible regulation of ordinary gene expression and enzyme reaction occur in the cells that accept proper signal (middle and lower panels). The figure is equal to Fig. 6-7 in [Chap. 6].

Figure 8-14. Schematic representation of chromatin conformation change code (4C) theory for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications via a lot of generations

The figure is equal to Fig. 6-6 in [Chap. 6].