Chapter 8

Personal closing remarks on the occasion of retirement: The real course and history of the 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 un-programmed and new cell functions by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications through various generations. The 4C theory had been studied under worst research environments, i.e., without any research grants, in our spare time from our main research projects and that only by a womanpower. The 4C theory is the typical fruit of my serendipity and also the outcome of some 10 years of my small research group. Here, I described the real course and the history of the 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 the chromatin structure are remarkably involved in regulations of gene expression, and DNA replication, repair and recombination and others [1-5]. Furthermore, in higher eukaryotes, such alterations decisively participate in the development and differentiation of cells, e.g., lymphocytes [6-12], and then determine specificity of diverse cell types. Among several epigenetic modifications of the chromatin structure, the chemical modification of specific Lys (K) residues of core histones H2A, H2B, H3 and H4 with acetyl group is major one [13-26]. Of various chromatin-modifying enzymes participating in alterations of the chromatin structure, histone acetyltransferase(s) (HATs) and histone deacetylase(s) (HDACs) cooperatively control acetylation and deacetylation levels of specific 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, two-dimensional gel electrophoresis (2D-PAGE) analysis of proteins, and structure and function of DNA binding proteins and others [48-59]. Figuratively speaking, if my whole researches are likened to a game, these studies must be the pre-run of the hop, step and jump.

In the midst of the above-mentioned studies on the structures of various histone genes, the ALV-transformed 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 can continue any of researches using the chicken DT40

cell line, although both of the start and the continuance of researches using model animals (such as mice and rats, etc.) are very hard for us because of insufficient grants and manpower. In addition, I was fully convinced that the peculiar knowledge concerning basic bio-systems obtained in DT40 cells can be universally expanded to understand fundamental and complex bio-systems in higher eukaryotes. It is because that almost all ways of the most essential life phenomena, including "central dogma", which had been elucidated in phage, prokaryote (especially E. coli) and/or yeast systems, can be thoroughly applicable to higher eukaryotic systems.

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 the elucidation of in vivo functions (and structures) of various members of chromatin-modifying enzymes (HATs and HDACs) and transcription factors, as well as histones and histone chaperones (CAF-1, HIRA, HAT1, ASF1), 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, in order to assess individual roles of various members of histones and histone chaperones, we had comprehensively generated numerous homozygous DT40 mutant cell lines, respectively, which were devoid of the corresponding specific genes. The results obtained from these resultant mutants, combined, showed that this approach is really useful and powerful to know individual physiological functions of these specific genes in vivo [69-79].

In parallel and/or continuously, based on these positive results and my intrinsic interest on universal and global roles of histone acetylation and deacetylation, in order to assess individual roles of members of HATs and HDACs, we had generated a number of homozygous DT40 mutant cell lines, each of which was devoid of a specific member of HATs and HDACs, such as GCN5, PCAF, HAT1, HDAC1, HDAC2, HDAC3, etc. [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 its gene expression and pre-mRNA alternative processing [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 specific functions of these transcription 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 indirectly regulates gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of Pax5, EBF1, Aiolos, Ikaros, E2A and also OBF1 [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 the life science field until then, with a few exceptions. I chiefly owed such feelings to the following facts. I have been fascinated by the word "life" from my boyhood; therefore, I had a great interest in fundamental concept on life phenomena but a slight interest in 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 (re-expression) experiments to further study the participation of HDAC2 in regulations of gene expressions of IgM H- and L-chains, I accidently became aware of the surprising and interesting 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 both 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 had different interpretations. One of colleagues intensely asserted not only that the accumulation of IgM H- and L-chains in HDAC2(-/-) is not truly depending on the HDAC2-deficiency and resulted from only unknown artificial cause of beneath our notice, but also that the accumulated immunoglobulin proteins are only spontaneously reduced during cultivation for the construction of HDAC2(-/-)tetHDAC2(+). Because the colleague was bigoted toward the vague biased (but ordinary in a sense) view that several properties of higher eukaryotic cells are easily changeable during cultivation. Similarly, another colleague conservatively insisted that the accumulation and diminution of IgM H- and L-chains in HDAC2(-/-) are not directly related to the HDAC2-lacking but based on only somatic mutations, which occur 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, the colleagues were not interested in these interesting findings.

However, intuitively, I believed that the accumulation of IgM H- and L-chains in HDAC2(-/-) is really caused by the HDAC2-deficiency itself, and their diminution during cultivation for the construction of HDAC2(-/-)tetHDAC2(+) is 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) are firstly increased in several independent HDAC2(-/-) mutant clones and thereafter reproducibly decreased, whenever they were cultivated for a long period (our unpublished data). Second,

by contrast, insignificant changes (increases and/or decreases) in protein and 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 mistaken speculations due to qualitative results in our previous paper as follows [27]. Re-experiments showed that protein and mRNA levels of HDAC2 in the heterozygous mutants HDAC2(-/+) are obviously decreased to ~50% of those as in DT40 cells (our unpublished data), whereas these two levels had been reported to be kept constant by means of the compensation mechanism [69-75]. Re-examined RNase protection assay (and Northern blotting) showed that total amounts of IgM H-chain proteins and the ratio of secreted to membrane-bound forms of its mRNA are certainly increased in HDAC2(-/+), compared with those as in DT40 cells (our unpublished data), though both of the amounts and the ratio had been reported to remain unchanged. Growth rate of initially generated HDAC2(-/-) mutant clones in the re-experiment (our unpublished data), as well as that of most clones of secondly generated HDAC2(-/-) mutants [Chap. 4], was slightly but truly different from that of DT40 cells, though growth rate of the initial mutants had been reported to be essentially identical with that of DT40 cells. Pulse-labeling/chase experiment and Northern blotting re-examined showed that the protein and mRNA of IgM L-chain really exist as two forms (large and small) and their amounts are surely 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 colleagues of my research group to study mechanisms of the increases and decreases in protein and mRNA levels of IgM H- and L-chains in HDAC2(-/-) as a part of their own research projects. However, several kinds of approaches gave only following negative data (unpublished). RT-PCR performed on various transcription factors (including some promoter and intron enhancer binding proteins) to regulate the IgM H-chain gene expression showed only insignificant (or no) changing patterns in their mRNA levels in both DT40 cells and HDAC2(-/-) mutants at any cultivation stages. MNase protection assay carried out on several regions within the chromatin surrounding the IgM H-chain gene showed insignificant changing patterns in nucleosome ladders in both DT40 cells and HDAC2(-/-) mutants at any cultivation stages. In addition, MNase protection assay performed on several genes encoding transcription factors (such as Pax5, Aiolos, Ikaros, EBF1, etc.) also showed insignificant changing patterns in nucleosome ladders for these genes in both DT40 cells and HDAC2(-/-) mutants 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 later 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. However, the above-mentioned findings were not reproducible; therefore, I decided that in our case these small RNA molecules are 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 are indirect but not direct targets of HDAC2 [85]. 2) MNase protection assay was regrettably carried out only on ORF regions (coding regions) but not on proximal 5'-upstream regions of the tested transcription factor genes. 3) HDAC2(-/-) mutants were really collected at a vague early cultivation stage. That is, although HDAC2(-/-) mutants were regarded to be collected at the early stage, those were actually collected at fairly later and 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 the key facts as follows. I resolved that we newly generate HDAC2(-/-) mutants, collect them at different cultivation periods from virtual early (as early as possible) until later stages, and systematically and closely analyze their various cellular properties to solve the above-mentioned 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 large 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 colleague. She had worked on this project and other projects full-time, in spite of a part-time contract. Further, another PhD joined in my group as an 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 colleague 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 only by a womanpower (of M. N.), we had undertaken the dreamiest study on chromatin conformation change code (4C) theory for gaining un-programmed and new cell functions by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications during various generations (cell divisions) [Chaps. 2 - 7]. At the start of the research, I had firmly resolved that my small research group (of T. N. and M. N.) made some contribution (even if a sole and small concept) to recent rapid development of the life science field, which had already become a big science, like the space sciences, experimental elementary particle physics, geophysics and others. On the other hand, it was also true that we must principally continue 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 and collaborative researches [27, 38-59, 63, 69-74, 121-144] and some unlisted researches, because minimum research conditions (i.e., grants, manpower and others) 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 randomly selected from them were listed as references [1-26, 28-37, 60-62] in this article, although many of the referred papers were cited in our previous papers [27, 38-59, 63-78, 80-94, 96-100]. If I had not come across this biological phenomenon, my life as a scientist must be ended in imperfect combustion, besides my failure in formation of excellent research group. 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 only a 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 an abnormal and uncomfortable intra- and/or extra-cellular environment change, when they firstly encounter with it in their lives. 2) Somatic cells, for gaining un-programmed and new cell functions, are pluri-potential, elastic and flexible, all of which are originated from pluri-potency, elasticity and flexibility of the chromatin structure. 3) New cell functions to adapt to and/or eliminate the abnormal environment change are acquired by means of irreversible creation of the chromatin structure plasticity surrounding proximal 5'-upstream chromatin region (named notch and also director) of each of various specific genes (principally encoding chromatin-modifying enzymes, transcription factors, and related enzymes and factors). 4) Diversity in the chromatin structure plasticity in individual cells of the same type is triggered by spontaneous unbalanced response to the abnormal environment change at the first encounter time with it and then accomplished by successive convergence of the response to it through various generations. 5) Irreversible creation of the chromatin structure plasticity occurs inevitably but not incidentally and/or neutrally. 6) The chromatin structure plasticity (from the tight to loose forms or vice versa) of the proximal 5'-upstream region is created through successive chromatin conformation change by epigenetic modifications with acetyl group and

others through various cell divisions. 7) Putative environment change recognition receptor/site (ECRR/ECRS) recognizes the abnormal environment change and putative chromatin conformation change complex (4C) machinery directly and irreversibly creates the chromatin structure plasticity. 8) Variety in irreversible creation of the chromatin structure plasticity fairly depends on antecedents of somatic cells until then. 9) Irreversible creation of the chromatin structure plasticity occurs in descendent cells through various generations but not in the cell that initially encounters with it. 10) The chromatin structure of the proximal 5'-upstream region(s) of the above-mentioned specific gene(s), as just dynamic and changeable three-dimensional conformation, receives the signal on the abnormal environment change. 11) The chromatin structure (the loose or tight form) of the proximal 5'-upstream region directs switch (on or off) for the latent transcription ability of the corresponding gene. 12) The number of codes in the 4C theory, which may determine complicated and diverse cell functions and types of higher eukaryotes, can be roughly estimated based on combination of the number of candidate genes and the number (probably two, i.e., the loose and tight forms of the chromatin structure) of codes for each of these candidate genes.

In any case, if we had been able to study this biological phenomenon in favorable research surroundings, some of questions (which were previously mentioned in [Chap. 6]) concerning the 4C theory on the exclusion of IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants during cultivation must have been clarified. Here, I pick out some momentous questions of them as follows. 1) Why do acetylation levels of specific Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) within the chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes decrease at the early stage of cultivation in HDAC2(-/-) mutants, in spite of the deficiency of HDAC2 activity. 2) Why do the decreased acetylation levels of these specific Lys residues within proximal 5'-upstream chromatin regions of the three genes increase in HDAC2(-/-) mutants during cultivation. Why the case of the OBF1 gene is opposite. 3) Steric and functional differences between the loose and tight forms of the chromatin structure surrounding proximal 5'-upstream regions of these four genes must be clarified precisely. 4) How does the chromatin conformation change complex (4C) machinery (participating in irreversible creation of the chromatin structure plasticity surrounding proximal 5'-upstream regions of the four specific genes) differ from the well-known chromatin-modifying machinery. 5) To demonstrate the 4C theory, both of the putative environment change recognition receptor/site (ECRR/ECRS) as a first player to recognize the abnormal environment change and the putative 4C machinery as a final player to create the 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 briefly express my personal interpretation of the conception about life phenomena as follows. How do higher eukaryotic cells cope with and/or overcome an abnormal, unexpected and disadvantageous environment change, when they firstly encounter it in their lives. Probably, this is possible by adhering to four typical countermeasures. 1) In the case of a very severe change, cells die because it is far ahead of their adaptation ability. 2) In the case of a considerably severe change, cells 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 through various generations. This mode is a basis for the evolution of species. 3) In the case of a moderate change, cells cope with or overcome it by means of irreversible creation of the chromatin structure plasticity caused by successive chromatin conformation changes with epigenetic modifications through various generations. This mode is a basis for the development and differentiation of cells. 4) In the case of a minor change, cells respond to it only through the already acquired regulation mechanisms. The exclusion of IgM H- and L-chains artificially and excessively accumulated in HDAC2(-/-) mutant cells (and also in Pax5(-) mutant cells) during cultivation is really an example of the third case of the above-mentioned countermeasures [see Chaps. 2 ~ 7].

In addition, I have a following harmless insight concerning the development and differentiation of higher eukaryotes, which are certainly related to irreversible creation of the chromatin structure plasticity. Whenever ovum and sperm are fertilized, all components of the chromatin (or chromosomes) (except DNA), which exist in the ovum, are generally considered to be evenly and symmetrically divided into two chromatin sets. However, I guess that an asymmetrical allotment of these chromatin components spontaneously becomes in a few unbalance. That is, two daughter cells after the first cell division of the fertilized ovum have already a slight quantitative and qualitative difference in constituent elements of their chromatin (chromosomes). Successively, the unbalanced distribution of the chromatin components is repeated and converged to create the varied chromatin structure plasticity surrounding proximal 5'-upstream regions of different sets of numerous specific genes in individual cells through various cell divisions. Furthermore, such spontaneous and asymmetrical allotment of the chromatin components at the first cell division (followed by its successive convergence) universally occurs in all fertilized ova. Consequently, both of the complexity and the diversity of the chromatin structure plasticity cause those of cell specificity, i.e., distinct functions of the chromatin, the nucleus and/or the cell, inducing irreversible creation of variety of specific cell (or tissue) types in higher eukaryotes.

Well, genome information, which is inherited to descendant generations through cell divisions (and of course, fertilization or pollination of generative cells), is generally preserved in nucleotide sequences of DNA and translated into amino acid sequences of proteins (or polypeptides) through transcription into nucleotide sequences of RNA. In eukaryotes, genome information and nuclear function are mainly protected by the nuclear membrane that acts as a cytoplasm-nucleus barrier; naturally, there are two other

typical and important barrier systems for conservation (protection) of life, e.g., a blood-brain barrier for conservation of individuals in higher animals and an insemination (sperm-ovum fertilization and stamen-pistil pollination) barrier for conservation of species in higher animals and plants. Moreover, communication and signal transduction between the nucleus and cytoplasm, both of which are essential for expressions of normal cell functions, are preferentially performed by the to and fro passage of large and/or small molecules via the nuclear pore that acts as a guard station at the barrier. By contrast, both the transport of useless molecules to the nucleus and the signal transduction on an abnormal and unexpected (and/or unfavorable) change in the intra- and/or extra-cellular environment to the chromatin are usually shielded by this barrier system.

Finally, I wished to digress from the subject on researches and to largely change the subject to my personal experiences and 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, into the theme of the formal symposium. Anyhow, I am proud of the 4C theory [Chaps. 2 ~ 7] and also of 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 started and accomplished only by myself and my small group, except some technical helps, and not directly succeeded to the researches of any related scientists and/or groups. In particular, I have a credit for the 4C theory, a new fundamental concept concerning life phenomena, which was created based on only a few and insufficient data in our solitary step, i.e., by a very small group (of M. N. and T. N.), in spare time from our other main projects and without grants.

Incidentally, as easily perceived by the above-mentioned facts, prior to the first paper on the iPS cells, we had already started the research on the 4C theory concerning the exclusion of IgM H- and L-chains artificially accumulated in HDAC2(-/-) mutants during cultivation. However, during my tenure of office, all data on the 4C theory for gaining un-programmed and new cell functions by means of irreversible creation of the chromatin structure plasticity could not be published as papers in Journals and presentations in Meetings. Because I thought it was the minimum requirement that besides all of our results obtained by that time, additional data must be published at the same time, in order to win recognition of the life scientist community. When I retired, to my regret, nobody took over the research on the 4C theory, therefore, I had to abandon all of my own old samples, finally submitted manuscripts and Experimental Notes (containing raw data, most of which had been already published), which had been kept from my graduate school (except some samples and all data of M. N. on the 4C theory),

because I lost both academic and private spaces for keeping them. It goes without saying that my colleagues 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 monograph on the 4C theory in original article's form.

Meanwhile, concerning the gaining the pluri-potency of higher eukaryotic cells, following the iPS cells, the analogous cells triggered by the simple stress of several reagent-treatments were reported in rapid succession. Nevertheless, to my great surprise, two papers on the STAP cells were splendidly and showily reported toward the end of the writing of our 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 what was worse, the Japanese science world, especially the Japanese life science field was thereby got a violent blow. Naturally, I was afraid that the scandal gives indirect effects (e.g., severe criticisms of reviewers) on our data concerning the 4C theory not a little, although it does not give direct effects at all.

Anyhow, regardless of the STAP scandal, I decided to publish our data on the 4C theory as my retirement commemorative monograph at my own expense as a scientist out of office in the worst case, since I could not obtain additional data any more and thereby I feared that our data should be rejected in the peer review by severe referees when those are submitted. Based on my own experiences, concerning a means for further efficient development of sciences, I should like to express my brief opinion as follows. First, when scientists retire thoroughly, I guess that most of them have lots of certain but immature and unpublished results that are taken over by nobody. To prevent the huge unquestionable data from being left over as unpublished and come to naught secretly, it is necessary for the scientist community to make suitable resorts to publish these unpublished data (of course, their reliabilities are essential) as the gemstone with a hidden potential, besides the so-called formal Journals, in which the mature results are published as papers and reports in complete forms. Although this idea may be contrary to the ordinary opinion of the scientist community and the general public now especially after the historic STAP scandal, the reason is as follows. If it is possible, 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 those are incompletely and immaturely published.

Next, I wished to change the subject to life scientist's types (styles), which have no direct connection with the 4C theory at all. Since old times, roughly speaking, there are three typical researcher's types (styles) depending on means of taking influential data in the life science field as follows. The first type is a category of researchers who find new biological concepts and/or phenomena depending on morphological nature observation. A typical scientist was C. R. Darwin. Most of primate biologists (in the fieldwork) are classified into the category. The second type is a category of researchers who find

new biological concepts, phenomena, functions and/or molecules depending on their own experimental results. A typical scientist was G. J. Mendel. Most of experimental biologists (in the wet laboratory) are classified into the category. The third type is a category of researchers who find new biological concepts, phenomena, functions and/or molecules depending on results and/or data of other scientists. The typical scientists were J. D. Watson and F. H. C. Crick, and M. Kimura. Most of molecular evolutionists and informatics biologists (in the dry/silico laboratory) are classified into the 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 this fact was one of the reasons and/or excuses for the above-mentioned my decision. As noted again, I decided to publish the retirement commemorative monograph at my own expense as if an amateur or an old fossil-like/analog-type scientist out of office.

I should like to just mention a following word in conclusion. When higher living things firstly encounter abnormal, unexpected, disadvantageous and/or even advantageous environment changes, they possess the ability to adapt to, cope with and/or overcome such environment changes by means of varied alterations in the chromatin structure, genome information and/or other bio-systems. Future Biology that I mean must preferentially focus on the bio-system (we tentatively named the 4C theory) for gaining un-programmed and new cell functions by means of irreversible creation of the chromatin structure plasticity (which inevitably occurs), rather than the bio-systems attributed to alterations in the genome information (which incidentally and/or neutrally occur). As a concrete approach to generalize the 4C theory, for instance, elucidation of influences of changes in temperature, atmosphere and/or nutrition on the ability to gain un-programmed and new cell functions in established cell lines through various generations (cell divisions) and in model animals (such as C. elegans, Drosophila, Xenopus, mice, rats, etc.) during development and differentiation is very helpful and significant, because these influences under the varied conditions can be easily studied by many research groups. Consequently, the 4C theory can open the door for acquisition of the adaptation ability of higher eukaryotes to the above-mentioned various environment changes and thereby innovate the common concept on the nature of somatic cells. Because the bio-system (the 4C theory) for gaining un-programmed and new cell functions by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications is one of the most fundamental and important phenomena for the life conservation and cell-type determination of higher eukaryotes.

POSTSCRIPT

On the occasion of the revision of the retirement commemorative monograph, we newly added the postscript and conflict of interest statement sections into Chapter 8 as follows. In 2015, we published

the first edition of the above-mentioned monograph at my own expense and registered as the university repository that is available from a following URL [145]. Thereafter, several scientists advised that we have better to publish our data on the 4C theory in suitable Journals. Therefore, concerning the contents of the monograph, we wrote the manuscripts of five original articles and a review article, and then negotiated with two open-access Journals on the submitting of these manuscripts several times. However, unfortunately, the two Journals will not be able to consider our submissions, because our manuscripts did not match with their guidelines. Reluctantly, we registered these six articles as the university repositories, all of which are available from respective URLs [146-151]. In 2017, fortunately, at length we had the opportunity to publish the researches on the 4C theory (especially the researches in Refs. 148-150) as three review articles in Current Topics in Biochemical Research [152-154]. In 2018, we revised the monograph and the six articles, and then registered their revisions as the university repositories, all of which are also available from the above-mentioned respective URLs [145-151]. Naturally, we added these publications as Refs. 145-154 in the References section of the revised edition of Chapter 8.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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FIGURE LEGENDS

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

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

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

Immuno-electron microscopy, using anti-chicken IgM H-chain antiserum, was performed on HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages and on DT40 cells (W). The figure is identical with 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 continuous cultivation

Western blotting, using anti-chicken IgM L- and H-chain antisera, was performed on total cellular proteins prepared from six HDAC2(-/-) mutant clones at indicated cultivation periods and from DT40 cells. The figure is identical with Fig. 4-2 in [Chap. 4].

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

RT-PCR using appropriate primers was performed on total RNAs prepared from four HDAC2(-/-) mutant clones at indicated cultivation periods and from DT40 cells. The figure is identical with Fig. 4-6 in [Chap. 4] and shown with slight modifications.

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

The figure is identical with Suppl. Fig. 6-S1 in [Chap. 6].

Figure 8-6. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of the Pax5 gene in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

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

Figure 8-7. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of the Aiolos gene in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

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

Figure 8-8. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of the EBF1 gene in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

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

Figure 8-9. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of the OBF1 gene in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

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

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

The 4C machinery contains a specific member of HATs and HDACs and others. The TFC machinery contains RNA polymerase, specific transcription factors and others. The figure is identical with Fig. 6-5 in [Chap. 6].

Figure 8-11. Localization of IgM H-chain proteins in 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 the peri-nuclear space, endoplasmic reticulum and cell surface. The figure is identical with 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 the peri-nuclear space of HDAC2(-/-) mutant cells. **Right panel:** Signal on accumulated IgM H- and L-chains in the peri-nuclear

space of HDAC2(-/-) mutant cells was transmitted to the chromatin, followed by the unbalanced correspondence and convergence for the signal to the proximal 5'-upstream region of each of specific transcription factor genes (Pax5, Aiolos, EBF1, OBF1, etc.) in individual HDAC2(-/-) mutant clones. ECRR/ECRS: environment change recognition receptor/site. 4C machinery: chromatin conformation change complex (4C) machinery. The figure is identical with Fig. 6-4 in [Chap. 6].

Figure 8-13. Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region with epigenetic modifications in descendent cells and reversible regulation of ordinary gene expression and enzyme reaction in cells that accept proper signal

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

Figure 8-14. Schematic presentation of chromatin conformation change code (4C) theory for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations

The figure is identical with Fig. 6-6 in [Chap. 6].