Chapter 6

Chromatin conformation change code (4C) theory: A bio-system for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations

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SUMMARY

When higher eukaryotic cells firstly encounter moderate intra- and/or extra-cellular environment change, they newly acquire the ability to cope with and/or overcome it by means of irreversible creation of the chromatin structure plasticity based on successive chromatin conformational change with epigenetic modifications. Putative environment change recognition receptor/site (ECRR/ECRS) recognizes the new environment change. Putative chromatin conformation change complex (4C) machinery irreversibly and separately creates the variety of the chromatin structure plasticity not only for various specific transcription factor genes but also in individual clones of the same cell type. We advance a chromatin conformation change code (4C) theory for a bio-system to gain un-programmed and new cell function(s) by means of irreversible creation of the chromatin structure plasticity by the 4C machinery through various generations, in order to adapt to the new environment change recognized by the ECRR/ECRS. The 4C theory is also suitable as an explanation for the ways to control development and differentiation of higher eukaryotes.

In eukaryotes, genome information and nuclear function are mainly protected by the nuclear membrane that acts as a cytoplasm-nucleus barrier. Additionally, communication and signal transduction between the nucleus and cytoplasm, both of which are necessary for expressions of normal cell functions, are preferentially carried out 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 signal transduction concerning an abnormal, unexpected, and/or disadvantageous change in the intra- and/or extra-cellular environment and the transport of useless molecules to nucleus are usually prevented by this barrier system. Then, how do the higher eukaryotic cells cope with and/or overcome an abnormal, unexpected, and/or disadvantageous environment change, when they firstly encounter it. Generally, this is possible by adhering to four typical countermeasures. First, in the case of a very severe change, cells die because it is far ahead of their adaptation ability. Second, 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 in the DNA molecule through various generations. This mode is a basis for the evolution of species. Third, 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 conformational (structural) changes with epigenetic modifications through various generations. This mode is a basis for the development and differentiation of cells. Fourth, in the case of a minor change, cells respond to it only through already acquired regulation mechanisms.

In chicken wild-type DT40 cells, HDAC2 as a supervisor regulates gene expressions of IgM H- and L-chains through opposite control of gene expressions of Pax5, Aiolos, EBF1, OBF1, and Ikaros plus

E2A [1, Chap. 4]. Furthermore, in HDAC2-deficient DT40 mutant cells, HDAC2(-/-), IgM H- and L-chains are excessively accumulated at the early cultivation stage by their dramatically increased gene expressions, caused by drastic decreases in gene expressions of Pax5, Aiolos and EBF1, all of which down-regulate gene expressions of the two immunoglobulin proteins [1, Chap. 4]. Remarkably, the accumulated IgM H- and L-chains are dramatically and distinctly decreased at the later cultivation stage in individual clones of HDAC2(-/-) mutants by their drastically decreased gene expressions, caused by dramatic increases or decreases in gene expressions of Pax5, Aiolos and EBF1 or OBF1, which up-regulates gene expressions of the two immunoglobulin proteins (details will be discussed later) [Chap. 4]. These results obtained from wild-type DT40 cells and HDAC2(-/-) mutants at the early and later cultivation stages are schematically shown in Supplementary Figure 6-S1. Such diminutions of the accumulated IgM H- and L-chains in distinct ways in all individual HDAC2(-/-) mutant clones during cultivation are really examples of the third case of the above-mentioned countermeasures. Presumably, the way to diminish the two immunoglobulin proteins excessively accumulated at the first cultivation stage in Pax5(-) mutant cells during cultivation fairly resembles this case [Chap. 3]. Here, we focused our attention only on HDAC2(-/-) mutant cells and concretely presented some of the results obtained from individual clones of them.

The HDAC2-deficiency in DT40 cells rapidly and dramatically increases mRNA (i.e., gene expression/transcription) levels of IgM H- and L-chains, followed by accumulation of the two immunoglobulin proteins probably within endoplasmic reticulum (Figs. 6-1, 6-2 and 6-3, and Suppl. Figs. 6-S2 and 6-S3). Surprisingly, excessively increased mRNAs and proteins of IgM H- and L-chains at the early stage of cultivation are gradually reduced until the later cultivation stage in almost similar pattern in all examined individual clones cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6 of secondly generated HDAC2(-/-) mutants (Suppl. Figs. 6-S4 and 6-S5). By contrast, interestingly, gene expressions of various specific transcription factors and chromatin-modifying enzymes change in distinct patterns among these individual clones of HDAC2(-/-) mutants (Suppl. Fig. 6-S5). To reduce the increased protein levels of IgM H- and L-chains resulted from their increased gene expressions, apparently, following three distinct ways based on changed gene expressions of some specific transcription factors exist at the later cultivation stage in the six individual mutant clones (Suppl. Fig. 6-S1(L)) [Chap. 4]. The way in clone cl.2-1 seems to be dependent on OBF1 and different from that in wild-type DT40 cells. The ways in clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be dependent on Pax5 plus Aiolos and slightly similar to that in DT40 in appearance. The way in clone cl.2-6 seems to be dependent on Pax5, Aiolos plus EBF1 and similar to that in DT40 in appearance.

To clarify these three distinct ways, we performed neighboring overlapping tiling chromatin immuno-precipitation (NotchIP: this abbreviation also means IP on notch of chromatin) assay on the proximal 5'-upstream chromatin region (named as notch of chromatin) of each of Pax5, Aiolos, EBF1

and OBF1 genes [Chap. 5]. The results obtained by the NotchIP assay revealed that such distinct ways should be fundamentally originating from irreversible creation of the distinct chromatin structure plasticity surrounding proximal 5'-upstream regions of corresponding transcription factor genes with epigenetic modifications through various generations during cultivation. To put it concretely, in wild-type DT40 cells having HDAC2 activity, the chromatin structure surrounding the proximal 5'-upstream regions of genes encoding Pax5, Aiolos and EBF1 (which down-regulate gene expressions of IgM H- and L-chains [1, Chap. 4]) and OBF1 (which probably up-regulates those of these two immunoglobulin proteins [2]) is in the loose (open) form due to no binding ability of histone H3 to DNA based on hyper- (high) acetylation levels of one or more of the specific Lys (K) residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) [Chap. 5]. Therefore, these four transcription factor genes are transcribed at high levels in DT40 cells [Chap. 4]. On the other hand, in all individual clones of HDAC2(-/-) mutants having no HDAC2 activity, the chromatin structure of the proximal 5'-upstream region of each of these four factor genes dramatically and severally change based on varied acetylation and deacetylation levels of one or more of the five specific Lys residues of histone H3 during cultivation, resulting in alterations in their gene expression levels as follows (Suppl. Fig. 6-S5) [Chap. 4].

In clone cl.2-1, at the early stage of cultivation, the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes is in the tight (closed) form due to the binding ability of histone H3 to DNA based on hypo- (low or no) acetylation levels of one or more of the five specific Lys residues (Suppl. Fig. 6-S6), and as a result, transcriptions of these three genes are almost completely suppressed to undetectable levels. However, the chromatin structure of the OBF1 gene is somewhat in the loose form due to the less binding ability of histone H3 to DNA based on slight (or considerably decreased) hyper-acetylation levels, thereby its gene expression is slightly decreased. By contrast, at the later cultivation stage, the chromatin structure of the proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes remains in the tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, their gene expressions also remain unchanged at an undetectable level. On the other hand, the chromatin structure of the proximal 5'-upstream region of the OBF1 gene changes to the tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, its gene expression is suppressed to almost undetectable or very low level. These results supported the above-mentioned inference (i.e., OBF1-dependent) on the ways of gene expressions of IgM H- and L-chain proteins at the later cultivation stage in clone cl.2-1.

In clones cl.2-2 and cl.2-4, at the early cultivation stage, as in clone cl.2-1, the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is in the tight or somewhat loose (or less tight) form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five specific Lys residues of histone H3 (Suppl. Figs. 6-S7 and 6-S8). Therefore, transcriptions of the first three genes are almost completely suppressed and that of

the last one gene is certainly decreased. At the later cultivation stage, contrary to this, the chromatin structure of the proximal 5'-upstream regions of Pax5, Aiolos and OBF1 genes changes to the loose form based on hyper-acetylation levels, and their gene expressions are dramatically or certainly increased. However, the chromatin structure of the EBF1 gene remains in the tight form based on hypo-acetylation levels, and its gene expression remains low at almost undetectable level. These results supported the above-mentioned inference (i.e., Pax5- and Aiolos-dependent) on the ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clones cl.2-2 and cl.2-4 (and also cl.2-3 and cl.2-5). Moreover, these four clones should be major type, since they resemble in several cellular characteristics to four initially generated HDAC2(-/-) clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) [3] [Chap. 2].

In clone cl.2-6, at the early stage of cultivation, as in clones cl.2-1, cl.2-2 and cl.2-4, the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is in the tight or somewhat loose form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five specific Lys residues of histone H3 (Suppl. Fig. 6-S9). Therefore, transcriptions of the first three genes are almost completely suppressed and that of the last one gene is certainly decreased. By contrast, at the later cultivation stage, the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes changes to the loose form based on hyper-acetylation levels, and their gene expressions are dramatically or certainly increased. These results supported the above-mentioned inference (i.e., Pax5-, Aiolos- and EBF1-dependent) on the ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clone cl.2-6.

Results on alterations in acetylation levels (hyper or hypo) of the five specific Lys (K) residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3), the form (loose or tight) of the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (gene expression/transcription) levels (high or low) during cultivation in individual HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 [Chaps. 4 and 5] are roughly and schematically presented in Supplementary Figure 6-S10. Concerning on the above-mentioned results on the suppression of gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivations, there are some important comments as follows. Excessively accumulated IgM H- and L-chain proteins resulting from their dramatically increased gene expressions in HDAC2(-/-) mutants just after their birth (by gene targeting techniques) [3, 4, Chap. 4] are probably unfavorable and useless for them; therefore, the mutant cells have acquired a new ability to diminish increased gene expressions of these two immunoglobulin proteins as an abnormal and uncomfortable environment change through various generations during cultivation. In all HDAC2(-/-) mutant clones, even in clone cl.2-6 [Chap. 4], the ways to suppress gene expressions of IgM H- and L-chains at the later cultivation stage are surely distinct from the ordinary and reversible

ways to regulate gene expressions of the two immunoglobulin proteins in wild-type DT40 cells, because gene expressions of IgM H- and L-chains are indirectly regulated by HDAC2 through opposite regulations of Pax5, Aiolos, EBF1, OBF1 and also E2A gene expressions in DT40 cells [1, 5]. In addition, as described previously [Chaps. 2 and 4], gene expressions of various transcription factors and chromatin-modifying enzymes (including Blimp1, PCAF, HDAC7, HDAC9 and others), besides Pax5, Aiolos, EBF1 and OBF1, dramatically and separately change in individual HDAC2(-/-) mutant clones during cultivation (Suppl. Fig. 6-S5). Therefore, remarkably, in addition to such alterations in gene expressions of IgM H- and L-chains (and Pax5, Aiolos, EBF1 and OBF1) and in cell morphology, some other unknown important cellular characteristics might be obviously changing in individual HDAC2(-/-) mutant clones during cultivation. Such presumable changeable characteristics may be complicated and diverse in individual clones of HDAC2(-/-) mutants, even though they are originally of the same cell type whose genotype is completely the same.

Here, based on our morphological but insufficient findings, together with previous results mentioned above and others [1-3, 5-7, Chaps. 2, 3, 4 and 5], we slightly revised our previous hypothesis and proposed an all-inclusive hypothetic way for gaining un-programmed and new cell function to diminish excessively accumulated IgM H- and L-chains by means of irreversible creation of the varied chromatin structure plasticity of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications during continuous cultivation in individual clones of HDAC2(-/-) mutants.

First, we supposed that the environment change recognition receptor/site (ECRR/ECRS) participates in the recognition of accumulation of IgM H- and L-chains as an abnormal and unfavorable environment change (and probably acts in part in the signal transduction concerning the accumulation to the chromatin structure) (Fig. 6-4). In addition, the chromatin conformation (structure) change complex (4C) machinery, which is diverse and consists of a member of each of HATs plus HDACs (most of which were roughly listed in [8]) and other factors, is supposed to directly and irreversibly create the chromatin structure plasticity surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes (and also to act in part in the signal transduction) (Figs. 6-4 and 6-5). Using the ECRR/ECRS, the 4C machinery and other components, the chain reaction of response to the abnormal environment change occurs as follows. Large amounts of IgM H- and L-chains artificially synthesized by their excessively increased gene expressions induced by the HDAC2-deficiency are first accumulated probably within the endoplasmic reticulum of HDAC2(-/-) mutant cells as mentioned above (Figs. 6-1, 6-2 and 6-3 and Suppl. Figs. 6-S2 and 6-S3). Most of these accumulated immunoglobulin proteins (probably exist as a high molecular weight complex of IgM H- and L-chains with each other [1]) are gradually secreted into the cultivation media (i.e., outside of cells), but as indicated by immuno-electron microscopy, remarkably, a part of them are transported to the nuclear envelope but not

inside of nucleus, and kept at the peri-nuclear space at the early and also later cultivation stages (Figs. 6-1, 6-2, 6-3 and 6-4). The excessively accumulated immunoglobulin proteins at the peri-nuclear space bind to the ECRR/ECRS localized at the inner nuclear membrane (where hetero-chromatin is possibly located) (Fig. 6-4). After the ECRR/ECRS recognizes the accumulation of IgM H- and L-chains as an abnormal and unfavorable environment change, the signal concerning it is genome-widely transmitted to the chromatin structure surrounding proximal 5'-upstream regions (notches of chromatin) of numerous genes (probably located at the several distinct chromosomes) encoding transcription factors, chromatin-modifying enzymes and related factors and enzymes. Following the initial signal transduction, a spontaneous unbalanced response to the abnormal environment change is consecutively and separately converged not only on the various specific genes but also in individual clones of HDAC2(-/-) mutants.

The 4C machinery for each of Pax5, Aiolos, EBF1 and OBF1 genes in wild-type DT40 cells probably consists of HDAC2 as HDAC activity, a specific HAT member (e.g., GCN5) as HAT activity and other factors. On the other hand, in all of individual clones of HDAC2(-/-) mutants at the very early stage of cultivation (just soon after their birth by gene targeting techniques), bulk conformation of the 4C machinery dramatically changes to remove or drastically reduce HAT activity (of the assumed member of HATs), attributed to the HDAC2-deficiency. Through the above-mentioned processes and following continuous cultivation, the 4C machinery newly consists of a different member of HDACs, a specific (same or different) member of HATs and other factors, and thereby becomes varied. The diversity of alterations in the chromatin structure surrounding the proximal 5'-upstream regions of the above-mentioned transcription factor genes is preferentially attributed to varied acetylation and deacetylation levels of one or more of the specific Lys residues at N-terminal tail of histone H3 [Chap. 5] caused by collaboration of a proper member of HATs and HDACs in the protean 4C machinery. These successive epigenetic modifications with acetyl group of K9/H3 and K27/H3 (and also K14/H3, K18/H3 and K23/H3) induce irreversible creation of the distinct chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes (Fig. 6-6). In general, the chromatin structure surrounding the proximal 5'-upstream region possessing hyper-acetylation levels of one or more of the specific Lys residues of histone H3 is in the loose form based on their no binding ability to DNA, but the chromatin structure surrounding the proximal 5'-upstream region possessing hypo-acetylation levels of one or more of the specific Lys residues of histone H3 is in the tight form based on their binding ability to DNA (Fig. 6-6). By contrast, probably, the 4C machinery cannot change the chromatin structure surrounding the ORF region (coding regions) of these specific target genes by much [Chap. 5]. Thus, as the need arises, the transcription factor complex (TFC) machinery (which consists of RNA polymerase, proper transcription factor(s), certain member of the HATs and HDACs and other factors) is able to bind to promoter regions (or elements) within the loose (but not

tight) form of the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned target (but not un-target) genes (which have become latently active (but not inactive) state), and thereby initiates their gene expressions (Fig. 6-6). Consequently, individual clones of HDAC2(-/-) mutants acquire flexible, elastic and pluri-potential ability, i.e., the same and new cell function to reduce increased gene expressions of IgM H- and L-chains (resulting in decreases in their protein levels) in distinct ways through increases and/or decreases in gene expressions of Pax5, Aiolos, EBF1 and OBF1, even though these clones are completely the same cell type and also established cell line [1, Chaps. 2 and 4]. Naturally, these distinct ways are not under the control of HDAC2 but based on irreversible creation of their distinct chromatin structure plasticity with epigenetic modifications during continuous cultivation [Chap. 5].

We expanded the above-mentioned hypothetic way on the exclusion of IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants to a universal hypothetic way for gaining un-programmed and new cell functions by means of irreversible creation of the varied chromatin structure plasticity of the specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications through various generations (cell divisions) in individuals of the same cell type of higher eukaryotes whose genotype is completely the same.

When higher eukaryotic cells firstly encounter an intra- and/or extra-cellular environment change in their lives, in order to adapt for or eliminate the change (if abnormal and/or uncomfortable), they gradually gain un-programmed and new cell functions through various generations. Namely, the cells acquire the ability to adapt themselves to newly encountered environment change and/or to exclude the abnormal and/or painful environment change. Using the ECRR/ECRS, the 4C machinery and other components, the cells create a chain reaction of response to the new environment change as follows (Figs. 6-4 and 6-5). First of all, the new environment change is recognized by means of ECRR/ECRS, which may be localized nearby the nuclear membrane as a cytoplasm-nucleus barrier (probably at the inner nuclear membrane where hetero-chromatin is possibly located) (Fig. 6-4). Naturally, there is a great possibility that at this step putative specific molecule(s) acts as the intermediary sensor in the way to recognize the new environment change. Next, putative signal(s) concerning the new environment change is genome-widely transmitted to the chromatin structure within nucleus through various generations (cell divisions). Following the initial acceptance of the putative signal(s) at the chromatin, gene expressions of various chromatin-modifying enzymes, transcription factors, and related enzymes and factors may slightly change, associated with a slight alteration in their chromatin structure. The transduction of the signal(s) and spontaneous unbalanced response to the environment change are successively repeated and converged into the restricted chromatin structure surrounding the proximal 5'-upstream regions (notches of chromatin) of the corresponding genes and related factor and enzyme

genes. Finally, this successive signal transduction concerning the environment change causes various epigenetic modifications of histone proteins and/or DNA within the restricted chromatin regions with acetyl, methyl, phosphate, ubiquitin and ADP ribose groups and/or others.

The 4C machinery, which consists of a specific member of the HATs plus HDACs and other factors, preferentially participates in these epigenetic modifications (Fig. 6-5). Of these various epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones may be major ones. Participating positions of specific Lys residues and kinds of core histones are diverse. For instance, in the above-mentioned case [Chap. 5], acetylation and deacetylation of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 are prominent. Consequently, epigenetic modifications of one or more of these specific Lys residues of histone H3 with acetyl group change within the chromatin structure surrounding the proximal 5'-upstream regions of the above-mentioned specific target genes through various generations (cell divisions). Distinct functions of the protean 4C machineries on such acetylation and deacetylation levels are preferentially based on different combinations of each member of HATs and HDACs as the components, because any HAT and HDAC members' own activities are probably unchangeable, just as activities of almost all enzymes in any biological reactions. By contrast, these protean 4C machineries cannot change the chromatin structure surrounding ORF regions (coding regions) of corresponding genes by much.

The binding ability of the N-terminal tail of histone H3 to DNA is tentatively and qualitatively deduced from the acetylation levels of one or more of the specific Lys residues, though which Lys residue(s) really and/or mainly participates in the binding is still undefined (Fig. 6-5). Namely, hyper- (high) or hypo- (low or no) acetylation levels induces no binding or full binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure. Thus, the chromatin structure plasticity is irreversibly created based on successive conformation changes with epigenetic modifications. Whenever the need arises, the TFC machinery is able to bind to promoter regions (or elements) within the loose form of the chromatin structure surrounding proximal 5'-upstream regions of specific target genes (which are in the latently active state), followed by initiation of their gene expressions (Fig. 6-5). By contrast, the TFC machinery cannot bind to promoter regions (or elements) within the tight form of the chromatin structure surrounding proximal 5'-upstream regions of un-target genes (which are in the latently inactive state), and thereby cannot initiate their gene expressions. Consequently, the loose and tight forms of the chromatin structure surrounding proximal 5'-upstream regions respectively cause high and low (or no) transcription levels of corresponding genes (Fig. 6-6). Notably, there is a great possibility that the ways for irreversible creation of the chromatin structure plasticity are distinct among individual cells of the same type having the same genotype, even though the new environment change and signal(s) on it are the same for all of them. That is, in order to gain un-programmed and new cell functions, individual cells possess the ability not only to complicatedly and diversely change the

chromatin structure surrounding proximal 5'-upstream regions of numerous specific genes but also to separately change even the same proximal 5'-upstream chromatin structure of a certain gene into varied forms. Thus, gene expressions of the specific chromatin-modifying enzymes and transcription factors diversely change in individual cells through various generations (cell divisions), in spite of the same environment change. In consequence, individual cells are newly able to gain the same or distinct un-programmed cell function(s) in different ways, in order to accommodate themselves to a new environment change.

In conclusion, in higher eukaryotes, in order to gain un-programmed and new cell function(s), somatic cells (and also tumor cells) of higher eukaryotes become pluri-potent, elastic and flexible, all of which basically originate from pluri-potency, elasticity and flexibility of the chromatin structure. Namely, in order to adapt to intra- and/or extra-cellular environment change, individual somatic cells of higher eukaryotes possess the ability to gain the same and/or distinct un-programmed and new cell function(s) in different ways by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications, i.e., from the tight to loose forms or vice versa of the chromatin structure surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme and related genes (Fig. 6-6). Such a tight or loose form of the proximal 5'-upstream chromatin structure should be in the latently inactive or active state for transcription ability of the corresponding gene, although the proximal 5'-upstream region as mere nucleotide sequences is potential but in the silent state for expressions of most of the genome functions. Creation of the varied chromatin structure plasticity in individual somatic cells is triggered by the spontaneous unbalanced response to the environment change when they firstly encounter it and then irreversibly accomplished by the successive unbalanced convergence of the response through various generations (cell divisions). Plasticity of the chromatin structure in somatic cells is created in distinct ways, which probably depend on their antecedents and successive unbalanced response to the change through various generations (cell divisions). Moreover, the chromatin structure plasticity, regardless of whether its creation is in the course or was already completed, is inherited to cells of descendant generations associated with or without additional structural change through cell divisions. Thus, remarkably, irreversible creation of the chromatin structure plasticity occurs in descendant cells but not in the cell which initially and/or directly accepts the signal on the environment change, although reversible regulations of ordinary gene expressions and enzyme reactions occur in the cell itself which accepts proper signal (Fig. 6-7). Probably, irreversible creation of the chromatin structure plasticity, with the intention of adapting to the environment change, occurs inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region (as loose or tight form) directs the switch (on or off) for latent transcription ability of the corresponding gene through irreversible creation of the chromatin structure plasticity; therefore, besides as a "notch" from a structural point of view as mentioned above,

the proximal 5'-upstream chromatin region can be regarded as a "director" from a functional point of view. Naturally, the notch (or director) covers specific nucleotide sequences of transcriptional elements (such as promoter, operator, enhancer, etc.) and also their neighboring nucleotide sequences. We should like to emphasize that the real recipient of the signal on the environment change is just the chromatin structure itself as the three-dimensional conformation, which is dynamic and changeable between the tight and loose forms, but not mere the chromatin and chromosomes themselves as one- (or two-) dimensional conformation, which are static and unchangeable. That is, the chromatin structure of proximal 5'-upstream chromatin region (notch or director), as dynamic and changeable three-dimensional conformation, possesses two fundamental abilities, i.e., to receive the signal concerning the intra- and/or extra-cellular environment change and to direct the switch (on or off) for latent transcription ability of the corresponding gene through its irreversible chromatin conformation change that is responsive to the signal. These ideas are based on the facts that almost all of macromolecules (such as proteins) generally exhibit characteristic steric conformations and thereby possess abilities to receive specific signals and to express their own biological functions. However, concrete data are not enough to support these ideas.

Finally, we named our theory on such a bio-system that gains un-programmed and new cell function(s) by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications, which is one of the most important ways for life conservation and cell-type determination of higher eukaryotes, as chromatin conformation (structure) change code (4C) theory. Therefore, the 4C theory can open the door for gaining un-programmed and new cell function(s) of higher eukaryotes and innovate the general notion on the nature of somatic cells. Probably, the supposed number of codes in the 4C theory, which determines complicated and varied characteristics of higher eukaryotic cells, can be roughly estimated based on the combination (multiplication) of the number of candidate genes and that of codes for each of these genes as follows. The most influential candidates are various specific genes encoding transcription factors, chromatin-modifying enzymes and related factors and enzymes, all of which are necessary for gaining un-programmed and new cell function(s) and cell-type determination of higher eukaryotes. The number of codes for each of these candidate genes is two. Because the loose or tight form of the proximal 5'-upstream chromatin region, as in the latently active or inactive state, directs the switch (on or off) for transcription ability of the corresponding gene. Moreover, the 4C theory is suitable as an explanation for the development and differentiation of higher eukaryotes, because action of putative signal(s) concerning extra-cellular environment change seems to fairly resemble that of certain players (such as hormone, cytokine, nerve-transmission substance, etc.), which participate in cell-cell, tissue-tissue and/or organ-organ interactions (communications) throughout these two fundamental life phenomena.

There are several crucial questions to be clarified in the 4C theory on the exclusion of excessive IgM

H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutant cells [Chaps. 2, 4 and 5]. 1) Despite the HDAC2-deficiency, why do the acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone 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(-/-). 2) Why do the decreased acetylation levels of one or more of the specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of these genes increase during cultivation. Why the case of the OBF1 gene is reverse. 3) Which Lys residue(s) of K9, K14, K18, K23 and K27 of histone H3 is really and/or mainly involved in its binding to DNA within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes must be determined. 4) Functional and steric differences between the loose and tight forms (based on hyper- and hypo-acetylation levels of one or more of the specific Lys residues of histone H3) of the chromatin structure surrounding proximal 5'-upstream regions of these four genes must be clarified more precisely. 5) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor gene during cultivation differ among individual mutant clones. 6) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor genes during cultivation differ within individual mutant clone. 7) How does the 4C machinery (which acts in irreversible creation of the chromatin structure plasticity of proximal 5'-upstream region) differs from the well-known chromatin-modifying machinery, although the TFC machinery (which acts in transcription of ORF region of the corresponding gene) may be almost the same as the well-known transcription machinery. 8) To demonstrate the 4C theory, it is essential to establish both the putative ECRR/ECRS as the first player to recognize the environment change and the putative 4C machinery as the final player to directly and irreversibly create the chromatin structure plasticity. 9) As a concrete approach to generalize the 4C theory, for instance, elucidation of the influences of changes in temperature, atmosphere and/or nutrition on the ability to gain un-programmed and new cell function(s) in established cell lines through various generations (cell divisions) and in model animals (such as C. elegans, Drosophila, Xenopus, mice and rats) during development and differentiation is very effective and powerful, because these influences under the varied conditions can be easily studied by various research groups.

METHODS

Electron microscopy and immuno-electron microscopy

Electron microscopy and immuno-electron microscopy (using rabbit anti-chicken IgM H-chain antiserum as primary antibody) were carried out on exponentially growing DT40 cells and HDAC2(-/-) mutants in some different ways as described [9 and Chaps. 2, 3 and 4].

For immuno-electron microscopy using the pre-embedding staining procedure, exponentially growing HDAC2(-/-) mutant cells at the early (E; ~10 days) and later (L; ~60 days) cultivation stages and DT40

cells (W) were suspended in 1% bovine serum albumin (BSA) and concentrated by a light centrifugation. The resultant cell pellets were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4 ℃ for 60 min. After washing with PB, the sample pellets were treated with or without 0.2% saponin in phosphate buffered saline (PBS) for 30 min to be permeable or non-permeable. After washing with PBS, the sample pellets were incubated with goat anti-chicken IgM H-chain antibody A30-102A (Bethyl Laboratories Inc., TX, USA; diluted 1:1000 with 1% saponin in PBS) at 4 ℃ for overnight. After washing with PBS, the sample pellets were incubated with horseradish peroxidase (HRP)-labeled affinity-purified F(ab')2 fragment Donkey anti-goat IgG(H+L) (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA; diluted 1:1000 in PBS) at 4 ℃ for overnight. After washing with PBS, the sample pellets were fixed with 1% glutaraldehyde in PBS at 4 ℃ for 5 min. After washing with 50 mM Tris-HCl buffer (pH 7.4) (TB), the sample pellets were incubated in 0.05% 3', 3'-diaminobenzidine (DAB) in TB for 20 min and complete DAB solution containing 0.005% H_2O_2 for 10 min. The sample pellets were post-fixed with 1% osmium tetroxide in TB containing 1% potassium ferrocyanide for 30 min, dehydrated with a series of ethanol and embedded in epoxy resin mixture. Ultrathin sections were contrasted with lead citrate for 20 sec and examined with a JEOL 1200 EX electron microscope (JEOL, Tokyo, Japan).

Immunocytochemistry using immuno-gold labeling was carried out as described [9]. Exponentially growing DT40 (W) and HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages were collected by a light centrifugation. The resultant cell pellets were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PB for 30 min. After washing with PB, the samples were post-fixed with 1% osmium tetroxide in PB for 60 min, washed with PB, dehydrated in ethanol and embedded in Epon. Ultrathin sections were picked up on 200-mesh gold grids coated with Formvar film and treated with 5% sodium meta-periodate in distilled water for 30 min. After rinsing in distilled water, the sections were treated with 5% normal horse serum (NHS) and 1% BSA in PBS for 10 min to block non-specific binding and incubated with goat anti-chicken IgM H-chain antibody A30-102A (Bethyl Laboratories Inc., TX, USA; diluted 1:1500 with 5% NHS, 1% BSA in PBS) at room temperature for 60 min. After rinsing in PBS, the sections were incubated with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1:200 with 1% BSA in PBS) for 40 min. After washing with PBS, the sections were incubated with 8 nm colloidal gold conjugated anti-biotin antibody (Vector Laboratories, Burlingame, CA, USA; diluted with 1% BSA in PBS) for 30 min. After washing with distilled water and drying, the sections were contrasted with 2% uranyl acetate in 70% methanol and Reynolds' lead citrate and observed in a JEOL 1200EX transmission electron microscope operating at 80 kV (JEOL, Tokyo, JAPAN). As a control, primary antibody was omitted or replaced by normal goat serum.

POSTSCRIPT

The studies in Chapter 6 were reviewed in Ref. 10.

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FIGURE LEGENDS

Figure 6-1. Localization of IgM H-chain proteins at the peri-nuclear space, endoplasmic reticulum and cell surface of HDAC2(-/-) DT40 mutant cells

HDAC2(-/-) mutant cells were collected at the early (E) stage of cultivation. Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out as in Methods. A) Immuno-electron microscopy after treatment without saponin. B) ~ E) Immuno-electron microscopy after treatment with saponin. Arrows P, E and S indicate positive signals of IgM H-chain proteins localized at the peri-nuclear space, endoplasmic reticulum and cell surface, respectively.

Figure 6-2. Localization of IgM H-chain proteins in the peri-nuclear space of HDAC2(-/-) DT40 mutant cells

HDAC2(-/-) mutant cells were collected at the early (E) stage of cultivation, and immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out as in Methods. A) and B) Immuno-electron microscopy of two individual single mutant cells. D) and E) Enlarged versions of parts indicated by arrows in A) and B), respectively. C) and F) Enlarged versions of immuno-electron microscopy of the peri-nuclear space of one DT40 cell and another HDAC2(-/-) mutant cell, respectively. Accumulated IgM H-chain proteins were observed in the peri-nuclear space (indicated by arrows) of all HDAC2(-/-) mutant cells.

Figure 6-3. Localization of IgM H-chain proteins in the peri-nuclear space of HDAC2(-/-) DT40 mutant cells at early and later stages of continuous cultivation

HDAC2(-/-) mutant cells were collected at the early (E) and later (L) stages of cultivation, and immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out as in Methods. A) Immuno-electron microscopy of one HDAC2(-/-) mutant cell at the early (E) cultivation stage. B) and C) Enlarged versions of parts indicated by arrows b and c in A). D), G) and J) Immuno-electron microscopy of three independent HDAC2(-/-) mutant cells at the later (L) cultivation stage. E), F), H), I) and K) Enlarged versions of parts indicated by arrows e, f, h, i and k in D), G) and J). The mutant cell in G) was the same one in [Chap. 2]. Accumulated IgM H-chain proteins were observed in the peri-nuclear space of all HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages.

Figure 6-4. Localization of IgM H-chain proteins in the peri-nuclear space and a model for signal transduction concerning the accumulation of IgM H- and L-chains to the chromatin structure in HDAC2(-/-) DT40 mutant cells

Left panel: A portion of the peri-nuclear space (where IgM H-chain proteins were accumulated) of the HDAC2(-/-) mutant cell (indicated by an arrow P at lower position in Fig. 6-1C) was reversely enlarged.

Right panel: A model of signal transduction concerning the accumulation of IgM H- and L-chains. Signal concerning artificially accumulated IgM H- and L-chains in the peri-nuclear space of the HDAC2(-/-) mutant cell was repeatedly transmitted to the chromatin structure, followed by unbalanced response and convergence for the signal to specific transcription factor genes in individual mutant clones. ECRR/ECRS: environment change recognition receptor/site. 4C machinery: chromatin conformation change complex (4C) machinery.

Figure 6-5. Summary of alterations in mRNA (gene expression/transcription) levels, acetylation levels of specific Lys residues of histone H3 and the chromatin structure of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in HDAC2(-/-) DT40 mutant clones during continuous cultivation

Alterations in mRNA (gene expression/transcription) levels (-, ++ or ++++), acetylation levels (Ac; -, ++ or ++++) of specific Lys residues of histone H3 and the chromatin structure (form; loose or tight) of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 at the early (E) and later (L) stages of cultivation and in DT40 cells (W) are schematically presented. Chromatin conformation change complex (4C) machinery generally contains a specific member of the HATs plus HDACs and other factors. Transcription factor complex (TFC) machinery generally contains RNA polymerase (RPase), specific member of transcription factors and others.

Figure 6-6. Chromatin conformation change code (4C) theory: A model for gaining un-programmed and new cell function(s) by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications through various generations

Irreversible creation of the chromatin structure plasticity with epigenetic modifications occurs within the proximal 5'-upstream region (notch of chromatin) but not in the ORF region of specific transcription factor gene(s) through various generations. The tight or loose form of the chromatin structure is based on hypo- or hyper-acetylation levels of specific Lys residues of histone H3, and causes low or high gene expression levels.

Figure 6-7. Irreversible creation of the chromatin structure plasticity surrounding proximal 5'-upstream region with epigenetic modifications in descendant cells, and reversible regulations of ordinary gene expression and enzyme reaction in initial cells that accept proper signal

Upper panel: Creation of the chromatin structure plasticity surrounding the proximal 5'-upstream region of a certain specific gene with epigenetic modifications irreversibly occurs in descendant cells but not in cells that initially accepts an environment change signal. Ac, Ac/2 and Ac/10 qualitatively

indicate hyper-, considerable hyper- and somewhat hyper-acetylation levels of specific Lys residues of core histones, respectively. Middle and lower panels: Regulations of both gene expression (at regulatory elements) and enzyme reaction reversibly occur in the cells that initially accept proper signal. Ac and P indicate acetylation, phosphorylation and/or other chemical modifications.

Supplementary Figure 6-S1. 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 mutants at early (E) and later (L) cultivation stages

The figure is a set of Figs. 4-8, 4-9 and 4-10 in [Chap. 4].

Supplementary Figure 6-S2. Localization of IgM H-chain proteins in DT40 and HDAC2(-/-) DT40 mutant cells

Upper panel: Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out on wild-type DT40 cells (left) and HDAC2(-/-) mutants at the early cultivation stage (center and right) as in Methods. Lower panel: Enlarged versions of DT40 and HDAC2(-/-) mutant cells indicated by arrows in the upper panel. Positive signals for IgM H-chain proteins were observed both on the cell surface and in the cytoplasm of HDAC2(-/-) mutant cells but only on the cell surface of DT40 cells.

Supplementary Figure 6-S3. Localization of dense fractions due to accumulated IgM H-chain proteins in HDAC2(-/-) DT40 mutant cells

Electron microscopy was carried out on DT40 (upper panel; left and center) and HDAC2(-/-) mutant cells at the early cultivation stage (lower panel; left and center) as in Methods. Enlarged versions of DT40 and HDAC2(-/-) mutant cells indicated by arrows in the centers are shown in the right of upper and lower panels, respectively. Dense fractions due to accumulated IgM H-chain proteins were observed only in HDAC2(-/-) mutant cells.

Supplementary Figure 6-S4. Alterations in protein levels of IgM H- and L-chains in six individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

The figure is identical with Fig. 4-2 in [Chap. 4].

Supplementary Figure 6-S5. Alterations in gene expressions of IgM H- and L-chains, and various members of HATs, HDACs and transcription factors in four individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

The figure is identical with Fig. 4-6 in [Chap. 4] and shown with some modifications.

Supplementary Figure 6-S6. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-1 of HDAC2(-/-) DT40 mutants during continuous cultivation

The figure is a set of Figs. 5-1, 5-5, 5-9 and 5-13 in [Chap. 5].

Supplementary Figure 6-S7. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-2 of HDAC2(-/-) DT40 mutants during continuous cultivation

The figure is a set of Figs. 5-2, 5-6, 5-10 and 5-14 in [Chap. 5].

Supplementary Figure 6-S8. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-4 of HDAC2(-/-) DT40 mutants during continuous cultivation

The figure is a set of Figs. 5-3, 5-7, 5-11 and 5-15 in [Chap. 5].

Supplementary Figure 6-S9. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-6 of HDAC2(-/-) DT40 mutants during continuous cultivation

The figure is a set of Figs. 5-4, 5-8, 5-12 and 5-16 in [Chap. 5].

Supplementary Figure 6-S10. Summary of alterations in mRNA (transcription/gene expression) levels (high or low), acetylation levels (hyper or hypo) of specific Lys residues of histone H3 and the chromatin structure (form; loose or tight) within proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in DT40 cells (W) and clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at early (E), middle (M) and later (L) stages of cultivation

The figure is identical with Fig. 5-21 in [Chap. 5] and shown with some modifications.