Chapter 6

Chromatin conformation change code (4C) theory: A bio-system for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications via a lot of generations

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SUMMARY

When higher eukaryotic cells firstly encounter moderate intra- and/or extra-cellular environment change, they newly acquire ability to cope with and/or overcome it through irreversible creation of chromatin structure plasticity based on successive chromatin conformational change with epigenetic modifications. Putative environment change recognition receptor/site (ECRR/ECRS) should recognize the environment change. Putative chromatin conformation change complex (4C) machinery should create irreversibly the variety of chromatin structure plasticity not only for various particular genes but also in each individual clone of the same cell type. We advance a chromatin conformation change code (4C) theory for a bio-system to gain new cell function through irreversible creation of chromatin structure plasticity by the 4C machinery via a lot of generations, in order to adapt to the environment change recognized by ECRR/ECRS. The 4C theory should be suitable as an explanation for the manner of cell development and differentiation of higher eukaryotes.

In eukaryotes, genome information and nuclear function should be mainly protected by nuclear membrane, which acts as cytoplasm-nucleus barrier. Additionally, communication and signal transduction between nucleus and cytoplasm, both of which are necessary for expressions of normal cell functions, should be preferentially carried out by the going and returning of large and/or small molecules through nuclear pore acting as a guard station at the barrier. By contrast, both of signal transduction on unexpected and/or disadvantageous change in intra- and/or extra-cellular environment and transport of useless molecules to nucleus must be usually prevented by this barrier system. Then, how higher eukaryotic cells cope with and/or overcome unexpected and/or disadvantageous environment change, when they firstly encounter it. Following four countermeasures should be generally possible. First, in the case of most severe change, cells should die because it is far ahead of their adaptation ability. Second, in the case of considerable severe change, cells should cope with or overcome it by means of alterations in genome information, such as point mutation, insertion, deletion, duplication and multiplication on the DNA molecule via a lot of generations. This mode should be a basis for evolution of species. Third, in the case of moderate change, cells should cope with or overcome it through irreversible creation of chromatin structure plasticity caused by successive chromatin conformational (structural) changes with epigenetic modifications via a lot of generations. This mode should be a basis for differentiation of cells. Fourth, in the case of minor change, cells should respond to it using only an already acquired regulation mechanism.

In chicken wild-type DT40 cells, HDAC2 as a supervisor regulates gene expressions of IgM H- and L-chains through opposite control of those of Pax5, Aiolos, EBF1, OBF1, and Ikaros plus E2A [1, Chap.

4]. On the other hand, 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 transcriptions of the two immunoglobulin genes [1, Chap. 4]. Remarkably, the accumulated IgM H- and L-chains are dramatically and distinctly decreased at the late 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 transcriptions of the two immunoglobulin genes (details will be discussed later) [Chap. 4]. These results obtained from wild-type DT40 cells and HDAC2(-/-) mutants at the early and late cultivation stages are schematically shown in Supplementary Figure 6-S1. Such diminutions of the accumulated IgM H- and L-chains in distinct manners in all of individual HDAC2(-/-) mutant clones during cultivation are really the above-mentioned third case. Presumably, the manner for diminutions of the two immunoglobulin proteins, which were accumulated in Pax5(-) mutant cells, during cultivation fairly resembles this case [Chap. 3]. Here, we focused our attention only on HDAC2(-/-) mutant cells and presented some of the results obtained from them in the concrete.

The HDAC2-deficiency in DT40 cells rapidly and dramatically increases gene expressions of IgM H- and L-chains, followed by accumulation of these 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 accumulated mRNAs and proteins of IgM H- and L-chains at the early stage of cultivation are gradually reduced until the late 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 HDAC2(-/-) mutants (Suppl. Figs. 6-S4 and 6-S5). By contrast, interestingly, gene expressions of several particular transcription factors and chromatin-modifying enzymes change in distinct patterns within these individual clones of HDAC2(-/-) mutants (Suppl. Fig. 6-S5). To reduce the accumulated protein levels of IgM H- and L-chains resulted from their increased gene expressions, apparently, following three distinct manners based on altered gene expressions of particular transcription factors exist at the late cultivation stage in these individual mutant clones (Suppl. Fig. 6-S1(L)) [Chap. 4]. The manner in clone cl.2-1 seems to be dependent on OBF1 and different from that in wild type DT40 cells. The manner in clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seems to be dependent on Pax5 plus Aiolos and slightly similar to that in DT40 in appearance. The manner 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 manners, we performed neighboring overlapping tiling chromatin immuno-precipitation (NotchIP: this abbreviation also means IP on notch of chromatin) assay on proximal 5'-upstream chromatin region (named as notch of chromatin) of each of these particular genes [Chap. 5]. The results obtained by the NotchIP assay revealed that such distinct manners should be fundamentally originated from irreversible creation of distinct chromatin structure plasticity surrounding

proximal 5'-upstream regions of corresponding transcription factor genes with epigenetic modifications via a lot of generations during cultivation. To put it concretely, in wild-type DT40 cells having HDAC2 activity, proximal 5'-upstream chromatin 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]) each are loose (open) form due to no binding ability of histone H3 to DNA based on hyper- (high) acetylation levels of one or more of their particular Lys (K) residues (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 individual clones of HDAC2(-/-) mutants having no HDAC2 activity, chromatin structure of proximal 5'-upstream region of each of these four factor genes is dramatically and severally altered based on changes in acetylation levels of one or more of the five particular Lys residues of histone H3 during cultivation, resulting in alterations in their transcription levels as follows (Suppl. Fig. 6-S5) [Chap. 4].

In clone cl.2-1, at the early stage of cultivation, chromatin structure of each of proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes is 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 particular Lys residues (Suppl. Fig. 6-S6), and as a result transcriptions of these three genes are almost completely suppressed to undetectable levels. However, that of the OBF1 gene is somewhat 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 late cultivation stage, chromatin structure of proximal 5'-upstream regions of the former three genes remains to be tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, their transcriptions also remain unchanged at undetectable level. On the other hand, chromatin structure of proximal 5'-upstream region of the latter one gene changes to tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, its transcription is suppressed to almost undetectable or very low level. These results supported the above-mentioned inference (i.e., OBF1-dependent) on the manner of transcriptions of IgM H- and L-chain genes at the late 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, chromatin structure of each of proximal 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is 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 particular Lys residues of histone H3 (Suppl. Figs. 6-S7 and 6-S8). Therefore, transcriptions of the former three genes are almost completely suppressed and that of the latter one gene is certainly decreased. At the late cultivation stage, contrary to this, chromatin structure of proximal 5'-upstream regions of Pax5, Aiolos and OBF1 genes changes to loose form based on hyper-acetylation levels, and their transcriptions are dramatically or certainly increased. However, that of the EBF1 gene remains tight form based on hypo-acetylation levels, and its

transcription remains low at almost undetectable level. These results supported the above-mentioned inference (i.e., Pax5- and Aiolos-dependent) on the manner of transcriptions of IgM H- and L-chain genes at the late 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 four initially generated HDAC2(-/-) clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) [3] resemble them in several cellular characteristics [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, chromatin structure of each of proximal 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is tight or somewhat loose form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five particular Lys residues of histone H3 (Suppl. Fig. 6-S9). Therefore, transcriptions of the former three genes are almost completely suppressed and that of the latter one gene is certainly decreased. By contrast, at the late cultivation stage, chromatin structure of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes changes to loose form based on hyper-acetylation levels, and their transcriptions are dramatically or certainly increased. These results supported the above-mentioned inference (i.e., Pax5-, Aiolos- and EBF1-dependent) on the manner for transcriptions of IgM H- and L-chain genes at the late cultivation stage in clone cl.2-6.

Results on alterations in acetylation levels of the five particular Lys (K) residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3), chromatin structure of proximal 5’-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their transcription levels 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 represented in Supplementary Figure 6-S10. To make sure, we should like to mention some following important comments on the above-described results concerning the suppression of excessive expressions of IgM H- and L-chain genes in HDAC2(-/-) mutants during cultivations. Excessively accumulated IgM H- and L-chain proteins resulted from their dramatically increased gene expressions in HDAC2(-/-) mutants just after their birth (by gene targeting techniques) [3, 4, Chap. 4] should be unfavorable and useless for them; therefore, the mutant cells have come to acquire a new ability to diminish increased gene expressions of these two immunoglobulin proteins as uncomfortable environment change via a lot of generations during cultivation. In all of HDAC2(-/-) mutant clones, even in clone cl.2-6 [Chap. 4], the manners to suppress transcriptions of IgM H- and L-chain genes at the late cultivation stage should be surely distinct from the ordinary and reversible transcription regulations of the two immunoglobulin genes in wild type DT40 cells, i.e., gene expressions of IgM H- and L-chains are indirectly regulated by HDAC2 through transcription regulations of Pax5, Aiolos, EBF1, OBF1 and also E2A genes 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, were also dramatically and separately altered 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 should be obviously altered in individual HDAC2(-/-) mutant clones during cultivation. Such presumable altered characteristics may be complicated and diverse in each of HDAC2(-/-) mutant clones, even though they are 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 hypothesis on manners to gain new cell function for diminution of the artificially accumulated IgM H- and L-chains through irreversible creation of varied chromatin structure plasticity of distinct genes with epigenetic modifications during continuous cultivation in individual clones of HDAC2(-/-) mutants.

First, we assume putative environment change recognition receptor/site (ECRR/ECRS), which participates in the recognition of accumulation of IgM H- and L-chains as unfavorable environment change (and probably acts in part in the signal transduction on the accumulation to chromatin structure) (Fig. 6-4). In addition, putative 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 alter directly and irreversibly chromatin structure surrounding proximal 5'-upstream regions of a set of particular genes (and also to act in part in the signal transduction) (Figs. 6-4 and 6-5). Using ECRR/ECRS, the 4C machinery and other components, the chain reaction of response for the environment change should occur as follows. Excessive amounts of IgM H- and L-chains artificially caused by the HDAC2-deficiency are first accumulated within 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 two accumulated immunoglobulin proteins (probably exist as a high molecular weight complex with IgM H- and L-chains each other [1]) are gradually secreted to outside of cells, whereas, as indicated by immuno-electron microscopy some of them are transported to nuclear envelope but not inside of nucleus and kept at peri-nuclear space at the early and also late cultivation stages (Figs. 6-1, 6-2, 6-3 and 6-4). These accumulated immunoglobulin proteins at peri-nuclear space should bind to ECRR/ECRS localized on inner nuclear membrane (where hetero-chromatin is possibly located) (Fig. 6-4). After ECRR/ECRS recognizes the accumulation of IgM H- and L-chains as unfavorable environment change, the signal concerning it is genome-widely transmitted to chromatin structure surrounding proximal 5'-upstream regions (notches of chromatin) of numerous genes (probably located on several distinct chromosomes) encoding transcription factors, chromatin-modifying enzymes and related factors/enzymes. Following the initial signal transduction,

spontaneous unbalanced response to the environment change is consecutively and separately converged not only on the particular 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 contains HDAC2 as HDAC activity, a particular 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 should be dramatically changed to remove or drastically reduce HAT activity (of the assumed member of HATs) associated with the HDAC2-deficiency. Through the above-mentioned processes during cultivation, the 4C machinery should come to newly contain a different member of each of HDACs (except HDAC2) plus HATs and other factors, and thereby becomes varied. The diversity of alterations in chromatin structure is preferentially originated from varied acetylation and deacetylation levels of one or more of the particular Lys residues at N-terminal tail of histone H3 [Chap. 5] caused by collaboration of proper member of each 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) lead to irreversible creation of distinct chromatin structure plasticity surrounding proximal 5'-upstream regions of the above-mentioned targeted genes (Fig. 6-6). Accordingly, chromatin structure of proximal 5'-upstream regions possessing hyper-acetylation levels of one or more of the particular Lys residues of histone H3 is loose form based on their no binding ability to DNA, but that of proximal 5’-upstream regions possessing hypo-acetylation levels of one or more of the particular Lys residues of histone H3 is tight form based on their binding ability to DNA (Fig. 6-6). By contrast, probably, the 4C machinery cannot change chromatin structure surrounding open reading frames (coding regions) of the targeted genes so much [Chap. 5]. Thus, as the need arises, transcription factor complex (TFC) machinery (which consists of RNA polymerase, proper transcription factor(s), certain member of each of HATs and HDACs and other factors) is able to bind to promoter regions (or elements) within loose (but not tight) form of chromatin structure surrounding proximal 5’-upstream regions of targeted (but not untargeted) genes (which have become latently active (but not inactive) state), and thereby initiates transcriptions of them (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 manners through increases and/or decreases in transcriptions of Pax5, Aiolos, EBF1 and OBF1 genes, even though these clones are completely the same cell type and was also established cell line [1, Chaps. 2 and 4]. Naturally, these distinct manners are not under the control of HDAC2 but based on irreversible creation of their distinct chromatin structure plasticity with epigenetic modifications during cultivation [Chap. 5].

We expanded the above-mentioned hypothesis on the exclusion of IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants to a universal hypothetic concept (principle) for gain of a new cell function through irreversible creation of varied chromatin structure plasticity of a set of particular genes with epigenetic modifications via a lot of generations (cell divisions) in individuals of the same cell type of higher eukaryotes whose genotype is completely the same.

When higher eukaryotic cells firstly in their life encounter the change in intra- and/or extra-cellular environment, in order to adapt for or eliminate the change (if uncomfortable), they have gradually gained new cell function via a lot of generations. Namely, the cells have acquired the ability to adapt themselves to newly encountered environment change and/or to exclude the painful environment change. Using ECRR/ECRS, the 4C machinery and other components, the cells should cause the chain reaction of response for the environment change (Figs. 6-4 and 6-5). First of all, the environment change should be recognized by means of ECRR/ECRS, which may be localized nearby nuclear membrane as a cytoplasm-nucleus barrier (probably at inner nuclear membrane where hetero-chromatin is possibly located) (Fig. 6-4). Naturally, there is a great possibility that at this step putative particular molecule(s) should act as intermediary sensor in the manner for the recognition of the environment change. As a next step, putative signal(s) concerning the change should be genome-widely transmitted to chromatin structure within nucleus via numerous generations (cell divisions). Following the initial acceptance of the putative signal(s) at chromatin structure, gene expressions of various chromatin-modifying enzymes, transcription factors and/or related enzymes/factors may slightly change, associated with a slight alteration in their chromatin structure. The transduction of the signal(s) and spontaneous unbalanced response for the environment change should be successively repeated and converged into restricted chromatin structure surrounding proximal 5'-upstream chromatin regions (notches of chromatin) of corresponding and/or related factor and/or enzyme genes. Finally, this successive signal transduction concerning the environment change should cause various epigenetic modifications of histone proteins and/or DNA within the restricted chromatin regions with acetyl group, methyl group, phosphate group and/or others.

The 4C machinery, which consists of particular member(s) of each of HATs plus HDACs and other factors, should preferentially participate in these epigenetic modifications (Fig. 6-5). Of these various epigenetic modifications, acetylation and/or deacetylation of particular Lys residues of core histones may be major ones. Participating positions of particular Lys residues and/or kinds of core histones should be diverse. For instance, in the above-mentioned case [Chap. 5], acetylation and/or 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 Lys residues of histone H3 with acetyl group change within proximal 5'-upstream chromatin regions of the above-mentioned particular targeted genes via a lot of generations (cell divisions). Distinct functions of the protean 4C machineries on such acetylation and/or

deacetylation levels should be preferentially based on different combinations of member of each of HATs and HDACs in them, because any HAT and HDAC members' own activities are probably unchangeable, just as those of almost all enzymes in any biological reactions. By contrast, these protean 4C machineries may not participate so much in alterations in chromatin structure surrounding open reading frames (coding regions) of corresponding genes.

The binding ability of the N-terminal tail of histone H3 to DNA is tentatively and qualitatively deduced from acetylation levels of one or more of these particular 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 should induce no binding or full binding ability, resulting in loose (open) or tight (closed) form of chromatin structure. Thus, chromatin structure plasticity should be 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 loose form of chromatin structure surrounding proximal 5'-upstream regions of targeted genes (which are latently active state), followed by initiation of their transcriptions (Fig. 6-5). By contrast, the TFC machinery cannot bind to promoter regions (or elements) within tight form of chromatin structure surrounding proximal 5'-upstream regions of untargeted genes (which are latently inactive state), and thereby cannot initiate their transcriptions. Consequently, loose and tight forms of chromatin structure surrounding proximal 5'-upstream regions should respectively cause high and low (or no) transcription levels of corresponding genes (Fig. 6-6). Notably, manners for irreversible creation of chromatin structure plasticity are distinct in individual clones of the same cell type having the same genotype, even though the environment change and signal(s) on it are the same for all of them. That is, to gain new cell function, individual cell clones each should possess ability not only to change complicatedly and diversely chromatin structure surrounding proximal 5'-upstream regions of a set of numerous particular genes but also to separately alter even proximal 5'-upstream chromatin structure of the same gene into varied forms. Thus, gene expressions of the particular enzymes and factors should be changed diversely in individual cell clones via a lot of generations (cell divisions), in spite of the same environment change. In consequence, individual cell clones are newly able to gain the same and/or distinct cell function(s) in different manners, to accommodate themselves for a new environment.

In conclusion, in higher eukaryotes, for gain of new cell function, somatic cells (and also tumor cells) exhibit pluri-potency, elasticity and flexibility, all of which should be basically originated from those of chromatin structure. Namely, in order to adapt to intra- and/or extra-cellular environment change, somatic cells of higher eukaryotes possess ability to gain new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications, i.e., from tight to loose forms or vice versa of chromatin structure surrounding proximal 5'-upstream region(s) of particular gene(s) (Fig. 6-6). Such tight or loose form of the proximal 5'-upstream chromatin structure should be latently inactive or

active state for transcription ability of the corresponding gene, although the proximal 5'-upstream region as mere nucleotide sequences may be potential but silent state for its transcription. Variety of chromatin structure plasticity in individual cell clones should be triggered by the spontaneous unbalanced response for the environment change when they firstly encounter with it and irreversibly accomplished by the successive unbalanced convergence of the response via a lot of generations (cell divisions). Plasticity of chromatin structure in somatic cells should be created in distinct manners, which probably depend on their antecedents and successive unbalanced response for the change via a lot of generations (cell divisions). Moreover, chromatin structure plasticity, regardless of whether its creation is in the course or was already completed, should be inherited to descendant generations associated with or without additional structural change via cell divisions. Thus, remarkably, irreversible creation of chromatin structure plasticity should occur in descendant cells but not in the cell which initially accepts the signal on the environment change, although reversible regulations of ordinary gene expressions and/or enzyme reactions occur in the cell itself which accepts proper signal (Fig. 6-7). Probably, irreversible creation of chromatin structure plasticity, with the intention of adapting to the environment change, should occur inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region (as loose or tight form) should direct the switch (on or off) for latent transcription ability of the corresponding gene through irreversible creation of chromatin structure plasticity; therefore, besides "notch" from a structural side-view as mentioned above, the proximal 5'-upstream chromatin region could be regarded as "director" from a functional side-view. Naturally, the notch (or director) covers specific nucleotide sequences of transcriptional elements (such as promoter, operator and others) and also their neighboring nucleotide sequences. We should like to emphasize that real recipient of the signal on the environment change may be just chromatin structure itself as three-dimensional conformation which is dynamic and changeable between tight and loose forms, but not mere chromatin and chromosome themselves as one- (or two-) dimensional conformation which are static and unchangeable. That is, 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 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 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 particular signals and to express their own biological functions. However, concrete data are not enough to support these ideas so far.

Finally, we name such bio-system for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications, which should be one of the most important manners for life conservation and cell-type determination of higher eukaryotes, a chromatin conformation

(structure) change code (4C) theory. Therefore, the 4C theory should open the door for acquisition of new cell function of higher eukaryotes and innovate the general notion on nature of somatic cells. Probably, the supposed number of codes in the 4C theory, which should determine complicated and varied characteristics of higher eukaryotic cells, can be roughly estimated based on combination (multiplication) of the number of candidate genes and that of codes for each of these genes as follows. Most influential candidates should be genes of transcription factors, chromatin-modifying enzymes and related factors/enzymes, which are necessary for gain of new cell function and cell-type determination in higher eukaryotes. The number of codes for each of these candidate genes should be two. Because loose or tight form of the proximal 5'-upstream chromatin region, as latently active or inactive state, directs the switch (on or off) for transcription ability of the corresponding gene. Moreover, the 4C theory should be suitable as an explanation for 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 exclusion of artificially accumulated IgM H- and L-chains in HDAC2(-/-) DT40 mutant cells [Chaps. 2, 4 and 5]. 1) Despite the HDAC2-deficiency, why acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes are reduced at the early stage of cultivation in HDAC2(-/-). 2) Why the decreased acetylation levels of one or more of the particular Lys residues of histone H3 within proximal 5'-upstream chromatin regions of these genes are increased during cultivation. Why the case of the OBF1 gene is reverse. 3) It must be determined 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. 4) Functional and steric differences between loose and tight forms (based on hyper- and hypo-acetylation levels of one or more of the particular Lys residues of histone H3) of chromatin structure surrounding proximal 5'-upstream regions of these genes must be clarified more minutely. 5) Why changing patterns in acetylation levels of the particular Lys residues of histone H3 during cultivation in individual transcription factor genes differ among individual mutant clones. 6) Why changing patterns in acetylation levels of the particular Lys residues of histone H3 during cultivation in each individual transcription factor gene differ within individual mutant clones. 7) How the 4C machinery (which acts in irreversible creation of 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 open reading frame of the corresponding gene) may be almost

the same as the well-known transcription machinery. 8) To demonstrate the 4C theory, both of putative ECRR/ECRS as a first player to recognize the environment change and putative 4C machinery as a final player to irreversibly create chromatin structure plasticity must be clarified. 9) As concrete approach to generalize the 4C theory, for instance, elucidation of influences of changes in temperature, atmosphere and/or nutrition on ability to gain new cell function in established cell lines via a lot of generations (cell divisions) and in model animals (such as mice and rats) during development and differentiation should be very effective and powerful.

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 manners as described [9, Chaps. 2, 3 and 4].

For immuno-electron microscopy using pre-embedding staining procedure, exponentially growing HDAC2(-/-) mutant cells at the early (E; ~10 days) and late (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 nonpermeable. 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 postfixed 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 late (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 postfixed 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 nonspecific 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.

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FIGURE LEGENDS

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

HDAC2(-/-) mutant cells were collected at the early 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 peri-nuclear space, endoplasmic reticulum and surface, respectively.

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

HDAC2(-/-) mutant cells were collected at the early 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 peri-nuclear space of one DT40 cell and another HDAC2(-/-) mutant cell, respectively. Accumulated IgM H-chain proteins were observed in peri-nuclear space (indicated by arrows) of all HDAC2(-/-) mutant cells.

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

HDAC2(-/-) mutant cells were collected at the early (E) and late (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 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 late 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 peri-nuclear space of all HDAC2(-/-) mutant cells at the early and late cultivation stages.

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

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

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

Alterations in mRNA levels (-, ++ or ++++), acetylation levels (Ac; -, ++ or ++++) of particular Lys residues of histone H3 and 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 late (L) stages of cultivation and DT40 cells (W) are schematically represented. Chromatin conformation change complex (4C) machinery should generally contain particular member of each of HATs plus HDACs and other factors. Transcription factor complex (TFC) machinery should generally contain RNA polymerase (RPase), particular transcription factors and others.

Figure 6-6. A model of chromatin conformation change code (4C) theory for gain of new cell function through irreversible creation of chromatin structure plasticity with epigenetic modifications via a lot of generations

Irreversible creation of chromatin structure plasticity with epigenetic modifications occurs in proximal 5'-upstream region (notch of chromatin) but not in open reading frame of particular gene via a lot of generations. Tight or loose form of chromatin structure is based on hypo- or hyper-acetylation levels of particular Lys residues of histone H3, and causes low or high transcription levels.

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

Upper panel: Creation of chromatin structure plasticity with epigenetic modifications surrounding proximal 5'-upstream region of particular gene occurs irreversibly in descendant cells but not in initial cell, which accepts environment change signal. Ac, Ac/2 and Ac/10 indicate qualitatively hyper-, considerable hyper- and somewhat hyper-acetylation levels of Lys residues of core histones, respectively. Middle and lower panels: Regulations of both gene expression (on regulatory elements) and enzyme reaction occur reversibly in the cell itself, which accepts proper signal. Ac and P indicate acetylation, phosphorylation and/or other chemical modifications.

Supplementary Figure 6-S1. Manners for control of gene expressions of IgM H- and L-chains through control of gene expressions of particular transcription factors in the presence or absence of HDAC2 in wild-type DT40 cells (W) or all and individual clones of HDAC2(-/-) mutants at early (E) and late (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-chains in DT40 and HDAC2(-/-) 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-chains are observed both at cell surface and cytoplasm of HDAC2(-/-) mutant cells but only at cell surface of DT40 cells.

Supplementary Figure 6-S3. Localization of dense fractions due to accumulated IgM H-chains 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-chains are 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 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, HATs, HDACs and transcription factors in four individual clones of HDAC2(-/-) DT40 mutants during 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 particular 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 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 particular 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 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 particular 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 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 particular 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 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 on alterations in transcription levels (high or low), acetylation levels (hyper or hypo) of particular Lys residues of histone H3 and chromatin structure (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 late (L) stages of cultivation

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