Chapter 7

All-inclusive review and history on the 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

In the chicken DT40 cell line, histone deacetylase-2 (HDAC2) indirectly regulates gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, OBF1, and Ikaros plus E2A. The HDAC2-deficiency induces dramatic accumulations of mRNAs and proteins of IgM H- and L-chains, and thereafter the accumulated mRNAs and proteins are gradually and dramatically reduced in almost similar changing pattern in all individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation. By contrast, gene expressions of Pax5, Aiolos, EBF1 and OBF1 remarkably show distinct changing patterns in individual HDAC2(-/-) mutant clones during continuous cultivation. At the later stage of cultivation, there exist at least three distinct ways of gene expressions of IgM H- and L-chains, i.e., OBF1-dependent, Pax5- plus Aiolos-dependent, and Pax5-, Aiolos- plus EBF1-dependent types. These distinct alterations in gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual clones of HDAC2(-/-) mutants are originating from irreversible chromatin conformation (structure) changes based on varied changes in acetylation levels of specific Lys residues of histone H3 within the chromatin surrounding their proximal 5'-upstream regions during continuous cultivation. Based on these results, universally, we named bio-system for gaining un-programmed and new cell functions by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications during various generations as the chromatin conformation change code (4C) theory.

Outline of the 4C theory is concretely as follows. 1) Somatic cells of higher eukaryotes are pluri-potent, elastic and flexible for gaining un-programmed and new cell functions to cope with and/or overcome an abnormal environment change, when they firstly encounter with it. 2) These pluri-potency, elasticity and flexibility are originating from those of the chromatin structure. 3) Un-programmed and new cell functions to adapt to and/or eliminate an abnormal environment change are gained by means of irreversible creation of the chromatin structure plasticity surrounding proximal 5'-upstream region(s) of specific transcription factor gene(s). 4) Plasticity of the chromatin structure (from the tight to loose forms or vice versa) is continuously and irreversibly created based on the chromatin conformation change with epigenetic modifications through various generations (cell divisions). 5) Diversity of the chromatin structure plasticity in individual cells of the same type is triggered by spontaneous unbalanced response to the environment change and accomplished by its successive convergence through various generations. 6) Irreversible creation of the chromatin structure plasticity probably depends on antecedents of somatic cells and successive response to the environment change, and occurs in descendent cells but not in the cell that initially meets with it. 7) Irreversible creation of the chromatin structure plasticity occurs inevitably but not incidentally and/or neutrally. 8) The environment change is recognized by putative environment change recognition receptor/site (ECRR/ECRS) and the chromatin structure plasticity is irreversibly created by putative chromatin conformation change complex (4C) machinery. 9) The chromatin structure of proximal 5'-upstream region(s) of the specific gene(s), as dynamic and changeable

three-dimensional conformation, receives the signal on the environment change. 10) The chromatin structure (the loose or tight form) of the proximal 5'-upstream region of a specific gene directs switch (on or off) for its latent transcription ability. 11) The proximal 5'-upstream chromatin region of a certain gene is regarded as a "notch of chromatin" from a structural point of view and a "director for gene expression" from a functional point of view. 12) The number of codes in the 4C theory, which determines varied cell functions and cell-types of higher eukaryotes, is estimated based on combination of the number of candidate genes and the number (probably two) of codes for each of these genes.

In eukaryotes, alterations in the chromatin structure are remarkably involved in regulations of gene expressions, and DNA replication, repair plus recombination and others [1-5, Chap. 1], and also in the lymphocyte development and differentiation [6-12]. On the other hand, numerous transcription factors, including Ikaros, PU.1, E2A, GATA-3, EBF, Pax5 and others, are involved in regulations of the development and differentiation of lymphocytes [13-22]. In addition, transcriptional regulation of the IgM H-chain gene requires USF, TFEB, Ig/EBP, NF-IL6, OCA-b and others as promoter binding proteins, and Ig/EBP, NF-IL6, YY-1, E2A, PU.1 and others as intron enhancer binding proteins. Meanwhile, of various chromatin-modifying enzymes participating in alterations in the chromatin structure, histone acetyltransferase(s) (HATs) and histone deacetylase(s) (HDACs) cooperatively control acetylation and deacetylation levels of specific Lys residues of core histones H2A, H2B, H3 and H4 [23-46]. To assess physiological roles of individual members of HDACs and HATs, we have systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines, each of which is devoid of a specific member of HDACs and HATs [47-63], by gene targeting techniques using two different targeting vectors [64-74]. Our previous findings in initially generated HDAC2-deficient mutant cells, HDAC2(-/-), revealed not only that HDAC2 controls the amount of IgM H-chain at the steps of its gene expression plus alternative processing of its pre-mRNA [47], but also that it down-regulates the IgM L-chain gene promoter activity [50]. Moreover, the HDAC2-deficiency has varied severe and/or moderate effects on several cellular characteristics. That is, the deficiency represses gene expressions of HDAC7, Pax5, Aiolos, Ikaros and EBF1, elevates gene expressions of HDAC4, HDAC9, PCAF and E2A, and changes bulk acetylation levels of several Lys residues of core histones [54].

To know individual roles of these changed chromatin-modifying enzymes and transcription factors on regulation of gene expressions of IgM H- and L-chains, we generated homozygous DT40 mutant cell lines: EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), respectively, devoid of EBF1, Aiolos, E2A and Helios genes [54, 75-79], and Pax5-deficient DT40 mutant cell line, Pax5(-), devoid of the Pax5 gene existing on Z sex chromosome that is monosomy in chickens (USCS Genome Browser data base) [Chap. 3]. In addition, we generated Ikaros-down DT40 mutant cell line, Ikaros(-/-/+), devoid of two alleles of the Ikaros gene existing on chromosome 2 that is trisomy in chickens (our unpublished data). Analyses

of these resultant mutants revealed that Pax5, EBF1, Aiolos plus Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates gene expressions of these two immunoglobulin proteins [54]. These results, together with others [80], indicated that in wild-type DT40 cells HDAC2 indirectly regulates gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, OBF1, Ikaros and E2A (Fig. 7-1(W)) [54, 57].

Hereafter, in all of our studies any cultivation periods (stages and/or days) were practically counted from the first day of cultivation from the stock at -80 °C, although until the stock approximately 15 ~ 16 days had already passed since the disruption of two HDAC2 alleles (or one Pax5 allele) [47, Chaps. 2, 3, 4 and 5]. Throughout further qualitative analyses of characteristics of initially generated HDAC2(-/-) mutant cells [47], which were cultivated for different periods, surprisingly, we accidentally noticed interesting and amazing phenomena as follows [Chap. 2]. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) showed that amounts of IgM H-chain and L-chain (detected as two spots) are dramatically and considerably increased at the early (~10 to 20 days) stage of cultivation, and thereafter gradually decreased through the middle (~30 to 40 days) stage and at the later (~60 days) cultivation stage reach comparable levels as in DT40 cells (Fig. 7-2). By contrast, insignificant changes are observed for most of other major cellular proteins during cultivation. Western blotting, which was carried out at shorter interval periods, using antibodies for chicken IgM H-chain and L-chain that cross-reacts with IgM H-chain, sufficiently confirmed the above-mentioned results obtained by 2D-PAGE. Immuno-electron microscopy using antibody specific for chicken IgM H-chain showed that IgM H-chain is obviously accumulated at the early stage and thereafter at the later stage reduced to almost the same level as in DT40 cells (Fig. 7-3). These results, together, indicated not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage (Fig. 7-1(E)), but also that these accumulated immunoglobulin proteins are gradually reduced during cultivation and finally at the later stage reach comparable levels as in DT40 cells (details will be shown later) (Fig. 7-1(L)). Reverse transcription-polymerase chain reaction (RT-PCR) using primers IgM Hc plus IgM Hs showed that whole and secreted forms of IgM H-chain mRNA are dramatically increased at the early stage, and thereafter gradually reduced during cultivation and at the later stage reach very close levels as in DT40 cells (Fig. 7-4). Remarkably, RT-PCR, using appropriate primers specific for various genes encoding chromatin-modifying enzymes and transcription factors, showed that gene expressions of HDAC7, HDAC9 and PCAF are gradually elevated, and those of EBF1, Pax5 and Aiolos are certainly change in distinct patterns during cultivation (details will be shown later). Immuno-blotting, using antibodies specific for various acetylated Lys (K) residues of core histones H2A, H2B, H3 and H4, showed that in spite of the HDAC2-deficiency, bulk acetylation levels of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) are gradually increased during cultivation, though insignificant changes are observed in those of most of remaining Lys residues of core histones.

Because the gene expression of Pax5 is controlled by HDAC2 and among the above-mentioned altered transcription factors, Pax5 mainly controls gene expressions of IgM H- and L-chains [54], we studied the molecular mechanism of the gene expression of Pax5 [Chap. 2]. Until we started this study, the fact that the chicken Pax5 gene exists on Z sex chromosome that is monosomy was not reported, and then nucleotide sequences of its 5'-upstream region were not yet deposited in any database, although those of its cDNA and several homologous genes could be cited from a databank. Therefore, first, we directly cloned the proximal ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques [54], including Southern blotting, colony hybridization and sub-cloning, and determined its nucleotide sequences (GenBank accession number: LC060666) [81, 82]. Dual-luciferase assay carried out on this Pax5 5'-upstream region, using various 5'- and 3'-deletion plasmid constructs, suggested that two distinct proximal 5'-upstream regions are presumably necessary to negatively control the gene expression, whereas clearly defined promoter region(s) or element(s) is not yet elucidated (our unpublished data). Moreover, using site-specific antibody for acetylated K9 residue of histone H3 (K9/H3) and several appropriate primers, we carried out chromatin immuno-precipitation (ChIP) assay on the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene in initially generated HDAC2(-/-) mutant cells collected at the early and later cultivation stages and in DT40 cells [Chap. 2]. These cultivation stages were vague and temporary, since these tested mutant cells had been already cultivated several times for other experiments. However, surprisingly, the preliminary ChIP assay suggested that in HDAC2(-/-) mutant cells acetylation levels of K9/H3 within some limited segments of the proximal 5'-upstream chromatin region of the Pax5 gene are decreased at the early stage and thereafter at the later stage increased to almost the same levels as in DT40 cells (details will be shown later). These results roughly agreed with the findings on changing patterns in the gene expression of Pax5 mentioned above.

As mentioned above, gene expressions of IgM H- and L-chains are mainly and indirectly regulated by HDAC2 through control of gene expressions of various transcription factors, especially Pax5 [54, Chaps. 2 and 3]. We performed time-course studies on some characteristics of Pax5(-) mutant cells at the early (~8 days), middle (~13 days) and later (~20 days) cultivation stages [Chap. 3], which were relatively shorter intervals than those adopted for HDAC2(-/-) mutant cells [Chap. 2]. 2D-PAGE revealed that protein levels of IgM H- and L-chains are drastically and considerably increased at the early stage, and thereafter gradually decreased during cultivation and at the later stage reach almost the same levels as in DT40 cells (our unpublished data). Western blotting using antibody for chicken IgM L-chain (which cross-reacts with IgM H-chain) showed that IgM H-chain and L-chain (detected as two bands) are dramatically and considerably increased at the early stage, and thereafter gradually decreased through the middle to later stages to almost the same levels as in DT40 cells (Fig. 7-5). Furthermore, electron

microscopy revealed that dense cytoplasmic fractions probably due to artificially accumulated IgM H- and L-chains are detected at the early stage in Pax5(-) mutant cells but not at the later stage as in DT40 cells. Immuno-electron microscopy using antibody specific for chicken IgM H-chain showed that the immunoglobulin proteins are surely accumulated at the early stage, and thereafter most of them disappeared at the later stage as in DT40 cells. Together, these results indicated not only that IgM H- and L-chains are dramatically and considerably accumulated at the early stage in Pax5(-), but also that these accumulated immunoglobulin proteins are rapidly reduced during cultivation and finally at the later stage reach comparable levels as in DT40 cells. Microscopy showed that in any different ranges of vision and their magnified visions, Pax5(-) is observed to be the dispersive form at both the early and later stages, similar to that of DT40 cells (Fig. 7-6). Such a morphological property of Pax5(-) and its changing pattern are clearly different from those of HDAC2(-/-) [Chap. 4], as will be discussed later. RT-PCR using primers IgM Hc and IgM Hs revealed that the whole and secreted forms of IgM H-chain mRNA are dramatically elevated at the first (~4 days) stage (prior to the early stage) in Pax5(-), and thereafter rapidly reduced through the early and middle stages and finally at the later stage reach almost the same levels as in DT40 cells (Fig. 7-7). On the other hand, RT-PCR using primers IgM Hm and IgM L showed that the membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA are considerably increased at the first stage, and thereafter slowly decreased through the early and middle stages and at the later stage reach almost the same levels as in DT40 cells. RT-PCR, using appropriate primers specific for various genes encoding chromatin-modifying enzymes and transcription factors, showed not only that PCAF and HDAC9 mRNA levels are gradually elevated from the first through early and middle stages and at the later stage reach almost plateau levels, but also that the mRNA level of HDAC7 moderately changes during cultivation. In addition, Aiolos and OBF1 mRNA levels are gradually reduced from the first through early to middle stages and become undetectable at the later stage. Ikaros and E2A mRNA levels are drastically elevated at the first stage, and thereafter gradually and certainly decreased through the early to middle stages and at the later stage reach almost the same levels as in DT40 cells. The EBF1 mRNA level is completely reduced at the first stage and remains unchanged as an undetectable level throughout cultivation. The PU.1 mRNA level is obviously reduced at the first stage, and thereafter gradually elevated during cultivation and at the later stage reach almost the same level as in DT40 cells.

Based on these findings obtained by qualitative analyses of initially generated HDAC2- and Pax5-deficient DT40 mutants, HDAC2(-/-) and Pax5(-) [47, 54, Chaps. 2 and 3], we revealed that in these two mutant cells IgM H- and L-chains artificially accumulated at the early (and first) cultivation stage are diminished based on their decreased gene expressions, associated with alterations in gene expressions of various transcription factors and/or chromatin-modifying enzymes through various generations during cultivation. However, several cellular characteristics are obviously different between HDAC2(-/-) and

Pax5(-). The characteristics of HDAC2(-/-) will be shown in further detail as follows.

To further clarify the above-mentioned results in detail and to eliminate effects of drug-resistant genes within targeting vectors, we newly generated HDAC2-deficient DT40 mutant cells HDAC2(-/-) (Fig. 7-8) [Chap. 4], using two targeting vectors different from those used previously [47]. By systematical analyses of these newly generated HDAC2(-/-) mutant cells, we obtained again following remarkable and noticeable results. As shown in Figures 7-9 and 7-10, in HDAC2(-/-) mutant cells, surely, proteins and mRNAs of IgM H- and L-chains are dramatically and considerably accumulated at the early (~3 to 7 days) cultivation stage, which was earlier than that adopted for initially generated HDAC2(-/-) mutant cells [Chap. 2], i.e., soon after the generation (birth). It is because HDAC2 as a supervisor mainly regulates gene expressions of these two immunoglobulin proteins through opposite controls of Pax5, Aiolos, EBF1, Ikaros, OBF1 and E2A gene expressions in wild-type DT40 cells (Fig. 7-1(W)) [54]. These results obtained in HDAC2(-/-) mutant cells at the early stage of cultivation are schematically shown in Figure 7-1(E). The majority of artificially accumulated IgM H- and L-chains in HDAC2(-/-) mutant cells exist as a native soluble form capable of building a high molecular weight complex with each other within endoplasmic reticulum (Fig. 7-11), since the HDAC2 mediated regulatory mechanisms do not function any longer and lacking of the mechanisms may be far superior to the capacity to secrete accumulated immunoglobulin proteins. In addition, HDAC2(-/-) mutant cells at the early stage exist as a morphologically aggregative and distorted form (Fig. 7-12), the reason for this is still unknown. Anyhow, both of the accumulation of the two immunoglobulin proteins and the aggregative form should be abnormal and/or uncomfortable (painful) for HDAC2(-/-) mutant cells themselves. Surprisingly but somewhat expectedly, in all individual clones of HDAC2(-/-) mutants, the artificially accumulated proteins and mRNAs of IgM H- and L-chains at the early stage are dramatically and gradually reduced through the middle (~30 days) stage and at the later (~60 days) stage reach almost the same levels as in DT40 cells (Figs. 7-9, 7-10 and 7-11). Agreed with these changes, the morphology of HDAC2(-/-) mutant cells also changes; i.e., the aggregative form at the early stage changes during cultivation until the later stage to the dispersive form, which should be normal and/or comfortable (peaceful) for the mutants, as for DT40 cells (Fig. 7-12). In addition to the above-mentioned findings, very recently, we first noticed following interesting and important facts on the results which had been already obtained by immuno-electron microscopy of initially generated HDAC2(-/-) mutant cells [Chap. 6]. IgM H- and L-chains artificially and excessively synthesized caused by the HDAC2-deficiency are first accumulated within endoplasmic reticulum of HDAC2(-/-) mutant cells as described above. In parallel and/or subsequently, most of these accumulated immunoglobulin proteins are gradually secreted to outside of mutant cells (into cultivation media), however, a part of them is transported to the nuclear envelope but not inside of nucleus, and kept in the peri-nuclear space not only at the early but also at the later stages of

cultivation. These findings will be discussed in detail later.

At this step, concerning the ways to eliminate large amounts of IgM H- and L-chains as an abnormal and uncomfortable intra-cellular (and/or extra-cellular if existence) environment change for HDAC2(-/-), we built up a following brief working hypothesis [Chap. 4]. Putative signal(s) concerning the accumulation of IgM H- and L-chain proteins (and probably the aggregative cell form) is transmitted to the chromatin (structure) within nucleus during cultivation, though the mechanism and the machinery still remain quite unknown. Gene expressions of numerous genes encoding chromatin-modifying enzymes and transcription factors change, associated with alterations in their chromatin structure. The putative signal(s) on and response(s) to the abnormal environment change are repeatedly converged into the chromatin structure of PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, OBF1, Oct2, Blimp1, XBP-1 and other genes during cultivation. Interestingly, as will be described below in detail, mRNA (i.e., gene expressions/transcription) levels of these altered transcription factors and chromatin-modifying enzymes show distinct changing patterns during cultivation in six 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 (Fig. 7-10) [Chap. 4], regardless of almost similar changing pattern in mRNA and protein levels of IgM H- and L-chains and in the cell morphology.

In clone cl.2-1, mRNA levels of Pax5, Aiolos and EBF1, all of which are high levels in DT40 cells and down-regulate gene expressions of IgM H- and L-chains [54], are almost completely suppressed at the early stage and thereafter remain unchanged until the later stage (Fig. 7-10). By contrast, the mRNA level of OBF1, which is a high level in DT40 cells and probably up-regulates gene expressions of these two immunoglobulin proteins [54, 80], is gradually and dramatically reduced from the early to later stages. Therefore, the way for gene expressions of IgM H- and L-chains at the later stage in clone cl.2-1 seems to be dependent on OBF1 and considerably differ from that in wild-type DT40 cells in appearance (Fig. 7-1(L)). In clones cl.2-2 and cl.2-4, mRNA levels of Pax5, Aiolos and EBF1 are almost completely suppressed at the early stage (Fig. 7-10). Thereafter, those of Pax5 and Aiolos are gradually increased through the middle to later stages but that of EBF1 remains unchanged as an undetectable level until the later stage. On the other hand, the mRNA level of OBF1 is slightly reduced at the early stage and thereafter slightly increased until the later stage. Therefore, the ways for gene expressions of IgM H- and L-chains at the later stage in clones cl.2-2 and cl.2-4 (and cl.2-3 and cl.2-5) seem to be dependent on Pax5 and Aiolos and slightly similar to that in DT40 cells in appearance (Fig. 7-1(L)). Moreover, these four clones are the major type, since four initially generated HDAC2(-/-) mutant clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) resembled these four clones in several cellular properties [Chap. 2]. In clone cl.2-6, mRNA levels of Pax5, Aiolos and EBF1 are almost completely decreased at the early stage and thereafter dramatically increased through the middle to later stages (Fig. 7-10). On the other hand,

the mRNA level of OBF1 is slightly reduced at the early stage and thereafter slightly increased until the later stage. Therefore, the way for gene expressions of IgM H- and L-chains at the later stage in clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 and most similar to that in DT40 cells in appearance (Fig. 7-1(L)), since the two immunoglobulin gene expressions in DT40 cells are directly and cooperatively regulated by these three transcription factors (and E2A) (see Fig. 7-1(W)). These three models for roles of Pax5, Aiolos, EBF1 and OBF1 in control of gene expressions of IgM H- and L-chains at the later stage of cultivation in individual clones of HDAC2(-/-) DT40 mutants are schematically shown in Figure 7-1(L). These three ways to suppress gene expressions of IgM H- and L-chains at the later cultivation stage in all individual HDAC2(-/-) mutant clones, including clone cl.2-6, are really distinct from the ordinary and reversible transcriptional regulations of the two immunoglobulin genes in DT40 cells. Because all of these mutant clones are lacking HDAC2, which controls gene expressions of Pax5, Aiolos, EBF1, OBF1 and also E2A. If additional independent clones of HDAC2(-/-) mutants are analyzed, besides the above-mentioned three ways, other distinct ways for gene expressions of IgM H- and L-chains will be probably added. Moreover, the above-mentioned results on varied alterations in gene expressions of various transcription factors and chromatin-modifying enzymes suggest that some other unknown cellular characteristics certainly change in individual HDAC2(-/-) mutant clones during cultivation.

Based on these findings [Chap. 4], we concluded that individual clones of HDAC2(-/-) mutants possess their own ability to gain the same and new cell functions in distinct ways through various generations during cultivation. Namely, the same and new cell functions of individual HDAC2(-/-) mutant clones that we mean are the excluding artificially accumulated IgM H- and L-chains and the ridding themselves free from the aggregative form, since these two are abnormal and uncomfortable for the mutant cells, themselves. Such distinct ways bring about distinct changing patterns in gene expression levels of Pax5, Aiolos, EBF1, OBF1 (and E2A and others) in individual mutant clones, though gene expressions of IgM H- and L-chains change in almost similar pattern in all of them. In addition, we would like to emphasize that changes of any characteristics of HDAC2(-/-) (and also Pax5(-)) mutant cells are more drastic just soon after their birth. This inference is based on the facts that we collected the mutant cells at 15-16 days after their birth by gene targeting techniques [Chaps. 2, 3 and 4] and their doubling times were ~12 hrs [47, 54, 64]; therefore, they should be populations of ~30 to 32 generations even at the early stage. Of various transcription factors whose gene expressions changed in HDAC2(-/-) mutants during cultivation, particularly, Pax5, Aiolos, EBF1 and OBF1 are influential candidates participating in diminutions of artificially elevated gene expressions of IgM H- and L-chains. Because these four factors were already reported to positively or negatively regulate the two immunoglobulin gene expressions [54]. In addition, the changing patterns of their gene expressions were reported to be in

anti-parallel or in parallel with those of IgM H- and L-chain gene expressions in one or more of individual HDAC2(-/-) mutant clones [Chap. 4].

Next, we studied how do individual HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 separately gain distinct ways for positive or negative gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF through various generations during continuous cultivation [Chap. 5]. To exclude this project, we newly developed a chromatin immuno-precipitation (ChIP) assay on the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of the above-mentioned specific genes, using site-specific antibodies for several acetylated Lys residues of histone H3 and various appropriate primers. All DNA fragments amplified by PCR using appropriate primers designed based on nucleotide sequences of the proximal ~2.0 kb 5'-upstream region of each of these five genes, are laid overlapping to each other with neighboring ones. Actually, we performed the ChIP assay on the proximal ~2.0 kb 5'-upstream chromatin regions, each of which consists of approximately 10 or 11 nucleosomes, and the several distal 5'-upstream and ORF chromatin regions of the above-mentioned five genes. Hereafter, we designated the new ChIP assay as the neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP; this abbreviation means also IP on notch of chromatin) assay. Surprisingly, acetylation levels of five specific Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) within the proximal ~2.0 kb 5'-upstream chromatin regions of these five genes in the four mutant clones separately change during cultivation (Figs. 7-13 to 7-16). We supposed that hyper- (high), considerable hyper-, somewhat hyper- or hypo- (low or no) acetylation levels of one or more of these five specific Lys residues (especially K9/H3 and K27/H3) within the proximal ~2.0 kb 5'-upstream chromatin regions of the five genes qualitatively induce no, weak, less or full binding ability (capacity) of histone H3 to DNA, resulting in the loose, considerable loose, somewhat loose or tight form of the chromatin structure (Fig. 7-17). In consequence, for example, the loose or tight form of the chromatin structure of the proximal ~2.0 kb 5'-upstream region of a certain gene leads its a high or low (or no) gene expression level. Results obtained by the NotchIP assay [Chap. 5] and those obtained by RT-PCR [Chap. 4] on Pax5, Aiolos, EBF1, OBF1 and PCAF genes in the four individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants are as follows.

In DT40 cells, the five Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -1923 to +30 of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-1, those except K23/H3 exhibit a full binding ability of histone H3 to DNA at any cultivation stages based on their hypo-acetylation levels (Fig. 7-13). Consequently, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells, but changes to the tight form at the early stage in clone

cl.2-1 and remains unchanged until the later stage. These facts agreed with the findings that the gene expression of Pax5, which is a high level in DT40 cells, is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2250 to +145 of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-1, those except K23/H3 exhibit a full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-13). Accordingly, the chromatin structure surrounding the proximal ~2.1 kb 5'-upstream region of the gene, which may consist of ~11 nucleosomes, is the loose form in DT40 cells, but changes to the tight (or somewhat loose) form at the early stage in clone cl.2-1 and remains unchanged until the later stage. These facts agreed with the findings that the gene expression of Aiolos, which is a high level in DT40 cells, is drastically suppressed to a low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2031 to +200 of the EBF1 gene based on their hyper-acetylation levels. However, in clone cl.2-1, especially K9/H3, K18/H3 and K27/H3 possess a full (or less) binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-13). Accordingly, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells, but changes to the tight (or somewhat loose) form at the early stage in clone cl.2-1 and remains unchanged until the later stage. These facts agreed with the findings that the gene expression of EBF1, which is a high level in DT40 cells, is almost completely suppressed to a low (or no) level at the early stage in clone cl.2-1 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2138 to +164 of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-1, in particular, K9/H3 and K27/H3 (and probably K23/H3) certainly possess a weak binding ability of histone H3 to DNA based on their considerable hyper-acetylation levels at the early stage. Further, the weak binding ability is dramatically increased at the middle stage and remains unchanged at the later stage based on their hypo-acetylation levels (Fig. 7-13). Accordingly, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells. On the other hand, in clone cl.2-1, the chromatin structure changes to the considerable loose form at the early stage and thereafter changes to the tight form at the middle and

later stages. These facts agreed with the findings that the gene expression of OBF1, which is a high level in DT40 cells, is slightly decreased at the early stage in clone cl.2-1 and thereafter dramatically suppressed to a very low (or no) level at the middle and later stages (see Fig. 7-10).

In DT40 cells and clone cl.2-1 at any cultivation stages, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no (or a low) binding ability of histone H3 to DNA within the chromatin surrounding the proximal 5'-upstream region from positions -2005 to +231 (and two distal 5'-upstream regions) of the PCAF gene based on their hyper-acetylation levels with insignificant changes in case of clone cl.2-1 [Chap. 5]. Accordingly, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the gene, which may consist of ~10 nucleosomes, is the loose form in both DT40 cells and clone cl.2-1. However, as in DT40 cells, so in clone cl.2-1 the gene expression of PCAF is really a very low at the early stage but thereafter gradually and dramatically increased until the later stage (see Fig. 7-10). Consequently, other unknown mechanisms, including further distal 5'-upstream regions, are expected to participate in the gene expression of PCAF, and the examined proximal and distal 5'-upstream regions are assumed to poorly correlate with its gene expression, regardless of their loose form in both DT40 cells and clone cl.2-1.

These results, together with the previous inference speculated from changing patterns in gene expressions of the four transcription factors [Chap. 4], surely indicated that at the later cultivation stage clone cl.2-1 seems to be dependent on OBF1 and considerably distinct from wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains in appearance (Fig. 7-1(L)).

As described above, in DT40 cells, the four Lys residues (K9/H3, K14/H3, K18/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-2, those exhibit a full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early stage. Thereafter, very surprisingly, these four Lys residues gradually lose the binding capacity of histone H3 to DNA to no binding ability based on their hyper-acetylation levels during cultivation until the later stage (Fig. 7-14). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-2. Thereafter, the chromatin structure changes to the loose form until the later stage. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-2 and thereafter gradually and certainly elevated to a high level until the later stage (see Fig. 7-10).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-2,

those, mainly K9/H3 and K27/H3 possess a less binding ability of histone H3 to DNA based on their somewhat hyper-acetylation levels at the early and middle stages, but mainly the binding capacity of K9/H3 is certainly weakened to no (or weak) binding ability based on the considerable hyper-acetylation levels at the later stage (Fig. 7-14). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-2 and thereafter changes to the loose (or considerable loose) form at the later stage. These facts agreed with the findings that the gene expression of Aiolos is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-2 and thereafter increased to a high level at the later stage (see Fig. 7-10).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-2, especially K9/H3, K14/H3 and K27/H3 (and probably K18/H3) exhibit a full binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-14). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-2 and remains unchanged through the middle to later stages. These facts agreed with the findings that the gene expression of EBF1 is almost completely suppressed to a low (or no) level at the early stage in clone cl.2-2 and thereafter remains unchanged (see Fig. 7-10).

As described above, in DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-2, mainly K9/H3 and K27/H3 exhibit a full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at the early stage. The less binding ability of K27/H3 is slightly increased and that of K9/H3 remains unchanged as a full binding ability based on their hypo-acetylation levels at the middle stage. Thereafter, the full binding ability of K9/H3 and K27/H3 is obviously reduced to no binding ability based on their hyper- or considerable hyper-acetylation levels at the later stage (Fig. 7-14). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the considerable tight form at the early stage in clone cl.2-2 and thereafter changes to the tight form at the middle stage. Subsequently, the tightened chromatin structure becomes the loose form at the later stage. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early and middle stages in clone cl.2-2 and thereafter obviously increased to a high level at the later stage (see Fig. 7-10).

In both DT40 cells and clone cl.2-2 at any cultivation stages, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin

surrounding the proximal ~2.0 kb 5'-upstream region of the PCAF gene based on their hyper-acetylation levels with insignificant changes in case of clone cl.2-2 (see Chap. 5). Therefore, the chromatin structure surrounding the proximal 5'-upstream region of the gene is the loose form in both DT40 cells and clone cl.2-2 at any cultivation stages. However, in clone cl.2-2 the gene expression of PCAF is really a very low at the early stage, as in DT40 cells, but is gradually and dramatically increased until the later stage (see Fig. 7-10). Accordingly, other unknown mechanisms, including further distal 5'-upstream regions, are assumed to participate in gene expression of PCAF, and the examined distal and proximal 5'-upstream regions do not correlate directly and closely with its gene expression, regardless of the loose form in both DT40 cells and clone cl.2-2.

These results, together with the previous inference speculated from changing patterns in gene expressions of the four transcription factors [Chap. 4], surely indicated that at the later cultivation stage clone cl.2-2 seems to be dependent on Pax5 and Aiolos, and somewhat similar to wild-type DT40 cells in the way for gene expressions of IgM H- and L-chains in appearance, and to be the major type (Fig. 7-1(L)), like clone cl.2-4, the reason for this will be mentioned later.

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-4, these five Lys residues possess a full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early stage and thereafter gradually lose the binding capacity to no binding ability based on their hyper-acetylation levels until the later stage (Fig. 7-15). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-4. Thereafter, the tightened chromatin structure changes to the loose form until the later stage. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-4 and thereafter gradually and certainly elevated to a high level until the later stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-4, mainly K9/H3 (and probably K18/H3 and K27/H3) possess a less binding ability of histone H3 to DNA based on somewhat hyper-acetylation levels at the early stage. Thereafter, mainly the binding capacity of K9/H3 is certainly weakened to no binding ability based on the considerable hyper-acetylation levels at the later stage (Fig. 7-15). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in

clone cl.2-4 and thereafter changes to the loose (or considerable loose) form at the later stage. These facts agreed with the findings that the gene expression of Aiolos is certainly decreased to a low (or no) level at the early stage in clone cl.2-4 and thereafter increased to a high level until the later stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-4, especially K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) exhibit a full (or less) binding ability based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages (Fig. 7-15). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-4 and remains unchanged until the later stage. These facts agreed with the findings that the gene expression of EBF1 is dramatically suppressed to a very low (or no) level at the early stage in clone cl.2-4 and thereafter remains unchanged during cultivation (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-4, mainly K9/H3 (and possibly K27/H3) possess a less binding ability of histone H3 to DNA based on somewhat hyper-acetylation levels at the early stage. The less binding ability remains unchanged at the middle stage but thereafter obviously decreased to no binding ability based on the hyper- (or considerable hyper-) acetylation levels at the later stage (Fig. 7-15). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the somewhat loose form at the early and middle stages in clone cl.2-4 and thereafter changes to the loose form at the later stage. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early and middle stages in clone cl.2-4 and thereafter certainly increased to a high level at the later stage (see Fig. 7-10).

In both DT40 cells and clone cl.2-4 at any cultivation stages, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the distal and proximal ~2.0 kb 5'-upstream regions of the PCAF gene based on their hyper-acetylation levels with insignificant changes in case of clone cl.2-4 (see Chap. 5). Accordingly, the chromatin structure surrounding the proximal (and distal) 5'-upstream region of the gene is the loose form in both DT40 cells and clone cl.2-4. However, as in DT40 cells, the gene expression of PCAF is really a very low at the early stage in clone cl.2-4 but gradually and dramatically increased from the early through middle to later stages (see Fig. 7-10). Consequently, other unknown mechanisms, including further distal 5'-upstream regions, are assumed to participate in the PCAF gene

expression, and the examined distal and proximal 5'-upstream regions are assumed to poorly correlate with the gene expression, regardless of their loose form in both DT40 cells and clone cl.2-4.

These results and those obtained in clone cl.2-2, together with the previous inference speculated from changing patterns in gene expressions of the four transcription factors in initially and secondly generated HDAC2(-/-) mutants [Chaps. 2, 4 and 5], surely indicated that at the later cultivation stage clones cl.2-4 and cl.2-2 (and also clones cl.2-3 and cl.2-5) seem to be dependent on Pax5 and Aiolos, and somewhat similar to wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains in appearance (Fig. 7-1(L)) and also to be the major type.

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene based on their hyper-acetylation levels. In clone cl.2-6, those exhibit a full binding ability based on their hypo-acetylation levels at the early stage and thereafter gradually lose the binding capacity to no binding ability based on their hypo-acetylation levels until the later stage (Fig. 7-16). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-6. Thereafter, the tightened chromatin structure changes to the loose form through the middle to later stages. These facts agreed with the findings that the gene expression of Pax5 is dramatically suppressed to a low (or no) level at the early stage in clone cl.2-6 and thereafter gradually and dramatically elevated to a high level until the later stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Aiolos gene based on their hyper-acetylation levels. In clone cl.2-6, mainly K9/H3 and K27/H3 certainly strengthen the binding capacity of histone H3 to DNA to a full or less binding ability based on their hypo- or somewhat hyper-acetylation levels at the early stage (Fig. 7-16). Thereafter, the full or less binding ability of these five Lys residues is gradually weakened to no binding ability based on their hyper-acetylation levels through the middle to later stages. Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight (or somewhat loose) form at the early stage in clone cl.2-6 and thereafter changes to the loose form at the later stage. These facts agreed with the findings that the gene expression of Aiolos is drastically decreased to a low (or no) level at the early stage in clone cl.2-6 and thereafter dramatically increased to a high level at the later stage (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) possess no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene based on their hyper-acetylation levels. In clone cl.2-6,

mainly K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) exhibit a full or less binding ability based on their hypo- or somewhat hyper-acetylation levels at the early stage and lose the binding capacity to no binding ability based on their hyper-acetylation levels through the middle until later stages (Fig. 7-16). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-6 and thereafter changes to the loose form through the middle until later stages. These facts agreed with the findings that the gene expression of EBF1 is drastically suppressed to a very low (or no) level at the early stage in clone cl.2-6 and thereafter gradually elevated until the later stage to almost the same levels in DT40 cells (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene based on their hyper-acetylation levels. In clone cl.2-6, especially K9/H3 and K27/H3 (and probably K23/H3) obviously exhibit a full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at the early stage. Thereafter, the full or less binding ability is slightly reduced at the middle stage and further decreased to no binding ability based on their hyper-acetylation levels at the later stage (Fig. 7-16). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene, which is the loose form in DT40 cells, changes to the tight form at the early stage in clone cl.2-6 and thereafter changes to the loose form through the middle to later stages. These facts agreed with the findings that the gene expression of OBF1 is slightly decreased at the early stage in clone cl.2-6 and thereafter certainly increased to a high level through the middle to later stages (see Fig. 7-10).

In DT40 cells, as described above, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibit no binding ability of histone H3 to DNA within the chromatin surrounding the distal and proximal ~2.0 kb 5'-upstream regions of the PCAF gene based on their hyper-acetylation levels. Interestingly, in clone cl.2-6, mainly K9/H3 and K18/H3 possess a less binding ability based on their somewhat hyper-acetylation levels at the early stage, but thereafter lose the binding capacity to no binding ability based on their hyper-acetylation levels at the middle stage and again gain a less binding ability based on their somewhat hyper-acetylation levels at the later stage (see Chap. 5). Accordingly, the chromatin structure surrounding the proximal 5'-upstream region of the gene is the loose form in both DT40 cells and clone cl.2-6 at the middle stage but a somewhat loose form at the early and later stages in clone cl.2-6. However, in clone cl.2-6, the gene expression of PCAF is also a very low at the early stage, and thereafter is gradually and obviously increased until near middle stages but again dramatically decreased to a very low level through the middle to later stages (see Fig. 7-10). Based on these results, other unknown mechanisms, including further distal 5'-upstream regions, are assumed to participate in the PCAF gene expression, and reversely the examined distal and proximal 5'-upstream regions are not

directly correlated with its gene expression by much, regardless of the loose form of the chromatin structure in both DT40 cells and clone cl.2-6.

These results, together with the previous inference speculated from changing patterns in gene expressions of the four transcription factors [Chap. 4], surely indicated that at the later cultivation stage clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 (Fig. 7-1(L)) and most similar to wild-type DT40 cells in the way of gene expressions of IgM H- and L-chains in appearance.

CONCLUSION

As finishing touches, based on the above-mentioned results (Figs. 7-1 to 7-17) [Chaps. 2, 3, 4 and 5], newly noticed morphological findings (Figs. 7-18 to 7-20) [Chap. 6] and other findings [41, 50, 54, 57, 77, 80], we revised the previous brief hypothesis [Chaps. 4 and 5] and newly proposed an all-inclusive hypothesis on ways for gaining un-programmed and new cell function to exclude artificially accumulated IgM H- and L-chains by means of irreversible creation of the distinct chromatin structure plasticity surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications in individual clones of HDAC2(-/-) mutants during continuous cultivation [Chap. 6]. We presumed that environment change recognition receptor/site (ECRR/ECRS) recognizes the accumulation of these two immunoglobulin proteins as an abnormal and unfavorable environment change (and partly acts in signal transduction on the accumulation to the chromatin structure). In addition, we assumed that chromatin conformation (structure) change complex (4C) machinery, which consists of a specific member of HATs, a specific member of HDACs and other components, directly and irreversibly creates the chromatin structure plasticity surrounding the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of the chromatin) of each of the above-mentioned specific transcription factor genes through successive conformation changes with epigenetic modifications (and partly acts in the signal transduction).

Large amounts of IgM H- and L-chains artificially caused by the HDAC2-deficiency are first accumulated (probably as their large protein complex with each other [54]) within endoplasmic reticulum of HDAC2(-/-) mutant cells. Most of the accumulated immunoglobulin proteins are gradually secreted to outside of cells (i.e., into cultivation media), but a part of them are transported to the nuclear envelope but not inside of nucleus and kept at the peri-nuclear space not only at the early but also at the later cultivation stages (Figs. 7-18 and 7-19) [Chap. 6]. The accumulated immunoglobulin proteins in the peri-nuclear space bind to ECRR/ECRS localized at the inner nuclear membrane (where the hetero-chromatin is possibly located) (Fig. 7-20). After the ECRR/ECRS recognizes the accumulation of IgM H- and L-chains as an abnormal and unfavorable environment change, the putative signal concerning it is genome-widely transmitted to the chromatin structure surrounding proximal 5'-upstream regions of numerous genes (probably exist on several distinct chromosomes) encoding transcription

factors, chromatin-modifying enzymes, and related factors and enzymes (Figs. 7-20 and 7-21). Successively, the spontaneous unbalanced signal transduction and the response to the environment change are consecutively and separately converged not only on the 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 contains HDAC2 as a HDAC activity and a specific HAT member (e.g., GCN5) as a HAT activity and many other factors (Fig. 7-21). By contrast, in all of individual HDAC2(-/-) mutant clones, at the very early stage (just soon after the birth by gene targeting techniques), bulk conformation of the 4C machinery dramatically changes to remove or drastically reduce the HAT activity (of the assumed HAT member) associated with the HDAC2-deficiency. Through the above-mentioned processes during cultivation, the 4C machinery newly contains another specific member of HDACs (except HDAC2), a specific member of HATs and many other factors, and thereby becomes diverse.

The diversity of alterations in the chromatin structure 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], which are cooperatively caused by a proper member of each of HATs and HDACs in the protean 4C machinery. These successive epigenetic modifications with acetyl group of the specific Lys residues, such as K9/H3 and K27/H3 (and also K14/H3, K18/H3 and K23/H3), induce irreversible creation of the chromatin structure plasticity surrounding the proximal 5'-upstream regions of the above-mentioned target genes. By contrast, probably, the protean 4C machinery can not change the chromatin structure surrounding ORF regions (coding regions) of these target genes by much. As a result, the proximal 5'-upstream chromatin region possessing hyper-acetylation levels of one or more of the specific Lys residues is in the loose (open) form based on no binding ability of histone H3 to DNA, and that possessing hypo-acetylation levels of one or more of the specific Lys residues is in the tight (closed) form based on the binding ability of histone H3 to DNA (Figs. 7-17 and 7-21). Thus, as the need arises, transcription factor complex (TFC) machinery, which consists of RNA polymerase, proper transcription factors, certain members of HATs and HDACs, and other factors (Fig. 7-21), 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 target (but not un-target) genes (i.e., Pax5, Aiolos, EBF1 and OBF1 genes), which have become latently active (but not inactive) state, followed by initiation of their gene expressions. Consequently, individual clones of HDAC2(-/-) mutants exhibit flexible, elastic and pluri-potent ability for gaining un-programmed and new cell function to diminish increased gene expressions of IgM H- and L-chains in distinct ways, i.e., increases and/or decreases in gene expressions of Pax5, Aiolos, EBF1 and OBF1 by means of irreversible creation of the distinct chromatin structure plasticity with epigenetic modifications during continuous cultivation, even though they are completely

the same cell type having the same genotype and are also established cell line (Fig. 7-21) [54, Chaps. 2, 4, 5 and 6].

We expanded the above-mentioned hypothetic way on the diminution of IgM H- and L-chains excessively accumulated in HDAC2(-/-) mutants to a universal hypothetic way (principle) for gaining un-programmed and new cell functions by means of irreversible creation of the distinct chromatin structure plasticity of specific transcription factor, chromatin-modifying enzyme, and related factor and enzyme genes with epigenetic modifications in higher eukaryotic cells through various generations (cell divisions) [Chap. 6]. When somatic cells of higher eukaryotes firstly encounter an intra- and/or extra-cellular environment change in their lives, in order to adapt to or eliminate the change (if abnormal and/or uncomfortable), they gradually gain un-programmed and new cell functions through various generations. Namely, the somatic cells of higher eukaryotes acquire the ability to adapt themselves to a newly encountered environment change and/or to eliminate the abnormal and/or painful environment change.

Using the ECRR/ECRS, the 4C machinery and other components, the somatic cells cause a chain reaction of response to the abnormal environment change as follows. First of all, the abnormal environment change is recognized by means of the ECRR/ECRS, which is localized nearby the nuclear membrane as a cytoplasm-nucleus barrier (probably exists at the inner nuclear membrane where the hetero-chromatin is possibly located) (Fig. 7-20). There is a possibility that the putative specific molecule(s) acts as intermediary sensor molecule(s) at this step in the way to recognize the abnormal environment change. As a next step, the putative signal(s) concerning the abnormal 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 structure, gene expressions of various chromatin-modifying enzymes, transcription factors, and related enzymes and factors slightly change, coupled with a slight alteration in their chromatin conformation. Both the signal transduction and the response to the abnormal environment change are successively repeated and converged into the restricted chromatin structure surrounding proximal 5'-upstream regions of specific target factor and/or enzyme genes. Finally, these successive signal transduction and response concerning the abnormal and environment change causes various epigenetic modifications of histones and/or DNA within the restricted chromatin regions with acetyl, methyl, phosphate, ubiquitin and ADP ribose groups and/or others. Of these various epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones (H2A, H2B, H3 and H4) are major ones [23-26, 28-37]. The 4C machinery, which comprises a specific member of HATs, a specific member of HDACs and other factors, is preferentially involved in such epigenetic modifications with acetyl group. Participating positions of specific Lys residues and kinds of core histones are 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. In consequence, epigenetic modifications of one or more of these specific Lys residues of histone H3 with acetyl group change within proximal 5'-upstream chromatin regions of specific target genes through various generations (cell divisions). Distinct functions of the protean 4C machinery on such acetylation and deacetylation levels are mainly 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 those of almost all enzymes in any biological reactions. By contrast, the protean 4C machinery cannot act in alterations in the chromatin structure surrounding ORF 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 acetylation and/or deacetylation levels of one or more of these specific Lys residues, though which Lys residue(s) is really and/or mainly involved in the binding is still undefined. Namely, hyper- (high) or hypo- (low or no) acetylation levels induces no binding or full binding ability of histone H3 to DNA, resulting in the loose (open) or tight (closed) form of the chromatin structure. Thus, the chromatin structure plasticity is irreversibly created based on the successive conformation changes with epigenetic modifications (Figs. 7-21 and 7-22). Whenever the need arises, the TFC machinery can bind to promoter regions (or elements) within the loose form of the chromatin structure surrounding proximal 5'-upstream regions of target genes (which are latently active state), and thereby can initiate their gene expressions (Fig. 7-21). 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 latently inactive state), followed by no initiation of their gene expressions. Consequently, the loose or tight form of the chromatin structure surrounding proximal 5'-upstream region causes the high or low (or no) transcription level of corresponding gene. Remarkably, the ways of irreversible creation of the chromatin structure plasticity are distinct among individual somatic cells of the same type having the same genotype, even though the abnormal environment change and signal(s) concerning the change are the same for all of them. That is, in order to gain un-programmed and new cell functions, in spite of the same abnormal environment change, individual somatic cells possess the ability not only to complicatedly and diversely change the chromatin structure surrounding proximal 5'-upstream regions of numerous genes but also to change the chromatin structure surrounding the proximal 5'-upstream region of the same gene into diverse forms. Consequently, gene expressions of various specific chromatin-modifying enzymes and transcription factors differently change among individual somatic cells through repeated cell divisions, and as a result they are able to newly and separately gain the same (or distinct) un-programmed cell function(s) in distinct ways through various generations.

As a result of the theoretical prediction mentioned above, in order to gain un-programmed and new

cell functions, somatic cells (and also tumor cells) of higher eukaryotes are pluri-potent, elastic and flexible, all of which are basically originated from pluri-potency, elasticity and flexibility of their chromatin structure. That is, individual somatic cells of higher eukaryotes possess the ability to gain the same and/or distinct un-programmed and new cell functions 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 genes, in order to adapt themselves to an abnormal and/or uncomfortable intra- and/or extra-cellular environment change (Fig. 7-23). Such the loose or tight form of the proximal 5'-upstream chromatin structure is latently active or inactive state for transcription of the corresponding gene, although the proximal 5'-upstream region (as mere nucleotide sequences) of the gene is probably potential but silent state for expressions of most genome functions. Irreversible creation of the varied chromatin structure plasticity in individual somatic cells of the same type is basically triggered by the spontaneous unbalanced response to the abnormal environment change when they firstly encounter with it and accomplished by the successive convergence of the unbalanced response through various generations (cell divisions). The chromatin structure plasticity in somatic cells is irreversibly created in distinct ways, which are probably dependent upon their antecedents. Moreover, the chromatin structure plasticity, regardless of whether its creation is in the course or was already completed, is inherited to cells of descendent generations associated with or without additional structural change through cell divisions. Remarkably, irreversible creation of the chromatin structure plasticity occurs in descendant cells but not in the cell which initially accepts the signal on the abnormal environment change, although reversible regulations of ordinary gene expressions and enzyme reactions occur in the cell itself which accepts proper signal (Fig. 7-22). With the intention of adapting to the abnormal environment change, irreversible creation of the chromatin structure plasticity occurs inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region, as if it is coxswain, directs the switch for latent transcription ability of the corresponding gene by means of irreversible creation of the chromatin structure plasticity (Fig. 7-23). Therefore, besides a "notch of chromatin" from a structural point of view as mentioned above, the proximal 5'-upstream chromatin region can be regarded as a "director for gene expression/transcription" from a functional point of view. Of course, the notch (or director) covers specific nucleotide sequences of transcriptional elements (such as promoter, operator, enhancer and others) and their neighboring nucleotide sequences. We should like to emphasize that real recipient of the signal on an abnormal environment change may be just the chromatin structure itself as three-dimensional conformation that is dynamic and changeable between the loose and tight forms, but not mere the chromatin and chromosomes themselves as one- (or two-) dimensional conformation that is static and unchangeable. Naturally, the chromatin structure of the proximal 5'-upstream chromatin

region (notch or director), as dynamic and changeable three-dimensional conformation, undertakes two fundamental abilities, i.e., to receive the signal concerning an abnormal intra- and/or extra-cellular environment change and to direct the switch (on or off) for latent transcriptional ability of the corresponding gene through its irreversible chromatin conformational 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 specific signal(s) and to express their own biological functions. However, concrete data are not enough to support these ideas.

Finally, we named our hypothetic theory on such a bio-system that gains un-programmed and new cell functions by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications, which is one of the most important ways for life maintenance and cell-type determination of higher eukaryotes, as the chromatin conformation (structure) change code (4C) theory [see Chap. 6]. The 4C theory opens the door for gaining un-programmed and new cell functions of higher eukaryotes and innovates the general concept on nature of somatic cells. Presumably, in the 4C theory, the supposed number of codes, 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. The most influential candidates are genes of chromatin-modifying enzymes, transcription factors, and related enzymes and factors, which are necessary for gaining un-programmed and new cell functions and cell-type determination of higher eukaryotes. The number of codes for each of these candidate genes should be two, i.e., the loose or tight form of the proximal 5'-upstream chromatin region, which directs the switch (on or off) for latent transcriptional ability of the corresponding gene. Incidentally, the 4C theory is suitable as an explanation for development and differentiation of higher eukaryotes, because a behavior of putative signal concerning the environment change seems to considerably resemble that of certain substances (such as hormone, cytokine and nerve-transmission substance), which participate in cell-cell, tissue-tissue and/or organ-organ interactions (communications) throughout these two fundamental life phenomena.

In the 4C theory on the exclusion of IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutants during cultivation, there are several crucial questions to be clarified as follows [Chaps. 2, 4, 5 and 6]. 1) Despite the deficiency of HDAC2 activity, why do 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 cultivation stage in HDAC2(-/-) mutant cells. 2) Why do the decreased acetylation levels of one or more of K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 within proximal 5'-upstream chromatin regions of these genes increase during cultivation. Why the case of the OBF1 gene is opposite. 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) Steric and functional differences between the loose and tight forms (which are 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 genes must be clarified more precisely. 5) Why do the changing patterns in acetylation levels of the above-mentioned specific Lys residues of histone H3 during cultivation in individual transcription factor gene differ among individual mutant clones. 6) Why do the changing patterns in acetylation levels of the above-mentioned specific Lys residues of histone H3 for 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 surrounding proximal 5'-upstream regions) differ from the well-known chromatin-modifying machinery, although the TFC machinery (which acts in transcriptions of ORF regions of corresponding genes) may be almost the same as the well-known transcription machinery. 8) To demonstrate the 4C theory, both of the ECRR/ECRS as a first player to recognize the abnormal environment change and the 4C machinery as a final player to directly and irreversibly creates the chromatin structure plasticity must be clarified. 9) Clarification of effects of changes in temperature, atmosphere and nutrition on ability to gain un-programmed and new cell functions in established cell lines through various cell divisions and in model animals (such as C. elegans, Drosophila, Xenopus, mice, rats and others) during development and differentiation may be effective and powerful as a concrete approach to generalize the 4C theory, because these effects under the varied conditions can be easily studied by various research groups.

POSTSCRIPT

The studies on the 4C theory were reviewed in Refs. 83, 84 and 85.

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FIGURE LEGENDS

Figure 7-1. Ways for control of 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] and also identical with Suppl. Fig. 6-S1 in [Chap. 6].

Figure 7-2. Alterations in amounts of IgM H- and L-chains in HDAC2(-/-) DT40 mutant cells during continuous cultivation

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

Figure 7-3. Alterations in amounts of IgM H-chain proteins in HDAC2(-/-) DT40 mutant cells during continuous cultivation

Immuno-electron microscopy was performed on HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages and on DT40 cells (W), using anti-chicken IgM H-chain antiserum. Large amounts of accumulated IgM H-chain proteins were detected only at the early (E) stage in mutant cells. The figure is identical with Fig. 2-3 in [Chap. 2].

Figure 7-4. Alterations in gene expressions of IgM H- and L-chains, and core histones in HDAC2(-/-) DT40 mutant cells during continuous cultivation

Total RNAs were extracted from three HDAC2(-/-) mutant clones at the early (E; ~20 days), middle (M; ~40 days) and later (L; ~60 days) cultivation stages and from DT40 cells (W). RT-PCR was performed on total RNAs, using appropriate primers for mRNAs of HDAC2, and whole, secreted plus membrane-bound forms of IgM H-chain and IgM L-chain, and core histones H2A, H2B, H3 and H4. The figure is identical with Fig. 2-4 in [Chap. 2].

Figure 7-5. Alterations in amounts of IgM H- and L-chains in Pax5(-) DT40 mutant cells during continuous cultivation

Western blotting was performed on total cellular proteins prepared from three Pax5(-) mutant clones at the early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W), using anti-chicken IgM L-chain antiserum that cross-reacts with IgM H-chain. The figure is identical with Fig. 3-3 in [Chap. 3].

Figure 7-6. Morphology of Pax5(-) DT40 mutant cells during continuous cultivation

Microscopy was performed on Pax5(-) mutant cells at the early (E) and later (L) cultivation stages and on DT40 cells (W). The figure is identical with Suppl. Fig. 3-S1 in [Chap. 3].

Figure 7-7. Alterations in gene expressions of IgM H- and L-chains in Pax5(-) DT40 mutant cells during continuous cultivation

RT-PCR was performed on total RNAs prepared from three Pax5(-) mutant clones at the first (F), early (E), middle (M) and later (L) cultivation stages and from DT40 cells (W), using appropriate primers for mRNAs of Pax5, and whole, secreted plus membrane-bound forms of IgM H-chain and IgM L-chain. The figure is identical with Fig. 3-5 in [Chap. 3].

Figure 7-8. Genomic organization of chicken HDAC2 gene, generation of HDAC2(-/-) DT40 mutant cells and alterations in protein and mRNA levels of IgM H- and L-chains in individual HDAC2(-/-) DT40 mutant clones during continuous cultivation

A) Schematic presentation of the chicken HDAC2 genomic locus (top) with enlarged drawing of the targeted region (middle) and its targeted alleles (two bottoms). **B)** Southern blotting of homologous recombination for DT40, one heterozygous mutant clone (-/+) and six homozygous mutant clones (-/-). **C)** Western blotting for six HDAC2(-/-) mutant clones at the early (E), middle (M) and later (L) cultivation stages and for DT40 cells (W). IgM H and L indicate IgM H-chain and IgM L-chain, respectively. **D)** RT-PCR for six HDAC2(-/-) mutant clones at the early (E), middle (M) and later (L) cultivation stages and for DT40 cells (W). The figure is identical with Fig. 4-1 in [Chap. 4].

Figure 7-9. Alterations in amounts of IgM H- and L-chains in individual HDAC2(-/-) DT40 mutant clones during continuous cultivation

Western blotting was performed on total cellular proteins prepared from six HDAC2(-/-) mutant clones at indicated cultivation periods, including the early (E), middle (M) and later (L) stages and from DT40 cells (W). Proteins were sequentially detected with anti-chicken IgM L-chain antiserum that cross-reacts with IgM H-chain (top and third) and anti-chicken IgM H-chain antiserum (second). The figure is identical with Fig. 4-2 in [Chap. 4] and shown with slight modifications.

Figure 7-10. Alterations in gene expressions of IgM H- and L-chains, members of HATs, HDACs and transcription factors in individual HDAC2(-/-) DT40 mutant clones during continuous cultivation

RT-PCR was performed on total RNAs prepared from four HDAC2(-/-) mutant clones at indicated

periods of cultivation, including the early (E), middle (M) and later (L) stages and from DT40 cells (W), using appropriate primers for mRNAs of IgM H- and L-chains, PCAF, HDAC9, Pax5, Aiolos, EBF1, Blimp1, OBF1, HDAC7, Ikaros, E2A, PU.1 and XBP-1. The figure is identical with Fig. 4-6 in [Chap. 4] and shown with slight modifications.

Figure 7-11. Alterations in amounts of IgM H-chain in individual HDAC2(-/-) DT40 mutant clones during continuous cultivation

Electron microscopy (upper panels) and immuno-electron microscopy using anti-chicken IgM H-chain antiserum (lower panels) were carried out on four HDAC2(-/-) mutant clones collected at the early (E; ~5 days) and later (L; ~60 days) cultivation stages and on DT40 cells (W). Dense cytoplasmic fractions due to accumulated IgM H-chain were observed only at the early (E) stage in four mutant clones (E in upper panels). Positive signals for IgM H-chains were observed only at the early (E) stage in four mutant clones (E in lower panels). The figure is identical with Fig. 4-4 in [Chap. 4].

Figure 7-12. Alterations of morphology of individual HDAC2(-/-) DT40 mutant clones during continuous cultivation

Microscopy was performed on four HDAC2(-/-) mutant clones at the early (E) and later (L) cultivation stages and on DT40 cells (W). Aggregative form was observed only at the early (E) stage in all of mutant clones. The figure is identical with Fig. 4-5 in [Chap. 4].

Figure 7-13. 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 HDAC2(-/-) mutant clone cl.2-1 during continuous cultivation

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

Figure 7-14. 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 HDAC2(-/-) mutant clone cl.2-2 during continuous cultivation

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

Figure 7-15. 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 HDAC2(-/-) mutant clone cl.2-4 during continuous cultivation

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

Figure 7-16. 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 HDAC2(-/-) mutant clone cl.2-6 during continuous cultivation

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

Figure 7-17. Summary of alterations in mRNA (gene expression/transcription) levels (high or low), acetylation levels (hyper or hypo) of specific Lys residues of histone H3 and the chromatin structure (loose or tight form) surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in four individual HDAC2(-/-) mutant clones at early (E), middle (M) and later (L) cultivation stages and in DT40 cells (W)

The figure is identical with Suppl. Fig. 6-S10 in [Chap. 6] and shown with some modifications.

Figure 7-18. Localization of IgM H-chain proteins at peri-nuclear space, endoplasmic reticulum and cell surface of HDAC2(-/-) DT40 mutant cells

Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was performed on HDAC2(-/-) mutant cells at the early (E) cultivation stage after treatment without (A) or with saponin (B ~ E). Arrows P, E and S indicate IgM H-chain proteins existing at the peri-nuclear space, endoplasmic reticulum and cell surface, respectively. The figure is identical with Fig. 6-1 in [Chap. 6].

Figure 7-19. Localization of IgM H-chain proteins in peri-nuclear space of HDAC2(-/-) DT40 mutant cells at early and later stages of continuous cultivation

Immuno-electron microscopy was carried out on HDAC2(-/-) mutant cells at the early (E) and later (L) cultivation stages, using anti-chicken IgM H-chain antiserum. A) Immuno-electron microscopy of one HDAC2(-/-) mutant cell at the early (E) 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 HDAC2(-/-) mutant cells at the later (L) 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) stages. The figure is identical with Fig. 6-3 in [Chap. 6].

Figure 7-20. A model for signal transduction on IgM H- and L-chains accumulated in peri-nuclear space to chromatin structure in HDAC2(-/-) DT40 mutant cells

Left panel: Immuno-electron microscopy revealed that artificially accumulated IgM H-chain proteins were localized in the peri-nuclear space of HDAC2(-/-) mutant cells. **Right panel:** A model for signal transduction on accumulation of IgM H- and L-chains. Signal on excessively accumulated IgM H- and

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

Figure 7-21. Summary of alterations in mRNA (gene expression/transcription) levels of IgM H- and L-chains, Pax5, Aiolos, EBF1 and OBF1 genes, and acetylation levels of specific Lys residues of histone H3 and chromatin structure of their proximal 5'-upstream regions in individual HDAC2(-/-) DT40 mutant clones during continuous cultivation

Alterations in mRNA (gene expression/transcription) levels of IgM H- and L-chains, Pax5, Aiolos, EBF1 and OBF1 genes, acetylation levels (Ac) of specific Lys residues of histone H3, and the chromatin structure (loose or tight) of the proximal 5'-upstream regions of the last four genes in four HDAC2(-/-) mutant clones at the early (E) and later (L) cultivation stages and in DT40 cells (W) are schematically presented. The 4C machinery contains a specific member of each of HATs and HDACs and others. The TFC machinery contains RNA polymerase, specific transcription factors and others. The figure is identical with Fig. 6-5 in [Chap. 6].

Figure 7-22. Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region in descendent cells and reversible regulations of gene expression and enzyme reaction in cells which initially accept proper signal

Irreversible creation of the chromatin structure plasticity surrounding proximal 5'-upstream region of certain specific gene occurs in descendent cells but not in initial cells that accept signal on the abnormal environment change (upper panel). Regulations of ordinary gene expression and enzyme reaction reversibly occur in the cells that accept proper signal (middle and lower panels). The figure is identical with Fig. 6-7 in [Chap. 6].

Figure 7-23. Schematic presentation of chromatin conformation change code (4C) theory for gaining un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations

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