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Review

Chromatin conformation change code (4C) theory: A bio-system to gain 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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ABSTRACT

In this article we review our recent studies on the ability of higher eukaryotes to gain un-programmed and new cell functions by means of irreversible creation of chromatin structure plasticity through various generations. Following the exclusion of excessively accumulated IgM H- and L-chains in histone deacetylase2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) as a concrete case, we proposed a universal hypothetic concept on the ability of higher eukaryotic cells to adapt to abnormal environment changes. When higher eukaryotic cells firstly encounter moderate intra- and/or extra-cellular environment change, they acquire the ability to cope with and/or overcome it through irreversible creation of plasticity of the varied chromatin structure surrounding proximal 5'-upstream regions of the specific transcription factor and chromatin-modifying enzyme genes. The basis for this striking biological event is their successive chromatin conformational change with epigenetic modifications through various generations. Putative environment change recognition receptor/site (ECRR/ECRS) recognizes the new environment change. Putative chromatin conformation change complex (4C) machinery irreversibly and separately creates plasticity of the varied chromatin structure surrounding the proximal 5'-upstream regions of the above-mentioned specific factor and enzyme genes in distinct ways in individual cells of the same cell type. We advance a chromatin conformation change code (4C) theory for the bio-system to gain un-programmed and new cell function(s) by means of irreversible creation of chromatin structure plasticity by the 4C machinery through various generations, in order to adapt to the new environment change recognized by ECRR/ECRS. The 4C theory is suitable as an explanation for the ways to control development and differentiation of higher eukaryotes.

KEYWORDS: chromatin conformation change code (4C) theory, gaining un-programmed and new cell function, irreversible creation of chromatin structure plasticity, pluri-potency, epigenetic modifications, various generations, immuno-electron microscopy study, elasticity and flexibility of somatic cells.

INTRODUCTION

In eukaryotes, genome information and nuclear function are mainly protected by the nuclear membrane that acts as a nucleus-cytoplasm barrier. Additionally, communication and signal transduction between the nucleus and cytoplasm, both of which are essential for expressions of normal cell functions,

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are preferentially carried out by the to and fro passage of large and/or small molecules *via* the nuclear pore that acts as a guard station at the barrier. By contrast, both the signal transduction concerning an unexpected and/or abnormal change in the intra- and/or extra-cellular environment and the transport of useless molecules to nucleus are usually prevented by this barrier system. Then, how do the higher eukaryotic cells cope with and/or overcome an unexpected and/or abnormal environment change, when they firstly encounter it. Generally, this is possible by adhering to four typical countermeasures. First, in the case of a very severe change, cells die because it is far ahead of their adaptation ability. Second, in the case of a considerably severe change, cells cope with or overcome it by means of alterations in genome information, such as point mutation, insertion, deletion, duplication and multiplication in the DNA molecule through various generations. This mode is a basis for the evolution of species. Third, in the case of a moderate change, cells cope with or overcome it through irreversible creation of chromatin structure plasticity caused by successive chromatin conformational (structural) changes with epigenetic modifications through various generations. This mode is a basis for the development and differentiation of cells. Fourth, in the case of a minor change, cells respond to it only through already acquired regulation mechanisms.

Since chemical modifications of histones with acetyl and methyl groups were first proposed to regulate RNA synthesis [1], the modulation of chromatin topology has been thought to be one of the most fundamental and important events for expression of normal cell functions in eukaryotes. The ways to modulate chromatin structure with acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation and others have been intensively studied in a variety of life science fields. Of these epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones (H2A, H2B, H3 and H4) catalyzed by chromatin-modifying enzymes, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), are undoubtedly the major ones. For the past several decades, countless numbers of research papers on acetylation and deacetylation (and also other epigenetic modifications) are accumulating without interruption in more diverse life science fields, e.g. transcription/gene expression, DNA replication, development, differentiation, memory, pluri-potency, clinical medicine, etc. [2-28].

Using gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [29, 30], we have systematically generated various mutants, all of which were devoid of specific members of HDACs, HATs and transcription factors [31-45], besides those lacking numerous members of histones and histone chaperones [46-77]. Systematic analyses of the resultant DT40 mutants HDAC2(-/-), Aiolos(-/-), Pax5(-), EBF1(-/-), Ikaros(-/-/+), Helios(-/-) and E2A(-/-) have revealed their interesting participation in gene expressions of IgM H- and L-chains. In wild-type DT40 cells, HDAC2 as a supervisor indirectly regulates gene expressions of IgM H- and L-chains through opposite control of gene expressions of Pax5, Aiolos plus EBF1, and Ikaros plus E2A [31, 35]. Furthermore, in HDAC2(-/-), IgM H- and L-chains are excessively accumulated based on their dramatically increased gene expressions caused by drastic decreases in gene expressions of Pax5, Aiolos and EBF1, all of which down-regulate gene expressions of the two immunoglobulin proteins [31, 33, 35]. On the other hand, OBF1 is strongly suggested to up-regulate gene expressions of IgM H- and L-chains [78].

Recently, we analyzed initially generated HDAC2-deficient mutants [79, 80], Pax5-deficient mutants [79, 81] and secondly generated HDAC2-deficient mutants [79, 82], all of which were continuously cultivated for varyingly long periods. In these studies, the cultivation stages and/or periods were practically counted from the first day of cultivation from the stock at -80 °C. At the outset, surprisingly, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of the initially generated HDAC2(-/-) mutants not only revealed that the IgM H- and L-chains are dramatically increased at the early stage of cultivation, but also that the two accumulated immunoglobulin proteins are gradually decreased throughout the middle stage and at the later cultivation stage reached comparable levels as in DT40 cells (Fig. 1) [79, 80]. Successively, as will be described in detail later, in individual clones of secondly generated HDAC2(-/-) mutants, the accumulated IgM H- and L-chains at the early cultivation stage are dramatically decreased, which is attributed to their drastically decreased gene

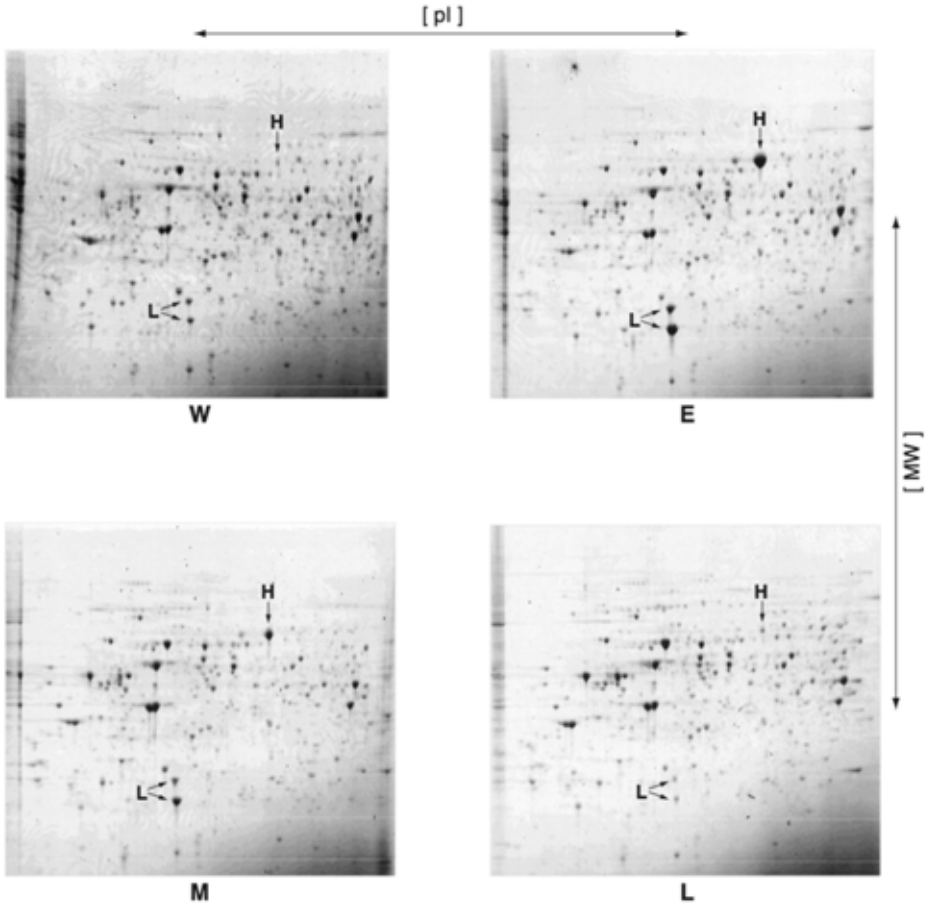

Fig. 1. Alterations in amounts of IgM H- and L-chains in HDAC2(-/-) DT40 mutants during cultivation.
2D-PAGE was performed on total cellular proteins prepared from DT40 cells (W) and the initially generated HDAC2(-/-) mutant clone at the early (E; ~10 days), middle (M; ~30 days) and later (L; ~60 days) stages of cultivation. Iso-electrofocusing in the first dimension (pI) and SDS-PAGE in the second dimension (MW) were performed on Immobiline DryStript gel (pH 4-7) and ExcelGel XL SDS gel (gradient 12-14), followed by the fluorostaining method. H and L indicate IgM H-chain and L-chain (detected as two spots), respectively. The figure is identical with fig. 1 of ref. 80.

expressions in almost the same changing pattern, caused by dramatic increases or decreases in gene expressions of Pax5, Aiolos and EBF1 or OBF1 in distinct ways during cultivation [79, 82]. Such diminutions of the accumulated IgM H- and L-chains in all individual HDAC2(-/-) mutant clones during cultivation are really examples of the third case of the above-mentioned countermeasures. In addition, acetylation levels of specific Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) within the chromatin surrounding proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes change in distinct patterns in all individual clones of HDAC2(-/-) mutants during continuous cultivation (details will be discussed later) [79, 83]. We also reported that the way for diminutions of IgM H- and L-chains accumulated in Pax5-deficient mutant cells Pax5(-) during cultivation moderately resembles the case in HDAC2(-/-) mutant cells, although detailed molecular mechanisms to decrease gene expressions of the two immunoglobulin proteins should be distinct between these two mutant cell lines [79, 81, 82]. Based on our previous and recent results on HDAC2(-/-) mutant cells [31, 35, 78-84], as a fundamental way for higher eukaryotes to adapt to environment change, we proposed a universal concept, which we named the chromatin conformation change code (4C) theory, for the bio-system to gain un-programmed and new cell function(s) through

irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations [84, 85]. This article is the review of the recent studies in [79-85].

IgM H- and L-chains excessively accumulated in HDAC2(-/-) DT40 mutants are dramatically decreased in distinct ways in individual mutant clones through various generations during continuous cultivation

The HDAC2-deficiency in chicken DT40 cells dramatically increases mRNA (i.e., transcription/ gene expression) levels of IgM H- and L-chains, followed by accumulation of the two immunoglobulin proteins (Figs. 1, 2 and 3) [79, 80, 82]. Surprisingly, excessively increased mRNAs and proteins of IgM H- and L-chains at the early stage of cultivation are gradually decreased until the later cultivation stage in almost similar pattern in all examined individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of secondly generated HDAC2(-/-) mutants (Figs. 2 and 3). By contrast, remarkably, gene expressions of various specific transcription factors and chromatin-modifying enzymes change in distinct patterns among these individual HDAC2(-/-) mutant clones (Fig. 3). To reduce the increased protein levels of IgM H- and L-chains resulted from their increased gene expressions, apparently, three distinct ways based on altered gene expressions of some specific transcription factors exist at the later cultivation stage in the six individual mutant clones [79, 82]. The way in clone cl.2-1 seems to be dependent on OBF1 and different from that in DT40 cells. The ways 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 cells in appearance. The way in clone cl.2-6 seems to be dependent on Pax5, Aiolos plus EBF1 and similar to that in DT40 cells in appearance.

Fundamental ways to irreversibly create plasticity of the chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications for gaining new cell functions to diminish IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation

To clarify these three distinct ways, we performed neighboring overlapping tiling chromatin immuno-

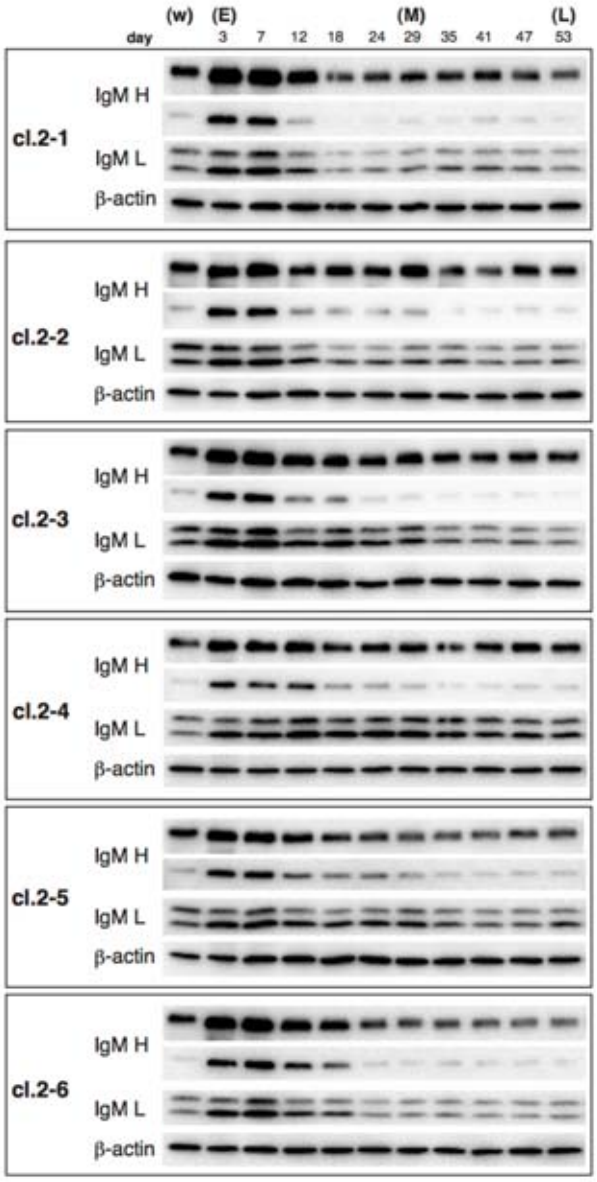

Fig. 2. Alterations in protein levels of IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants during cultivation.

Western blotting was performed on total cellular proteins prepared from DT40 cells (W) and secondly generated HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) at the indicated cultivation periods, including the early (E; 3 days), middle (M; 29 days) and later (L; 53 days) cultivation stages. Upper and lower bands (indicated by IgM H) correspond to the IgM H-chain detected by antibodies for IgM H- and L-chains, respectively. Double bands (indicated by IgM L) correspond to IgM L-chain detected by the antibody for IgM L-chain. The figure is identical with fig. 2 of ref. 82 and also suppl. fig. S4 of ref. 84.

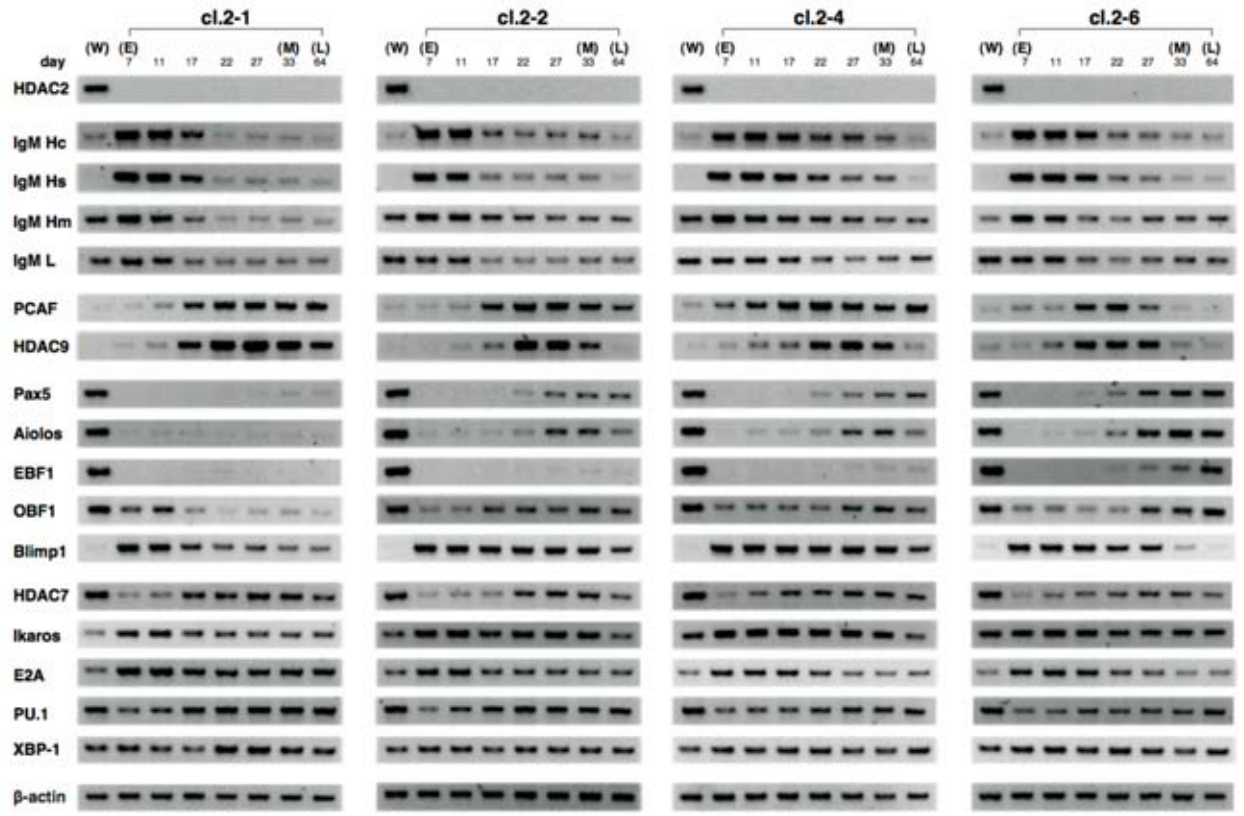

Fig. 3. Alterations in gene expressions of IgM H- and L-chains and specific members of HATs, HDACs and transcription factors in individual clones of HDAC2(-/-) DT40 mutants during cultivation.

RT-PCR was performed on total RNAs prepared from DT40 cells (W) and four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the indicated periods of cultivation, including the early (E; 7 days), middle (M; 33 days) and later (L; 64 days) cultivation stages, using appropriate primers for IgM H- and L-chains, PCAF, HDAC9, Pax5, Aiolos, EBF1, Blimp1, OBF1, HDAC7, Ikaros, E2A, PU.1 and XBP-1, because the alterations in gene expressions of these factors and enzymes during cultivation were previously reported [82]. The figure is identical with fig. 6 of ref. 82 and also with suppl. fig. S5 of ref. 84.

precipitation (NotchIP or Notch-IP: this abbreviation also means IP on notch of chromatin) assay on the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) surrounding the Pax5, Aiolos, EBF1 and OBF1 genes (Figs. 4, 5, 6 and 7) [79, 83]. The results obtained by the NotchIP assay revealed that such distinct ways should be fundamentally originating from irreversible creation of plasticity of the varied chromatin structure surrounding proximal ~2.0 kb 5'-upstream regions of these transcription factor genes with epigenetic modifications through various generations during cultivation. That is, in wild-type DT40 cells having HDAC2 activity, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of genes encoding Pax5, Aiolos and EBF1 (which down-regulate gene expressions of IgM H- and L-chains [35, 79, 80, 82]) and OBF1 (which probably up-regulates those of the two immunoglobulin proteins [78, 79, 82]) is in the loose (open) form due to no binding ability of histone H3 to DNA based on hyper- (high) acetylation levels of one or more of the five specific Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) [79, 83]. Therefore, these four transcription factor genes are transcribed at high levels in DT40 cells [35, 79, 80, 82]. On the other hand, in all individual clones of HDAC2(-/-) mutants having no HDAC2 activity, the chromatin structure surrounding proximal ~2.0 kb 5'-upstream regions of these four factor genes changes dramatically and severally based on varied acetylation

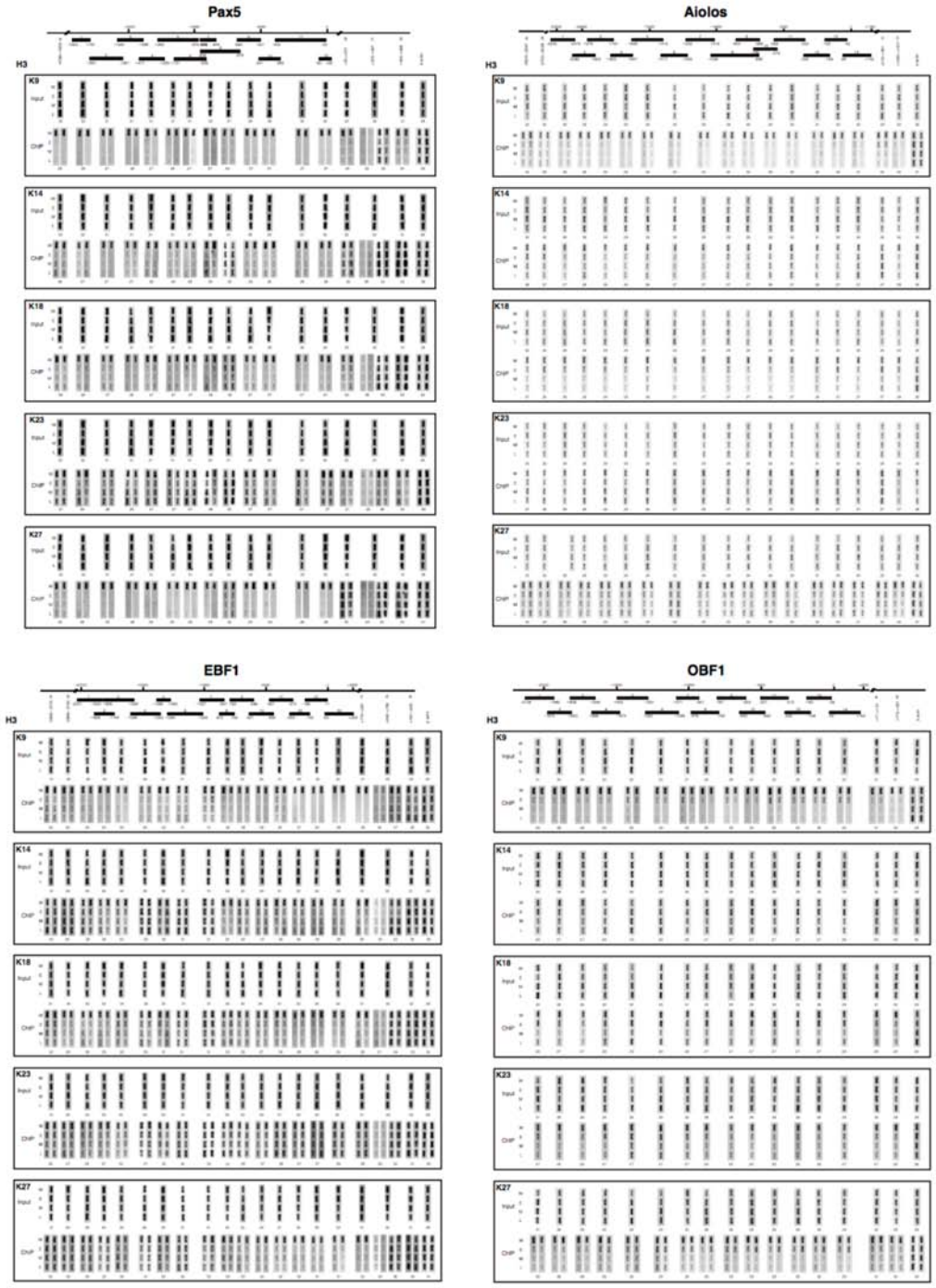

Fig. 4. Alterations in acetylation levels of five specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-1 of HDAC2(-/-) DT40 mutants during cultivation.

The NotchIP assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes. Cross-linked chromatins were prepared from DT40 cells (W) and clone cl.2-1 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages and co-precipitated by five antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers for the indicated segments of the Pax5, Aiolos, EBF1 and OBF1 genes. The figure is a set of figs. 1, 5, 9 and 13 of ref. 83 and also identical with suppl. fig. S6 of ref. 84.

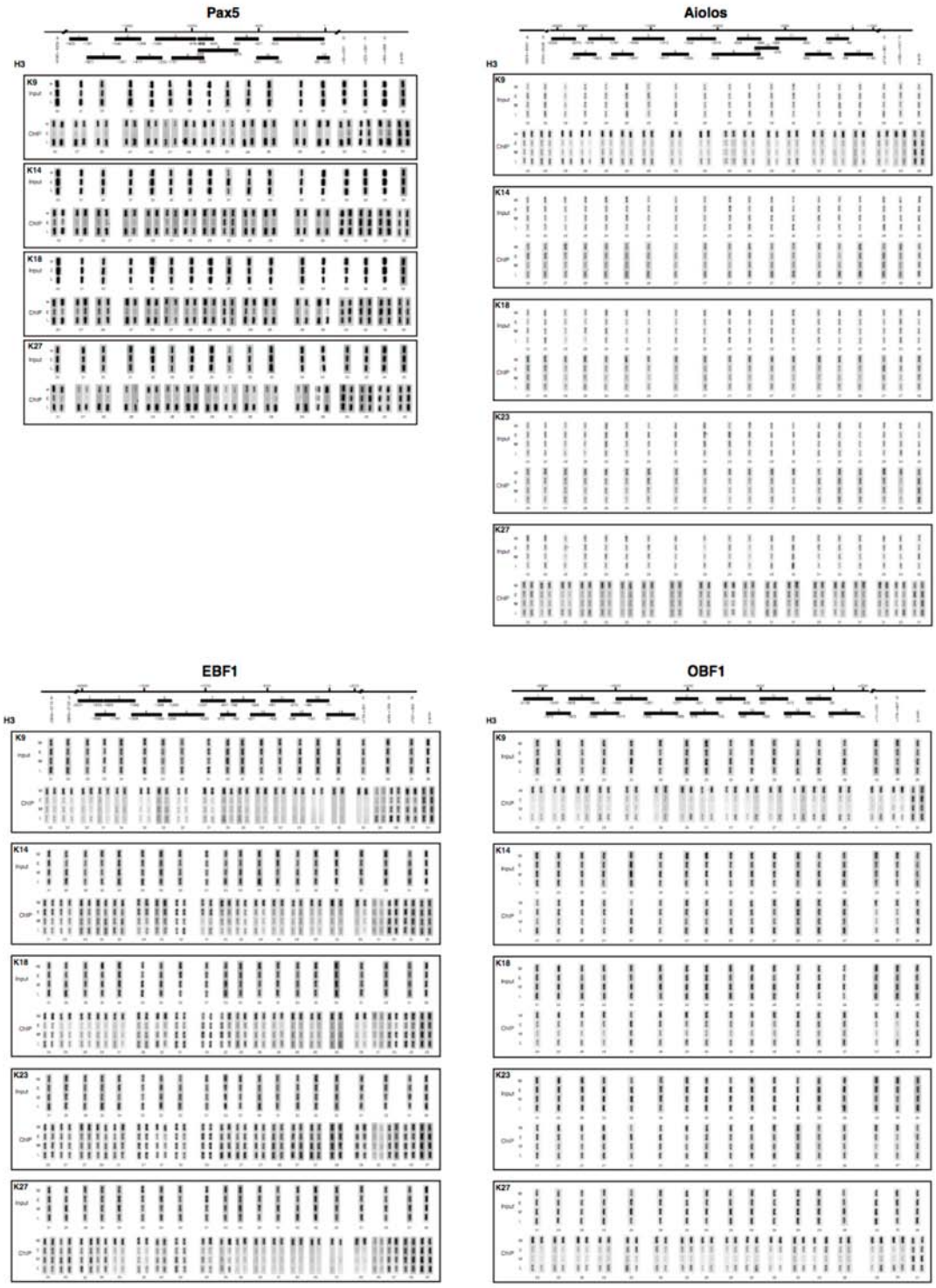

Fig. 5. Alterations in acetylation levels of five specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-2 of HDAC2(-/-) DT40 mutants during cultivation.

The NotchIP assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes in DT40 cells (W) and clone cl.2-2 of HDAC2(-/-) mutants at the early (E), middle (M) and late (L) cultivation stages as described in the legend to fig. 4. The figure is a set of figs. 2, 6, 10 and 14 of ref. 83 and also identical with suppl. fig. S7 of ref. 84.

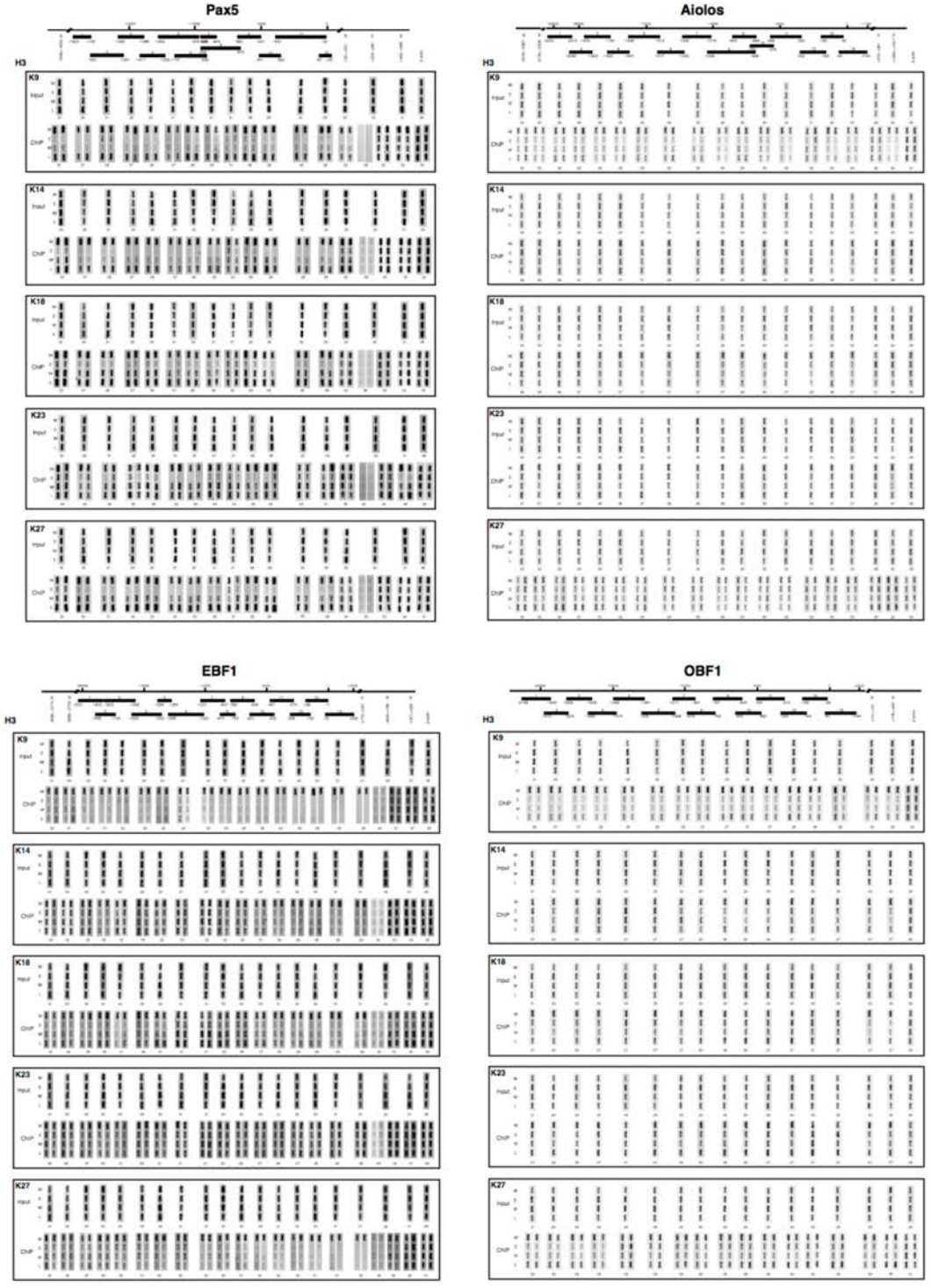

Fig. 6. Alterations in acetylation levels of five specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-4 of HDAC2(-/-) DT40 mutants during cultivation.

The NotchIP assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes in DT40 cells (W) and clone cl.2-4 of HDAC2(-/-) mutants at the early (E), middle (M) and late (L) cultivation stages as described in the legend to fig. 4. The figure is a set of figs. 3, 7, 11 and 15 of ref. 83 and also identical with suppl. fig. S8 of ref. 84.

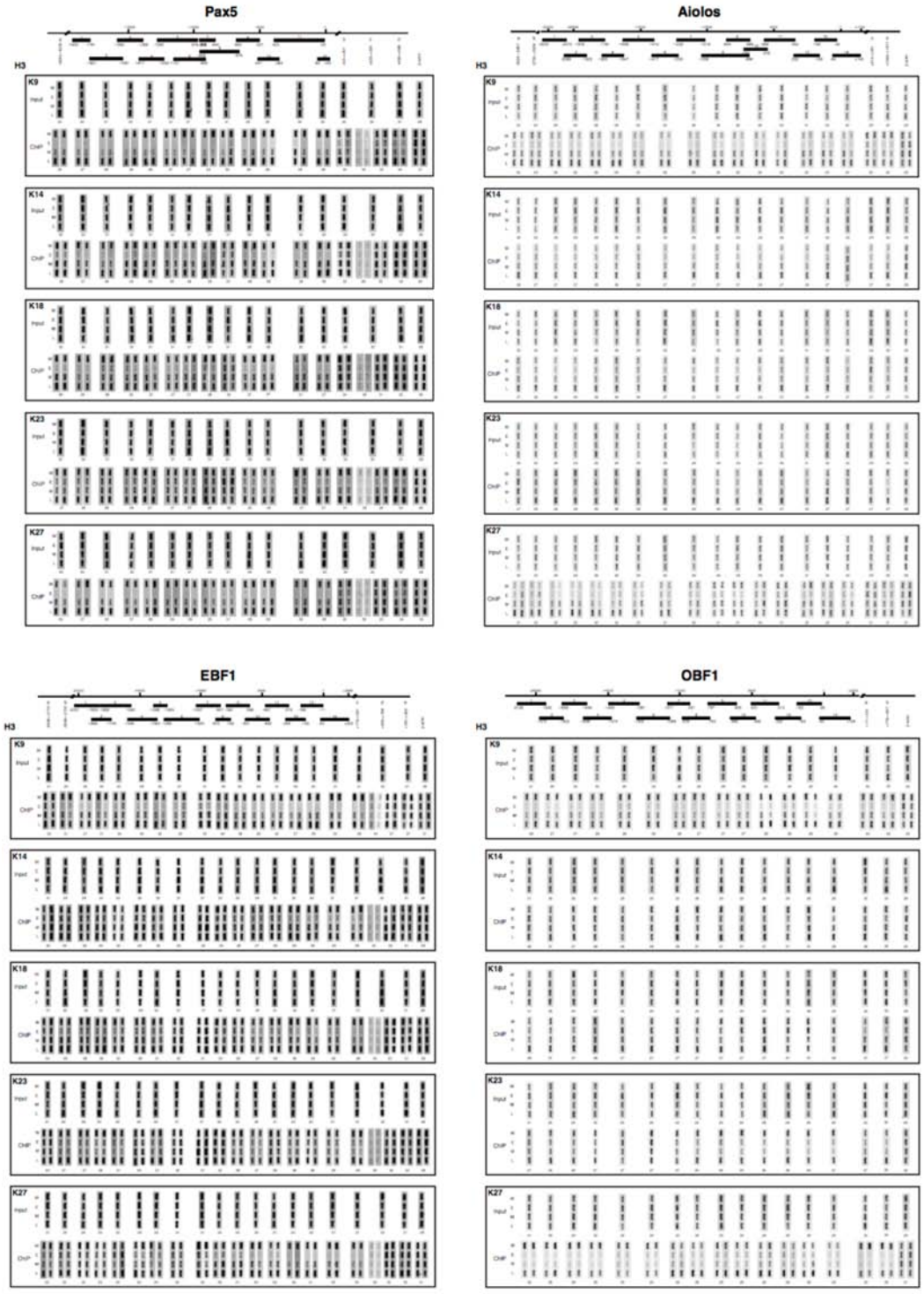

Fig. 7. Alterations in acetylation levels of five specific Lys residues (K) of histone H3 within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in clone cl.2-6 of HDAC2(-/-) DT40 mutants during cultivation.

The NotchIP assay was carried out on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of Pax5, Aiolos, EBF1 and OBF1 genes in DT40 cells (W) and clone cl.2-6 of HDAC2(-/-) mutants at the early (E), middle (M) and late (L) cultivation stages as described in the legend to fig. 4. The figure is a set of figs. 4, 8, 12 and 16 of ref. 83 and also identical with suppl. fig. S9 of ref. 84.

levels of one or more of the five specific Lys residues of histone H3 during cultivation, resulting in alterations in their gene expression levels as follows [79, 80, 82, 83].

In clone cl.2-1, at the early stage of cultivation, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos and EBF1 genes is in the tight (closed) form due to the binding ability of histone H3 to DNA based on hypo- (low or no) acetylation levels of one or more of the five specific Lys residues (Fig. 4), and as a result, transcriptions of these three genes are almost completely suppressed to undetectable levels (Fig. 3). However, the chromatin structure of the OBF1 gene is somewhat in the loose form due to the less binding ability of histone H3 to DNA based on slight (or considerably decreased) hyper-acetylation levels, thereby its transcription is slightly decreased. By contrast, at the later cultivation stage, the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes remains in the tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, their transcriptions also remain unchanged at an undetectable level. On the other hand, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene changes to the tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, its transcription is suppressed to almost undetectable or very low level. These results supported the above-mentioned inference (i.e., OBF1-dependent) on the ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clone cl.2-1 [79, 82-84].

In clones cl.2-2 and cl.2-4, at the early stage of cultivation, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is in the tight or somewhat loose (or less tight) form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five specific Lys residues of histone H3 (Figs. 5 and 6). Therefore, transcriptions of the first three genes are almost completely suppressed and that of the last one gene is certainly decreased (Fig. 3). At the later cultivation stage, contrary to this, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos and OBF1 genes changes to the loose form based on hyper-acetylation levels, and their transcriptions are dramatically increased. However, the chromatin structure of the EBF1 gene remains in the 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 ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clones cl.2-2 and cl.2-4 (and also cl.2-3 and cl.2-5) [79, 82-84]. Moreover, these four clones should be the major types, since they resemble in several cellular characteristics to four initially generated HDAC2(-/-) clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) [31, 35, 79, 80, 82].

In clone cl.2-6, at the early stage of cultivation, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is in the tight or somewhat loose form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five specific Lys residues of histone H3 (Fig. 7). Therefore, transcriptions of the first three genes are almost completely suppressed and that of the last one gene is certainly decreased (Fig. 3). By contrast, at the later cultivation stage, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes changes to the loose form based on hyper-acetylation levels, and their transcriptions are dramatically increased. These results supported the above-mentioned inference (i.e., Pax5-, Aiolos- and EBF1-dependent) on the ways of gene expressions of IgM H- and L-chains at the later cultivation stage in clone cl.2-6 [79, 82-84].

Results on alterations in acetylation levels (hyper or hypo) of the five specific Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3), the form (loose or tight) of the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (transcription/gene expression) levels (high or low) during cultivation in individual HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 [79, 82, 83] will be schematically presented later. Concerning the above-mentioned results on the suppression of gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivation, there are some important comments as follows.

Excessively accumulated IgM H- and L-chains resulting from their dramatically increased gene expressions in HDAC2(-/-) mutants just after their birth (by gene targeting techniques) [31, 35, 79, 80, 82] are probably useless for them; therefore, the mutant cells have acquired a new ability to diminish a large amount of the two immunoglobulin proteins as abnormal environment change through various generations during cultivation. The ways to suppress gene expressions of IgM H- and L-chains at the later cultivation stage in all of HDAC2(-/-) mutant clones [79, 82] are surely distinct from the ordinary and reversible way to regulate their gene expressions in DT40 cells, because in DT40 cells gene expressions of IgM H- and L-chains are indirectly regulated by HDAC2 through opposite regulations of Pax5, Aiolos, EBF1, OBF1 and E2A gene expressions [35, 37]. In addition, gene expressions of several transcription factors and chromatin-modifying enzymes (i.e., Blimp1, PCAF, HDAC7, HDAC9, etc.), besides those of Pax5, Aiolos, EBF1 and OBF1, dramatically and separately change in individual HDAC2(-/-) mutant clones during cultivation (Fig. 3) [79, 80, 82]. Therefore, remarkably, some other unknown important cellular characteristics might be changing among individual HDAC2(-/-) mutant clones during cultivation. Such presumable changeable characteristics may be complicated and diverse in individual clones of HDAC2(-/-) mutants, even though they are originally of the same cell type.

A part of excessively accumulated IgM H- and L-chains is transported to nuclear envelope but not inside of nucleus and kept at peri-nuclear space of HDAC2(-/-) DT40 mutant cells at early and later stages of continuous cultivation

Large amounts of IgM H- and L-chains artificially created by their excessively increased gene expressions induced by the HDAC2-deficiency (Figs. 1, 2 and 3) are first accumulated probably within the endoplasmic reticulum of HDAC2(-/-) mutant cells (Figs. 8 and 9) [35, 79, 82, 84]. Most of the accumulated IgM H- and L-chains (which probably exist as a high-molecular weight complex) [35]) are gradually secreted into the cultivation media, whereas a part of them is transported to the nuclear envelope but not inside of nucleus, and kept at peri-nuclear space at the early and later cultivation stages (Figs. 8 and 9) [79, 84]. This inference should be surely supported by other results obtained from immuno-electron microscopy study [79, 84].

Proposed ways for gaining un-programmed and new cell functions to diminish excessively accumulated IgM H- and L-chains through irreversible creation of varied chromatin structure plasticity of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

Based on the above-mentioned results and others [31, 33, 35, 37, 78-84], we proposed an all-inclusive hypothetic way for gaining un-programmed and new cell functions to diminish excessively accumulated IgM H- and L-chains through irreversible creation of varied chromatin structure plasticity of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications during continuous cultivation in individual clones of HDAC2(-/-) mutants [84, 85]. First, we supposed that the environment change recognition receptor/site (ECRR/ECRS) participates in the recognition of accumulation of IgM H- and L-chains as an abnormal environment change (and probably acts in part in the signal transduction concerning the IgM H- and L-chain accumulation on the chromatin) (Figs. 9 and 10). In addition, chromatin conformation (structure) change complex (4C) machinery, which is diverse and consists of a member of each of the HATs plus HDACs and other factors, is supposed to directly and irreversibly create plasticity of the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned transcription factor genes (and also act in part in the signal transduction) (Figs. 9 and 10). Using the ECRR/ECRS, the 4C machinery and other components, the chain reaction of response to the abnormal accumulation of IgM H- and L-chains occurs as follows. In wild-type DT40 cells, in which various members of HATs and HDACs are largely expressed [35, 79, 82], the 4C machinery for each of the Pax5, Aiolos, EBF1 and OBF1 genes probably consists of HDAC2, a specific HAT member and other factors (Fig. 10). On the other hand, in all of the individual clones of HDAC2(-/-) mutants at the very early stage of cultivation (just soon after their birth by gene targeting techniques),

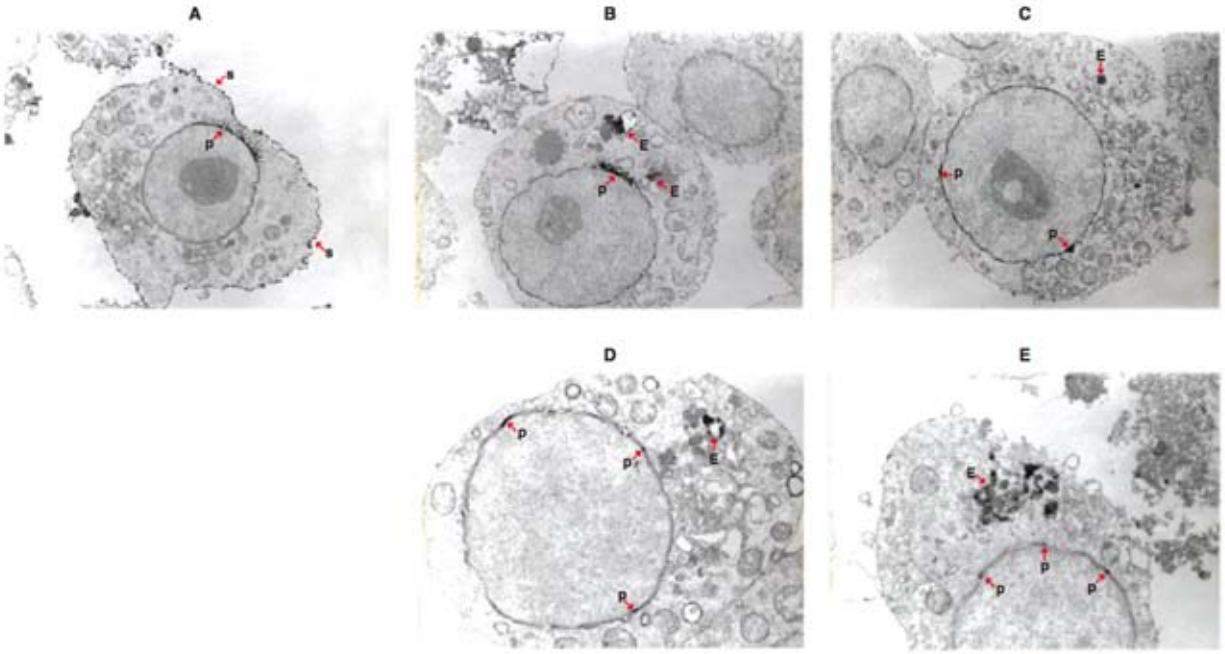
A
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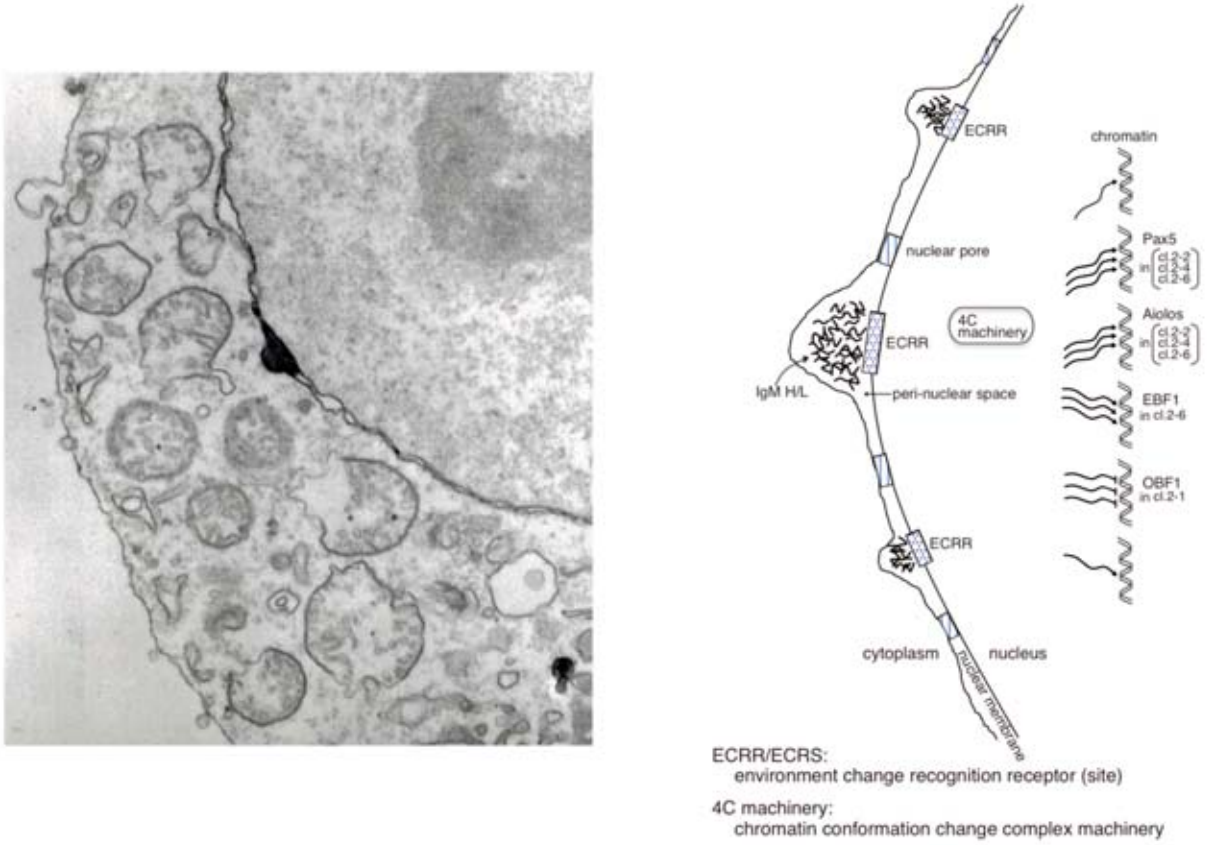
Fig. 8


ECRR
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ECRR
cytoplasm
nucleus
nuclear membrane
chromatin
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Aiolos
EBF1
in cl.2-6
OBF1
in cl.2-1
ECRR/ECRS:
environment change recognition receptor (site)
4C machinery:
chromatin conformation change complex machinery

Fig. 9

bulk conformation of the 4C machinery dramatically changes to remove or drastically reduce HAT activity (of the assumed member of HATs), attributed to the HDAC2-deficiency. Through the above-mentioned process and following continuous cultivation, the 4C machinery newly consists of a different member of HDACs, a specific (same or different) member of HATs and other factors, and thereby becomes varied.

The excessively accumulated IgM H- and L-chains lying at the peri-nuclear space bind to the ECRR/ECRS localized at the inner nuclear membrane (where hetero-chromatin is possibly located) (Fig. 9). After the ECRR/ECRS recognizes the accumulation of IgM H- and L-chains as an abnormal environment change, the signal concerning it is genome-widely transmitted to proximal 5'-upstream chromatin regions of numerous genes (probably located at several distinct chromosomes) encoding transcription factors, chromatin-modifying enzymes, and related factors and enzymes. Following the initial signal transduction, a spontaneous unbalanced response to the abnormal environment change is consecutively and separately converged to the proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1, OBF1 and other genes in individual clones of HDAC2(-/-) mutants. The diversity of alterations in the chromatin structure surrounding proximal 5'-upstream regions of these transcription factor genes is preferentially attributed to varied acetylation and deacetylation levels of one or more of the five specific Lys residues at N-terminal tail of histone H3 [79, 83] caused by the collaboration of proper members of the HATs and HDACs in the protean 4C machinery. These successive epigenetic modifications of K9/H3 and K27/H3 (and also K14/H3, K18/H3 and K23/H3) with acetyl group lead to irreversible creation of plasticity of the chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes (Figs. 4, 5, 6, 7 and 10). In general, chromatin structure surrounding proximal 5'-upstream regions possessing hyper-acetylation levels of one or more of the specific Lys residues of histone H3 is in the loose form based on its no binding ability to DNA, but the chromatin structure surrounding proximal 5'-upstream regions possessing hypo-acetylation levels of one or more of the specific Lys residues of histone H3 is in the tight form based on its binding ability to DNA (Figs. 10 and 11). By contrast, the 4C machinery cannot change the chromatin structure surrounding open reading frames (coding regions) of these specific transcription factor genes by much [79, 83]. As the need arises, transcription factor complex (TFC) machinery (which consists of RNA polymerase, proper transcription factor(s), certain members of the HATs and HDACs, and other factors) is able to bind to promoters (or elements) within loose form of chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes (which have become latently active state), and thereby initiates their gene expressions (Figs. 9, 10 and 11).

Legend to Fig. 8. Localization of IgM H-chain at the peri-nuclear space, endoplasmic reticulum and cell surface of HDAC2(-/-) DT40 mutant cells.

Immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out on initially generated HDAC2(-/-) mutant cells at the early cultivation stage. A) Immuno-electron microscopy without saponin-treatment. B)–E) Immuno-electron microscopy with saponin-treatment. Arrows P, E and S indicate positive signals of IgM H-chain localized at the peri-nuclear space, endoplasmic reticulum and cell surface, respectively. A large amount of accumulated IgM H-chain were observed in the peri-nuclear space of all HDAC2(-/-) mutant cells. The figure is identical with fig. 1 of ref. 84.

Legend to Fig. 9. Localization of IgM H-chain at the peri-nuclear space, and a model of signal transduction concerning the accumulation of IgM H- and L-chains on the chromatin in HDAC2(-/-) DT40 mutants during cultivation.

Left panel: A portion of the peri-nuclear space (where IgM H- and L-chains were accumulated) of the HDAC2(-/-) mutant cell (indicated by an arrow P at lower position in Fig. 8C) was reversely enlarged. Right panel: A model of signal transduction concerning the accumulation of IgM H- and L-chains on the chromatin. Signal concerning IgM H- and L-chains artificially accumulated in the peri-nuclear space of HDAC2(-/-) mutants was repeatedly transmitted to the chromatin structure, followed by unbalanced response to the signal and its convergence to various specific genes (for 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. 4 of ref. 84.

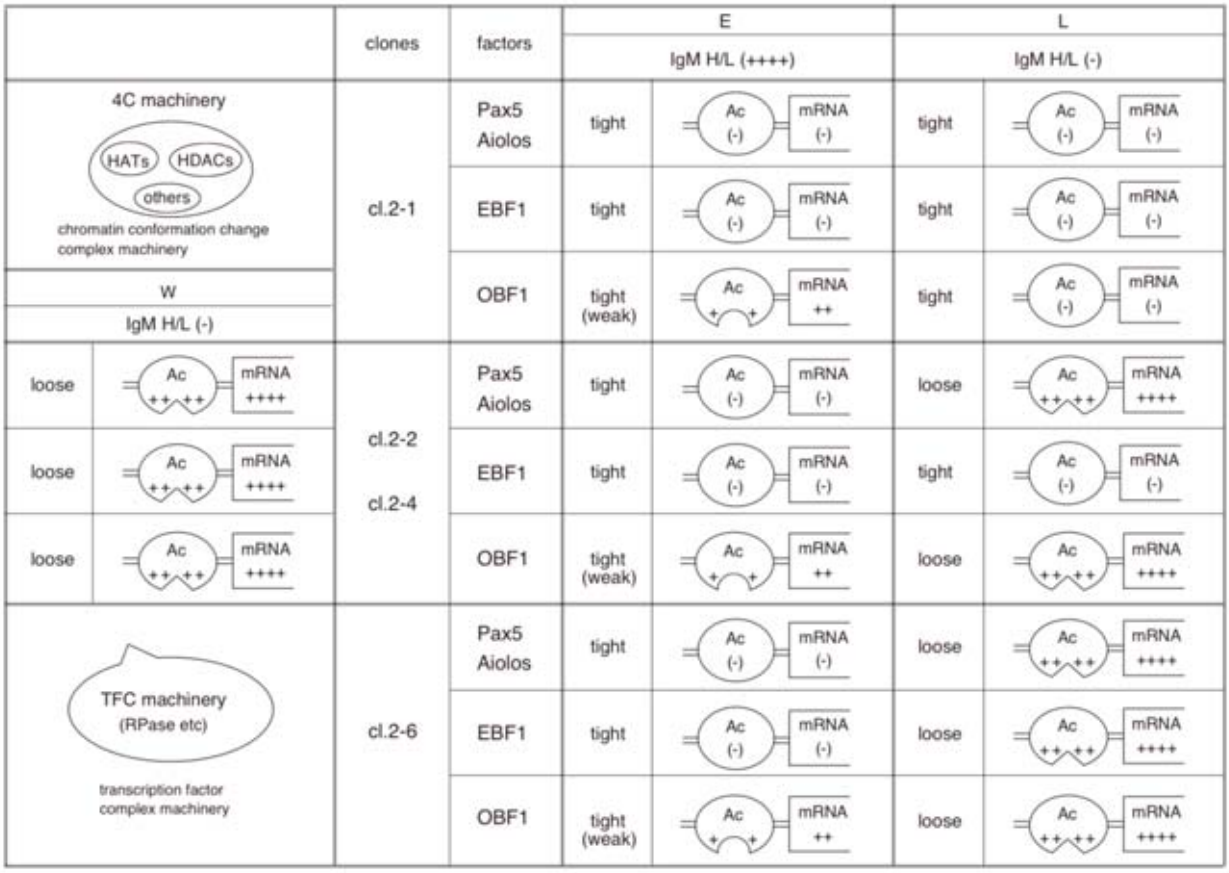

Fig. 10. Summary of alterations in gene expression levels, and acetylation levels of five specific Lys residues (K) of histone H3, and the chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in individual clones of HDAC2(-/-) DT40 mutants during cultivation.

Alterations in mRNA (gene expression) levels (-, ++ or ++++), and acetylation levels (Ac; -, ++ or ++++) of five specific Lys residues (K) of histone H3 and the chromatin structure (form; loose or tight) of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in DT40 cells (W) and four HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E) and late (L) cultivation stages are schematically presented. Alterations in mRNA levels (- or ++++) of IgM H- and L-chains are also presented. The 4C machinery should generally contain specific member of the HATs plus HDACs and other factors. The TFC machinery should generally contain RNA polymerase (RPase), specific transcription factors and others. The figure is identical with fig. 5 of ref. 84.

As a concrete result, individual HDAC2(-/-) mutant clones gain the same, un-programmed and new cell function to reduce increased gene expressions of IgM H- and L-chains (resulting in their decreased protein levels) in almost the same changing pattern through increased or decreased gene expressions of Pax5, Aiolos plus EBF1 or OBF1 in distinct ways during simple continuous cultivation under the same conditions (Fig. 3) [35, 79, 80, 82]. Such distinct ways are originally based on irreversible creation of their distinct chromatin structure plasticity with epigenetic modifications during continuous cultivation [79, 83]. Remarkably, the six individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants can be clearly classified into the above-mentioned three different cell types exhibiting distinct functions, because they show three dissimilar ways for gene expressions of transcription factors and chromatin-modifying enzymes (such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC9, etc.) at the later cultivation stage (Fig. 3). Besides, in one or more of these six mutant clones, gene expression patterns of PCAF, HDAC7, HDAC9, Ikaros and OBF1 are spontaneously and complicatedly reversed in the midst of simple continuous cultivation,

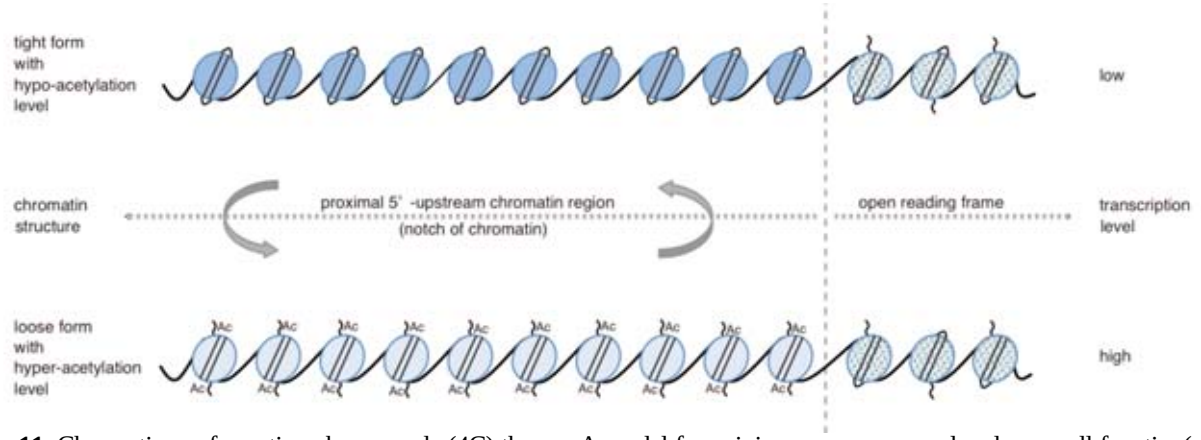

Fig. 11. Chromatin conformation change code (4C) theory: A model for gaining un-programmed and new cell function(s) through irreversible creation of chromatin structure plasticity with epigenetic modifications through various generations. Irreversible creation of chromatin structure plasticity with epigenetic modifications occurs within proximal 5'-upstream but not ORF regions of specific transcription factor gene(s) through various generations (cell divisions). Tight or loose form of the chromatin structure is based on hypo- or hyper-acetylation levels of specific Lys residues (K) of histone H3, and causes low or high transcription (gene expression) levels. The figure is identical with fig. 6 of ref. 84.

although those of Pax5, Aiolos, EBF1, E2A, PU.1 and Blimp1 do not change while passing from the early to later cultivation stages. Furthermore, if other individual mutant clones [79, 82] are analyzed, there is a possibility that additional different cell types might exist. Consequently, individual clones of HDAC2(-/-) mutants acquire flexible, elastic and pluri-potential ability not only to adapt in distinct ways to an abnormal environment change but also to branch off into diverse derivative cell types, which may exhibit varied characteristics (functions), even though they are originally of the same cell type.

Chromatin conformation change code (4C) theory: A universal way to gain un-programmed and new cell functions through irreversible creation of varied chromatin structure plasticity of specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications through various generations in higher eukaryotes

We expanded the above-mentioned hypothetic way on the exclusion of excessively accumulated IgM H- and L-chains to a universal hypothetic way for gaining un-programmed and new cell function(s) through irreversible creation of varied chromatin structure plasticity of the specific transcription factor and chromatin-modifying enzyme genes with epigenetic modifications through various generations (cell divisions) in higher eukaryotes [84, 85]. When higher eukaryotic cells firstly encounter an intra- and/or extra-cellular environment change in their lives, they gradually acquire the ability to adapt themselves to the new environment change or to exclude it (if abnormal). Using the ECRR/ECRS, the 4C machinery and other components, the eukaryotic cells create a chain reaction of response to the new environment change as follows (Figs. 8, 9 and 10). First of all, the new environment change is recognized by means of the ECRR/ ECRS, which may be localized nearby the nuclear membrane as a nucleus-cytoplasm barrier (probably at the inner nuclear membrane where hetero-chromatin is possibly located) (Fig. 9). Naturally, there is a possibility that putative specific molecule(s) acts as the intermediary sensor at this step to recognize the new environment change. Next, putative signal(s) concerning the new environment change is genome-widely transmitted to the chromatin within the nucleus through various generations (cell divisions). Following the initial acceptance of the putative signal(s), the 4C machinery induces a slight alteration in the chromatin structure of numerous genes encoding chromatin-modifying enzymes, transcription factors, and related enzymes and factors with epigenetic modifications. The transduction of the signal(s) and spontaneous unbalanced response to the new environment change are successively repeated and converged into the restricted chromatin structure surrounding

proximal 5'-upstream regions of several specific members of the above-mentioned factors and enzyme genes. Finally, this successive signal transduction concerning the new environment change causes various epigenetic modifications of histones and/or DNA within the restricted chromatin structure of the aforesaid specific genes with acetyl, methyl, phosphate, ubiquitin and ADP ribose groups and/or others. Of these various epigenetic modifications, acetylation and deacetylation of several specific Lys residues of core histones H2A, H2B, H3 and H4 may be the major ones.

The 4C machinery, which consists of a specific member of the HATs and HDACs and other factors, preferentially participates in the acetylation and deacetylation among such epigenetic modifications (Figs. 9 and 10). Positions of specific Lys residues and kinds of core histones are diverse. For instance, in the above-mentioned case [79, 83], acetylation and deacetylation of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 are prominent. Consequently, epigenetic modifications of one or more of these Lys residues of histone H3 with acetyl group change within the chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned specific target genes through various generations. 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

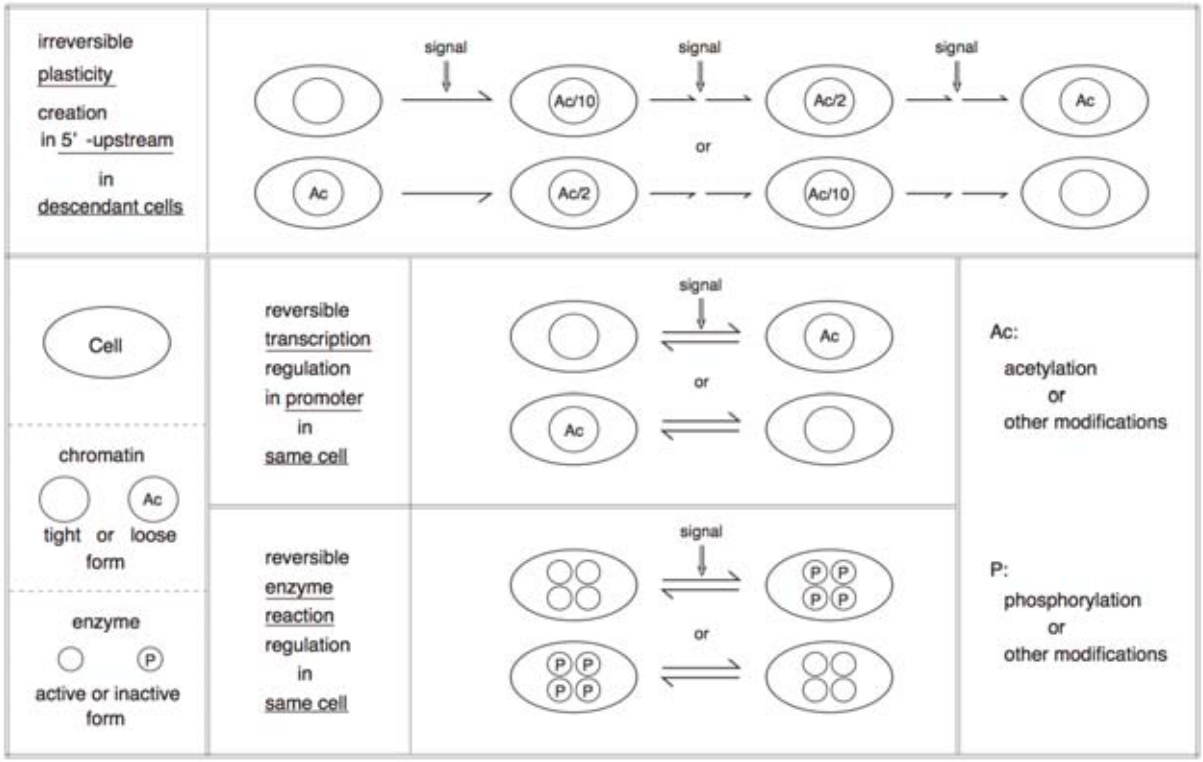

Fig. 12. Irreversible creation of plasticity of the chromatin structure surrounding proximal 5'-upstream region with epigenetic modifications in descendent cells, and reversible regulation of ordinary gene expression and enzyme reaction in cells that accept proper signal.

Upper panel: Irreversible creation of plasticity of the chromatin structure surrounding proximal 5'-upstream region of specific gene with epigenetic modifications occurs in descendant cells but not in cells that initially accept environment change signal. Ac, Ac/2 and Ac/10 indicate qualitatively hyper-, considerably hyper- and somewhat hyper-acetylation levels of specific Lys residues (K) of core histones (e.g., histone H3), respectively. Middle and lower panels: Regulation of ordinary gene expression (on transcriptional regulatory elements) and enzyme reaction occur reversibly in cells that accept proper signal. Ac and P indicate acetylation, and phosphorylation and/or other chemical modifications, respectively. The figure is identical with fig. 7 of ref. 84.

HDACs as the components. By contrast, the protean 4C machinery cannot alter the chromatin structure surrounding open reading frames (coding regions) of corresponding genes by much. The binding ability of the N-terminal tail of histone H3 to DNA is tentatively and qualitatively deduced from the acetylation levels of one or more of the specific Lys residues, though which Lys residue(s) really and/or mainly participates in the binding is still undefined. That is, hyper- (high) or hypo- (low or no) acetylation levels induces no binding or full binding ability, resulting in loose (open) or tight (closed) form of the chromatin structure (Figs. 10 and 11). Thus, the chromatin structure plasticity is irreversibly created based on successive conformation changes with epigenetic modifications. These ways to gradually and tardily create the chromatin structure plasticity for gaining un-programmed and new cell function(s) are obviously different from those that immediately and rapidly cause the chromatin conformation change for expressing programmed and ordinary cell function(s) (Figs. 11 and 12).

Whenever the need arises, the TFC machinery is able to bind to promoters (or elements) within the loose form of the chromatin structure surrounding proximal 5'-upstream regions of specific target genes (which are in the latently active state), followed by initiation of their gene expressions (Fig. 10). By contrast, the TFC machinery cannot bind to promoters (or elements) within the tight form of the chromatin structure surrounding proximal 5'-upstream regions of un-target genes (which are latently in the inactive state), and thereby cannot initiate their gene expressions. Consequently, the loose or tight form of the chromatin structure surrounding proximal 5'-upstream regions causes high or low (or no) transcription levels of the corresponding genes (Figs. 10 and 11). Notably, there is a great possibility that the ways for irreversible creation of chromatin structure plasticity are distinct among individual cells of the same type, even though the new environment change and signal(s) on the environment change are the same for all of them. That is, in order to gain un-programmed and new cell function(s), individual cells possess the ability not only to complicatedly and diversely alter the chromatin structure surrounding proximal 5'-upstream regions of various specific genes but also to separately alter the chromatin structure of the same proximal 5'-upstream region of a certain gene into varied forms. Thus, gene expressions of the specific transcription factors and chromatin-modifying enzymes change diversely among individual cells through various generations, in spite of the same environment change. As a consequence, individual cells of the same type are able to newly gain the same and/or distinct un-programmed cell function(s) in different ways, in order to accommodate themselves to a new environment change [84, 85].

CONCLUSION

In order to gain un-programmed and new cell function(s), somatic cells of higher eukaryotes become pluripotent, elastic and flexible, all of which basically originate from pluri-potency, elasticity and flexibility of the chromatin structure. That is, in order to adapt to an intra- and/or extra-cellular environment change, individual somatic cells possess the ability to newly gain the same and/or distinct un-programmed cell function(s) in different ways through irreversible creation of varied chromatin structure plasticity with epigenetic modifications, i.e., from loose to tight forms or vice versa of the chromatin structure surrounding proximal 5'-upstream regions of specific transcription factor and chromatin-modifying enzyme genes (Fig. 11). Such a loose or tight form of the chromatin structure surrounding the proximal 5'-upstream region is in the latently active or inactive state for transcription of the corresponding gene, although the proximal 5'-upstream region as mere nucleotide sequences is in the silent state for expressions of most of the genome functions. The varied chromatin structure plasticity in individual somatic cells is triggered by the spontaneous unbalanced response to the environment change when they firstly encounter it and then irreversibly accomplished by the successive unbalanced convergence of the response through various generations. The different ways to create varied chromatin structure plasticity in somatic cells are certainly dependent on their antecedents. Moreover, chromatin structure plasticity, regardless of whether its creation is in the course or was already completed, is inherited to descendant generations associated with or without additional structural change through cell divisions. Remarkably, irreversible creation of chromatin structure plasticity occurs 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 enzyme reactions occur in the cell itself which accepts proper signal (Fig. 12). Probably, irreversible creation of chromatin structure plasticity, with the intention of adapting to the environment change, occurs inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region (as loose or tight form) directs the switch (on or off) for latent transcription ability of the corresponding gene through irreversible creation of chromatin structure plasticity; therefore, the 5'-upstream chromatin region can be regarded as a "director" from a functional point of view, besides as a "notch" from a structural point of view as mentioned above. The notch (or director) covers specific nucleotide sequences of transcriptional elements (such as promoter, operator, enhancer, etc.). Remarkably, the real recipient of the signal concerning the environment change is the chromatin structure itself, which is dynamic and changeable (between loose and tight forms), but not the chromosomes, which are static and unchangeable. That is, the chromatin structure of proximal 5'-upstream region (notch or director), as a dynamic and changeable three-dimensional conformation, possesses two fundamental abilities, i.e., to receive the signal concerning the intra- and/or extra-cellular environment change and to direct the switch (on or off) for latent transcription ability of the corresponding gene through its irreversible chromatin conformation change that is responsive to the signal. Naturally, concrete data are not enough to support these ideas. As a consequence of these complicated biological events, higher eukaryotes acquire pluri-potential, elastic and flexible ability to create diverse derivative cell types possessing varied characteristics (functions) in distinct ways from the same type of somatic cells, in order to accommodate themselves to new intra- and/or extra-cellular environment. In some cases, even neighboring cells derived from the same cell type are probably regarded as extra-cellular environment for themselves. Moreover, in higher eukaryotes diverse kinds of somatic cells share and express cooperatively vital functions with each other.

Finally, we named our theory on such a bio-system that gains un-programmed and new cell function(s) through irreversible creation of chromatin structure plasticity with epigenetic modifications, which is one of the most fundamental and important ways for life conservation and cell type determination in higher eukaryotes, as the chromatin conformation (structure) change code (4C) theory [79, 84, 85]. Probably, the supposed number of codes in the 4C theory, which determines complicated and varied characteristics (functions) of higher eukaryotic cells, can be roughly estimated based on the combination of the number of candidate genes and that of codes for each of these genes. The most influential candidates are various specific genes encoding transcription factors, chromatin-modifying enzymes, and related factors and enzymes, all of which are necessary for gaining varied cell functions and specificities of higher eukaryotes. The number of codes for each of these candidate genes is two. This is because the proximal 5'-upstream chromatin region (the loose or tight form) directs the switch (on or off) for transcription activity of the corresponding gene. Therefore, the 4C theory can open the door for gaining un-programmed and new cell function(s) of higher eukaryotes and innovate the general notion on the nature of somatic cells. On the other hand, programmed, complicated and diverse cell functions are orderly and systematically expressed throughout their development and differentiation. However, the 4C theory is suitable as an explanation for the development and differentiation of higher eukaryotes, because action of putative signal(s) concerning environment changes seems to fairly resemble that of certain players (such as hormone, cytokine, nerve-transmission substance, etc.), which participate in cell-cell, tissue-tissue and/or organ-organ interactions (communications) throughout these two fundamental life phenomena.

There are several crucial questions to be clarified in the 4C theory on the exclusion of excessive IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutant cells [79-85]. 1) Despite the HDAC2-deficiency, why do the acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within the chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes decrease at the early stage of cultivation in HDAC2(-/-) mutants. 2) Why do the decreased

acetylation levels of one or more of the specific Lys residues of histone H3 within proximal 5'-upstream chromatin regions of these three genes increase during cultivation. 3) Why do the acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within the chromatin structure (of ~10 nucleosomes) surrounding the proximal 5'-upstrean region of the OBF1 gene decrease during cultivation. 4) 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. 5) Functional and steric differences between loose and tight forms (based on hyper- and hypo-acetylation levels of one or more of the specific Lys residues of histone H3) of the chromatin structure surrounding proximal 5'-upstream regions of these four genes must be clarified more precisely. 6) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor gene during cultivation differ among individual mutant clones. 7) Why do the changes in acetylation levels of the specific Lys residues of histone H3 for individual transcription factor genes during cultivation differ within individual mutant clone. 8) How does the 4C machinery (which acts in the 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 the transcription of open reading frame of the corresponding gene) may be the same as the well-known transcription machinery. 9) To demonstrate the 4C theory, it is essential to establish both the putative ECRR/ECRS as the first player to recognize the environment change and the putative 4C machinery as the final player to directly and irreversibly create chromatin structure plasticity. 10) As a concrete approach to generalize the 4C theory, for instance, elucidation of the influences of changes in temperature, atmosphere and/or nutrition on the ability to gain un-programmed and new cell function(s) in established cell lines through various generations and in model animals (such as *C. elegans*, *Drosophila*, Xenopus, mice, rats and others) during development and differentiation is very helpful and significant, because these influences under the varied conditions can be easily studied by various research groups.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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