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

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Abstract

When higher eukaryotic cells firstly encounter moderate intra- and/or extra-cellular environment change, they newly acquire ability to cope with and/or overcome it through irreversible creation of chromatin structure plasticity based on successive chromatin conformational change with epigenetic modifications via a lot of generations. Putative environment change recognition receptor/site (ECRR/ECRS) should recognize the environment change. Putative chromatin conformation change complex (4C) machinery should create irreversibly and separately varied chromatin structure plasticity of proximal 5'-upstream regions of various transcription factor and/or chromatin-modifying enzyme genes in individual cells of the same cell type. We advance a chromatin conformation change code (4C) theory for a bio-system to gain unprogramed and new cell function(s) through irreversible creation of chromatin structure plasticity by the 4C machinery via a lot of generations, in order to adapt to the environment change recognized by ECRR/ECRS. The 4C theory should be suitable as an explanation for the manner of development and differentiation of higher eukaryotes.

Keywords

Chromatin conformation change code (4C) theory; Gain of unprogramed and new cell function; Irreversible creation of chromatin structure plasticity; Epigenetic modifications; Lots of generations; Immuno-electron microscopy study; Pluri-potency, elasticity and flexibility of somatic cells

Introduction

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

(structural) changes with epigenetic modifications via a lot of generations. This mode should be a basis for development and differentiation of cells. Fourth, in the case of minor change, cells should respond to it using only 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. Manners to modulate chromatin structure with acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation and so on have been intensively studied in a variety of life science fields. Of these epigenetic modifications, acetylation and deacetylation of particular 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 major ones. For the last several decades, countless numbers of researches on the acetylation and deacetylation (and also other epigenetic modifications) have been accumulated without interruption in more diverse life science fields; transcription/gene expression, DNA replication, development, differentiation, memory, pluri-potency, clinical medicine and so on [2-28].

By 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 particular members of HDACs, HATs and transcription factors [31-45], besides those lacking numerous members of histones and histone chaperones. Systematic analyses of resultant DT40 mutants HDAC2(-/-), Aiolos(-/-), Pax5(-), EBF1(-/-), Ikaros(-/-/+), Helios(-/-) and E2A(-/-) revealed their interesting participations in gene expressions of IgM H- and L-chains as follows. In wild-type DT40 cells, HDAC2 as a supervisor regulates indirectly gene expressions of IgM H- and L-chains through opposite control of those of Pax5, Aiolos and 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 [46].

Very recently, we reported following remarkable and important phenomena by analyzing initially generated HDAC2-deficient mutants [47], Pax5-deficient mutants [48] and secondly generated HDAC2-deficient mutants [49], all of which were continuously cultivated for varying long periods. In individual clones of HDAC2(-/-) mutants, the accumulated IgM H- and L-chains at the early cultivation stage are dramatically decreased attributed to their drastically decreased gene 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 manners during cultivation (details will be discussed later) [49]. These results obtained from wild-type DT40 cells and HDAC2(-/-) mutants at the early and late cultivation

stages are schematically shown in Supplementary Figure S1. Such diminutions of the accumulated IgM H- and L-chains in all individual HDAC2(-/-) mutant clones during cultivation are really the third case of the above-mentioned countermeasures. Presumably, the manner 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 as a whole, although detailed molecular mechanisms to decrease gene expressions of the two immunoglobulin proteins should be distinct between these two mutant cells [48, 49]. In addition, acetylation levels of particular Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) within chromatin surrounding proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes are altered in distinct manners in all individual clones of HDAC2(-/-) mutants during continuous cultivation (details will be discussed later) [50].

Here, based on these previously and newly obtained results from HDAC2(-/-) mutant cells, we propose a universal concept, which we named the chromatin conformation change code (4C) theory, for the bio-system to gain unprogramed and new cell function(s) through irreversible creation of chromatin structure plasticity with epigenetic modifications via a lot of generations in higher eukaryotes.

Methods

Electron microscopy and immuno-electron microscopy

Electron microscopy and immuno-electron microscopy (using anti-chicken IgM H-chain antiserum as primary antibody) were carried out on DT40 and initially generated HDAC2(-/-) mutant cells stocked at -80°C [31, 35] in some different manners essentially as described [47-51]. For instance, immuno-electron microscopy using pre-embedding staining procedure was done as follows. Exponentially growing HDAC2(-/-) mutant cells at the early (E; ~10 days) and late (L; ~60 days) cultivation stages and DT40 cells (W) were suspended in 1% bovine serum albumin (BSA) and collected by a light centrifugation. The resultant cell pellets were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4℃ for 60 min. After washing with PB the cell pellets were treated with or without 0.2% saponin in phosphate buffered saline (PBS) for 30 min to be permeable or non-permeable. After washing with PBS the cell pellets were incubated with goat anti-chicken IgM H-chain antibody A30-102A (Bethyl Laboratories Inc., TX, USA; diluted 1:1000 with 1% saponin in PBS) at 4℃ for overnight. After washing with PBS the cell pellets were incubated with horseradish peroxidase (HRP)-labeled affinity-purified F(ab')2 fragment Donkey anti-goat IgG(H+L) (Jackson ImmunoResearch Lab. Inc., West Grove, PA, USA; diluted 1:1000 in PBS) at 4℃ for overnight. After washing with PBS the cell pellets were fixed with 1% glutaraldehyde in PBS at 4℃ for 5 min. After washing with 50 mM Tris-HCl buffer (pH 7.4) (TB) the cell pellets were incubated in 0.05% 3', 3'-diaminobenzidine (DAB) in TB for 20 min and complete DAB solution containing 0.005% H_2O_2 for 10 min. The cell pellets were post-fixed with 1% osmium tetroxide in TB containing 1% potassium ferrocyanide for 30 min,

dehydrated with a series of ethanol and embedded in epoxy resin mixture. Ultrathin sections were contrasted with lead citrate for 20 sec and examined with a JEOL 1200 EX electron microscope (JEOL, Tokyo, Japan).

Immunocytochemistry using immuno-gold labeling was carried out as described [51]. Exponentially growing DT40 (W) and HDAC2(-/-) mutant cells at the early (E) and late (L) cultivation stages were collected by a light centrifugation. The resultant cell pellets were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PB for 30 min. After washing with PB the cells were post-fixed with 1% osmium tetroxide in PB for 60 min, washed with PB, dehydrated in ethanol and embedded in Epon. Ultrathin sections were picked up on 200-mesh gold grids coated with Formvar film and treated with 5% sodium meta-periodate in distilled water for 30 min. After rinsing in distilled water the sections were treated with 5% normal horse serum (NHS) and 1% BSA in PBS for 10 min to block nonspecific binding and incubated with goat anti-chicken IgM H-chain antibody A30-102A (diluted 1:1500 with 5% NHS, 1% BSA in PBS) at room temperature for 60 min. After rinsing in PBS the sections were incubated with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1:200 with 1% BSA in PBS) for 40 min. After washing with PBS the sections were incubated with 8 nm colloidal gold conjugated anti-biotin antibody (Vector Laboratories, Burlingame, CA, USA; diluted with 1% BSA in PBS) for 30 min. After washing with distilled water and drying the sections were contrasted with 2% uranyl acetate in 70% methanol and Reynolds' lead citrate and observed in a JEOL 1200EX transmission electron microscope operating at 80 kV (JEOL, Tokyo, Japan). Primary antibody was omitted or replaced by normal goat serum as control.

Results

New findings and previous results [31, 35, 47-50] are as follows. The HDAC2-deficiency in DT40 cells rapidly and dramatically increases mRNA (i.e., transcription/gene expression) levels of IgM H- and L-chains, followed by accumulation of the two immunoglobulin proteins probably within endoplasmic reticulum (Figs. 1, 2 and 3, and Suppl. Figs. S2 and S3). Surprisingly, excessively increased mRNAs and proteins of IgM H- and L-chains at the early stage of cultivation are gradually decreased until the late cultivation stage in almost similar pattern in all examined individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants (Suppl. Figs. S4 and S5). By contrast, remarkably, gene expressions of various particular transcription factors and chromatin-modifying enzymes change in distinct patterns among these individual HDAC2(-/-) mutant clones (Suppl. Fig. S5). To reduce the increased protein levels of IgM H- and L-chains resulted from their increased gene expressions, apparently, following three distinct manners based on altered gene expressions of particular transcription factors exist at the late cultivation stage in the six individual mutant clones (Suppl. Figs. S1(L) and S5) [49]. The manner in clone cl.2-1 seems to be dependent on OBF1 and different from that in DT40 cells.

The manner in clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seems to be dependent on Pax5 plus Aiolos and slightly similar to that in DT40 cells in appearance. The manner in clone cl.2-6 seems to be dependent on Pax5, Aiolos plus EBF1 and similar to that in DT40 cells in appearance.

To clarify these distinct manners, we performed neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP: this abbreviation also means IP on notch of chromatin) assay on proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of these particular transcription factor genes (Suppl. Figs. S6, S7, S8 and S9) [50]. The results obtained by the NotchIP assay revealed that such distinct manners should be fundamentally originated from irreversible creation of varied chromatin structure plasticity surrounding proximal ~2.0 kb 5'-upstream regions of corresponding transcription factor genes with epigenetic modifications via a lot of generations during cultivation. To put it concretely, in wild-type DT40 cells having HDAC2 activity, chromatin structure of each of 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, 47, 49]) and OBF1 (which probably up-regulates those of the two immunoglobulin proteins [46, 49]) is 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 particular Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) [50]. Therefore, these four transcription factor genes are transcribed at high levels in DT40 cells [35, 47, 49]. On the other hand, in all individual clones of HDAC2(-/-) mutants having no HDAC2 activity, chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of these four factor genes is dramatically and severally altered based on varied acetylation levels of one or more of the five particular Lys residues of histone H3 during cultivation, resulting in alterations in their gene expression levels as follows [47, 49, 50].

In clone cl.2-1, at the early stage of cultivation, chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos and EBF1 genes is tight (closed) form due to the binding ability of histone H3 to DNA based on hypo- (low or no) acetylation levels of one or more of the five particular Lys residues (Suppl. Fig. S6), and as a result, transcriptions of these three genes are almost completely suppressed to undetectable levels (Suppl. Fig. S5). However, that of the OBF1 gene is somewhat loose form due to the less binding ability of histone H3 to DNA based on slight (or considerably decreased) hyper-acetylation levels, thereby its transcription is slightly decreased. By contrast, at the late cultivation stage, chromatin structure of each of proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes remains to be tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, their transcriptions also remain unchanged at undetectable level. On the other hand, chromatin structure of proximal ~2.0 kb 5'-upstream region of the OBF1 gene changes to tight form due to the binding ability of histone H3 to DNA based on hypo-acetylation levels; therefore, its transcription is suppressed to almost undetectable or very low level. These results supported the above-mentioned

inference (i.e., OBF1-dependent) on the manner of gene expressions of IgM H- and L-chains at the late cultivation stage in clone cl.2-1.

In clones cl.2-2 and cl.2-4, at the early stage of cultivation, as in clone cl.2-1, chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is tight or somewhat loose (or less tight) form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five particular Lys residues of histone H3 (Suppl. Figs. S7 and S8). Therefore, transcriptions of the former three genes are almost completely suppressed and that of the latter one gene is certainly decreased (Suppl. Fig. S5). At the late cultivation stage, contrary to this, chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos and OBF1 genes changes to loose form based on hyper-acetylation levels, and their transcriptions are dramatically or certainly increased. However, that of the EBF1 gene remains tight form based on hypo-acetylation levels, and its transcription remains low at almost undetectable level. These results supported the above-mentioned inference (i.e., Pax5- and Aiolos-dependent) on the manner of gene expressions of IgM H- and L-chains at the late cultivation stage in clones cl.2-2 and cl.2-4 (and also cl.2-3 and cl.2-5). Moreover, these four clones should be major type, since they resemble four initially generated HDAC2(-/-) clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) in several cellular characteristics [31, 35, 47, 49].

In clone cl.2-6, at the early stage of cultivation, as in clones cl.2-1, cl.2-2 and cl.2-4, chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos plus EBF1 genes or the OBF1 gene is tight or somewhat loose form based on hypo- or slight (or considerably decreased) hyper-acetylation levels of one or more of the five particular Lys residues of histone H3 (Suppl. Fig. S9). Therefore, transcriptions of the former three genes are almost completely suppressed and that of the latter one gene is certainly decreased (Suppl. Fig. S5). By contrast, at the late cultivation stage, chromatin structure of each of proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes changes to loose form based on hyper-acetylation levels, and their transcriptions are dramatically or certainly increased. These results supported the above-mentioned inference (i.e., Pax5-, Aiolos- and EBF1-dependent) on the manner of gene expressions of IgM H- and L-chains at the late cultivation stage in clone cl.2-6.

Results on alterations in acetylation levels (hyper or hypo) of the five particular Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3), form (loose or tight) of chromatin structure of 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 [49, 50] are roughly and schematically represented in Supplementary Figure S10. Concerning the above-stated results on the suppression of excessive gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivation, we should like to mention

some following important comments. Excessively accumulated IgM H- and L-chains resulted from their dramatically increased gene expressions in HDAC2(-/-) mutants just after their birth (by gene targeting techniques) [31, 35, 47, 49] should be unfavorable and useless for them; therefore, the mutant cells have come to acquire a new ability to diminish a large amount of the two immunoglobulin proteins as uncomfortable environment change via a lot of generations during cultivation. The manners to suppress gene expressions of IgM H- and L-chains at the late cultivation stage in all of HDAC2(-/-) mutant clones, even in clone cl.2-6 [49], should be surely distinct from the ordinary and reversible manner to regulate their gene expressions in DT40 cells. Because gene expressions of IgM H- and L-chains are indirectly regulated by HDAC2 through opposite regulations of Pax5, Aiolos, EBF1, OBF1, and E2A gene expressions in DT40 cells [35, 37]. In addition, gene expressions of various transcription factors and chromatin-modifying enzymes (i.e., Blimp1, PCAF, HDAC7, HDAC9 and others), besides Pax5, Aiolos, EBF1 and OBF1, are dramatically and separately altered in individual HDAC2(-/-) mutant clones during cultivation (Suppl. Fig. S5) [47, 49]. Therefore, remarkably, besides the alterations in gene expressions of IgM H- and L-chains (and Pax5, Aiolos, EBF1 and OBF1) and in cell morphology, some other unknown important cellular characteristics should be obviously altered among individual HDAC2(-/-) mutant clones during cultivation. Such presumable altered characteristics may be complicated and diverse in individual clones of HDAC2(-/-) mutants, even though they are originally and completely the same cell type.

Conclusions

Based on our morphological but insufficient findings, together with previous results mentioned above and others [31, 33, 35, 37, 46-50], we slightly revised our previous hypothesis [50] and proposed an all-inclusive hypothesis on manners for gain of unprogramed and new cell function to diminish artificially accumulated IgM H- and L-chains through irreversible creation of varied chromatin structure plasticity of several particular transcription factor genes with epigenetic modifications during continuous cultivation in individual clones of HDAC2(-/-) mutants.

First, we assume putative environment change recognition receptor/site (ECRR/ECRS), which participates in the recognition of accumulation of IgM H- and L-chains as unfavorable environment change (and probably acts in part in the signal transduction on the accumulation to chromatin structure) (Fig. 4). In addition, putative chromatin conformation (structure) change complex (4C) machinery, which is diverse and consists of a member of each of HATs plus HDACs and other factors, is supposed to create directly and irreversibly chromatin structure plasticity surrounding proximal 5'-upstream regions of a set of particular genes (and also to act in part in the signal transduction) (Figs. 4 and 5). Using ECRR/ECRS, the 4C machinery and other components, the chain reaction of response to the environment change should occur as follows. Large amounts of IgM H- and L-chains artificially caused by the

HDAC2-deficiency (Suppl. Fig. S4) are first accumulated within endoplasmic reticulum of HDAC2(-/-) mutant cells (Figs. 1, 2 and 3, and Suppl. Figs. S2 and S3). Most of the accumulated immunoglobulin proteins (which probably exist as a high molecular weight complex of IgM H- and L-chains each other [35]) are gradually secreted to outside of cells, whereas, as indicated by immuno-electron microscopy some of them are transported to nuclear envelope but not inside of nucleus, and kept at peri-nuclear space at the early and also late cultivation stages (Figs. 1, 2, 3 and 4). The accumulated immunoglobulin proteins at peri-nuclear space should bind to ECRR/ECRS localized on inner nuclear membrane (where hetero-chromatin is possibly located) (Fig. 4). After ECRR/ECRS recognizes the accumulation of IgM H- and L-chains as unfavorable environment change, the signal concerning it is genome-widely transmitted to chromatin structure surrounding proximal 5'-upstream regions of numerous genes (probably located on several distinct chromosomes) encoding transcription factors, chromatin-modifying enzymes, and related factors and enzymes. Following the initial signal transduction, spontaneous unbalanced response to the environment change is consecutively and separately converged not only on the above-mentioned particular genes but also in individual clones of HDAC2(-/-) mutants.

In wild-type DT40 cells in which various members of HATs and HDACs are largely expressed [35, 49], the 4C machinery for each of Pax5, Aiolos, EBF1 and OBF1 genes probably contains HDAC2 as HDAC activity, a particular HAT member (e.g., GCN5) as HAT activity, and other factors (Fig. 5). On the other hand, in all of individual clones of HDAC2(-/-) mutants, at the very early stage of cultivation (just soon after their birth by gene targeting techniques), bulk conformation of the 4C machinery should be dramatically changed to remove or drastically reduce HAT activity (of the assumed member of HATs), attributed to the HDAC2-deficiency. Through the above-mentioned process and following continuous cultivation, the 4C machinery should come to newly contain a different member of HDACs, a particular (same or different) member of HATs, and other factors, and thereby becomes varied. The diversity of alterations in chromatin structure is preferentially attributed to varied acetylation and deacetylation levels of one or more of the particular Lys residues at N-terminal tail of histone H3 [50] caused by collaboration of proper member of each of HATs and HDACs in the protean 4C machinery. These successive epigenetic modifications with acetyl group of K9/H3 and K27/H3 (and also K14/H3, K18/H3 and K23/H3) lead to irreversible creation of distinct chromatin structure plasticity surrounding proximal 5'-upstream regions of the above-mentioned targeted genes (Suppl. Figs. S6, S7, S8, S9 and S10, and Fig. 6). Accordingly, chromatin structure of proximal 5'-upstream regions possessing hyper-acetylation levels of one or more of the particular Lys residues of histone H3 is loose form based on its no binding ability to DNA, but that of proximal 5'-upstream regions possessing hypo-acetylation levels of one or more of the particular Lys residues of histone H3 is tight form based on its binding ability to DNA (Fig. 6). By contrast, probably, the 4C machinery cannot change chromatin structure surrounding open reading frames (coding regions) of the targeted genes so much [50]. Thus, as the need arises,

transcription factor complex (TFC) machinery (which consists of RNA polymerase, proper transcription factor(s), certain member of each of HATs and HDACs, and other factors) is able to bind to promoter regions (or elements) within loose (but not tight) form of chromatin structure surrounding proximal 5'-upstream regions of the targeted (but not untargeted) genes (which have become latently active (but not inactive) state), and thereby initiates their gene expressions (Figs. 5 and 6). As a concrete result, individual HDAC2(-/-) mutant clones gain the same, unprogramed 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 manners during simple continuous cultivation under the same conditions (Suppl. Fig. S5) [35, 47, 49]. Naturally, such distinct manners are not under the control of HDAC2 but originally based on irreversible creation of their distinct chromatin structure plasticity with epigenetic modifications during continuous cultivation [50]. Remarkably, the six tested clones of HDAC2(-/-) mutants can be clearly classified into three different cell types exhibiting distinct functions, because they show three dissimilar manners for gene expressions of transcription factors and chromatin-modifying enzymes (such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC9 and others) at the late cultivation stage (Suppl. Figs. S1 and S5). Besides, in one or more of these six mutant clones, as a whole, respective gene expression patterns of PCAF, HDAC7, HDAC9, Ikaros and OBF1 are spontaneously and complicatedly reversed in the midst of simple continuous cultivation, although those of Pax5, Aiolos, EBF1, E2A, PU.1 and Blimp1 are unchanged from the early to late cultivation stages. Furthermore, there is a great possibility that additional different cell types exist, if other individual mutant clones obtained [49] are analyzed. Consequently, individual clones of HDAC2(-/-) mutants acquire flexible, elastic and pluri-potential ability not only to adapt in distinct manners to an environment change but also to branch off into diverse derivative cell types, which should exhibit varied characteristics (functions), even though they are originally the same cell type and also established cell line.

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

When higher eukaryotic cells firstly in their life encounter the change in intra- and/or extra-cellular environment, in order to adapt to or eliminate the change (if uncomfortable), they gradually gain unprogramed and new cell function(s) via a lot of generations. Namely, the cells acquire the ability to adapt themselves to newly encountered environment change and/or to exclude the painful environment change. Using ECRR/ECRS, the 4C machinery and other components, the cells should cause the chain reaction of response to the environment change (Figs. 4 and 5). First of all, the environment change should be recognized by means of ECRR/ECRS, which may be localized nearby nuclear membrane as a

nucleus-cytoplasm barrier (probably at inner nuclear membrane where hetero-chromatin is possibly located) (Fig. 4). Naturally, there is a possibility that putative particular molecule(s) should act as intermediary sensor at this step in the manner to recognize the environment change. As a next step, putative signal(s) concerning the change should be genome-widely transmitted to chromatin structure within nucleus via a lot of generations (cell divisions). The initial acceptance of the putative signal(s) may induce a slight alteration in chromatin structure of numerous 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 environment change should be successively repeated and converged into restricted chromatin structure surrounding proximal 5'-upstream regions of a set of particular members of the above-mentioned factor and enzyme genes. Finally, this successive signal transduction concerning the environment change should cause various epigenetic modifications of histones and/or DNA within the restricted chromatin structure of the aforesaid particular genes with acetyl group, methyl group, phosphate group and/or others. Of these various epigenetic modifications, acetylation and deacetylation of particular Lys residues of core histones H2A, H2B, H3 and H4 may be major ones.

The 4C machinery, which consists of a particular member of each of HATs and HDACs, and other factors, should preferentially participate in acetylation and/or deacetylation among such epigenetic modifications (Fig. 5). Participating positions of particular Lys residues and/or kinds of core histones should be diverse. For instance, in the above-mentioned case [50], acetylation and/or deacetylation of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 are prominent. Consequently, epigenetic modifications of one or more of these Lys residues of histone H3 with acetyl group are changed within chromatin structure surrounding proximal 5'-upstream regions of the above-mentioned particular targeted genes via a lot of generations. Distinct functions of the protean 4C machinery on such acetylation and/or deacetylation levels should be 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. By contrast, the protean 4C machinery may not alter so much chromatin structure surrounding open reading frames (coding regions) of corresponding genes. The binding ability of the N-terminal tail of histone H3 to DNA is tentatively and qualitatively deduced from acetylation levels of one or more of these particular Lys residues, though which Lys residue(s) really and/or mainly participates in the binding is still undefined. Namely, hyper- (high) or hypo- (low or no) acetylation levels should induce no binding or full binding ability, resulting in loose (open) or tight (closed) form of chromatin structure (Fig. 5). Thus, chromatin structure plasticity should be irreversibly created based on successive conformation changes with epigenetic modifications. These manners to create gradually and tardily chromatin structure plasticity for gain of unprogramed and new cell function(s) should be obviously different from those to cause immediately and rapidly chromatin conformation change for gain

of programed and ordinary cell function(s).

Whenever the need arises, the TFC machinery is able to bind to promoter regions (or elements) within loose form of chromatin structure surrounding proximal 5'-upstream regions of targeted genes (which are latently active state), followed by initiation of their gene expressions (Fig. 5). By contrast, the TFC machinery cannot bind to promoter regions (or elements) within tight form of chromatin structure surrounding proximal 5'-upstream regions of untargeted genes (which are latently inactive state), and thereby cannot initiate their gene expressions. Consequently, loose or tight form of chromatin structure surrounding proximal 5'-upstream regions should respectively cause high or low (or no) transcription levels of corresponding genes (Fig. 6). Notably, there is a great possibility that manners for irreversible creation of chromatin structure plasticity are distinct among individual cells of the same type, even though the environment change and signal(s) on it are the same for all of them. That is, in order to gain unprogramed and new cell function(s), individual cells each should possess ability not only to change complicatedly and diversely chromatin structure surrounding proximal 5'-upstream regions of various particular genes but also to alter separately chromatin structure of the same proximal 5'-upstream region of a certain gene into varied forms. Thus, gene expressions of the particular chromatin-modifying enzymes and transcription factors should be changed diversely among individual cells via a lot of generations, in spite of the same environment change. In consequence, individual cells of the same type are able to newly gain the same and/or distinct unprogramed cell function(s) in different manners in order to accommodate themselves to a new environment.

In conclusion, for gain of unprogramed and new cell function(s), somatic cells (and even tumor cells) of higher eukaryotes are pluri-potential, elastic and flexible, all of which should be basically originated from those of chromatin structure. Namely, in order to adapt to intra- and/or extra-cellular environment change, individual somatic cells of higher eukaryotes possess ability to newly gain the same and/or distinct unprogramed cell function(s) in different manners through irreversible creation of varied chromatin structure plasticity with epigenetic modifications, i.e., from loose to tight forms or vice versa of chromatin structure surrounding proximal 5'-upstream regions of particular transcription factor and chromatin-modifying enzyme genes (Fig. 6). Such loose or tight form of chromatin structure surrounding the proximal 5'-upstream region should be latently active or inactive state for transcription of the corresponding gene, although the proximal 5'-upstream region as mere nucleotide sequences may be silent state for expressions of most genome functions. Variety of chromatin structure plasticity in individual somatic cells should be triggered by the spontaneous unbalanced response to the environment change when they firstly encounter with it and then irreversibly accomplished by the successive unbalanced convergence of the response via a lot of generations. Naturally, different manners to create varied chromatin structure plasticity in somatic cells should be certainly dependent on their antecedents. Moreover, chromatin structure plasticity, regardless of whether its creation is in the course or was already

completed, should be inherited to descendant generations associated with or without additional structural change via cell divisions. Thus, remarkably, irreversible creation of chromatin structure plasticity should occur in descendant cells but not in the cell which initially accepts the signal on the environment change, although reversible regulations of ordinary gene expressions and enzyme reactions occur in the cell itself which accepts proper signal (Fig. 7). Probably, irreversible creation of chromatin structure plasticity, with the intention of adapting to the environment change, should occur inevitably but not incidentally and/or neutrally.

The proximal 5'-upstream chromatin region (as loose or tight form) should direct the switch (on or off) for latent transcription ability of the corresponding gene through irreversible creation of chromatin structure plasticity; therefore, besides "notch" from a structural side-view as mentioned above, the 5'-upstream chromatin region could be regarded as "director" from a functional side-view. Naturally, the notch (or director) covers specific nucleotide sequences of transcriptional elements (such as promoter, operator and others) and also their neighboring nucleotide sequences. Remarkably, the real recipient of the signal on the environment change may be just chromatin structure possessing self-reformation ability itself as three-dimensional conformation which is dynamic and changeable between loose and tight forms, but not mere chromatin and chromosome themselves as one- (or two-) dimensional conformation which are static and unchangeable. That is, the chromatin structure of proximal 5'-upstream region (notch or director), as dynamic and changeable three-dimensional conformation, possesses two fundamental abilities, i.e., to receive the signal concerning intra- and/or extra-cellular environment change and to direct the switch (on or off) for latent transcription ability of the corresponding gene through its irreversible chromatin conformation change responsive to the signal, although concrete data are not enough to support these ideas so far. In 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 manners from the same type of somatic cells, in order to accommodate themselves to new intra- and/or extra-cellular environment. Naturally, in some cases, even neighboring cells derived from the same cell type are probably regarded as extra-cellular environment each other. Moreover, in higher eukaryotes diverse kinds of somatic cells should share and express cooperatively vital functions each other.

Finally, we name such bio-system for gain of unprogramed and new cell function(s) through irreversible creation of chromatin structure plasticity with epigenetic modifications, which should be one of the most fundamental and important manners for life conservation and cell type determination of higher eukaryotes, a chromatin conformation (structure) change code (4C) theory. Probably, the supposed number of codes in the 4C theory, which should determine complicated and varied characteristics (functions) of higher eukaryotic cells, can be roughly estimated based on combination (multiplication) of the number of candidate genes and that of codes for each of these genes as follows.

Most influential candidates should be genes of transcription factors, chromatin-modifying enzymes, and related factors and enzymes, which are necessary for gain of varied cell functions and specificities of higher eukaryotes. The number of codes for each of these candidate genes should be two. Because loose or tight form of proximal 5'-upstream chromatin region, as latently active or inactive state, directs the switch (on or off) for transcription ability of the corresponding gene. Therefore, the 4C theory should open the door for acquisition of unprogramed and new cell function(s) of higher eukaryotes and innovate the general notion on nature of somatic cells. Naturally, programed, complicated and diverse cell functions are orderly and systematically gained throughout their development and differentiation. Nevertheless, the 4C theory should be 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 exclusion of excessive IgM H- and L-chains artificially accumulated in HDAC2(-/-) DT40 mutant cells [47, 49, 50]. 1) Despite the HDAC2-deficiency, why acetylation levels of one or more of K9, K14, K18, K23 and K27 residues of histone H3 within chromatin structure (of ~10 nucleosomes) surrounding proximal 5'-upstream regions of Pax5, Aiolos and EBF1 genes are decreased at the early stage of cultivation in HDAC2(-/-) mutants. 2) Why the decreased acetylation levels of one or more of the particular Lys residues of histone H3 within proximal 5'-upstream chromatin regions of these three genes are increased during cultivation. Why the case of the OBF1 gene is reverse. 3) It must be determined which Lys residue(s) of K9, K14, K18, K23 and K27 of histone H3 is really and/or mainly involved in its binding to DNA within proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes. 4) Functional and steric differences between loose and tight forms (based on hyper- and hypo-acetylation levels of one or more of the particular Lys residues of histone H3) of chromatin structure surrounding proximal 5'-upstream regions of these four genes must be clarified more minutely. 5) Why changing patterns in acetylation levels of the particular Lys residues of histone H3 during cultivation in individual transcription factor genes differ among individual mutant clones. 6) Why changing patterns in acetylation levels of the particular Lys residues of histone H3 during cultivation in each individual transcription factor gene differ within individual mutant clones. 7) How the 4C machinery (which acts in irreversible creation of chromatin structure plasticity of proximal 5'-upstream region) differs from the well-known chromatin-modifying machinery, although the TFC machinery (which acts in transcription of open reading frame of the corresponding gene) may be the same as the well-known transcription machinery. 8) To demonstrate the 4C theory, both of putative ECRR/ECRS as a first player to recognize the environment change and putative 4C machinery as a final player to irreversibly create chromatin structure

plasticity must be clarified. 9) As concrete approach to generalize the 4C theory, for instance, elucidation of influences of changes in temperature, atmosphere and/or nutrition on ability to gain unprogramed and new cell function(s) in established cell lines via a lot of generations and in model animals (such as C. elegans, Drosophila, Xenopus, mice, rats and others) during development and differentiation should be very effective and powerful.

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Figure legends

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

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

Figure 2. Localization of IgM H-chain at peri-nuclear space of HDAC2(-/-) DT40 mutants

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

Figure 3. Localization of IgM H-chain at peri-nuclear space of HDAC2(-/-) DT40 mutants at early and late stages of cultivation

HDAC2(-/-) mutant cells were collected at the early (E) and late (L) stages of cultivation, and immuno-electron microscopy using anti-chicken IgM H-chain antiserum was carried out. A) Immuno-electron microscopy of one HDAC2(-/-) mutant cell at the early cultivation stage. B) and C) Enlarged versions of parts indicated by arrows b and c in A). D), G) and J) Immuno-electron microscopy of three independent HDAC2(-/-) mutant cells at the late cultivation stage. E), F), H), I) and K) Enlarged versions of parts indicated by arrows e, f, h, i and k in D), G) and J). The mutant cell in G) was identical with that of Ref. 47. Accumulated IgM H-chain proteins were observed in peri-nuclear space of all HDAC2(-/-) mutant cells at the early and late cultivation stages.

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

Left panel: A portion of peri-nuclear space (where IgM H-chain proteins were accumulated) of the HDAC2(-/-) mutant cell (indicated by an arrow P at lower position in Figure 1C) was reversely enlarged. Right panel: Signal concerning artificially accumulated IgM H- and L-chains in peri-nuclear space of the HDAC2(-/-) mutant cell was repeatedly transmitted to chromatin structure, followed by unbalanced

response for the signal and its convergence to various particular genes in individual mutant clones. ECRR/ECRS: environment change recognition receptor/site. 4C machinery: chromatin conformation change complex (4C) machinery.

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

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

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

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

Upper panel: Irreversible creation of chromatin structure plasticity surrounding proximal 5'-upstream region of particular gene with epigenetic modifications occurs in descendant cells but not in initial cell that accepts environment change signal. Ac, Ac/2 and Ac/10 indicate qualitatively hyper-, considerable hyper- and somewhat hyper-acetylation levels of particular Lys residues of core histones (e.g., histone H3), respectively. Middle and lower panels: Regulations of both gene expression (on transcriptional regulatory elements) and enzyme reaction occur reversibly in the cell itself, which accepts proper signal. Ac and P indicate acetylation, phosphorylation and/or other chemical modifications, respectively.

Supplementary Figure S1. Manners for control of gene expressions of IgM H- and L-chains through control of gene expressions of particular transcription factors in the presence or absence of HDAC2 in wild-type DT40 cells (W) or all and individual clones of HDAC2(-/-) DT40 mutants at early (E) and late (L) stages of cultivation

The figure is a set of Figures 4-8, 4-9 and 4-10 of Ref. 49.

Supplementary Figure S2. Localization of IgM H-chain in wild-type DT40 cells and HDAC2(-/-) DT40 mutants

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

Supplementary Figure S3. Localization of dense fraction due to accumulated IgM H-chain in HDAC2(-/-) DT40 mutants

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

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

The figure is identical with Figure 4-2 of Ref. 49.

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

The figure is identical with Figure 4-6 of Ref. 49 and shown with some modifications.

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

The figure is a set of Figures 5-1, 5-5, 5-9 and 5-13 of Ref. 50.

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

The figure is a set of Figures 5-2, 5-6, 5-10 and 5-14 of Ref. 50.

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

The figure is a set of Figures 5-3, 5-7, 5-11 and 5-15 of Ref. 50.

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

The figure is a set of Figures 5-4, 5-8, 5-12 and 5-16 of Ref. 50.

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

The figure is identical with Figure 5-21 of Ref. 50 and shown with some modifications.