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Generation of Pax5-deficient DT40 mutant cells, Pax5(-), and protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in Pax5(-) are rapidly reduced via a lot of generations during continuous cultivation**

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Abstract

We generated Pax5-deficient DT40 mutant cells, Pax5(-), devoid of the Pax5 gene located on Z chromosome that is monosomy in chickens and analyzed characteristics of Pax5(-) by Western blotting, microscopy, electron microscopy, immuno-electron microscopy and RT-PCR.   Protein levels of IgM H- and L-chains in Pax5(-) were drastically increased at the early stage of cultivation and thereafter dramatically decreased during cultivation and at the late stage reached to comparable levels in DT40 cells. Whole and secreted forms of IgM H-chain mRNA in Pax5(-) were dramatically elevated at the first stage and thereafter rapidly reduced until the late stage to comparable levels in DT40 cells.   Membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage and thereafter decreased slowly during cultivation.   In addition, PCAF and HDAC9 mRNA levels were gradually elevated during cultivation, and HDAC7 mRNA level changed moderately.   Aiolos and OBF1 mRNA levels were gradually reduced from the first to late stages, whereas Ikaros and E2A mRNA levels were drastically elevated at the first stage and gradually decreased until the late stage.   EBF1 mRNA level was completely decreased at the first stage and remained unchanged during cultivation.   PU.1 mRNA level was remarkably reduced at the first stage and gradually elevated until the late stage.

These results that Pax5 down-regulates dramatically gene expressions of IgM H- and L-chains, together with our previous findings, revealed not only that gene expressions of these two immunoglobulin proteins are indirectly regulated by HDAC2 through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, Ikaros and E2A in DT40 cells, but also that the decreases in the elevated immunoglobulin protein and mRNA levels during cultivation are more rapid in Pax5(-) than in HDAC2(-/-).   In addition, influences of Pax5-deficiency on several cellular characteristics should be certainly different from those of HDAC2-deficiency.

Keywords

Introduction

For the last several decades, mechanisms to modulate chromatin structure have been vigorously studied in diverse life science fields by numerous research groups, because the modulation of chromatin topology with epigenetic modifications is one of the most important events for expression of cell functions in eukaryotes.   Alterations in chromatin structure are strongly involved in regulations of gene expression/transcription, and replication, repair plus recombination of DNA in eukaryotes [1-4], and also concerned in regulations of lymphocyte development and differentiation [5-9].   Among various chromatin modifying enzymes participating in alterations in chromatin structure, histone

2

acetyltransferase(s) (HATs) and histone deacetylase(s) (HDACs) cooperatively control acetylation and deacetylation levels of particular Lys residues (K) of core histones H2A, H2B, H3 and H4 [10-19].   To assess their individual roles in expressions of cell functions, we have systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines, each of which is devoid of a particular member of HDACs and HATs [20-28], by gene targeting techniques [29-35].   Our previous analyses of HDAC2-deficient DT40 mutant cell line, HDAC2(-/-), showed that HDAC2 regulates the amount of IgM H-chain at the steps of its gene expression plus alternative processing of its pre-mRNA [20], and down-regulates IgM L-chain gene promoter activity [23].   Moreover, the HDAC2-deficiency represses gene expressions of HDAC7, Pax5, Aiolos, Ikaros plus EBF1, elevates those of HDAC4, HDAC9, PCAF plus E2A, and changes bulk acetylation levels of several particular Lys residues of core histones H3, H4 and H2B [25].   Therefore, to know individual roles of these altered transcription factors, we subsequently generated and analyzed homozygous DT40 mutant cell lines, EBF1(-/-), Aiolos(-/-), E2A(-/-) and Helios(-/-), devoid of EBF1, Aiolos, E2A and Helios genes, respectively [25, 36, 37], and Ikaros-down DT40 mutant cell line, Ikaros(-/-/+), devoid of two alleles of the Ikaros gene located on chromosome 2 that is trisomy in chickens (data will be shown elsewhere).   Results obtained showed that EBF1, Aiolos plus Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates these two immunoglobulin gene expressions [25].   Taken together, these findings indicated that HDAC2 regulates indirectly gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of EBF1, Aiolos plus Ikaros, and E2A [25].

Throughout the process of analyzing several characteristics of HDAC2(-/-) cultivated for different periods, we casually noticed following interesting phenomena [38].   Results obtained by 2D-PAGE, Western blotting and immuno-electron microscopy, together, showed that IgM H- and L-chains were dramatically accumulated at the early stage of cultivation in HDAC2(-/-), and thereafter gradually reduced during cultivation and at the late stage reached to comparable levels in DT40 cells.   RT-PCR showed that whole and secreted forms of IgM H-chain mRNA in HDAC2(-/-) were dramatically increased at the early stage and thereafter gradually decreased during cultivation and at the late stage reached to very close levels in DT40 cells.   Further, RT-PCR carried out on chromatin modifying enzymes and transcription factors revealed that the mRNA level of PCAF in HDAC2(-/-) was drastically elevated from the early to late stages, and those of HDAC7 and HDAC9 changed slightly during cultivation.   The mRNA level of EBF1 in HDAC2(-/-) was almost completely reduced at the early stage and thereafter remained unchanged, and that of E2A was slightly elevated at the early stage and remained unchanged.   Interestingly, the mRNA level of Pax5 in HDAC2(-/-) was obviously decreased at the early stage and thereafter increased until the late stage.   This changing pattern in the Pax5 gene expression was in anti-parallel with those in IgM H- and L-chain gene expressions as a whole.   These results revealed not only that the gene expression of Pax5 was certainly controlled by HDAC2, but also that Pax5

3

was expected to control mainly gene expressions of IgM H- and L-chains among various transcription factors.   In addition, immunoblotting using site-specific antibodies for various acetylated Lys residues (K) of core histones (H2A, H2B, H3 and H4) showed that during cultivation bulk acetylation levels of K9, K14, K18, K23 and K27 residues of histone H3 were gradually increased, and those of K5 residue of histone H4 and K16 residue of histone H2B changed differently.

Successively, we studied molecular mechanism of the Pax5 gene expression [38].   Since detailed information on the 5'-upstream region of the chicken Pax5 gene was not available from any database at the start of this study, we directly cloned and sequenced ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques (GenBank accession no.: LC060666). Interestingly, our preliminary chromatin immunoprecipitation (ChIP) assay showed that in HDAC2(-/-) acetylation levels of Lys-9 residues of histone H3 (K9/H3) within some chromatin segments surrounding proximal ~2.0 kb 5'-upstream region of the Pax5 gene were decreased at the early stage but at the late stage restored to comparable levels in DT40 cells.   These results qualitatively agreed with the above-mentioned findings on alterations in the Pax5 gene expression, whereas these early and late cultivation stages were convenience sake in fact, since the mutant cells used were already cultivated several times for the use of other experiments.

In this study, we generated and analyzed Pax5-deficient DT40 mutant cell line, Pax5(-), devoid of the Pax5 gene located on Z chromosome that is monosomy in chickens (USCS Genome Browser data base). Protein levels of IgM H- and L-chains in Pax5(-) were drastically elevated at the early stage and thereafter rapidly reduced via the middle until late stages to comparable levels in DT40 cells.   Whole and secreted forms of IgM H-chain mRNA in Pax5(-) were dramatically increased at the first stage and thereafter rapidly decreased until the late stage to comparable levels in DT40 cells.   Membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage and thereafter decreased until the late stage to comparable levels in DT40 cells.   In addition, interestingly, mRNA levels of PCAF, HDAC7, HDAC9, Aiolos, OBF1, Ikaros, E2A, EBF1 and PU.1 in Pax5(-) changed in different patterns during cultivation.   These results, together with others, indicated not only that Pax5 should be certainly different from HDAC2 in the participation in gene expressions of IgM H- and L-chains, but also that Pax5(-) must be obviously distinct from HDAC2(-/-) in several cellular properties.

Methods

Cell cultures

All positive Pax5-related mutant clones (generated as below) were picked up on 96-well plates at ~11 days after knockout of one Pax5 allele, cultivated for another several days to be ~10^6 cells/ml and stocked at -80°C until use as described [20].   Pax5(-) mutant clones were continuously cultivated and collected

at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and late (L; 20 days) stages of cultivation as described [20, 25, 38].   These cultivation stages were practically counted from the first day of cultivation from the stock at -80°C.   To determine growth rate of Pax5(-) mutant clones, cell numbers were counted at indicated times nearby the first cultivation stage.   DT40 (W) was used as control.

Generation of Pax5-deficient DT40 cells, Pax5(-)

   To generate Pax5-deficient DT40 mutants, Pax5(-), we used only the cassette carrying hygromycin (hyg) transcribed by the chicken β-actin promoter [39], since the Pax5 gene is located on Z chromosome that is monosomy in chickens (USCS Genome Browser data base).   Partial Pax5 fragment was obtained from DT40 genomic DNA by PCR using appropriate primers synthesized based on nucleotide sequences from a database, and those of the PCR amplified product were confirmed by the PCR sequencing protocol as described [24, 25].

   We first constructed the ΔPax5/hyg vector for disruption of the Pax5 gene as follows.   The 5'-arm, a HindIII digested 1.8 kb PCR fragment (obtained using sense primer 5'-GGTGTGAACCAGCTGGGGGGCGTTTTTGTG-3' from exon 2 and antisense primer 5'-TGGGCACGGTGTCGTTATCGCACACTCGTT-3' from exon 3), and the 3'-arm, a 4.7 kb PCR fragment (obtained using sense primer 5'-ACCGGGAGCATTAAGCCTGGAGTGATTGGA-3' from exon 3 and antisense primer 5'-AGACTGGGACTTGCTGATTGGGCGGCTGCT-3' from exon 4) were ligated to the pBluescript II vector.   The hyg cassette flanked by a loxP site [21] was inserted between the 5'-arm and 3'-arm (5'-upstream and 3'-downstream fragments).   In the resultant targeting vector, therefore, the genomic DNA sequences corresponding to exon 3 of the Pax5 gene were replaced with the hyg-carrying cassette (Fig. 1A).

   To obtain Pax5-deficient mutant cells, we transfected the ΔPax5/hyg vector into DT40 cells and selected three hyg-resistant transfectants in medium containing 2.5 mg of hyg per ml [21].   Southern blotting showed that all of these three transfectants carrying the hyg cassette, which had the positive 11.5 kb fragment hybridized with probe Pax5 and lost completely the endogenous positive 7.8 kb fragment at the same time.   Therefore, we regarded these mutants as the Pax5-deficient DT40 mutant cells, Pax5(-).

Southern blotting

   Genomic DNAs were isolated from three Pax5(-) mutant clones, one Pax5(+) clone and DT40 cells, digested with SacI, separated in 0.8% agarose gel, electro-transferred to Hybond N+ membrane, and hybridized with ^{32}P-labeled probe Pax5 as described [20].   The probe Pax5, corresponding to the 5'-end region of intron 4 (and 3'-outer side of exon 4) of the Pax5 gene, comprised the 0.47 kb fragment obtained by PCR using sense primer 5'-CCAGTCACAGCATAGGTGA-3' and antisense primer 5'-TGGCTCGAGATCGCAAAGT-3' from intron 4.

RT-PCR

Total RNAs were isolated from exponentially growing DT40 and mutant cells as described [20, 25]. Mutant cells were three wild-type Pax5(+) clones (cl.1, cl.2 and cl.3) containing the Pax5 allele plus hyg cassette, and three Pax5 (-) mutant clones (cl.46, cl.58 and cl.62) at the first, early, middle and late stages of cultivation. Total RNAs obtained were subjected to synthesize first-strand cDNA using a kit (ReverTra Ace-α-™), and mRNA levels were measured by RT-PCR using equal amounts of total RNAs and appropriate primers listed in our previous reports [24, 25, 40]. RT-PCR products were subjected to 15% agarose gel electrophoresis. Nucleotide sequences of all amplified products were confirmed by the PCR sequencing protocol as described [24, 25]. Data analyses were carried out by Multi Gauge Ver3.X software using a luminescent image analyzer LAS-1000plus (FUJIFILM). Data calibrated with internal control in these three Pax5(-) clones are indicated as percentages of control values obtained from DT40 cells. Chicken glyceraldehydephosphate dehydrogenase (GAPDH) and Pax5 genes were used as positive and negative controls, respectively.

Western blotting

Western blotting was performed as described [20, 25, 38]. Whole cellular proteins were prepared from DT40 and Pax5(-) mutant clones (cl.46, cl.58 and cl.62) at the early, middle and late stages of cultivation, whose intervals were shorter than corresponding those for HDAC2(-/-) mutant cells [38]. In brief, cells (1 x 10^7) were treated with 10% trichloroacetic acid and lysed in 100 μl of SDS buffer. Aliquots (10 μl) of 1:50 dilutions of the resultant cell extracts were subjected to 12% SDS-PAGE and electro-transferred to membranes, and proteins were detected with rabbit anti-chicken IgM L-chain antiserum (as primary antibody) that cross-reacts with IgM H-chain. β-actin was used as control. Antibodies used were: A30-100A (Bethyl Laboratories Inc., TX, USA) for IgM H- and L-chains and ab6276 (Abcam) for β-actin. The relative amounts of IgM H-chain and large (high) and small (low) forms of IgM L-chain were measured as described [24, 25].

Microscopy, electron microscopy and immuno-electron microscopy

Microscopy was carried out on exponentially growing DT40 cells and Pax5(-) mutant clone (cl.62) at the early and late stages of cultivation in distinct ranges of vision with magnified visions as described [26, 36, 37]. Electron microscopy and immuno-electron microscopy using immuno-gold labeling were carried out as described [38, 41]. Exponentially growing DT40 and Pax5(-) clone (cl.58) at the early and late 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 phosphate buffer (PB) for 30 min. After washing with PB, the samples were postfixed with 1% osmium tetroxide in PB for 60 min, washed with

6

PB, dehydrated in graded 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% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 10 min to block nonspecific binding and incubated with goat anti-chicken IgM H-chain antibody A30-102A (Bethyl Laboratories Inc., TX, USA; diluted 1:1500 with 5% NHS, 1% BSA in PBS) for 60 min.   After rinsing in PBS, the sections were incubated with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1:200 with 1% BSA in PBS) for 40 min.   After washing with PBS, the sections were incubated with 8 nm colloidal gold conjugated anti-biotin antibody (Vector Laboratories, Burlingame, CA, USA; diluted with 1% BSA in PBS) for 30 min.   After washing with distilled water and drying, the sections were contrasted with 2% uranyl acetate in 70% methanol and Reynolds' lead citrate and observed in a JEOL 1200EX transmission electron microscope operating at 80 kV (JEOL, Tokyo, Japan).   As control, primary antibody was omitted or replaced by normal goat serum.

Results

Generation of Pax5-deficient DT40 mutant cells, Pax5(-)

To generate the Pax5-deficient DT40 mutant cells, we transfected the targeting ΔPax5/hyg vector into DT40 cells.   After integration of the targeting vector into one Pax5 allele, three stable transfectant clones (cl.46, cl.58 and cl.62) were selected in the presence of hyg based on the positive 11.5 kb SacI fragment hybridized with probe Pax5 (Fig. 1A).   Surprisingly, all of these three transfectants lost completely the endogenous 7.8 kb SacI fragment at the same time (Fig. 1B).   To verify whether the Pax5 gene was really disrupted in these mutant clones, we carried out RT-PCR using appropriate primers specific for Pax5 on total RNAs.   The mRNA level of Pax5 was not detected in three independent mutant clones (cl.46, cl.58 and cl.62), whereas in three Pax5(+) clones (cl.1, cl.2 and cl.3) having the endogenous Pax5 gene and randomly integrated hyg-gene the mRNA level was comparable to that of DT40 cells (Fig. 2). These results indicated that the Pax5 gene exists as a single allele and agreed with the fact that the Pax5 gene is located on Z chromosome, which is monosomy in chickens as mentioned above, though its some homologues are located on other chromosomes.   Hereafter, we regarded these three transfectants as Pax5-deficient DT40 mutant cells, Pax5(-).

Pax5-deficiency affects severely or moderately on gene expressions of IgM H- and L-chains, chromatin modifying enzymes and transcription factors

We studied effects of the Pax5-deficiency on several cellular characteristics as follows.   Three independent Pax5(-) clones (cl.46, cl.58 and cl.62) proliferated and reached to a maximum cell density comparable to that of DT40 cells, suggesting that the Pax5-deficiency had an insignificant effect on

growth rate and maximum cell density (Fig. 1C).   To know whether the Pax5-deficiency affects on gene expressions of IgM H- and L-chains, chromatin modifying enzymes and transcription factors, we carried out RT-PCR, using primers IgM Hc, IgM Hs plus IgM Hm and IgM L (which are specific for whole, secreted plus membrane-bound forms of IgM H-chain mRNA and IgM L-chain mRNA), and primers specific for PCAF, HDAC2, HDAC4, HDAC7, HDAC9, Ikaros, Aiolos, E2A, EBF1 and CstF-64, on total RNAs prepared from three Pax5(-) clones (cl.46, cl.58 and cl.62), together with DT40 (W) and three Pax5(+) clones (cl.1, cl.2 and cl.3) as control (Fig. 2).   In all three Pax5(-) clones, the levels of whole and secreted forms of IgM H-chain mRNA were dramatically elevated, whereas those of membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were slightly and considerably elevated.   Concerning chromatin modifying enzymes, in these three Pax5(-) clones, mRNA levels of PCAF and HDAC9 were dramatically elevated, that of HDAC7 was slightly reduced, and those of HDAC2 and HDAC4 remained unchanged.   On the other hand, regarding transcription factors, mRNA levels of Ikaros and E2A were slightly elevated but that of Aiolos mRNA was slightly reduced.   Interestingly, the mRNA level of EBF1 was almost completely reduced in all of three Pax5(-) clones, whereas that of CstF-64 remained unchanged.   These results indicated that Pax5 exhibits severe effects on gene expressions of IgM H- and L-chains and also EBF1, PCAF and HDAC9, but moderate and/or insignificant effects on those of residual enzymes and factors examined.

IgM H- and L-chains artificially accumulated in Pax5(-) mutant cells are gradually reduced during continuous cultivation

We studied changing patterns in several properties of Pax5(-) clones (cl.46, cl.58 and cl.62) at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and late (L; 20 days) stages of cultivation.   These cultivation stages were shorter intervals than corresponding those employed for HDAC2(-/-) mutants [38].   To know changing patterns in cellular protein levels, we first analyzed total cellular proteins prepared from Pax5(-) clone (cl.58) at the early and late stages by 2D-PAGE.   The amounts of IgM H- and L-chains were dramatically and certainly elevated at the early stage and thereafter at the late stage reduced to almost similar levels in DT40 cells (data not shown).   Western blotting, using antibody for chicken IgM L-chain that cross-reacts with IgM H-chain, revealed that protein levels of IgM H- and L-chains (detected as two bands) were dramatically elevated at the early stage in these three Pax5(-) clones (cl.46, cl.58 and cl.62) and thereafter gradually reduced via the middle to late stages to comparable levels in DT40 cells (Fig. 3).   Electron microscopy showed that electron-dense materials (probably immunoglobulin proteins) were accumulated in irregularly elaborated and vesiculated rough endoplasmic reticula in Pax5(-) clone (cl.58) at the early stage, but not at the late stage like in DT40 cells (Fig. 4A).   Immuno-electron microscopy revealed not only that colloidal gold immuno-labeling for chicken IgM H-chain was vividly observed in electron-dense materials accumulated in the dilated and vesiculated

rough endoplasmic reticula only at the early stage, but also that the heightened protein levels were obviously reduced at the late stage to almost similar levels in DT40 cells (Fig. 4B). These results, together, indicated that protein levels of IgM H- and L-chains in Pax5(-) are dramatically and obviously elevated at the early stage of cultivation, and thereafter gradually reduced during cultivation and at the late stage reached to comparable levels in DT40 cells.

Morphological property of Pax5(-) mutant cells slightly changes during continuous cultivation

Microscopy showed that Pax5(-) mutant clone (cl.62) at both the early and late cultivation stages were morphologically observed to be dispersive form, like that of DT40 cells, in any distinct ranges of vision within microscopy and their magnified visions (Fig. 5). In addition, Pax5(-) mutant cells were observed to be mature plasma B cell-like form at the early stage but thereafter to change to normal form at the late stage, like that of DT40 cells. The morphology of Pax5(-) and its changing patterns during cultivation were clearly different from those of HDAC2(-/-) (data will be shown in our next paper [42]).

Gene expressions of IgM H- and L-chains in Pax5(-) mutant cells change during continuous cultivation

To clarify changing patterns in gene expressions of IgM H- and L-chains in Pax5(-) during cultivation, we carried out RT-PCR, using appropriate primers IgM Hc, IgM Hs, IgM Hm and IgM L, on total RNAs prepared from three Pax5(-) clones (cl.46, cl.58 and cl.62) at the first, early, middle and late cultivation stages (Fig. 6). Whole and secreted forms of IgM H-chain mRNA in Pax5(-) were dramatically increased at the first stage and thereafter rapidly decreased via the early and middle stages and at the late stage reached to almost the same levels in DT40 cells. On the other hand, membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage and thereafter gradually and slowly decreased via the early and middle stages and at the late stage reached to almost the same levels in DT40 cells. These results meant that gene expressions of IgM H- and L-chains are dramatically and considerably increased at the first stage and thereafter gradually and surely decreased via the early and middle to late stages to almost similar levels in DT40 cells.

Gene expressions of HDAC9 plus PCAF or HDAC7 in Pax5(-) mutant cells dramatically or moderately change during continuous cultivation

We carried out RT-PCR, using various appropriate primers specific for chromatin modifying enzymes; HDAC1, HDAC2, HDAC3, HDAC4, HDAC7, HDAC8, HDAC9, PCAF, GCN5, HAT1, Elp3, p300, MORF, MOZ and TIP60, on total RNAs prepared from three Pax5(-) clones (cl.48, cl.58 and cl.62) at the first, early, middle and late cultivation stages (Fig. 7A). Gene expressions of HDAC9 and PCAF in Pax5(-) were dramatically increased from the first via early and middle to late stages. The gene

expression of HDAC7 was slightly decreased at the first stage but thereafter slightly increased until the late stage to almost similar level in DT40 cells.   On the other hand, gene expressions of residual HATs and HDACs tested remained unchanged during cultivation.

Gene expressions of Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1 and OBF1 in Pax5(-) mutant cells differently change during continuous cultivation

We carried out RT-PCR, using various appropriate primers specific for transcription factors, such as Oct1, Oct2, OBF1, NF-kB, RelB, YY1, NF-AT, Blimp1, XBP-1, EBF1, PU.1, E2A, Aiolos, Ikaros and CstF-64, on total RNAs prepared from three Pax5(-) clones (cl.46, cl.58 and cl.62) at the first, early, middle and late cultivation stages (Fig. 7B).   Gene expressions of Aiolos and OBF1 in Pax5(-) were gradually decreased from the first via early and middle to late stages to undetectable levels.   On the other hand, gene expressions of Ikaros, E2A and Blimp1 were dramatically increased at the first stage and thereafter gradually decreased via the early and middle stages and finally at the late stage reached to almost the same levels in DT40 cells.   The gene expression of EBF1 was completely decreased at the first stage and remained unchanged as undetectable level until the late stage.   The gene expression of PU.1 was remarkably decreased at the first stage and thereafter gradually increased to the late stage to slightly higher level than that in DT40 cells.   Relative mRNA levels of these altered transcription factors, together with those of altered chromatin modifying enzymes, are shown in Figure 8.

Discussion

The modulation of chromatin topology with epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and so on, is one of the most important events to express cell functions in eukaryotes.   Mechanisms to alter chromatin structure have been vigorously studied in diverse research fields of life science for the last several decades [1-4, 10-19].   Even now, surely, such researches on the epigenetic modifications have been intensively continued without interruptions in more diverse life science fields, e.g., transcription/gene expression, DNA replication, differentiation, development, pluripotency, memory and clinical medicine [43-70].   We have also systematically studied in vivo roles of numerous members of histones, histone chaperones, transcription factors and chromatin modifying enzymes (HATs and HDACs) [20-32, 36-38, 40, 42], by gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [33-35].

In this study, to explore the physiological participation of Pax5 in gene expressions of IgM H- and L-chains, we generated and analyzed Pax5-deficient DT40 mutant cells, Pax5(-), devoid of the Pax5 gene (as a single allele) located on Z chromosome that is monosomy in chickens (Fig. 1).   Results obtained from Pax5(-) (Fig. 2), together with those from DT40 mutant cell lines, EBF1(-/-), Aiolos(-/-), Helios(-/-),

E2A(-/-) and Ikaros(-/-/+), all of which were generated by us [25], revealed that Pax5, EBF1, Aiolos and Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates gene expressions of these two immunoglobulin proteins. Thus, these results, combined with those from HDAC2(-/-), indicated that HDAC2 regulates indirectly gene expressions of IgM H- and L-chains through controlling those of Pax5, Ikaros, EBF1 plus Aiolos, and E2A in opposite manners [25, 38]. Surprisingly, 2D-PAGE, Western blotting, immuno-electron microscopy and/or RT-PCR analyses of four HDAC2(-/-) clones (cl.33-12, cl.33-28, cl.33-30 and cl.45-28) cultivated for different periods revealed not only that protein and mRNA levels of IgM H- and L-chains are dramatically and considerably elevated at the early (~10 days) stage of cultivation, but also that these elevated immunoglobulin proteins and mRNAs are obviously reduced at the late (~60 days) cultivation stage to comparable levels in DT40 cells [38]. In addition, gene expressions of HDAC7, HDAC9, PCAF, EBF1, E2A, Pax5 and Aiolos each were certainly altered in different patterns in these mutant clones during cultivation. Interestingly, in these four HDAC2(-/-) mutant clones, the changing pattern in the gene expression of Pax5 during cultivation was anti-parallel with those in gene expressions of IgM H- and L-chains, whereas those in gene expressions of residual altered factors (such as EBF1, E2A and Aiolos) were neither parallel nor anti-parallel so much. These results, together with previous findings [25], suggested that in DT40 cells Pax5 should control preferentially gene expressions of IgM H- and L-chains among various transcription factors.

Therefore, we next studied several characteristics of Pax5(-) clones (cl.46, cl.58 and cl.62), which were cultivated for several distinct periods, by Western blotting, RT-PCR, microscopy, electron microscopy and immuno-electron microscopy. Protein levels of IgM H- and L-chains in Pax5(-) were drastically and considerably elevated at the early (8 days) stage of cultivation and thereafter gradually reduced via the middle (13 days) until late (20 days) stages to comparable levels in DT40 cells (Figs. 3 and 4). Concerning mRNA levels of these two immunoglobulin proteins, whole and secreted forms of IgM H-chain mRNA were dramatically elevated at the first (4 days) stage (prior to the early stage), and thereafter these elevated two type mRNAs were rapidly reduced via the early and middle stages until the late stage to comparable levels in DT40 cells (Fig. 6). Membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were considerably increased at the first stage and thereafter slowly decreased until the late stage to almost the same levels in DT40 cells. Thus, changing patterns in protein and mRNA levels of IgM H- and L-chains in Pax5(-) during cultivation were similar to those in HDAC2(-/-) on the whole, however, the changing speeds in Pax5(-) were obviously faster than those in HDAC2(-/-) [38]. In addition, Pax5(-) was dispersive form at both the early and late stages, like that of DT40 cells (Fig. 5), whereas HDAC2(-/-) was aggregative form at the early stage but dispersive form at the late stage (data will be shown in our next paper [42]).

Concerning chromatin modifying enzymes, mRNA levels of PCAF and HDAC9 in Pax5(-) were gradually increased from the first to late stages, and that of HDAC7 changed moderately during

cultivation (Figs. 7A and 8). These changing patterns were similar to those in HDAC2(-/-) as a whole [38]. On the other hand, concerning transcription factors, Pax5(-) showed different changing patterns from those in HDAC2(-/-) as follows (Figs. 7B and 8). That is, mRNA levels of Aiolos and OBF1 were gradually decreased from the first to late stages to undetectable levels, and those of Ikaros, E2A and Blimp1 were drastically increased at the first stage and thereafter gradually decreased until the late stage to almost similar or slightly higher levels in DT40 cells. The EBF1 mRNA level was completely decreased at the first stage and remained unchanged as undetectable level throughout cultivation. The PU.1 mRNA level was remarkably decreased at the first stage and gradually increased until the late stage: the level being slightly higher than that in DT40 cells.

In summary, the reason for the differences in the changing speeds of protein plus mRNA levels of IgM H- and L-chains between Pax5(-) and HDAC2(-/-) is probably based on the fact that Pax5 should participate at down-stream of HDAC2 in regulation of gene expressions of these two immunoglobulin proteins [35, 38]. In addition, both of the above-mentioned differences and those in the cell morphology between Pax5(-) and HDAC2(-/-) during cultivation must be due to diverse alterations in gene expressions of particular transcription factors; Pax5, Aiolos, OBF1, Ikaros, E2A, EBF1, Blimp1, PU.1 and so on, but not to alterations in gene expressions of chromatin modifying enzymes; PCAF, HDAC7 and HDAC9 and so on. As a result, Pax5(-) mutants should be obviously different from HDAC2(-/-) mutants in several cellular characteristics.

Acknowledgments

References

[1] Kornberg, R. D.: Structure of chromatin. Annu. Rev. Biochem. 46, 931-954, 1977.

[2] Felsenfeld, G.: Chromatin. Nature 271, 115-121, 1978.

[3] van Holde, K. E.: Chromatin. New York: Springer-Verlag, 1989.

[4] Workman, J. L. and Kingston, R. E.: Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. 67, 545-579, 1998.

[5] Staudt, L. M. and Lenardo, M. J.: Immunoglobulin gene transcription. Annu. Rev. Immunol. 9, 373-398, 1991.

[6] Calame, K. L., Lin, K. I. and Tunyaplin, C.: Regulatory mechanisms that determine the development and function of plasma cells. Annu. Rev. Immunol. 21, 205-230, 2003.

[7] Busslinger, M.: Transcriptional control of early B cell development. Annu. Rev. Immunol. 22, 55-79, 2004.

[8] Nutt, S. L. and Kee, B. L.: The transcriptional regulation of B cell lineage commitment. Immunity 26, 715-725, 2007.

[9] Ramirez, J., Lukin, K. and Hagman, J.: From hematopoietic progenitors to B cells: mechanisms of lineage restriction and commitment. Curr. Opin. Immunol. 22, 177-184, 2010.

[10] Brownell, J. E., Zhou, J., Rannali, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y. and Allis, C. D.: Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5 linking histone acetylation to gene activation. Cell 84, 843-851, 1996.

[11] Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. and Nakatani, Y.: The transcriptional coactivators p300 and CPB are histone acetyltransferases. Cell 87, 953-959, 1996.

[12] Taunton, J., Hassig, C. A. and Schreiber, S. L.: A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272, 408-411, 1996.

[13] Hassig, C. A. and Schreiber, S. L.: Nuclear histone acetylases and deacetylases and transcriptional regulation: HATs off to HDACs. Curr. Opin. Chem. Biol. 1, 300-308, 1997.

[14] Strahl, B. D. and Allis C. D.: The language of covalent histone modifications. Nature 403, 41-45, 2000.

[15] Jenuwein, T. and Allis, C. D.: Translating the histone code. Science 293, 1074-1080, 2001.

[16] Khochbin, S., Verdel, A., Lemercier, C. and Seigneurin-Berny, D.: Functional significance of histone deacetylase diversity. Curr. Opin. Genet. Dev. 11, 162-166, 2001.

[17] Roth, S. Y. Denu, J. M. and Allis, C. D.: Histone acetyltransferases. Annu. Rev. Biochem. 70, 81-120, 2001.

[18] Carrozza, M. J., Utley, R. T., Workman, J. L. and Cote, J.: The diverse functions of histone acetyltransferase complexes. Trend. Genet. 19, 321-329, 2003.

[19] Yang, X. J. and Seto, E.: Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. Curr. Opin. Genet. Dev. 13, 143-153, 2003.

[20] Takami, Y., Kikuchi, H. and Nakayama, T.: Chicken histone deacetylase-2 controls the amount of the IgM H-chain at the steps of both transcription of its gene and alternative processing of its pre-mRNA in the DT40 cell line. J. Biol. Chem. 274, 23977-23990, 1999.

[21] Takami, Y. and Nakayama, T.: N-terminal region, C-terminal region, nuclear export signal and deacetylation activity of histone deacetylase-3 are essential for the viability of the DT40 chicken B cell line. J. Biol. Chem. 275, 16191-16201, 2000.

[22] Nakayama, T. and Takami, Y.: Participation of histones and histone-modifying enzymes in cell functions through alterations in chromatin structure. J. Biochem. 129, 491-499, 2001.

[23] Takechi, S., Adachi, M. and Nakayama, T.: Chicken HDAC2 down-regulates IgM light chain gene promoter activity. Biochem. Biophys. Res. Commun. 299, 263-267, 2002.

[24] Kikuchi, H., Takami, Y. and Nakayama, T.: GCN5: a supervisor in all-inclusive control of vertebrate cell cycle progression through transcription regulation of various cell cycle-related genes. Gene 347, 83-97, 2005.

[25] Nakayama, M., Suzuki, H., Yamamoto-Nagamatsu, N., Barman, H. K., Kikuchi, H., Takami, Y., Toyonaga, K., Yamashita, K. and Nakayama, T.: HDAC2 controls IgM H and L-chain gene expressions via EBF1, Pax5, Ikaros, Aiolos and E2A gene expressions. Genes Cells 12, 359-373, 2007.

[26] Kikuchi, H. and Nakayama, T.: GCN5 and BCR signaling collaborate to induce pre-mature B cell apoptosis through depletion of ICAD and IAP2 and activation of caspase activities. Gene 419, 48-55, 2008.

[27] Kikuchi, H., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, Y., Takami, Y. and Nakayama, T.: GCN5 protects vertebrate cells against UV-irradiation via controlling gene expression of DNA polymerase η. J. Biol. Chem. 287, 39842-39849, 2012.

[28] Kikuchi, H., Nakayama, M., Kuribayashi, F., Imajoh-Ohmi, S., Nishitoh, H., Takami, Y. and Nakayama T.: GCN5 is essential for IRF-4 gene expression followed by transcriptional activation of Blimp-1 in immature B cells. J. Leukoc. Biol. 95, 399-404, 2014.

[29] Takami, Y., Takeda, S. and Nakayama, T.: Targeted disruption of an H3-IV/H3-V gene pair causes increased expression of the remaining H3 genes in the chicken DT40 cell line. J. Mol. Biol. 250, 420-433, 1995.

[30] Takami, Y. and Nakayama, T.: A single copy of linker H1 genes is enough for proliferation of the DT40 chicken B cell line, and linker H1 variants participate in regulation of gene expression. Genes Cells 2, 711-723, 1997.

[31] Sanematsu, F., Takami, Y., Barman, H. K., Fukagawa, T., Ono, T., Shibahara, K. and Nakayama, T.: Asf1 is required for viability and chromatin assembly during DNA replication in vertebrate cells. J. Biol. Chem. 281, 13817-13827, 2006.

[32] Takami, Y., Ono, T., Fukagawa, T., Shibahara, K. and Nakayama, T.: Essential role of CAF-1-mediated rapid nucleosome assembly for DNA replication and cell division in vertebrate cells. Mol. Biol. Cell 18, 129-141, 2007.

[33] Baba, T. W., Giroir, B. P. and Humphries, E. H.: Cell lines derived from avian lymphomas exhibit two distinct phenotypes. Virology 144, 139-151, 1985.

[34] Buerstedde, J.-M. and Takeda, S.: Increased ratio of targeted to random integration after transfection of chicken B cell lines. Cell 67, 179-188, 1991.

[35] Takeda, S., Masteller, E. L., Thompson, C. B. and Buerstedde, J.-M.: RAG-2 expression is not essential for chicken immunoglobulin gene conversion. Proc. Natl. Acad. Sci. USA 89, 4023-4027, 1992.

[36] Toyonaga, K., Kikuchi, H., Yamashita, K., Nakayama, M., Chijiiwa, K. and Nakayama, T.: E2A participates in a fine control of pre-mature B cell apoptosis mediated by B cell receptor signaling via transcription regulations of survivin, IAP2 and caspase-8 genes. FEBS J. 276/5, 1418-1428, 2009.

[37] Kikuchi, H., Yamashita, K., Nakayama, M., Toyonaga, K., Tsuneyoshi, I., Takasaki, M. and Nakayama, T.: Lacking of Aiolos accelerates pre-mature B cell apoptosis mediated by BCR signaling through elevation in cytochrome c release. BBA-Molecular Cell Research 1793, 1304-1314, 2009.

[38] Nakayama M. and Nakayama T.: Protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in HDAC2-deficient DT40 mutants are gradually reduced via a lot of generations during continuous cultivations. In: Chromatin Conformation Change Code (4C) Theory on Gain of New Cell Function through Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications via a Lot of Generations, Nakayama, T. and Nakayama, M. (Eds.), Miyakonojoh-Insatsu, Inc. Miyazaki, Japan, pp. 11-44, 2015. Available from two URLs (http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995 and/or http://hdl.handle.net/10458/5293). The article with a slight modification is in preparation for publication.

[39] Miyazaki, J., Takaki, S., Araki, K., Tashiro, F., Tominaga, A., Takatsu, K. and Yamamura, K.: Expression vector system based on the chicken b-actin promoter directs efficient production of interleukin-5. Gene 79, 269-277, 1989.

[40] Ahmad, A., Kikuchi, H., Takami, Y. and Nakayama, T.: Different roles of N-terminal and C-terminal halves of HIRA in transcription regulation of cell cycle-related genes that contribute to control of vertebrate cell growth. J. Biol. Chem. 280, 32090-32100, 2005.

[41] Bendayan, M. and Zollinger, M.: Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J. Histochem. Cytochem. 31, 101-109, 1983.

[42] Nakayama, M. and Nakayama, T.: Artificially and excessively accumulated IgM H- and L-chains in HDAC2(-/-) DT40 mutants are gradually and dramatically reduced in distinct manners in individual mutant clones via a lot of generations during continuous cultivation. In: Chromatin Conformation Change Code (4C) Theory on Gain of New Cell Function through Irreversible Creation of Chromatin Structure Plasticity with Epigenetic Modifications via a Lot of Generations, Nakayama, T. and Nakayama, M. (Eds.), Miyakonojoh-Insatsu, Inc. Miyazaki, Japan, pp. 71-103, 2015. Available from two URLs (http://opac2.lib.miyazaki-u.ac.jp/webopac/TC10123995 and/or http://hdl.handle.net/10458/5293). The article with a slight modification is in preparation for publication.

[43] Biel, M., Wascholowski, V. and Giannis, A.: Epigenetics - an epicenter of gene regulation: histones and histone-modifying enzymes. Angew. Chem. Int. Ed. Engl. 20, 3186-3216, 2005.

[44] Margueron, R., Trojer, P. and Reinberg, D.: The key to development: interpreting the histone code? Curr. Opin. Genet. Dev. 15, 163-176, 2005.

[45] Saha, A., Wittmeyer, J. and Cairns, B. R.: Chromatin remodelling: the industrial revolution of DNA

around histones. Nat. Rev. Mol. Cell Biol. 7, 437-447, 2006.

[46] Berstein, B. E., Meissner, A. and Lander, E. S.: The mammalian epigenome. Cell 128, 669-681, 2007.

[47] Goldberg, A. D., Allis, C. D. and Bernstein, B. E.: Epigenetics: a landscape takes shape. Cell 128, 635-638, 2007.

[48] Espada, J. and Esteller, M.: Epigenetic control of nuclear architecture. Cell. Mol. Life Sci. 64, 449-457, 2007.

[49] Shahbazian, M. D. and Grunstein, M.: Functions of site-specific histone acetylation and deacetylation. Annu. Rev. Biochem. 76, 75-100, 2007.

[50] Kouzarides, T.: Chromatin modifications and their function. Cell 128, 693-705, 2007.

[51] Allis, C. D., Berger, S. L., Cote, J., Dent, S., Jenuwien, T., Kouzarides, T., Pillus, L., Reinberg, D., Shi, Y., Shiekhattar, R., Shilatifard, A., Workman, J. and Zhang, Y.: New nomenclature for chromatin-modifying enzymes. Cell 131, 633-636, 2007.

[52] Lee, K. K. and Workman, J. L.: Histone acetyltransferase complexes: one size doesn't fit all. Nat. Rev. Mol. Cell Biol. 8, 284-295, 2007.

[53] Berger, S. L.: The complex language of chromatin regulation during transcription. Nature 447, 407-412, 2007.

[54] Esteller, M.: Cancer epigenomics: DNA methylomes and histone-modification maps. Nat. Rev. Genet. 8, 286-298, 2007.

[55] Suganuma, T. and Workman, J. L.: Crosstalk among histone modifications. Cell 135, 604-607, 2008.

[56] Kohn, K. W., Aladjem, M. I., Weinstein, J. N. and Pommier, Y.: Chromatin challenges during DNA replication: A systems representation. Mol. Biol. Cell 19, 1-7, 2008.

[57] Clapier, C. R. and Cairns, B. R.: The biology of chromatin remodeling complexes. Annu. Rev. Bioch. 78, 273-304, 2009.

[58] Selvi, R. B. and Kundu, T. K.: Reversible acetylation of chromatin: implication in regulation of gene expression, disease and therapeutics. Biotech. J. 4, 375-390, 2009.

[59] Talbert, P. B. and Henikoff, S.: Histone variants-ancient wrap artists of the epigenome. Nat. Rev. Mol. Cell Biol. 11, 264-275, 2010.

[60] Javierre, B. M., Hemando, H. and Ballestar, E.: Environmental triggers and epigenetic deregulation in autoimmune disease. Discov. Med. 12, 535-545, 2011.

[61] Bannister, A. J. and Kouzarides, T.: Regulation of chromatin by histone modifications. Cell Res. 21, 381-395, 2011.

[62] Verrier, L., Vandromme, M. and Trouche, D.: Histone demethylases in chromatin cross-talks. Biol. Cell 103, 381-401, 2011.

[63] Butler, J. S., Koutelou, E., Schibler, A. C. and Dent, S. Y.: Histone-modifying enzymes: regulators

16

of developmental decisions and drivers of human disease. Epigenomics 4, 163-177, 2012.

[64] Kooistra, S. M. and Helin, K.: Molecular mechanisms and potential functions of histone deacetylases. Nat. Rev. Mol. Cell. Biol. 13, 297-311, 2012.

[65] Oliner, J. R. and Winston, F.: Chromatin and transcription in yeast. Genetics 190, 351-387, 2012.

[66] Narlikar, G. J.: Mechanisms and functions of ATP-dependent chromatin-remodeling enzymes. Cell 154, 490-503, 2013.

[67] Graff, J. and Tsai, L.-H.: Histone acetylation: molecular mnemonics on the chromatin. Nat. Rev. Neurosci. 14, 97-111, 2013.

[68] Chen, T. and Dent, S. Y. R.: Chromatin modifiers and remodellers: regulators of cellular differentiation. Nat. Rev. Genet. 15, 93-106, 2014.

[69] T. W.-W. and Reinberg, D.: Chromatin futures and the epigenetic regulation of pluripotency states in ESCs. Development 141, 2376-2390, 2014.

[70] Morgan, M. A. and Shilatifard, A.: Chromatin signatures of cancer. Genes and Dev. 29, 238-249, 2015.

Figure legends

Figure 1.   Genomic organization of chicken Pax5 gene and generation of Pax5-deficient DT40 mutants, Pax5(-)

A. Schematic representation of the chicken Pax5 gene locus (top) with enlarged drawing of its intact allele (middle) and targeted allele (bottom).   Locations of exons are indicated by solid boxes with appropriate designations.   White box indicates drug resistance cassette (hyg).   Location of probe Pax5 is indicated by a gray box.   Only relevant restriction sites are indicated.   Possible relevant fragments obtained from SacI digestion are shown with their length in kb.

B. Southern blotting of homologous recombination event.   Genomic DNAs were prepared from DT40, one mutant clone cl.1(+) integrated randomly with ΔPax5/hyg construct and three Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-).   The SacI fragments were analyzed with probe Pax5.

C. Growth rate of Pax5(-) mutant cells.   Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-) and DT40 cells were grown, and cell numbers were determined at indicated times.   The numbers are plotted on a log phase.   The values are averages for three independent experiments.   Symbols for these mutant clones are shown in the figure.

Figure 2.   Effects of Pax5-deficiency on gene expressions of IgM H- and L-chains, and several members of HATs, HDACs and transcription factors

Total RNAs were extracted from DT40 cells (W), three Pax5(+) clones (cl.1, cl.2 and cl.3) randomly integrated with ΔPax5/hyg construct and three Pax5(-) clones (cl.46, cl.58 and cl.62).   RT-PCR was performed on equal amounts of total RNAs, using appropriate primers for whole IgM H-chain mRNA (IgM Hc), its secreted form (IgM Hs), its membrane-bound form (IgM Hm), IgM L-chain mRNA (IgM L) and also for mRNAs of several members of HATs, HDACs and transcription factors (indicated by appropriate designations).   Chicken GAPDH was used as control.

Figure 3.   Alterations in amounts of IgM H- and L-chains in Pax5(-) DT40 mutants during cultivation

Total cellular proteins were prepared from Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-) at the early (E; 8 days), middle (M; 13 days) and late (L; 20 days) cultivation stages and compared with those from DT40 cells (W) by Western blotting.   Aliquots of cell extracts were subjected to 12% SDS-PAGE and transferred to membrane filter, and proteins were detected with anti-chicken IgM L-chain antiserum (as primary antibody) that cross-reacts with IgM H-chain.   Chicken β-actin was used as internal control. Typical patterns are shown in the upper panel.   Relative levels of IgM H-chain and large (high) plus small (low) forms of IgM L-chain are shown in the lower panel.   Symbols for IgM H- and L-chains are shown in the bottom.

Figure 4.　Alterations in amounts of IgM H-chain in Pax5(-) DT40 mutants during cultivation

　　Pax5(-) mutant cells (clone cl.58) collected at the early (E; 8 days) and late (L; 20 days) cultivation stages and DT40 cells (W) were fixed.　Electron microscopy (A) and immuno-electron microscopy using anti-chicken IgM H-chain antiserum (B) were carried out.　Dense cytoplasmic fraction (indicated by arrow) due to accumulated IgM H-chain was observed only at the early (E) cultivation stage in Pax5(-) (E in A).　A large number of IgM H-chain were observed only at the early (E) cultivation stage in Pax5(-) (E in B).

Figure 5.　Morphology of Pax5(-) DT40 mutant cells during cultivation

　　Microscopy on Pax5(-) mutant cells (clone cl.62) at the early (E) and late (L) cultivation stages and DT40 cells (W) was performed in distinct ranges of vision with magnified visions.　Typical ones are shown.

Figure 6.　Alterations in gene expressions of IgM H- and L-chains in Pax5(-) DT40 mutants during cultivation

　　Total RNAs were extracted from Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-) at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and late (L; 20 days) cultivation stages and DT40 cells (W). RT-PCR was performed on equal amounts of total RNAs, using appropriate primers for Pax5, whole IgM H-chain mRNA (IgM Hc), its secreted form (IgM Hs), its membrane-bound form (IgM Hm) and IgM L-chain (IgM L).　Chicken GAPDH was used as control.　Typical patterns are shown in the upper panel. Relative mRNA levels of whole, secreted and membrane-bound forms of IgM H-chain and IgM L-chain are shown in the lower panel.　Symbols for mutant clones are shown in the bottom.

Figure 7.　Alterations in gene expressions of HDACs, HATs and transcription factors in Pax5(-) DT40 mutants during cultivation

　　RT-PCR was performed on total RNAs identical with those in Figure 6, using appropriate primers for members of HDACs and HATs (A), and transcription factors (B), instead of primers for IgM H- and L-chains.

Figure 8.　Relative mRNA levels of altered genes in Pax5(-) DT40 mutants during cultivation

　　Relative mRNA levels of altered genes in Figure 7 are shown.　Symbols for Pax5(-) mutant clones are shown in the lower right.