Chapter 3

Generation of Pax5-deficient DT40 mutants, Pax5(-), and protein and mRNA levels of IgM H- and L-chains artificially accumulated in Pax5(-) are rapidly and dramatically reduced through various generations during continuous cultivation

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SUMMARY

We generated Pax5-deficient DT40 mutant cells, Pax5(-), devoid of the Pax5 gene existing on Z sex chromosome that is monosomy in chickens, and analyzed characteristics of Pax5(-) by Western blotting, microscopy, electron microscopy, immuno-electron microscopy and reverse transcription-polymerase chain reaction (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 finally at the later stage reached comparable levels as 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 later stage to comparable levels as in DT40 cells. The 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 the HDAC7 mRNA level moderately changed. Aiolos and OBF1 mRNA levels were gradually reduced from the first to later stages, whereas Ikaros and E2A mRNA levels were drastically elevated at the first stage and thereafter gradually decreased until the later stage. The EBF1 mRNA level was completely decreased at the first stage and remained unchanged during cultivation. The PU.1 mRNA level was remarkably reduced at the first stage and thereafter gradually elevated until the later stage.

These results, together with our previous findings, revealed not only that Pax5 dramatically down-regulates gene expressions of IgM H- and L-chains, which are indirectly regulated by histone deacetylase-2 (HDAC2) through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, Ikaros and E2A in DT40 cells, but also that during cultivation the decreases in elevated protein and mRNA levels of these two immunoglobulin molecules are more rapid in Pax5(-) than in HDAC2(-/-). In addition, the influences of the Pax5-deficiency on several cellular characteristics are certainly different from those of the HDAC2-deficiency.

INTRODUCTION

Alterations in the chromatin structure are strongly involved in regulations of gene expressions, and DNA replication, repair plus recombination and others in eukaryotes [1-5], and also concerned in regulations of the lymphocyte development and differentiation [6-12]. Among various chromatin modifying-enzymes participated in alterations in the chromatin structure, specific members of histone acetyltransferases (HATs) and histone deacetylases (HDACs) cooperatively control acetylation and deacetylation levels of specific Lys residues of core histones H2A, H2B, H3 and H4 [13-27]. To assess their individual roles in expressions of normal cell functions, we systematically generated a number of homozygous (or conditional) chicken DT40 mutant cell lines, each of which is devoid of a specific member of HDACs and HATs [28-44], by gene targeting techniques [45-55]. Our previous analyses of

the 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 its alternative pre-mRNA processing [28], and down-regulates IgM L-chain gene promoter activity [31]. 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 specific Lys residues of core histones H3, H4 and H2B [35]. Therefore, to know individual roles of these changed 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 [35, 56-60], and Ikaros-down DT40 mutant cell line, Ikaros(-/-/+), devoid of two alleles of the Ikaros gene existing on chromosome 2 that is trisomy in chickens (data will be shown elsewhere). The results obtained showed that EBF1, Aiolos plus Ikaros down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates gene expressions of these two immunoglobulin proteins [35]. Taken together, these findings indicated that HDAC2 indirectly regulates gene expressions of IgM H- and L-chains through opposite regulations of gene expressions of EBF1, Aiolos plus Ikaros, and E2A [35, 38].

Throughout the process of analyzing several characteristics of HDAC2(-/-) cultivated for different periods, we accidently noticed following interesting and remarkable phenomena as presented in the Chapter 2 [Chap. 2]. The results obtained by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), Western blotting and immuno-electron microscopy, together, showed that IgM H- and L-chains are dramatically accumulated at the early stage of cultivation in HDAC2(-/-), and thereafter gradually reduced during cultivation and finally at the later stage reached comparable levels as in DT40 cells. RT-PCR showed that whole and secreted forms of IgM H-chain mRNA in HDAC2(-/-) are dramatically increased at the early stage and thereafter gradually reduced during cultivation and finally at the later stage reach very close levels as in DT40 cells. Further, RT-PCR carried out on chromatin-modifying enzymes and transcription factors revealed that the mRNA level of PCAF in HDAC2(-/-) is drastically elevated from the early to later stages, and those of HDAC7 and HDAC9 slightly change during cultivation. The mRNA level of EBF1 in HDAC2(-/-) is almost completely reduced at the early stage and thereafter remains unchanged, and that of E2A is slightly elevated at the early stage and remains unchanged. Interestingly, the mRNA level of Pax5 in HDAC2(-/-) is obviously decreased at the early stage and thereafter increased until the later stage. This changing pattern in the Pax5 gene expression is in anti-parallel with those in gene expressions of IgM H- and L-chains as a whole. These results revealed not only that the gene expression of Pax5 is certainly controlled by HDAC2, but also that Pax5 is expected to mainly control gene expressions of IgM H- and L-chains among various transcription factors. In addition, immunoblotting using site-specific antibodies for various acetylated Lys residues 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 are gradually increased, but those of K5

residue of histone H4 and K16 residue of histone H2B change in the different patterns.

Next, we studied molecular mechanism of the gene expression of the Pax5 gene in HDAC2(-/-) [Chap. 2]. Since the detailed information on the chicken Pax5 gene could not be cited from any database at the start of this study, we directly cloned the proximal ~4.9 kb 5'-upstream region of the Pax5 gene from DT40 genomic DNA by our original gene walking techniques. 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 the proximal ~2.0 kb 5'-upstream region of the Pax5 gene are decreased at the early stage but at the later stage restored comparable levels as in DT40 cells. These results qualitatively agreed with the above-mentioned findings on alterations in the Pax5 gene expression, whereas these early and later 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 the Pax5-deficient DT40 mutant cell line, Pax5(-), devoid of the Pax5 gene existing on Z sex chromosome that is monosomy in chickens (USCS Genome Browser data base). Protein levels of IgM H- and L-chains in Pax5(-) were drastically increased at the early stage and thereafter rapidly decreased through the middle until later stages to comparable levels as 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 later stage to comparable levels as in DT40 cells. The 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 later stage to comparable levels as in DT40 cells. In addition, interestingly, the 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 is certainly different from HDAC2 in the participation in gene expressions of IgM H- and L-chains, but also that Pax5(-) is obviously distinct from HDAC2(-/-) in several cellular properties.

RESULTS

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

To generate the Pax5-deficient DT40 mutant cell line, 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 hygromycin (hyg) based on the positive 11.5 kb SacI fragment hybridized with probe Pax5 (Fig. 3-1A). Surprisingly, all of these transfectants completely lost the endogenous 7.8 kb SacI fragment at the same time (Fig. 3-1B). To verify whether or not 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 these three independent mutant clones (cl.46, cl.58 and cl.62), whereas in three Pax5(+) clones (cl.1, cl.2 and cl.3)

having both the endogenous Pax5 gene and the randomly integrated hyg-gene, it was comparable to that as in DT40 cells (Fig. 3-2). These results indicated that the Pax5 gene exists as a single allele and agreed with the fact that the Pax5 gene exists on Z sex chromosome, which is monosomy in chickens as mentioned above, distinct from its some homologues exist on other chromosomes. Hereafter, we regarded these three transfectants as the Pax5-deficient DT40 mutant cells, Pax5(-).

Pax5-deficiency severely or moderately affects on gene expressions of IgM H- and L-chains, and various members of 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 a maximum cell density comparable to that of DT40 cells, suggesting that the Pax5-deficiency had a slight effect on growth rate and maximum cell density (Fig. 3-1C). To know whether or not 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 also 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), and from DT40 (W) and three Pax5(+) clones (cl.1, cl.2 and cl.3) as controls (Fig. 3-2). In all three Pax5(-) clones, the levels of whole and secreted forms of IgM H-chain mRNA were dramatically elevated, whereas those of the membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA were slightly and considerably elevated. Concerning chromatin-modifying enzymes, in three Pax5(-) clones, the mRNA levels of PCAF and HDAC9 were dramatically increased, that of HDAC7 was slightly reduced, and those of HDAC2 and HDAC4 remained unchanged. On the other hand, regarding transcription factors, the mRNA levels of Ikaros and E2A were slightly elevated but the mRNA level of Aiolos was slightly decreased. Interestingly, the mRNA level of EBF1 was almost completely reduced in all of three Pax5(-) clones, whereas that of CstF-64 unchanged. These results indicated that Pax5 exhibits severe effects on gene expressions of IgM H- and L-chains, and also on those of EBF1, PCAF and HDAC9, but moderate effects on those of residual enzymes and factors examined.

IgM H- and L-chains artificially accumulated in Pax5(-) DT40 mutant cells are gradually reduced during continuous cultivation

We studied changing patterns in several cellular properties of three Pax5(-) clones (cl.46, cl.58 and cl.62) at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and later (L; 20 days) stages of cultivation. These cultivation stages were practically counted from the first day of cultivation from the stock at -80 °C and at shorter intervals than corresponding those employed for HDAC2(-/-) mutants

[Chap. 2]. To know changing patterns in cellular protein levels, we first analyzed total cellular proteins prepared from Pax5(-) clone (cl.58) at the early and later 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 later stage reduced to almost similar levels as 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 increased at the early stage in three Pax5(-) clones (cl.46, cl.58 and cl.62), and thereafter gradually reduced through the middle to later stages to comparable levels as in DT40 cells (W) (Fig. 3-3). Electron microscopy showed that electron-dense materials were accumulated in irregularly elaborated and vesiculated rough endoplasmic reticula in Pax5(-) at the early stage, but not at the later stage like in DT40 cells (Fig. 3-4A). Immuno-electron microscopy revealed not only that the 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 later stage to almost similar levels as in DT40 cells (Fig. 3-4B). These results, together, indicated that protein levels of IgM H- and L-chains in Pax5(-) are dramatically and obviously accumulated at the early stage of cultivation, and thereafter gradually reduced during cultivation and finally at the later stage reach comparable levels as in DT40 cells.

Morphological property of Pax5(-) DT40 mutant cells slightly changes during continuous cultivation

Microscopy showed that Pax5(-) clone (cl.62) at both the early and later cultivation stages was observed to be morphologically dispersive form, like that of DT40 cells, in any distinct ranges of vision within microscopy and their magnified visions (Suppl. Fig. 3-S1). In addition, Pax5(-) was observed to be mature plasma B cell-like form at the early stage but thereafter to change to normal form at the later 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 [Chap. 4]).

Gene expressions of IgM H- and L-chains in Pax5(-) DT40 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 later cultivation stages and from DT40 cells (Fig. 3-5). Whole and secreted forms of IgM H-chain mRNA in Pax5(-) were dramatically increased at the first stage, and thereafter rapidly decreased through the early and middle stages and finally at the later stage reached almost the same levels as in DT40 cells. On the other

hand, the 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 through the early and middle stages and finally at the later stage reached almost the same levels as in DT40 cells.

Gene expressions of HDAC9 plus PCAF or HDAC7 in Pax5(-) DT40 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 the three Pax5(-) clones at the first, early, middle and later cultivation stages and from DT40 cells (Fig. 3-6A). Gene expressions of HDAC9 and PCAF in Pax5(-) were dramatically increased from the first through early and middle to later stages. The gene expression of HDAC7 was slightly decreased at the first stage but thereafter slightly increased until the later stage to almost similar level as 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(-) DT40 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 the three Pax5(-) clones at the first, early, middle and later cultivation stages and from DT40 cells (Fig. 3-6B). Gene expressions of Aiolos and OBF1 in Pax5(-) were gradually reduced from the first through early and middle to later 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 through the early and middle stages and finally at the later stage reached almost same levels as in DT40 cells. The gene expression of EBF1 was completely decreased at the first stage and remained unchanged as undetectable level until the later stage. The gene expression of PU.1 was remarkably reduced at the first stage and gradually increased at the later stage to slightly higher level than that as in DT40 cells. The relative mRNA levels of these changed transcription factors and those of the changed chromatin-modifying enzymes are shown in Supplementary Figure 3-S2.

DISCUSSION

In this study, to explore the participation of Pax5 in gene expressions of IgM H- and L-chains, we first generated and analyzed the Pax5-deficient DT40 mutant cell line, Pax5(-), devoid of the Pax5 gene (as a single allele) existing on Z sex chromosome that is monosomy in chickens (Fig. 3-1). The results obtained from Pax5(-) (Fig. 3-2), together with those from chicken DT40 mutant cell lines, EBF1(-/-),

Aiolos(-/-), Helios(-/-), E2A(-/-) and Ikaros(-/-/+), which were generated by us, revealed that Pax5, Ikaros, EBF1 and Aiolos down-regulate gene expressions of IgM H- and L-chains, and E2A up-regulates gene expressions of these two immunoglobulin proteins [35, 38, 58]. Thus, these results, combined with those from HDAC2(-/-), indicated that HDAC2 indirectly regulates gene expressions of IgM H- and L-chains through controlling those of Pax5, Ikaros, EBF1 plus Aiolos and that of E2A in opposite ways [35, 38, Chap. 2]. Surprisingly, as shown in [Chap. 2], our qualitative 2D-PAGE, Western blotting, immuno-electron microscopy and RT-PCR analyses of initially generated HDAC2(-/-) mutant 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 accumulated at the early (~10 days) stage of cultivation, but also that these accumulated immunoglobulin proteins and mRNAs are obviously reduced at the later (~60 days) cultivation stage to comparable levels as in DT40 cells. In addition, gene expressions of HDAC7, HDAC9, PCAF, EBF1, E2A, Pax5 and Aiolos certainly change in different patterns in HDAC2(-/-) mutants during cultivation. Interestingly, the changing pattern in the gene expression of Pax5 in HDAC2(-/-) during cultivation is in anti-parallel with those in gene expressions of IgM H- and L-chains, whereas those in gene expressions of residual changed factors (such as EBF1, E2A plus Aiolos and others) were neither in parallel nor in anti-parallel by much. These results, together with others [35, 38], suggested that Pax5 preferentially controls gene expressions of IgM H- and L-chains among various transcription factors tested.

Therefore, we next studied several characteristics of three 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(-) are drastically and considerably increased at the early (8 days) stage of cultivation, and thereafter gradually decreased through the middle (13 days) until later (20 days) stages to comparable levels as in DT40 cells (Figs. 3-3 and 3-4). Concerning mRNA levels of these two immunoglobulin proteins, whole and secreted forms of IgM H-chain mRNA are dramatically elevated at the first (4 days) stage (prior to the early stage), and thereafter these accumulated two type mRNAs are rapidly reduced until the later stage to comparable levels as in DT40 cells (Fig. 3-5). The membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA are considerably increased at the first stage, and thereafter slowly decreased until the later stage to almost the same levels as in DT40 cells. Thus, the changing patterns in protein and mRNA levels of IgM H- and L-chains in Pax5(-) during cultivation are similar to those as in HDAC2(-/-) on the whole, however, the changing speeds in Pax5(-) are obviously faster than those in HDAC2(-/-) [see Chap. 2]. In addition, Pax5(-) is observed to be dispersive form at both the early and later stages, like that of DT40 cells (Suppl. Fig. 3-S1), whereas HDAC2(-/-) is seemed to be aggregative form at the early stage but dispersive form at the later stage (data will be shown in [Chap. 4]).

The mRNA levels of PCAF and HDAC9 in Pax5(-) are gradually elevated from the first to later

stages, and that of HDAC7 moderately changes during cultivation (Fig. 3-6A and Suppl. Fig. 3-S2). These changing patterns are similar to those observed in HDAC2(-/-) as a whole [see Chap. 2]. On the other hand, the changing patterns of several transcription factors in Pax5(-) are obviously different from those in HDAC2(-/-) as follows (Fig. 3-6B and Suppl. Fig. 3-S2). The mRNA levels of Aiolos and OBF1 are gradually reduced from the first to later stages to undetectable levels, and those of Ikaros, E2A and Blimp1 are drastically elevated at the first stage and thereafter gradually decreased until the later stage to almost same or slightly higher levels as in DT40 cells. The EBF1 mRNA level is completely decreased at the first stage and remains unchanged as an undetectable level throughout cultivation. The PU.1 mRNA level is remarkably reduced at the first stage and gradually elevated until the later stage: the level being slightly higher than that as 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 depending on the fact that Pax5 participates at the down-stream of HDAC2 in regulation of gene expressions of these two immunoglobulin proteins [35, 38]. In addition, both 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 specific transcription factors; Pax5, Aiolos, OBF1, Ikaros, E2A, EBF1, Blimp1, PU.1 and others, but not to alterations in gene expressions of chromatin-modifying enzymes; PCAF, HDAC7 and HDAC9 and others. As a result, Pax5(-) mutants are obviously different from HDAC2(-/-) mutants in several cellular characteristics.

METHODS

Cell cultures

DT40 and Pax5(-/-) mutant cells were grown as described [28, 35, Chap. 2]. Cell numbers were counted at indicated times to determine growth rate. Pax5(-) mutant clones were continuously cultivated and collected at indicated periods, i.e., at the first (F; 4 days), early (E; 8 days), middle (M; 13 days) and later (L; 20 days) stages of cultivation. These cultivation stages were practically counted from the first day of cultivation from the stock at -80 °C, although all positive mutant clones 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. DT40 (W) was used as a control.

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

In this study we used only the cassette carrying hyg transcribed by the chicken β-actin promoter [61], since the Pax5 gene exists on Z sex chromosome that is monosomy in chickens (USCS Genome Browser data base), although two other cassettes carrying each of hisD and neo had been prepared. Partial genomic 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 [32, 35].

We generated 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 [29] 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.

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 [29]. Southern blotting showed that all of these three transfectants carrying the hyg cassette had positive 11.5 kb fragment hybridized with probe Pax5 and completely lost the endogenous positive 7.8 kb fragment at the same time. Therefore, we regarded these mutants as the Pax5-deficient DT40 mutant cells, Pax5(-).

RT-PCR

Total RNAs were isolated from exponentially growing DT40 cells (W) and mutant cells as described [28, 35]. Mutant cells were three wild-type Pax5(+) clones (cl.1, cl.2 and cl.3) containing the endogenous Pax5 allele plus the hyg cassette, and three 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 later (L; 20 days) stages of cultivation. Total RNAs were subjected to synthesize first-strand cDNA using a kit (ReverTra Ace-α-™). The mRNA levels were measured by RT-PCR, using equal amounts of total RNAs and appropriate primers listed in our previous papers [32, 35, 62]. 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 [32, 35]. Data analyses were carried out by Multi Gauge Ver3.X software using a luminescent image analyzer LAS-1000plus (FUJIFILM). Data calibrated with an internal control in the three Pax5(-) clones are indicated as percentages of control values obtained from DT40 cells. Chicken glyceraldehydephosphate dehydrogenase (GAPDH) was used as a control.

Southern blotting

Genomic DNAs were isolated from the three Pax5(-) mutant clones, one Pax5(+) clone and DT40 cells, digested with the indicated enzyme (SacI), separated in 0.8% agarose gel, electro-transferred to

Hybond N+ membrane, and hybridized with ^{32}P-labeled probe Pax5 as described [28]. 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.

Western blotting

Western blotting was performed as described [28, 35, Chap. 2]. Whole cellular proteins were prepared from DT40 cells and Pax5(-) mutant clones (cl.46, cl.58 and cl.62) at the early, middle and later stages of cultivation. These stages were shorter than corresponding those for HDAC2(-/-) [see Chap. 2]. 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 a 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 [32, 35].

Microscopy, electron microscopy and immuno-electron microscopy

Three Pax5(-) mutant clones (cl.46, cl.58 and cl.62) at the early and later stages of cultivation and DT40 cells were exponentially growing. Microscopy was carried out in distinct ranges of vision with magnified visions as described [36, 56, 57]. Electron microscopy and immuno-electron microscopy using immuno-gold labeling were carried out as described [63, Chap. 2]. Exponentially growing DT40 and Pax5(-) clone cl.58 at the early and later 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 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 a control, primary antibody was omitted or replaced by normal goat serum.

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FIGURE LEGENDS

Figure 3-1. Genomic organization of chicken Pax5 gene and generation of Pax5-deficient DT40 mutant cells, Pax5(-)

A. Schematic presentation of the chicken Pax5 gene locus (top) with enlarged drawing of the targeted region (middle) and its targeted allele (bottom). Locations of exons are indicated by solid boxes with appropriate designations. White box indicates the 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(+) randomly integrated with the ΔPax5/hyg construct and three homozygous 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 3-2. 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 homozygous Pax5(-) clones (cl.46, cl.58 and cl.62). RT-PCR was performed using equal amounts of total RNAs and appropriate primers for whole IgM H-chain mRNA (IgM Hc), its secreted form (IgM Hs), its membrane-bound form (IgM Hm) and 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 a control.

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

Total cellular proteins were prepared from DT40 cells (W) and Pax5(-) mutant clones cl.46(-), cl.58(-) and cl.62(-) at the early (E; 8 days), middle (M; 13 days) and later (L; 20 days) cultivation stages and analyzed 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 an 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 3-4. Alterations in amounts of IgM H-chains in Pax5(-) DT40 mutant cells during continuous cultivation

Pax5(-) mutant cells (cl.58) collected at the early (E; 8 days) and later (L; 20 days) cultivation stages and from 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 of Pax5(-) (E in A). A large number of IgM H-chain proteins were observed only at the early (E) cultivation stage of Pax5(-) (E in B).

Figure 3-5. Alterations in gene expressions of IgM H- and L-chains in Pax5(-) DT40 mutant cells during continuous cultivation

Total RNAs were extracted from DT40 cells (W) and three 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 later (L; 20 days) cultivation stages. RT-PCR was performed using equal amounts of total RNAs and appropriate primers for Pax5, and 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 a 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 the mutant clones are shown in the bottom.

Figure 3-6. Alterations in gene expressions of various members of HDACs, HATs and transcription factors in Pax5(-) DT40 mutant cells during continuous cultivation

Total RNAs used were the same ones as in Fig. 3-5. RT-PCR was performed in the same way using appropriate primers for various members of HDACs and HATs (A), and transcription factors (B), instead of primers used in Fig. 3-5.

Supplementary Figure 3-S1. Morphology of Pax5(-) DT40 mutant cells during continuous cultivation

Microscopy on Pax5(-) mutant cells at the early (E) and later (L) cultivation stages and on DT40 cells (W) was performed in distinct ranges of vision with magnified visions.

Supplementary Figure 3-S2. Relative mRNA levels of changed genes in Pax5(-) DT40 mutant cells during continuous cultivation

Relative mRNA levels of changed genes in Fig. 3-6 are shown. Symbols for three Pax5(-) mutant clones are shown in the right.