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Coordinated change of a ratio of methylated H3-lysine 4 or acetylated H3 to acetylated H4 and DNA methylation is associated with tissue-specific gene expression in cloned pig

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Abbreviations: ChIP, chromatin immunoprecipitation; FISH, fluorescence *in situ* hybridization; GFP, green fluorescence protein; H, histone; NT, nuclear transfer

Abstract

Various cell types in higher multicellular organisms are genetically homogenous, but are functionally and morphologically heterogeneous due to the differential expression of genes during development, which appears to be controlled by epigenetic mechanisms. However, the exact molecular mechanisms that govern the tissue-specific gene expression are poorly understood. Here, we show that dynamic changes in histone modifications and DNA methylation in the upstream coding region of a gene containing the transcription initiation site determine the tissue-specific gene expression pattern. The tissue-specific expression of the transgene correlated with DNA demethylation at specific CpG sites as well as significant changes in histone modifications from a low ratio of methylated H3-lysine 4 or acetylated H3-lysine 9, 14 to acetylated H4 to higher ratios. Based on the programmed status of transgene silenced in cloned mammalian ear- derived fibroblasts, the transgene could be reprogrammed by change of histone modification and DNA methylation by inhibiting both histone deacetylase and DNA methylation, resulting in high expression of the transgene. These findings indicate that dynamic change of histone modification and DNA methylation is potentially important in the establishment and maintenance of tissue-specific gene expression.

Keywords: DNA methylation; DNA modification methylases; gene expression; open reading frames; organ specificity

Introduction

Various cell types in higher multicellular organisms have in essence an identical genotype. However, they are functionally and morphologically different. This is due to tissue-specific, temporal and spatial gene expression patterns which are controlled by genetic and epigenetic mechanisms. DNA methylation and histone modifications are two major epigenetic mechanisms that are crucial for tissue- specific gene expression and global gene silencing (Bird, 2002; Li, 2002). It is now generally accepted that the precise regulation of gene expression by epigenetic mechanisms is required to maintain normal development of mammals. In fact, disruption of epigenetic mechanisms which is closely linked to aberrant gene expression leads to abnormal development and, potentially, malignant transformation (Jaenisch and Bird, 2003).

Epigenetic code most likely consists of both DNA methylation and histone modifications. Although numerous studies have been carried out on DNA methylation and histone modifications in the regulation of expression of diverse genes, there still remains controversy on the role of epigenetic code in tissue-specific gene expression during normal development of mammals. The DNA methylation code appears to be a critical factor: CpG methylation near the promoter region correlates with suppression of transcription. In contrast, a variety of histone modifications including acetylation and methylation at various lysine residues and other modifications, constitutes the more complex histone code (Jenuwein and Allis, 2001). In general, acetylation of histone H3 and H4 correlates with gene activation, while deacetylation correlates with gene silencing (Fry and Peterson, 2001). Methylation of Lys9 of H3 (H3-K9) is involved in the formation of stable repressive heterochromatin (Peters *et al.*, 2001; Hall *et al.*, 2002), whereas methylation of H3-K4, -K36 and -K79 is associated with transcriptional activity. However, there are many exceptions to this fundamental rule (Briggs *et al.*, 2001; Deckert and Struhl, 2001); combinations of histone modifications at different residues within each histone molecule (Fischle *et al.*, 2003; Schneider *et al.*, 2004) as well as between histones are highly dynamic and complex (Kurdistani *et al.*, 2004; Schubeler *et al.*, 2004), and currently little is known about the importance of these modifications in higher eukaryotes. Furthermore, the processes underlying differentiation and regulation of tissue-specific gene expression remain as unresolved problems in developmental and molecular biology. Therefore, unraveling the physical basis of control processes through alterations in epigenetic modifications will ultimately result in a deeper understanding of the complex development of eukaryotic organisms. Employing cloned transgenic pigs, we investigated how a gene is programmed in a tissue-specific manner by epigenetic modifications, and whether the programmed gene can be reprogrammed by alterations of epigenetic modifications, such as inhibition of both histone deacetylase and DNA methylation, or by re-nuclear-transfer. Here, we show that dynamic changes in histone modifications between histone molecules in combination with DNA methylation determine the tissue-specific expression pattern of a transgene.

Materials and Methods

Preparation of fetal fibroblast cell line

A porcine fetus was obtained from a pregnant sow at day 35 after insemination, and the tissue was cut into small pieces with fine blades. All cells were transfected with the pCX-EGFP/neo vector (Figure 1A). The neomycin gene was isolated from pPNT vector by *Hin*dIII digestion and ligated with pCX-EGFP vector (Okabe *et al.*, 1997), which was kindly provided by Dr. Masaru Okabe, Osaka University. pCX-EGFP/neo was linearized with *Bam*HI and *Sal*I. Transfected cells were selected with 250 μg/ml of G418 (Gibco-BRL 250 μg/ml) for 14 days. GFP expression of the surviving cells was examined under an epi-fluorescent microscope using a standard fluorescein isothiocyanate (FITC) filter set. GFP-expressing cells were then frozen and used for nuclear transfer; a subset of cells was used for PCR analysis.

Nuclear transfer

Nuclear Transfer (NT) was carried out using the procedure described by Park *et al.* (2001) with a slight modification. For nuclear transfer, the cumulus-free oocytes matured *in vitro* were enucleated by aspirating the first polar body and the adjacent cytoplasm using fine glass pipette. Enucleated oocytes were stained with Hoechst 33342 to identify the nuclei using epi-fluorescent microscope. And a single donor cell was placed in the perivitelline space of the oocyte to contact the oocyte membrane. Injected oocytes were placed between two 0.2 mm diameter platinum electrodes 1 mm apart in a medium, and fusion/activation was simultaneously induced with two successive DC pulses of 1.1 kV/cm for 30 μsec using a BTX Elector-Cell Manipulator 2001 (BTX, San Diego, CA). After 6 days of culture, all embryos were stained with Hoechst 33342, and the number of nuclei was determined by epi- fluorescent microscopy. To detect GFP expression, embryos were examined by an epi-fluorescent microscope, using a standard FITC filter set. After 1 or 2 days of culture, NT embryos were surgically transferred into one oviduct of each sow (Cheil Breeding Stock Co., Ltd). Pregnancy status was monitored using an ultrasound scanner (Mysono 201, Medison Co., Korea).

Microsatellite analysis

Genomic DNA was extracted from NT piglets, donor cells, and surrogates and subjected to PCR analysis. The microsatellite analysis consisted of 4 polymorphic porcine loci consisting of different multimers of dinucleotide repeats. PCR products were concurrently analyzed on 6% polyacrylamide gels on an automatic sequencer by using Genescan and Genotyper software.

Observation of green fluorescence in piglets

GFP images were obtained using a Las 3000 (FUJI FILM, Tokyo, Japan) equipped with a highly sensitive cooled CCD camera. After the piglet GF2-4 was placed in the dark-box, gray-scale body surface images were taken. Afterwards, GFP images were taken using blue light (460 nm) for excitation and an 510DF10 filter. The piglets were dissected and various organs were removed. Specimens were also placed in the dark box, and GFP expression was captured.

Chromosome analysis and Fluorescence *in situ* Hybridization (FISH) analysis

GFP probes were labeled with Cy3, using the random prime method (Invitrogen), and purified using QIAquick PCR purification kit (Qiagen). Two microliters of probe (250 ng/μl), 2 μl of Cot-1 DNA (1 mg/ml) and 1 μl of salmon sperm DNA (1 mg/ml) were mixed with 10 μl of hybridization solution. Metaphase slides were treated in 70% formamide/2x SSC for 5 min at 75°C, followed by a series of ethanol washes (70%, 85%, 100%, 2 min each). The probe was applied to denatured pig metaphase slides after overnight hybridization under a coverslip in a humidified chamber at 37°C. The metaphases were counterstained with DAPI and examined under a DMRX2 fluorescence microscope (Leica) equipped with a CoolSNAP (Photometrics), CCD camera and an image-analysis system (QFISH, Leica). Images were captured using appropriate filters for the detection of Cy3 and DAPI. At least 20 metaphases were analyzed.

Chemical treatments

Cells were plated onto 6 well dishes 18 to 24 h prior to experiments. They were then treated with Decitabine (0.03-8 μM) or mock-treated with the same volume of PBS. Apicidin (0.1-1 μM) from a 1 mM DMSO dissolved stock was added to the medium or cells were mock-treated with an identical volume of DMSO. Decitabine (5-aza-2'-deoxycytidine, A-3656) was purchased from Sigma Chemical Co., and apicidin, cyclo(N-O-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl), was prepared from *Fusarium* sp. Strain KCTC 16677 according to the method previously described (Park *et al.*, 1999).

Immunoblotting

Cells were lysed in lysis buffer (50 mM Tris-HCL, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.15% Igepal CA-630, 1.5 mM phenylmethylsulfonyl fluoride). Equal amounts of proteins (20 to 30 μg) were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide gels, and proteins were then transferred onto nitrocellulose membranes. The antibodies used were 0.2 μg of anti-GFP (Santa Cruz, SC-8334) and 0.3 μg of β-actin (Abcam, ab8227)/ml.

Reverse transcription-PCR

Total RNA was extracted using TRIZOL reagent (Gibco-BRL). One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) using an RNA PCR kit (Perkin Elmer); 2 μl of cDNA was used for PCR analysis. A 25-cylcle PCR was used to achieve linear amplification using the following conditions: 96°C for 15 s 55°C for 30 s, and 72°C for 30 s. GFP and GAPDH were amplified by PCR using the following specific primers: (GFP for forward primer 5'-GCTGACCCTGAAGTTCATCTG-3' and reverse primer 5'-TGCTCAGGTAGTGGTTGTCG-3'; GAPDH forward primer for 5'-TGATGACATCAAGAAGGTGGTGAAG-3' and reverse primer 5'-TCCTTGGAGGCCATGTAGGCCAT-3').

Bisulfite genomic sequencing of EGFP coding regions

Genomic DNA was isolated from pig fetal fibroblasts, cloned ear cells and various tissues (kidney, liver, skeletal muscle, tongue), and purified genomic DNA (1 μg) was treated with sodium bisulfite. The GFP coding region was amplified by PCR with specific primers (5'-TTGTTTTATTATTTTGGTAAAGAATT-3' and 5'-AAATAAACTTCAAAATCAACTTACC-3'). During the reaction, cytosine was converted to uracil. The PCR product was ligated into the T-plasmid using the TA cloning system. Five to ten subclones were picked and sequenced.

Chromatin Immunoprecipitation (ChIP) assays

The ChIP assays for donor and ear fibroblast cells (Upstate Biotechnology) and for histone methylation and acetylation in various tissues (Sakamoto *et al.*, 2004) were performed essentially as described. Antibodies obtained from Upstate Biotechnology included: anti-H3 dm K4 (di-methyl lysine 4 of histone H3, catalog no. 07-030), and anti-AcH3 (acetyl lysine 9 and 14, catalog no. 06-599), and anti-AcH4 (acetyl lysine 5, 8, 12 and 16, catalog no. 06-866). After immunoprecipitation (IP), recovered chromatin fragments were subjected to 35 cycles of PCR using primers 5'-CTGTCTCATCATTTTGGCAAAGAA-3' and 5'-AGATGAACTTCAGGGTCAGCTTG-3' to amplify a 178 bp segment corresponding to the GFP coding

region, and primers 5'-GTTACTCCCACAGGTG-AGC-3' and 5'-GCCCTTTAAGGCTTTCAC-3' to amplify a 113 bp DNA fragment corresponding to the chicken β-actin promoter region (see schematic in Figure 3C). The intensity of the PCR bands was quantitated using Image Quant software. The relative intensity of PCR was calculated using the formula: (intensity of each sample/intensity of each Input)/(intensity of reference/intensity of reference Input). Results were obtained from at least three separate PCRs of three independent ChIP assays.

Real-Time PCR analysis of immunoprecipitated DNA

Differences in the DNA content from the immunoprecipitated (bound) and input fractions were determined by real-time PCR using the ABI 7700 sequence detector and the Taqman PCR Master Mix protocol (Applied Biosystems, Warrington, UK). PCR reactions were carried out in triplicate using fixed amounts of template DNA from each fraction at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The 5' to 3' sequences of the primers used to detect the presence of GFP coding region fragments were: forward 5'-CTGTCTCATCATTTTGGCAAAGAA-3' and reverse 5'-AGATGAACTTCAGGGTCAGCTTG-3'. The GFP probe sequence was 5'-ACGTAAACGGCC-ACAAGTTCAGCGT-3'. With each set of PCR, titrations of known amounts of DNA were included as a standard for quantitation. DNAs from the ChIP samples immunoprecipitated with anti-H3 dm K4, anti-AcH3, anti-AcH4 and from the ChIP samples immunoprecipitated with no antibody (negative control) were included in each PCR set. The fraction of immunoprecipitated DNA was calculated as (amount of immunoprecipitated sample with antibody - amount of negative control) / (amount of input DNA (5%) - amount of negative control).

Results

Production of transgenic cloned pigs

With an exception of lymphocytes, the various cell types in higher multicellular organisms, which are

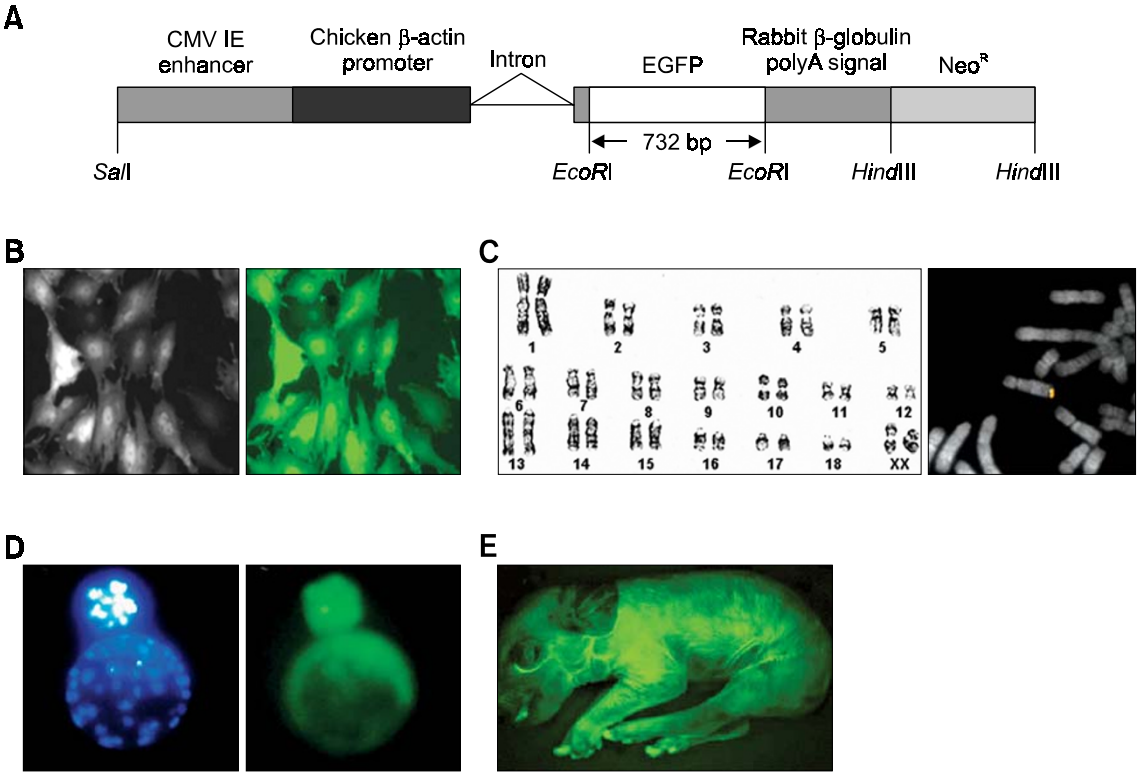

Figure 1. Cloning of Pigs with GFP as a transgene. (A) Structure of the pCX-EGFP/neo gene. (B) GFP expression in donor fetal fibroblast cells transfected with pCX-EGFP/neo vector (×200). (C) Chromosome assay and FISH of donor cells. The GFP gene integration (orange dots) is located close to the telomere of chromosome 7. (D) An NT blastocyst cultured for 6 days *in vitro*. Left, an embryo stained with Hoechst 33342 under UV light to identify the nuclei number; Right, GFP expression was examined under blue light (×200). (E) GFP expression in an NT piglet (GF2-4).

derived from totipotent fertilized oocyte, have basically an identical genotype. Nevertheless, each of these cells is functionally and morphologically different due to differences of gene expression patterns arising from specific temporal and spatial factors (Li, 2002). To understand the epigenetic regulation of tissue-specific gene expression, we employed a transgene, the green fluorescence protein (GFP) gene, which was engineered to be expressed in cloned transgenic pigs. To this end, porcine fetal fibroblast cells were transfected with the pCX-EGFP/neo vector (Figure 1A), to establish and isolate a GFP-expressing clonal cell line (PCX-8) (Figure 1B). Analysis of PCX-8 cell using fluorescence in situ hybridization (FISH) probes for the GFP gene revealed that the transgene was integrated into chromosome 7 close to the telomere (Figure 1C). Next, we carried out nuclear transfer (NT) of PCX-8 cell into enucleated oocytes and determined its *in vitro* developmental ability during 6 days of culture. As shown in Figure 1D, the percentage of blastocyst formation was 12.9%, and the mean cell number of blastocysts was 23.8 ± 2.0 (Table 1). Subsequently, the reconstructed embryos (1- or 2-cell stage) were transferred to 18 surrogate sows (Table 2), and 12 healthy female piglets were delivered from three surrogate sows. Microsatellite analysis using 4 markers revealed that all piglets were derived from the donor cells (Table 3). Furthermore, the GFP gene was found to be expressed in embryos as well as in all piglets (Figure 1E).

Table 1. Developmental ability of nuclear transfer embryos.

Reconstructed embryos	224
Blastocysts to develop	29 (12.9%)
Nuclei of blastocysts (range)	23.8 ± 2.0 (10-40)

Table 2. Embryo transfer.

Embryos transferred*	2578
Recipients	18
Pregnancy at day 50	5 (27.8%)
Term pregnancy	3 (16.7%)
Live offspring	12
% Alive from embryos transferred	0.47%

* After 1 or 2 d culture, NT embryos (1- or 2-cell stage) were surgically transferred into one oviduct of each sow.

Table 3. Microsatellite analysis of donor cells, surrogates and offspring.

Pigs	Microsatellite			
	S0097	S0301	SW902	S0005
Donor	217	253	199/201	202
#31-157(Surrogate)	217	253	199/205	202/216
GF1-1	217	253	199/201	202
GF1-2	217	253	199/201	202
GF1-3	217	253	199/201	202
#19-55(Surrogate)	205/217	253/259	201/205	216/232
GF2-1	217	253	199/201	202
GF2-2	217	253	199/201	202
GF2-3	217	253	199/201	202
GF2-4	217	253	199/201	202
GF2-5	217	253	199/201	202
#34-135(Surrogate)	205	251/253	201	202/232
GF3-1	217	253	199/201	202
GF3-2	217	253	199/201	202
GF3-3	217	253	199/201	202
GF3-4	217	253	199/201	202

12 healthy female piglets (GF1-1~3, GF2-1~5 and GF3-1~4) were delivered from three surrogate sows (#31-157, #19-55 and #34-135).

Silencing of transgene in primary ear fibroblasts of cloned pig

To find out whether the GFP gene was also expressed in ear-derived fibroblasts of cloned pig as in donor fibroblast cells, we examined the expression of the GFP gene in primary cultures of ear fibroblasts from cloned pigs by fluorescence microscopy. Interestingly, the GFP gene in ear-derived fibroblasts obtained from all of the cloned offspring was not expressed (Figure 2A). This was the case even though the GFP transgene was under the control of the β-actin promoter packaged in an active chromatin structure with highly acetylated H3 and H4 histones (Hashimshony *et al.*, 2003). In addition, all piglets carried the chromosome 7 with the transgene integration identical to that found in the metaphase spreads of the PCX-8 cell line (Figure 2C), and PCR analysis confirmed that the cells carried the GFP gene (Figure 2B). Since the GFP mRNA transcript was not detectable in the cells (Figure 4A), the absence of transgene expression was likely due to gene silencing. Furthermore, the silencing of GFP expression persisted during subcultures, indicating that the silencing was comparatively stable. These results suggest that the GFP transgene of donor cells packaged in an active chromatin structure is silenced by epigenetic regulation during the development of donor cells into ear fibroblast cells following NT.

Differential epigenetic modification of the transgene in donor cells and primary ear fibroblasts

Epigenetic programming of the genome by DNA methylation and histone modifications ensures proper gene expression during development (Jenuwein and Allis, 2001; Jones and Takai, 2001). DNA methylation is one of the best-studied epigenetic programming of DNA in all organisms and essential for the development of mammals (Kang *et al.*, 2002). Epigenetic silencing of gene expression is often correlated with extensive DNA methylation at cytosine residues in the promoter and the coding region of silenced genes (Magdinier *et al.*, 2002; Richards and Elgin, 2002). To examine how the GFP gene was silenced during the development of donor cells into ear fibroblast cells, we first investigated the status of DNA methylation and histone modifications

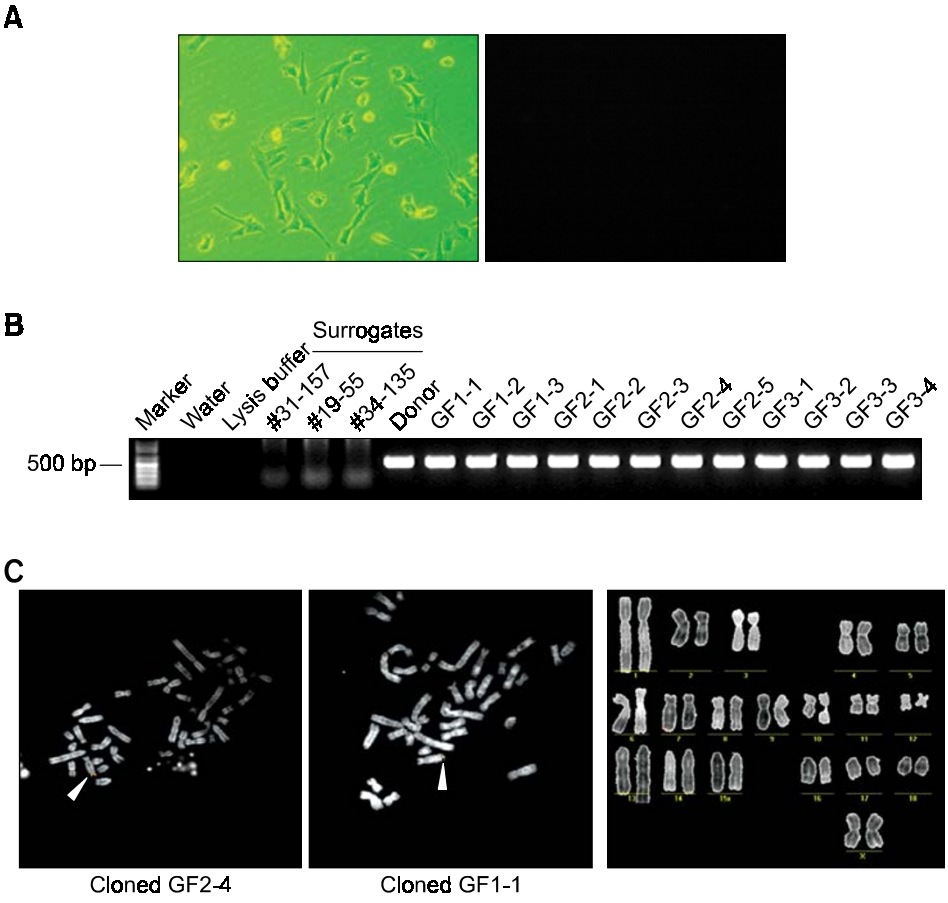

Figure 2. Transgene silencing in primary ear fibroblasts of cloned pigs. (A) GFP silencing in cultured primary ear fibroblast cells (GF2-4; ×100). (B) PCR analysis of surrogates, donor, and ear cells of offspring (GF1-1 to GF3-4). 12 healthy female piglets (GF1-1~3, GF2-1~5 and GF3-1~4) were delivered from three surrogate sows (#31-157, #19-55 and #34-135). (C) FISH analysis of ear fibroblast cells of cloned piglet. In all cloned piglets, the GFP gene integration (orange dots, arrowheads) is located close to the telomere of chromosome 7.

in the promoter region of the GFP transgene. For analysis of DNA methylation, we used the methyl-sensitive restriction enzyme *Hpa*II rather than bisulfite sequencing, because due to high CpG content in the β-actin promoter (12 CpG sites within 148 bp) and β-actin promoter-encompassing region, the MethPrimer software (Li and Dahiya, 2002), could not design specific primers for bisulfite sequencing of PCR fragments in the promoter region. As shown in Figure 3A, *Hpa*II-sensitive CpG site in the promoter region was methylated in both donor and ear fibroblast cells. In addition, the chromatin immunoprecipitation (ChIP) assay showed that the levels of H3K4 methylation and acetylation of histones H3 and H4 in the promoter region did not differ between donor and ear fibroblast cells (Figure 3B). This result indicates that differential expression of GFP in donor and ear fibroblast cells does not appear to be governed by histone modifications and DNA methylation in the promoter region of the gene. We next attempted to investigate the status of DNA methylation and histone modifications in the coding region containing the transcription initia tion site of the GFP gene. As seen in Figure 3C, sodium bisulfite mapping revealed that most CpG sites in donor cells, except 3 sites, were unmethylated, while all

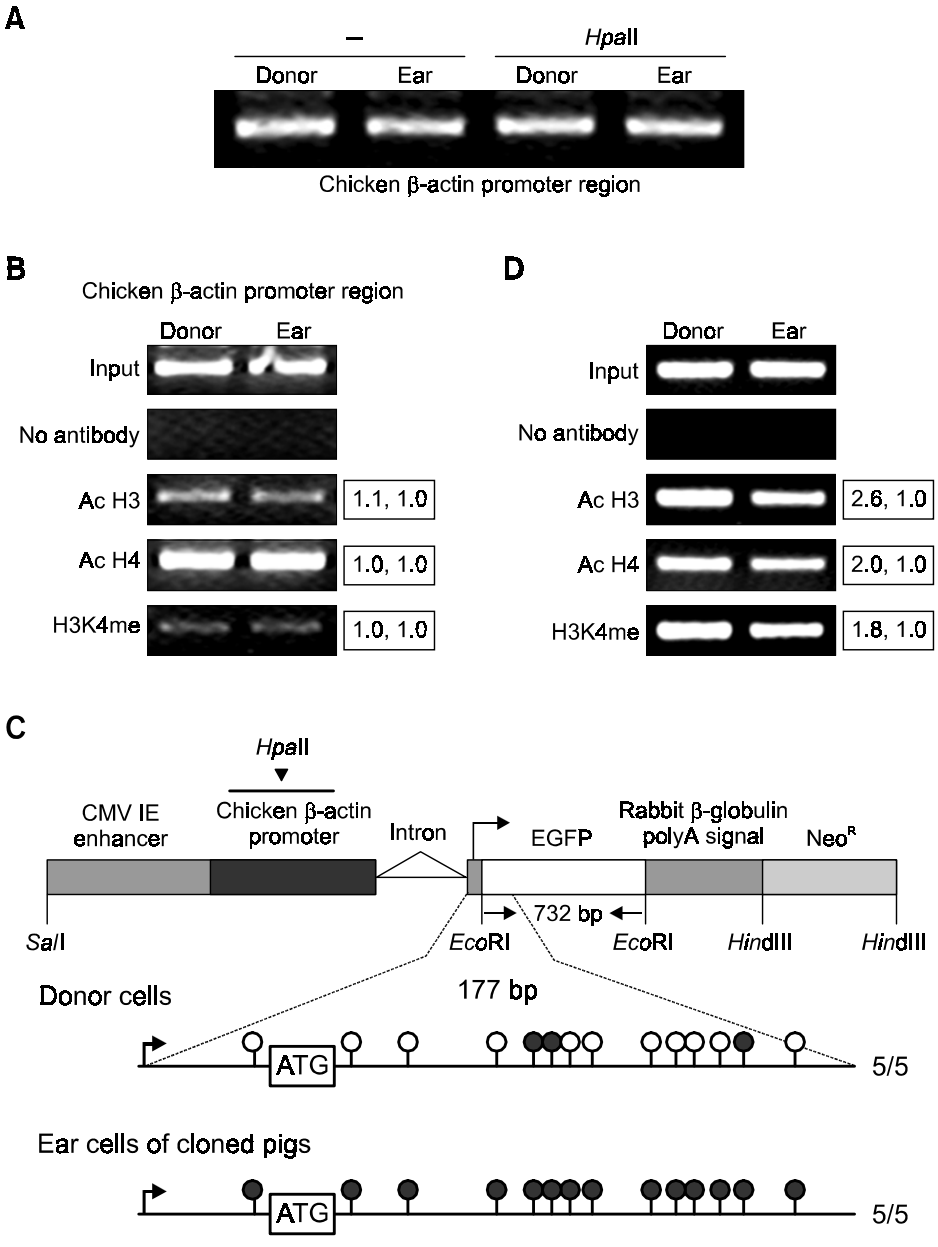

Figure 3. Differences in epigenetic modifications of transgene between donor cells and ear fibroblast (GF2-4). (A) Genomic DNA samples of donor and cloned ear cells were divided into 2 aliquots that were subjected to digestion with empty enzyme and methyl-sensitive enzyme *Hpa*II. The PCRs were carried out with 5 ng of DNA using chicken β-actin promoter primers (horizontal bar in (C)). Black arrowheads indicate *Hpa*II restriction sites in (C). (B) ChIP assays of chicken β-actin promoter region in donor and cloned ear cells. (C) The bisulfite genomic sequencing of GFP coding region. 5 clones were analyzed. The bent arrow represents the transcription initiation site. The CpG sites are represented by closed circles (methylated) or opened circles (unmethylated). (D) ChIP assays in donor and cloned ear cells.

CpG sites in ear fibroblast cells were methylated. This result is in agreement with the observation above indicating that GFP was not expressed (Figure 2A) and suggests that the hypermethylation of CpG sites in the upstream coding region of GFP gene was responsible for its silencing. In addition, H3-K4 methylation and acetylation of H3-K9/K14 and H4 in donor cells were higher than in ear fibroblast cells (Figure 3D). These results indicate that differential expression of GFP might be regulated by histone modifications and DNA methylation in the upstream coding region containing the transcription initiation site rather than in the β-actin promoter region of the gene.

Synergistic effects of DNA demethylation and histone deacetylase inhibition on expression of the transgene

Based on the above results, we next examined whether the GFP gene of ear fibroblast cells programmed to be silenced could be reprogrammed through alterations of histone modifications and DNA methylation. Thus, the ear fibroblast cells were treated with apicidin, a potent inhibitor of histone deacetylase. However, RT-PCR and fluorescence microscopy showed that apicidin (0.1- 0.5 μM) was not able to reprogram the expression of the GFP gene (Figure 4A), and further treatment with a higher dose (1 μM) of apicidin also failed to reprogram the gene. On the other hand, treatment of the cells with decitabine, a cytidine analogue that inhibits methylation by incorporating into DNA (Liang *et al.*, 2004), resulted in the expression of the GFP gene in a dose-dependent manner (Figure 4A). To further investigate the relationship between DNA methylation and histone acetylation in the reprogramming of the GFP gene in ear fibroblast cells, we induced partial demethylation of the gene in the presence or absence of the histone deacetylase inhibitor. Apicidin treatment of the cells following the addition of low dose of decitabine (120 nM), which has been shown to induce partial demethylation of CpG methylation (Cameron *et al.*, 1999), led to a synergistic increase in the expression level of GFP (Figure 4A), indicating that the GFP gene of ear fibroblast cells packaged in an inactive chromatin structure could be completely reprogrammed by a

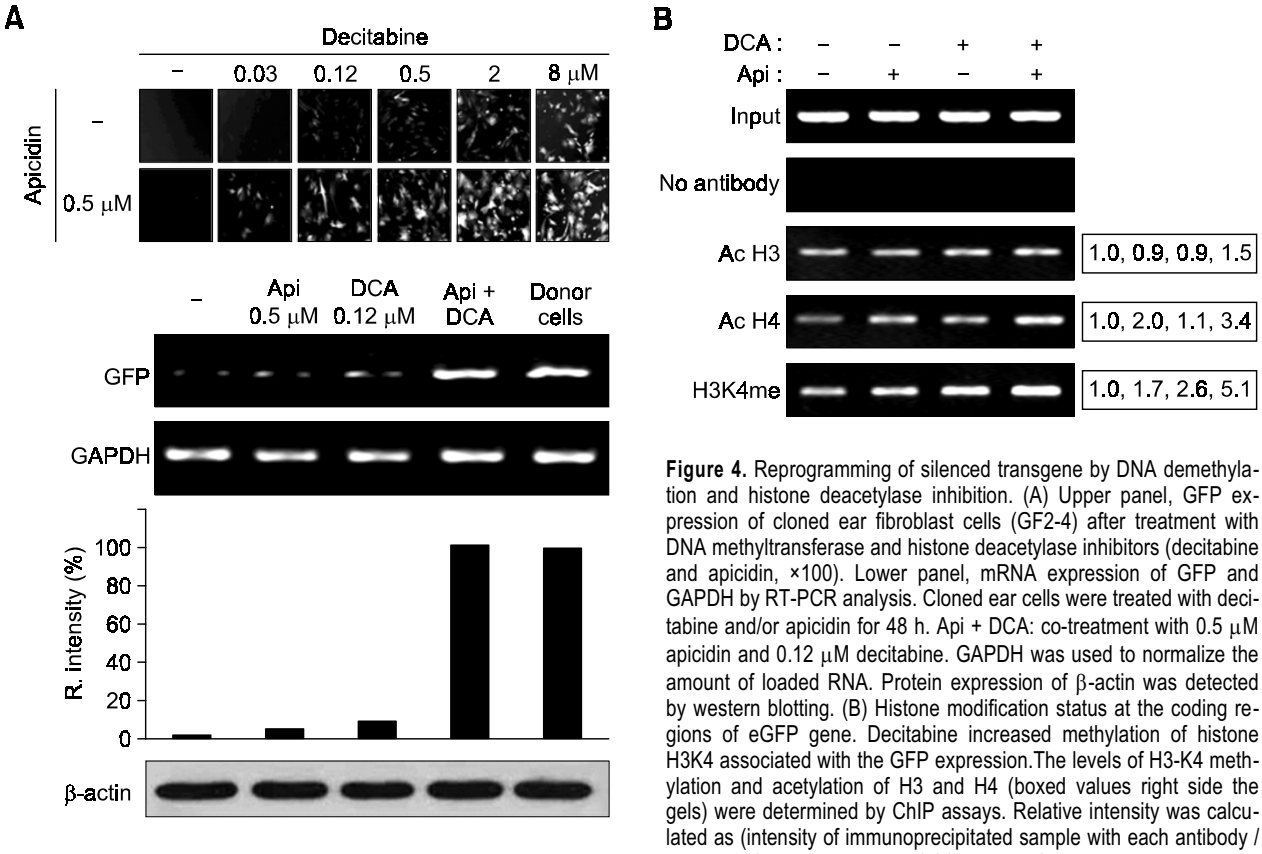

Figure 4. Reprogramming of silenced transgene by DNA demethylation and histone deacetylase inhibition. (A) Upper panel, GFP expression of cloned ear fibroblast cells (GF2-4) after treatment with DNA methyltransferase and histone deacetylase inhibitors (decitabine and apicidin, ×100). Lower panel, mRNA expression of GFP and GAPDH by RT-PCR analysis. Cloned ear cells were treated with decitabine and/or apicidin for 48 h. Api + DCA: co-treatment with 0.5 μM apicidin and 0.12 μM decitabine. GAPDH was used to normalize the amount of loaded RNA. Protein expression of β-actin was detected by western blotting. (B) Histone modification status at the coding regions of eGFP gene. Decitabine increased methylation of histone H3K4 associated with the GFP expression.The levels of H3-K4 methylation and acetylation of H3 and H4 (boxed values right side the gels) were determined by ChIP assays. Relative intensity was calculated as (intensity of immunoprecipitated sample with each antibody / intensity of each sample Input) / (intensity of immunoprecipitated control with each antibody / intensity of control Input) from three independent experiments.

combination of DNA demethylation and histone acetylation, and that DNA demethylation alone was not sufficient for complete reprogramming of the gene. As expected, endogenous β-actin was constitutively expressed in both donor cells and primary ear fibroblasts, and treatment of the cells with either decitabine or apicidin did not affect the expression of the β-actin gene (Figure 4A), indicating that expression of GFP gene might be regulated by epigenetic modifications in the upstream coding region containing the transcription initiation site.

To more closely elucidate the role of histone modification in GFP gene reprogramming, we examined the status of histone acetylation and methylation in nucleosomes associated with the coding region of the GFP gene in the presence or absence of histone deacetylase inhibition using the ChIP procedure. Figure 4B shows that apicidin treatment in the absence of DNA methylation inhibition marginally increased histone H4 acetylation, whereas apicidin-induced histone H3 and H4 acetylation was increased by about 1.5 fold upon pretreatment with decitabine, which failed to increase H3 and H4 acetylation alone. Histone H3 tails can be modified by both acetylation and methylation at specific lysine residues (Li *et al.*, 2004; Liang *et al.*, 2004). Therefore, to examine the role of methylation at H3-K4 in GFP gene expression, we carried out ChIP analysis on nucleosomes associated with the upstream coding region of GFP gene using antibodies to these methylated H3-K4 moieties. Nucleosomes associated with the coding region of the gene in ear fibroblast cells were found to be relatively depleted of methylated H3-K4, whereas they were enriched following decitabine treatment. The level of methylated H3-K4 was further increased by a combination treatment of apicidin subsequent to decitabine (Figure 4B). These results indicate that GFP gene reprogramming is closely correlated with a change of histone modifications in the upstream coding region in combination with that of DNA methylation.

Tissue-specific gene expression by epigenetic modification

An emerging consensus from recent studies is that the regulation of higher-order chromatin structures by DNA methylation and histone modifications in the promoter and the coding region of genes (Magdinier *et al.*, 2002) is crucial for tissue-specific gene expression and global gene silencing (Li, 2002). Therefore, we first compared the GFP expression patterns in 21 tissues which were separated from the GF2-4. Examination of the GFP expression patterns under fluorescence light revealed that GFP was expressed in a tissue-specific manner: GFP was expressed in the hoof, nose, heart, tongue, skin and skeletal muscle tissues, but not in the ear, liver, kidney, ovary and bone tissues (Figure 5). To examine whether tissue-specific gene expression could be attributed to the difference in the status of histone modification and DNA methylation, we first determined the state of DNA methylation in tissues which were expressing GFP and those that were not. Si-

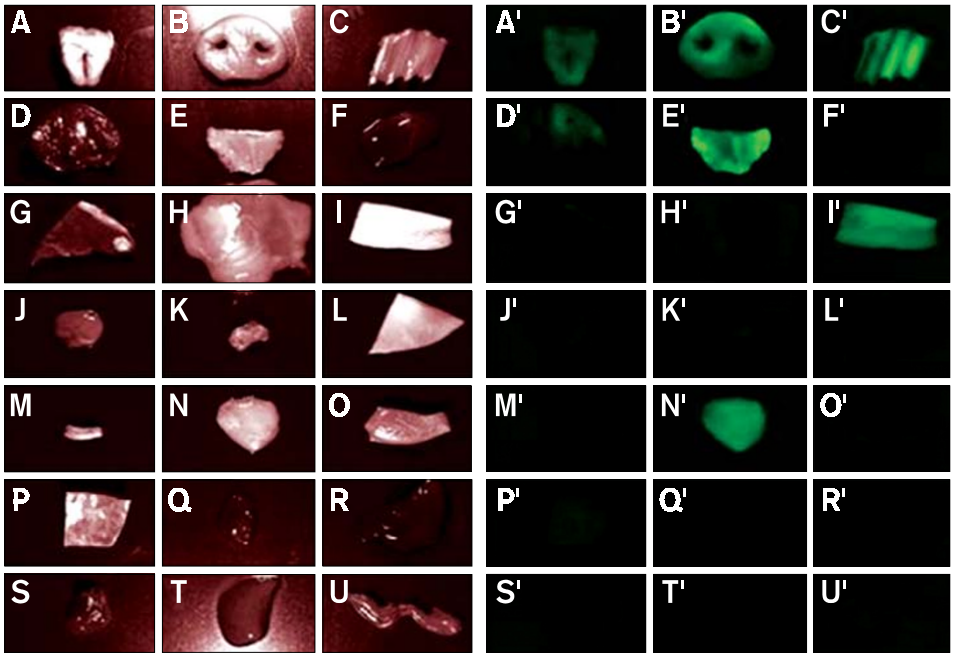

Figure 5. GFP expression patterns in the tissues of piglet (GF2-4). GFP was mainly expressed in muscle related tissues. (A-A') Hoof, (B-B') nose, (C-C') Ribs with skeletal muscle, (D-D') Heart, (E-E') Tongue, (F-F') Kidney, (G-G') Skeletal muscle (non-transgenic, negative control), (H-H') Brain, (I-I') Skin, (J-J') Thymus, (K-K') Ovary, (L-L') Ear, (M-M') Spinal cord, (N-N') Skeletal muscle, (O-O') Umbilical cord, (P-P') Frontal bone, (Q-Q') Pancreas, (R-R') Liver, (S-S') Thyroid gland, (T-T') Lung, (U-U') Small intestine. (A-U) Tissues under normal light. (A'-U') Tissues under blue light.

milar to the results obtained from the primary culture of the ear fibroblast cells, most CpG sites in the coding region of GFP gene in the kidney and liver tissues, which were not expressing GFP, were methylated. However, the DNA methylation status in the skeletal muscle and tongue tissues was different

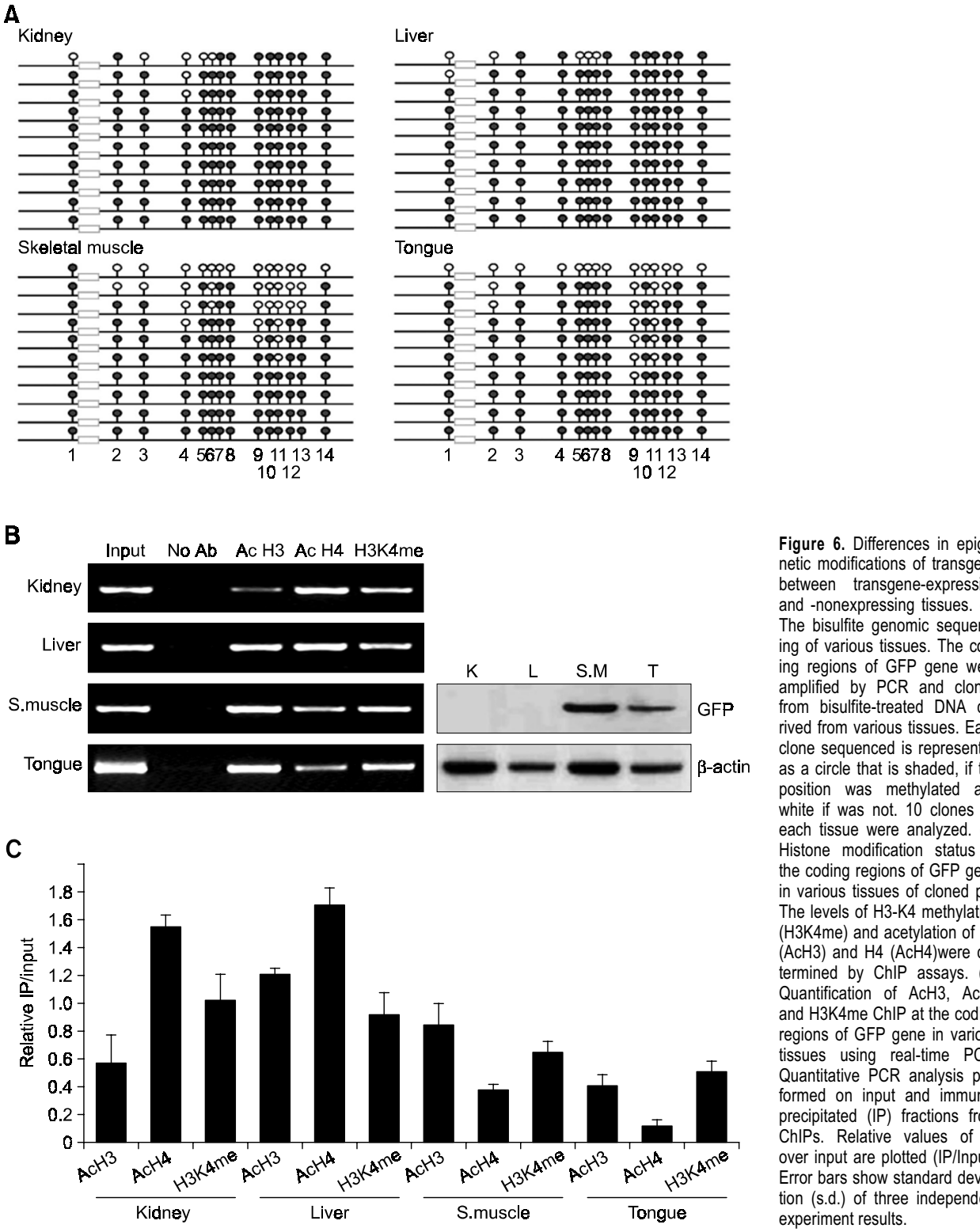

Figure 6. Differences in epigenetic modifications of transgene between transgene-expressing and -nonexpressing tissues. (A) The bisulfite genomic sequencing of various tissues. The coding regions of GFP gene were amplified by PCR and cloned from bisulfite-treated DNA derived from various tissues. Each clone sequenced is represented as a circle that is shaded, if the position was methylated and white if was not. 10 clones for each tissue were analyzed. (B) Histone modification status at the coding regions of GFP gene in various tissues of cloned pig. The levels of H3-K4 methylation (H3K4me) and acetylation of H3 (AcH3) and H4 (AcH4)were determined by ChIP assays. (C) Quantification of AcH3, AcH4 and H3K4me ChIP at the coding regions of GFP gene in various tissues using real-time PCR. Quantitative PCR analysis performed on input and immunoprecipitated (IP) fractions from ChIPs. Relative values of IP over input are plotted (IP/Input). Error bars show standard deviation (s.d.) of three independent experiment results.

from that of donor cells, although the tissues and donor cells expressed GFP. Unmethylated CpG sites in donor cells were further methylated by epigenetic programming during development (Figure 6A). GFP expression correlated with unmethylation of 9th CpG site (50% in skeletal muscle and 70% in tongue) and 11th CpG site (60% in skeletal muscle and tongue).

We then examined how the GFP gene in GFP-nonexpressing or -expressing tissues is programmed by histone modification of the nucleosomes in the upstream coding region. To this end, we determined the levels of H3-K4-methylation and acetylation of H4 and H3-K9/K14 between the tissues. However, the active markers of H3/H4 modificationsin the coding region and GFP expression did not correlate with the level of methylated H3-K4 (data not shown). Therefore, we next examined changes in the ratio of H3-K4-methylation and H3-K9/K14 acetylation over H4 acetylation in each tissue, in order to assess the relative contribution of the combination of histone modifications between histone H3 and H4 to gene activation. As seen in Figure 6B, the levels of H3-K9/K14 acetylation and H3-K4-methylation in GFP-expressing tissues were higher than that of H4 acetylation, whereas the ratios of H3-K9/K14 acetylation and H3-K4-methylation over H4 acetylation were decreased in GFP-nonexpressing tissues. To more closely quantitate the relative contribution of combination of histone modifications between histone H3 and H4 to gene activation, we employed real-time PCR. As seen in Figure 6C, the levels of H3-K9/K14 acetylation and H3-K4-methylation in GFP-expressing tissues were higher than that of H4 acetylation, whereas the ratios of H3-K9/K14 acetylation and H3-K4-methylation over H4 acetylation were decreased in GFP-nonexpressing tissues. Again, the quantification of each histone modification using a real-time PCR further confirmed the inverse correlation between the active markers of H3/H4 modifications in the coding region and GFP expression (Figure 6C): The levels of H3-K4-methylation and acetylation of H4 and H3-K9/K14 were slightly higher in GFP-nonexpressing tissues than in GFP-expressing tissues. Taken together, the results indicate that the increase of the ratios of H3-K4 Me/H4-Ac and H3-K9,14 Ac/H4-Ac is correlated with GFP gene expression.

Discussion

Using the green fluorescence protein (GFP) transgene engineered to be expressed in cloned transgenic pigs, we observed a strong correlation between tissue-specific patterns of gene expression and dynamic changes in histone modification and DNA methylation in the coding region harboring the transcription initiation site. Tissue-specific expression of the transgene coincided with DNA unmethylation at specific CpG sites and a change of histone modification from a low to high ratio of H3-K4 Me/H4-Ac or H3-K9,14 Ac/H4-Ac; however, it did not correlate with the level of H3-K4 Me.

At least 9 acetylatable lysine positions are presently known to reside in the N-termini of histone H3 (K9, K14, K18, K23 and K27) and H4 (K5, K8, K12 and K16), and 6 methylatable lysine positions in those of histone H3 (K4, K9, K27, K36 and K79) and H4 (K20) in the nucleosome associated with the promoter region of genes (Kimura *et al.*, 2004). In general, acetylation of histone H3 and H4 correlates with gene activation, while deacetylation correlates with gene silencing (Fry and Peterson, 2001). Methylation of H3-K4 also marks active chromatin, which contrasts with the modulation of inactive chromatin by methylation of H3-K9 (Lachner *et al.*, 2003). In yeast, histone H-K9/14 acetylation and H3-K4 methylation have been shown to be associated not only with the promoter regions, but also with coding regions, suggesting that these histone modifications may also play an important role in transcriptional elongation (Bernstein *et al.*, 2002; Santos-Rosa *et al.*, 2002; Ng *et al.*, 2003; Schaft *et al.*, 2003; Xiao *et al.*, 2003). However, the mechanisms by which epigenetic modification patterns in the coding region of genes in mammals are established remain unclear. In order to elucidate this, a genome-wide chromatin analysis technique, which couples the chromatin immunoprecipitation assay with the scanning capabilities of microarray, has recently been developed (Kurdistani *et al.*, 2004; Liang *et al.*, 2004). Kurdistani *et al.* (2004) compared acetylation of each lysine in the promoter and coding regions with the expression of its immediate downstream gene, and observed that hyperacetylation of histone H3K9/18/27, but hypoacetylation of H4K6 and H2BK11/16, are correlated with transcription in both regions, indicating global correlations between individual acetylation sites of the core histone in both regions and gene activity. Furthermore, methylated K4 and acetylated K9, K14 of histone H3 were highly localized in the 5' regions of transcriptionally active human genes, suggesting that these histone H3 modifications in the transcription initiation site containing coding region play an important role as active chromatin markers in gene expression (Liang *et al.*, 2004). This notion supports our observations that tissue-specific expression of the transgene was regulated by modifications of histone H3 and H4 in the nucleosome associated with the upstream coding region which contained the transcription initiation

site. The question remains whether endogenous genes of normal pigs behave in an epigenetic modification manner similar to that of the transgene of cloned pigs in this study. Futscher *et al.* (2002) provide evidence that cytosine methylation of the maspin gene (SERPINB5) promoter controls normal cell type-specific SERPINB5 expression. Further studies would be required.

There has been an attempt to decipher the histone code in the promoter region by analyzing the combination of histone modifications at different residues within each histone protein. However, decryption of histone code has not yet been perfected. There are exceptions to the basic rule of the histone code which is generally accepted (Briggs *et al.*, 2001; Deckert and Struhl, 2001). In fact, H3-K4 methylation, a general mark of gene activation, has been reported to be required for rDNA silencing in *Saccharomyces cerevisiae* (Briggs *et al.*, 2001). In addition, Gal4-dependent gene activation in yeast has been demonstrated to be associated with a dramatic decrease of histone H4 acetylation without alteration of histone H3 acetylation at promoters of genes regulated by the Gal4 activator protein (Deckert and Struhl, 2001). Moreover, a recent study, which determined the relation of each acetylation site to transcription by using genome-wide chromatin analysis, found a negative correlation between the acetylation of histone H4-K8 and -K16 in the coding region of genes and increased transcription (Kurdistani *et al.*, 2004). Indeed, contrary to the simple rule of the histone code, we also observed in the present study that the levels of H3-K4 methylation and acetylation of H4 and H3-K9/K14 in the coding region containing transcription initiation site were not correlated with transcription of the transgene. Therefore, reading the combination of various modifications of different histones appears to be important in determining the role of histone modification in gene expression. Indeed, an increase of the ratios of H3-K4 Me/H4-Ac and H3-K9,14 Ac/H4-Ac has been shown to correlate with GFP gene expression: In addition to histone code on individual histones, combination of various modifications of histone H3 and H4 should be considered in order to gain a more complete interpretation of histone code.

In summary, our findings provide evidence of an essential role of dynamic changes of histone modification and DNA methylation in the upstream coding regions, which contains transcription initiation site, in the establishment of tissue-specific gene expression during development and maintenance. In addition, the ratio of various modifications of histone proteins in each tissue might help interpreting the histone code for tissue-specific gene expression, which is under intense investigation. A better understanding of epigenetic molecular mechanisms for tissue-specificity is expected to provide crucial clues for therapeutic strategies of diseases, resulting from dysregulation of normal epigenetic control, such as numerous developmental disorders (Jaenisch and Bird, 2003) and cancers (Egger *et al.*, 2004) in human, and also improve the inefficiency of cloning animals from somatic cells, arising from incomplete reprogramming of the transplanted somatic cell nucleus (Solter, 2000).

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