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TRANSGENE INSERTION AFFECTS TRANSCRIPTION AND EPIGENETIC MODIFICATION OF FLANKING HOST SEQUENCE IN TRANSGENIC PIGS

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Abstract

Transgenic technology has been used for years to study gene function, produce important proteins, and generate models for the study of human diseases. However, the efficiency of producing transgenic animal lines that retain normal function is extremely low. The low efficiency can be mainly attributed to the integrated transgene. A further understanding of the effects of transgene integration on transcription and epigenetic modification of the host genome would improve the transgenic efficiency. Therefore, we utilized three transgenic pigs produced by SCNT expressing GFP, to identify alterations of transcription, DNA methylation and histone acetylation resulting from integration of the GFP gene. Multiple copies of the transgene integrated into a single site of the three transgenic pigs were verified by TAIL-PCR and the integration sites were different in each pig. We observed that the integrated transgene frequently resulted in significantly low transcription of flanking sequences in various tissues of transgenic pigs in comparison with wild-type pigs. Corresponding with the low transcription, DNA hypermethylation and loss of acetylation of histone H3 and H4 were detected. Our results demonstrate that the abnormal transcription and epigenetic modification of sequences flanking the transgene were not correlated with the expression of the transgene. However, the disturbance caused by the insertion of the transgene, was dependent upon the integration site. This suggests that some sequences in the host genome could permit integration and expression of transgene without causing defects in the host.

Key words: Transgene disturbance, DNA methylation, histone acetylation, transgenic pig.

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Abbreviations: **SCNT**: somatic cell nuclear transfer; **NT**: nuclear transfer; **GFP**: green fluorescent protein; **TAIL-PCR**: thermal asymmetric interlaced PCR.

INTRODUCTION

Animal genomes allow insertion of foreign DNA both naturally and experimentally. Many DNA and RNA viruses are able to naturally integrate their genomes into a host (21). Under experimental conditions, transgenic technology has been used for years to study gene function, produce important proteins, and generate models for the study of human diseases by various transfection, microinjection and NT protocols (6). However, the efficiency of producing transgenic cell and animal lines with normal function is extremely low (11). This can be attributed to the genome wide disturbance of transcription and methylation (17, 20). The integrated transgene can cause disruption of global transcription by inducing chromatin remodeling as a mechanism of immune-mediated protection (1, 19).

Chromatin remodeling is thought to involve epigenetic modifications, especially DNA methylation and histone acetylation. DNA methylation is important for the maintenance of

genome stability. Transcriptionally repressive chromatin states are established through methylation of CpG dinucleoitides that are clustered to form CpG islands. Therefore, the appropriate patterns of DNA methylation in the genome is essential for normal development and gene expression (7). Following the stable integration of a transgene into host genome, *de novo* methylation of the transgene and alteration of the DNA methylation pattern in flanking and remote sequences have been observed, suggesting more general changes in chromatin structure (27). It is also well established that acetylation of histones H3 and H4 is associated with chromatin remodeling and gene expression. Hypoacetylation of histone H3 and H4 leads to chromatin condensation as a result of interactions between the free lysine residues, leading to suppression of gene expression (15). Therefore, alterations of the DNA methylation and histone acetylation patterns can serve as reliable indicators of structural rearrangements in the host genome.

A more clear understanding of the effects of transgene integration on transcription and epigenetic modification would lead to improvement in transgenic efficiency. Furthermore, there has been no previous report demonstrating the influence of transgene integration on the pattern of histone acetylation in the host genome. In the study, three transgenic pigs produced by SCNT expressing GFP driven by the CMV promoter were used to examine changes in transcription, DNA methylation and acetylation of histone H3 and H4.

MATERIALS AND METHODS

Establishment of transgenic pigs

Plasmid pEGFP-C1 (Clontech) containing eGFP driven by the CMV promoter was introduced to fibroblast cells derived from E32 fetuses by liposome-mediated transfection. Integration was based on a random insertion of the nonhomologous DNA vector into the host genome. After G418 selection, surviving cells were used as nuclear donors, and nuclear transfer was preformed as described. Positive transgenic pigs were identified by PCR and Southern blot (13, 16).

Integration site analysis

TAIL-PCR, which relies on a series of PCR reactions with transgene specific and degenerate primers, was used for amplifying the integration site of the transgene as described (18). Junction PCR combined with transgene specific and integration site primers was performed to confirm the integration sites. The sequences of primers and detailed methods were described in the previous report (14).

Q-PCR analysis of flanking sequence transcription

Total RNAs were extracted from each sample using the PureLink™ Micro-to-Midi system (Invitrogen), and PrimeScript™ RT Reagent Kit (TaKaRa) was used to generate cDNAs. Real-time PCR was performed using SYBR Premix Ex Taq™ (TaKaRa) and the 7500 Real-Time PCR System (Applied Biosystems) with standard parameters. Primers amplifying the three flanking sequences of the transgene were 5' -ATGTTATGGGTACTTTGATAGGG-3' (forward) and 5'-CAATCAAGGAAAATAAAGAGGATTC-3' (reverse), 5'-TTGATTTTGGTGCCAAAACAAACTC-3' (forward) and 5'-CATGGTGATGCGGTTTAGGCAGAAC-3' (reverse), and 5'-GGGGATAACGCAGGAAAGAACAT-3' (forward) and 5'-GGGTTTCGCCACCTCTGACTTGA-3' (reverse). The sizes of the amplification products were 144bp, 118bp and 149bp, respectively. The β-actin gene was used as a reference and the primer sequences were 5'-AGATCGTGCGGGACATCAAG-3' (forward) and 5'-GCGGCAGTGGCCATCTC-3' (reverse), producing a 93bp product. For each cDNA sample, both target and reference genes were always amplified independently in triplicate on the same plate and in the same experimental run. A melting curve analysis showed that all reactions were free of primer–dimers or other non-specific products (data not shown). C_t values were calculated by the Sequence Detection System software (Applied Biosystems), and the amount of target sequence normalized to the reference sequence was calculated as $2^{-\triangle\triangle Ct}$.

Bisulfite sequencing analysis of flanking sequence methylation

Bisulfite modification was performed on 0.3ug of DNA from each sample using the EZ DNA Methylation-Gold™ Kit (Zymo research). PCR primers to amplify the flanking sequences of the transgene were designed by MethPrimer software available on line (http://www.urogene.org/ methprimer/). This software was also used to predict CpG islands and CpG sites in the sequence. Because there are no predicted CpG islands in the integration site of K25-2, we could only examine the methylation status of the flanking sequences in K28-1 and K25-3. A 286bp sequence in K28-1 containing one CpG island with 25 CpG sites was amplified using the primer pair psK28-1: 5'AAGTGTAAAGTTTGGGGTGTTTAAT3' (forward) and 5'ACCTTTTACTCACATATTCTTTCC TAC3' (reverse). A 173bp sequence in K25-3 containing a CpG island with eight CpG sites was amplified using the primer pair psK25-3: (forward) 5'AGTTTTTGAAGTGGTGGTTTAATTA3' and 5'AAAAAATCTTCTTAAAATCCTTTTT3' (reverse). The amplification of bisulfited-modified DNA was performed using Hot Start Taq™ polymerase (TaKaRa) with the following conditions: 94 °C for 5 min, followed by 40 three steps cycles at 94 °C for 30 sec, 50 °C for 30 sec and at 72 °C for 1 min. The PCR products were purified and sequenced (Invitrogen). The presence of a cytosine residue after bisulfite treatment shows that the cytosine residue was protected from bisulfite modification by methylation. For each DNA sample, the number of protected cytosine residues was counted and expressed as a percentage of the CpG cytosines present in the regions that were analyzed. As a control, the DNA sample from a wild type pig was treated and analyzed. At least ten clones were sequenced and analyzed for each sample.

Chromatin immunoprecipitation analysis of histone acetylation in flanking sequences

Tissue ChIP assays was carried out using the Acetyl-Histone Immunoprecipitation (CHIP) Assay Kit (Millipore) with the following specific details. Cells were harvested and mixed with formaldehyde at a final concentration of 1.0% for 10 min at 37℃ to cross-link protein to DNA. Cells were rinsed with cold PBS containing 1 mM PMSF (Amresco) and resuspended in SDS lysis buffer. Lysates were sonicated using an Ultrasonic Homogenizer 4710 series to shear DNA into 200–1000 bp fragments. Protein-DNA complexes were immunoprecipitated using antibodies to acetyl-H3/H4 (Millipore). Five microliters of each specific antibody were added to lysates and incubated overnight at 4℃. To reduce nonspecific background, Immunocomplexes were blocked with a salmon sperm DNA/Protein A Agarose Slurry by incubating for one hour at 4℃ before chromatin immunoprecipitation. Beads were washed once with each of the following: low salt buffer, high salt buffer, LiCl buffer, and twice with TE buffer. Immunocomplexes were eluted by incubating beads at room temperature for 15 minutes in 250ul elution buffer (0.1 M $NaHCO_3$, 1% SDS), and the cross-links were reversed by incubating at 65℃ for 4 hours. After incubation with 0.2 mg/ml RNase A at 37℃ for 2 hours and 0.2 mg/ml Proteinase K at 45℃ for 1 hour, DNA was purified using TOPure™ Gel/PCR Purification Kit (Gene Tech). Q-PCR was used to analyze the immunoprecipitated DNA as described above. Primers used for both the three transgene flanking sequences and the β-actin gene (control) were the same as those described above.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for MicroSoft™ Windows. Data are shown as mean ± SD. One-way ANOVA was used to assess differences between groups. The Duncan method was employed for pairwise comparison, followed by Bonferroni correction. $P < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

Generation of transgenic pigs

A total of 1978 reconstructed embryos were transferred to ten recipients, and four founder GFP-positive transgenic pigs were born at full term. Two transgenic pigs, named K28-1 and K25-1, died at birth, and the other two, named K25-2 and K25-3, survived to adults, but K25-3 was dead during delivery. In this study, three transgenic pigs, K28-1, K25-2 and K25-3, were examined.

Expression of the transgene

Expression of GFP mRNA was determined by Q-PCR analysis in various tissues studied for K25-2 and K25-3. Intestine, ovary, lung, liver, tongue, kidney, heart, spleen, stomach, brain and ear were examined (Figure 1A). In K25-2, mRNA levels were significantly higher in liver, heart and stomach compared to ovary, tongue, spleen and brain ($p<0.001$). In K25-3, significantly higher levels of mRNA expression were detected in the liver, kidney, brain, spleen and ear compared to the intestine, ovary and lung ($p<0.001$). Expression of GFP mRNA levels was also examined in the ear of K28-1 (Figure 1B).

Integration site of the transgene

The integration sites were cloned and determined by TAIL-PCR and junction PCR. Three integration sites, named TgInS1 (1440bp), TgInS2 (1263bp) and TgInS3 (1861bp), were detected, respectively in K28-1, K25-2 and K25-3 by conducting BLAST searches of the pig genome and EST database, and junction PCR confirmed the integration sites (14). Moreover, multiple copies of the transgene were detected by absolute quantitative real-time PCR, 23.64, 18.87, 30.58 copies were respectively in K28-1, K25-2 and K25-3 (13). The integration site in K28-1 significantly matched CV877820 in the pig EST database (98%) and it is homologous with *Embigin*. This gene is mainly expressed

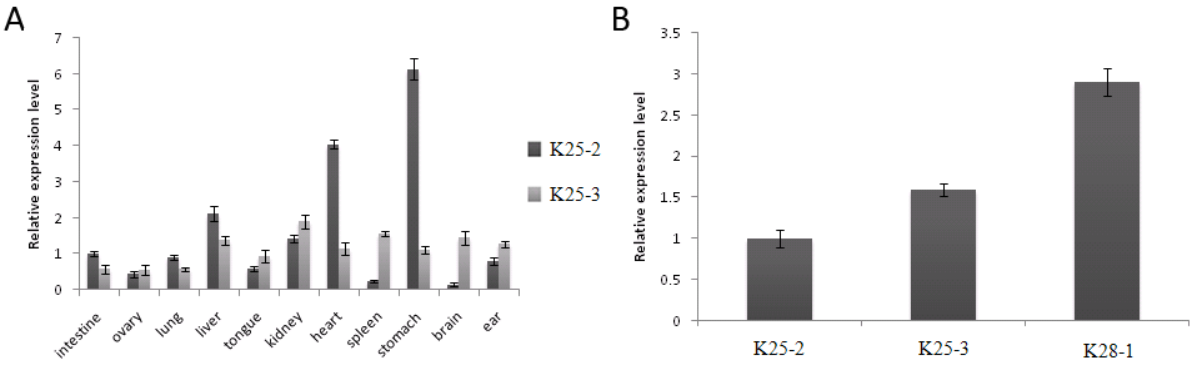

Figure 1. Expression of GFP in various tissues of transgenic pigs. GFP mRNA expression levels were detected by Q-PCR analysis in various tissues of K25-2 and K25-3 (A), and in ear of K28-1 (B). Significant differences of GFP mRNA expression were observed in these tissues of K25-2 ($p<0.001$) and K25-3 ($p=0.016$), and in ear of the three transgenic pigs ($p<0.001$). Error bars denote standard deviations.

during embryogenesis in endoderm, future brain, yolk sac and so on. It is also expressed in pancreas and epithelium (26). In K25-2, the integration site was within an L1M LINE element that is known to be involved with retrotransferase expression. Due to the repetitive nature of these sequences, the L1M LINE element could be transcribed at an extremely low level, so it is not an active element (25). The integration site in K25-3 significantly matched the pig EST EV932945 (99%), which is homologous with *Aatf*. This gene is expressed in oocytes, embryos, ear, brain, liver and so on (2).

Transcription of sequences flanking the transgene

It has been demonstrated that an integrated transgene primarily influences transcription of upstream sequences (17). Therefore, we examined the transcription of the 5' upstream flanking sequence by Q-PCR in the three transgenic pigs. In K25-2 and K25-3, the transcription of 5' upstream flanking sequence in various tissues including intestine, ovary, lung, liver, tongue, kidney, heart, spleen, stomach, brain and ear, were detected. In K25-2, alterations of transcription of the sequence flanking the transgene were restricted to ear and intestine where transcription was significantly high compared to WT ($p<0.001$). There was no significantly abnormal transcription observed in other tissues (Figure 2A). However, in K25-3, significantly lower levels of transcription were detected in various tissues ($p<0.001$) (Figure 2B). Transcription of the 5' upstream flanking sequence in the ear of K28-1 was also tested and found to be significantly low in comparison with WT ($p<0.001$) (Figure 2C). Transcription patterns and epigenetic modifications are functionally related and the abnormal transcription could be mainly due to the spreading of epigenetic modifications marking on the sequences (8). Therefore, we analyzed DNA methylation and histone acetylation of these sequences in the transgenic pigs.

Methylation of sequences flanking the transgene

DNA methylation is involved with genome stability and can regulate gene expression (4, 23). Therefore, the methylation status of a 286bp and 173bp region of the sequences flanking the transgene in K28-1 and K25-3 were examined. These regions contain one CpG island and have 25 and 8 CpG sites, respectively. There are no CpG islands predicted in the transgene flanking sequence of K25-2. The bisulfite sequencing method employed can reveal the methylation status of all the cytosine residues in a DNA region of interest (9).

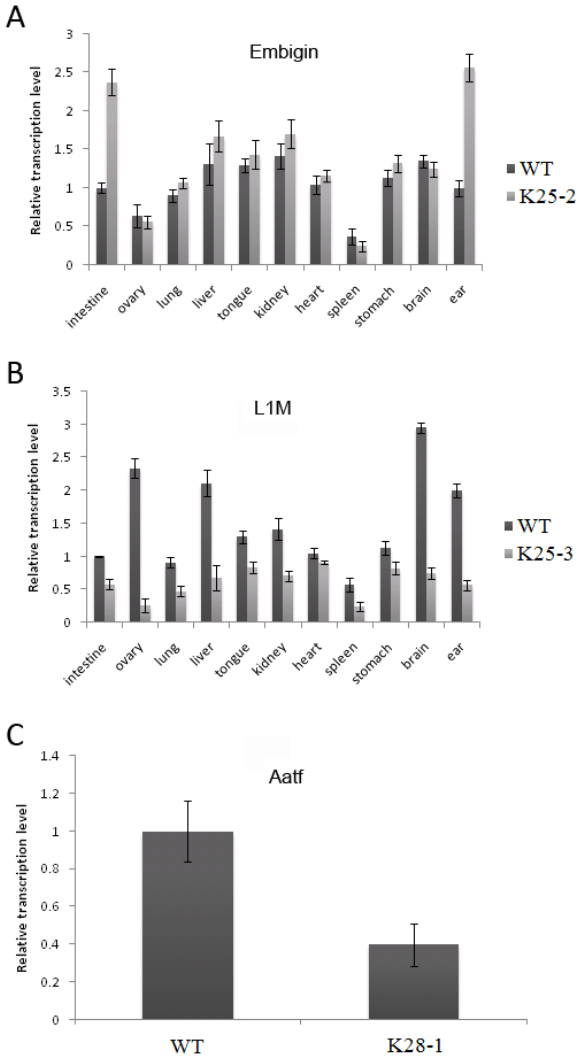

Figure 2. Transcription of sequences flanking of the transgene in various tissues of transgenic pigs. The transcription of the 5' upstream flanking sequence was detected by Q-PCR analysis and compared to wild-type (WT) in various tissues of K25-2 (A), K25-3 (B) and in ear of K28-1 (C). In K25-2, abnormal transcription of the flanking sequence in ear and intestine had a significantly higher level of transcription compared to WT ($p<0.001$) and there was no significantly abnormal transcription observed in other tissues. However, in various tissues of K25-3 and ear of K28-1, significantly lower transcription was observed in comparison with WT ($p<0.001$). Error bars denote standard deviations.

The methylation status of the 5' upstream flanking sequence in various tissues of K25-3 was detected and compared with WT (Figure 3A). The increases in DNA methylation levels were apparent. Specifically, in ovary, the sequence was converted from an unmethylated status (0%) to an absolutely methylated status (100%). Except in stomach ($p=0.057$), the

differences in DNA methylation were significant in the other tissues examined. Similar to our observations in K25-3, methylation of the 5' upstream flanking sequence in the ear of K28-1 (Figure 3B) was significantly increased in comparison with WT (p=0.046). We conclude that the sequences flanking the transgene were converted from a hypomethylated to a hypermethylated state, suggesting a mechanism of methylation-mediated protection to maintain the stability of the host genome (4).

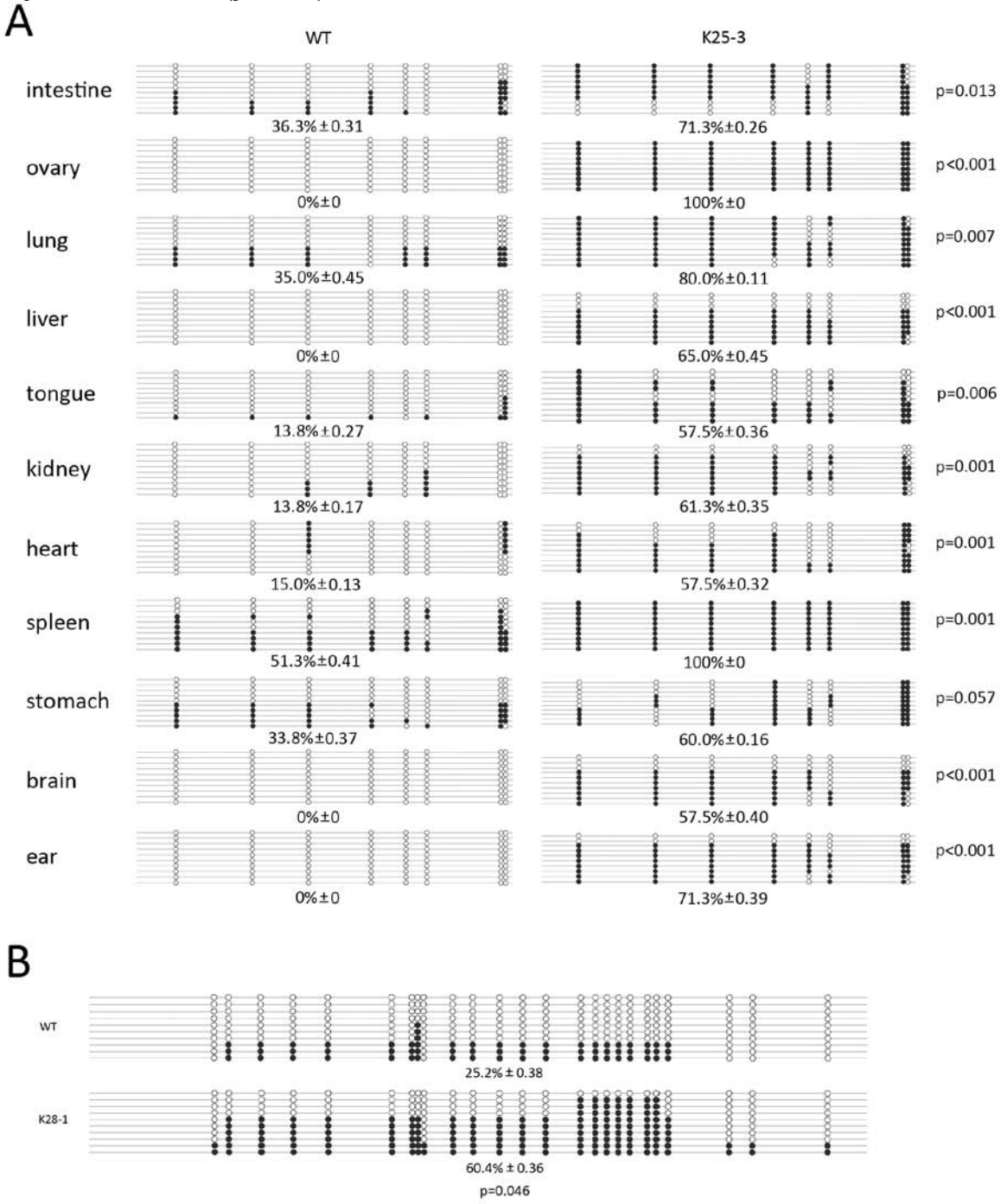

Figure 3. Methylation of sequence flanking the transgene in various tissues of transgenic pigs. The methylation status of the flanking sequence in various tissues of K25-3 and ear of K28-1 was detected by the bisulfite sequencing method. Methylated and non-methylated CpG dinucleotides of each clone are illustrated with closed and open circles, respectively. (A) The methylation status of a 173bp region of the sequence flanking the transgene in K25-3 containing one CpG island with 8 CpG sites was examined and compared to a wild-type pig (WT). The increases in DNA methylation levels were apparent, and specifically in ovary, the sequence was converted from an unmethylated status (0%) to an absolutely methylated status (100%). With the exception of stomach (p=0.057), the differences were significant in other tissues. (B) The methylation status of a 286bp region of the transgene flanking sequences in K28-1 containing one CpG island with 25 CpG sites was examined and compared to wild-type pig (WT). The methylation status of the 5' upstream flanking sequence in ear of K28-1 was significantly increased in comparison with WT (p=0.046).

Histone acetylation at sequences flanking the transgene

In general, histone acetylation plays an important role in the regulation of chromatin remodeling and gene expression (24, 28). The enrichment of acetyl-H3/H4 suggests a functional role in maintenance of transcriptionally permissive DNA domains and direct regulation of gene transcription and was examined in the sequences flanking the transgene by chromatin immunoprecipitation (ChIP) analysis.

Levels of histone H3 and H4 acetylation in the 5' upstream flanking sequences relative to acetylation levels in the active β-actin gene were measured in various tissues of transgenic pigs (Figure 4). The level of acetyl-H3 enrichment was significantly high in ear and intestine of K25-2 compared to WT ($p<0.001$) (Figure 4A), and abnormal H4 acetylation at the flanking sequence in various tissues of K25-2 was detected in intestine, lung, spleen and ear ($p<0.001$) (Figure 4A'). Moreover, there was no significantly abnormal histone acetylation observed in other tissues of K25-2. In contrast, histone H3 and H4 acetylation at the flanking sequence in most tissues of K25-3 was significantly low in comparison with WT ($p<0.001$) with the exception of H4 acetylation in heart (Figure 4B'). Specifically, the level of H3 acetylation was extremely low (Fig. 4B). Similarly, modification of H3 and H4 acetylation at the 5' upstream flanking sequence in the ear of K28-1 was significantly decreased compared to WT ($p<0.001$) (Figure 4C and C'). The results demonstrate that transcription, DNA methylation and histone acetylation were disrupted in sequences flanking the transgenic insertion.

DISCUSSION

Insertion of foreign DNA into established animal genomes is considered to be a frequent event. Virus infection, microinjection and transfection can result in the insertion of foreign DNA into a host genome. In most cases, multiple copies of foreign DNA arrayed in a head-to-tail manner and comprising a total sequence of up to one megabases or more are randomly inserted into the recipient genome (3). The acquisition of megabases of foreign DNA can lead to perturbations of transcription and epigenetic modifications in the host genome. However, it is still unclear whether the perturbations are due to the products or the insertion of foreign DNA. Therefore, we sought to investigate these changes of sequences flanking a GFP transgene integrated at different sites in three transgenic pigs.

We observed alterations in transcription, DNA methylation and histone acetylation of sequences flanking the transgene in the three transgenic pigs. Our findings are consistent with previous observations of genome-wide alterations in transcription and DNA methylation in transgenic cells (5, 10, 12). In the study, we further characterized the alterations in various tissues of transgenic animals. Specifically, we detected markedly low levels of transcription corresponding to DNA hypermethylation and histone hypoacetylation in various tissues of K25-3 and in the ear of K28-1. This is consistent with the hypothesis that a host genome can maintain its stability by inducing epigenetic modification of transgene in order to inhibit its expression. Furthermore, the epigenetic modifications that mark transgene can spread to neighboring sequences leading to disruption. We propose that these disturbances may have result in defects in the host, leading to the death of K28-1 at birth and K25-3 during delivery.

However, in K25-2, the alterations were not found in most tissues. This observation could be attributed to the fact that the site of integration in K25-2 was an L1M LINE element. L1M LINE elements are repetitive in nature and probably integrated into mammalian genomes several million years ago (21). This sequence formed a heterochromatic structure and lost its activity during evolution. This may be the reason why the integrated transgene did not significantly induce the perturbations at the integration site. We demonstrate that the effect of transgene integration on transcription and epigenetic modification in the recipient genome is integration site dependent. Different sequences in host genome have specific structures and functions that may allow or prevent integration and expression of transgene. Therefore, some sequences in the host genome could permit integration and expression of transgene without causing host defect.

Transgene expression as a result of transcription perturbation in a host genome has been previously reviewed (30). Also, the products of transgene may contribute to alteration of host genome transcription and epigenetic modification (29). However, in the study, a high level of transgene expression in these tissues did not significantly correspond with disturbances of transcription or epigenetic modification, or vice verse. Thus, this possibility is unlikely. Due to multiple copies of transgene integrated, it is

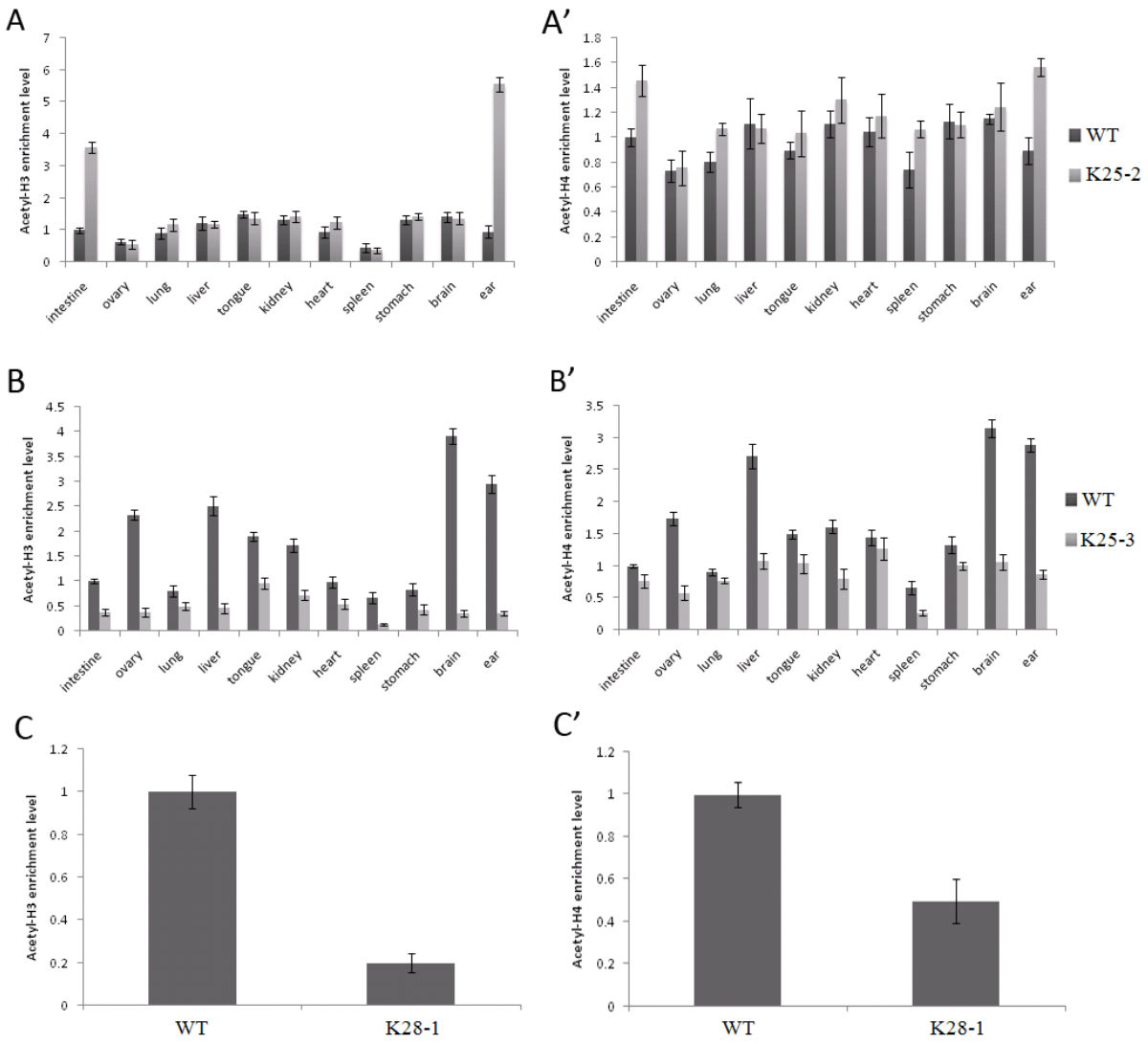

Figure 4. Histone acetylation at sequence flanking the transgene in various tissues of transgenic pigs. ChIP analysis measuring acetylated histone H3 and H4 in various tissues of K25-2, K25-3 and K28-1. In K25-2, the level of acetyl-H3 enrichment was significantly high in ear and intestine of K25-2 compared to WT ($p<0.001$) (A). The abnormal H4 acetylation at the flanking sequence was detected in intestine, lung, spleen and ear of K25-2 with a significantly higher level ($p<0.001$). Moreover, there was no significantly abnormal histone acetylation observed in other tissues of K25-2 (A'). The histone acetylation at the flanking sequence in most tissues of K25-3 was significantly low in comparison with WT ($p<0.001$), with the exception of H4 acetylation in heart (B'). Specifically, H3 acetylation was observed at an extremely low level compared to WT (B). H3 (C) and H4 (C') acetylation at the 5' upstream flanking sequence of the transgene in ear of K28-1 were significantly decreased compared to WT ($p<0.001$). Immunoprecipitated DNA levels were quantified by Q-PCR relative to the modification levels measured at the active β-actin gene. Error bars denote standard deviations.

conceivable that severe structural perturbations may lead to the alterations.

In the study, we described the alterations in transcription, DNA methylation and acetylation of histone H3 and H4 resulting from transgene integration. We characterized these changes *in vivo* and analyzed the reasons for the alterations. In summary, our results provide a clear demonstration that the abnormal transcription and epigenetic modification of sequences flanking the transgene do not correlate with the expression of transgene, but caused by insertion of the transgene and the disruption is dependent upon the integration site, suggesting that some sequences in a host genome could permit transgene integration and expression without causing defects in the host.

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