Age-dependent Alteration of Transgene Expression and Cytomegalovirus Promoter Methylation in Transgenic Cloned and Recloned Dogs

The production of transgenic animals through a combination of genetic engineering and somatic cell nuclear transfer (SCNT) has increased, yet limitations in the use of these clonal populations continues to constrain the application of these technologies (Kishigami et al., 2008). In addition, the mosaic pattern of transgene integration and the varied copy number of transgenes integrated at different chromosome locations both disturb the homogeneity among transgenic animals and can increase the variegation of transgene expression with age, which further complicate the interpretation of how these transgenes are developmentally regulated (Robertson et al., 1996). One strategy to overcome the obstacles associated with transgene positional effects is to reclone animals from founder animals.

Here, we evaluated the genetic identity between a cloned and a recloned dog, specifically asking whether red fluorescent protein (RFP) expression and cytomegalovirus (*CMV*) promoter methylation change with cloning generation and/or age. 'Ruppy1', a female, cloned dog transgenic for a *CMV-RFP* construct, and 'Magic', a recloned dog produced by SCNT using a somatic cell of Ruppy1 (Oh et al., 2011), were used in this study. Southern-blot analysis revealed that both cloned and recloned dogs have a single copy of the RFP gene that was stably integrated into their genomes, specifically into chromosome 25 based on DNA Walking SpeedUp^{TM} analysis (Seegene, Seoul, Korea). Western-blot analysis showed no significant difference in RFP abundance between 1-year-old Ruppy1 and 1-year-old Magic. At 4 years of age, each animal showed a proportional and significant increase in RFP levels compared to their 1-year-old selves ($P < 0.05$) (Fig. 1 A, B). To determine the source of this age-related change in expression, bisulfite-modified genomic DNA was prepared using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA), and converted genomic DNA was used for methylation analyses of the *CMV* promoter region by bisulfite pyrosequencing (see supplemental Materials and Methods). Promoter methylation percentage was calculated from the average methylation values at 4 cytosine-phosphate-guanine (CpG) sites (Fig. 2A). The mean methylation levels of 1-year-old Ruppy1 (26%) and 1-year-old Magic (33%) decreased significantly at 4 years of age (Ruppy1, 17%; Magic, 25%; respectively a 1.5- and 1.3- fold decrease compared with the 1-year-old levels; $P < 0.05$) (Fig. 2B).

We therefore conclude that recloning can provide a valuable tool to provide an unlimited supply of identical nuclei from transgenic, cloned dogs. Furthermore, the same integration site and copy number of a transgene also inversely correlates with patterns of promoter methylation in these animals. The current results provide a paradigm to study the role of epigenetics in gene expression during aging of transgenic dogs and other mammals.

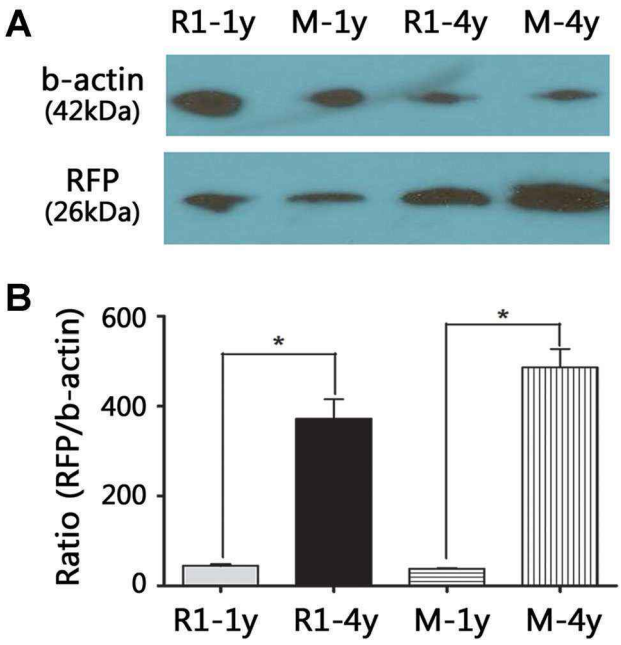

Figure 1. Age-dependent expression of *RFP* in tissue samples from Ruppy1 and Magic. **A**: Western-blot analysis for RFP abundance in each dog at ages 1 and 4 years. **B**: RFP expression, normalized to beta-actin level in each sample. Analysis was replicated three times. Data are presented as the mean ± standard deviation. Statistical analysis was performed with. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc testing, using GraphPad Prism version 5 (Graphpad Incorporation, San Diego, USA). Asterisks (*) indicates $P \leq 0.05$. R1-1y, 1-year-old Ruppy1; R1-4y, 4-year-old Ruppy1; M-1y, 1-year-old Magic; M-4y, 4-year-old Magic.

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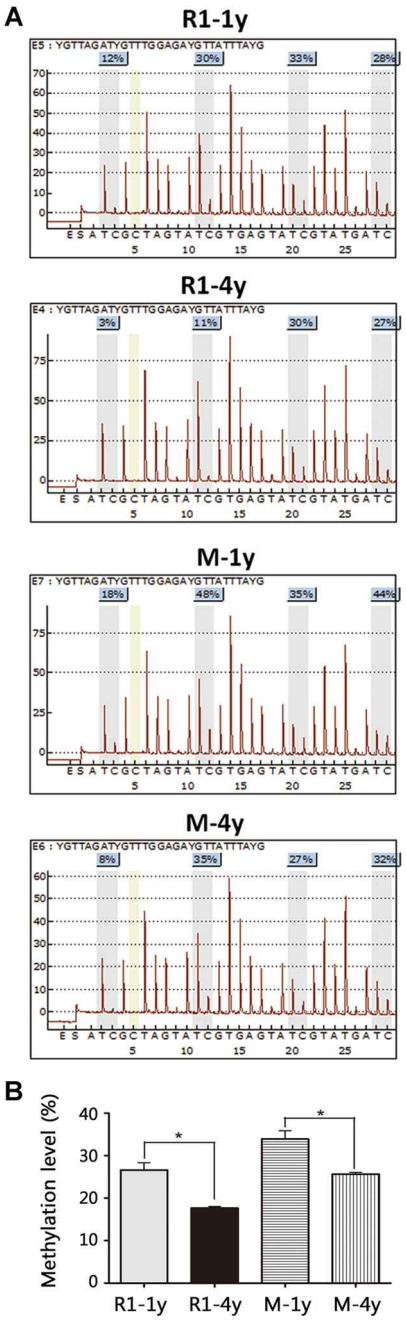

Figure 2. Age-dependent expression of *CMV* promoter methylation in tissue samples from Ruppy1 and Magic. **A**: Representative pyrograms obtained from samples for 4 select cytosine-phosphate-guanine (CpG) sites in the *CMV* promoter. Each gray shaded column indicates the assayed CpG dinucleotide, and the percentage of methylation at that CpG dinucleotide is indicated above. The yellow shaded boxes are internal bisulfite-modification control assessments. **B**: The average methylation percentages of cytosine-phosphate-guanine (CpG) in the *CMV* promoter of Ruppy1 and Magic, measured at ages 1 and 4 years. Statistical analysis was performed with. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc testing, using GraphPad Prism version 5 (Graphpad Incorporation). Asterisks (*) indicates $P \leq 0.05$. R1-1y, 1-year-old Ruppy1; R1-4y, 4-year-old Ruppy1; M-1y, 1-year-old Magic; M-4y, 4-year-old Magic.

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REFERENCES

Kishigami S, Wakayama S, Hosoi Y, Iritani A, Wakayama T. 2008. Somatic cell nuclear transfer: Infinite reproduction of a unique diploid genome. Exp Cell Res 314:1945–1950.

Oh HJ, Park JE, Kim MJ, Hong SG, Ra JC, Jo JY, Kang SK, Jang G, Lee BC. 2011. Recloned dogs derived from adipose stem cells of a transgenic cloned beagle. Theriogenology 75:1221–1231.

Robertson G, Garrick D, Wilson M, Martin DI, Whitelaw E. 1996. Age-dependent silencing of globin transgenes in the mouse. Nucleic Acids Res 24:1465–1471.

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