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授与した学位	博　士
専攻分野の名称	歯　学
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学位授与の要件	医歯薬学総合研究科機能再生・再建学専攻 （学位規則第４条第１項該当）
学位論文の題目	Challenges of Generation and Analysis of Knock-in Mice Harboring a Primate-Specific Long Non-Coding RNA, UCA1 with CRISPR-Cas9 System (CRISPR-Cas9 システムを用いた霊長類特異的長鎖ノンコーディング RNA, UCA1 ノックインマウスの作製と解析への挑戦)
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学位論文内容の要旨

Background: Urothelial cancer associated 1 (UCA1) gene is a primate-specific long non-coding RNA which is upregulated in the bladder cancer. Our previous work has showed that UCA1 accelerates chondrogenesis and endochondral ossification. CRISPR-Cas9 system is recently developed and enables efficient production of knock-out animals, however knock-in of genes to modify a target remains extremely low efficient than the knockout, because of the difficulty of homologous recombination (HR) and delivery of donor DNA into the pronucleus with Cas9 nucleases and guide RNA.

Materials and methods: To demonstrate the physiological role of UCA1 gene *in vivo*, knock-in mice harboring UCA1 gene which was drived by CMV promoter in rosa26A locus was generated. For each increment of recombination efficiency 1.5kb of 5' and 3' homologous region to rosa26A besides of PAM site were linked to 3.8 kb knock-in fragment, and double stranded target DNA was denatured and purified as long single stranded oligonucleotides (lsODN): LsODN, Cas9 nuclease and guide RNA were transferred into zygotes with electroporation to increase the efficiency of genome editing. To identify founder mice F0 the targeted insertion of knock-in gene of UCA1 was detected by PCR using several specific primers. Some of the PCR amplicons were cloned into PUC118 vector after kination reaction using blunting kination kit or purified with PCR purification kit to analyze the sequences. To analyze the phenotypes, skeletons were analyzed at the embryonic stages, and their livers were postnatally analyzed histologically by Masson trichrome staining.

Results: The insertion of the knock-in genes in the founder mouse lines were detected by PCR. 1) Boundary between 5' arm and UCA1 knock-in gene; positive in F0 mice #32 and #44 and the sequencing indicated the existence of knock-in gene. 2) From upstream of 5 arm to knock-in gene F0 mice #32 and #44 showed amplification and with direct sequencing of 5' side showed rosa26A sequencing without BsrD1 sequence, and 3' side showed same sequence as 1) indicating targeted knock-in in rosa26A locus. 3) Full length UCA1 gene from CMV promoter to BGHpA sites; positive in F0 mouse #32, as well as F1 mouse #17, F2 mice # 4 and #14. The amplification was

observed in F0 mice #24, #35, indicated very few cells has knocked-in and some of the primer sets could not reach to the threshold of the amplification. 4) Internal region of UCA1; positive in mice F0 #32 and #44 with faint bands observed in F0 mice #24 and #35. The sequencing showed UCA1 gene, indicating the amplification of knock-in gene. 5) Boundary between UCA1 knock-in gene; and 3' arm positive in F0 mouse #32, as well as F1 mouse #17 and several F2 mice. The sequencing analysis showed linked sequence of pcDND3.1 (+) followed by next of PAM site of rosa26A sequence in summary, at least F0 mice #32 was successfully knocked-in. The founder line mice F0 #32 and 44 showed hair loss on skin and the livers of #32 showed fibrotic changes.

Discussion: To demonstrate the effects in vivo, UCA1, was planned to be ubiquitously expressed and knocked-in to rosa26A using CRISPR-Cas9 system and lsODN with electroporation. The PCR analysis of genomic DNA from obtained mice showed successful knock-in at least in F0 #32 and #44, however #44 could not yield the following generation. Other F0-#24, #35 mice which existed showed knock-in amplification at least once with primer sets, however, not all the primer sets could detect knock-in gene. This could be due to the mosaicism of the genomic DNA and low amplification efficiency of UCA1 gene due to the three-dimensional structure created by the base pair of RNA strand in UCA1 gene.

Conclusion: UCA1, primate-specific lncRNA was successfully inserted to the mouse genome by knock-in to rosa26locus with CRISPR-Cas9 system and electroporation. The founder lines showed senescent phenotypes in several organs such as hair loss on skin and fibrotic changes in liver, indicating that over expression of long-UCA1 may induce cell senescence in mouse organs.

論文審査結果の要旨

Introduction: Urothelial cancer associated 1 (UCA1) is a long non-coding RNA (lncRNA) exclusively found in primates, and has 2 major transcript variants: short (1.4 kb) and long (2.3 kb) forms. The department of oral biochemistry previously demonstrated that UCA1 enhanced chondrocyte differentiation using cultured cells. Here Ms. Kavitha aimed to investigate the function of UCA1 *in vivo*. UCA1 was knocked-in under the control of the CMV promoter into the *rosa26A* locus of UCA1-null mice using the CRISPR-Cas9 system.

Materials and methods: To increase the efficiency of knock-in, 1.5-kb upstream and downstream franking regions of the PAM in *rosa26A* were used as arms for homologous recombination, and nick endonuclease sequences were added to both ends of the arms. The obtained transgene constructs were treated with nick endonuclease, denatured, and electrophoresed. The target fragments were recovered to make long single-stranded oligodeoxynucleotides (lsODNs), and the target lsODNs were then introduced into fertilized eggs by electroporation together with Cas9 and guide RNA. Ms. Kavitha identified founder animal by PCR amplification using specific primer sets. PCR amplicons were cloned into pUC118 vector and the sequences were analyzed. After establishment of the founder line, homozygous knock-in mice were generated and their skeleton phenotypes were analyzed at embryonic stages, and the livers were analyzed postnatally with Masson trichrome staining.

Results: The founder lines from the obtained F0 mice were identified by PCR detection of 1) the junction between the 5' end of the CMV-UCA1 expression cassette and *Rosa26A*, 2) the inside of the 5' arm from the outside of the CMV-UCA1 expression cassette, 3) the entire CMV-UCA1 expression cassette, 4) the inside of the UCA1 gene, and 5) the junction between the 3' end of the CMV-UCA1 expression cassette and *Rosa26A*, and two founder lines were obtained out of 48 F0 mice. One of the lines died at 10 months of age and no new offsprings could be obtained, but the other line was crossed with a wild type to obtain the next generation. Both F0 founder lines showed hair loss and fibrosis in the liver, and in homozygotes of knock-in embryos slightly shorter epiphyseal cartilages with less staining by Alcian blue compared to that of wild types.

Discussion: These results suggest that UCA1 is involved in hair formation on skin, fibrotic changes in the liver of adult F0 founders, as well as slight changes in skeleton during embryonic stage, possibly by affecting the endochondral ossification.

This study demonstrated that the function of lncRNA UCA1 *in vivo* by generating knock-in mice using CRISPR-Cas9 system. Techniques and methodology are novel. The phenotype results can provide a basis for understanding the functions of UCA1 in vivo. Therefore, the defense committee hereby accepts this article as a doctoral dissertation in dentistry.