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Generation of *mmp15b* Zebrafish Mutant to Investigate Liver Diseases

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ABSTRACT : Upon gene inactivation in animal models, the zebrafish (*Danio rerio*) has become a useful model organism for many reasons, including the fact that it is amenable to various forms of genetic manipulation. Genome editing is a type of genetic engineering in which DNA is inserted, deleted, modified, or replaced in the genome of a living organism. Mainly, CRISPR (clustered regularly interspaced short palindromic repeats) Cas9 (CRISPR-associated protein 9) is a technology that enables geneticists to edit parts of the genome. In this study, we utilized this technology to generate an *mmp15b* mutant by using zebrafish as an animal model. MMP15 is the membrane-type MMP (MT-MMP) which is a recently identified matrix metalloproteinase (MMP) capable of degrading all kinds of extracellular matrix proteins as well as numerous bioactive molecules. Although the newly-established *mmp15b* zebrafish mutant didn't exhibit morphological phenotypes in the developing embryos, it might be further utilized to understand the role of MMP15 in liver-related diseases, such as liver fibrosis, and associated pathogeneses in humans.
Key words : *mmp15b*, Zebrafish, Liver diseases, Regeneration, Fibrosis, CRISPR/Cas9

INTRODUCTION

Before the application of CRISPR/Cas9(clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9) technology in the zebrafish research field, targeted gene knockouts were not possible in zebrafish, and its utility for validation studies of candidate genes was limited. At that time, researchers using the zebrafish animal model have been experienced difficulty using ENU technology to generate mutants for interesting genes. This challenge was eliminated with the development of novel gene targeting approaches including ZFNs, TALENs, and CRISPR/Cas9 (Bedell et al., 2012; Jinek et al., 2012; Mali et al., 2013; Hsu et al., 2014; Varshney et al., 2015). Among these, the CRISPR/Cas9 approach now offers an efficient method to target any gene of interest. Although off-target effects of CRISPR/Cas9 technology remains a hurdle for consideration, several screens and engineering methods have been developed to reduce genome-wide off-target mutations including nuclease mutation, protospacer adjacent motif (PAM) sequence modification, single guide RNA (sgRNA) truncation and novel nuclease discovery. For example, Kleinstiver et al. (2015, 2016) recently reported that manipulation of the Cas9 protein (i.e., SpCas9) and sgRNA target not only improved specificity but also reduced off-target effects (Fu et al., 2014).

Matrix metallopeptidases, also known as matrix metalloproteinases (MMPs), are metalloproteinases that are calcium-dependent, zinc-containing endopeptidases. These enzymes are capable of degrading all kinds of extracellular

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matrix (ECM) proteins, but can also process numerous bioactive molecules. The most common categorizations of MMP are partly based on the historical assessment of substrate specificity and cellular localization of MMP, which include collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs). Among them, MT-MMPs constitute a growing subclass of recently identified MMPs; however, MT-MMPs are yet to be fully understood. Previously, Choi et al. (2017) reported that *mmp15b* was highly expressed in the regenerating liver after severe hepatocyte ablation. In addition, MMPs are involved in the degree of initial injury and repair, the onset and resolution of inflammation, the activation and deactivation of myofibroblasts, and the deposition and breakdown of ECM. In other words, MMPs are involved in both augmenting and attenuating many processes that impact fibrosis (Giannandrea & Parks, 2014). Thereby, a zebrafish mutant model of *mmp15b* would be an effective tool to investigate the underlying mechanism during liver fibrosis after severe hepatocyte injury.

In this study, we generated a zebrafish *mmp15b* mutant using CRISPR/Cas9 technology as a model to explore liver disease with human fibrosis.

MATERIALS AND METHODS

1. Zebrafish

We used wild type (TÜ and AB) zebrafish. Wild-type zebrafish were obtained from the Zebrafish Center for Disease Modeling (ZCDM). Embryos and adult fish were raised and maintained under standard laboratory conditions (Westerfield, 2000).

2. Zebrafish reverse transcriptase polymerase chain reaction (RT-PCR)

cDNA from embryos at various stages (as indicated in Fig. 1) was used as a template for PCR to amplify *mmp15a* and *mmp15b* genes. The primer sequences, *mmp15a* (407 bp) and *mmp15b* (556 bp), used for RT-PCR were 5'-GACTTCATGGGATGCCGAGT-3', 5'- ACGACAAGGT GTGTGTCTCG-3', and 5'-CCGGTTACCCTCAAGAGC TG3', 5'-GTGCGCTCCGTCTCATCTAT-3'; respectively.

3. Generation of the *mmp15b* mutant line

mmp15b sgRNAs (5'-ATGACCCGTTCACGTTGCTG TGG-3' and 5'- TTGATGGGTGCGCGCACCTTTGG-3') were generated as previously described (Irion et al., 2014). *mmp15b* target sequences are located in exon 1. *mmp15b* gRNAs (500 ng each) and Cas9 protein (1 ug) were mixed and injected into 1-cell stage embryos which were raised to adulthood. Cas9 protein was purchased from Toolgen. Either the adult zebrafish tail fin or the whole embryo at 1–2 dpf was used to obtain genomic DNA for PCR-mediated genotyping. PCR products were sequenced to identify frameshift mutation. F1 fish containing a 14 bp deletion

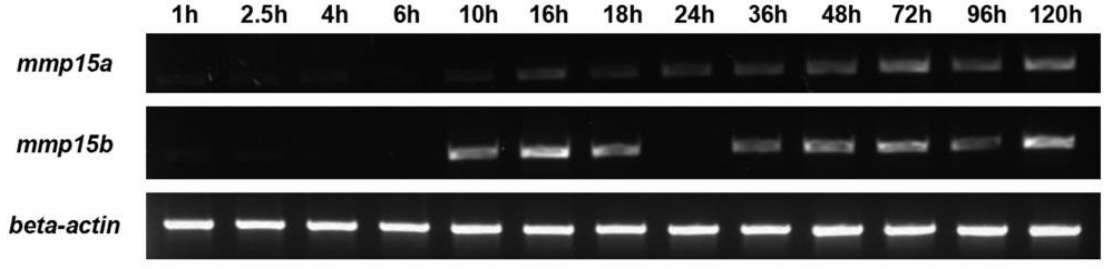

Fig. 1. Developmental expression of *mmp15b* mRNA. Differential mRNA expression of *mmp15b* in the developing zebrafish embryo. RNA extracted from the embryo at the indicated embryonic stage was used for RT-PCR analysis. RT-PCR, reverse transcriptase polymerase chain reaction.

were selected to establish the *mmp15b* mutant line.

4. Genotyping of *mmp15b* mutants

For *mmp15b* genotyping, genomic DNA was amplified with the forward (5'-GATCCCGGTGGGAATGATGGC-3') and reverse (5'-AGAACCCTGTAGAACTGACTTG -3') primers. The wild-type allele generates a band of 148 bp, whereas the *mmp15b* mutant allele generates a band of 134 bp.

RESULTS AND DISCUSSION

We recently established a zebrafish liver regeneration model in which severe hepatocyte loss resulted in the regeneration of hepatocytes from BECs. Using this model, we performed RNA-Seq analysis to identify pathways or factors involved in the regulation of liver regeneration (Choi et al., 2017). Among them, we selected *mmp15b* which was highly expressed in early liver regeneration. MMPs are a family of extracellular endopeptidases defined by conserved catalytic domains (Ra & Parks, 2007) and have long been considered to be primarily responsible for turnover and degradation of ECM substrates. However, they are now recognized for immunity and repair; being involved in such processes as cell migration, leukocyte activation, antimicrobial defense, chemokine processing and more (Yoshifum, 2015). Thereby, *mmp15b* might play a regulatory role in zebrafish liver regeneration or liver fibrosis.

Due to genome duplication in zebrafish, we found that two *mmp15* genes, *mmp15a* and *mmp15b*, searched. We first examined the expression pattern of *mmp15* genes during development. Expression of *mmp15a* and *mmp15b* was detected as early as 10 hpf (hour post-fertilization), indicating zygotic but not maternal expression (Fig. 1). Based on their expression patterns, *mmp15a* and *mmp15b* likely play an important role in normal animal development and in other physiological settings. Of note, gene expression profiling after RNA-Seq analysis during early liver regeneration showed that *mmp15b* was highly induced in the regenerating liver, whereas *mmp15a* was not (Choi et al., 2017). These results suggest a role of *mmp15b* during liver regeneration.

We generated *mmp15b* zebrafish mutant utilizing CRISPR/Cas9 technology which is a simple yet powerful tool for editing genomes by altering DNA sequences and modifying gene function. We first designed sgRNAs (Fig. 2B) after predicting the critical domain of Mmp15b (Fig. 2A). As an MT-MMP, the Mmp15b protein contains a membrane linker, cytoplasmic tail, and catalytic domain for the degradation of ECM substrates (Fig. 2A). Therefore, we synthesized two sgRNAs in exon 1 of *mmp15b* (Fig. 2B) and tested the efficiency by co-injecting with Cas9 protein into one-cell stage embryos. After performing T7E1 assay for DNA sequence mismatch in co-injected embryos, we found that the sgRNAs efficiently generated mismatched DNA, suggesting successful generation of the *mmp15b* mutant zebrafish (Fig. 2C).

The resulting mismatched DNA from the two sgRNAs suggested germline transmission and were used to generate the *mmp15b* knockout mutation (Fig. 2C). To determine the role of Mmp15b, we examined (1) outcross with wild type raised to adulthood and (2) identification of mutation by PCR and DNA sequencing in the F1 zebrafish (Fig. 3). We performed PCR after isolation of genomic DNA from 42 zebrafish offspring (Fig. 3B bottom). Approximately 52% (22/42) of zebrafish offspring were *mmp15b* knockout mutants showing 13, 14, or 16 bp deletions, suggesting that frameshift occurred in the 22 F1 zebrafish (Fig. 3B). Frameshift mutation of *mmp15b* resulted in termination-site change and early termination of the Mmp15b protein, indicating that the Mmp15b variant was generated with 32 amino acid of the Mmp15b protein. Of note, the *mmp15* mutant exhibited no obvious morphological phenotypes in the developing embryo or adult (data not shown). Despite the absence morphological changes, the *mmp15b* mutant would be a useful tool for the study of liver regeneration.

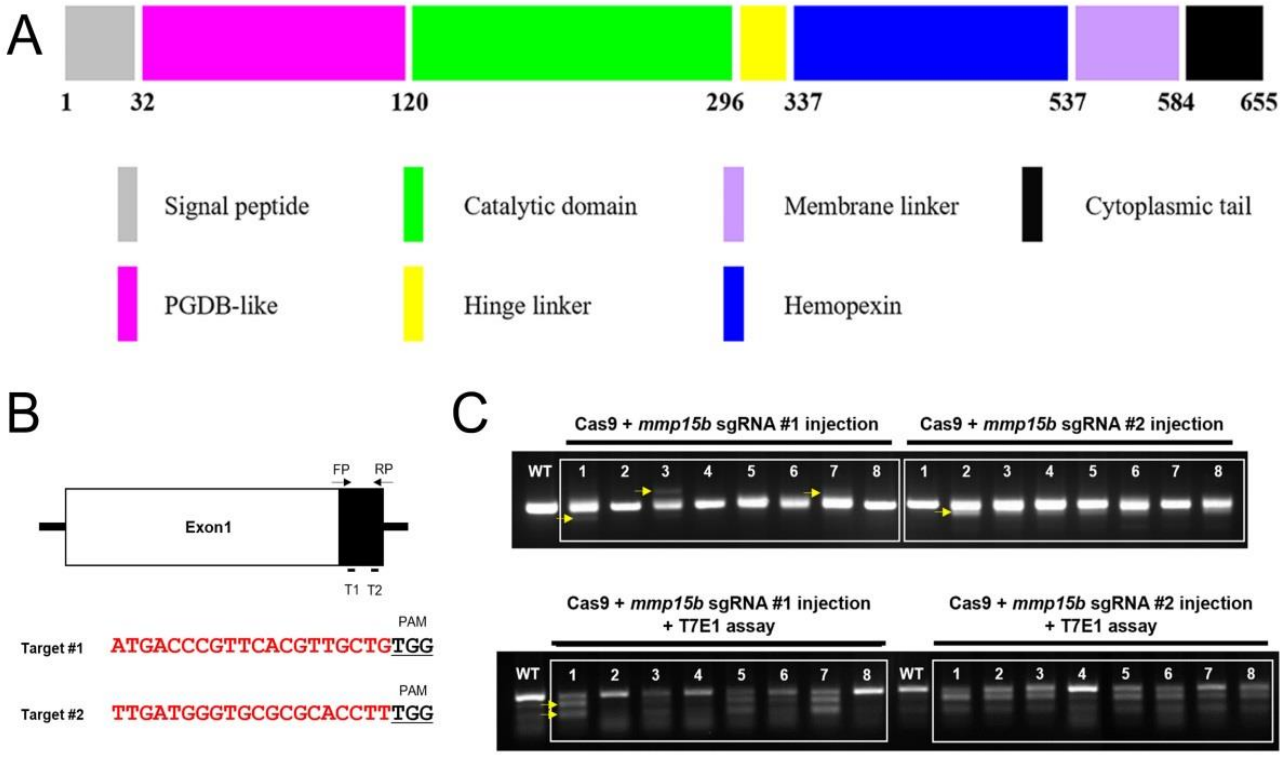

Fig. 2. Generation of the *mmp15b* mutant in zebrafish. (A) Scheme of predicted domains for the Mmp15 protein. The main domain for the Mmp15b protein is shown in the green box, which is a catalytic domain with zinc ion binding. Mmp15b possesses membrane linker and cytoplasmic domains as an MT-MMP. (B) To generate *mmp15b* mutant zebrafish, two sgRNAs with PAM sequence were designed for exon 1 of the *mmp15b* gene. (C) To check the efficiency of the sgRNAs, we co-injected sgRNA and Cas9 protein. Co-injected embryos showed that the two sgRNAs efficiently generated mismatched DNA when T7 endonuclease I (T7E1) assay was performed. MT-MMP, membrane-type matrix metalloproteinase; PAM, protospacer adjacent motif; sgRNA, single guide RNA.

The zebrafish liver is fully functional by day 5, which provides us an opportunity to investigate liver regeneration by combining other transgenic fishes; namely hepatocyte ablation lines, e.g., *fabo10a*:CFP-NTR, or macrophage ablation lines, such as *mpeg1*:NTR-mCherry. As our *mmp15b* mutant zebrafish could be utilized in this regard, future experiments would be of interest to explore the role of *mmp15b* underlying fibrosis mechanism during liver regeneration.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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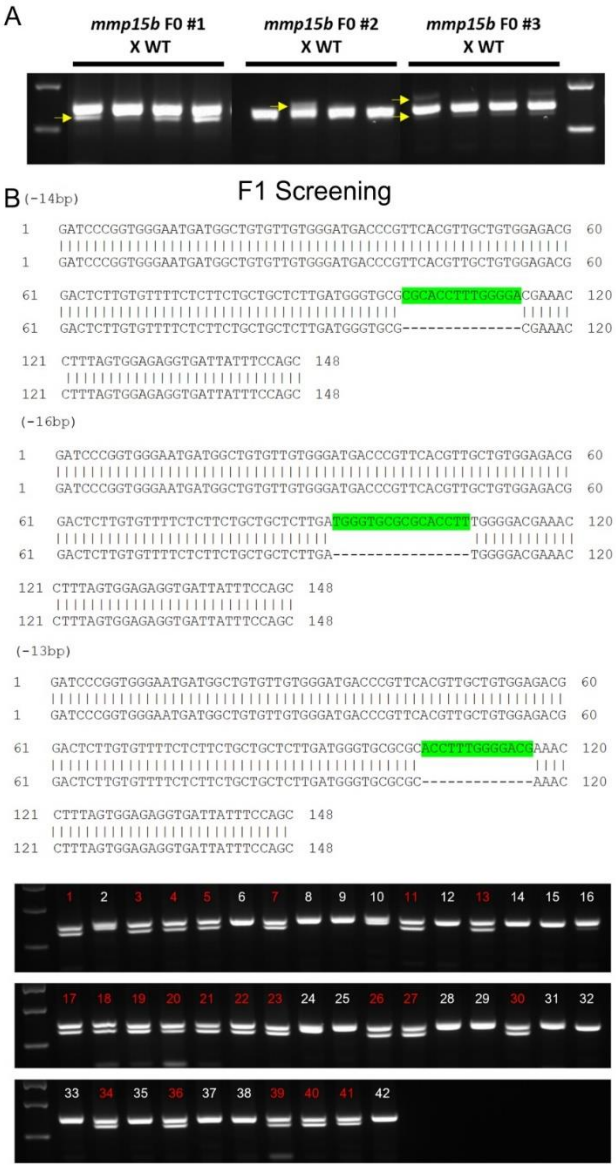

Fig. 3. CRISPR/Cas9-mediated gene editing for *mmp15b* resulted in germline transmission in zebrafish. (A) Most F0 individuals, when raised to adulthood, transmitted the *mmp15b* knockout mutation to their offspring, demonstrating a very high efficiency of targeted mutagenesis in both soma and germline. (B) Sequencing after *mmp15b* PCR found that F1 individuals exhibited 13, 14, or 16 bp deletions, thereby frameshift mutation of *mmp15b* resulted in change of termination-site and early termination of Mmp15b protein. The Mmp15b variant is generated with 32 amino acids of the Mmp15b protein. CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9.

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Investigation: Kim OH, Choi TY
Writing original draft: An HS, Choi TY
Writing review & editing: An HS, Choi TY.

ETHICS APPROVAL

This article does not require IRB/IACUC approval because there are no human and animal participants.

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