Research Article

A Crucial Role of Bone Morphogenetic Protein Signaling in the Wound Healing Response in Acute Liver Injury Induced by Carbon Tetrachloride

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Received 30 December 2011; Revised 14 March 2012; Accepted 12 April 2012

1. Introduction

The mammalian liver is the main organ for metabolism of nutrients and drugs, as well as storage of glycogen and lipids, synthesis and secretion of serum proteins, as well as detoxification, and production of biochemicals necessary for digestion [1, 2]. Moreover, the liver has a robust ability to self-regenerate from the remaining tissue after two-third partial hepatectomy [3, 4]. Chronic liver injury caused by hepatitis viruses, autoimmune responses, hepatotoxin intake, or cholestatic and metabolic diseases progresses to liver cirrhosis or fibrosis through stimulation of quiescent hepatic stellate cells to proliferate and transform into fibroblast cells [5]. However, the earliest stage of these processes is thought to consist of repeated cycles of injury and repair in liver cells [6, 7]. Acute liver injury can be caused by various pharmacological toxicants. A dynamic regeneration or tissue repair response similar to that after partial hepatectomy occurs following cell death and tissue injury caused by exposure to toxic chemicals. An intricate signal transduction network consisting of chemokines, cytokines, growth factors, and hormones has been revealed for liver regeneration after partial hepatectomy [8, 9].

Acute liver injury induced by carbon tetrachloride (CCl4) is widely studied as a model of liver injury in rats. CCl4 is metabolized by cytochrome P450 IIIE1 [10] in mature hepatocytes and converted to trichloromethyl radicals, resulting in acute but reversible damage to the centrilobular hepatocytes that is followed by liver regeneration. Recently, we observed that BMP2 or BMP4 were transiently expressed in the early stage of CCl4 injury in rats [11]. However, the role of Bmp2 or Bmp4 in CCl4 liver injury was not clarified [11]. Bmp2 and Bmp4 are members of the transforming growth factor-β (TGF-β) superfamily and are involved in the development of many organs, including the liver. In hepatogenesis, Bmp2 is secreted from the cardiac mesoderm and participates in morphogenetic growth of the hepatic endoderm into a liver bud. In mice, Bmp4 has an important
role in hepatogenesis in early embryos [12, 13]. Therefore, BMPs are thought to be involved in cell proliferation and determination of progenitor cell fate.

BMP2 and BMP4 signals are transduced by heteromultimers of two types of transmembrane serine/threonine kinases, Bmp type 1A and type 2 receptors (Bmpr1a and Bmpr2, resp.). Mice homozygous for a Bmpr1a-null allele die at embryonic day 8.0 without mesoderm formation [14].

In the present study, to examine whether the BMP signaling transiently expressed in CCl4 liver injury is involved in liver regeneration, we investigated the liver injury and healing in Bmpr1a conditional knockout (Bmpr1a-KO) mice induced by the Cre/loxP system [15, 16]. In these mice, intravenous injection of a recombinant Cre adenovirus efficiently induces transgene expression in most of liver cells, and genomic knockout of the Bmpr1a gene is specifically induced in the liver. If BMP signaling serves as a regenerative cue in the early stage of liver injury, Bmpr1a-KO mice should exhibit retarded restoration of liver function.

2. Materials and Methods

2.1. Ethics Statement. All of the animal experiments described were approved by the Institutional Animal Care and Use Committee of Tottori University (permission numbers: 18-2-39 and 06-5-80). All the mice received humane care in compliance with Tottori University’s guidelines for the care and use of laboratory animals in research.

2.2. Animals. The mice in this study were fed ad libitum and housed in a room maintained at a constant temperature of 22 °C, with 50% humidity and a 12-h/12-h light/dark cycle.

Eight-week-old male ICR mice were purchased from Nihon Clea (Tokyo, Japan). Bmpr1a+/− mice and Bmpr1alox/lox mice were obtained from Dr. Yuji Mishina (National Institute of Environmental Health Sciences, Research Triangle Park, NC; present in University of Michigan School of Dentistry) [15, 16]. Male Bmpr1alox/− mice weighing 30 g were generated by breeding between Bmpr1a+/− and Bmpr1alox/lox mice and used at 10 weeks of age. The genotypes of the mice were determined by PCR of genomic DNA. The following primer sets were used: flox allele detection, forward 5′-CTGACCCT-3′ and 5′-TGGATA-3′, null allele detection, forward 5′-AGACTGCCTTGGGAAAAGCGC and reverse 5′-ACGATAGTGGGATGCAGTA-3′, and genomic knockout of the Bmpr1a gene is specifically induced in the liver. If BMP signaling serves as a regenerative cue in the early stage of liver injury, Bmpr1a-KO mice should exhibit retarded restoration of liver function.

2.3. Biochemical Measurements. The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum samples from the treated mice were determined using L-Type WAKO AST/ALT J2 assay kits (Wako Pure Chemicals Co. Ltd., Osaka, Japan), according to the manufacturer’s instructions.

2.4. Recombinant Adenovirus. A recombinant adenovirus vector (Ad-Cre) was constructed with human adenovirus type 5 by replacing the E1A and E1B genes with the bacteriophage P1 Cre recombinase gene under the control of the CAG promoter. Likewise, Ad–LacZ was constructed with the Escherichia coli LacZ gene as a control. The recombinant adenoviruses were propagated in HEK293 cells, which are human embryonic kidney cells transformed by the E1A and E1B genes. Virions were purified by CsCl equilibrium centrifugation, dialyzed against 10 mM HEPES containing 1 mM EDTA and 10% glycerol, and titrated with HEK293 cells.

2.5. CCl4 Injury and Infection of the Adenovirus In Vivo. ICR mice were treated with 5 μL of CCl4/liquid paraffin (1:4 mixture) per gram of body weight and euthanized at 24 or 72 h postinjection for total RNA extraction. Bmpr1a−/− mice were infected with 100 μL (1.5 × 108 pfu) of the purified recombinant adenovirus Ad-Cre or Ad-LacZ via the tail vein by single injection. Mock-infected mice were injected with phosphate-buffered saline (PBS). The infected mice were treated with CCl4 at 14 days postinfection. Subsequently, the mice were euthanized after 24 or 72 h to obtain livers for tissue sections and genomic DNA and total RNA isolation.

2.6. RNA Preparation, RT-PCR, and Real-Time PCR. Total RNA was isolated from mouse tissues by acid phenol-guanidinium thiocyanate-chloroform extraction. Total RNA (2 μg) was converted to complementary DNA and the target genes were amplified using Taq DNA polymerase (Bio Academia, Osaka, Japan) in a PCR thermal cycler using the primer sets as follows: Bmp2: 5′-GACGGACTGCAGGCTCTCCTAAAG and 5′-TCTGCAAGATGTGAAACACTGTC; Bmp4: 5′-GAGGATTTCTCATCAGGAGA and 5′-GCTCTGCGAGGAAGATCA, Bmpr1a: 5′-GAAAGCAGAGTGAAATGCT and 5′-CTATAATGGCAAAAGCATGTG, Id1: 5′-GGATCATGAAGGTCGCCAGT and 5′-TTGCTCAGTCCTGGGTTCTG, Id2: 5′-GGGTCTCCTCTCAGGAGCAG and 5′-ACGATATGCGATGCGAGGT, Id3: 5′-AGCTCACTCCCGGAACCTTGTG and 5′-GGGACAGAGTGCAGTTGC, Albumin: 5′-GAAAGCACCAGTGTGAGAC and 5′-CAGTCAAGGAGCAGCTTGC, AldolaseB: 5′-ATTTCATTGTCCTTTGCTCTAT and 5′-ATGCAAGTCTGGCTTATCA, Tdo2: 5′-AAGGTGAACGGAGCAGCTTCA and 5′-AGTTGAACGAGTTAATGAT, PECK: 5′-GACCTCTTCTTGGCTACAC and 5′-CTGATTCTCTGAGTTACCT, Transferrin: 5′-GGTGGTAAAGCCTACTGAC and 5′-TAAGGGCAGACGAGGAGAC, PCNA: 5′-CTTACTTGGCCTCCAGG and 5′-CAATTTCCCGGAGCCAT, GAPDH: 5′-AAGGGTGCTGGCAAGGTTCAT and 5′-CACCACCTGTGGCTTACG. Quantitative analyses were also performed to measure the mRNA levels by real-time PCR (ABI 7900HT; Applied Biosystems Co., Foster City, CA) with TaqMan probes (Applied Biosystems Co.) according to the manufacturer’s protocol.

2.7. Immunohistochemistry. Livers were fixed in 10% formalin and embedded in paraffin. De-paraffinization in xylene and rehydration in a graded ethanol series, 7-μm sections were immersed in a vessel containing 10 mmol/L citrate buffer (pH 7.0) and autoclaved at 121 °C for 15 min. The sections were then treated with 3% (v/v) H2O2 for 10 min at room temperature, blocked with 10% (v/v) goat serum or rabbit serum (Nichirei, Tokyo, Japan) for 30 min at
room temperature, and incubated with a rabbit monoclonal antibody against Ki-67 (Thermo Fisher Scientific Inc., San Jose, CA) diluted 1:200 for 1 h at room temperature. After washing with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Nichirei) or biotinylated rabbit anti-mouse IgG (Nichirei) for 30 min. The sections were washed with PBS, incubated with a solution of streptavidin-conjugated horseradish peroxidase (Nichirei) for 15 min according to the manufacturer’s recommendations and washed again with PBS for 5 min. Peroxidase activity was detected with H2O2/diaminobenzidine substrate solution and the sections were counterstained with hematoxylin before dehydration and mounting. The percentage of Ki-67-positive hepatocytes was determined by counting positively stained hepatocyte nuclei in 40 random fields at 40× magnification and calculating the mean value. The value was expressed as a fraction of the total number of hepatocytes in a 40× field, which averaged 30 cells/field.

2.8. β-Galactosidase Staining. Determination of the expression of the lacZ gene was carried out according to Jaffe et al. [17]. Briefly, the fixed specimens were rinsed three times with PBS and incubated in a reaction mixture containing 5 mM β-D-galactopyranoside (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in PBS for 2 h at 37°C. Subsequently, these specimens were counterstained with eosin.

2.9. Immunofluorescence. Liver sections prepared as in immunohistochemistry were blocked with 10% (v/v) goat serum (Nichirei, Tokyo, Japan) for 30 min at room temperature and incubated with a rabbit polyclonal antibody against BMPR1A (ABGENT Inc, San Diego, CA) diluted 1: 50 for 1 h at room temperature. After washing with PBS, the sections were incubated with Alexa fluor 488 conjugated goat anti-rabbit IgG (Invitrogen, Austin, TX) diluted 1: 1000 for 1 h at room temperature. The sections were washed with PBS and counterstained with DAPI before mounting. Images were acquired with OLYMPUS Laser Confocal Scannig Microscope FV1000D Spectral Type (inverted microscope 40×).

2.10. Statistical Analysis. Statistical analysis was performed using StatView (SAS Institute Inc., Cary NC). The Student's t-test was used to analyze the difference between the study and control groups; P values less than 0.05 were considered statistically significant.

3. Results

3.1. Transient Expression of BMP4 in Mice Treated with CCl4. Previously, we reported that Bmp2 or Bmp4 are transiently expressed in CCl4-treated rats in the early stage of liver injury [11]. To confirm whether this transient expression of Bmp4 is also observed in mice treated with CCl4, we examined the expressions of liver-specific genes and the Bmp2 or Bmp4 genes in the liver-injured mouse model by RT-PCR and real-time RT-PCR. Albumin mRNA expression, which was examined as a marker for liver function, was decreased at 3–36 h and recovered at 48 h after treatment with CCl4 (Figures 1(a) and 1(b)). Likewise, we determined the expressions of the Bmp2 or Bmp4 genes in liver-injured mice, because Bmp4, especially Bmp4, play an important role in liver development during mouse embryogenesis. Bmp4 mRNA was significantly and transiently induced at 3–6 h after treatment with CCl4, while Bmp2 mRNA was slightly induced (Figures 1(a) and 1(b)). These findings are similar to the wound and repair responses in the liver injury model in rats, and they suggest that Bmp4 is also involved in the wound healing response in the injured liver of mice.

3.2. Bmpr1a Knockout in Liver by Cre Recombinase. To determine the role of Bmp4 in acute liver injury, we analyzed the wound healing response in injured conditional knockout mice with inhibited Bmp4 signaling by deletion of Bmpr1a. Since Bmpr1a-null mice (Bmpr1a−/−) show embryonic lethality [14], we used Bmpr1a-floxed mice in which both sides of exon 4 in the Bmpr1a gene were flanked by loxP sites and generated liver-specific Bmpr1a knockdown using the Ad-Cre adenovirus expressing Cre recombinase. Removal of exon 4 of the Bmpr1a gene is known to delete the biological function of Bmpr1a [16]. To obtain complete deletion of the Bmpr1a gene in the mouse genome, we generated Bmpr1alox/− heterozygotic mice by mating between Bmpr1alox/lox mice and Bmpr1a+/− mice. It is known that an adenovirus can efficiently infect liver cells through blood circulation from a peripheral vein [18]. First, we confirmed that most of liver cells are efficiently infected with adenovirus vectors carrying LacZ gene by intravenous injection (Figure 2(a)). As the result, single injection of Ad-Cre into the floxed mice induced deletion of the Bmpr1a gene in Bmpr1a−/− mice livers. The wild-type (or floxed) Bmpr1a gene is 2298 bp in length in genomic PCR, while the deleted allele is 214 bp in length (Figure 2(b)). Cre-mediated recombination occurred in the liver. Furthermore, when exon 4 of the Bmpr1a gene was excised from the genome by Cre recombinase, the shortened mRNA lacking exon 4 should be transcribed. The RT-PCR product is 390 bp before recombination and 227 bp after recombination. Expression of Bmpr1a mRNA lacking exon 4 was confirmed in Ad-Cre-injected mice (Figure 2(c)). Furthermore, expression of Bmpr1a protein was significantly decreased in Ad-Cre-infected mouse liver (Figure 2(d)). Small and strong signals without nuclei in each panels were derived from erythrocytes remained in liver tissue. These results indicate that Cre-mediated recombination resulted in the removal of the Bmpr1a gene from the liver of Ad-Cre-injected mice.

3.3. Liver Injury in Bmpr1a-KO Mice. To determine whether BMP signaling is involved in the wound healing response in liver injury, we induced CCl4 liver injury in Bmpr1a-KO mice generated by single injection of Ad-Cre for 14 days when inflammatory response by adenovirus infection should be cured (Figure 3(a)). The extent of the liver injury was determined histologically by hematoxylin and eosin staining of tissue sections. In the control mock-infected Bmpr1alox/− mice, severe damage to the centrilobular hepatocytes was observed at 24 h after CCl4 injection, and
Figure 1: Time course of gene expressions in the liver of mice treated with CCl4. (a) RT-PCR analyses of BMP2, BMP4 and albumin were performed using the primer sets shown in Section 2. Albumin expression is decreased at 3–36 h and recovers at 48 h. BMP4 is transiently expressed at 3–6 h after CCl4 injection. (b) Real-time PCR analyses of albumin and BMP4 were performed using primer sets and TaqMan probes provided by Applied Biosystems Co. All data are shown as the means ± SE from three independent experiments.

Figure 2: Time course of gene expressions in the liver of mice treated with CCl4. (a) RT-PCR analyses of BMP2, BMP4 and albumin were performed using the primer sets shown in Section 2. Albumin expression is decreased at 3–36 h and recovers at 48 h. BMP4 is transiently expressed at 3–6 h after CCl4 injection. (b) Real-time PCR analyses of albumin and BMP4 were performed using primer sets and TaqMan probes provided by Applied Biosystems Co. All data are shown as the means ± SE from three independent experiments.

most of the necrotic hepatocytes had disappeared at 72 h (Figure 3(b)). Similar observations were noted in the injured liver of Bmpr1a<sup>lox/−</sup> mice infected with Ad-LacZ as a control (Figure 3(b)). In contrast to these control mice, Bmpr1a<sup>lox/−</sup> mice infected with Ad-Cre (Bmpr1a-KO mice) showed a low level of amelioration of the injured liver histologically at 72 h after CCl4 injection (Figure 3(b)). On the other hand, the serum AST and ALT activities of the mice were increased at 24 h after CCl4 treatment and recovered to the basal levels at 72 h in both the control and knockout mice (Figure 3(c)). However, AST activity in KO mice was shown at little bit high level compared to Mock and Ad-LacZ-infected mice without CCl4 by unknown reason. These findings suggest that a single injection of CCl4 induced transient liver damage regardless of the presence or absence of BMP signaling, but it did not induce additional damage. However, amelioration of the wound healing response was dependent on the presence of BMP signaling.

To determine whether BMP signaling is involved in the wound healing response at the molecular level in the CCl4 liver injury model, we examined the expressions of various hepatic genes in Bmpr1a-KO mice by RT-PCR. Initially, we evaluated the expression of Bmpr1a mRNA carrying exon 4 deletion after Ad-Cre infection. As shown in Figure 4(a), a shorter Bmpr1a mRNA (shown with arrowhead) was expressed in the liver of Bmpr1a-KO mice, while the full-length Bmpr1a mRNA was observed in both mock-infected and Ad-LacZ-infected Bmpr1a<sup>lox/−</sup> mice. Interestingly, the levels of Bmpr1a expression were less affected by CCl4 treatment. Furthermore, the expressions of Id1, Id2, and Id3, as target genes for BMP signaling, were induced by CCl4 treatment, but 24 and 72 h in mock-infected and Ad-LacZ-infected
**Bmpr1a**

**Bmpr1a** flox− mice, but they were significantly reduced in Bmpr1a-KO mice (Figure 4(a)). These observations are consistent with our finding that Bmp4 was induced by CCl4 treatment.

To evaluate the hepatic function in the CCl4-treated liver of Bmpr1a-KO mice, we determined the expressions of hepatic genes by RT-PCR (Figure 4(a)). The reduced expression of the albumin gene in the injured liver was increased at 72 h posttreatment with CCl4 in mock-infected and Ad-LacZ-infected Bmpr1a flox− mice as controls, whereas little restoration was observed in Bmpr1a-KO mice. Similarly, the expressions of the aldolase B and tryptophan 2,3-dioxygenase (Tdo2) genes in Bmpr1a-KO mice were hardly recovered, compared with control mice. These observations were confirmed by quantitative real-time RT-PCR (Figure 4(b)). Meanwhile, the reduced expression of the aldolase B and
Figure 3: Liver injury in BMPR1A-KO mice. (a) Time course for the experiment. BMPR1A<sup>flox/−</sup> mice were infected with mock, Ad-Cre or Ad-LacZ for 14 days, followed by intraperitoneal administration of CCl<sub>4</sub> for the indicated time. (b) Hematoxylin and eosin staining of tissue sections from the injured liver in BMPR1A<sup>flox/−</sup> mice. The hepatotoxicity of CCl<sub>4</sub> causes necrotic damage to the centrilobular hepatocytes at 24 h. The recovery from the liver injury in BMPR1A-KO mice is retarded at 72 h after CCl<sub>4</sub> injection compared with control mice. Scale bars: 50 μm. (c) AST activities in serum samples from CCl<sub>4</sub>-treated mice. All data are shown as the means ± SE from three independent experiments.
Figure 4: Gene expressions in the CCl₄-injured liver of BMPRIA-KO mice. (a) RT-PCR was performed in duplicate with total RNA from CCl₄-injured livers of BMPRIA<sup>flox/⁻</sup> mice infected with mock, Ad-LacZ or Ad-Cre. Short size PCR products in BMPRIA (arrowhead in top panel) shows the deletion in BMPRIA mRNA generated by Cre recombination. (b) Real-time RT-PCR of Id1, albumin, and Tdo2. Relative expression was shown by ratio of albumin expression levels normalized by β-actin internal control to the value in 0-time of CCl₄-treated liver. All data are shown as the means ± SE from three independent experiments. *P < 0.05, significant difference by Student’s t-test.

Phosphoenolpyruvate carboxykinase (Pepck) gene by CCl₄ treatment was not restored in either the control mice or Bmpr1a-KO mice. Moreover, the expression of the transferrin gene was barely or not influenced by CCl₄ treatment and the absence of BMP signaling. These findings show that some of the hepatic gene expressions reduced by the hepatotoxin were recuperated through BMP signals expressed in the wounded liver, suggesting a role for the transient expression of Bmp4.

3.4. Decreased Hepatocyte Proliferation in Bmpr1a-KO Mice. Hepatocyte proliferation and differentiation are necessary
Figure 5: Proliferative activity in the CCl₄-injured liver of BMPR1A-KO mice. (a) Immunohistochemistry for Ki-67, a cell cycle marker, in the CCl₄-injured liver. BMPR1A<sup>−/−</sup> mice were infected with mock or Ad-Cre for 14 days, and BMPR1A<sup>+/−</sup> mice were infected with Ad-Cre as a control. Brown nuclei indicate Ki-67-positive cells. Scale bars: 25 μm. (b) Quantitative analysis of Ki-67-positive cells. Ki-67-positive cells were counted in 10 randomly taken microscopic photos, and are shown as percentages relative to the total cell number. All data are shown as the means ± SE from three independent experiments. *P < 0.05, significant difference by Student’s t-test.
for the healing process after liver injury. We determined the proliferative activity in the injured liver by evaluating the expression of proliferating cell nuclear antigen (PCNA) by RT-PCR (Figure 4(a)) and the cell cycle marker Ki-67 by immunohistochemistry (Figure 5). Bmprrla-KO mice treated with CCl4 showed significantly decreased expression of PcnA at 24 and 72 h post-treatment, compared with mock-infected and Ad-LacZ-infected mice. Furthermore, Ki-67-positive cells were markedly fewer in number in the liver of Bmprrla-KO mice at 72 h after CCl4 treatment, while the number of proliferating cells was increased in the liver of control mice after CCl4 injection (Figure 5). These findings indicate that BMP signaling was involved in the cell proliferation during the wound healing response in the CCl4-injured liver.

4. Discussion

Chronic liver diseases are aggravated by repeated cycles of injury and repair in liver cells [7, 19]. Therefore, understanding the mechanism and regulation of the elementary processes in the wound healing response may lead to novel therapeutic methods for these liver diseases. In this study, we observed that a single injection of CCl4 into mice induced transient expression of Bmp4, which is involved in hepatogenesis in early embryos. This finding suggests that the processes involved in liver development are tightly associated with the repair of acute liver injury. BMP4 is also involved in hepatogenesis, while Bmp7 was reported to facilitate regeneration of the injured kidney [20].

Previously, we reported that Bmp2 or Bmp4 were induced in hepatocyte progenitor or oval-like cells, but not in Kupffer or macrophage cells, during liver injury [11]. Oval cells are hepatic stem-like cells (progenitor cells) derived from bone-marrow cells [21–25]. The mechanism underlying the induction of Bmp4 expression after liver injury remains unknown, and it needs to be clarified. In this study, we have shown a crucial role of BMP signaling in the proliferation and differentiation of hepatic cells, including progenitor cells, in the response to liver injury induced by CCl4 using Bmprrla-KO mice. In the early stage of embryonic development, Bmp2 or Bmp4 derived from the cardiac mesoderm or septum transversum mesenchyme are required for morphogenetic movement of the liver bud, including hepatic competence and endodermal patterning in the foregut ventral endoderm expressing Gata-4 [12, 26]. Therefore, we can consider that the BMP signaling in the wound healing response to liver injury in adult rodents may imitate hepatogenesis in the early embryo.

Previously, we showed that hepatic stem-like cells differentiate in a stepwise manner in vitro in response to a series of cytokines and extracellular matrix components, such as type I collagen, TGF-β, hepatocyte growth factor, and oncostatin M [27]. This process also mimics hepatocyte differentiation in the early step of embryogenesis. In the present study, we have shown a pivotal role of BMP signaling in the wound healing of acute liver injury, and also that hepatic genes such as albumin and Tdo2 respond significantly to BMP signaling, although aldolase B and Pepck did not recover from the injury. Interestingly, transferrin gene expression was independent of the injury and BMP signaling. This observation suggested to us that the proliferation and differentiation of hepatocytes are regulated by BMP signaling partially or only in one of the steps. However, it still remains a possibility that the deletion in BMP signaling enhanced CCl4 injury by some metabolic alteration resulting in a delayed healing response.

Regarding the role of BMP signaling, our results are consistent with recent reports that regeneration in Bmprrla-KO zebrafish is delayed after partial hepatectomy [28]. Furthermore, Id3, a target gene of BMP signaling, was reported to have an important role in the proliferation and differentiation of hepatoblasts during chick liver development [29]. Liver-specific knockout of the Bmprrla gene after Ad-Cre infection is a very useful tool for elucidating the important role of BMP signaling in the wound healing response, and for the development of therapeutic protocols for hepatic disease based on the mechanism of the healing process.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The work was supported by KAKENHI (20590282) from JSPS. The authors would like to thank Drs. Naohiro Hori and Haruaki Ninomiya, Tottori University, for their many helpful discussions.

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