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Promotion of Liver Regeneration/Repair by Farnesoid X Receptor in Both Liver and Intestine

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Abstract

FXR is a member of the nuclear receptor superfamily and is the primary bile acid receptor. We previously showed that FXR was required for the promotion of liver regeneration/repair after physical resection or liver injury. However, the mechanism by which FXR promotes liver regeneration/repair is still unclear. Here we showed that both hepatic-FXR and intestine-FXR contributed to promoting liver regeneration/repair after either 70% partial hepatectomy or CCl₄-induced liver injury. Hepatic FXR, but not intestine FXR, is required for the induction of Foxm1b gene expression in liver during liver regeneration/repair. In contrast, intestine FXR is activated to induce FGF15 expression in intestine after liver damage. Ectopic expression of FGF15 was able to rescue the defective liver regeneration/repair in intestine-specific FXR null mice.

Conclusion—These results demonstrate that, in addition to the cell-autonomous effect of hepatic FXR, the endocrine FGF15 pathway activated by FXR in intestine also participates in the promotion of liver regeneration/repair.

Keywords

FXR; FGF15/19; bile acids; liver regeneration; CCl₄

Introduction

FXR (Farnesoid X receptor) is a member of the nuclear receptor superfamily. It is now well known that FXR plays a critical role to protect cells against bile acid-induced toxicity. FXR is not only a master regulator of bile acid homeostasis and detoxification^{1–4}, but also mediates a novel role of bile acid signaling in promoting liver regeneration/repair^{5,6}.

Liver regeneration or repair is a compensatory re-growth of liver after liver damage, including physical resection or toxic injury. Many genes and signaling pathways, such as cytokines and growth factors, have been identified to initiate or promote this process of liver re-growth. We recently showed that bile acid signaling was activated after 70% partial hepatectomy (PH) and FXR was required to promote liver regeneration^{5–7}. FXR has at least two roles during liver regeneration. One role is to suppress cholesterol 7 α -hydroxylase (CYP7a1) expression and reduce bile acid stress. The other role is to promote hepatocyte

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proliferation by directly activating Foxm1b that is a key regulator of hepatic cell cycle progression⁷. Moreover, FXR is critical to promote liver repair after injury induced by a liver toxin, carbon tetrachloride (CCl₄)^{8,9}. More interestingly, FXR null mice spontaneously develop liver tumors when they age^{10,11}. Because bile acids are known to cause DNA damage and induce cell transformation if their levels are not controlled, FXR's roles in suppressing bile acid synthesis as well as promoting liver repair could be an intrinsic mechanism to protect liver from tumorigenesis^{12,13}.

Although bile acids are synthesized in the liver, FXR in both liver and intestine are required to control levels of bile acids. FXR represses CYP7a1 gene expression through the coordinated induction of fibroblast growth factor 15 (FGF15) in intestine and short heterodimer partner (SHP) in liver. FGF15 and SHP then act cooperatively to repress CYP7a1 transcription through a mechanism that is not yet understood¹⁴. Mice with deletion of either FGF15 or SHP have markedly elevated basal CYP7a1 expression. Mice with intestine-specific deletion of FXR lost the suppression of CYP7a1 expression after treatment with a FXR ligand, GW4064, suggesting that FXR in the gut is key to regulate bile acid synthesis in the liver¹⁵. Moreover, FGF15 has been shown to promote hepatocyte proliferation through its receptor (FGFR4) in liver¹⁶. FGFR4-deficient mice exhibited increased liver injury and delayed liver repair after injury¹⁷. All these results highlight an endocrine role of FGF15 from intestine to the liver. However, whether FGF15 has a role in liver regeneration/repair is unclear.

In this study, we took advantage of liver- and intestine-specific FXR null mice and showed that both hepatic FXR and intestinal FXR contributed to promoting liver regeneration/repair. We further demonstrated that FGF15 induced by intestine FXR was an endocrine pathway to promote liver regrowth.

Materials and Methods

Animal maintenance and treatments

FXR whole body knockout mice (KO) were described previously [5]. Liver specific FXR null mice (Δ L-FXR) and intestine specific FXR null mice (Δ IN-FXR) were generated by University of Southern California. All procedures followed the NIH guidelines for the care and use of laboratory animals. Mice were housed in a pathogen-free animal facility under standard 12h light/dark cycle and fed standard rodent chow and water ad libitum. Male mice between 8 and 10 weeks old were used in each group of experiments. 3–6 mice were used in each group of the experiment.

Characterization and genotyping protocol

Total proteins from livers or ileal mucosa of liver-specific (Δ L-FXR) and intestine-specific (Δ IN-FXR) FXR-null mice and FXR flox/flox (FXR Fl/Fl) controls were extracted and subjected to Western blot analysis. Tail biopsies from animals were analyzed by PCR. The presence of cre allele was detected by primers ML136 and ML137, resulting in a 500-bp PCR product. Primer sequence information is provided in supplemental table 1.

Hepatectomy and liver regeneration

Partial hepatectomy (PH) was done according to the method of Higgins and Anderson¹⁸. Left lateral, caudate and median lobes were completely excised and the gallbladder was left intact as previously described⁵.

CCl₄ and liver regeneration

For acute CCl₄-induced liver damage and liver regeneration study, a single dose of 1.5 ml/kg of body weight was administered by intraperitoneal (IP) injection as previously described¹³.

Liver histology

As described by Huang et al.⁵ and Zhang et al.⁷, briefly to say, after mice were euthanized, their livers were removed and small pieces from different lobes of the livers were fixed in 4% formaldehyde-PBS solution, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E). For 2-bromodeoxy-uridine (BrdU) staining, mice were i.p. injected with BrdU solution (10mg/kg body weight) 2 hours before euthanasia. Liver sections were prepared and stained using a BrdU staining kit (Roche, Indianapolis, IN). The number of positively stained cells was counted in at least 3 randomly selected fields for each tissue section. The percentage of liver necrosis areas was assigned a score on a semiquantitative scale where 0 is defined as no necrosis area at 0h after CCl₄ treatment: 1 is mild (30–40%), 2 is moderate (40–50%), 3 is severe (50–60%) and 4 is the most severe (60–80%).

Adenovirus FGF15 purification and mice treatment

Viruses were propagated in 911 cells as reported previously¹⁴ and purified by using the adenovirus purification kit (Clontech). Mice were infected with adenovirus by injection into the tail vein as previously described¹⁴. Each mouse received 1.0×10^9 particles/10g body weight in 0.1 ml of saline. 3 days later, mice were either euthanized as control group (0h), treated with CCl₄ (40h) or subjected to 70% PH (40h). Total RNA and liver sections were prepared at 0h and 40h after liver regeneration.

RNA analysis

Total liver RNA was extracted using TRIzol Reagent from Invitrogen((Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative real time PCR was performed using SYBR Green PCR Master Mix and an ABI prim 7300 Sequence Detection System (Applied Biosystems, Foster city, CA, USA). Murine 36B4 was used as internal controls. PCR primers specific for each gene are listed in supplemental table 1

Western Blotting

Livers or ileums were homogenized in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF and proteinase inhibitor cocktail). Proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane and detected by chemiluminescence (Supersignal, Pierce). Western blotting was performed by using antibodies (anti-FXR and β -Actin) from Santa Cruz Biotechnology company (Santa Cruz, CA).

Serum bile acids

Serum after 70% PH or CCl₄ treatment were collected and the bile acids were measured using a kit from Diagnostic Chemicals Limited (Carlottetown, PE, Canada).

Statistic analysis

Data are expressed as means $\pm$ SD. Two-tailed Student's *t* test was used to determine significant differences between data groups. All analyses were performed using one-way analysis of variance (ANOVA). *P* < 0.05 was considered statistically significant.

Results

Generation of the Δ L-FXR mice and Δ IN-FXR mice

A conditional FXR gene allele (FXR neo-flox) was generated in ES cells. Two loxP sequences flank exons 4, 5 and 6 of the murine FXR allele (FXR Fl/Fl). FXR Fl/Fl mice were crossed with the albumin-cre or villin-cre mice to delete FXR gene specifically in liver or intestine respectively. After correct genotyping, western blotting to measure FXR protein were performed using total protein extracted from liver and ileum. The results indicated no FXR expression in the liver of Δ L-FXR mice. However, liver FXR protein levels were comparable between the FXR Fl/Fl and Δ IN-FXR mice (Fig. 1A). Similarly, no ileum FXR expression was detected in the Δ IN-FXR mice (Fig. 1B).

Defective liver regeneration in Δ L-FXR mice

We previously showed that FXR in liver was required for promoting liver regeneration. To confirm the previous observation that hepatic FXR is required to promote liver regeneration, we compared the liver regeneration after 70% PH in FXR Fl/Fl, Δ L-FXR and FXR KO mice. As expected, a significant delay in hepatocyte proliferation was observed in Δ L-FXR animals compared to FXR Fl/Fl mice at 24h, 36h and 72h after surgery. Less BrdU positive hepatocytes were presented in Δ L-FXR mice than in FXR Fl/Fl mice (Fig. 2A). In FXR Fl/Fl mice, the hepatocyte proliferation peaked at 36 h after 70% PH, but this peak was strongly reduced in Δ L-FXR mice compared to the FXR Fl/Fl mice (Fig. 2A). These results suggest that hepatic FXR is required to promote liver regeneration. However, to our surprise, comparing to Δ L-FXR mice, FXR KO mice showed significant decreased BrdU incorporation in the liver at 36h and 72h (Fig. 2A), suggesting that FXR in other tissues may also contribute to a maximum effect on promoting liver regeneration.

We also compared the serum bile acid levels in FXR Fl/Fl, Δ L-FXR and FXR KO mice. As expected, serum bile acid levels were significantly higher in the FXR KO and Δ L-FXR mice comparing to the FXR Fl/Fl mice at 24h and 36h after 70% PH. On day 3, serum bile acid levels in Δ L-FXR mice returned to a comparable level compared to the control mice. However, bile acid levels were still significantly higher in FXR KO mice at day3 (Fig. 2B). This suggests that, although hepatic FXR plays a role in suppressing bile acid levels after 70% PH, FXR in other tissues such as intestine may be required to suppress bile acid levels at later stages after 70% PH. Consistently, the gene encoding the rate limiting enzyme of bile acids synthesis, CYP7a1, was suppressed in all three groups of mice after 70% PH, but CYP7a1 mRNA levels were much higher in Δ L-FXR and FXR KO mice compared to the FXR Fl/Fl mice (Fig. 2C). FXR was shown previously to directly activate the Foxm1b gene after 70% PH⁶. We measured the mRNA levels of Foxm1b after 70% PH and found that the induction of Foxm1b was blocked in both Δ L-FXR and FXR KO mice comparing to that in FXR Fl/Fl mice at 36h after PH (Fig. 2D). Therefore, hepatic FXR is responsible for the hepatic induction of Foxm1b gene expression during liver regeneration.

Impaired liver repair in Δ L-FXR mice after CCl₄-induced injury

We previously showed that FXR is also required to promote liver repair following CCl₄-induced liver injury^{8,19}. We therefore ask whether the hepatic FXR plays the same role in this liver repair model. Following a single dose of CCl₄ injection, both Δ L-FXR and FXR KO mice displayed defective liver regeneration comparing to the FXR Fl/Fl mice during the first 3 days (Supp. Fig. 1A). But there was a significant less BrdU positive hepatocytes in FXR KO mice compared to Δ L-FXR mice (Fig. 3A). We next analyzed the serum bile acid levels after CCl₄ treatment and found that FXR KO mice had much higher bile acid levels in serum than the other 2 groups of mice during the first day after CCl₄ injection (Fig. 3B). But in all three groups of mice, CYP7a1 mRNA levels were dramatically suppressed (Fig. 3C).

Consistently, the induction of Foxm1b gene expression also mainly depended on hepatic FXR activation because its mRNA levels were significantly lower in both Δ L-FXR and FXR KO mice comparing to FXR Fl/Fl mice (Fig. 3D). Similarly, the Cyclin D1 expression levels were much lower in Δ L-FXR and FXR KO mice than that in FXR Fl/Fl mice (Fig. 3E).

However, H&E staining showed that FXR KO mice displayed much extensive liver injury comparing to that in Δ L-FXR mice (Supp. Fig. 1B). Scores of the liver necrosis areas showed significant differences between Δ L-FXR and FXR KO mice (Fig. 3F). These results suggest that, in addition to liver, FXR in other tissues may be important to protect liver injury and promote liver repair.

Intestine FXR contributes to the liver regeneration/repair after either 70% PH or CCl₄-induced injury

Since FXR in intestine is crucial for the feedback regulation of bile acid synthesis in liver and FXR KO mice display more severe defect of liver regeneration compared to Δ L-FXR mice, we hypothesize that intestine FXR may also play roles in the liver regeneration/repair. Therefore, we compared the liver regeneration between Δ IN-FXR and FXR Fl/Fl mice after 70% PH. The hepatic BrdU incorporation was significantly lower in Δ IN-FXR mice compared to FXR Fl/Fl mice at 48h after 70% PH (Fig. 4A). Consistent with a key role of intestine FXR in regulating bile acid levels, Δ IN-FXR mice had higher serum bile acid levels comparing to that in FXR Fl/Fl mice (Fig. 4B). Although the CYP7a1 gene was suppressed in both Δ IN-FXR and FXR Fl/Fl mice, the expression levels were much higher in Δ IN-FXR mice at 48h and 72h after 70% PH (Fig. 4C). Interestingly, we observed a strong induction of FGF15 and SHP gene expression in the intestine of FXR Fl/Fl mice on first two days of liver regeneration (Fig. 4D and E). However, this induction was absent in Δ IN-FXR mice (Fig. 4D and E).

Similarly, the number of BrdU positive hepatocytes was lower on first 2 days after CCl₄-induced liver injury. At 40h, there were significant less proliferative hepatocytes in Δ IN-FXR mice compared to that in FXR Fl/Fl mice (Fig. 5A). We also compared the necrosis areas in liver induced by CCl₄. As shown in Fig. 5B and Supp Fig. 2, CCl₄ caused more severe liver injury in Δ IN-FXR mice than in FXR Fl/Fl mice. Though the CYP7a1 expression levels were decreased in both Δ IN-FXR and FXR Fl/Fl mice after CCl₄ injection, the expression levels of CYP7a1 in the Δ IN-FXR mice were significantly higher comparing to that in FXR Fl/Fl mice (Fig. 5C). This confirms that intestine FXR plays an important role in the regulation of CYP7a1 expression. We next measured the FGF15 expression levels in intestine and found that the induction of the FGF15 in the FXR Fl/Fl mice was blocked in Δ IN-FXR mice (Fig. 5D).

Ectopic expression of FGF15 rescues the defective liver regeneration/repair in Δ IN-FXR mice

FGF15 is a hormone that can mediate the effect of intestine FXR to regulate bile acid levels in liver. Since we observed that intestine-specific deletion of FXR resulted in greater defective liver regeneration/repair induced by 70% PH and CCl₄, therefore, we used both of the models to ask whether FGF15 plays a role in promoting liver regeneration/repair. Δ IN-FXR and FXR KO mice were injected with either a recombinant adenovirus that expresses FGF15 or a control adenovirus, and then 70% PH was performed or a single dose of CCl₄ were administered. We first confirmed that the FGF15 adenovirus infection increased FGF15 expression in Δ IN-FXR and FXR KO mice (Fig. 6A and 6B). We then observed that hepatic BrdU incorporation was significantly increased in Δ IN-FXR and FXR KO mice after FGF15 adenovirus injection comparing with the control mice receiving the adenovirus

alone after 70% PH at 40 h (Fig. 6C). Similar results were also observed in a toxic CCl₄ induced liver injury model (Fig. 6D and Supplemental Fig.3). BrdU incorporation was significantly increased in adenovirus FGF15 expression group comparing with the control group in Δ IN-FXR and FXR KO mice. CYP7a1 expression levels were down regulated in the FGF15 infected mice compared to the controls in either the 70% PH model (Fig. 6E) or CCl₄ model (6F). These results indicate that FGF15 activated by intestine FXR indeed participates in promoting liver regeneration/repair.

Discussion

We previously showed that FXR was required for normal liver regeneration and liver repair after injury. However, the mechanism by which FXR regulates this process is still unclear. In this report, we show that hepatic and intestine FXR use distinct mechanism to promote liver regeneration/repair.

Liver regeneration is regulated by many signals from hepatic environment. Different signal pathways will lead to the activation of transcription factors that either stimulate hepatocyte proliferation or promote cell survival to promote liver regrowth^{5,20}. We previously showed that FXR bound to an FXRE in Foxm1b intron 3 and induced Foxm1b gene transcription during liver regeneration⁶. In FXR KO mice, this Foxm1b induction was blocked and liver regeneration was delayed. Consistent with these results, we observed that the induction of Foxm1b expression is dramatically reduced in Δ L-FXR mice comparing with the control mice after either 70% PH or CCl₄-induced liver injury. In contrast, the induction of Foxm1b was not affected in Δ In-FXR mice after liver damage, indicating the requirement of a cell autonomous mechanism for hepatic FXR to activate Foxm1b and potential other factors that are involved in regulating cell cycle in liver.

Bile acids are potentially toxic and substantial increases in hepatic bile acid levels will induce hepatocyte death²¹. We previously demonstrated that FXR was activated by elevated bile acid influx during liver regeneration⁵. The importance for a stringent control of bile acid levels is highlighted by a delicate regulation of CYP7a1 expression. The identified regulators of CYP7a1 expression include cytokines, growth factors^{22–26} and nuclear receptors^{27,28}. During liver regeneration, hepatic bile acid levels need to be suppressed rapidly to prevent the toxic effect of increased bile acids in liver as shown by a dramatic down regulation of CYP7a1 mRNA levels^{5,7}. We previously showed that, in addition to FXR-SHP axis, hepatocyte growth factor and JNK pathways were involved in suppressing CYP7a1 expression during the acute phases of liver regeneration⁷. In current study, we now further demonstrate that, during liver regeneration/repair, FXR also activates the expression of FGF15 in the intestine to suppress CYP7a1 transcription. Consistently, several reports also suggest that FGF15 secreted from ileum has profound effects on the liver metabolism^{14,29,30,31}. Because we previously showed that the suppression of CYP7a1 expression and decreased bile acid synthesis was beneficial for liver regeneration, we therefore conclude that FGF15 induction after liver damage may also contribute to the normal liver regeneration.

The most novel observation in this report is the delayed liver regeneration/repair and increased liver injury in Δ IN-FXR mice compared to FXR Fl/Fl control mice after either 70% PH or CCl₄ injection. These results identify an unexpected role of intestine FXR in regulating liver regeneration/repair. It is clear that intestine FXR is key to control the bile acid levels. Thus, higher levels of bile acids in Δ IN-FXR mice after liver injury may hamper the normal liver regeneration/repair. Besides its effect on bile acid levels, the metabolic and mitogenic activities of FGF15 cannot be excluded. Moreover, the hydrophobic bile acid, deoxycholic acid (DCA) is significantly increased in fecal extracts from intestine FXR null

mice but not from FXR KO or liver FXR null mice¹⁵, and DCA may cause hepatocyte apoptosis and the colon inflammation and necrosis^{32,33,34}. This may also be a protective function of intestine FXR during liver regeneration/repair. We further showed that intestine FXR induced FGF15 expression after liver injury, which in turn suppressed the CYP7a1 transcription and lowered serum bile acid levels. Exogenous delivery of FGF15 rescued the defect of liver repair in Δ IN-FXR and FXR KO mice. However, we would like to mention that ectopic expression of FGF15 via adenovirus, which is an effective model to overexpress FGF15, may generate some side effect and liver toxicity due to virus infection. A better delivery approach of FGF15 will be needed in the future.

Our results strongly suggest a promotive effect of FGF15 in liver regeneration/repair. FGF15 has also been shown to down-regulate the Foxo1 gene expression and the Foxo1 is associated with cell cycle arrest and growth inhibition³⁵. This may also contribute to the overall effect of intestine FXR and FGF15 in promoting liver regeneration/repair. Furthermore, a recent report indicates that selective activation of intestine FXR or treating mice with FGF19 could reduce liver necrosis and inflammatory cell infiltration in cholestasis mouse models³⁶. Taken together, we conclude that intestine FXR and its induction of FGF15 may have more important roles in liver protection than we previously thought.

In summary, our results confirm a critical role of hepatic FXR in inducing Foxm1b expression and promoting liver regeneration/repair. Moreover, our studies demonstrate that intestine FXR activates FGF15 expression in the intestine to promote liver regeneration/repair. Therefore, in addition to the cell-autonomous effect of hepatic FXR, the endocrine FGF15 pathway induced by FXR in intestine also participates in the promotion of liver regeneration/repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Generation of Δ L-FXR and Δ In-FXR mice

Western blot analysis of FXR protein levels for the indicated mice. Liver protein extracts (A) and ileum protein extracts (B) were prepared and immunoblotted with FXR and β -actin antibodies.

Fig 2. Defective liver regeneration in Δ L-FXR and FXR KO mice after 70% PH
 BrdU incorporation were counted on FXR FI/FI, Δ L-FXR and FXR KO mice at indicated times after 70% PH (24h, 36h, 48h and 72h) (A). Total bile acid levels were measured in serum (B). CYP7a1 (C) and Foxm1b (D) gene expression were measured at indicated times after PH. Δ L-FXR: Δ L; FXR KO: KO.

Fig 3. Impaired liver regeneration of Δ L-FXR mice and FXR KO mice after CCl_4 -induced liver injury

(A) Brdu positive hepatocytes were counted at 24h, 40h, 48h and 72h after CCl_4 injection. For each group of mice, 3–7 mice were used for each time points. Total serum bile acids were measured (B). Gene expression of CYP7a1 (C), FOXm1b (D) and CyclinD1 (E) were measured in FXR FI/FI, Δ L-FXR and FXR KO mice after CCl_4 treatment at indicated time points. (F) Semi-quantification of liver necrosis areas of H&E staining in FXR FI/FI, Δ L-FXR and FXR KO mice at 24h, 40h, 48h and 72h after CCl_4 injection.

Fig 4. Defective liver regeneration in Δ IN-FXR mice after 70% PH

PH was performed in FXR FI/FI and Δ IN-FXR mice and BrdU staining was measured at 24, 48 and 72h after PH (A), and total serum bile acids was measured at the indicated time (B). Liver CYP7a1 mRNA levels were measured (C). Ileum FGF15 (D) and SHP (E) were measured by quantitative real-time PCR.

Fig 5. Impaired liver regeneration in Δ IN-FXR mice after CCl_4 -induced liver injury
 (A) Quantification of BrdU staining of hepatocytes after CCl_4 injection in FXR Ff/Ff and Δ IN-FXR mice. (B) Semi-quantification measurements of necrosis areas of H&E staining liver sections from FXR Ff/Ff and Δ IN-FXR mice at indicated time points after CCl_4 treatment. Total liver RNA was isolated for quantitative real-time PCR analysis of liver CYP7a1 (C) and ileum FGF15 (D) mRNA levels at 24h, 40h, 48h and 72h after CCl_4 injection in FXR Ff/Ff and Δ IN-FXR mice.

Fig 6. Ectopic expression of FGF15 rescued defective liver repair

Measurement of FGF15 levels after FGF15 adenovirus infection in Δ IN-FXR and FXRKO mice before and after 70% PH (40h) (Fig. 6A) and measurement of FGF15 levels after FGF15 adenovirus infection in Δ IN-FXR and FXR KO mice before and after CCl₄ (40h) injection (Fig. 6B). Quantification of the BrdU positive hepatocytes at 0h and 40h after 70% PH in Δ IN-FXR and FXR KO mice (Fig. 6C) and quantification of the BrdU positive hepatocytes at 40h after CCl₄ injection with and without FGF15 adenovirus injection (Fig. 6D). CYP7a1 gene expression levels were analyzed in Δ IN-FXR and FXR KO mice after virus infection in 70% PH model (Fig. 6E) and in CCl₄ liver toxic model (Fig. 6F).