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Functional expression of human type I interferon receptors in the mouse liver

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Abstract

We expressed human type I interferon (IFN) receptors (IFNAR) in mice and investigated their signaling. Using a hydrodynamics-based delivery method, vectors containing the genes for IFNAR1 and IFNAR2 were transferred into mice. Six hours after gene-transfer, mice were intravenously injected with human IFN-α at 10,000 IU. IFNAR1 and IFNAR2 were both expressed in the liver, but not spleen or lung. The receptors were coexpressed in single liver cells. One hour after IFN-α injection, the phosphorylation status of signal transducer and activator of transcription factor 1 (STAT1), a key molecule of IFN signaling, was determined. Phosphotyrosine-STAT1 (p-STAT1), localized to the nucleus of IFNAR-expressing cells, was increased in the livers of IFNAR gene-transferred mice but not in control vector-transferred animals. In conclusion, functional human IFNAR can be delivered to the mouse liver, resulting in an increase in p-STAT1 levels following human IFN-α stimulation.

Keywords: Interferon receptor; IFNAR1; IFNAR2; Gene transfer; Hydrodynamics-based transfection; Interferon; STAT1; Phosphorylation; Mice; In vivo

Type I interferon (IFN) is a clinically effective drug against viral infectious diseases such as hepatitis B, hepatitis C, and malignant cancers such as chronic myelogenous leukemia and melanoma [1–3]. The multifunctional activities of type I IFNs are mediated by its binding to specific receptors on the cell surface; consequently, the number of receptors on the cell surface is an important factor in the efficacy of IFNs against several diseases.

Type I IFN receptors (IFNAR) are composed of 2 subunits: IFNAR1 and IFNAR2. IFNAR1 exists in a single molecular form, and IFNAR2 in 3 molecular forms: a soluble receptor protein (IFNAR2a), and short (IFNAR2b) and long (IFNAR2c) transmembrane forms. It has been suggested that both IFNAR1 and IFNAR2c are required to be coexpressed to form a high-affinity receptor and exert a full-biological response to IFN-α [4,5].

When IFN binds to its receptors, they are activated by IFN receptor-associated tyrosine kinases, and the activated receptor-tyrosine kinase complexes phosphorylate signal transducer and activator of transcription factor 1 (STAT1) and 2 (STAT2). Phosphorylated STAT1 (p-STAT1) and STAT2 (p-STAT2) form heterotrimers with p48, and are translocated into the nucleus to activate the transcription of target genes, including those for several antiviral proteins, tumor suppressors, and apoptotic proteins.

To predict the clinical response to IFN-α therapy, IFNAR levels were measured in patients with hepatitis, hepatocellular carcinoma (HCC), renal cancer, or leukemia. Several investigators have suggested that IFNAR levels in the livers of patients with hepatitis C were significantly higher in sustained responders than in nonresponders [6–9]. It was also reported that protein and mRNA expression levels of IFNAR2 correlate well with IFN-α response in patients with chronic myelogenous leukemia [10,11].

These studies suggested that more efficient IFN-α therapy might be achieved through upregulation of IFN receptors.

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In fact, when IFN receptors were upregulated in a hepatoma cell line, by treatment with IFN-γ [12] or 9-*cis* retinoic acid [13], combined treatment with IFN-α and IFN-γ or 9-*cis* retinoic acid increased the intracellular levels of the antiviral protein 2′,5′-oligoadenyl-5′-triphosphate synthetase and decreased levels of hepatitis C virus RNA. Additionally, in vitro transfection and enhanced expression of IFNAR2c in cancer cell lines markedly increased the sensitivity of these cells to the antiproliferative effects of IFN in a xenograft tumor model [14]. However, a demonstration of in vivo augmentation of IFN receptors has not yet been reported.

In the present study, we transferred human IFNAR1 and IFNAR2 genes into mice using a hydrodynamics-based delivery method, and demonstrate that upregulated IFNARs augment IFN action in vivo. The species specificity of IFN action makes it possible to study human IFNAR without any effects due to endogenous IFNAR; hence, transferred human IFNAR was detected in mice using anti-human IFNAR antibodies, and its function was determined by measuring levels of phosphorylated STAT1 following human IFN injection.

Materials and methods

Animals. Male ICR mice, weighing 30–40 g at the time of use, were purchased from Japan SLC, Inc. (Shizuoka, Japan). All animals were kept at room temperature and given sufficient water and food. All experimental procedures were performed under the Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd. (version revised in April, 2004).

Construction, amplification, and purification of plasmid DNA. To construct an expression vector, the human ubiquitin B (UBB) promoter, and intron were amplified by polymerase chain reaction (PCR) as previously described [15], then inserted into the pcDNA3.1(+) vector (Invitrogen, CA, USA). The pcDNA3.1(+) vector sequence from the 3′ end of the cytomegalovirus (CMV) enhancer region to the *Hin*dIII site of the multiple cloning region (bases 722–910) was replaced with the human UBB promoter and intron (pCMVenhUBBI). To construct pCMVenhUBBI-IFNAR1 and pCMVenhUBBI-IFNAR2 vectors, the coding regions of human IFNAR1 and IFNAR2c cDNAs were amplified from Daudi cells using reverse transcriptase (RT)-PCR, then inserted into the *Eco*RI–*Xba*I site of the multiple cloning region of the pCMVenhUBBI vector. Plasmid DNAs were purified using the EndoFree Plasmid Mega Kit (Qiagen, Germantown, MD, USA).

In vivo IFNAR gene transfection, IFN administration, and sampling of organs. Mice were rapidly injected with 20 μg of control plasmid DNA (pCMVenhUBBI) or 10 μg each of the IFNAR1 (pCMVenhUBBI-IFNAR1) and IFNAR2 (pCMVenhUBBI-IFNAR2) plasmid DNAs, in 3 mL of saline via the tail vein. Six hours after injection, the animals were intravenously injected with 10,000 IU of human IFN-α (OIF™, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) or its vehicle, phosphate-buffered saline (PBS). Human IFN-α (OIF™) was derived from Sendai virus-induced BALL-1 cells and was composed mainly of IFN-α2 and IFN-α8 [16]. When p-STAT1 in the liver was assayed at 0.5, 1, 2, 18, and 24 h after mouse IFN-α injection, a peak increase in p-STAT1 was observed after 1 h. Therefore, mice were anaesthetized with ether 1 h after human IFN-α injection, and the quadrate liver, spleen, and right lung were carefully dissected. Tissues were washed with PBS containing 10 U/mL heparin. For Western blotting analysis, the organs were stored at −20 °C. For immunohistochemical analysis, the quadrate liver was cut into 4 pieces and immediately frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan) using dry ice.

Measurement of IFNAR1, IFNAR2, p-STAT1, and STAT1 by Western blotting analysis. A volume of 1.5 mL of a lysing buffer (50 mM Tris–HCl, pH 8.0, 280 mM NaCl, 0.2 mM EDTA, 2 mM EGTA, 100 μM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40™, and 10% glycerol) was added to each of the frozen organs, which were homogenized and centrifuged for 30 min at 30,000*g* at 4 °C. Protein concentrations in the supernatants were determined using the Lowry assay (Bio-Rad Laboratories, CA, USA). The supernatant was mixed with a loading buffer, boiled for 5 min, and subjected to SDS–PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies for 16 h. The primary antibodies anti-IFNAR1, anti-IFNAR2, anti-p-STAT1 (Cell Signaling, MA, USA), and anti-STAT1 antibodies were used at 1:1000, 1:5000, 1:1000, and 1:1000 dilutions, respectively. Anti-IFNAR1 and anti-IFNAR2 were obtained by immunizing rabbits with the extracellular domains of human IFNAR1 and IFNAR2 [17]. Nitrocellulose membranes were washed with 0.9% NaCl containing 0.05% Tween 20 and incubated in a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibodies (Cell Signaling, MA) for 1 h. Protein bands were visualized by enhanced chemiluminescence (Cell Signaling, MA). Images of p-STAT1 and STAT1 bands were scanned and analyzed using Scion Image (Scion Corporation, MD, USA).

Immunohistochemical staining. Frozen liver samples embedded in OCT compound were cut to a thickness of 5 μm using a cryostat (Leica microsystems, Wetzlar, Germany). Sections were fixed in 4% paraformaldehyde for 1 h and stained using an indirect immunoperoxidase method for detecting IFANAR1 and IFANR2. First, they were incubated in 3% cold hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase. After washing, they were incubated in PBS containing 5% fetal bovine serum for 2 h, then a 1:500 dilution of anti-IFNAR1 antibody or a 1:2000 dilution of anti-IFNAR2 antibody at room temperature for 2 h. After washing, sections were incubated in a 1:500 dilution of horseradish peroxidase-goat anti-rabbit IgG (H + L) (Zymed Laboratories, CA, USA) at room temperature for 2 h, followed by extensive washing with PBS. After a visualization solution containing 3,3′-diaminobenzidine (Nichirei, Tokyo, Japan) was applied, sections were counterstained with Mayer's hematoxylin (Nichirei, Tokyo, Japan).

For double labeling of p-STAT1 and IFNAR2, indirect methods using immunoperoxidase (p-STAT1) and immunoalkaliphosphatase (IFNAR2) were used. Sections, which had been treated with paraformaldehyde and hydrogen peroxide, were incubated in a TBS solution (20 mM Tris–HCl, pH 8.0, 137 mM NaCl) containing 5% bovine serum albumin and 0.1% Tween 20 for 2 h, and then incubated in a 1:100 dilution of anti-p-STAT1 antibody at room temperature overnight. After washing, they were incubated in a 1:500 dilution of horseradish peroxidase-conjugated secondary antibody (Cell Signaling, MA) at room temperature for 2 h, followed by extensive washing with PBS. p-STAT1 was visualized using a solution containing 3,3′-diaminobenzidine. Subsequently, sections were incubated in a 1:2000 dilution of anti-IFNAR2 antibody. After washing, they were incubated in a 1:500 dilution of alkaliphosphatase-conjugated goat anti-rabbit IgG (H + L) (Zymed Laboratories) for 2 h, followed by extensive washing with PBS. IFNAR2 was visualized by Vector Red Alkaline Phosphatase Substrate Kit I (Vector, CA, USA).

Statistical analysis. Values were expressed as means ± SEM. For comparing values obtained from p-STAT1 and STAT1 bands, the Student's *t* test was used, and *p* values less than 0.05 were considered significant.

Results

Expression of human IFNAR1 and IFNAR2 genes transferred into mice

When human IFNAR1 and IFNAR2 genes were separately transferred into mice, only each transferred IFNAR was observed in the liver by Western blotting analysis (data not shown). When both human IFNAR1

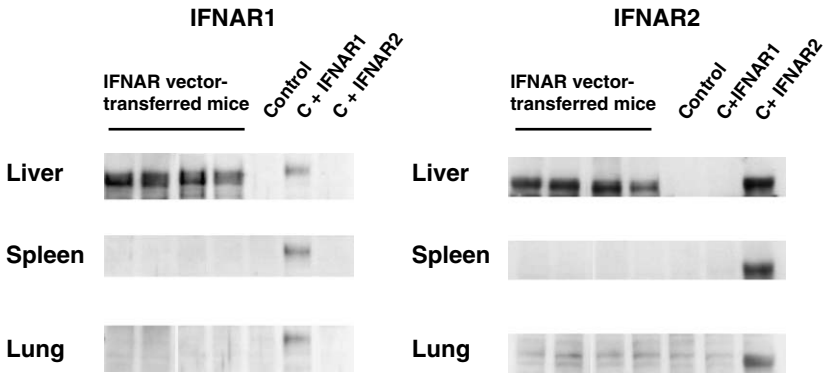

Fig. 1. Expression of IFNAR1 and IFNAR2 in the liver, spleen, and lung of IFNAR-transferred mice. IFNAR1 and IFNAR2 vectors were transferred into mice using a hydrodynamics-based gene delivery method. After 6 h, livers, spleens, and lungs were isolated, and expression of IFNAR1 and IFNAR2 was determined using specific antibodies. Positive and negative controls were loaded to compare with samples, including normal mouse lysate (Control), lysate plus recombinant IFNAR1 (C + IFNAR1), and lysate plus recombinant IFNAR2 (C + IFNAR2).

and IFNAR2 genes were transferred into mice, both IFNARs were detected in the liver, but not in the spleen or lung (Fig. 1). In individual mice, the amounts of IFNAR1 and IFNAR2 expression were correlated with each other.

Immunohistochemical analysis of IFNAR1 and IFNAR2 expression in the liver

Localization of human IFNAR in the liver was elucidated by immunohistochemical staining with anti-IFNAR antibodies. In contrast to the livers of animals receiving the control vector, positive cells, which were labeled with anti-IFNAR1 or anti-IFNAR2 antibodies, were observed in patches in the livers of IFNAR-transferred animals (Fig. 2A). These cells were localized around areas of necrosis, which was thought to be induced by the hydrodynamics-based procedure. Interestingly, analysis of serial sections revealed that these cells, which were identified as hepatocytes by their polygonal shape and round nuclei, expressed both IFNAR1 and IFNAR2. Immunoreactivity for IFNAR1 and IFANR2 was diffuse within cells, with strong labeling at cell membranes (Fig. 2B). The proportion of IFNAR2-positive cells was less than 5% (Fig. 2C).

Effect of human IFN-α on STAT1 phosphorylation in IFNAR-transferred mice

To examine the function of transferred human IFNARs in mice, vehicle or 10,000 IU of human IFN-α was intravenously injected into mice 6 h after transfection. After another hour, p-STAT1 levels were determined. As shown in Fig. 3, p-STAT1 levels were significantly increased by IFN-α in IFNAR-transferred mice, but unchanged in control vector-transferred mice. In contrast, non-phosphorylated STAT1 was almost the same in all groups. Human IFN-α injection did not affect the expression of either IFNAR1 or IFNAR2 in IFNAR-transferred mice (data not shown).

Double labeling of liver sections from IFNAR-transferred mice, with anti-IFNAR2 and anti-p-STAT1 antibodies, revealed the presence of IFNAR2 (red) at membranes and in the cytoplasm of several cells, and p-STAT1 (brown) in the nucleus of the same cells (Fig. 4).

Discussion

In the present study, we simultaneously transferred plasmids containing the genes for human IFNAR1 and IFNAR2c into mice, using a hydrodynamics-based gene delivery method. Human IFNAR1 and IFNAR2 were both detected in the plasma membranes of the same hepatocytes. It has been suggested that a high-affinity receptor complex of human IFNAR1 and IFNAR2 is expressed in the liver of mice [4,5].

To examine the function of the transferred IFNAR, we assayed the phosphorylation of STAT1, which is a key molecule downstream of IFN binding. When IFN-α binds to IFNAR, both STAT1 and STAT2 are phosphorylated, and p-STAT1 and p-STAT2 associate with p48 to form a heterotrimer that is translocated to the nucleus where they activate the transcription of genes that are regulated by an IFN-stimulated response element. This signal cascade has been supported by the observation that the antiviral and anti-tumor effects of IFN-α are abrogated in STAT1-deficient mice [18–20]. In the present study, we showed that an injection of human IFN-α increased the levels of p-STAT1 in the mouse liver 6 h after human IFNAR vector transfer. p-STAT1 levels were higher in IFNAR-transferred mice than in control vector-transferred mice. However, STAT1 levels were similar. Phosphorylation of STAT1 following injection of human IFN-α was mediated by transferred human IFNAR, because human IFN-α is unable to stimulate endogenous mouse IFNAR. Additionally, the

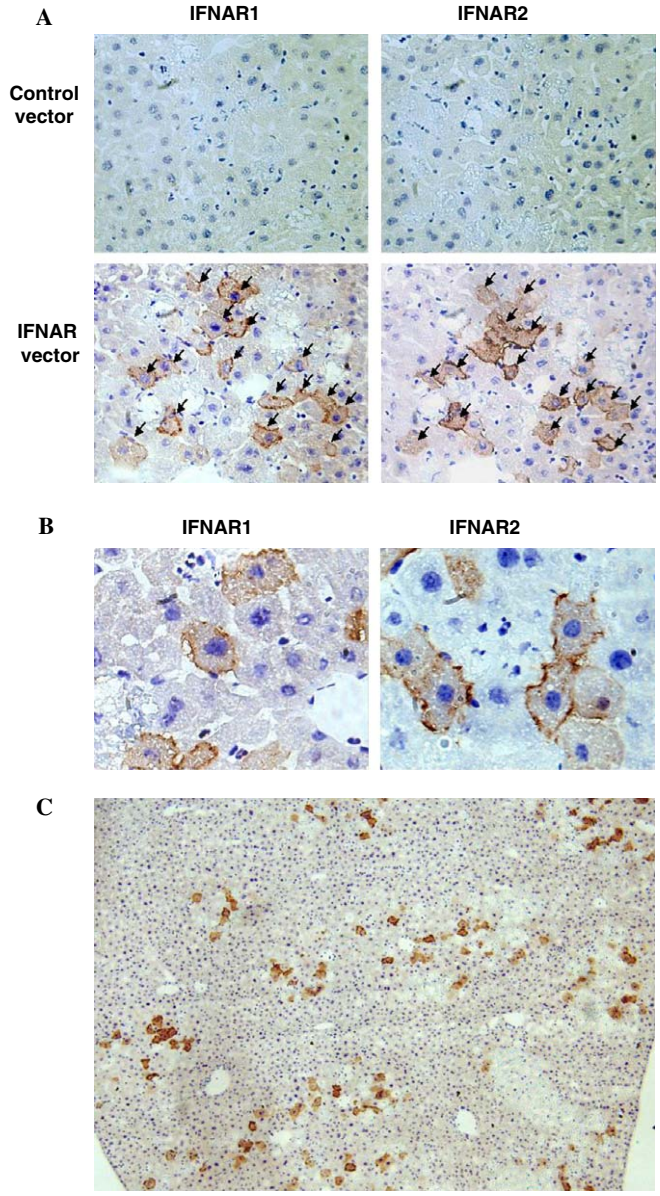

Fig. 2. Photomicrographs of IFNAR1 and IFNAR2 expression in the livers of IFNAR-transferred mice. (A) Liver sections from mice that received control vector (upper) or both IFNAR1 and IFNAR2 vectors (lower). Sections were stained with anti-IFNAR1 (left) or anti-IFNAR2 (right) antibodies. Arrows show positive cells in serial sections. (B) IFNAR1 and IFNAR2 subunits at liver cell surfaces. (C) The distribution of IFNAR2-expressing cells in liver following transfer of both IFNAR1 and IFNAR2 vectors.

results of immunohistochemical analysis showing that p-STAT1 and IFNAR were expressed in the same cells strongly supports the above idea.

In the present study, a hydrodynamics-based gene delivery method [21] was used to transfer IFNAR genes. Hydrodynamic injection induces a sharp increase in venous pressure, enlargement of fenestrae, and enhancement of the membrane permeability of hepatocytes [22]. In our experiments, IFNAR-expressing cells were mainly found in the liver, not in the spleen or lung, as previously reported. Although hydrodynamics-based delivery achieved expression of IFNAR1 and IFNAR2 in the same cells, the efficiency of the transfection was not especially high. Additionally, the transferred gene products were observed in peripheral areas of the liver that received only minor damage induced by the hydrodynamics-based procedure. Twenty-four hours after the hydrodynamics-based procedure, we observed that areas of necrosis were extended compared with those 6 h after the procedure (data not shown). At that time, levels of STAT1 were increased even in the control vector-transferred liver, and we could not detect an increase in p-STAT1 levels following injection of human IFN-α into the mice (data not shown). The hydrodynamics-based gene transfer procedure is a conventional method for expressing gene products in in vivo animal experiments, but a progression of tissue damage accompanies this procedure over time. It is therefore important to consider the limitations of the method; namely, low efficiency of transfection and accompanying injury of transferred cells.

As resistance to IFN therapy remains a serious problem in clinical settings, different types of combination therapies have been introduced. In chronic hepatitis C, the molecular mechanisms underlying failure of IFN-α treatment are not yet fully understood, but several lines of evidence indicate that both viral and host factors are involved. Of several host factors, expression of IFNAR1 and IFNAR2c is key to the antiviral activity of IFN-α in the treatment of viral hepatitis [23]. Numerous clinical reports have shown that high levels of IFNAR1 and IFNAR2 in the liver correlate with a sustained response to IFN-α therapy [6–9]. In HCC, IFNAR2 is also expressed in tumors [24,25], and the efficacy of combination therapy with IFN-α and 5-fluorouracil for advanced HCC might be related to expression levels of IFNAR2 [26,27]. These studies suggest that more efficient IFN therapy might be achieved by upregulating IFNAR on the cell surface. One approach to IFNAR upregulation might be to use agents such as IFN-γ and 9-*cis* retinoic acid [12,13], but IFNAR upregulation by agents has not been demonstrated in vivo. Another approach might be to use IFNAR gene transfer [14,26]. In the present study, we transferred human IFNAR1 and IFNAR2 genes into mice. Expression of IFNAR1 and IFNAR2 was observed in vivo, and the receptors responded to exogenous human IFN-α. Our results suggest that combination therapy with IFN-α and IFNAR genes might be useful, and that it would be worthwhile examining this type of therapy in a virus infection model or tumor-bearing animal model in the future.

In conclusion, we showed that human IFNAR can be expressed in the mouse liver by hydrodynamics-based transfection, and that human IFNAR subunits can form functional receptors in mice. In the future, it should be determined how an increase in IFNAR increases the sensitivity of IFN in vivo.

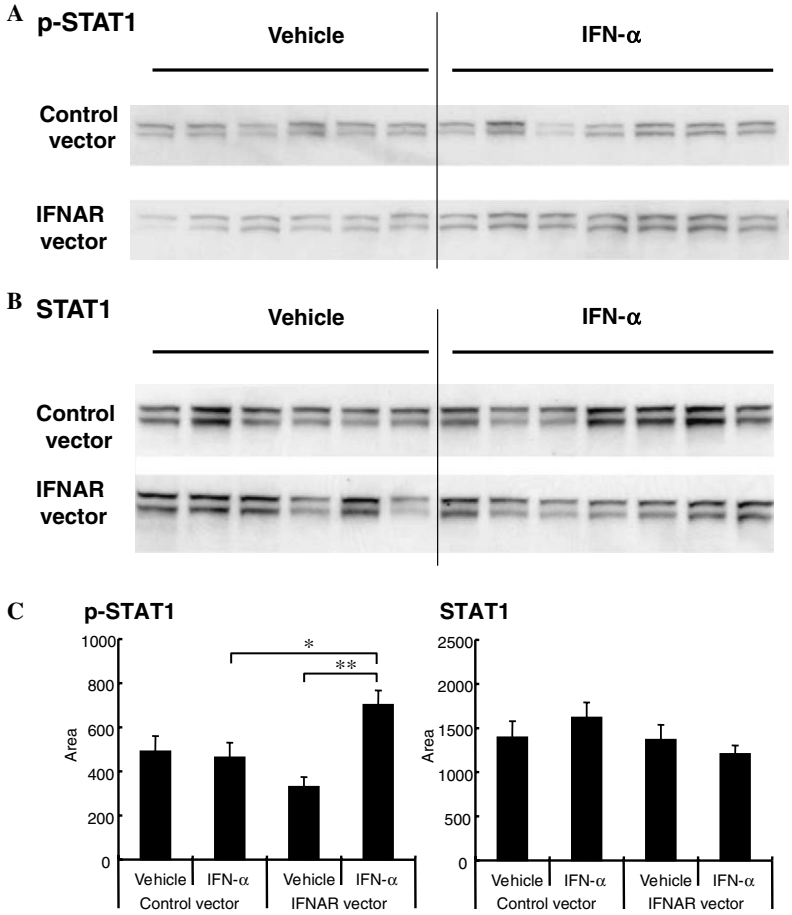

Fig. 3. Phosphorylated-STAT1 (p-STAT1) and STAT1 expression in IFNAR-transferred mice after IFN-α administration. Mice received either control vectors, or both IFNAR1 and IFNAR2 vectors. After 6 h, mice were injected intravenously with vehicle or 10,000 IU IFN-α. After another hour, livers were isolated and levels of p-STAT1 (A) and STAT1 (B) were determined. Images of p-STAT1 and STAT1 bands were scanned, and the amounts of p-STAT1 and STAT1 were assessed as area (mean ± SEM) by analysis of Scion Image (C). $*p < 0.05$, $**p < 0.01$ (by Student's t test).

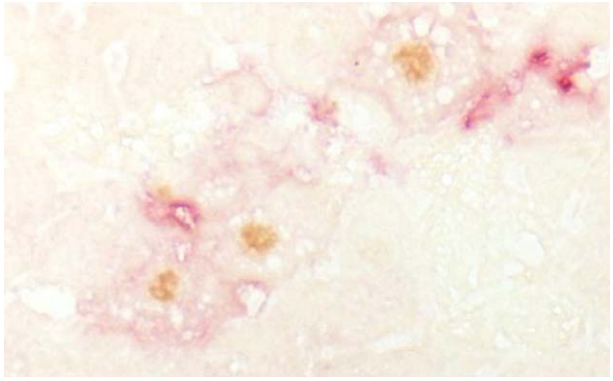

Fig. 4. Liver cells double-immunolabeled with anti-IFNAR2 (red) and anti-p-STAT1 (brown) antibodies. Mice that received both IFNAR1 and IFNAR2 vectors were injected with 10,000 IFN-α. After 1 h, livers were dissected and used for immunohistochemical analysis.

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