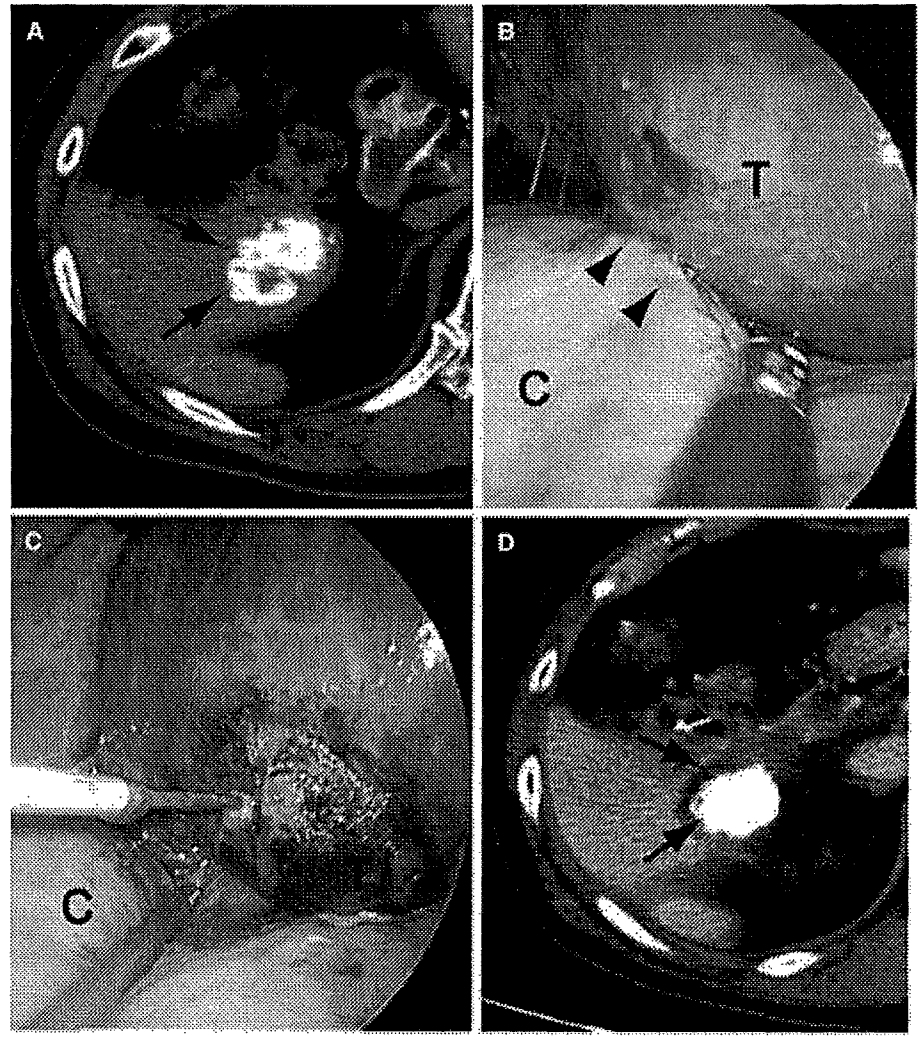

Fig. 3. Laparoscopic radiofrequency ablation. (a) Computed tomography (CT) reveals tumor (arrows) in contact with the colon. (b) Adhesion is demonstrated between the tumor (T) and the colon (C) (arrowheads). (c) After the tumor was separated from the colon by detaching the adhesion, the tumor could be safely ablated. (d) After only one ablation, CT reveals complete tumor ablation (arrows).

Table 1. Baseline characteristics of 74 patients

Percutaneous RFA group (37 patients: 44 nodules)		Laparoscopic RFA *P* (37 patients: 42 nodules)	
Mean age (years)	70.2 ± 8.5	66.6 ± 7.9	0.115
Sex (M/F)	27 : 10	20 : 17	0.091
Tumor size (mm)	24.9 ± 4.6	26.0 ± 6.9	0.473
Etiology (HBV : HCV : other)	6 : 24 : 7	6 : 26 : 5	0.393
Child–Pugh (A : B)	29 : 8	25 : 12	0.129

HBV, hepatitis B virus; HCV, hepatitis C virus; RFA, radiofrequency ablation.

The present results show that LRFA represents a better treatment choice for treating HCC nodules >2 cm in diameter. As these patients were under anesthesia, ablation could be continued because pain and other subjective symptoms did not hinder treatment. The efficacy of other methods such as RFA assisted by laparoscopy,[14,15] thoracoscopy[17] and open surgery[18] has been reported.

In small HCC nodules, PRFA with artificial ascites is superior to LRFA, as this method of inducing ascites is easily carried out without general anesthesia. Artificial ascites was able to be properly induced in all except two patients. Adequate safety margins were attained. Duration of hospitalization was thus shorter for LRFA than for PRFA (Table 2). If tumors are larger, the low numbers of interventions required and the safety margin of LRFA are advantageous. As targeted tumors and changes in the colored area following ablation can be confirmed directly under laparoscopy, a larger safety margin is readily obtained despite the larger size of the tumor. TAE remains necessary to prevent cooling effects.[19,20] Moreover, if the HCC nodules are located near the liver surface, bleeding and dissemination are apprehended after RFA alone. In these nodules, TAE is useful in order to avoid these complications. After TAE, adhesions are often seen. Kondo *et al.* have noted that if adhesion is

Table 2. Comparison between percutaneous RFA and laparoscopic RFA

Percutaneous RFA (37 patients: 44 nodules)		Laparoscopic RFA *P* (37 patients: 42 nodules)	
No. sessions	2.1 ± 1.0	1.0 ± 0.0	<0.001
Safety margin (<5 mm : 5–9 mm : 9 mm<)	9 : 33 : 2	2 : 8 : 32	<0.001
Hospital stay (days)	22.0 ± 11.6	15.9 ± 8.4	0.031
Local recurrence (1 year : 2 years)	0 : 17.5	0 : 0	0.010

RFA, radiofrequency ablation.

confirmed when inducing artificial ascites, PRFA should be avoided to prevent damage to the surrounding organs[10]. In cases with tumor >2 cm or adhesions near the tumor, LRFA should be carried out.

In conclusion, LRFA is a better treatment option for ablation of HCC nodules more than 2.0 cm in diameter.

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Editorial

Fish model leads to new findings in liver disease

See article in *Hepatology Research* **39**: 633–644
Liver development: lessons from knockout mice and mutant fish
Takashi Nakamura, Hiroshi Nishina

In liver research, rodent models have primarily been used to identify disease mechanisms, diagnostic criteria and therapies. Tissue-specific knockout systems have also recently been developed. On the other hand, small fish such as zebrafish (*Danio rerio*)[1] and medaka (*Oryzia laptipes*) have been applied in new research models.[2,3] Medaka and zebrafish compare favorably to rodents as experimental animals for drug screening because medaka and zebrafish have a high reproductive rate, mature rapidly and cost little in terms of rearing space and daily maintenance due to their small size.

In a previous issue of the Journal, Nakamura *et al.* reported a comparative analysis of mice and medaka with regard to liver development findings.[2] In the field of liver development, mouse models were analyzed using a reverse genetic approach to identify phenotypic changes using specific gene targets.[2] Various genes, including *BMP4*, *Hhex*, *Xbp1*, *NF-κβ* and *c-jun*, were found to be important in the generation and differentiation of hepatoblasts, and new monoclonal antibodies have been found to be useful in monitoring lineage commitment during hepatocyte differentiation. Liv8/CD44 are particularly useful markers in understanding hepatoblast differentiation.[4–6]

On the other hand, medaka could be applied to forward genetic screening using N-acethyl-N-nitrosourea (ENU) treatment.[2,3] Previous forward ENU screening in Japan has established five groups of medaka liver mutants, while 19 medaka liver mutants have been established and their mutant genes are now being analyzed. In addition, 15 types of zebrafish liver mutants have been established.[1] The merit of forward genetic screening is powerful because forward screening is an unbiased approach to identify genes essential for the process of liver development.[1] In zebrafish and medaka, specific gene analysis for early development through morpholine knockdown has been shown to be effective.[7] TILLING[8] and zinc finger nucleases have recently been developed to find target gene mutants in zebrafish and medaka.[9]

We believe that new systems for drug screening using medaka and zebrafish will greatly assist in the search for new candidate drugs. Previous research using medaka and zebrafish has focused on developmental biology, but there remains an urgent need for research concerning the mechanisms and treatment of liver disease. In Japan, the history of medaka research began with a hepatocellular carcinoma model developed in the 1980s.[10] Disease models such as liver tumors in zebrafish combined with ultrasonography[11] and a non-alcoholic steatohepatitis (NASH) medaka model[12] have also recently been developed, and these models will be useful for drug screening.

We have proposed two drug screen methods using medaka and zebrafish. As shown in Figure 1(a), medaka and zebrafish liver mutants were used to screen new candidate compounds to reduce the deleterious effects of mutant genes in liver mutants. Embryos of liver mutants were bred on culture plates with various chemical compounds. Any specific chemical compounds that allow liver mutants to grow normally would thus be candidates to compensate for the effects of mutant genes. This drug screening system is specific for identifying candidate drugs for liver development. An alternative system is shown in Figure 1(b); wild-type and specific mutants showing liver disease,[3] and medaka and zebrafish with specific promoter transgenes are bred and raised on specific diets, such as high-fat, choline-deficient or N-nitrodiethylamine (DEN)-containing diets. Combination studies using liver mutants and TG

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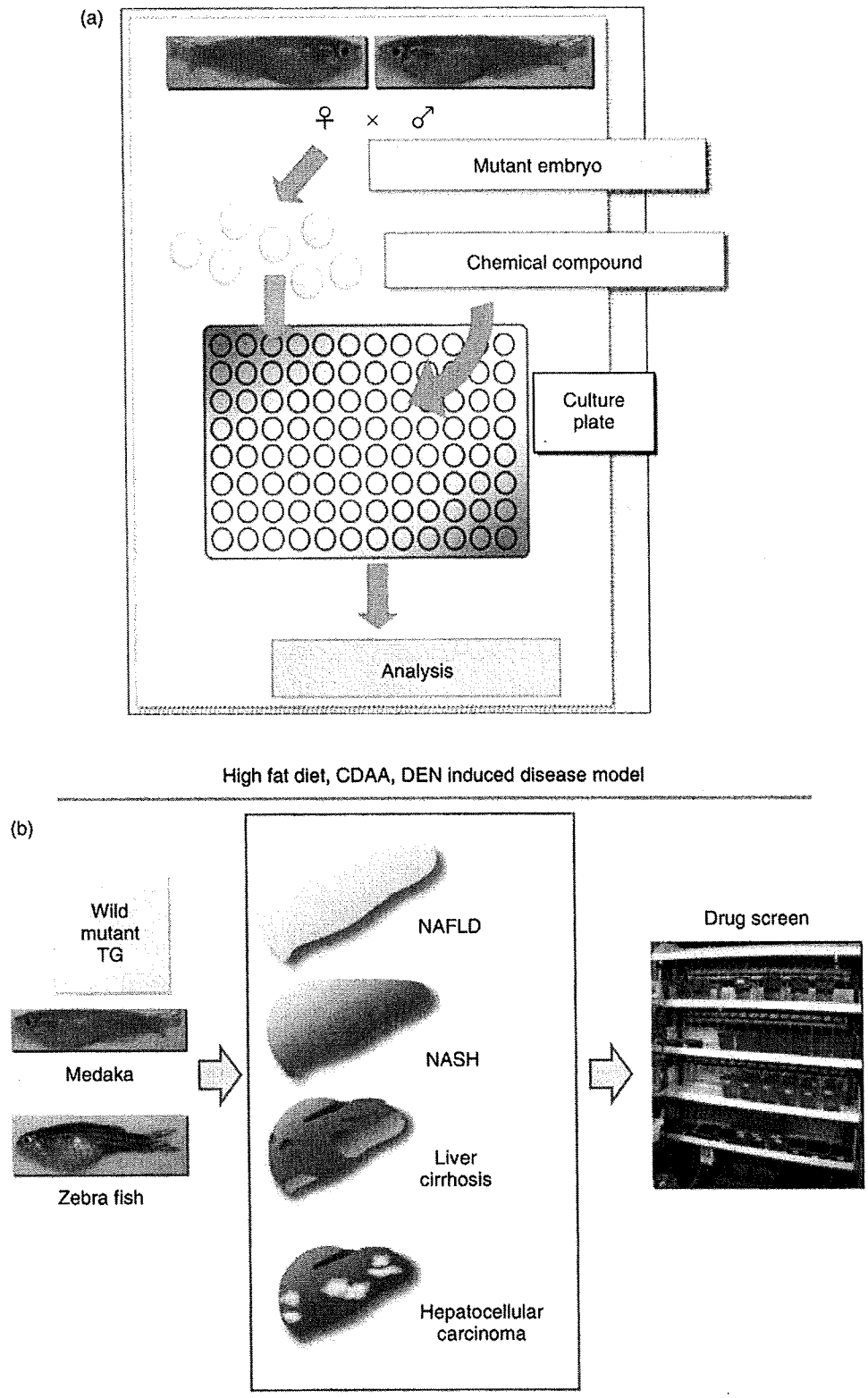

Figure 1 Drug screening using medaka and zebrafish. NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

fish with specific diets are thus useful. Using this system, candidate drugs for NASH, liver cirrhosis and hepatocellular carcinoma will be developed. Sequencing of the medaka and zebrafish genome has been completed and techniques for producing transgenic and knockout animals have been established.[1,13,14] Thus, an increasing number of genetic mechanisms can now be analyzed more efficiently.

At present, new approaches should be developed in order to incorporate the new techniques in medaka and zebrafish research. Fish models will readily allow new findings to be translated into clinical research.

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特集II 肝細胞癌の予防

肝発癌制御におけるMaidシグナルの重要性について*

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坂井田功**

Key Words: human homologue of maid (HHM), Maid, hepatocarcinogenesis, Zebrafish

はじめに

肝細胞癌に対して新たな分子標的治療剤としてsorafenibの有効性が報告された．この分子標的剤の有効性の報告に伴い，肝発予防の実現に肝発癌の分子制御機構の研究がさらに注目されてきている[1]．肝臓は再生する臓器として知られ，その増殖・再生システムには肝臓独自の特徴があるため，その肝発癌機序を明らかにするためには，持続炎症下(肝線維化，肝脂肪化環境下)における肝細胞の増殖，分化，癌化の接点に着目して解析していく必要がある(図1)．われわれは肝発癌機構を明らかにするには，特に肝細胞の細胞分化系譜を明らかにすることが重要と考え研究を推進してきた．今回の特集ではわれわれが同定し研究を行ってきたHHM (human homologue of maid)，〔別名GCIP1 (grap2 and cyclin D1 binding protein)，DIP1 (cyclin D1binding protein 1)〕の解析を通じたMaidシグナルの研究結果について報告する．

HHMの同定と機能評価

筋肉細胞においては1つのマスター遺伝子が，筋肉特異的遺伝子の細胞の系譜を制御することが知られている．特にhelix-loop-helix型の転写因子であるMyoDがマスター遺伝子の1つとして知られている[2]．われわれは，肝幹細胞の分化制御機構の解明，肝臓の発生分化を制御する肝臓特異的なhexix-loop-helix型転写制御因子(マスター遺伝子)の同定を目指し研究を行ってきた．E12蛋白をおとり蛋白としたtwo hybrid screenによりinhibitor of differentiation (Id)とは違う新規のdominant inhibitory型HLH型転写制御分子をクローニングした．その結果，胎児肝のcDNAライブラリーから新規のdominant inhibitory型helix-loop-helix (HLH)型の転写制御分子であるHHMをクローニングした．HHMは360個のアミノ酸から形成されるId2と比べ，大きい新規のdominant Inhibitoryタイプの HLH型転写制御分子である(図2)．Maid自体は，生誕直後の2細胞期に発現が増加し，さらにわれわれの解析においてHHM

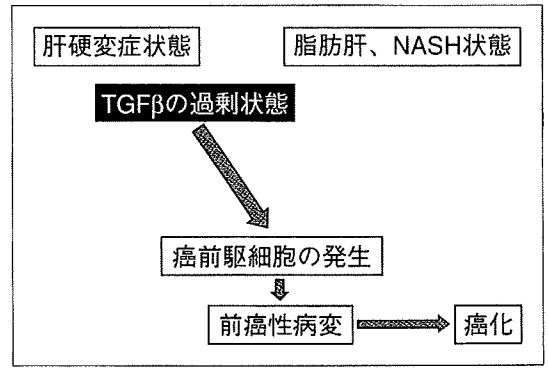

図1　肝細胞癌は肝硬変の進行の過程で発生してくる

* Maid reglate hepatocarcinogenesis.

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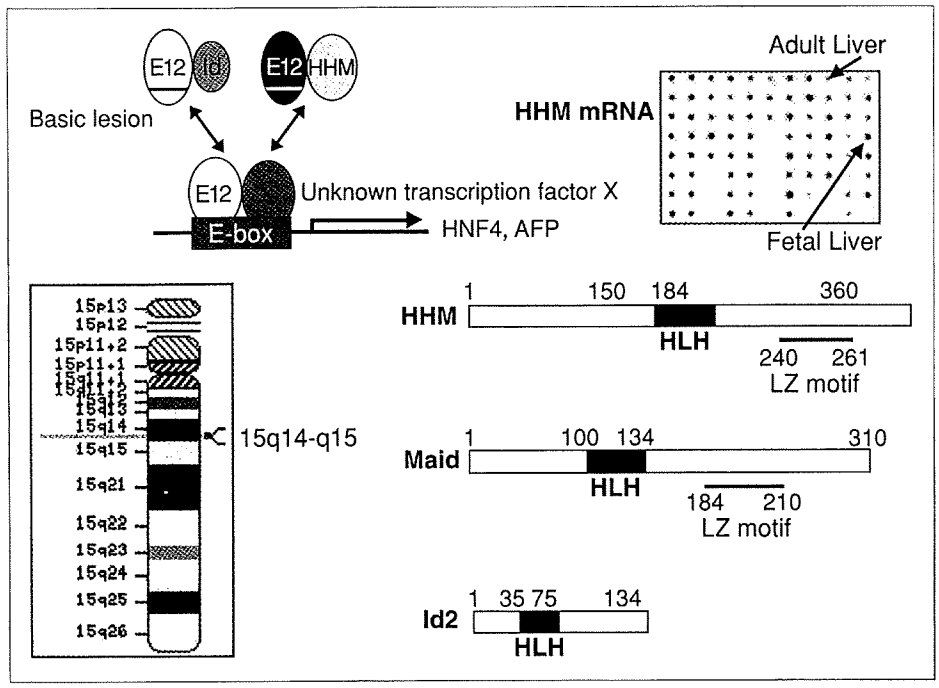

図2　Human homologue of maid(HHM)
GCIP1(grap2 and cyclin D1 biding protein)，DIP1(cyclin D1 biding protein 1)．
（文献[3]より引用）

CyclinD1；Xia C, J Biol Chem. 2000 Jul 7；275（27）：20942-8. GCIP, a novel human grap2 and cyclin D interacting protein, regulates E2F-mediated transcriptional activity.

P29；Chang MS, Biochem Biophys Res Commun. 2000 Dec 20；279（2）：732-7. p29, a novel GCIP-interacting protein, localizes in the nucleus.

CT847；Chellas-Géry B, Cell Microbiol. 2007 Oct；9（10）：2417-30. Epub 2007 May 28. Human GCIP interacts with CT847, a novel Chlamydia trachomatis type III secretion substrate, and is degraded in a tissue-culture infection model.

Ribosomal phosphoprotein P0；Chang TW, Oncogene. 2008 Jan 10；27（3）：332-8. Epub 2007 Jul 9. Ribosomal phosphoprotein P0 interacts with GCIP and overexpression of P0 is associated with cellular proliferation in breast and liver carcinoma cells.

Olig1；Ikushima H, EMBO J. 2008 Nov 19；27（22）：2955-65. Epub 2008 Oct 16. An Id-like molecule, HHM, is a synexpression group-restricted regulator of TGF-beta signaling.

E12；Terai S, Hepatology. 2000 Aug；32（2）：357-66. Human homologue of maid：A dominant inhibitory helix-loop-helix protein associated with liver-specific gene expression.

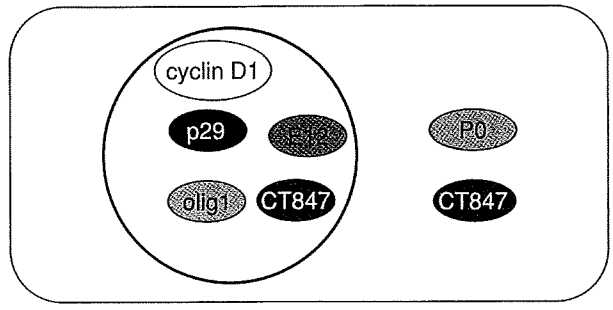

図3　HHM(Maid)結合蛋白

が肝細胞の分化成熟に関与するhepatocyte nuclear factor 4(HNF-4)の発現を特異的に制御し，アセトアミオフルレン肝部分切除モデルにおいてoval細胞の肝細胞の分化過程に出現するalpha-fetopro-

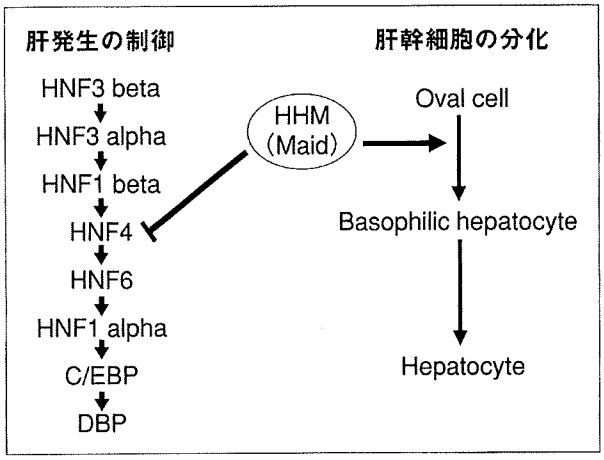

図4　HHM(Maid)の機能

tein(AFP)陽性のFociに一致し，HHMの発現が特異的に高いことが明らかになっており肝臓の発生また，Ovalcellからの肝細胞への分化に関与する(図3)[3]．一方で，HHMはcyclin D1[4]，p29[5]，CT847[6]，ribosomal phosphoprotein P0[7]，Olig1[8]などさまざまな細胞増殖，分化を制御する分子と結合することが知られている(図4)．最近ではTGF-betaシグナルの制御分子として注目されている[8]．

HHMと肝発癌との関連

肝発癌とHHM(Maid)の関連であるが，HHMはヒトの肝前癌性病変や肝細胞癌の癌部で高発現し，前癌性病変のマーカー蛋白である[9]．肝発癌における機構として，HHMの強制発現により細胞のreplicationを促進し，肝前癌性病変adenomatous hyperplasiaが発生する可能性を考えている．一方で，MaidKOマウスは肝発癌を特異的に発生することが報告され[10]，Maid自身の肝発癌への関与についての詳細な分子機構は不明であるが，Maidシグナルの重要性が明らかになってきている．さらに最近明らかになったTGF-betaシグナルの制御分子である特徴は，肝発癌は高度の肝線維化状態で起きることより，TGF-beta過剰状態下における細胞の生存に関与する可能性も示唆され，今後はこのTGF-betaとの関連について解析していく予定がある．

小型魚類動物を用いた解析システム

メダカおよびゼブラフィッシュなどの小型魚類を用いた研究が進められている．小型魚類は，マウス，ラットと同じ脊椎動物である．小型魚類の研究は主に発生分野を中心に行われてきたが，最近ではさらに病態モデルを用いた解析が注目されている[11][12]．小型魚類のメリットとしては，大量飼育が可能でありマウス，ラットモデルに比べると薬物スクリーニングについて大幅なコストダウンが考えられ，効率的な薬物スクリーニングが可能になることである．また多く

小型魚類であるゼブラフィッシュはNIH（米国立衛生研究所）によって人間、ラットに次いで重要度３位のモデル生物に指定された．
また、小型で大量に飼育が可能であり、現状のマウスやラットに比べると大幅なコストダウンが見込めることから新しいモデル動物として注目されている．

生物的なメリット	遺伝学的なメリット	コスト的なメリット
■脊椎動物 ■寿命は２～３年 ■世代サイクルが短い（２～３か月） ■たくさんの卵を産む ■母体外で発生し、体が透明なため生体での細胞動態を継続して観察できる ■卵が大きく細胞の移植などの胚操作がしやすい	■人とほぼ相同な遺伝子を持っているとされている（7～8割） ■変異体が作成されている ■ゲノム配列が公表されている	■小型であるため、大量の個体を飼育できる ■マウスの10分の１以下のコストで飼育ができる

図5　ゼブラフィッシュなどの小型魚類の動物モデルとしての利点

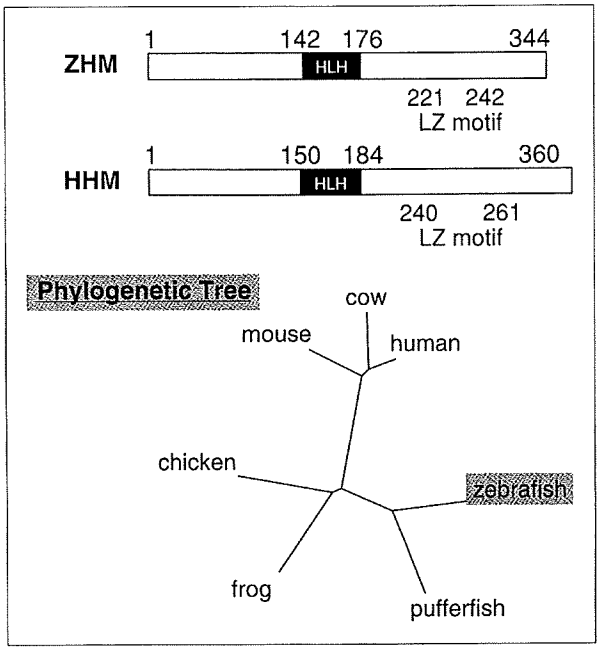

図6 Zebrafish homologue of maid(ZHM)
(ID：EF581817)

の遺伝子の変異体が存在し，それを利用してさらに新規薬物のスクリーングが可能になる(図5)．肝臓についての特徴としては，ゼブラフィッシュでは肝部分肝切除[13]，また肝発癌の誘導が可能である[14]．メダカモデルについては日本独自に研究者が発癌モデルとして研究を行ってきた素晴らしい歴史がある[15]．われわれは小型魚類動物としてメダカおよびゼブラフィッシュを用いて研究解析を行ってきたが，Maidに肝発癌における意義の解析さらにモデルを用いた薬物スクリーニングを検討するため，Maidについてはゼブラフィッシュを用いて解析を行った．われわれはzebrafish homologue of maid(ZHM)をクローニングした(図6)(gene accession number, ID：EF581817)．このZHMについてゼブラフィッシュモデルを用いて解析した所，肝再生，肝発癌状態において発現が増加しており，肝細胞の増殖分化に密接に関連のあることが明らかになってきている(論文投稿中)．この結果は，ヒト，マウス等の解析と同様にゼブラフィッシュでもMaidシグナルは肝発癌と密接な関連があることが明らかになった．今後はさらにゼブラフィッシュを用いてMaidシグナルに着目し，効率的な肝発癌の病態解析，スクリーニングを行っていく予定である(図7)．

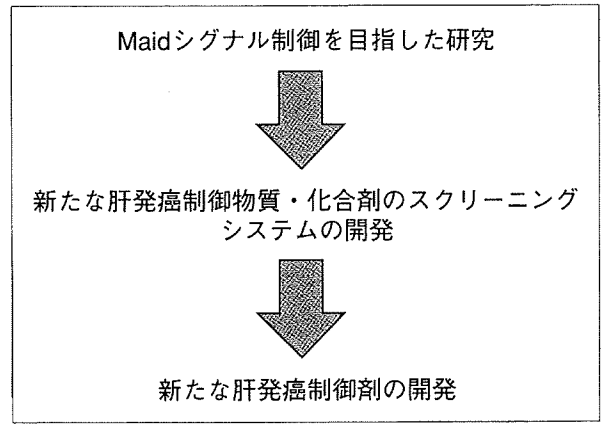

図7 今後の展望

今後の展望

新しい予防剤の開発には，肝発癌の分子制御機構を基盤として進めていく必要がある．従来のモデルとは違うモデル生物を使うことで，より有効な薬物のスクリーニングが可能になると考えられる．今後はゼブラフィッシュ，メダカモデルを用いた解析はさらに進んでいくと考えられ，肝発癌予防剤の開発への期待が高まる．

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ORIGINAL ARTICLE

Natural killer cell is a major producer of interferon γ that is critical for the IL-12-induced anti-tumor effect in mice

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Abstract Although the anti-tumor effect of IL-12 is mediated mostly by IFNγ, which cell types most efficiently produce IFNγ and therefore initiate or promote the anti-tumor effect of IL-12 has not been clearly determined. In the present study, we demonstrated hydrodynamic injection of the IL-12 gene led to prolonged IFNγ production, NK-cell activation and complete inhibition of liver metastasis of CT-26 colon cancer cells in wild-type mice, but not in IFNγ knockout mice. NK cells expressed higher levels of STAT4 and upon IL-12 administration displayed stronger STAT4 phosphorylation and IFNγ production than non-NK cells. Adoptive transfer of wild-type NK cells into IFNγ knockout mice restored IL-12-induced IFNγ production, NK-cell activation and anti-tumor effect, whereas transfer of the same number of wild-type non-NK cells did not. In conclusion, NK cells are predominant producers of IFNγ that is critical for IL-12 anti-tumor therapy.

Keywords IFNγ · Innate immunity · Liver tumor · IL-12 · NK

A. Uemura and T. Takehara contributed equally to this work.

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Introduction

IL-12 is a 70-kDa heterodimer protein, composed of p35 and p40 subunits, mainly produced by antigen-presenting cells. IL-12 was originally found as a "natural killer-stimulating factor" and a "cytotoxic lymphocyte maturation factor" [1, 2]. IL-12 has multi-potent effects, inducing a Th1 response, enhancing the CD8 T-cell response, activating natural killer cells and inducing production of IFNγ [3, 4]. Therapeutic use of IL-12, either using its recombinant protein or gene, can induce an efficient anti-tumor effect on primary or metastatic tumors in various murine models and humans [5, 6].

Research has shown that IL-12 mediates anti-tumor effects in a variety of ways. They include anti-proliferative effects, anti-angiogenic effects [7, 8] and cytotoxic effects of effector lymphocytes. A variety of effector cells has been reported to be required for IL-12-mediated anti-tumor effects: they include CD8 T cells [9], NKT cells [10], CD4 T cells [11] and NK cells [12]. The relative contribution of these cells may differ among IL-12 doses and types of tumor models [13]. Endogenous IFNγ production is required for most, if not all, of the anti-tumor effects of IL-12 administration [14, 15]. IL-12 stimulates a variety of immune cells, such as T cells [16], B cells [17] and NK cells [18], to produce IFNγ. However, which cell types are most critical for producing IFNγ during IL-12 therapy is not clearly known.

In the present study, we used a murine model of liver metastasis of CT-26 colon cancer cells and found that NK cells highly expressed the IL-12 signaling molecule STAT4 and most efficiently produced IFNγ. IFNγ was essential for the anti-tumor effect of IL-12, and NK-cell production of IFNγ sufficed to produce the full-blown anti-tumor effects. These results demonstrated that NK cells

serve not only as an effector but also as an important mediator producing IFNγ that is critical for the anti-tumor effects of IL-12.

Materials and methods

Mice

Specific pathogen-free female Balb/c mice were purchased from Clea Japan, Inc (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice with a Balb/c background were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/c background were kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo). All mice used were at the age of 6 to 10 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intra-splenic injection of tumor cells was used to establish micro-disseminated liver tumors in mice [19]. CT-26 colon cancer cells originating from Balb/c mice were maintained in RPMI1620 supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 200 μl of PBS and injected into the spleen.

Injection of naked plasmid DNA

A plasmid coding the murine IL-12 gene, pCMV-IL-12, was generously provided by Dr. M Watanabe (Laboratory of Experimental Immunology, Division of Basic Sciences, National Cancer Institute-Frederick Cancer Research and Development Center) [20]. Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany,) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described [21]. In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 26-gauge needle. DNA injection was completed within 5 to 8 s.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IL-12 p70, IFNγ (BD Biosciences-Pharmingen, San Diego, CA), IFNγ-inducible protein 10 (IP-10) and monokine induced by IFNγ (MIG) (R&D Systems, Inc, Minneapolis, MN) were measured using commercially available ELISA kits in accordance with the manufacturer's instructions.

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described. The NK activity of mononuclear cells was assessed by a standard 4-h ^{51}Cr-releasing assay using Yac1 cells as targets. In some experiments, mononuclear cells were separated into $DX5^+$ cells (NK cells) and $DX5^-$ cells (non-NK cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated NK-cell population was found to be greater than 90% by FACS analysis.

Flow cytometric analysis

Liver mononuclear cells were isolated 2 days after pCMV-IL-12 injection. Cytokine secretion was then blocked by the addition of brefeldin A for 4 h. Next, liver mononuclear cells were stained with FITC-conjugated anti-TCRβ antibody and biotin-conjugated anti-CD49b antibody (DX5), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with PE-conjugated anti-INFγ antibody or corresponding isotype controls. Analysis was performed using a FACSCalibur (Becton Dickinson), with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as $DX5^+/TCR\beta^-$ lymphocytes, NKT cells as $DX5^+/TCR\beta^+$ lymphocytes and T cells as $DX5^-/TCR\beta^+$ lymphocytes.

Adoptive transfer

For adoptive transfer experiments, GKO mice were injected intravenously 1 day before plasmid DNA injection with 2.0×10^8 whole mononuclear cells or 4.0×10^6 NK cells, or non-NK cells or whole mononuclear cells, all of which had been harvested from wild-type mice that can produce IFNγ.

Western blotting

Mouse recombinant IL-12 was purchased from R&D Systems, Inc (Minneapolis, MN). Mononuclear cells were treated with or without IL-12. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to the PVDF membrane. The membrane was stained with anti-STAT4 antibody (BD biosciences),

anti-phospho-specific STAT4 (pY693) antibody (BD biosciences), anti-STAT1 antibody (Cell Signaling), anti-phospho-specific STAT1 antibody (Cell Signaling) and visualized by chemiluminescence.

NK-cell depletion

For depletion of NK cells in vivo, anti-asialoGM1 antibody (WAKO, Osaka, Japan) was intraperitoneally administered. We determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. The percentage of $DX5^+/TCR\beta^-$ cells (NK cells) is 12.6 ± 2.4% in IgG-injected liver, whereas it decreased to 0.76 ± 0.04% one day after anti-asialo GM1 antibody injection ($N = 3$/group). This effect remained at least 3 days after anti-asialo GM1 antibody injection. NKT cells were less affected than NK cells, because 90% of $DX5^+/TCR\beta^+$ cells (NKT cells) still remained in the liver after the treatment. Anti-asialoGM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Histology

The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Acetone-fixed fresh frozen liver sections were immunostained with anti-mouse CD4 (H123.19), anti-mouse CD8α (53-6.7) or anti-CD31 (390) monoclonal antibody (all from BD Biosciences), using a VECSTAIN ABC kit (Vector Laboratories, Burlingame, California, USA).

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired *t*-test with Welch's correction. $p < 0.05$ was considered statistically significant.

Results

Hydrodynamic injection of IL-12-expressing plasmid led to prolonged production of IFNγ

Hydrodynamics-based gene delivery into mice establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes. Serial measurement of serum IL-12 demonstrated that pCMV-IL-12 injection led to substantial IL-12 production on day 1. The levels of serum IL-12 then rapidly declined (Fig. 1a). We also measured IFNγ production in serum, since IL-12 is known to activate IFNγ production. pCMV-IL-12 and, to a lesser extent, pCMV injection increased serum IFNγ on day 1. In contrast to the pCMV injection group, high levels of serum IFNγ were maintained at later time points in the pCMV-IL-12 injection group (Fig. 1a). Thus, hydrodynamic injection of pCMV-IL-12 led to prolonged production of IFNγ. Transient IFNγ production followed by control plasmid may be an indirect effect of liver injury caused by bolus injection of saline or DNA injection.

IL-12 therapy induced NK activation and anti-metastatic effects, both of which are critically dependent on IFNγ

To examine the biological effects of the produced IL-12, we evaluated the NK activity of mononuclear cells from the liver. pCMV-IL-12 injection, but not control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells (Fig. 1b). When GKO mice were injected with pCMV-IL-12 or pCMV, the hepatic mononuclear cells did not display any lytic ability to Yac1 cells, suggesting that IL-12-mediated NK-cell activation required IFNγ.

To examine the anti-metastatic effect of IL-12, pCMV-IL-12 or pCMV was injected into wild-type mice 2 days after intrasplenic injection of CT-26 cells. At 14 days after tumor injection, the mice were killed for evaluation of liver tumor (Fig. 1c). While pCMV-injected mice displayed huge liver tumors, pCMV-IL-12-injected mice did not show any macroscopic or microscopic tumor (Fig. 1d). Liver weight was significantly higher in pCMV-injected mice than pCMV-IL-12-injected mice, reflecting liver tumor formation. To examine the involvement of IFNγ in the IL-12-induced anti-tumor effect, we injected pCMV or pCMV-IL-12 into GKO mice 2 days after CT-26 injection. At 14 days after CT-26 injection, both groups showed similar degrees of tumor formation and there was no significant difference in liver weight between the two. This indicated that IL-12-induced anti-metastatic effect was strictly dependent on IFNγ.

NK cells were the most potent producer of IFNγ during IL-12 therapy

To evaluate which cell types most efficiently produced IFNγ, we isolated hepatic mononuclear cells from mice 2 days after plasmid injection and then stained cell surface TCRβ and DX5 as well as intracellular IFNγ (Fig. 2). $TCR\beta^-/DX5^+$ NK cells, $TCR\beta^+/DX5^+$ NKT cells and $TCR\beta^+/DX5^-$ T cells from pCMV-IL-12-injected mice showed significant levels of IFNγ production compared

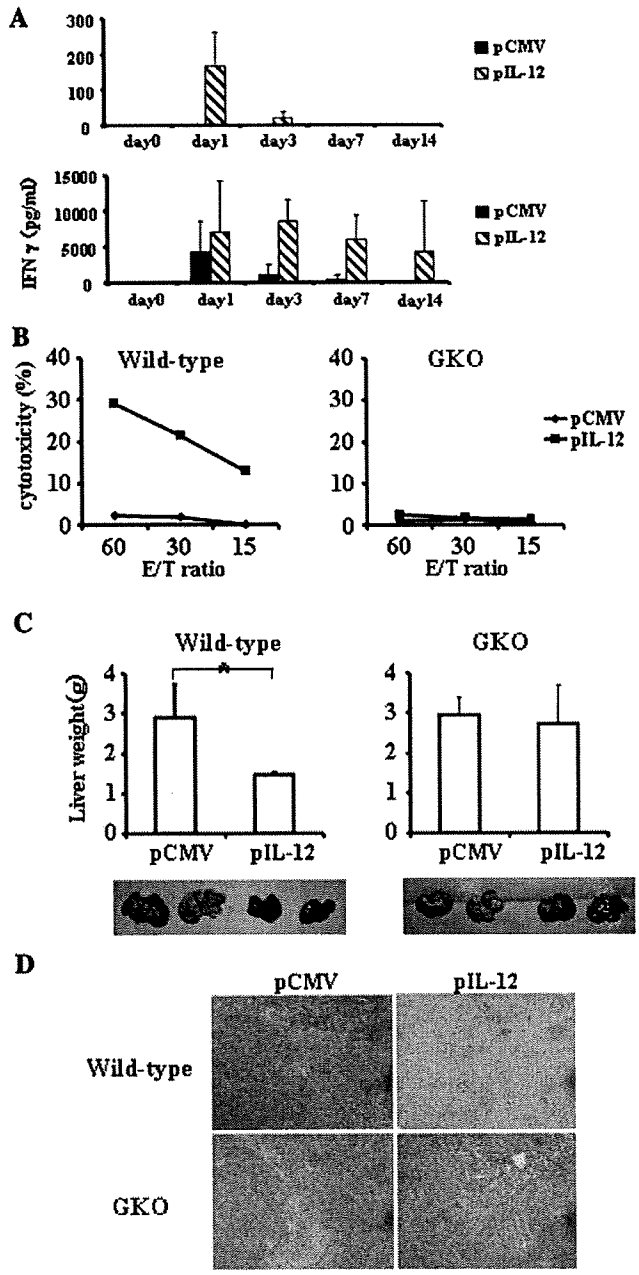

Fig. 1 Effects of hydrodynamic injection of IL-12-encoding plasmid. **a** Wild-type mice were hydrodynamically injected with either pCMV-IL-12 (*hatched bars*) or pCMV (*closed bars*) and bled at the indicated time points to measure the levels of serum IL-12 and IFNγ. Results are indicated as mean and SD ($n = 6$/group). **b** NK-cell activation after IL-12 administration. Hepatic mononuclear cells were isolated from wild-type mice (*left*) or GKO mice (*right*) which had been injected with pCMV-IL-12 (*closed squares*) or pCMV (*closed diamonds*) 4 days earlier. Yac1 lytic ability was measured by a standard ^{51}Cr-release assay at the indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown. **c** and **d** Anti-metastatic effects of IL-12 therapy. Wild-type mice (*left*) or GKO mice (*right*) were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. At 14 days after the plasmid injection, the mice were killed to examine liver tumor development. **c** Data are indicated as mean and SD of the liver weight at the top ($n = 6$/group) and a representative picture of the liver in each group is shown at the bottom. *$p < 0.001$. **d** Representative histology of liver sections

with those from naive mice or pCMV-injected mice. The levels of IFNγ production were highest in NK cells among those cells. Even at a later time point, 7 days after plasmid injection, NK cells were found to produce the highest levels of IFNγ (data not shown).

IL-12-induced STAT4 signaling and IFNγ production increased in NK cells

IL-12 activates Janus kinases Tyk2 and Jak2, STAT4 as well as other STATs. To examine the activation of STAT1 and STAT4, we isolated splenocytes from wild-type mice and GKO mice and stimulated them with IL-12 and/or IFNγ in the presence or absence of anti-IFNγ Ab (Fig. 3a). IL-12 led to phosphorylation of both STAT1 and STAT4 in wild-type splenocytes. In contrast, the same treatment led to phosphorylation of STAT4, but not of STAT1, in GKO splenocytes. Addition of IFNγ restored STAT1 phosphorylation in GKO splenocytes. Furthermore, adding anti-IFNγ inhibited STAT1 phosphorylation in wild-type cells. These findings demonstrated that phosphorylation of STAT4 is a direct effect of IL-12 but phosphorylation of STAT1 is indirect, via an autocrine or paracrine IFNγ-dependent manner.

To examine STAT1 and STAT4 activation and IFNγ production in NK cells and non-NK cells, we prepared whole mononuclear cells as well as NK and non-NK populations from wild-type spleens and stimulated the cells with IL-12 (Fig. 3b). NK cells expressed higher levels of STAT4 than non-NK cells. Upon IL-12 treatment, STAT4 was rapidly phosphorylated in NK cells, but to a lesser extent in non-NK cells. In contrast, NK cells expressed lesser levels of STAT1 than non-NK cells. STAT1 was similarly phosphorylated in NK cells and non-NK cells upon IL-12 treatment. Both NK cells and non-NK cells produced significant levels of IFNγ, but the levels were much higher in NK cells than non-NK cells (Fig. 3c). These results indicated that compared with non-NK cells, NK cells possessed higher levels of STAT4, a direct signaling molecule of IL-12, and produced higher levels of IFNγ than non-NK cells.

NK cells were sufficient for IL-12-mediated anti-tumor effects

The above observation indicated that NK cells are a predominant producer of IFNγ, which was critical for the IL-12-induced anti-tumor effects. To examine whether NK

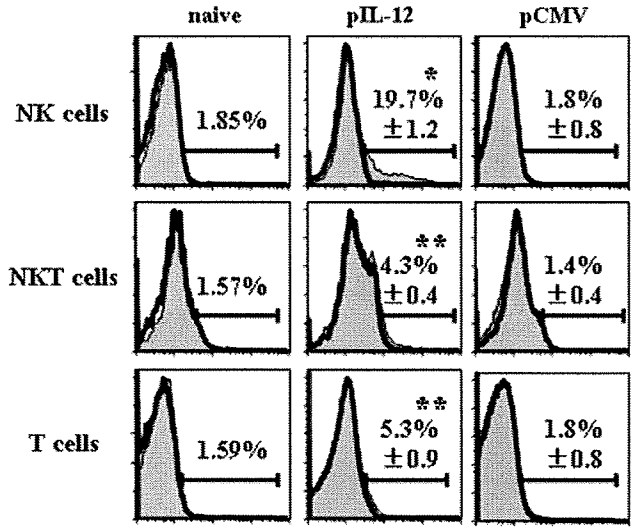

Fig. 2 IFNγ expression of mononuclear cells after IL-12 administration. Wild-type mice were injected with pCMV-IL-12 or pCMV, or were untreated (naive). Mononuclear cells were isolated from the liver 2 days after plasmid injection and stained with anti-TCRβ mAb, anti-DX5 mAb and anti-IFNγ mAb. *Closed histograms* show the IFNγ expression in the gated populations ($TCR\beta^-/DX5^+$ cells for NK cells, $TCR\beta^+/DX5^+$ cells for NKT cells and $TCR\beta^+/DX5^-$ cells for T cells). Isotype control stainings are shown by *open histograms*. Numbers in histograms represent averages ± SD of percentages of positive cells ($n = 3$ mice/group). $^*p < 0.0001$ vs. mock in NK populations. $^{**}p < 0.05$ vs. mock in each population

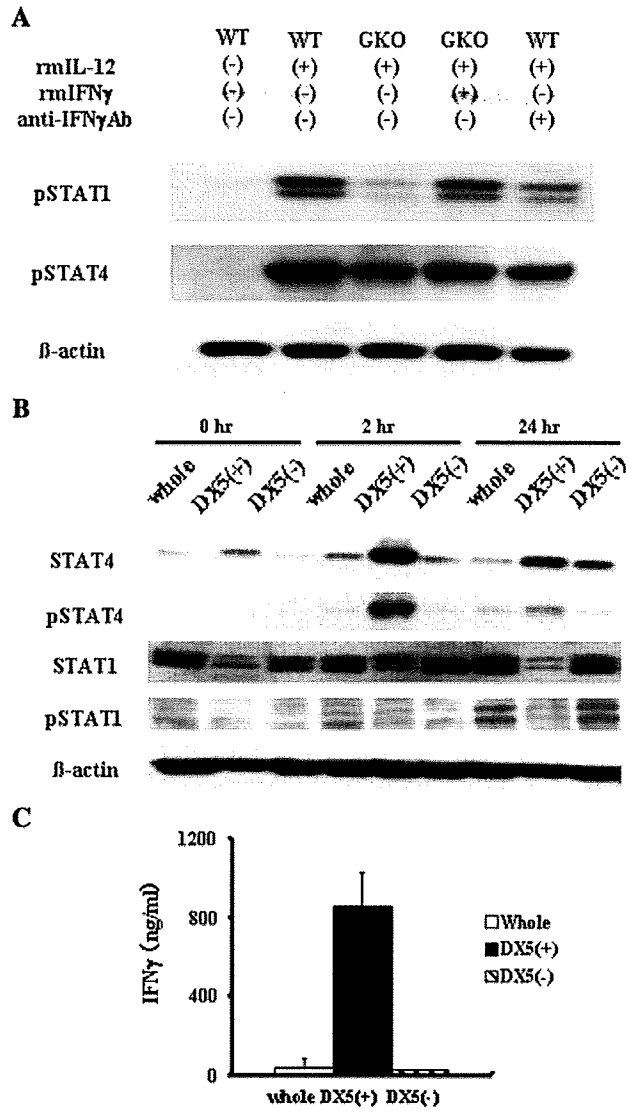

Fig. 3 STAT signaling and IFNγ production of mononuclear cells in vitro treated with IL-12. **a** STAT1 and STAT4 activation of splenocytes in vitro treated with IL-12. Splenocytes were isolated from wild-type mice or GKO mice and treated with or without recombinant IL-12 (20 ng/mL) in the presence or absence of recombinant IFNγ (500 ng/mL) or anti- IFNγ antibody (20 μg/mL) for 24 h. Cellular lysates were analyzed by Western blot for the expression of phospho-STAT1, phospho-STAT4 and β-actin. **b** and **c** STATs expression and signaling of NK cells and non-NK cells. Splenocytes were isolated from wild-type mice. Whole splenocytes were further purified into $DX5^+$ cells and $DX5^-$ cells. Each cell population was cultured with recombinant IL-12 (20 ng/mL) for the indicated times. **b** The cells were lysed to examine expression of whole STAT and phospho-STAT by Western blot. **c** The levels of IFNγ in the culture supernatant at 24 h were determined by ELISA. Data are expressed as mean and SD ($n = 3$)

cells are sufficient for the anti-metastatic effects of IL-12, we examined the anti-metastatic effect in Rag2 KO mice which lack T cells, B cells and NKT cells. pCMV-IL-12 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in Rag2 KO mice higher than in wild-type mice (Fig. 4a). To examine whether NK cells are sufficient for IL-12-mediated rejection of hepatic metastasis, we injected pCMV-IL-12 or pCMV into mice that had been intra-splenically injected with CT-26 cells 2 days earlier. Serum IFNγ levels of Rag2 KO mice were about 4 times higher than those of wild-type mice (Fig. 4b). pCMV-IL-12 completely suppressed hepatic metastasis in Rag2 KO mice (Fig. 4c).

Adoptive transfer of wild-type NK cells into GKO mice restored the anti-tumor effects of IL-12

Since NK cells were sufficient for producing IL-12-induced anti-tumor effects, we postulated that their production of IFNγ may play an important role in these effects. To test this, we performed adoptive transfer experiments with GKO mice. First, whole mononuclear cells isolated from the spleens of wild-type mice (2.0×10^8 cells) were adoptively transferred to GKO mice 1 day before plasmid injection. pCMV-IL-12 injection increased Yac1 lytic activity of hepatic mononuclear cells in the adoptively transferred group, but not in the untreated group (Fig. 5a). pCMV-IL-12 induced significant increase in serum IFNγ levels 4 days after plasmid injection in the adoptive transferred group, but not in the other groups (Fig. 5b). The

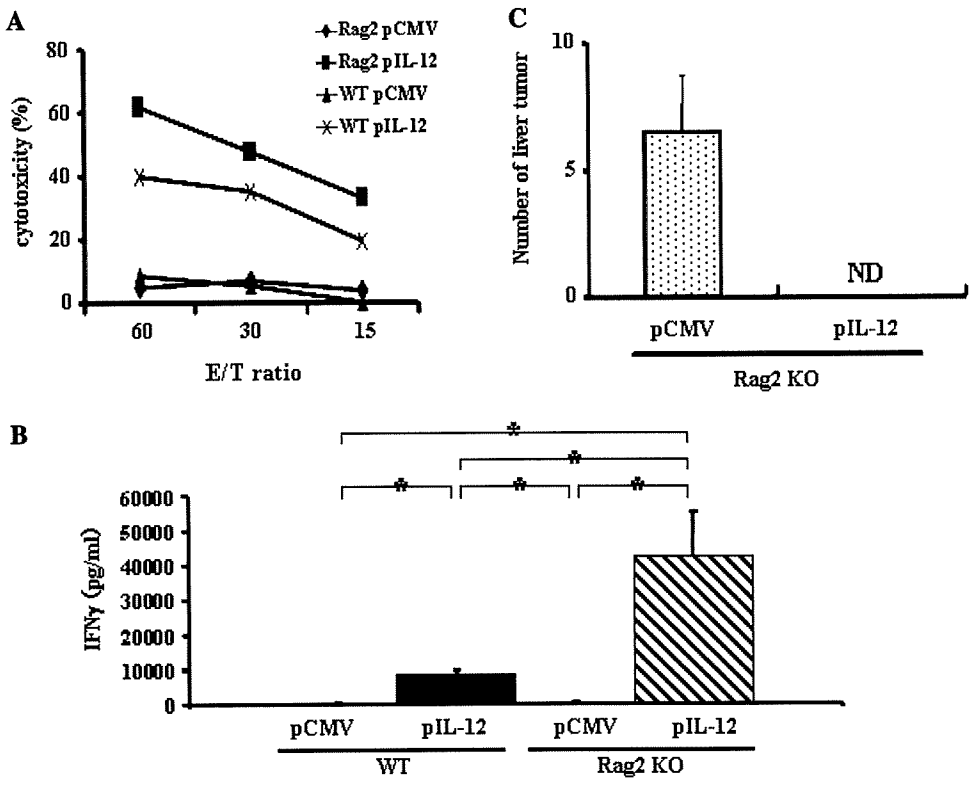

Fig. 4 Anti-tumor effects of IL-12 in Rag2 KO mice. Serum IFNγ levels and NK-cell activation. Wild-type or Rag2 KO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV and killed at 4 days. **a** Yac1 lytic ability of hepatic mononuclear cells was determined by Cr releasing assay as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ were determined by ELISA. Data are expressed as mean and SD ($n = 7$/group). *$p < 0.0001$. **c** Anti-metastatic effect. Rag2 KO mice were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Fourteen days after plasmid injection, mice were killed to examine tumor development in the liver. The numbers of hepatic tumors in each group are expressed as mean and SD ($n = 7$/group). *ND* not detectable

anti-metastatic effect of IL-12 was restored in GKO mice when whole mononuclear cells from wild-type mice were adoptively transferred (Fig. 5c).

To evaluate the contribution of IFNγ production from each subset of mononuclear cells to the anti-metastatic effect of IL-12, we adoptively transferred the same number of whole mononuclear cells, NK cells or non-NK cells from wild-type mice (4.0×10^6 cells) 1 day before pCMV-IL-12 injection and analyzed liver tumor formation. Only in the NK-cell-transferred group, pCMV-IL-12 injection induced NK cytolytic ability in the liver and IFNγ elevation in serum 4 days after plasmid injection, but not in the other groups (Fig. 5d, e). No liver tumor formed in the NK-cell-transferred group. In contrast, livers in other groups had massive tumors, and the liver weights were significantly heavier than those in the NK-cell-transferred group (Fig. 5f). These results clearly demonstrated the strong impact of IFNγ produced from NK cells on IL-12-induced anti-tumor effects compared with that from non-NK cells.

Anti-tumor effects of IL-12 deteriorated slightly in mice depleted of NK cells

To examine the involvement of NK cells in the tumor deletion by IL-12 therapy, we induced depletion of NK cells by repeatedly injecting anti-asialoGM1 antibody. The cytolytic ability of NK cells was completely abolished in the anti-asialoGM1 antibody-injected group (Fig. 6a). Serum IFNγ induction by IL-12 in the NK depletion group was about half of that in the control immunoglobulin injected group (Fig. 6b). Unexpectedly, pCMV-IL-12 injection inhibited macroscopic liver metastasis of CT-26 cells in NK cell-depleted mice (Fig. 6c). However, a number of microscopic tumor regions were observed after IL-12 therapy in NK cell-depleted mice but not in control IgG-injected mice (Fig. 6d). This finding indicated that NK cells are required for a full-blown IL-12 anti-tumor effect, but IL-12's anti-tumor effect was still observed even if the NK cells were knocked down. To examine the underlying mechanisms of anti-tumor effect in NK cell-depleted mice,

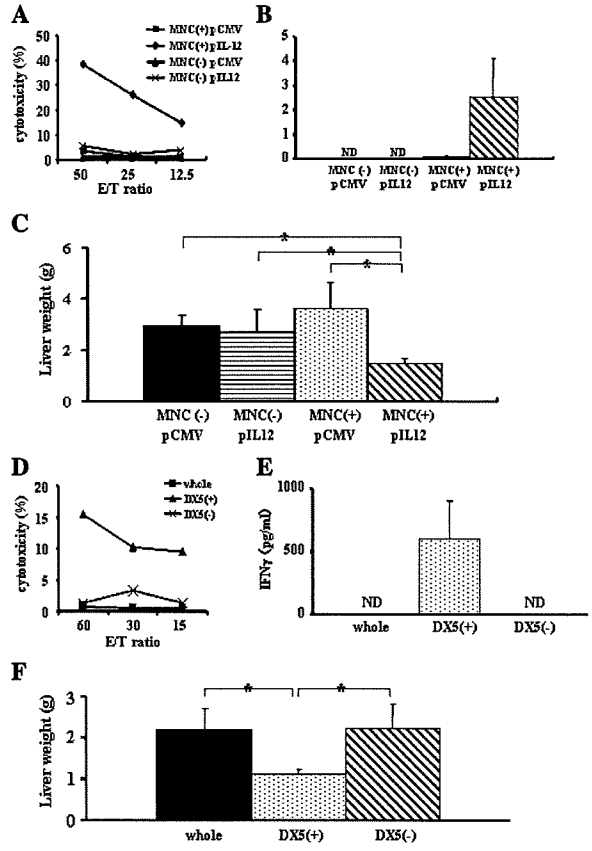

Fig. 5 Adoptive transfer of wild-type cells into GKO mice. Adoptive transfer of wild-type splenocytes restored anti-tumor effects of IL-12 in GKO mice. **a** GKO mice were intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. Yac1 lytic ability of hepatic mononuclear cells was expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **b** and **c** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice. Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **b** The levels of serum IFNγ 4 days after plasmid injection are expressed as mean and SD ($n = 6$/group). **c** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are indicated as mean and SD ($n = 6$/group). *ND* not detectable. $^*p < 0.01$. Adoptive transfer of wild-type NK cells, but not non-NK cells, restored anti-tumor effects of IL-12 in GKO mice. **d** Wild-type splenocytes were purified into $DX5^+$ cells and $DX5^-$ cells. GKO mice were intravenously injected with 4.0×10^6 whole mononuclear cells or $DX5^+$ cells or $DX5^-$ cells and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after hydrodynamic injection. Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **e** and **f** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with whole mononuclear cells, $DX5^+$ cells or $DX5^-$ cells (4.0×10^6/mouse). Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **e** The levels of serum IFNγ are expressed as mean and SD ($n = 6$/group). **f** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $^*p < 0.001$

serum levels of IP-10 and MIG, chemokines downstream of IFNγ, were measured after IL-12 therapy (Fig. 6e). pCMV-IL-12-injected mice showed significant increase in both levels compared with pCMV-injected mice. Significant increase after pCMV-IL-12 injection was also found in NK cell-depleted mice, but not in GKO mice. This result suggests that production of these chemokines was not completely suppressed in NK cell-depleted mice in our experimental condition. Immunohistochemical analysis revealed that tumoral accumulation of CD4-positive cells and CD8-positive cells was observed in pCMV-IL-12-injected mice but not in pCMV-injected mice. On the other hand, similar levels of CD31 expression were observed in tumors of pCMV-injected mice and pCMV-IL-12-injected mice (Fig. 6d). These results suggest that IL-12's anti-tumor effects might be mediated by T-cell accumulating in the tumor rather than anti-angiogenesis.

Discussion

IL-12 is recognized as a master regulator of adaptive type 1, cell-mediated immunity. One major action of IL-12 is its induction of other cytokines, particularly IFNγ. A large amount of evidence has indicated that IL-12 administration leads to IFNγ production from a variety of immune cells, such as T cells [16], B cells [17], NK cells [18] and NKT cells [22]. The relative impact of each immune cell as the source of IFNγ has been controversial. The present study highlighted NK cells as a most efficient producer of IFNγ that is critical for IL-12-induced anti-tumor effects.

Flow cytometric analysis revealed higher in vivo production of IFNγ of NK cells than that of other cell types. The levels of serum IFNγ were around fourfold higher in Rag2 KO mice which only possess NK cells than in wild-type mice. On the other hand, NK-cell depletion in wild-type mice led to twofold reduction of serum IFNγ levels. These data indicate substantial contribution of NK cells in IFNγ production in vivo. Previous research has demonstrated that the specific cellular effects of IL-12 are due mainly to activation of STAT4 [23, 24]. IL-12-induced STAT4 phosphorylation leads to the production of IFNγ [25]. In agreement with these reports, our in vitro analysis showed that, in contrast to STAT1, STAT4 was directly phosphorylated upon IL-12 stimulation, being independent of IFNγ. Of interest is the finding that NK cells express higher levels of STAT4 than non-NK cells, suggesting that NK cells possess an ideal expression profile of STATs for producing IFNγ upon IL-12 stimulation. Indeed, in vitro analysis revealed that NK cells, upon IL-12 exposure, displayed higher levels of IFNγ production as well as STAT4 phosphorylation than non-NK cells. These in vitro

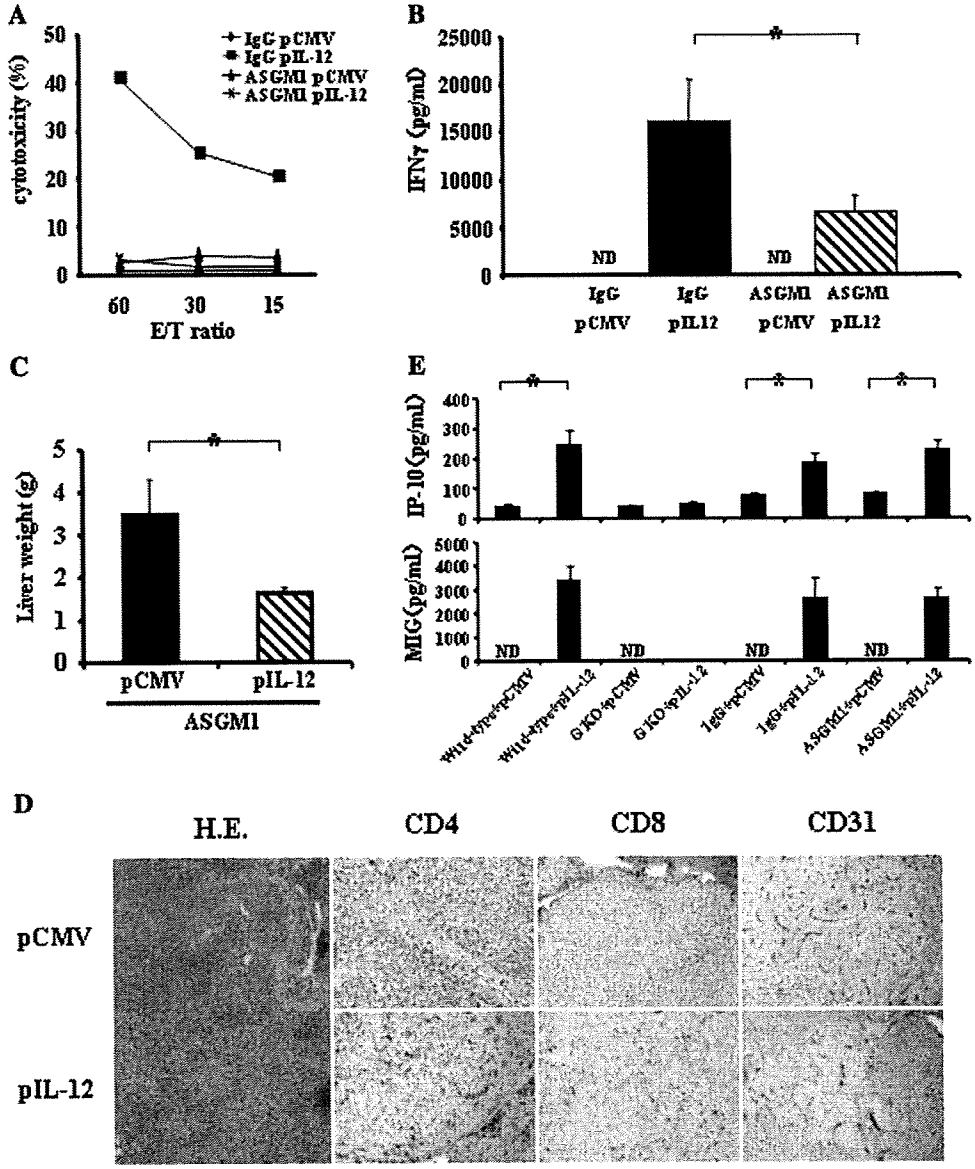

Fig. 6 Anti-tumor effects of IL-12 in NK-cell-depleted mice. Serum IFNγ levels and NK-cell activation. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and, 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. **a** Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $^*p < 0.005$. Anti-metastatic effects. Wild-type mice were intrasplenically injected with CT-26 cells and, 1 day later and then every 5 days, intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and hydrodynamically injected with either pCMV-IL-12 or pCMV 2 days after CT-26 injection. Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. **c** The results are indicated as mean and SD ($n = 6$/group). $^*p < 0.001$. **d** Representative histology of liver sections analyzed by hematoxylin-eosin staining and immunohistochemistry of CD4, CD8 and CD31. **e** Serum levels of IP-10 and MIG. Wild-type or GKO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Four days later, each mice were bled to measure the levels of serum IP-10 and MIG. Results are expressed as mean and SD ($n = 6$/group). *ND* not detectable. $^*p < 0.001$

data are consistent with the in vivo observation that NK cells are efficient producers of IFNγ during IL-12 therapy.

Many studies have demonstrated that IFNγ production is required for the anti-tumor effects of IL-12 [14, 26, 27]. In fact, we have demonstrated that deletion of IFNγ abolished NK cytotoxicity and the anti-metastatic effect of IL-12 therapy in the liver. A large amount of evidence supports the concept that a major action of IL-12 is to promote the differentiation of naïve CD4 + T cells into Th1 cells, which produce IFNγ. Previous research reported that CD4

T-cell depletion caused inhibition of anti-tumor effects. More recent studies have supported a critical role of IFNγ as a third signal for CD8 T-cell differentiation. There have been many reports focusing on IFNγ production from T cells induced by IL-12 for the anti-tumor effect of IL-12 [28]. Segal et al. performed an elegant study showing a critical role of T-cell production of IFNγ in the anti-tumor effect by adoptively transferring T cells into GKO mice in a subcutaneous tumor model [29]. However, apart from this study, little is known about the contribution of each immune cell as a producer of IFNγ in terms of an anti-tumor effect. In our model, T-cell mediated adaptive responses were not required for the anti-metastatic effect of IL-12. More importantly, the anti-metastatic effects of IL-12 were restored in GKO mice by an adoptive transfer of wild-type NK cells. The same number of non-NK cells could not provoke IL-12-induced anti-tumor effects in GKO mice. The present study demonstrated for the first time a potent effect of NK cells on producing IFNγ that was critical for anti-metastatic effect during IL-12 therapy.

Our study showed that the main IFNγ producer of IL-12 was NK cells. So we focused on NK cells which were activated by IL-12 in an IFNγ-dependent manner to examine the cellular mechanism of protection against hepatic metastasis. Many studies have shown the importance of each subset (NK- [12], NKT- [10] and T [9, 30] cells) for anti-tumor effects of IL-12. In the present study, NK cells were sufficient while T cells, B cells, NKT cells were dispensable for IL-12-mediated NK-cell activation and anti-metastatic effects as IL-12 therapy showed Yac1 lytic ability and antimetastatic effects in Rag2 KO mice. On the other hand, NK-cell depletion by a repeated injection of anti-aialoGM1 antibody protected wild-type mice from macroscopic liver metastasis, but did not from microscopic liver metastasis. Thus, although NK cells were required for a full-blown IL-12 anti-tumor effect, other anti-tumor pathways are activated by IL-12 in the absence of NK cells. Serum levels of IP-10 and MIG suggest that production of these chemokines downstream of IFNγ was not suppressed in NK-cell-depleted mice in our experimental condition. When compared with the experiment on GKO mice, accumulation of CD4-positive cells and CD8-positive cells were more evident in NK-cell-depleted mice than in GKO mice (Supplementary Figure). On the other hand, there was no remarkable difference in the expression of CD31 between pCMV injection and pCMV-IL-12 injection. These results suggested that in NK-cell-depleted mice IL-12 may exert anti-tumor effect via T-cell accumulation rather than anti-angiogenesis.

Since the liver contains an abundance of immune cells (especially NK cells) [31], the cytokine-mediated activation of these cells may be a promising approach toward anti-tumor therapy in this organ [32]. IL-12 is a cytokine known to elicit a potent anti-tumor effect in mouse experimental models. However, clinical trials attempted to date were interrupted by fatal adverse effects. Systemic IL-12 therapy has been associated with dose-limiting toxicity [33]. IL-12 induces activation of the pro-inflammatory pathway which causes the complications of high dose cytokine, independent of the action of IFNγ [34]. On the other hand, the levels of immunosuppressive cytokine, for example, TGF-β1 or IL-10 were significantly higher in patients with hepatocellular cancer and colon cancer [35–38]. In particular, TGF-β1 in serum can limit NK-cell IFNγ production [39]. Thus, in patients with advanced disease, IL-12 may not be able to exert its potent anti-tumor immune-effects because IFNγ, which is an important mediator of the IL-12-induced immune response, is less effective in a tumor environment. In the present study, we demonstrated that NK-cell IFNγ production induced by IL-12 was sufficient for the anti-metastatic effect of IL-12 in the liver. Thus, a strategy of efficiently producing IFNγ from NK cells may be important for avoiding toxicity of IL-12 therapy.

IL-12 gene therapy has an advantage to allow local production of the cytokine at the tumor sites with low serum concentration. Studies demonstrated that intratumoral administration of adenovirus encoding IL-12 to animals with different types of carcinoma caused complete tumor eradication and increased long-term survival [40, 41]. Moreover, injection of IL-12-encoding adenovirus in one nodule of liver tumor resulted in regression of distant nodules in the liver [41]. However, in a clinical trial anti-tumor activity of IL-12-encoding adenovirus was only observed in the injected tumor sites, but not in distant tumors [42]. The present study shed light on hydrodynamic transfection of hepatocytes as a promising strategy to eradicate disseminated tumors from whole liver.

In summary, NK cells are not just an effector for innate immunity but a mediator producing IFNγ that is critical for the IL-12 anti-tumor effects. Extremely higher expression of STAT4 may be a basis for efficient production of IFNγ from NK cells.

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