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H. 知的財産権の出願・登録状況（予定を含む。）

1.特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

研究成果の刊行に関する一覧表 I

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represent a subpopulation of oval cells negative for typical oval cell markers such as alpha-fetoprotein, biliary-type cytokeratins albumin, and also negative for leukocyte common antigen (CD45) and desmin. Because this pattern of cellular reaction was more prominent in models of periportal injury, rather than the more typical centrilobular APAP injury, it suggests that they may be related to hepatobiliary regeneration when the canal of Hering stem cell niche is disrupted or obliterated along with the destruction of the periportal hepatocytes [147].

Liver Stem/Progenitors Cells and Cell Therapy

In spite of the extensive regenerative capacity of the liver against diverse types of injuries, alternative methodologies to treat end-stage liver diseases are still urgently needed. Liver transplantation is the standard of care for end-stage liver disease and many liver-based metabolic conditions. Techniques involve whole organ replacement, split or reduced donor liver, and auxiliary liver transplantation. However, transplantation has serious limitations, such as donor scarcity, immunologic incompatibilities, high cost, significant morbidity and mortality associated with the procedure, and death while waiting for the transplant [148]. Furthermore, considerable long-term side effects have been reported [149-152]. Hepatocyte transplantation (HT) was thought to be a promising alternative to orthotopic liver transplantation (OLT) for treating liver-based inborn errors of metabolism where the aim is to replace a single deficient enzyme or its product [153-156]. The aim of this kind of procedure is to maintain liver function while the patient awaits OLT or until regeneration of the native liver occurs. The procedure is less invasive than OLT and can be performed repeatedly. The number of cells transplanted usually represents approximately 5% of theoretic liver mass, and either fresh or cryopreserved cells have been used. The safety of the procedure has been well established, and the clinical results are encouraging with clear improvement in disease phenotype. However, cell function often declines after about 9 months with the result that patients then undergo OLT. Problems with immunosuppression and rejection may be an important factor. Intraportal injection is the main cell delivery route for clinical HT with the portal venous system accessed by percutaneous transhepatic puncture or inferior mesenteric vein catheterization [157]. However, mature hepatocyte transplantation has been performed for more than 15 years in humans and there is still lack of evidence of success and reproducibility in large scale [158,159]. The main problems related to this approach are the fact that these harvested cells normally do not show optimal condition as well as the lack of standardized protocols to assess the cell's quality, the low proliferation/engraftment rate, the poor cell viability after cryopreservation methods, and the lack of hepatic metabolic functions after routine culture [160-162]. Moreover, it is known that mature hepatocytes exhibit increased

rates of polyploidy that contribute to proliferation decrease and cell senescence. Furthermore the latter events might presumably impair the regenerative capacity of these cells [163,164]. On the other hand, extrahepatic stem cells have been exhaustively tested. Stem cells obtained from different tissues (i.e., fetal annex, adipose tissue, bone marrow) have been successfully utilized in diverse settings of experimental chronic liver diseases [165-169].

A number of animal studies show that adult bone marrow cells could be applied to therapeutic purposes in certain liver diseases. Transplantation of adult bone marrow stem cells (BMSCs), either the mononuclear/hematopoietic cell fraction or mesenchymal stem cells, has therapeutic effects of restoration of liver function and mass, alleviation of fibrosis, and correction of inherited liver diseases. Although some controversial issues exist in relation to the results obtained by the different groups, mainly in relation with the beneficial effect on fibrosis, the restoration of liver function is evident in almost all animal studies [165,168,170,171-174]. Some of the discrepancies are thought to lie either in the differences between the experimental protocols or in the techniques employed to validate the effects [170].

Other sources of extrahepatic stem cells, such as embryonic stem cells and umbilical cord blood cells, have been tested and have demonstrated a potential for hepatic repopulation [166,170]. However, because of the ethics controversy and source shortages, their availability is limited. Therefore, BMSCs have unique advantages over other stem cell sources, particularly those BMSCs from the autologous source.

It must be noticed that the high prevalence of chronic liver disease and the increased number of patients reaching end-stage disease and requiring OLT may lead to a shortage of donor livers. This clinical scenario has driven forward a number of trials of autologous stem cell therapy. Cell therapy has several potential advantages when compared to OLT, because transplantable cells can be expanded in vitro and cryopreserved, genetically manipulated to correct inborn errors of metabolism, cryopreserved for future use and infused without major surgery, or obtained from the same patient, thereby avoiding risk of rejection and the need for lifelong immunesuppression.

Many of the clinical trials for liver diseases are still pilot studies and are therefore unrandomized and uncontrolled, but they show some interesting results. Studies from Terai and colleagues (2012) in Japan and Lyra and collaborators (2007) have confirmed the safety and efficacy of autologous bone marrow cell infusion (ABMi) therapy applied to patients with liver cirrhosis [175,176].

Terai and Sakaida, (2003) et al. have developed an in vivo murine model (the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model) and reported that GFP-positive bone marrow cells infused via a tail vein (peripheral vein) efficiently repopulated cirrhotic liver. Repopulated bone marrow cells ameliorated liver fibrosis through higher expression of matrix metalloproteinase-9, consistent with

improved liver functions and survival rate [165,177]. They also confirmed that the number of A6-positive cells in GFP-positive bone marrow cell infused livers increased, suggesting the activation of the HPC compartment by the bone marrow cell infusion [170]. Based on these findings, they started a clinical trial using autologous bone marrow cell infusion (ABM*i*) therapy for decompensated liver cirrhotic patients. As a result, at 6 months after ABM*i*, the average levels of serum albumin and Child-Pugh score significantly improved in nine patients (hepatitis B virus–related: three cases, hepatitis C virus–related: five cases, unknown: one case). The average proliferating cell nuclear antigen (PCNA)-labeling index also increased in biopsied livers after ABM*i*, suggesting induced proliferation of resident hepatocytes by ABM*i* [178]. In addition, Kim et al. confirmed that ABM*i* improved serum albumin levels, Child-Pugh score, liver volume measured by abdominal magnetic resonance imaging (MRI), and accumulation of ascites in 10 patients with hepatitis B virus–related decompensated liver cirrhosis, and histologic observations of liver biopsies taken over time showed increased CK-7 positive cells after ABM*i*, suggesting the possibility of HPC activation as the underlying mechanism [171].

In this scenario, studies focusing on intrahepatic stem/progenitors cells have shown promising results to overcome the present limitations. Because they are able to proliferate and give rise to hepatocytes and cholangiocytes [164,184], liver stem/progenitor cells could make a better choice for long-term repopulation and sustained metabolic activity as well as an efficient alternative for treating liver disorders.

Tanimizu et al. showed that Dlk-1 (delta-like1, a cell surface transmembrane protein highly expressed in human and rodent fetal liver, but not in the adult) is useful for enriching a progenitor population harvested from fetal liver. They described culture condition standardization for these cells (which they called hepatoblasts by their characteristic AFP expression), evidencing the important role of extracellular matrix proteins for cell behavior. Furthermore, they proved successful engraftment of Dlk+ cells harvested from GFP+ mice in recipient damaged livers [179,180]. In accordance with Tanamizu's reports, Oertel et al. also isolated Dlk-1+ cells from fetal liver and injected them in hepatectomized rats. These cells (but not Dlk-1− cells) were able to repopulate damaged liver [181].

Likewise, aiming at future clinical applications, Weiss et al. isolated Thy-1/CD90+ cells from human adult liver and transplanted them into immunodeficient mice. The group was able to verify engraftment and human hepatic marker production [182]. It is important to note that the Thy-1 (CD90) marker is absent in Dlk-1+ cells, as previously reported by Oertel M et al. [183].

Another recent and interesting study discussed other advantages of the hepatic stem/progenitor cells for future use in therapy. Steatotic livers, discarded for orthotopic liver transplantation, could be a good source of large number of these

cells [184]. In this study, Tolosa et al. used EpCAM, Thy-1, and OV-6 markers to select cells from both human and rat livers and verified significant proliferation of these cells. The group suggests that steatotic liver could be used to isolate stem/progenitor liver cells and transplant them in large scale. EpCAM has proved to be an important marker of stem/progenitor liver cells. It was demonstrated that purified EpCAM+/AFP− cells from fetal and postnatal livers are able to engraft the livers of immunodeficient adult mice and give rise to mature human liver parenchymal cells. Interestingly, these cells showed multipotencity and self-renewal [121].

In 2008, McClelland et al. demonstrated that the use of differential culture conditions can successfully isolate HpSCs, but not hepatoblasts, their immediate descendants, which died after few days. The tools used to differentiate them were size (HpSCs ~ 7 to 9 μm; HBs ~10 to 12 μm), morphology (HpSCs have high nucleus/cytoplasm ratio; HBs produce colonies with cordlike morphology), and markers (HBs express AFP and ICAM-1, but not NCAM or claudin 3). Furthermore, they identified high telomerase activity in their HpSC cultures, suggesting self-replication and proliferation [185].

More recently, new advances were achieved in understanding the relationship between HpSCs and their niche [184,185]. Wang et al. elicited the relationship among HpSCs [140] (EpCAM+/NCAM+) and their neighbors (angioblasts, endothelial and stellate cells) and focused on the paracrine signals, in particular those elicited by the ECM components able to regulate the parenchymal lineage stages. Co-culture of the hHpSCs with the different subpopulations of mesenchymal cells elicited distinct biologic responses. The hHpSCs co-cultured with angioblasts resulted in the maintenance of stem cell phenotype, whereas the co-culture of hHpSCs with endothelia and precursors of stellate cells led to hepatoblasts. Moreover, the most extensive effect on differentiation was found in the culture conditions that produced the highest levels of heparan sulfate proteoglycans and was also correlated with tri-dimensionality, the ratio of type I collagen to other collagen types, the ratio of fibronectin to laminin isoforms, the presence of proteoglycans with moderate to high levels of sulfation such as HS-PGs isoforms, and the rigidity of the hydrogels.

Yet another relevant clinical approach was proposed: the use of tissue scaffolds to seed stem cells. The main goal of this approach is to load cells onto a synthetic or natural three-dimensional scaffold in order to induce hepatic differentiation with enhanced cell viability, proliferation, and function before transplantation [186]. This methodology, however, needs more long-term studies to verify feasibility and efficacy.

We conclude that the use of liver stem cells in clinical practice still faces obstacles. It is necessary to identify good markers to isolate the appropriate cell fractions. Moreover, methodologies to maintain and expand these cells in culture have to be developed. But these hurdles do not diminish the excitement about the future use of HpSCs to reduce the suffering of patients waiting for liver transplantation.

16 Hippoシグナリング

Hippo signaling

畠 星治，仁科博史

1. はじめに

Hippo〔Hippopotamus（かば）〕シグナルは，ショウジョウバエにおいて見い出された器官サイズを制御するシグナル伝達経路である．細胞増殖を抑制するとともに細胞死を誘導することで，器官における細胞数の調節を行い，器官サイズを制御する（図1）．ヒトを含む哺乳動物にまで高度に保存されており，器官の形成や再生においても重要な役割を果たしている．また，腫瘍抑制シグナル伝達経路としても機能し，さまざまな組織において発がんの抑制に寄与している．

2. 基本メカニズム

Hippoシグナルの主要構成因子および基本メカニズムは，ショウジョウバエと哺乳動物においておおむね類似している．Hippoシグナルは，上流制御分子，中核キナーゼカスケード，下流標的分子の3つに分けて考えることができる．主に細胞-細胞間接触の刺激によって活性化し，中核のキナーゼカスケードが転写共役因子を負に制御することで，核内での遺伝子発現を調節するシグナル伝達経路である（図2）．

1）中核キナーゼカスケード

Hippoシグナルの中核を成すのは，セリン/スレオニンキナーゼのHippoとWartsによるキナーゼカスケードである．この2つのキナーゼに加えて，活性化因子もしくは足場タンパク質として働くSav（Salvador）とMats（Mob as tumor suppressor）により，中核のキナーゼカスケードが構成される．Hippoが活性化するとSalvadorやMatsと協調してWartsをリン酸化することにより活性化させる．

ショウジョウバエにおけるHippo，Warts，Salvador，Matsが，哺乳動物ではそれぞれのホモログであるMst1（Mammalian Ste20-like kinase 1）とMst2，Lats1（Large tumor suppressor 1）とLats2，Sav1（Salvador 1），Mob1に対応する．

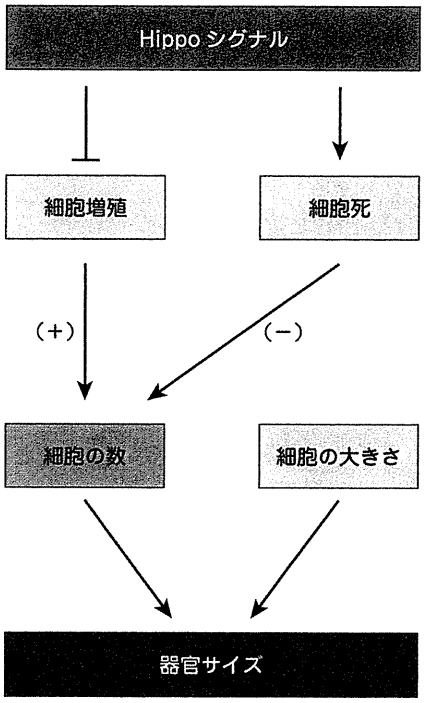

図1 Hippoシグナルによる器官サイズの制御
器官のサイズは，器官を構成する細胞の数とその大きさによって規定される．Hippoシグナルは，細胞増殖の抑制と細胞死の促進を誘導することにより細胞の数を調節し，器官サイズを制御する

2）下流標的分子

活性化したWartsは，Hippoシグナルの下流標的分子である転写共役因子Yki（Yorkie）をリン酸化する．非リン酸化型のYkiは，核内に局在し，転写因子Sd（Scalloped）による標的遺伝子の転写を促進する．しかし，Wartsによりリン酸化されると，リン酸化型のYkiと14-3-3タンパク質との結合が誘導され，細胞質に保持さ

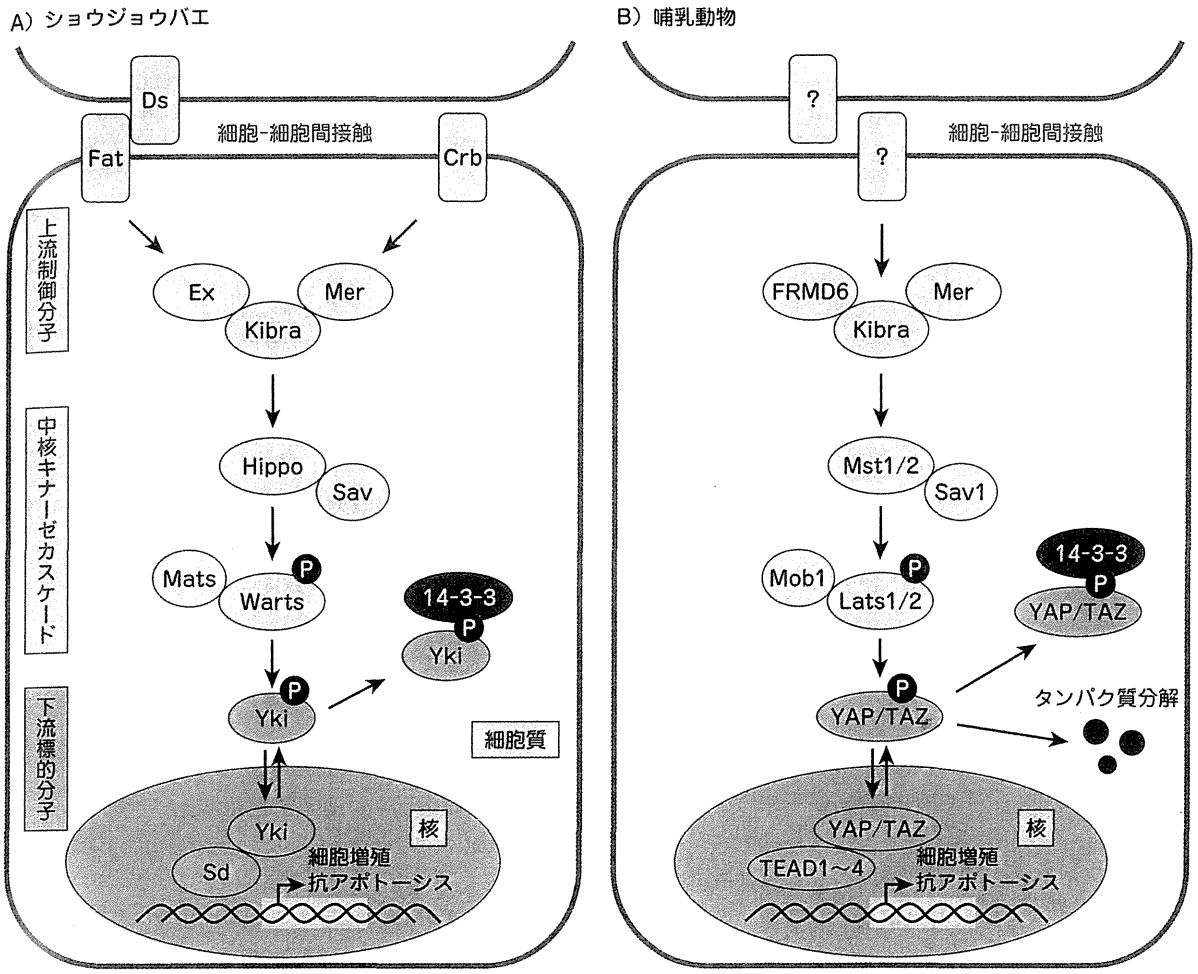

図2 ショウジョウバエと哺乳動物におけるHippoシグナルの概略図

A) ショウジョウバエのHippoシグナル．細胞-細胞間接触などの刺激に応答し，上流制御分子を介して中核キナーゼカスケードが活性化する．活性化したWartsにより，下流標的分子Ykiの核内局在が制限される．これにより，Yki依存的な遺伝子発現誘導が抑制され，細胞増殖の抑制と細胞死の促進が誘導される．B) 哺乳動物のHippoシグナル．主要構成因子および基本メカニズムはショウジョウバエと類似しているが，未解明な部分も多い

れる．その結果，Ykiの核内局在が抑制され，遺伝子発現が負に制御される．

Ykiの哺乳動物ホモログは，YAP（yes-associated protein）およびTAZ（transcriptional coactivator with PDZ-binding motif）であり，Sdの哺乳動物ホモログはTEAD（TEA domain transcription factor）1～4である．哺乳動物でのLats1/2によるYAP/TAZのリン酸化は，細胞内局在の制御に加えて，ユビキチン・プロテアソーム経路を介したタンパク質分解を誘導することにより，YAP/TAZの機能を抑制する．

3) 上流制御分子

中核キナーゼカスケードは，細胞-細胞間接触に関与する上流制御分子により活性化される．ショウジョウバエにおいては，接着分子であるプロトカドヘリンのFatが同じくプロトカドヘリンであるDs（Dachsous）との結合を介して，Hippoシグナルを活性化させる．また，細胞間の接着などにより形成される上皮細胞極性に重要な役割を果たすCrb（Crumbs）もHippoシグナルの活性化に寄与する．FatおよびCrbのいずれも細胞膜の裏打ちタンパク質であるEx（Expanded），Mer（Merlin），Kibra（kidney and brain expressed protein）による複合体を介して，中核キナーゼカスケードを活性化させると考えられている．

一方，哺乳動物では，Fatの関与は明らかではないが，Exのホモログであるfrmd6やMer，Kibraの機能は保存されており，ショウジョウバエと同様にこれらの複合

体が中核キナーゼカスケードの上流に位置すると考えられている．

4）その他の制御分子

上記の構成分子以外にも，Hippoシグナルの制御分子が次々と明らかになってきている．ショウジョウバエでは，Ras結合タンパク質であるdRASSF（Ras association domain family）やLimドメインタンパク質dJubaが，中核キナーゼの活性を制御する．哺乳動物においても，それぞれのホモログであるRASSFファミリー分子とAjubaが，類似の機能を有している．さらに，哺乳動物では，接着分子であるCD44や，細胞間接着に関与するα-カテニンおよびAngiomotinなどが，Hippoシグナルを制御することが知られている．

3. 機能

Yki/YAP/TAZはSd/TEAD1～4などを介して，細胞増殖および抗アポトーシスに関与する遺伝子の転写を誘導する．このため，Hippoシグナルの活性が亢進すると，Yki/YAP/TAZの機能が抑制され，細胞増殖の停止やアポトーシスの誘導が生じる．このように，Hippoシグナルは，器官における細胞数を調節することで器官サイズを制御している．さらに，細胞増殖の制御だけでなく，種々の幹細胞や前駆細胞の分化制御も担っており，胚発生や器官の形成や再生においても重要な役割を果たしている．

Hippoシグナルは，培養細胞株でみられる接触抑制（contact inhibition）に関与することも知られている．接触抑制は，細胞同士の接触により細胞の増殖が停止する現象であり，がん化した細胞株ではみられないことから，腫瘍抑制機構の1つとして考えられている．加えて，Hippoシグナルの構成因子が変異または欠損したショウジョウバエやマウスでは，細胞の過増殖が生じて発がんに至ることから，Hippoシグナルは腫瘍抑制シグナル伝達経路としても機能していることが明らかとなっている．実際に，ヒトのがん症例においてHippoシグナルの破綻が高頻度に認められている．

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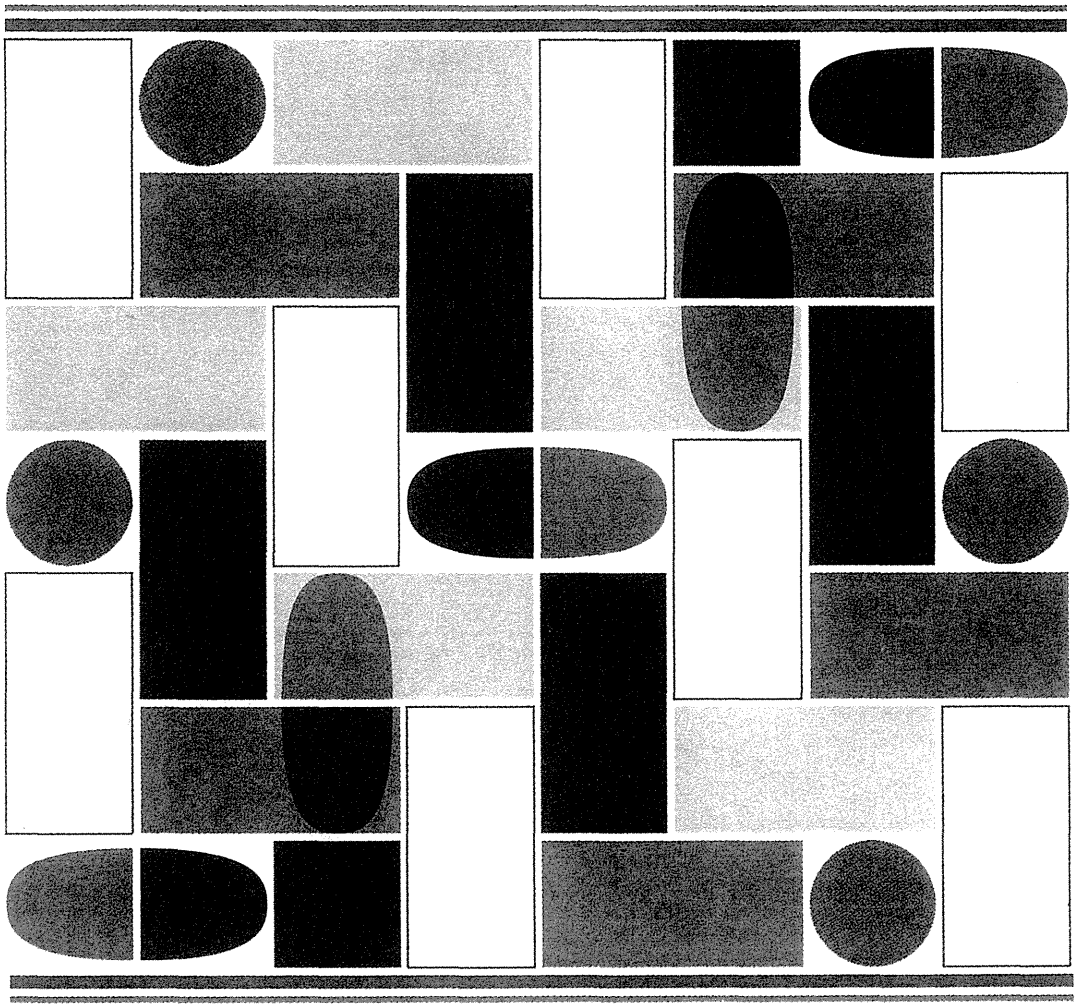

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Advanced Therapies Using Autologous Bone Marrow Cells for Chronic Liver Disease

TARO TAKAMI, SHUJI TERAI, AND ISAO SAKAIDA

Abstract: The radical treatment currently for decompensated liver cirrhosis is still liver transplantation. However, liver transplants are not widely performed worldwide and development of genuine regeneration therapy for liver cirrhosis is an urgent task. We have developed a novel murine model [the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model], and reported that infused GFP-positive bone marrow cells repopulated cirrhotic liver. Moreover, repopulated bone marrow cells ameliorated liver fibrosis through higher expression of matrix metalloproteinase-9, consistent with improved liver functions and better survival rate. Based on these findings, we started a clinical trial of autologous bone marrow cell infusion (ABM*i*) therapy for decompensated liver cirrhotic patients, and reported the efficacy and the safety of this approach. On the other hand, various other clinical studies for liver disease have been also reported, including hepatic administration of autologous CD34-positive cells induced by granulocyte colony-stimulating factor (G-CSF), portal vein administration of CD133-positive mononuclear cells, and administration of autologous bone marrow derived mesenchymal stem cells (MSCs). Effectiveness of these approaches has been shown in some patients. We provided here an overview of the current status of liver regeneration therapies including our results of the murine GFP/CCl_4 model and ABM*i* therapy for liver cirrhosis and future prospects. [*Discovery Medicine* 14(74):7-12, July 2012]

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Introduction

When decompensated liver cirrhosis occurs, the only radical treatment currently is still liver transplant. However, liver transplants are not performed worldwide, due to various problems including a chronic donor shortage, surgical invasiveness, risk of immunological rejection, and medical costs. Therefore, development of regeneration therapies for liver cirrhosis is an urgent task. Theise *et al.* (2000) previously reported the existence of Y chromosome-positive hepatocytes and cholangiocytes in autopsied women who had received therapeutic bone marrow transplantations from male donors, suggesting the existence of pluripotent stem cells in bone marrow cells. Since then, attention has been focused on bone marrow stem cells as a cell source for liver regeneration therapies (Houlihan and Newsome, 2008; Souza *et al.*, 2009; Stutchfield *et al.*, 2010; Muraca, 2011). We have reported improved liver fibrosis and liver functions after the infusion of autologous bone marrow cells in basic studies using a novel murine model [the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model], and based on those lines of evidence started autologous bone marrow cell infusion (ABM*i*) therapy for liver cirrhotic patients in November 2003. Since then the efficacy and the safety of ABM*i* therapy have also been reported by other institutions.

During this same period, other novel therapies have been reported, including cell therapies by administration of CD34-positive cells induced with granulocyte-colony stimulating factor (G-CSF) (Gordon *et al.*, 2006; Pai *et al.*, 2008), and portal administration of CD133-positive mononuclear cells (am Esch *et al.*, 2005; Furst *et al.*, 2007).

We here show the current status of liver regeneration therapies including our evidence from basic studies (the murine GFP/CCl_4 model) and clinical studies (ABM*i* therapy) for liver cirrhosis and future prospects.

Basic Studies Using the Murine GFP/CCl_4 Model

We have developed an *in vivo* murine model (the GFP/CCl_4 model), and reported that GFP-positive bone marrow cells infused via a tail vein (peripheral vein) efficiently repopulated cirrhotic liver and then reduced liver fibrosis (Terai *et al.*, 2003; Sakaida *et al.*, 2004). This GFP/CCl_4 model has the following characteristics: 1) intraperitoneally administering CCl_4 (1.0 ml/kg body) was performed over 4 weeks (2 times per week) in female mice to induce liver cirrhosis with chronic liver injury, 2) infused whole bone marrow cells were isolated from the femurs of syngeneic GFP transgenic male mice and whole bone marrow cells were washed and then injected into the recipient mice via a tail vein, and 3) the inflammatory condition is maintained by repeated administering CCl_4 after bone marrow cell infusion. In these processes, elevation in serum albumin levels (Terai *et al.*, 2003), a significant increase in survival rate, and reduced liver fibrosis assessed by Sirius red staining were seen following infusion of whole GFP-positive bone marrow cells (Sakaida *et al.*, 2004). Repopulated GFP-positive bone marrow cells were also confirmed to produce collagenases including matrix metalloproteinase (MMP)-9 (Sakaida *et al.*, 2004). Based on the above basic studies, infusion of autologous bone marrow cells via a peripheral vein in a chronic liver injury environment is thought to reduce liver fibrosis and improve liver functions, and to significantly improve vital prognosis in CCl_4-induced cirrhotic mice. We have also confirmed two kinds of GFP-expressing murine bone marrow derived cells repopulated in cirrhotic livers. The first one was macrophage's cell surface marker (F4/80) positive cells. The other was mesenchymal stromal cell's surface marker positive cells, suggesting that these were candidates of the cell-fraction involved in reducing liver fibrosis.

Moreover, we confirmed that the number of A6-positve cells [oval cells, hepatic progenitor cells (HPC)] in GFP-positive bone marrow cell infused livers was increased, suggesting the activation of HPC compartment by the bone marrow cell infusion (Terai *et al.*, 2003). Another study showed the up-regulated expressions of fibroblast growth factor (FGF) receptors after bone marrow cell infusion and enhanced repopulation of GFP-positive bone marrow cells with increased Liv-2 positive cells after administrating FGF-2, indicating that FGF-2 had important functions as a growth factor contributing to these processes (Ishikawa *et al.*, 2006). Our investigation also revealed that splenectomy before bone marrow cell infusion enhanced the repopulation of bone marrow cells into the cirrhotic liver and improved the liver fibrosis by higher expressed MMP-9 from GFP-positive cells, consistent with the absence of trapped bone marrow cells in the enlarged spleen following splenectomy (Iwamoto *et al.*, 2012).

As liver cirrhosis itself is still oncogenic, we recently investigated the effect of bone marrow cell infusion on the mechanisms of hepatocarcinogenesis using our developed hepatocarcinogenic mice with liver cirrhosis (the DEN/GFP-CCl_4 model). This DEN/GFP-CCl_4 model was developed by intraperitoneally administering *N*-nitrosodiethylamine (DEN) once to 2-week-old mice, followed by repeated twice-weekly intraperitoneal administration of CCl_4 from 1 month later. Syngeneic GFP-positive bone marrow cells were infused via a tail vein biweekly from 2 months after DEN treatment. Kinetics of hepatocarcinogenesis was histologically evaluated at 4.5 months after DEN treatment based on the incidence, number, and size of foci and tumors (adenoma + hepatocellular carcinoma). As a result, in frequent bone marrow cell infused livers, both foci and tumors showed significantly lower incidence and smaller number; moreover, both foci and tumor size were almost equal, consistent with significant lower hepatic 8-hydroxy-2-deoxyguanosine (8-OHdG). Higher superoxide dismutase (SOD) activity and increased nuclear translocation of erythroid 2 p45-related factor 2 (Nrf2) were also confirmed after frequent bone marrow cell infusions. In addition, many SOD3-positive cells in non-tumorous liver tissue were positive for GFP protein, suggesting that bone marrow cell infusion might contribute to suppressed tumor initiation during stages of hepatocarcinogenesis through the stabilization of redox homeostasis directly (Maeda *et al.*, 2011).

ABM*i* Therapy for Liver Cirrhotic Patients

Based on the evidence from the murine GFP/CCl_4 model, our clinical study of ABM*i* therapy for decompensated liver cirrhotic patients was started in November 2003 (Terai *et al.*, 2006). All protocols were approved by the Ethics Committee of Yamaguchi University, and the written informed consent was obtained from every patient. Indications of the ABM*i* therapy are shown as follows: 1) total bilirubin: $\leq$3.0 mg/dL, 2) platelet count: $\geq 5.0\times10^{10}$/L, 3) good control of esophagogastric varices and hepatocellular carcinoma, 4) good cardiopulmonary function and no serious comorbidities, and 5) no presence of any viable hepatocellular carcinoma on abdominal computed tomography (CT), abdominal magnetic resonance imaging (MRI), or other diagnostic imaging modalities. For indicated patients, around 400 mL of autologous bone marrow fluid was collected under general anesthesia, in the same manner as for bone marrow transplantation for leukemic disease. We washed and concentrated bone

marrow mononuclear cells according to standard operating procedures at the regenerative and cell therapy center fully equipped with good manufacturing practice (GMP)-grade facilities. Finally, 5.20±0.63 x 10^9 bone marrow mononuclear cells were infused into the same patient by drip infusion via a peripheral vein within the same hospital day. The treatment course was observed for 6 months after ABM*i*, and the efficacy and the safety were evaluated using blood biochemistry tests, liver biopsy, abdominal ultrasonography (US), abdominal CT, and so on. During this observation period, there were no changes in oral medications, antiviral drugs, or other agents. As a result, at 6 months after ABM*i*, the average levels of serum albumin, serum total protein, and Child-Pugh score were significantly improved in 9 patients (hepatitis B virus-related 3 cases, hepatitis C virus-related 5 cases, and unknown 1 case) for whom the course could be observed for 6 months after ABM*i*. The average of proliferating cell nuclear antigen (PCNA)-labeling index was also increased in biopsied livers after ABM*i*, suggesting induced proliferation of resident hepatocytes by ABM*i* (Terai *et al.*, 2006). Moreover, similar improvements were confirmed in the same patients at 15 months after ABM*i* (Terai and Sakaida, 2008).

In addition, a multicenter clinical trial of liver regeneration with cell transplantation (LRCT study) was started in 2005. Kim *et al.* (2010) joined in LRCT study and reported that ABM*i* improved serum albumin levels, Child-Pugh score, liver volume measured by abdominal MRI and accumulation of ascites in 10 patients with hepatitis B virus-related decompensated liver cirrhosis, and histological observations of liver biopsies taken over time showed increased cytokeratin (CK)-7 positive cells after ABM*i*, suggesting the possibility of HPC activation as the underlying mechanism. Moreover, Saito *et al.* (2011) joined in LRCT study and also reported improvements of serum albumin levels, prothrombin time, and Child-Pugh score in alcoholic liver cirrhotic patients after ABM*i*. Therefore, we believe that ABM*i* therapy represents a promising treatment for decompensated liver cirrhotic patients (Figure 1).

Other Clinical Studies Using Non-cultured Autologous Bone Marrow Cells

Other reports to date on cell therapies using bone marrow cells for liver cirrhosis include not only those on our ABM*i* therapy, but also reports by Lyra *et al.* (2007; 2010) on the effectiveness of infusion of bone marrow cells. Clinical studies by Lyra *et al.* suggested the feasibility and safety of autologous bone marrow cell infusion through a hepatic artery rather than a peripheral vein for chronic liver disease patients awaiting liver transplantation. In a trial studying patients with hepatitis B virus-related decompensated liver cirrhosis, 53 patients received 120 mL of autologous bone marrow fluid via a hepatic artery, and 105 patients matched for some parameters including age, gender, albumin, total bilirubin, prothrombin time, and Model for End-Stage Liver Disease (MELD) score were selected as controls who received regular treatments (reduced glutathione, glycyrrhizin, ademetionine, polyene phosphatidylcholine, alprostadil, and human serum albumin). Results of analysis showed no adverse effects from bone marrow administration (Peng *et al.*, 2011). Patients were also divided into a short-term observation group (up to 48 weeks) and a long-term observation group (until 192 weeks), and the results of analysis showed improved hepatic function in the early period. Long-term observation showed lower tendency ($p=0.107$) of the hepatocellular carcinoma development after the administration of bone marrow cells (Peng *et al.*, 2011), consistent with suppressed hepatocarcinogenesis that we observed in our murine DEN/GFP-CCl_4 model (Maeda *et al.*, 2011).

In other clinical studies, increased volumes of left lateral hepatic segments were reported with intraportal administrating CD133-positive bone marrow cells after portal venous embolization of right liver segments (am Esch *et al.*, 2005; Furst *et al.*, 2007). Conversely, death due to radiocontrast nephropathy has been reported as a result of infusion of concentrated CD34-positive cells from 200 mL of bone marrow fluid through hepatic artery into patients with decompensated liver cirrhosis, and that clinical study was discontinued (Mohamadnejad *et al.*, 2007a). This indicates the need for clarification of the treatment indication criteria and full investigation of administration routes, cell concentrations, and speed of drip infusion.

Other Clinical Studies Using Cultured Autologous Bone Marrow-derived Cells

Our ABM*i* therapy involves bone marrow aspiration under general anesthesia, and is not indicated for patients for whom general anesthesia is difficult. We therefore aimed to develop a new liver regeneration therapy in which cells having a curative effect on liver cirrhosis are isolated and cultured from a small amount of autologous bone marrow aspirated under local anesthesia and infused back into the same patient.

Mohamadnejad *et al.* (2007b) have reported improvements of the MELD score in 2 of 4 decompensated liver cirrhotic patients with peripheral vein administration of 3.2×10^7 cultured autologous bone marrow derived mesenchymal stem cells (MSCs) as phase I study.

Kharaziha *et al.* (2009) also reported that liver function assessed by the MELD score decreased significantly from 17.9±5.6 to 10.7±6.3 after administration of cultured autologous bone marrow derived MSCs in 8 cirrhotic patients (hepatitis B-related 4 cases, hepatitis C-related 1 case, alcoholic-related 1 case, and cryptogenic 2 cases). They aspirated around 20 mL of autologous bone marrow fluid from both posterior and superior iliac spines under local anesthesia. The mononuclear cells were separated by the Ficoll separation method. Separated bone marrow mononuclear cells were cultured for 2 weeks, and then were collected. They infused about 3 to 5×10^7 cells expressing CD44, CD73, and CD105, consistent with MSC's characteristics, to the same subject via a portal vein or peripheral vein. Moreover, Amer *et al.* (2011) reported the clinical study for 40 patients including 20 controls with hepatitis C virus-related liver failure. They aspirated around 120 mL of autologous bone marrow fluid from the posterior-superior iliac crest under local anesthesia, and then injected autologous cultured bone marrow-derived MSCs ,which had been stimulated to expand the hepatic lineage using hepatocytes growth factor (HGF)-containing medium, into spleen or liver directly using a needle gauge 18 under abdominal ultrasound guidance. In these patients, the MELD score and Child-Pugh score were significantly lower than those in controls at from 2 weeks to 6 months after the injection. No difference between intrasplenic route and intrahepatic route was observed.

Clinical Studies Using G-CSF

Gordon *et al.* (2006) collected CD34-positive cells from peripheral blood after induction with G-CSF, then administered these cells via a hepatic artery, and reported improved levels of serum bilirubin and serum albumin in some patients, despite a short observation period of only 60 days. In addition, Spahr *et al.* (2008) administered G-CSF to patients with alcoholic liver cirrhosis and reported increased proliferation of HPCs, while Pai *et al.* (2008) reported improvements in serum

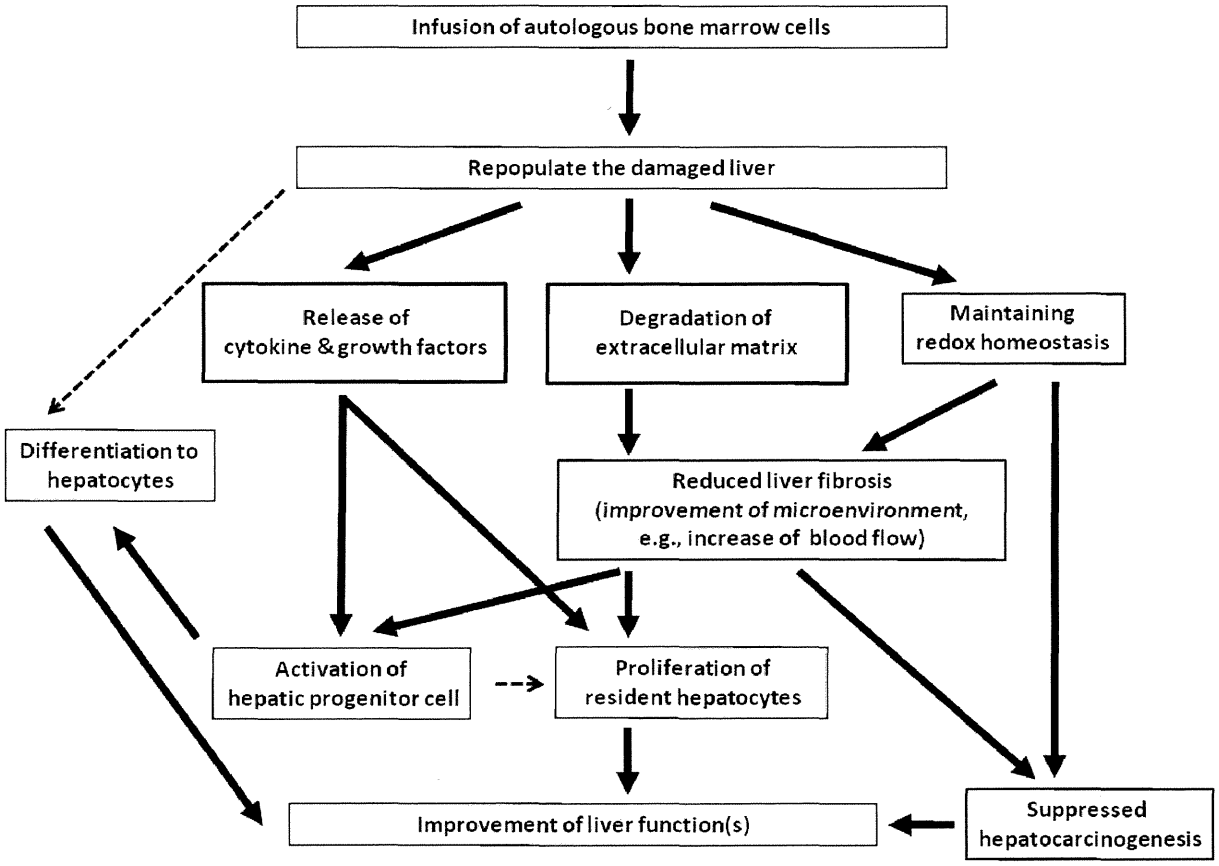

Figure 1. Possible mechanisms of autologous bone marrow cell infusion on liver cirrhosis based on evidences from the murine GFP/CCl_4 model and human ABM*i* studies.