Liver Cancer and Its Tumor Microenvironment: the Role of Mesenchymal Stromal Cells and SMADs

Pratika Yuhyi Hernanda

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Liver Cancer and Its Tumor Microenvironment: the Role of Mesenchymal Stromal Cells and SMADs

Leverkanker en de Micro-omgeving van de Tumor: de Rol van Mesenchymale Stromale Cellen en SMADs

Thesis

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Pratika Yuhyi Hernanda

born in Lumajang, East Java, Indonesia

ERASMUS UNIVERSITEIT ROTTERDAM

Doctoral Committee

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Cover:

Tumor microenvironment of the liver play an important role in the growth of liver tumor cells. [P.Y Hernanda, modified image @shutterstock.com]

Chapter 1

General Introduction and Aim of the Thesis

Hepatocellular carcinoma (HCC): pathogenesis and current treatment

Liver cancer is one of most devastating malignancies. Hepatocellular carcinoma (HCC) accounts for >90% of primary liver malignancies and is the third leading cause of cancer-related deaths worldwide. Major risk factors for hepatocellular carcinoma include infection with hepatitis B (HBV) or C (HCV) virus, alcoholic liver disease, and probably nonalcoholic fatty liver disease[1]. Especially chronic infection with HBV or HCV is a main risk factor for HCC, the recurrent viral infection characteristic for these diseases causes the body's immune system to attack liver cells is associated by repetitive damage of the genomic material, which leads to mistakes during its repair and in turn provoke carcinogenesis[2]. For patients with early-stage hepatocellular carcinoma, they may undergo resection for curative treatment and for patients who are not candidates for resection, liver transplantation should be offered with specific criteria[3]. In the case of unresectable limited disease, systemic treatment may be preceded and aided by locoregional therapies such as ablation (ie, radiofrequency, cryoablation, percutaneous alcohol injection, or microwave), transarterial chemoembolization, radioembolization, or stereotactic body radiotherapy and external-body radiotherapy. Systemic treatments are recommended for unresectable and advanced metastatic disease in patients with a Child-Pugh score of A or B (moderate operative risk).

For the majority of advanced HCC cases, curative treatments are not possible and the prognosis is dismal because of underlying cirrhosis as well as poor tumor response to standard chemotherapy[4]. For patients with advanced disease, representing the majority of patients at diagnosis, the only tumor-directed palliative option is sorafenib (Nexavar), an oral multi-kinase inhibitor, which increases patient survival with approximately 3 months[5]. Evidently, new therapeutic options are urgently needed for advanced or metastatic HCC. In this thesis I aim to explore the possibility of targeting of the intra-tumoral stromal compartment

as well as modulation of the cross-talk between the stromal compartment and the tumor cell through SMAD proteins as novel avenues for the potential treatment in this disease.

A specific tumor microenvironment characterizes the pathogenesis of liver cancer

The behavior of cancer cells is not only driven by genetic alterations in the cancer cells alone but also by tumor microenvironment, drives the surrounding milieu that the tumor cells often require for survival, growth, proliferation, and metastasis[6]. The tumor microenvironment is a complex product of an evolving crosstalk between different cell types, including the cancer cells themselves. Typically , in epithelial tumors the cancer bulk includes both invasive carcinoma and prominent stromal elements. These stromal elements consist of the extracellular matrix (ECM) as well as fibroblasts of various phenotypes, and a scaffold composed of immune and inflammatory cells, blood and lymph vessels, and nerves[6].

Remodeling of the liver microenvironment is a hallmark in the pathogenesis of liver cancer[7]. In cancer, the microenvironment which is also referred to as stroma, undergoes drastic changes, including the recruitment and the activation of stromal cells and the remodeling of the ECM. Co-evolution of tumor cells with their microenvironment during tumorigenesis suggests that tumor-stroma crosstalk may likely influence the phenotype of tumor cells and may provide a selective pressure for tumor initiation, progression and metastasis[8]. In addition, the liver provides a distinct immunological environment and the ultimate effects of this environment on cancer progression may differ in the liver as compared to other organs[9]. Nevertheless, clinical targeting of the tumor stroma or the cross-talk between the tumor cell environment and the cancer cell themselves have no place yet in clinical practice with regard to liver cancer, also because of lack of fundamental knowledge of the mechanisms involved. In chapter 2 of this thesis I shall aim to provide a comprehensive overview of the information available to date and argue that the absence of specific information as to the action of the stroma in liver cancer warrants a moratorium on clinical trials directed at stroma-tumor cell interaction until further data becomes available.

Mesenchymal stromal cells as a pivotal part of tumor stroma

Mesenchymal stem/stromal cells (MSCs) were initially identified as a heterogeneous population of stromal cells in the bone marrow (BM) that supports hematopoietic stem

cells[10]. Further studies demonstrated that MSCs possess multilineage differentiation potential, can exert anti-inflammatory function, immunomodulatory properties and can influence other cells through the production of paracrine factors[11]. MSCs attract attention as a possible cell-based therapy, especially in immune-related diseases and over 300 trials have been registered (January, 2014, clinicaltrials.gov). The role of MSCs in pathogenesis has been less well studied. Recent evidence has come forward in various pre-clinical models that MSCs can migrate into certain types of tumors and even using MSC as anti-cancer drug/gene delivery has been proposed[12,13]. The role of MSCs in cancer development, however, remains unclear. Several studies indicated that MSCs restrain cancer growth and exert a benign influence on disease course[14-16]; whereas other studies have shown that MSCs are able to promote tumor progression and metastasis in experimental cancer models[17-20]. Thus, it remains largely elusive whether MSCs have a beneficial or detrimental role in the cancerous process[21] and experimentation with MSCs directly obtained from human cancer is deemed necessary to obtain answers here. Such experimentation will be directly provided in chapters 3 and 4, where I shall show that the main action of MSCs is pro-carcinogenic, again argueing against the use of MSCs as therapeutic vehicles in liver cancer clinical trials.

SMAD signaling in the tumor microenvironment

The unique etiology as well as the distinct environment that the liver holds may govern a differential signaling network compared to other cancers. SMAD signaling is an important process operative in the tumor microenvironment. SMAD proteins are recognized as central mediators of Transforming Growth Factor Beta (TGFB) and/or Bone Morphogenetic Protein (BMP) signaling pathways, which regulate a plethora of physiological processes including cell growth and differentiation.[22] These two pathways signal through the family of SMAD proteins to exert their effects. In mammals, there are 8 SMADs that are subdivided into 3 distinct classes: receptor-regulated SMADs (R-SMADs) comprising SMAD2 and SMAD3 (transduce TGFB signaling) and SMAD1, SMAD5, and SMAD8 (transduce BMP signaling); a common SMAD called SMAD4; and 2 inhibitory SMADs, namely, SMAD6 and SMAD7.[23] SMAD proteins are highly conserved within their family and across species, with SMAD4 representing a somewhat divergent subtype which still retains about 40% identity with other family members.[24]

Accordingly, deregulation of TGFB/BMP pathways almost invariably leads to developmental defects and/or diseases, in particular cancer.[25] TGFB/SMAD signaling might also play a role in epigenetic silencing of critical ephitelial mesenchymal transition (EMT) genes.[26] EMT is a key event in tumor invasion process in the tumor microenvironment. The other pathway which also link to SMAD4 is the BMP pathway. BMPs are now considered to constitute a spectrum of pivotal biological functions in various types of cells[27]. Deregulation of BMP/SMAD pathway is often associated with developmental defects and/or diseases, in particular cancer[25]. Overexpression of BMP inhibitors was decribed in HCV associated HCC tumors[28]. Given the potential importance of the biological and clinical implications associated with SMAD signalling in general and its role in stroma-cancer cell interaction in particular, further investigation the role of SMAD signaling in liver cancer is neccessary. Experimentation addressing this issue is provided in chapters 5 and 6.

Therapy and the mesenchymal stromal compartment: do we make the right clinical choices?

Possibly the most important interaction between the tumor stroma and the cancer cell compartment consists of the immune-evasive landscaping provided by these cells. Recent work performed in our laboratory by Perdozo *et al.*[29] has shown that the tumor stroma in liver cancer appears highly capable of recruiting and regulatory T cells and immunosuppressive cell types and that these cells functionally impede tumor-specific immune responses of cytotoxic T cells and possibly also natural killer cells. Apart from suggesting novel regulatory T cell-directed therapies, like anti-PD1 or ipilimumab, these data raise important questions with respect to current clinical practice. Orthotopic liver transplantation is in many cases an integral and essential component of disease and requires immunosuppressing, which in turn possibly hampers subsequent immunological responses towards resident cancer cells. Especially in the context of liver cancer, characterized by an immunosuppressive stromal micro-environment this might be a problem. In chapter 7 I show that this is indeed the case as the choice of the immunosuppressive regimen employed after orthotopic liver transplantation in HCC has a large influence on outcome. Furthermore I identify immunosuppressive regimes associated with improved survival in these patients. These studies show that even simple therapeutic adjustments that take into account the

cross-talk of liver cancer with its surrounding cells can have an important effect on disease course. Chapter 8, which provides a general discussion on the work presented in this thesis explores this angle further and positions the studies provided in this work within the corpus of contempary biomedical literature.

Summary of aims and goals of this thesis

This thesis aims to functionally and molecularly dissect the mechanisms and interactions between tumor cells and its surrounding tumor stroma in liver cancer. I shall try to establish how these interactions result in an altered tumor microenvironment in liver cancer and provide a compelling against the use of MSCs as a therapeutic vehicle in the treatment of liver cancer. The definition of SMAD-mediated signaling as an important pro-carcinogenic component of the reciprocal between cancer and its milieu should allow the development of new therapies here, but I shall also show that even small changes in the pharmacological treatment of patients can have substantial effects on the outcome. Thus my thesis will show that targeting the tumor microenvironment is highly promising in liver cancer but simultaneously will demonstrate that substantial new studies are now neccessary to comprehensively characterize the biology of liver cancer as these might result in substantial patient benefit.

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Chapter 2

Multipotent Mesenchymal Stromal Cells in Liver Cancer: Implications for Tumor Biology and Therapy

Pratika Y. Hernanda[1,2], Alexander Pedroza-Gonzalez[1], Dave Sprengers[1], Maikel P. Peppelenbosch[1], Qiuwei Pan[1]

[1]Department of Gastroenterology & Hepatology, Erasmus MC-University Medical Center, Rotterdam, Netherlands, [2]Laboratory of Medical Genetics, Biomolecular Research Centre, Wijaya Kusuma University, Surabaya, Indonesia

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Abstract

Remodeling of tumor microenvironment is a hallmark in the pathogenesis of liver cancer. Being a pivotal part of tumor stroma, multipotent mesenchymal stromal cells (MSCs), also known as mesenchymal stem cells (MSCs), are recruited and enriched in liver tumors. Owing to their tumor tropism, MSCs are now emerging as vehicles for anticancer drug/gene delivery against liver cancer. However, the exact impact of MSCs on liver cancer remains elusive, as a variety of effects of these cells have been reported included a plethora of tumor-promoting effects and anti-oncogenic properties. This review aims to dissect the mechanistic insight regarding this observed discrepancy in different experimental settings of liver cancer. Furthermore, we call for caution using MSCs to treat liver cancer or even premalignant liver diseases, before conclusive evidence for safety and efficacy having been obtained.

Keywords: *multipotent mesenchymal stromal cells, liver cancer, dual role*

Highlights :

- MSCs are recruited and enriched in liver tumors
- MSCs can exert tumor-promoting or tumor-suppressive effect in liver cancer
- Tumor microenvironment could in turn affect the ultimate function of MSCs
- We call for caution of using MSCs to treat patients with liver cancer or premalignant liver diseases

1. Introduction

Liver malignancies including hepatocellular carcinoma (HCC), cholangiocarcinoma and hepatoblastoma are jointly the fifth most prevalent form of cancer and globally the third leading cause of cancer-related death, immediately after mortality due to lung and colon cancer [1]. In addition, the liver is a favorite site for metastasis of other cancers, in particular colorectal cancer (CRC), esophageal cancer and pancreatic cancer. The liver microenvironment is favorable for growth and invasion of cancer cells, with increased extracellular matrix remodeling being considered a hallmark of malignant liver disease [2]. Although many of the details are still sketchy, it is now generally assumed that within this microenvironment, reciprocal tumor-stroma crosstalk influences the phenotype of tumor cells, progression and metastasis [3]. Being a pivotal part of the tumor stroma, multipotent mesenchymal stromal cells (MSCs), also known as mesenchymal stem cells (MSCs), were found to be present and play important roles in various types of cancers [4] and recently more insight into their role in liver malignancies has been revealed.

MSCs were initially identified to reside within the stromal compartment of bone marrow (BM) and characteristically have multi-lineage differentiation potential [5]. In addition to BM, MSCs now have been identified in various postnatal organs, where they often occupy a perivascular niche [6, 7]. A recent study demonstrated that the adult human liver harbors resident MSCs that are phenotypically and functionally similar to BM MSCs [8]. Intriguingly, the evidence suggesting that MSCs are involved in both primary [9] and secondary liver cancer is gaining momentum [10]. Nevertheless, despite the extensive investigations being done, the exact impact of MSCs on liver cancer remains elusive.

Frustratingly, whereas various studies report tumor promoting effects of MSCs, others provide evidence for anti-oncogenic role of these cells. The specific accumulation of these cells in the tumor environment is not in doubt and thus owing to this tumor tropism, MSCs are now emerging as vehicles for anticancer drug/gene delivery [11] and clinical trials are being proposed. This review aims to dissect the mechanistic insight regarding this observed discrepancy in different experimental settings of liver cancer as to provide possible guidance to the appropriateness of clinical trials. Given the complexity of MSC action, we call for caution on such trials in humans with respect to therapeutic applications of these cells in liver malignancy until better evidence for safety and efficacy has been obtained.

1. What is the source of the MSC compartment in liver cancer?

a. Identification of MSCs in various organs/tissues

Mesenchymal stem cells (MSCs) were initially identified by placing whole bone marrow cells in plastic culture dishes and the subsequent expansion of a rare population of plastic-adherent cells [12]. However, the recognized biologic properties of the unfractionated population of cells do not seem to exactly meet the general criteria for stem cell properties. Therefore, these cells are also termed as multipotent mesenchymal stromal cells (MSCs) [13]. The characterization and definition of MSCs still relies solely on *in vitro* culture-expanded cell populations and consequently, both the spatial distribution and properties of native MSCs within their organ/tissue *in vivo* are much less known [14]. The identification of MSCs in various other organs/tissues (e.g. adipose, kidney, umbilical cord, brain, liver, lung, bone marrow, etc) [6, 15, 16], which have the common MSC features but also carry unique properties depending on their sources, has raised a lively debate regarding the origin of MSCs. Similarly, a resident population of MSCs has also been identified within the human adult liver that are phenotypically and functionally similar to BM MSCs but express a unique gene signature [8]. The question whether these MSCs are BM-derived hepatotropic cells with MSC-like properties that have subsequently acquired location-specific gene expression, or whether they resided locally throughout their developmental stages remains unanswered.

b. Migratory capacity of MSCs

In general, MSCs are proficient with respect to migratory capacity and nomadic in nature. They tend to be recruited by injured tissue where they are thought to contribute to tissue repair and wound healing [17]. As tumors are often considered to have many characteristics of "injured tissue", it is probably not surprising to find MSCs in the tumor. Recent evidence has come forward in various pre-clinical models that MSCs can migrate into certain types of tumors and this is one of the rationales put forward for using MSCs as vehicles for anti-cancer drug/gene delivery [18, 19]. This tumor-tropic migratory property of MSCs is attributed to two main determinants: their intrinsic properties and stimuli produced by the tumor [20]. Human MSCs express chemokine receptors CCR1, CCR2, CCR4, CCR6, CCR7, CCR8, CCR9, CCR10, XCR, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6 and CX3CR [17]. Production of their respective ligands is shared characteristic of inflamed tissue and malignant transformed tissue and thus these receptors are likely involved in the specific accumulation of MSCs in both processes. Accordingly, the cognate ligands of these receptors are efficient chemotactic stimuli for MSCs. Additional receptors implicated in MSCs migration is Toll-like receptors (TLR). TLR1–6 have been identified on primary human MSCs and have been reported that TLR stimulation enhanced the migratory function of MSCs [21]. MSCs are relatively resistant to ischemia because in the absence of oxygen. MSCs can survive by anaerobic adenosine triphosphate production [22], which should give these cells a competitive advantage in tumor microenvironment. Intravenous infusion of MSCs has indeed been shown to result in specific accumulation of these cells in liver cancer-derived structures, indicating that liver tumors are able to recruit them at high efficiency [23]. Consistently, HCC has been shown to produce relatively high amount of *bona-fide* MSC chemo-attractants, including hepatocyte growth factor (HGF), SDF-1, basic fibroblast growth factor (bFGF), vascular endothelial growth factor A (VEGF-A) and vascular cell adhesion molecule 1 (VCAM-1) [23-25]. Thus, these data suggest that *ex vivo* expanded MSCs will likely display at least some specificity with regard to MSC accumulation in liver neoplasms.

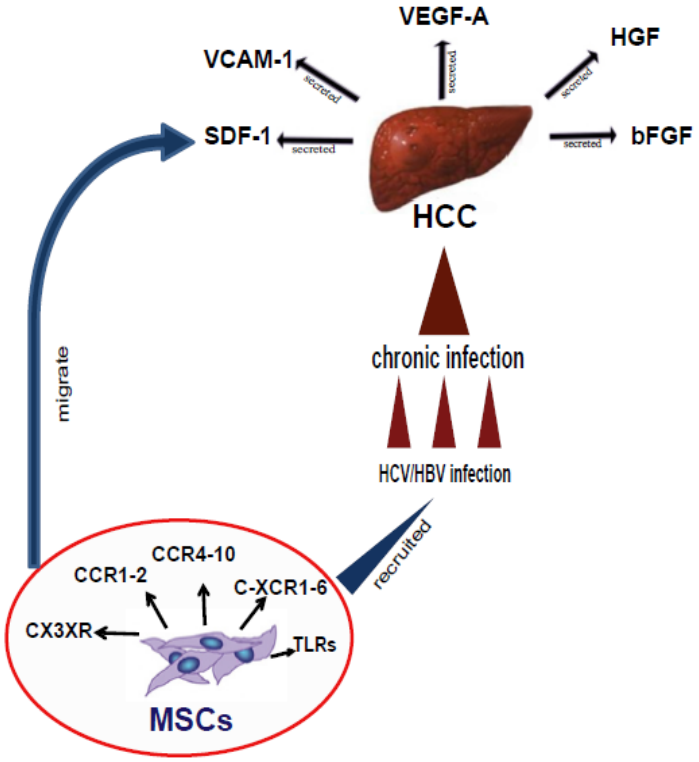

Figure 1. A proposed model for MSCs recruitment into liver tumor. MSCs were shown to express chemokine receptors CX3XR, CCR1-2, CCR4-10, C-XCR1-6 and TLRs. HCC tumors can release various cytokines, chemokines and growth factors, including HGF, bFGF, SDF-1, VCAM-1 and VEGF-A, which have been described as chemoattractants for MSCs. We propose that both liver or circulating MSCs (released from BM or other organ/tissue) are possibly recruited into liver tumor and MSCs may be constantly recruited in the stages from chronic viral hepatitis to liver cancer development.

c. Dualistic origin of MSCs in liver cancer?

The enrichment of MSCs in the tumor environment were reported for both human primary liver cancer [9] and liver metastases from colorectal cancer [10]. An intriguing question is to what extent the MSC compartment observed in liver tumor is derived from local sources (liver MSCs) or from the circulation in turn supplied by the BM (BM MSCs). In response to injury or infection, MSCs can be released from BM into the blood circulation and migrate towards the injured sites to promote tissue regeneration [26]. High frequencies of MSCs were found in liver tumor with extensive inflammation suggesting the recruitment of MSCs in response to infection/inflammation [9]. Moreover, high circulating levels of BM originated cells, such as endothelial progenitor cells, have been observed in HCC patients, which might subsequently home into the tumor and promote tumor growth [27]. We thus propose a dualistic origin of the MSC compartment in liver cancer, with MSCs constantly being recruited locally and from the circulation (Figure 1). Future studies using somatic genomic signatures may provide a definite answer.

2. Dual roles of MSCs in liver cancer

The intrahepatic microenviroment is substantially different from other organs, and this may affect cancer development [28]. MSCs constitute an important component within the microenvironment of both the normal liver as well liver tumors and they appear to have pleiotropic functionality. This is reflected in the results obtained in experimental liver tumor models. Depending on the exact experimental conditions, MSCs can exert a tumor-promoting or a tumor-limiting effects (Table 1) [9, 29-42]. Various hypotheses have been postulated to explain the dualistic behavior of MSCs in cancer. One school of thought attributes to an important role for TLRs and subsequent immuno-polarization of MSCs [43]. MSCs express several TLRs and their capabilities to migrate, invade, and secrete immune modulating factors are tightly regulated by specific TLR-agonist engagement. TLR4-primed MSCs are polarized into a pro-inflammatory MSC1 phenotype; whereas TLR3-primed MSCs are polarized into the classical immunosuppressive MSC2 phenotype [43]. In cancer models, MSC1-based treatment of established tumors in an immune competent model attenuates tumor growth and metastasis but MSC2-treated animals would display increased tumor

Table 1. Studies reporting effect of MSCs on liver cancer

References	Source of MSCs	Effects
Hernanda et al (9)	Human liver tumor/adjacent	Promote tumor growth
Yan et al (29)	Human liver tumor/adjacent	Promote tumor growth
Gong et al (30)	Human bone marrow	Promote tumor microvascular
Jing et al (31)	Human bone marrow	Promote tumor metastasis
Bhattacharya et al (32)	Human bone marrow	Promote tumor metastasis
Li et al (33)	Human bone marrow	Promote tumor growth Inhibit tumor metastasis
Zhao et al (34)	Human adipose tissue	Inhibit tumor growth
Abdel aziz et al (35)	Human bone marrow	Inhibit tumor growth
Li et al (36)	Human bone marrow	Inhibit tumor growth
Qiao et al (37 & 38)	Human dermal tissue	Inhibit tumor growth
Bruno et al (39)	Human bone marrow	Inhibit tumor growth
Hou et al (40)	Not indicated	Inhibit tumor growth
Ma et al (41)	Murine bone marrow	Inhibit tumor growth
Abd-Allah et al (42)	Murine bone marrow	Inhibit tumor growth

growth and metastasis [44]. The second hypothesis postulates a developmental phase-dependent MSC functionality [4, 45]. MSCs appear to promote tumor growth when co-injected with tumor cells, but inhibit tumor progression when administered into established tumors [4]. Thus, the presence of MSCs during the early phase of tumorigenesis may contribute to angiogenesis that is required for tumor initiation. Indeed, an increase in vessel density was observed when MSCs were co-injected with HCC or other tumor cell lines [30, 46]. Of note, tumor cells and the tumor microenvironment will in turn affect the ultimate function of these recruited MSCs. As both hypotheses are not mutually exclusive likely both are concomitantly true, making prediction as to the effects of MSCs on the cancerous process extremely difficult.

3. Mechanisms of MSC-dependent tumor suppression in liver cancer

A variety of processes possibly implicated in MSC-dependent tumor suppression have been identified. Wnt signaling is aberrantly activated in a subset of HCC tumors. In chemically induced murine HCC tumors the administration of MSCs has been demonstrated to have tumor suppressive effects associated with Wnt signaling target genes being down regulated, especially those related to anti-apoptosis, mitogenesis, cell proliferation and cell cycle regulation [35]. A mechanistic explanation is found by the active secretion of Wnt inhibitors, such as dickkopf-1, by MSCs [37, 40] and is supported by the MSC-dependent inhibition of NF-kB signaling in cancer cells [38]. In addition, TLR signals can stimulate down-stream effectors that may interfere LPS-TLR4 pathway and inhibit NF-kB activation during liver fibrosis [47].

Additionally, microvesicles released by MSCs have been shown to inhibit cell cycling and induce apoptosis or necrosis of different HCC cell lines *in vitro* and to inhibit growth of established tumors *in vivo* [39, 48], providing a further anti-oncogenic MSC effector pathway. Conversely, MSCs pulsed with tumor-derived microvesicles exert an enhanced antitumor activity against HCC [41]. Although it is still unclear which factors are the direct effectors, the secretome of MSCs appears to play an important role in their tumor suppressing function.

4. Tumor promoting effects of MSCs in liver cancer

The reported context-dependent tumor promoting roles of MSCs have been attributed to their abilities of supporting angiogenesis, promoting tumor growth and metastasis, and modulating immune response (detailed disused in the following section) via paracrine or direct mechanisms [4]. Support of tumor angiogenesis by MSCs could be via their direct differentiation into pericytes or perhaps endothelial cells [49], or indirectly by secreting pro-angiogenic factors and inhibition of apoptosis in vascular smooth muscle cells and endothelial cells [50]. The process of angiogenesis involves a large number of proteases. For instance, a protease named SERPINE1, which is abundantly secreted by MSCs, has been shown to regulate proliferation, migration, and apoptosis of vascular smooth muscle cells and endothelial cells [48, 51]. In mouse model, transplantation of BM MSCs promoted growth of microvascular in HCC tumor [30].

In addition, direct effects of MSCs on the tumor cells may contribute to HCC pathogenesis. MSCs have been shown to accelerate HCC metastasis, due to the induction of such epithelial-mesenchymal transition (EMT) [31], an effect which is even further enhanced by an inflammatory milieu (which characterizes many liver cancers). During EMT epithelial (cancer) cells lose cell polarity and cell-cell adhesion, and gain migratory and invasive properties. Supporting the existence of such effect is the observation of increased expression of cancer associated fibroblast (CAF) and EMT markers in a co-culture model of hepatoma cells and MSCs [32]. In HCC patients, MSC-dependent EMT induction is associated with a shorter tumor free survival and a worse overall survival [31], demonstrating the clinical relevance of this effect.

Secreted factors from patient HCC tumor-derived MSCs have been shown to promote tumor growth in xenograft mouse model associated with up-regulation of cell growth and proliferation-related processes and down-regulation of cell death-related pathways in HCC cells [9]. MMPs proteases as well as various others factors secreted by MSCs, are capable of remodeling extracellular matrix and facilitate tumor progression [9, 29]. Glycoproteins, such as osteonectin that is important in remodeling extracellular matrix, are highly expressed in the stromal myofibroblast of HCC patients and have been reported to promote HCC progression [52].

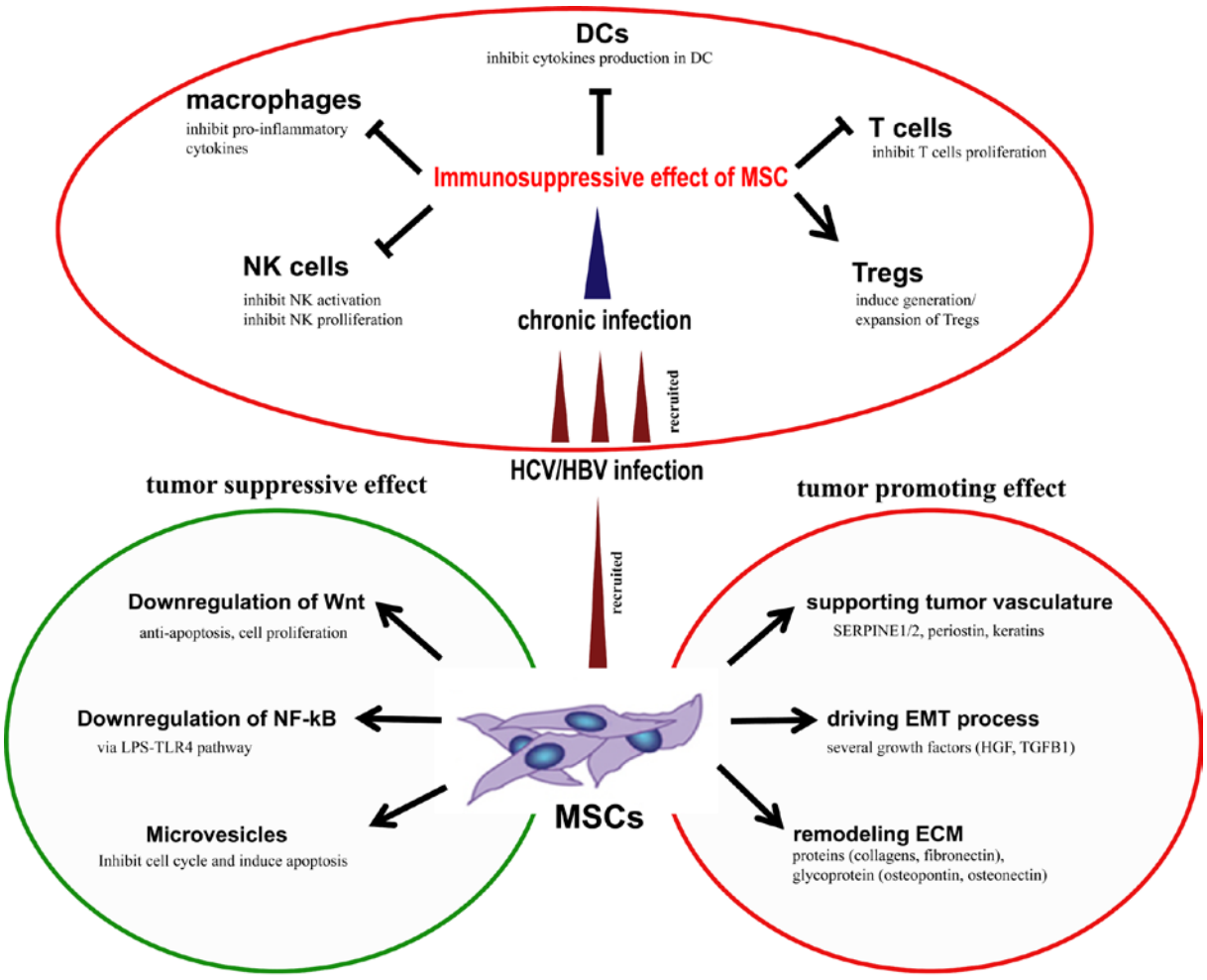

Figure 2. Proposed mechanisms for the tumor-suppressive and tumor-promoting effects of MSCs. The properties of MSCs and the particular tumor microenvironment may resulted in dual roles of MSCs that can suppress or promote tumor progression. The tumor-suppressive mechanisms are mainly due to secreted factors and downregulation of Wnt and NF-kB pathway. The tumor-promoting mechanisms are mainly attributed to both secreted factors and direct effects via 1) supporting tumor vasculature 2) EMT transition and 3) ECM remodeling. The immunosuppressive effect of MSCs (inhibit NK cells, macrophages, dendritic cells, T cells and support regeneration of Tregs) conceivably lead to tumor-promoting effect.

5. Potential immunomodulation by MSCs in liver cancer

a. Immune microenvironment in liver tumors

Immune surveillance plays key roles in protecting against cancer. The liver constitutes a relatively immunoprivileged microenvironment, and thus cancer cells may take advantage of the immunoregulatory mechanisms that are established in the liver [53]. Furthermore, HCC can use multiple mechanisms to evade host antitumor immunity, leading to disease progress

even in the presence of tumor-specific immune responses [54]. In liver tumor, the composition as well as the function of immune cells have been dramatically altered [53, 55]. In general, the frequency and functionality of anti-tumor immune cells are decreased [53, 55]. In contrast, variety of immunosuppressive cells with high activity are accumulated in the tumor, which can impede immunosurveillance and facilitate tumor growth [56]. Although MSCs have never officially joined the immune cell club, they are well-recognized for their potent immunomodulatory capacity.

b. Immunomodulation by MSCs

MSCs can modulate the function of several cell types of the immune system including those from innate immunity including natural killer (NK) cells [57] and macrophages [58]. MSCs are also capable of modulating the differentiation, activation and function of dendritic cells (DCs) [59], the most efficient antigen presenting cells. The key function of dendritic cells (DCs) is translating innate to adaptive immunity and these cells are thought to have important link with HCC progression [60]. T cells are the main components of adaptive immune system and are crucial in controlling malignant disease, mediating both cytotoxicity of cancer cell themselves and release of anti-oncogenic cytokines [61, 62]. MSCs can effectively inhibit T cell function through multiple pathways [63, 64].

Regulatory T cells (Tregs) are a specialized subset of T cells which suppress activation of the immune system to maintain homeostasis and tolerance to self-antigens. In patient HCC tumor, increased frequencies of highly activated Tregs are infiltrating the tumor milieu and they are mainly localized in the stroma compartment of the tumors [55]. Furthermore, the frequency of Tregs in HCC has been associated with poor prognosis [65-67]. In contrast to suppress cytotoxic T cells, MSCs can induce the generation and expansion of Tregs [68]. Additionally, MSCs have been reported to induce the production of IL-10 by plamacytoid dendritic cells (pDCs), which in turn triggered the generation of Tregs [63]. However, potential interactions between these immune cells with MSCs or tumor stroma in general, have been poorly studied in the context of liver cancer, which certainly deserve more attention for further research.

6. Therapeutic application of MSCs in liver cancer: call for caution

a. Potential therapeutic application

Evidence from various preclinical models showing that MSCs can migrate into certain types of tumors has inspired the use of MSCs as vehicle for anticancer drug/gene delivery [11]. This notion was further supported by the fact that several studies have demonstrated potential anti-cancer effects of MSCs [31, 35, 45, 69]. In experimental HCC models, genetically modified MSCs have been used to deliver anti-cancer gene and inhibition of HCC cell proliferation was demonstrated *in vitro* and *in vivo* [70, 71]. Another approach is to deliver oncolytic viruses (e.g. measles virus) by MSCs into the tumor, in order to avoid pre-existing immunity against the virus [72]. These observation encourage clinical investigators to design trials for treating HCC, which remains an unusually deadly disease, using MSCs as a vector.

MSCs have been extensively investigated in clinical trials to treat various diseases [73, 74]. For treating cancer, trials have also been initiated to treat ovarian cancer (NCT02068794), head and neck cancer (NCT02079324) and prostate cancer (NCT01983709). Although MSCs have not been used for treating liver cancer yet (to our knowledge), over 30 trials have been registered at ClinicalTrials.gov for treating various liver diseases (Table 2).

b. Reasons for caution

Harnessing the hepatic differentiation potential and anti-inflammatory function of MSCs, most of the current clinical studies aim to treat liver cirrhosis, a premalignant state [75, 76]. Given the immunomodulatory properties of these cells, MSCs are also used for immunomodulation therapy of patients after liver transplantation [77]. Such studies almost unavoidably involve patients who are positive for hepatitis B or C virus infection. These infections are, however, important drivers of cirrhosis and HCC [78]. In addition, HCC is an important indication for liver transplantation and liver transplant patients also have increased incidence of developing de novo cancer [79].

Another concern is that the cellular fate and distribution of transplanted MSCs *in vivo* remain unclear. MSCs subcutaneously engrafted into immunodeficient mice were detectable

Table 2. Registered trials of mesenchymal stem cells (MSCs) in various liver diseases

Disease	Phase	Source of MSCs	Status	Registered ID
Liver cirrhosis	1/2	Human umbilical cord MSCs	Not yet recruiting	NCT01573923
	1/2	Human bone marrow & umbilical cord MSCs	Not yet recruiting	NCT01877759
	2	Allogeneic MSCs	Recruiting	NCT01591200
	3	Autologous bone marrow MSCs	Enrolling by invitation	NCT01854125
	-	Autologous bone marrow MSCs	Unknown	NCT01499459
	1	Human umbilical cord MSCs	Unknown	NCT01224327
	1/2	Autograft MSCs	Completed	NCT00420134
	1	Human umbilical cord MSCs	Recruiting	NCT01728727
	1/2	Human umbilical cord MSCs	Completed	NCT01342250
	1	Human menstrual blood MSCs	Enrolling by invitation	NCT01483248
	2	Human umbilical cord MSCs	Recruiting	NCT01233102
	2	Autologous bone marrow MSCs	Unknown	NCT00993941
	2	Autologous bone marrow MSCs	Unknown	NCT00976287
	2	Autologous bone marrow MSCs	Unknown	NCT00476060
	1	Human umbilical cord MSCs	Recruiting	NCT01220492
	1	Autologous bone marrow MSCs	Unknown	NCT01454336
	1	Autologous adipose tissues MSCs	Terminated	NCT00913289
	-	Autologous adipose tissues MSCs	Enrolling by invitation	NCT01062750
	2	Allogeneic bone marrow MSCs	Unknown	NCT01223664
	2	Autologous bone marrow MSCs	Recruiting	NCT01741090
	2	Autologous bone marrow MSCs	Recruiting	NCT01875081
Liver failure	1	Human umbilical cord MSCs	Recruiting	NCT01218464
	1	Human umbilical cord MSCs	Recruiting	NCT01724398
	2	Allogeneic bone marrow MSCs	Unknown	NCT01322906
	1	Third party bone marrow MSCs	Recruiting	NCT01429038
	1/2	Allogeneic bone marrow & umbilical cord MSCs	Recruiting	NCT01844063
	2	Autologous bone marrow MSCs	Completed	NCT00956891
	2	Allogeneic bone marrow MSCs	Unknown	NCT01221454
Liver transplantation	1	Human umbilical cord MSCs	Recruiting	NCT01690247
Primary biliary cirrhosis	1	Human umbilical cord MSCs	Recruiting	NCT01662973
	1	Allogeneic bone marrow MSCs	Unknown	NCT01440309
Autoimmune hepatitis	1	Human umbilical cord MSCs	Recruiting	NCT01661842

NOTE: The trials are registered at ClinicalTrials.gov. Searched on 24th, April, 2014

up to 25 days [8]. In patients with liver cirrhosis, intravenously infused MSCs accumulated in the liver and spleen, which were detectable up to 10 days [80]. Magnetic resonance imaging (MRI) and radioactive labeling are commonly used for tracking infused stem cells [81]. These technics however suffer from low sensitivity [82], and therefore are not able to precisely

trace cell distribution and survival. Further, the functionalities of infused MSCs, including differentiation status and cytokines production, are not able to be defined *in vivo*.

Because of unclear clinical benefits in liver disease patients [75, 76, 80], uncertainty of infused MSCs *in vivo* and the potential tumor-prompting effects of MSCs as demonstrated in various experimental liver cancer models as well as potential malignant transformation may occur during *ex vivo* expansion of MSCs [83], we thus call for caution of using MSCs to treat liver cancer or even premalignant liver diseases.

Summary

The tumor tropism property of MSCs has been demonstrated both in experimental liver cancer models and in patients. However, as discussed above, MSCs may not only be tumor tropic but also tumor trophic. Both tumor-promoting and tumor-suppressive roles of MSCs have been described depending on the particular liver cancer models and methodologies used (Figure 2). Because of their tumor tropism, the use of MSCs as vehicles for anticancer drug/gene delivery has reached clinical investigation. However, we call for caution in using MSCs to treat liver cancer or even premalignant liver diseases. Given their potent immunosuppressive and tumor promoting properties, MSCs may in fact represent a target for anti-cancer therapy.

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Chapter 3

Tumor Promotion Through the Mesenchymal Stem Cell Compartment in Human Hepatocellular Carcinoma

Pratika Y. Hernanda[1], Alexander Pedroza-Gonzalez[1], Luc J. W. van der Laan[2], Mirelle E. E. Bröker[2], Martin J. Hoogduijn[3], Jan N. M. IJzermans[2], Marco J. Bruno[1], Harry L. A. Janssen[1, 4], Maikel P. Peppelenbosch[1], Qiuwei Pan[1]

[1]Department of Gastroenterology and Hepatology, [2]Department of Surgery, [3]Department of Internal Medicine Erasmus University Medical Center, Rotterdam, The Netherlands. [4]Division of Gastroenterology, University Health Network, Toronto, Canada

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ABSTRACT

Although the infiltration of mesenchymal stem/stromal cells (MSCs) into different tumors is widely recognized in animal models, the question whether these MSCs have a positive or negative effect on disease progression remains unanswered. The aim of this study is to investigate whether human hepatocellular carcinoma (HCC) harbors MSCs and whether these MSCs affect tumor growth. We observed that cells capable of differentiation into both adipocyte and osteocyte lineages and expressing MSCs markers can be cultured from surgically resected HCC tissues. *In situ* staining of human HCC tissues with a STRO-1-antibody showed that the tumor and tumor-stromal region are significantly enriched with candidate MSCs as compared to adjacent tissue (n=12, $P<0.01$). In mice, co-engraftment of a human HCC cell line (Huh7) with MSCs resulted in substantially larger tumors as compared to paired engraftment of Huh7 alone (n=8, $P<0.01$). Consistently, co-culturing Huh7 with irradiated MSCs significantly increased the number and the size of formed colonies. This enhancement of Huh7 colony formation was also observed by treatment of MSCs conditioned medium (MSC-CM), suggesting that secreted trophic factors are contributing to the growth promoting effects. Genome-wide gene expression array and pathway analysis confirmed the up-regulation of cell growth and proliferation-related processes and down-regulation of cell death-related pathways by treatment of MSC-CM in Huh7 cells. In conclusion, these results show that MSCs are enriched in human HCC tumor compartment and could exert trophic effects on tumor cells. Thus, targeting of HCC tumor MSCs may represent a new avenue for therapeutic intervention.

INTRODUCTION

Liver cancer is one of most devastating malignancies. Hepatocellular carcinoma (HCC) accounts for >90% of primary liver malignancies and is the third leading cause of cancer-related deaths worldwide. Most cases of HCC are found in patients with cirrhosis caused by chronic hepatitis B (HBV) or C (HCV) infection (1). It develops in particular when chronic infection with HBV or HCV repeatedly causes the body's immune system to attack liver cells followed by repetitive damage of cell cycle which leads to mistakes during its repair and in turn leads to carcinogenesis (2). For the majority of advanced HCC cases, curative treatments are not possible and the prognosis is dismal because of underlying cirrhosis as well as poor tumor response to standard chemotherapy (3). For patients with advanced disease, representing the majority of patients at diagnosis, the only option includes sorafenib (Nexavar), an oral multi-kinase inhibitor, which increases patient survival with approximately 3 months (4). Evidently, new therapeutic options are urgently needed for advanced or metastatic HCC.

Remodeling of the liver microenvironment is a hallmark in the pathogenesis of liver cancer (5). In cancer, the microenvironment which is also referred to as stroma, undergoes drastic changes, including the recruitment and the activation of stromal cells and the remodeling of extra cellular matrix (ECM). Co-evolution of tumor cells with their microenvironment during tumorigenesis suggests that tumor-stroma crosstalk may likely influence the phenotype of tumor cells and may provide a selective pressure for tumor initiation, progression and metastasis (6). In addition, the liver provides a distinct immunological environment and the ultimate effects of this environment on cancer progression may differ in the liver as compared to other organs (7).

Mesenchymal stem/stromal cells (MSCs) were initially identified as a heterogeneous population of stromal cells in the bone marrow (BM) that support hematopoietic stem cells (8). Further studies demonstrated that MSCs possess multilineage differentiation potential, can exert anti-inflammatory function, have immunomodulatory properties and influence other cells through the production of paracrine factors (9). MSCs attract attention as a possible cell-based therapy, especially in immune-related diseases and over 300 trials have

been registered (January, 2013, clinicaltrials.gov). The role of MSCs in pathogenesis has been less well studied. Recent evidence has come forward in various pre-clinical models that MSCs can migrate into certain types of tumors and even using MSC as anti-cancer drug/gene delivery has been proposed (10,11). The role of MSCs in cancer development, however, remains unclear. Several studies indicated that MSCs restrain cancer growth (12-14); whereas other studies have shown that MSCs are able to promote tumor progression and metastasis in experimental cancer models (15-18). Thus, it remains largely elusive whether MSCs have a beneficial or detrimental role in the cancerous process (19) and experimentation with MSCs directly obtained from human cancer is deemed necessary to obtain answers here.

Previously, we have identified a resident population of MSCs within the human adult liver which are phenotypically and functionally similar to BM MSCs (20). This raises obvious questions as to the potential role of these cells in liver cancer. In this study, we demonstrated that human HCC indeed harbors MSCs. Further more, these HCC-derived MSCs are highly trophic for tumor growth and therefore represent an interesting target for novel therapy.

MATERIAL & METHODS

Patients

For culturing MSCs, tissue samples from 7 individuals who were eligible for surgical resection of HCC were collected. Paired fresh liver tumor and tumor-free liver tissue at the maximum distance from the tumor were used. For immunohistochemical staining of MSCs maker, paraffin-embedded patient HCC (n=12) tissues were collected at the tissue bank at the Erasmus MC Rotterdam (Supplementary Table 1). The use of patient materials was approved by the medical ethical committee of Erasmus MC (Medisch Ethische Toetsings Commissie Erasmus MC) (21).

Isolation and culture of MSCs

Single cell suspensions from adjacent liver tissue and tumor were obtained by tissue digestion. Briefly, fresh tissue was cut into small pieces and digested with 0.5 mg/ml of collagenase (Sigma-Aldrich, St. Louid, MO) and 0.1 mg/ml of DNase I (Roche, Indianapolis, IN) for 30 minutes at 37 ºC. Cell suspensions were filtered through cell-strainers and mononuclear cells (MNC) were obtained by Ficoll density gradient centrifugation. Cells were cultured in Alpha Dulbecco's modified Eagle's medium (Alpha DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 IU/mL penicillin, and 100 lg/mL streptomycin. Adjacent liver tissue and tumor were also cut into small pieces for culturing MSCs in 12-well plates. Tissues were cultured in the Alpha DMEM medium as above. If MSCs emerged after 2-3 weeks, the tissues were removed and MSCs were sub-cultured in conditions as described above.

Flow cytometry

Cells were stained for 30 min at 4°C with directly-labeled mouse monoclonal antibodies directed against human CD13-PECy7, CD34-APC, CD45-PERCP, HLA-I-APC (all BD Biosciences), CD73-PE, CD166-PE (BD Pharma, San Jose, CA) and CD105-FITC (R&D Systems, Abingdon, UK) for human MSCs and rat antibodies directed against mouse CD90 & CD105 (R&D Systems, Abingdon, United Kingdom) for mouse MSCs. Flow cytometric analysis was performed using the FACSCanto II (BD Biosciences) and 10 000 events were collected for analysis performed using FlowJo software.

Adipogenic and osteogenic differentiation

For adipogenic differentiation, MSCs were cultured for 3 weeks in DMEM supplemented with 10% fetal bovine serum, 1 □00 dexamethasone, □M isobut □g/mL insulin, and 60 □M indomethacin (Sigma-Aldrich). Oil Red O staining (Sigma-Aldrich) was used for the detection of adipocytes. For osteogenic differentiation, cells were cultured for 3 weeks in DMEM with 10% fetal bovine serum supplemented with 0.2 mM ascorbic acid, 100 nM dexamethasone, and 10 mM b-glycerol phosphate (Sigma-Aldrich). Alizarin Red S staining (Sigma-Aldrich) was performed to detect deposited calcium phosphates.

Colony forming assay of Huh7 cells

MSCs were plated in 12-wells reaching ≈30% confluence. After 24 hours, MSCs were irradiated with 4 Gy of ^{60}Co gamma radiation. Subsequently, 2000 Huh7 cells, a validated human HCC cell line (22), were added to the wells and were cultured in Alpha DMEM medium.

The colony formation assay was also performed in Huh7 cells treated with MSCs conditioned mediums (MSC-CM). MSC-CM was prepared by culturing MSCs with 70-90% confluence. CM medium was collected after 48 hours culture. As control, colony formation array was performed with Huh7 cells only. Huh7 colonies were counterstained with haematoxylin & eosin after two weeks. The colony numbers were counted and their sizes were measured by microscope.

Western Blot analysis

Cell suspensions were lysed in lysis buffer (130mM Tris-HCl pH 8, 20% glycerol, 4.6% SDS, 0.02% Bromophenol Blue, 2% dithiothreitol (DTT)) and boil in 95ºC for 5 minutes. Twenty five microliter of lysates were electrophoretically separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Invitrogen USA), transferred onto Nitrocellulose transfer membranes and the membranes were incubated with the following primary antibodies: STRO-1 (Invitrogen corporation) and CD146 (Abcam). The immune complexes were detected using horseradish peroxidase-linked anti-mouse or anti-rabbit conjugates as appropriate (DAKO, Denmark) and visualized using enhanced chemiluminescence detection system (Amersham Biosciences, Amersham, UK).

Immunohistochemistry

Paraffin embedded liver tumor tissue slides were deparaffined in xylene, rehydrated in graded alcohols, and rinsed once in phosphate-buffered saline (PBS) plus Tween 0.05%. For antigen retrieval, slides were boiled in Tris/EDTA pH 9.0 for 10 min; 1.5% H2O2 was used to block endogenous peroxidase for 10 min at room temperature. The slides were incubated in 5% milk blocking solution followed by overnight incubation in mouse monoclonal antibody STRO-1 (Invitrogen Corporation) with concentration of 1:200 and then counterstained with

haematoxylin. As negative control, the primary antibody was replaced by PBS; the positive controls were taken from other slides that had successfully stained before. STRO-1 staining was scored by two independent observers. The protocol for CD146 staining is similar as mentioned above.

HCC xenograft tumor in NOD/SCID mice

HCC xenograft tumor model in NOD/SCID mice was established as we previously described (23). Eight mice aged 6-8 weeks were subcutaneously engrafted with human hepatoma Huh7 cells ($1X10^6$) with or without MSCs ($1X10^6$) into the lower left or right flank, respectively. Seven mice were injected with tumor-derived MSCs and one was injected with MSCs from adjacent liver tissue. Huh7 cells were labeled luciferase reporter gene for 3 out of the 8 mice, as previously described (19). Luciferase activity was measured by IVIS camera (Caliper Life Sciences, USA) in living animals. Data was analyzed with Living Image 4.0 software.

At day 19 or 21 post-engraftment, mice were sacrificed and tumors were harvested, imaged and weighted. Part of the tumor were fixed with formalin and embedded in paraffin for histology evaluation or immunohistochemistry. The use of animals was approved by the institutional animal ethic committee (Dier Experimenten Commissie).

Genome-wide gene expression analysis

The total RNA of 3 independent Huh7 cell lines cultures treated or untreated with MSCs conditioned medium was used for genome-wide microarray analysis with the Affymetrix GeneChip HuGene 1.0 ST.v1 array (Affymetrix, Santa Clara, CA) according to the manufacturer's procedures. Transcript-level expression measures were generated with the robust multi-array average procedure as implemented in the Affymetrix Gene Expression Console, and probe set annotations were retrieved from NetAffx with the same software. Principal component analysis, forest plot and pathway analysis were performed with Partek (Partek, Inc., Saint Louis, MO).

Statistical analysis

Statistical analysis was performed by using the paired nonparametric test, the unpaired nonparametric Mann–Whitney test or paired T-test using GraphPad InStat software (GraphPad Software Inc., San Diego, USA). P-values <0.05 were considered statistically significant.

RESULTS

Obtain MSCs by culturing resected human HCC tissues

Following surgical resection, patient HCC tissues were subjected to collagenase digestion followed by the MSCs-specific culture protocol previously validated (20). This protocol yielded ample colonies with an apparently MSCs morphology (Figure 1A and 1B). Furthermore, this stringent MSCs culture protocol was successful in 6/7 HCC cases, but only in 2/6 cases when applied to adjacent not-transformed liver tissue, which suggests a possible enrichment of MSCs in HCC. An even more stringent protocol failed to grow MSCs from normal liver (Figure 1C) but still yielded MSCs from HCC tumors (Figure 1D). To confirm that these cells represent *bona fide* MSCs, we assessed the capacity of these cells to yield multilineage progeny. As evident from figure 1E and 1F, the cells had capacity for both adipogenic and osteogenic differentiation. Furthermore, FACS analysis of their antigenic profiles confirmed that these cells are positive for the common mesenchymal markers CD13, CD73, CD105 and CD166 and are negative for the common hematopoietic markers CD34 and CD45 (Figure 1G).

Enrichment of STRO-1 positive cells in human HCC

STRO-1 is the best-known MSCs marker (24,25), in particular for in vivo immunohistochemical staining of candidate MSCs (11). Western blotting analysis showed that STRO-1 protein is abundantly expressed in HCC-derived MSCs (cell culture expanded) as

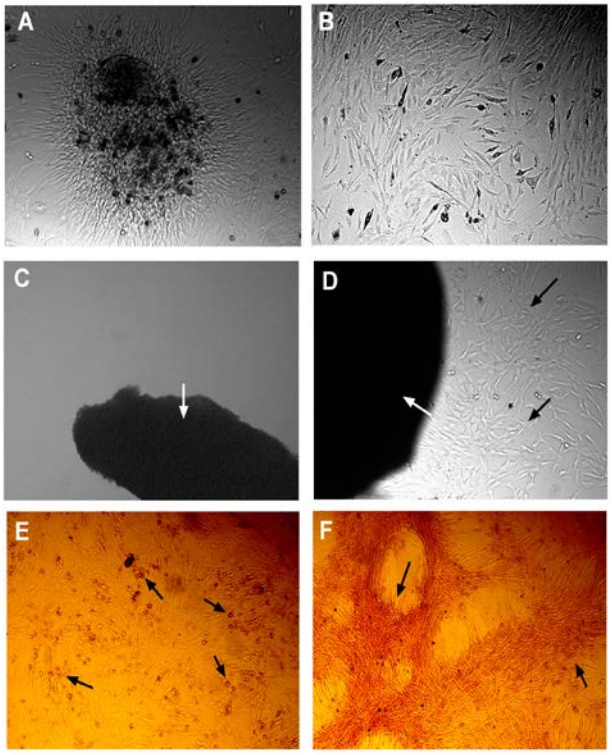


Fig. 1. Culture and characterization of MSCs from human liver carcinomas. With a method of collagenase digestion of surgical resected human HCC and LM-CRC tissues, colonized cell clusters were appeared **(A)** and these cells could rapidly grow out and expand by subculture showing typical fibroblast-like morphology **(B)**. With another method of culturing tiny tissue specimen, MSCs-like cells could only be obtained from tumor tissues **(D)** but not from adjacent liver tissue **(C)**. (E) Adipogenic differentiation of liver carcinoma derived MSC, detected by Oil red O staining for lipid droplet (Arrow). (F) Osteogenic differentiation of these cells was evaluated by detection of deposited calcium phosphates using Alizarin Red S staining (Arrow).

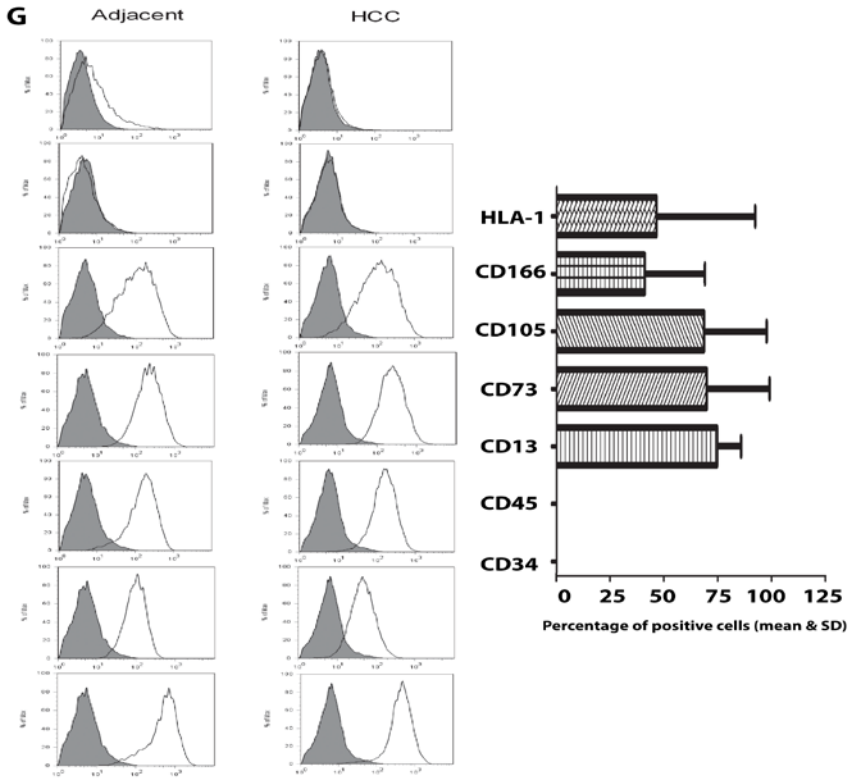


Fig. 1 (G) FACS staining confirmed that these cells are positive for common mesenchymal markers CD13, CD73, CD105 and CD166 and are negative for the common hematopoietic markers CD34 and CD45.

BM- or liver-derived MSCs, whereas it is hardly detectable in whole lysates of liver tumor or adjacent liver tissue (Supplementary Figure 1A). STRO-1 protein expression was further confirmed by immunohistochemical staining of cultured tumor MSCs (Figure Supplementary 1B).

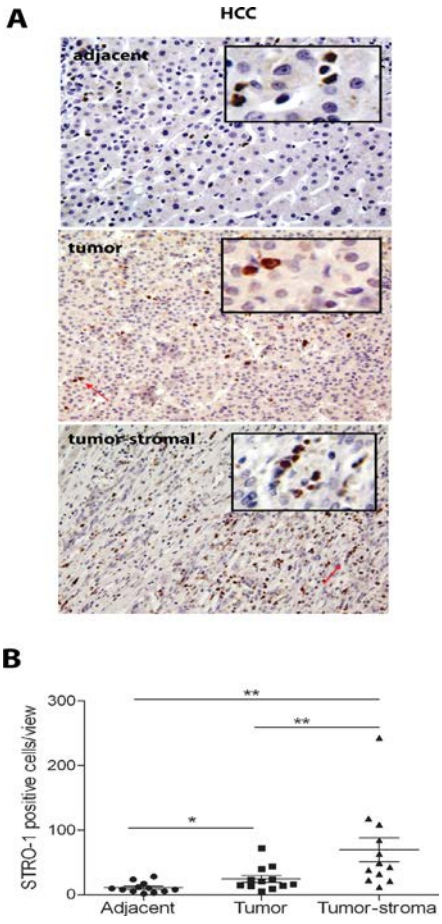


Fig. 2. *In situ* localization of STRO-1-positive cells in paraffin-embedded patient HCC tissues. (**A**) Distribution of STRO-1 cells in the adjacent, tumor and tumor-stromal regions in HCC tissues. (**B**) STRO-1-positive cells are significantly enriched in the tumor, in particular the tumor-stromal region, compared with the adjacent area in HCC tissues (n = 12, $*P < 0.05$, $**P < 0.01$).

Thus, we chose to employ a STRO-1 antiserum to quantify the candidate MSCs in HCC and to compare cell numbers to adjacent tissue. For this study, 12 cases of confirmed HCC were obtained for immunohistochemical investigation of both candidate MSCs number and histospatial distribution. STRO-1 positive cells were readily observed in both tissues adjacent to the cancer and within the tumor (Figure 2A). In the normal region, STRO-1 positive cells were mainly located in liver sinusoid or veins. The frequency of STRO-1 positive cells in adjacent tissue appears low (11±8 positive cells/view, mean±SD, n=12), except for livers with extensive inflammation (Supplementary Figure 2). Morphometric analysis of the samples confirmed that the tumor stroma is significantly enriched with STRO-1 positive cells as compared to adjacent non-transformed tissue (Figure 2B). We conclude that HCC is enriched with candidate MSCs, suggesting active sequestration of these cells by the tumor and a possible role of MSCs in HCC tumorigenesis.

Human hepatoma cells formed tumors in mice are infiltrated with MSCs-like cells

To further understand the enrichment of MSCs in human HCC, we evaluated whether MSCs can infiltrate into HCC tumors that are formed in immunodeficient mice by engraftment of human HCC cell line (1^{x} 10^{6} Huh7 cells). Upon subcutaneously injection of Huh7 cells into NOD/SCID mice, solid tumors were formed during a period of 2-4 weeks. When Huh7 cells were labeled with luciferase reporter gene, the solid tumor under the skin could be visualized by IVIS camera (Figure 3A). Subsequently, the tumors were harvested, digested and cultured *in vitro*. After 3-7 days culture, Huh7 cells were colonized (Figure 3B, indicated by Red arrow) but surprisingly were surrounded by fibroblast-like cells (Figure 3B, indicated by White arrow). All the tumors obtained from six mice contained substantial number of these cells shown by cell culture expansion. FACS analysis using anti-mouse antibodies against typical MSCs markers CD90 (Figure 3C) and CD105 (Figure 3D) measured approximately 1% (mean of two batches) of cells to be positive. This suggests that human HCC tumors may actively attract MSCs and can transcend the species barrier. Subsequently, we initiated experimentation to address the potential role of these MSCs in HCC progression.

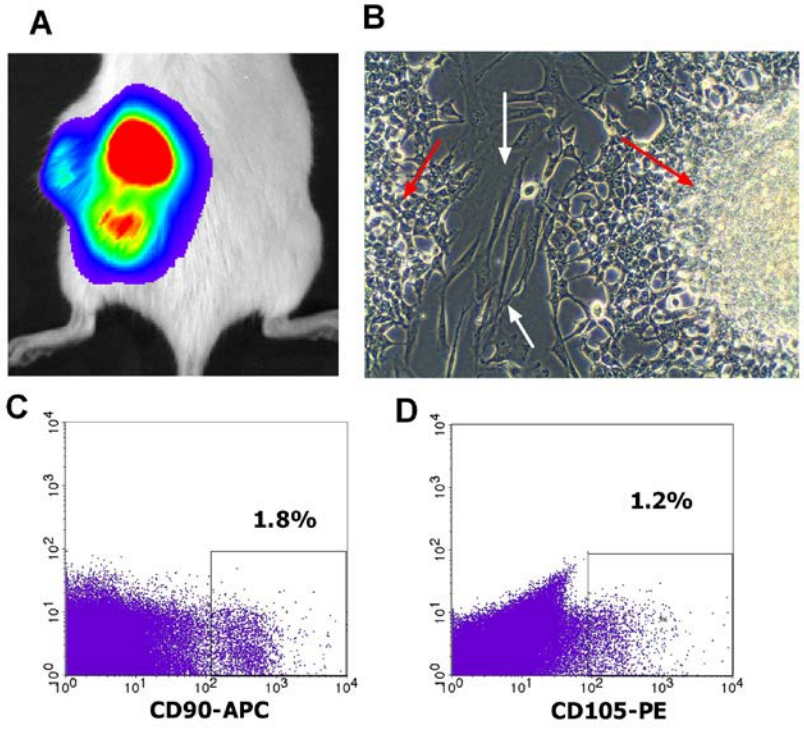


Fig. 3. Infiltration of MSCs into human hepatoma cell-derived solid tumors in mice. (**A**) Subcutaneous engraftment of human hepatoma Huh7 cells was able to form solid tumors in mice. By luciferase labeling of Huh7 cells, the tumors can be visualized by IVIS cameras in living animals. (**B**) Cell culture of digested tumors resulted in colonized Huh7 cells (grey arrow) surrounded by fibroblast-like cells (white arrow; *n* = 6). Fluorescence-activated cell-sorting analysis using anti-mouse antibodies against the typical MSC markers CD90 (**C**) and CD105 (**D**) showed ~1% of cells to be positive (*n* = 2).

Tumor MSCs enhance colony unit formation and growth of hepatoma cells through secretion of trophic factors

To explore the possible effects of MSCs on HCC, we performed co-culture experiments, in which Huh7 cells were grown in the presence or absence of irradiated HCC-derived MSCs (Figure 4A). Co-culture with MSCs significantly increased the number (196 ± 29 Vs 123 ± 36 clonies/2000 Huh7, Mean ± SD, n = 5, P < 0.05) and the size (1329 ± 258 Vs 570 ± 155 pixels, n = 10, P < 0.01) of Huh7 formed colonies (Figure 4B). To investigate whether the effects of

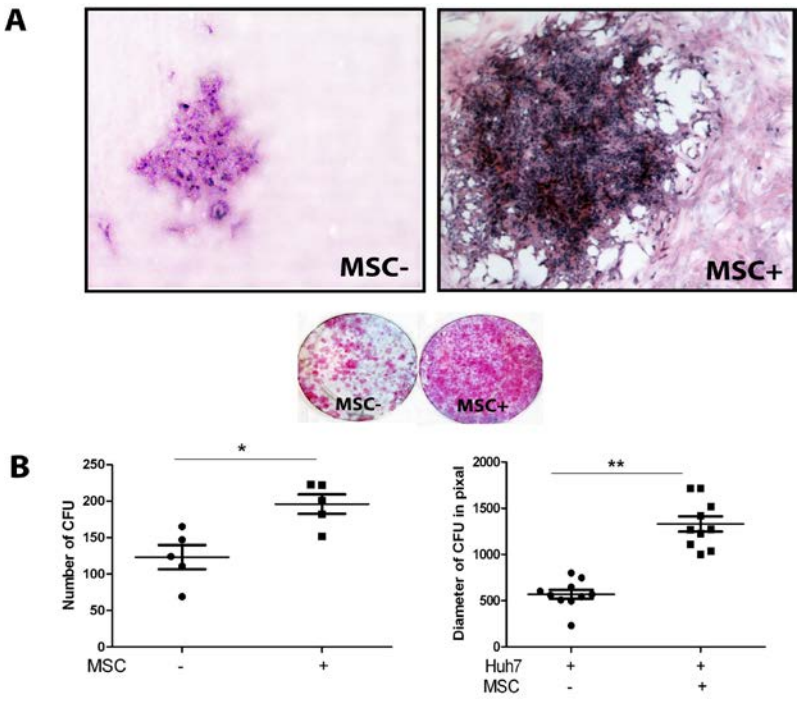


Fig. 4. MSCs promote colony formation and growth of hepatoma cells. (**A**) Coculturing 2000 Huh7 cells in 12-well plates with irradiated MSCs increased the size and the number of colonies formed. (**B**) The number of colonies formed was 196±29 (mean ± SD) in Huh7 cocultured with MSCs versus 123±36 in Huh7 alone (*n* = 5, **P* < 0.05). The average size was 1329±258 pixels versus 570±155 pixels (*n* = 10, ***P* < 0.01).

MSCs in this model system are mediated by cell-to-cell contact or through paracrine mechanisms, the experiment was also performed using MSCs-conditioned medium (MSC-CM). It showed that such conditioned medium increased both the number and size of the Huh7 colonies (Figure 5A and 5B), demonstrating that trophic factors of MSCs are powerful stimulus to support tumor cell growth.

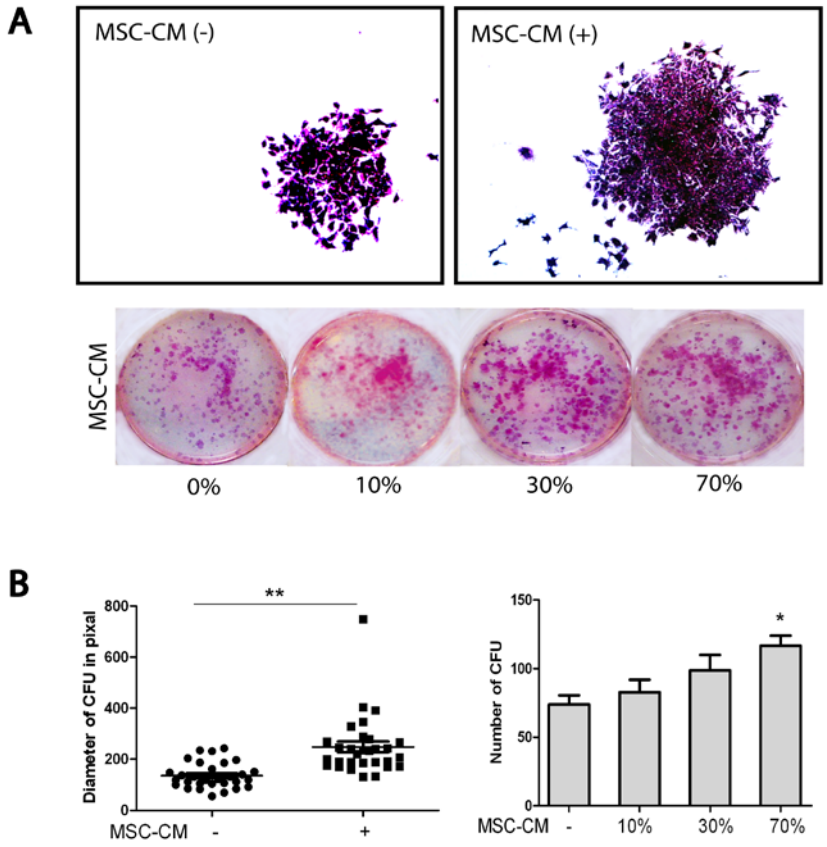


Fig. 5. The trophic factors secreted by MSCs promote colony formation and growth of hepatoma cells. (**A**) Huh7 treated with MSC-conditioned medium resulted in formation of more and larger colonies. (**B**) The size and the number of colonies were significantly increased by treatment with MSC-CM.

To map the molecular regulation of Huh7 cells by MSCs trophic factors, genome-wide expression arrays were performed on Huh7 cells treated with (n=3) or without MSC-CM (n=3). The principal component analysis of genome-wide expression profiles separated both treatment groups into two clusters, reflecting the effects of MSC-CM treatment (Supplemental Figure 3). Furthermore, forest plot (Figure 5C) and gene set enrichment analysis (Figure 5D) confirmed the up-regulation of cell growth and proliferation-related processes and down-regulation of cell death-related pathways by treatment of MSC-CM in Huh7 cells. These data further highlight the powerful trophic action of MSCs on the liver cancer growth.

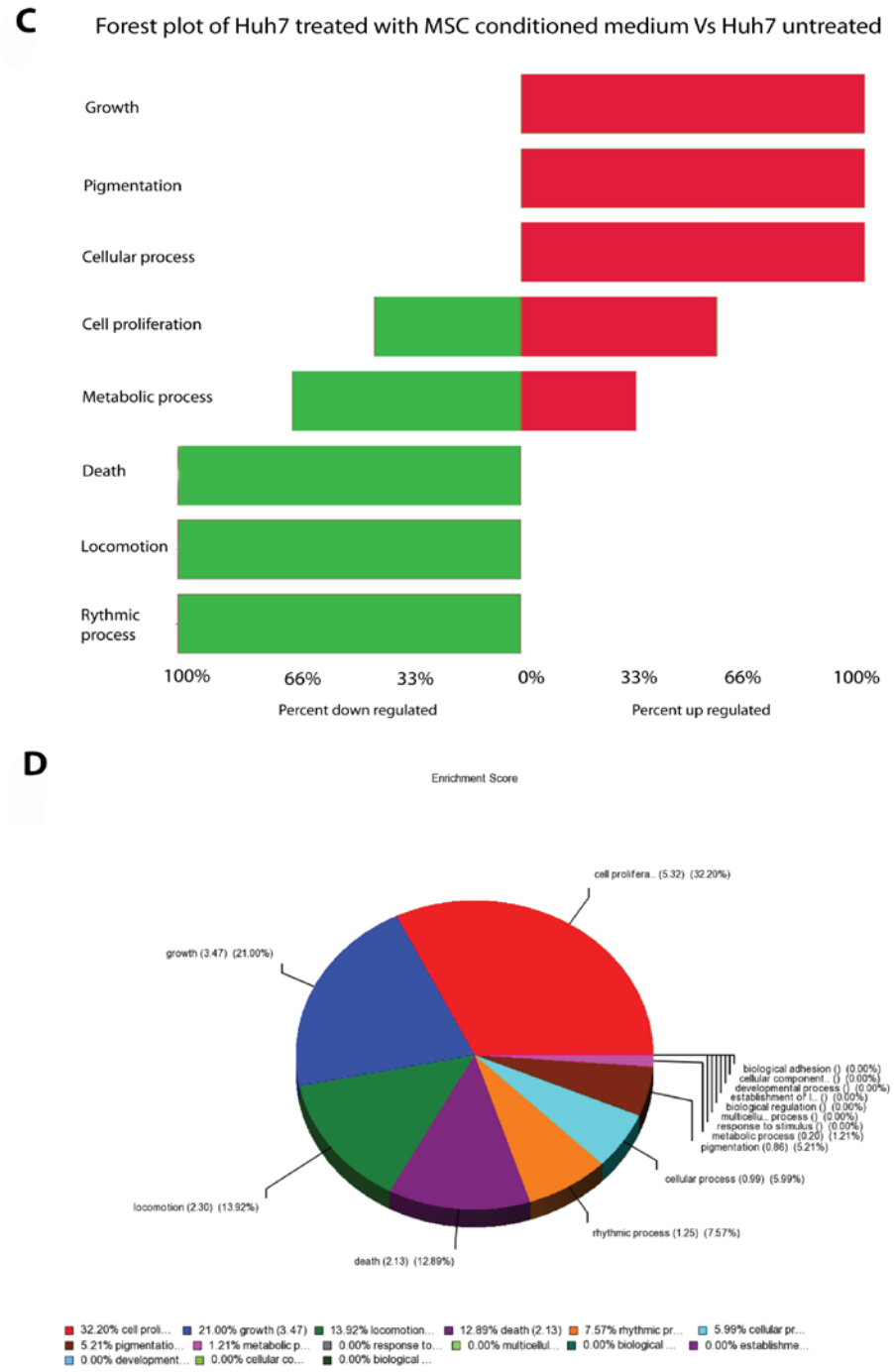


Fig. 5 (C) Forest plot and gene set enrichment analysis (**D**) of genome-wide gene expression array confirmed both the upregulation of cell growth and proliferation-related processes and downregulation of cell death-related pathways by treatment of Huh7 cells with MSC-CM. **P* < 0.05; ***P* < 0.01.

MSCs promote tumor growth in mice

The *in vitro* studies described above strongly support the notion that MSCs can enhance tumor growth. To prove it, Huh7 cells with or without MSCs were subcutaneously injected in NOD/SCID mice at the left or right side of the same mouse. Among 8 mice, 3 mice were injected with luciferase gene-labeled Huh7 cells. Therefore, the formation of tumors involved could be visualized in living animals by IVIS camera (Supplemental Figure 4). After engraftment of 19-21 days, mice were sacrificed for analysis of the formed solid tumors. As shown in figure 6A, the tumors from the co-injected side (Huh7 with MSCs) are markedly bigger than tumors formed in the other side. This is consistent with the observation that the weight of the tumors co-injected with MSCs is significantly higher. The tumor weight was 1.56 ± 0.27 g (mean ± SEM) in the co-engraftment group Vs 0.44 ± 0.19 g in the Huh7 alone group (n = 8, P < 0.01) (Figure 6B). Thus cancer-derived MSCs can support tumor growth to a large extent.

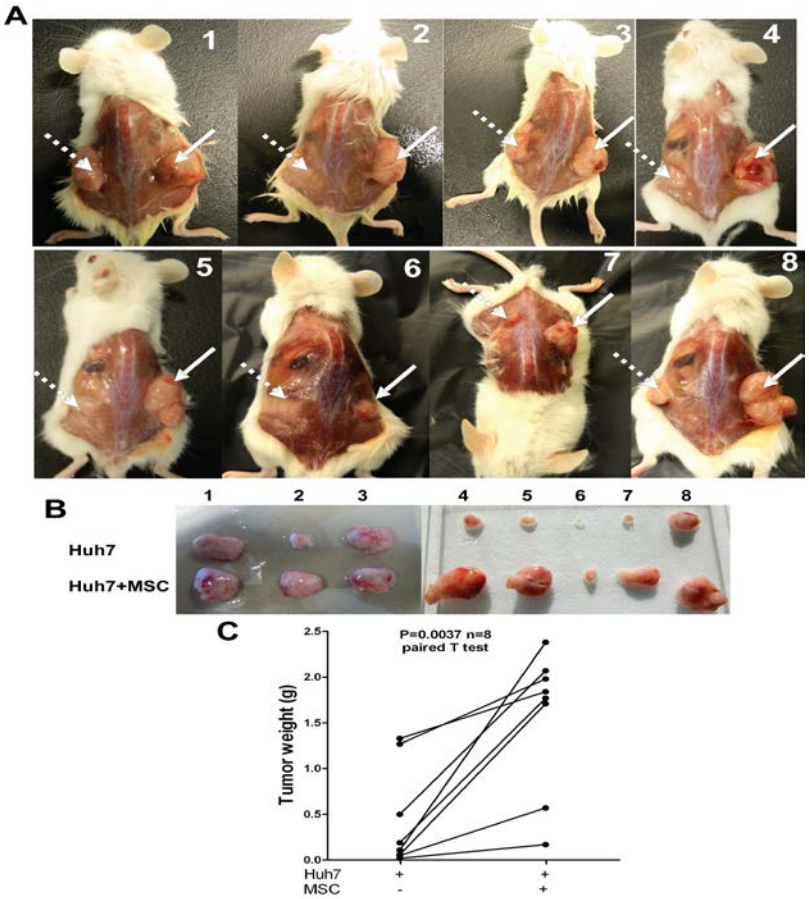


Fig. 6. MSCs promote tumor growth in mice. (**A** and **B**) Coengraftment of Huh7 with MSCs in mice resulted in larger tumors (right part) than engraftment of Huh7 alone (left part). (**C**) The tumor weight was 1.56±0.27g (mean ± SEM) in the coengraftment group versus 0.44±0.19g in the Huh7 alone group (n = 8, *P < 0.01).

DISCUSSION

It is well-recognized that the biology and pathology of cancer can only be understood by investigating the individual specialized cell types and their crosstalk within the tumor microenvironment (6). Recent studies have shown a possible involvement of MSCs as an important cellular element within the tumor microenvironment (26). In this study, we add to the existing knowledge by showing that STRO-1-positive MSCs are present in HCC at levels that are clearly higher as those observed in the surrounding tissue. Culturing these cells from resection material and subsequent investigation of their differentiation potential and cell surface marker repertoire confirmed the MSCs status of these cells. The most straightforward interpretation of these results is that HCC actively recruits MSCs. This notion was supported by xenograft experiments, which shows the human HCC model cell line may actively recruit murine MSCs. We speculate that HCC are subject to selection pressure that favors the acquisition of MSCs attracting properties. Although the mechanisms by which MSCs infiltrate into HCC are likely complicated, a potential key factor in this process could be Hepatocyte Growth Factor (HGF), well known to be a potent chemo-attractant for MSCs (27), which is produced at high levels by most HCC cell lines (28). Furthermore, human HGF is active in mice and can thus transcend the species barrier, as we observed the presence of mouse MSCs in human HCC cell line formed tumors in mice (Figure 3). Other cytokines are likely to contribute to HCC-dependent recruitment of MSCs as well (29). The observation that HCC are under apparent selection pressure to recruit MSCs into the tumor environment already provides a first hint at the importance of these cells for the HCC growth.

There is a lively debate in the literature whether MSCs exert a pro- or anti-cancer action (19). Several studies reported anti-tumor effects (12-14) whereas others demonstrated tumor promoting effects (15-17) of MSCs, depending on the particular cancer model and the methodologies applied. Our observation that HCC enriched with MSCs points to an important pro-oncogenic action. In addition, in this study we show that tumor-associated MSCs provide trophic effects on HCC through the production of soluble factors. Genome-wide gene expression profiles confirmed the up-regulation of cell growth and proliferation-related processes and down-regulation of cell death-related pathways by

treatment of MSC-CM in hepatoma cells. Finally, co-engraftment of human HCC-associated MSCs substantially promoted tumor growth in a xenograft model of HCC. Thus for at least HCC, the role of MSCs in the cancer process seems unequivocally pro-oncogenic. MSCs secrete paracrine factors including a variety of growth factors that are known to influence tumor proliferation, migration, and angiogenesis (19), which may explain the tumor support by MSCs observed in the present study. Although MSCs have been reported to support the tumor vasculature, directly by differentiating into pericytes and perhaps endothelial cells (30) and through indirect mechanisms by secreting vasculogenic growth factors (31), immunohistochemical staining and western blotting analysis of CD146 (angiogenesis marker) in the tumors formed in mice however showed no clear difference between the groups engrafted with or without MSCs (Supplemental Figure 5). The effects of MSCs on HCC thus do not seem to involve improved vascularisation and the effects seen on colony growth are more dominant. IL-6, one of the cytokines, secreted by MSCs, has been showed to promote formation of colorectal tumors in mice (32). We confirmed the secretion of IL-6 by MSCs using proteomic analysis of the secretome of MSCs (data not shown). However, neither adding exogenous IL-6 (Supplemental Figure 6) nor naturalizing MSCs produced IL-6 (Supplemental Figure 7) by antibody affected colony formation of Huh7 cells. These results excluded the involvement of IL-6 in our models. Conceivably, the pathways by which MSCs affect tumor growth are rather complicated as MSCs secret over 500 proteins as revealed by our proteomic analysis (data not shown). Innovative and high-throughput technologies are likely required to further elucidate this system biological interaction between MSCs and tumor cells.

In addition, other mechanisms such as exosomes (or microvesicles) produced by MSCs may also be involved in this process. Several studies have demonstrated that MSCs can secret exosomes that can result in a cell-to-cell transfer of mRNA, microRNA and proteins (33). Exosmes derived from BM MSCs have been shown to facilitate multiple myeloma progression (34) and promote gastric carcinoma growth (35). Whereas others have shown that exosomes from BM MSCs are able to inhibit growth of glioma (36), hepatoma, Kaposi's sarcoma or ovarian tumor in animal models (37). However, the exact roles and mechanisms of MSCs produced exosomes in tumor biology remain largely elusive. Immunomodulation,

another important feature of MSCs, could also have drastic influence on tumor microenvironment (38), although the current tumor models (mainly xenograft models in immunodeficient mice) are not able to properly evaluate these effects. Extensive studies have demonstrated the expression of several Toll-like receptors (TLR) by MSCs (39), which are known to be critically linked with innate and adaptive immunity. It has been described that the activation of certain TLRs can polarize MSCs to switch from a predominantly immune suppressive MSC2 (TLR3-primed) to a pro-inflammatory MSC1 (TRL4-primed) phenotype (40,41). Further study has shown that MSC1-based therapy attenuates tumor growth whereas MSC2-treatment promotes tumor growth and metastasis (42). Thus, the immunomodulatory property of MSCs deserves more attention in tumor biology.

Many clinical applications of MSCs are proposed, either as therapeutic agents in their own right (immunomodulating and favoring outcome in transplantation and autoimmune medicine for instance) or as anti-cancer drug/gene vehicles. The present study argues for caution. Clinical application of MSCs for treating liver diseases is currently being investigated in efforts to harness the hepatic differentiation potential, anti-inflammatory function and immunomodulatory properties of these cells. An early study involved the infusion of autologous bone marrow cells, which include the MSCs population, for treating decompensated liver cirrhotic patients, including HBV and HCV infected patients (43). More recently, using *ex vivo* cell expanded MSCs, either hepatic-differentiated or undifferentiated MSCs, were used to treat liver cirrhotic patients, (10,44). In addition, MSCs were also used for immunomodulation therapy of patients after liver transplantation (45). Such studies almost unavoidably involve patients who are positive for HBV or HCV. These infections are, however, not only important drivers of cirrhosis, but also for developing HCC (46). Of note, HCC is an important indication for liver transplantation (47) but also liver transplant patients have increased incidence of developing *de novo* cancer (48). Despite the short-term safety reported by these clinical trials, the findings of the present study caution the application of MSCs in such patients, and call for vigilant surveillance in patients with high risk of developing HCC but already treated with MSCs.

In summary, this study demonstrated that HCC are enriched with MSCs, which in turn provide trophic support for tumor growth. These results shed new light on the crosstalk

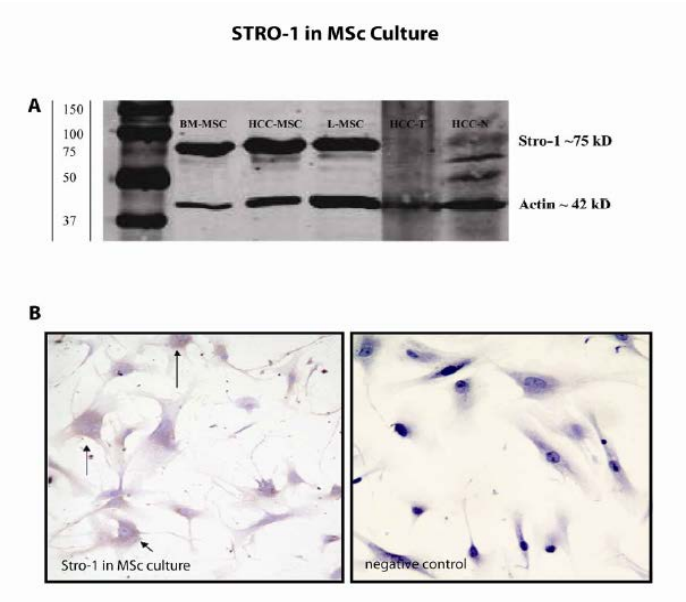
between MSCs with liver cancer cells and caution the therapeutic application of MSCs for liver cancer as well as other liver diseases with high risk of developing malignancy. Conceivably, targeting tumor MSCs may represent an innovative therapeutic approach against liver cancer.

SUPPLEMENTARY MATERIAL

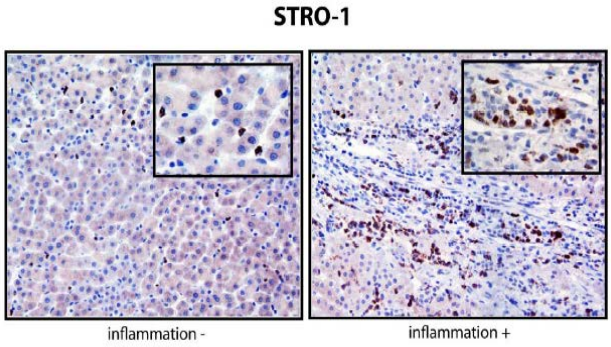
Supplemental table 1. Characteristics of hepatocellular carcinoma patients.

Patients characteristics

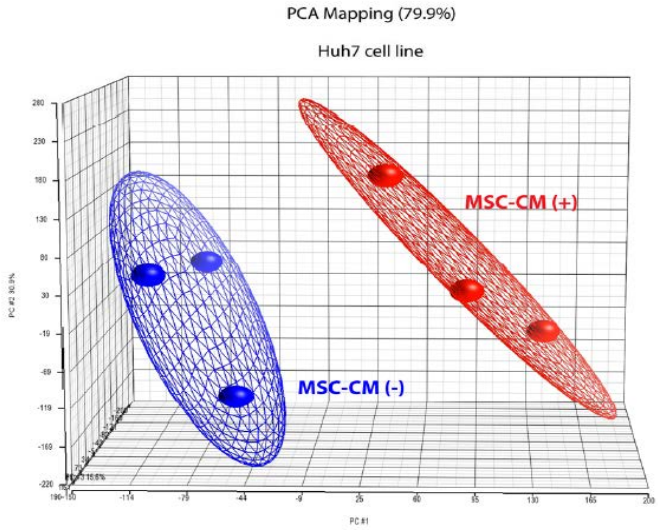
Patient no	Gender	Date of operated	Died	Recurrence	Differentiation	Cirrhosis	Hepatitis
1	M	18-10-2011	Unknown	Yes	Moderate	Yes	No
2	M	29-08-2008	Yes	Yes	Poor	Yes	No
3	F	26-06-2009	Unknown	Unknown	Poor	Yes	Yes
4	M	05-07-2009	No	No	Good	Yes	No
5	F	08-10-2009	Yes	No	Moderate	Yes	No
6	F	08-10-2009	Yes	No	Poor	Yes	No
7	M	04-12-2009	No	Yes	Moderate	No	No
8	M	15-12-2009	No	Yes	Moderate	No	Yes
9	M	28-07-2010	Yes, other reason	No	Moderate	Yes	Yes
10	M	14-09-2010	Unknown	Yes	Moderate	Yes	Yes
11	M	29-09-2010	No	No	Good	Yes	Yes
12	M	11-02-2011	No	Yes	Good	Yes	No



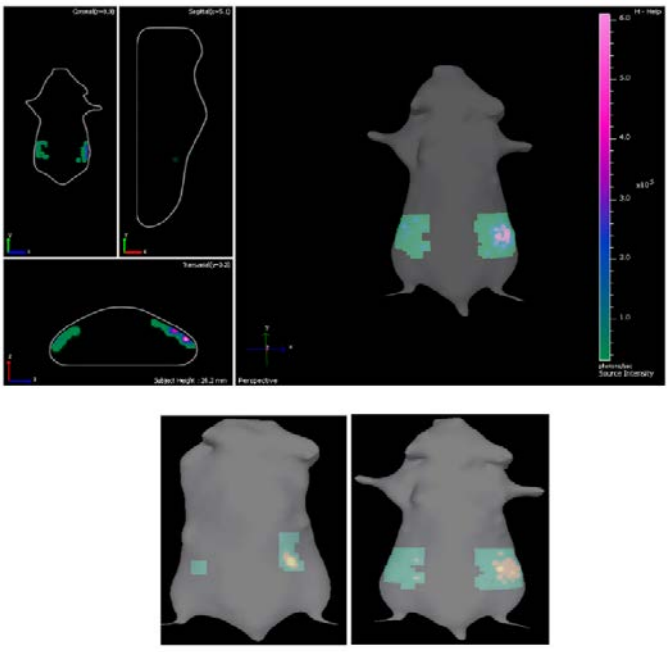
Supplemental Figure 1. (A) Western blot analysis showed abundant expression of STRO-1 protein in culture expanded liver tumor MSCs as well as liver and bone marrow-derived MSCs. But STRO-1 protein level is very low in whole lysates of liver and liver tumor tissues. (B) Immunohistochemistry staining confirmed the expression of STRO-1 in MSCs culture.



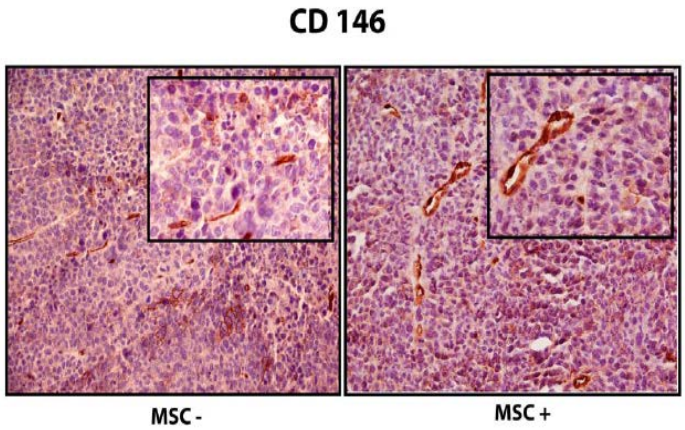
Supplemental Figure 2. Immunohistochemistry staining showed higher frequency of STRO-1 positive cells in liver tissues with massive inflammation, compared with the regular liver tissues.



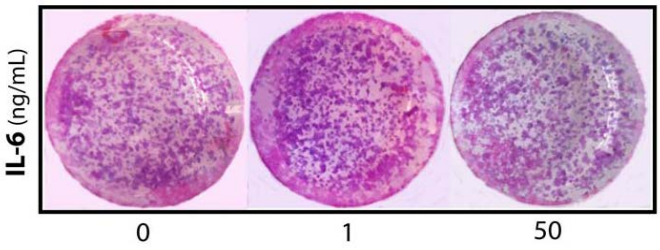
Supplemental Figure 3. The principal component analysis of genome-wide expression profiles of Huh7 cells treated with or without MSCs conditional medium (MSC-CM). The analysis separated the two treatment groups into two clusters, reflecting the effects of MSC-CM treatment in Huh7 cells.



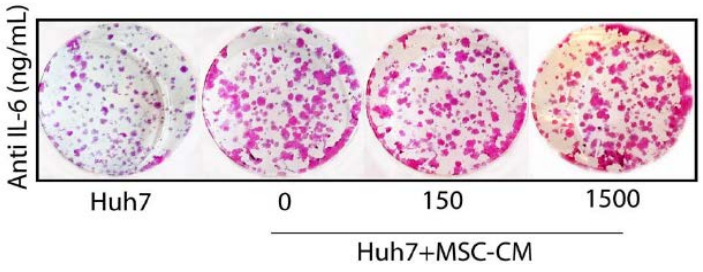
Supplemental Figure 4. The formation of tumors in mice injected with luciferase gene-labeled Huh7 cells. The right side is co-engrafted Huh7 with MSCs and the left side is Huh7 only. The luciferase signal is apparently higher in the right side, measured two weeks after engraftment.



Supplemental Figure 5.
Immunohistochemical staining and western blotting analysis of CD146 (angiogenesis marker) in the tumors formed in mice. No clear difference was observed between the groups co-engrafted Huh7 with or without MSCs.



Supplemental Figure 6. The effect of exogenous cytokine IL-6 on growth of Huh7 cells. We found secretion of IL-6 by MSCs using proteomic analysis of the secretome of MSCs. However, adding exogenous IL-6 did not affect colony formation of Huh7 cells.



Supplemental Figure 7. The effect of naturalizing cytokine IL-6 on growth of Huh7 cells. Naturalizing MSCs produced IL-6 by antibody did not affect colony formation of Huh7 cells.

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Chapter 4

Mesenchymal Stem/Stromal Cells Exert a Trophic Effect on Colorectal Cancer Metastasis to the Liver

Pratika Y. Hernanda[1], Alexander Pedroza-Gonzalez[1], Luc J. W. van der Laan[2], Martin J. Hoogduijn[3], Maikel P. Peppelenbosch[1] and Qiuwei Pan[1]

[1]Department of Gastroenterology & Hepatology, [2]Department of Surgery and Laboratory of Experimental Transplantation and Intestinal Surgery, [3]Department of Internal Medicine, Erasmus MC-University Medical Center, Rotterdam, Netherlands

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Abstract: Colorectal cancer (CRC) is the third most common cancer in the world. CRC tends to metastasize to the liver, which may occur in 20% to 70% of patients and represents the major cause of death. Mesenchymal stem/stromal cells (MSCs) have shown to be able to migrate to CRC site and play an important role in tumor progression. We have previously identified a resident MSC population in the liver. Therefore, this study aims to investigate whether there is infiltration of MSCs into patient CRC liver metastasis (CRC-LM) and their potential effects on tumor cell growth. By culturing resected patient CRC-LM tissue, we observed the emerging of fibroblast-like cells. Further phenotype and functional characterization confirmed their bona fide MSCs features. In situ staining with a well-established MSCs marker showed a significant enrichment of candidate MSCs in patient CRC-LM, particularly the tumor-stromal area. Moreover, MSCs secreted trophic factors significantly increased colony formation and growth of a metastatic CRC cell line. In summary, we found infiltration and enrichment of MSCs in CRC-LM patient, which could in turn nourish tumor cells.

Keywords: mesenchymal stem/stromal cells; colorectal liver metastasis; Trophic action; patients

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world. An estimated 1.24 million people worldwide were diagnosed with colorectal cancer in 2008, accounting for 10% of the total cancer patients (http://globocan.iarc.fr/). In approximately 75–80% of cases, patients have potentially resectable disease at the time of diagnosis [1]. Liver confined metastases (synchronous metastases) are found in 77% of CRC patients presenting stage IV disease at diagnosis [2]. The treatment of CRC has evolved greatly in the last 10 years, involving complex combined chemotherapy protocols and, in more recent times, new biologic agents. Evidence to date suggests potentially distinct roles for bevacizumab and EGF receptor-targeted biological agents (cetuximab and panitumumab) in the treatment of metastatic CRC. Advances in adjuvant therapy have been limited to the addition of oxaliplatin and the substitution of oral fluoropyrimidine (e.g., capecitabine) with intravenous 5-fluorouracil, however there was no evidence for improved outcome with biological agents [3]. Therefore, new therapeutic options are urgently needed for advanced or metastatic CRC.

Mesenchymal stem/stromal cells (MSCs) were first found in the bone marrow (BM) that act as stromal cells supporting hematopoietic stem cells [4]. In recent years, MSCs have been extensively demonstrated possessing multilineage differentiation potential, anti-inflammatory function and immunomodulatory properties [5]. More recently, the tumor-trophic and migratory properties of MSCs demonstrated in various pre-clinical models has emerged an interesting concept of using MSCs as carrier for anti-cancer drug/gene delivery [6, 7]. This notion also appears to be supported by several studies showing anti-tumor effects of MSCs [8-10]. In contrast, others reported that MSCs could promote tumor progression or metastasis in animal models [11-13]. Thus, it is still unclear, regarding the pro- or anti-cancer role of MSCs, in particular the effects in patients [14].

We have previously identified a resident MSC population within the human adult liver that are phenotypically and functionally similar to BM MSCs [15]. We subsequently demonstrated that MSCs are enriched in human primary liver cancer (hepatocellular

carcinoma; HCC) and could exert trophic effects on tumor cells [16]. This prompted us to further investigate the presence and the roles of MSCs in liver metastatic CRC (CRC-LM).

Materials and Methods

Patient Samples

Nine paired fresh CRC-LM and tumor-free liver tissue were collected for MSCs culturing. 12 paraffin-embedded patient CRC-LM tissues were collected at the tissue bank at the Erasmus MC Rotterdam for immunohistochemical staining of MSC marker (Table 1). The medical ethical committee of Erasmus MC proved the use of patient materials.

CRC Patients characteristics

Patient no	Gender	Recurrence	Cirrhosis	Hepatitis
1	M	yes	no	no
2	M	yes	no	no
3	M	yes	no	no
4	F	yes	no	no
5	F	no	no	no
6	F	no	no	no
7	M	no	no	no
8	M	yes	no	no
9	M	yes	no	no
10	M	no	no	no
11	F	no	no	no
12	M	no	no	no

Procedure of Culturing MSCs

Adjacent liver tissue or CRC-LM tumor were digested into single cell suspensions or cut into small pieces. Single cell suspension or small pieces of tissues were cultured for MSCs as previously described [16].

Flow Cytometric analysis

Cells were stained with mouse monoclonal antibodies against CD13-PECy7 (BD Biosciences), CD34-APC (BD Biosciences), CD45-PERCP (BD Biosciences), CD73-PE (BD

Pharma, San Jose, CA), CD105-FITC (R&D Systems, Abingdon, UK), CD166-PE (BD Pharma, San Jose, CA) and HLA-I-APC (BD Biosciences), and flow cytometric analysis was performed.

Assay of Adipogenic and Osteogenic Differentiation

Adipogenic and osteogenic differentiation were performed as previously described [16]. Oil Red O staining was used for adipocytes detection. Alizarin Red S staining was used to detect deposited calcium phosphates.

Colony Forming Assay of SW620 cells

The colony formation assay was performed in SW620 cells, the metastasis colorectal cancer cell lines [17], 1000 cells were plated in each well of 6 wells plate and were treated with MSCs conditioned medium (MSC-CM), which was prepared by culturing MSCs until 70-90% confluence and medium was collected 48 hours post-culture. Colony formation assay was also performed with SW620 cells only in DMEM medium with 10% fetal bovine serum with or without MSC-CM. SW620 colonies were stained with haematoxylin & eosin after two weeks.

Immunohistochemistry staining

Paraffin embedded LM-CRC tumor tissue slides were used to stain with the mouse monoclonal antibody STRO-1 (Invitrogen Corporation) and then counterstained with haematoxylin [16].

Statistical Analysis

Statistical analysis was performed by using the GraphPad Software with nonparametric Mann–Whitney test or paired t-test. P-values <0.05 were considered statistically significant.

Results

Culture of MSCs from Resected Human CRC-LM Tissues

To investigate whether MSCs are present in patient CRC-LM tumors, surgical resection of CRC-LM tissues of 9 patients were collected and submitted to an MSC culturing protocol previous validated for liver tissue [14, 16]. Indeed, mesenchymal like cells were easily identified in the resulting cultures (Figure 1A). Upon sub-culturing, rapid grow out and expansion of this cell type was validated (Figure 1B). To confirm the bona fide MSCs nature of these cells, a functional analysis was performed and demonstrated that these tumor-derived cells had a multilineage potential with a capacity for adipogenic (Figure 1C) and osteogenic differentiation (Figure 1D). In addition, FACS analysis confirmed that these cells are positive for common mesenchymal markers CD13, CD73, CD105 and CD166 and are negative for the common hematopoietic markers CD34 and CD45 (Figure 2A). Finally, STRO-1 is considered as the well-known MSCs marker [18], in particular for *in vivo* immunohistochemical staining of candidate MSCs [7]. The STRO-1 protein is highly expressed in MSCs cultured from CRC-LM tissues, as evident from immunohistochemical staining (Figure 2B).

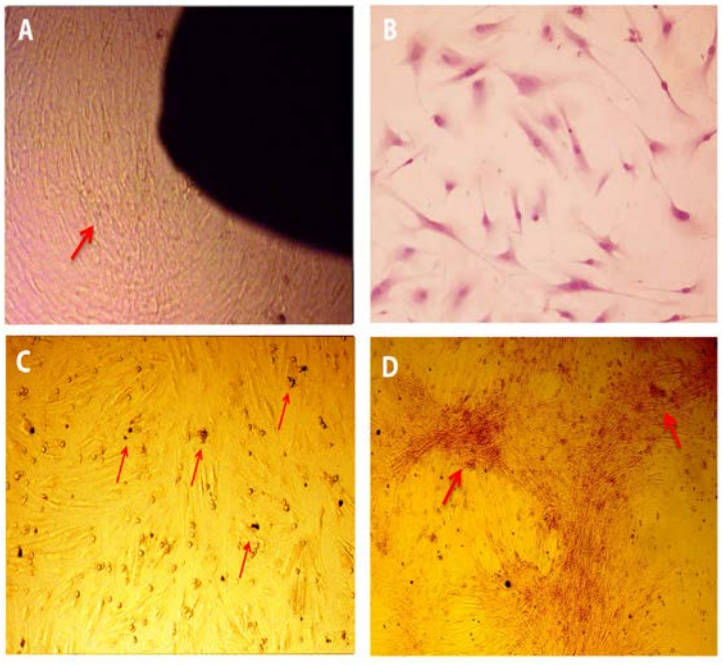

Figure 1. Culture and lineage differentiation of MSCs from human CRC-LM tissues. With a method of culturing tiny tissue specimen from surgical resected human CRC-LM tissues, fibroblast-like cells appeared (**a**) and these cells could rapidly grow out and expand by subculture showing typical MSCs morphology (**b**). (**c**) Adipogenic differentiation of CRC-LM derived MSCs, detected by Oil red O staining for lipid droplet (Arrow). (**d**) Osteogenic differentiation of these cells was evaluated by detection of deposited calcium phosphates using Alizarin Red S staining (Arrow).

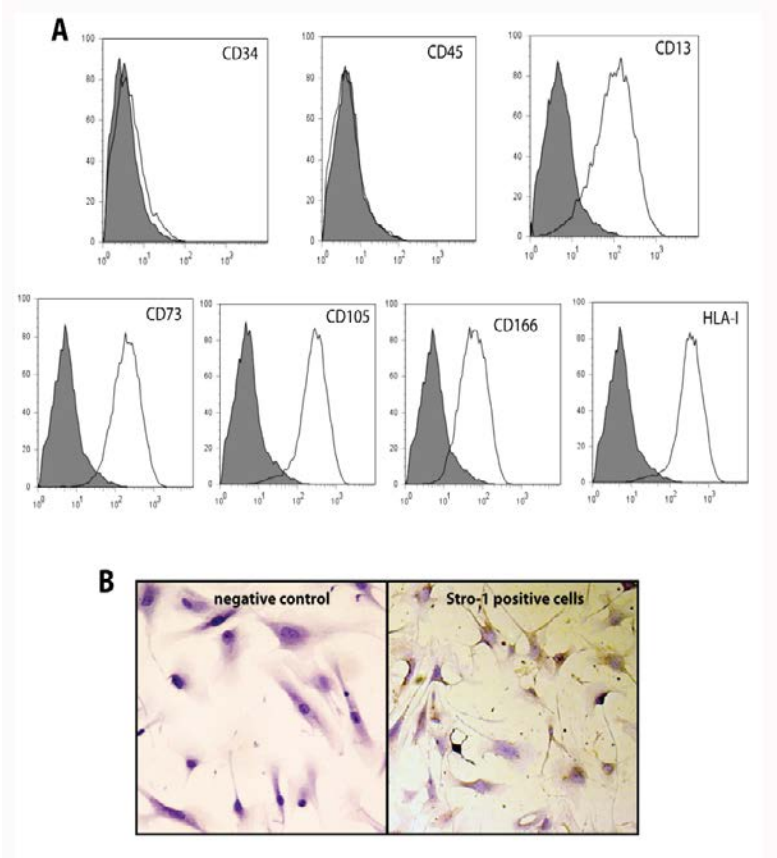

Figure 2. Antigenic profiling of cultured MSCs. (**a**) FACS staining confirmed that these cells are positive for common mesenchymal markers CD13, CD73, CD105 and CD166 and are negative for the common hematopoietic markers CD34 and CD45. (**b**) Expression of STRO-1 protein, the best-known MSCs marker, confirmed by immunohistochemical staining of cultured tumor MSCs.

STRO-1 Cells Are Enriched in Human CRC-LM Tumors

To further characterize whether the presence of MSCs is a general phenomenon in CRC metastasis to the liver, we investigated a further cohort of patients using paraffin-embedded patient CRC-LM (n=12, Table 1) and immunohistochemical staining of STRO-1. STRO-1 positive cells were detected in both adjacent tissue and tumor areas (Figure 3A). In the regions of the liver apparently unaffected by the cancerous process, STRO-1 positive cells mainly locate in liver sinusoid or blood veins. Interestingly, the frequency of STRO-1 in tumor, in particular the tumor stromal region, is significantly higher than adjacent tissue site (Figure 3B). Thus the presence of MSCs is not only a characteristic of CRC metastasis to the

liver, there is also active recruitment of MSCs to the tumor and these cells are locally enriched in the metastasis. MSCs are also present in the primary CRC tumor but hardly detectable in the adjacent colon tissue (Figure 3C).

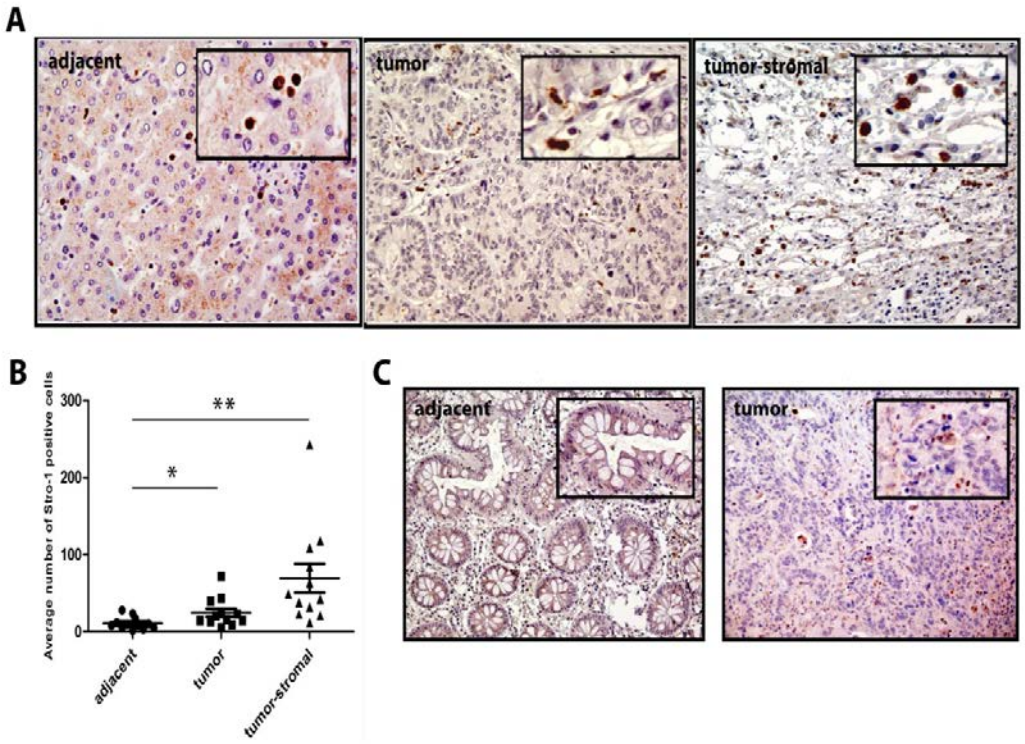

Figure 3. In situ localization of STRO-1 positive cells in paraffin-embedded patient primary CRC and CRC-LM tissues. (**a**) Distribution of STRO-1 cells in the adjacent, tumor and tumor-stroma regions in CRC-LM tissues. (**b**) STRO-1 positive cells (an average from 3 HP fields) are significantly enriched in the tumor, in particular the tumor-stroma region, compared with the adjacent area in CRC-LM tissues (n = 12, *P < 0.05, **P < 0.01). (**c**) In primary CRC tissues, STRO-1 positive cells are present in the tumor but hardly found in the normal adjacent area.

MSCs Secret Trophic Factors That Can Enhance Colony Unit Formation and Growth of Human Metastatic CRC Cells

Previously, we showed that human liver MSCs can exert trophic action on HCC cells [16], but whether these cells have the capacity to foster CRC cell is unknown. Thus we decided to investigate whether factors secreted by MSCs influence colony unit formation and growth of the SW620 CRC cell line. This cell line was initially isolated from a lymph node during a wide-

spread tumor metastasis from the colon to an abdominal mass [17]. SW620 cells were seed for colony forming assay and subsequently treated with MSC-conditioned medium (MSC-CM). This treatment significantly increased both the number and size of the SW620 colonies (251.3 ± 46.9 Vs 282.1 ± 55.6 colonies/ 500 SW620, Mean ± SD, n = 6, $P < 0.05$) and the size (184.0 ± 33.6 Vs 222.8 ± 57.8 pixels, n = 30, $P < 0.01$) of SW620 formed colonies (Figure 4C and 4D). These results indicate that trophic factors secreted by human liver MSCs provide a potent stimulus to support CRC growth in the liver environment.

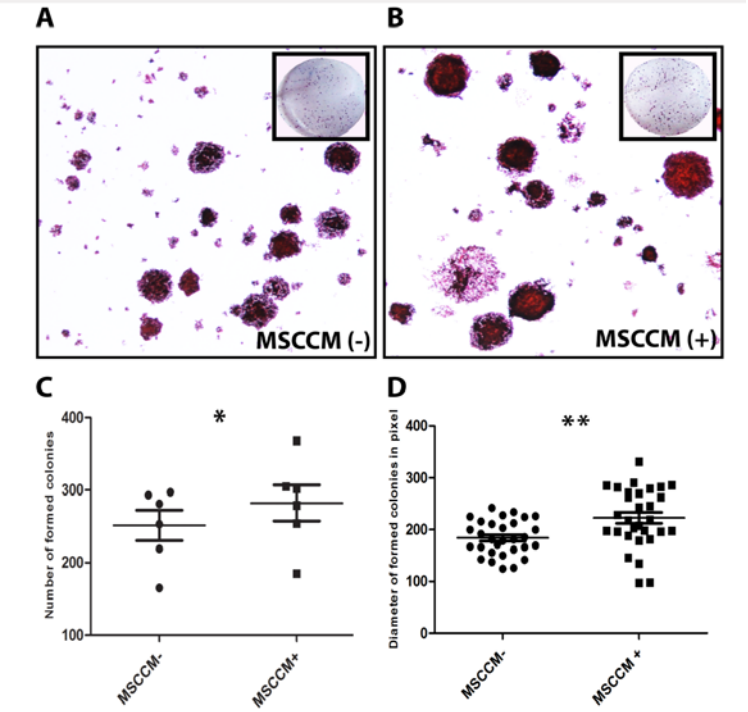

Figure 4. Trophic factors secreted by MSCs promote colony formation and growth of metastasis CRC cells. SW620 treated with MSCs conditioned medium (MSC-CM) resulted in formation of more and larger colonies (A & B). Treatment of SW620 with MSC conditioned medium significantly increased both the number (251.3 ± 46.9 Vs 282.1 ± 55.6 colonies/ 500 SW620, Mean ± SD, n = 6, $P < 0.05$) (C) and the size (184.0 ± 33.6 Vs 222.8 ± 57.8 pixels, n = 30, $P < 0.01$) (D) of SW620 formed colonies.

Discussion

CRC tends to metastasize to the liver, which may occur in 20% to 70% of patients and represents the major cause of death [19]. The liver holds a distinct cellular and molecular

environment [20] and structural changes of tumor microenvironment of CRC often occur once metastasis in the liver [21]. Recent studies have demonstrated the involvement of MSCs as an important cellular element within tumor microenvironments [22].

MSCs were initially discovered in the BM stromal compartment, but lately were identified in number of organs/tissues, including the liver [14]. Extensive studies have demonstrated that MSCs can migrate to the tumor site and incorporate into its microenvironment [23, 24]. It has also been reported that MSCs migrate to colorectal tumors [25, 26]. The common approach to study the homing of MSCs into tumor is transplanting culture expanded MSCs into animal models bearing tumor [24]. In this study, we have successfully located candidate MSCs in patient CRC-LM and adjacent tissue by immunohistochemical staining with a well-established MSCs Marker, STRO-1. Interestingly, the frequency of STRO-1 cells is significantly higher in the tumor region, in particular the tumor-stromal area of CRC-LM, compared with paired adjacent liver tissues. These cells can cultured from resected CRC-LM tissues and expanded in vitro. This tumor-homing property of MSCs is likely regulated by several specific danger signals and chemotactic factors [27, 28], although the exact mechanisms remain elusive.

There is constant debate whether MSCs suppress or support tumor growth and progression [14]. Several studies demonstrated anti-tumor effects [8-10] whereas others showed tumor promoting effects [11-13] of MSCs in different tumor models. Regarding the effects on CRC, most of the studies have shown a pro-cancer effects of MSCs [29-31]. Consistently, we observed that trophic factors secreted by MSCs can promote colony formation and growth of a metastasis CRC cell line. MSCs secrete paracrine factors including a number of growth factors that are known to influence cancer cell proliferation, migration, and angiogenesis [14].

In summary, this study has demonstrated the presence and local selective enrichment of MSCs in human CRC-LM. Trophic factors produced by MSCs can favor the growth of metastasis CRC cells. These results may help to understand the role of MSCs in favoring CRC liver metastasis. In addition, it cautions against the application of anti-cancer therapy or anti-cancer gene/drug delivery using MSCs as these may intrinsically favor the cancerous process.

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Chapter 5

SMAD4 Exerts a Tumor Promoting Role in Hepatocellular Carcinoma

Pratika Y. Hernanda[1, 2#], Kan Chen[1, 3#], Asha M. Das[4#], Kostandinos Sideras[1#], Steven J. A. Bots[1], Liudmila L. Kodach[5], Robert A. de Man[1], Jan N.M Ijzermans[6], Harry L. A. Janssen[1,7], Dave Sprengers[1], Marco J. Bruno[1], Herold J. Metselaar[1], Timo L. M. ten Hagen[4], Jaap Kwekkeboom[1], Maikel P. Peppelenbosch[1], Qiuwei Pan[1]

[1]Department of Gastroenterology and Hepatology, Erasmus MC Cancer Institute, Erasmus University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, The Netherlands. [2]Laboratory of Medical Genetics, Biomolecular Research Centre, Wijaya Kusuma University, Surabaya, Indonesia [3]Bio-X Center, College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou, China. [4]Laboratory of Experimental Surgical Oncology Section Surgical Oncology, Department of Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands. [5]Department of Gastroenterology and Hepatology, Leiden University Medical Centre, Leiden, The Netherlands. [6]Department of Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands. [7]Division of Gastroenterology, University Health Network, Toronto, Canada.

[#]Contribute equally

Oncogene, conditionally accepted

ABSTRACT

Objective: Molecular targeted therapy against hepatocellular carcinoma (HCC) has only demonstrated a temporary treatment response. Thus, further understanding of the molecular biology and pathogenesis of HCC is crucial for future therapeutic development. SMAD4, recognized as an important tumor suppressor, is a central mediator of Transforming Growth Factor Beta (TGFB) and Bone Morphogenetic Protein (BMP) signaling. This study investigated the role of SMAD4 in HCC.

Design: Nuclear expression of SMAD4 were stained in a cohort of 140 HCC patients using paraffin embedded liver tumor tissue in tissue microarray (TMA). HCC cell lines were used for functional assay *in vitro* and in immune-deficient mice.

Results: Nuclear SMAD4 levels were significantly increased in patient HCC tumors as compared to adjacent tissues. Furthermore, knockdown of SMAD4 significantly reduced the efficiency of colony formation and migratory capacity of HCC cells *in vitro* and was incompatible with HCC tumor initiation and growth in mice. Knockdown of SMAD4 partially conferred resistance to the anti-growth effects of BMP ligand in HCC cells. Importantly, simultaneous elevation of SMAD4 and phosphorylated SMAD2/3 is significantly associated with poor patient outcome after surgery. Although SMAD4 can also mediated an anti-tumor function by coupling with phosphorylated SMAD1/5/8, this signaling however is absent in majority of our HCC patients.

Conclusion: This study revealed a highly non-canonical tumor promoting function of SMAD4 in HCC. The drastic elevation of nuclear SMAD4 in sub-population of HCC tumors highlights its potential as an outcome predictor for patient stratification and a target for personalized therapeutic development.

Introduction

SMAD proteins are recognized as central mediators of Transforming Growth Factor Beta (TGFB) and/or Bone Morphogenetic Protein (BMP) signaling pathways, which regulate a plethora of physiological processes including cell growth and differentiation.[1] Accordingly, deregulation of TGFB/BMP pathways almost invariably leads to developmental defects and/or diseases, in particular cancer.[2] These two pathways signal through the family of SMAD proteins to exert their effects. In mammals, there are 8 SMADs that are subdivided into 3 distinct classes: receptor-regulated SMADs (R-SMADs) comprising SMAD2 and SMAD3 (transduce TGFB signaling) and SMAD1, SMAD5, and SMAD8 (transduce BMP signaling); a common SMAD called SMAD4; and 2 inhibitory SMADs, namely, SMAD6 and SMAD7.[3] SMAD proteins are highly conserved within their family and across species, with SMAD4 representing a somewhat divergent subtype which still retains about 40% identity with other family members.[4] SMAD4 binds to receptor-regulated SMADs and forms heteromeric complexes and facilitating the translocation of these heteromeric complexes into the nucleus. In the nucleus, the heteromeric complex binds to promoters and interacts with transcriptional activators[2, 5] and the

SUMMARY BOX

What are already known about this subject

- SMAD4 is originally identified as a candidate tumor suppressor gene
- SMAD4 loss or inactivation is associated with several types of cancers
- SMAD4 mutation in hepatocellular carcinoma (HCC) appears rare

What are the new findings

- Drastic elevation of nuclear SMAD4 level in patient HCC tumors.
- High SMAD4 expression has been screwed towards tumor promoting effects due to simultaneous elevation of p-SMAD2/3 in subset of HCC patients.
- SMAD4 can also mediate an anti-tumor signaling by coupling p-SMAD1/5/8, this complex however is absent in majority of HCC patients

How might it impact on clinical practice in the foreseeable future

- This is an unexpected results in a view of the dogma that SMAD4 is a potent tumor suppressor and have certainly shed a new light on the molecular biology of HCC. More importantly, SMADs-based molecules may have potential as outcome predictors for patient stratification and targets for personalized therapeutic development.

presence of nuclear SMAD4 protein has profound consequences for gene expression.

Originally identified as a candidate tumor suppressor gene at 18q21.1 decades ago,[6] the tumor suppressive function of SMAD4 has now almost achieved dogmatic status and loss of its activity has been implicated in the in the initiation and progression of a multitude of cancer types.[2, 7-10] Loss or inactivation of both normal gene copies is associated with carcinoma in several organ systems, including approximately 55% of pancreatic adenocarcinomas,[6] 15% to 55% of extrahepatic cholangiocarcinomas[11] and a smaller percentage of gastrointestinal and other carcinomas.[12, 13] Strikingly, loss of SMAD4 expression in hepatocellular carcinoma (HCC) has not been observed, prompting investigations into role and importance of this tumor suppressor in this disease.

HCC which accounts for the majority of primary liver malignancies is the fifth most prevalent neoplasm and the third most frequent cause of cancer-related death and is characterized by a remarkable failure of conventional treatments.[14] Most cases of HCC are found in patients with cirrhosis caused by chronic hepatitis B or C virus infection, in particular in Asian countries.[15] The unique etiology as well as the distinct environment that the liver holds may govern a differential signaling network compared to other cancers. One study reported a very small percentage of HCC displaying a Asp332Gly mutation in *SMAD4* gene, but it is unknown whether this mutation affects its function; whereas another study failed to identify mutations in *SMAD4*, and thus functionality-corrupting mutations in *SMAD4* appear rare.[16, 17] Hence, we endeavored to establish the role of SMAD4 in HCC which we uncovered a non-conventional function of SMAD4 in HCC as a tumor promoter.

Material and Methods

Tissue microarray (TMA)

To make tissue microarray, paraffin-embedded HCC patient tissues (n = 140, between 2004 to 2013) were collected from the pathology department of Erasmus Medical Centre (Erasmus MC) Rotterdam. The use of patient materials was approved by the medical ethical committee of Erasmus MC (Medisch Ethische Toetsings Commissie Erasmus MC).[17]

Immunohistochemistry

Paraffin embedded liver tumor tissue in tissue microarray (TMA) slides were deparaffinized in xylene, rehydrated in graded alcohols, and rinsed once in phosphate-buffered saline (PBS) plus Tween 0.05%. For antigen retrieval, slides were boiled in Tris/EDTA pH 9.0 for 30 min (for SMAD4 antibody) and 10 min for other antibodies; 3% H2O2 was used to block endogenous peroxidase for 10 min at room temperature. The slides were incubated in 5% milk blocking solution followed by overnight incubation in mouse SMAD4 antibody (1:100 dilution, Santa Cruz Biotechnology, Inc), goat p-SMAD2/3 antibody (1:250 dilution, Santa Cruz Biotechnology, Inc) and rabbit p-SMAD1/5/8 (1:500 dilution, Cell Signaling) and then counterstained with haematoxylin. The SMAD4 scoring was based on the nuclear staining and the p-SMAD2/3 and p-SMAD1/5/8 scoring were based on cytoplasm and/or nuclear staining. The following scores were applied: score 0 for 0-10% positive staining, score 1 for 10-30% positive staining, score 2 for 30-70% positive staining, score 3 for >70% positive staining, and score 4 for >70% positive staining + high intensity. The scorings were done by two investigators and the difference of scoring was valued by Kappa test.

Lentiviral shRNA vectors

Lentiviral backbone vectors for *SMAD4* knockdown and non-targeting control were obtained from the Erasmus Center for Biomics (the Sigma–Aldrich TRC library). A vectors expressing shRNA targeting GFP (not expressed in HCC cell lines) served as control (CTR). Lentiviral viral particles were generated as described previously.[18]

Cell culture and reagents

Human hepatoma cell lines (BEL-7404 and Huh7) were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich) and 1% penicillin/streptomycin (p/s) (Gibco). SMAD4 knockdown cells and control cells were generated by inoculation of lentiviral vectors and subsequently selected and maintained in DMEM with 10% FBS, 1% p/s and 2 μg/ml puromycin (Sigma). Recombinant human BMP4 protein (100μg/ml, Merck Millipore) and recombinant human noggin (50μg/ml, R&D System) were used to treat cells, respectively.

Colony forming assay

Colony formation was performed in BEL-7404 and Huh7 cells as described previously.[19] After trypsinizing, 1000 cells were added to each well of 6-well plate and were cultured in DMEM medium as previously described. The colonies formed are counterstained with haematoxylin & eosin after two weeks.

Western blotting

Subconfluent cells were lysed in Laemmli sample buffer containing 0.1 M dithiothreitol and incubated for 5 min at 96°C. Immunoblotting was performed using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE). Antibodies used were mouse SMAD4 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc), goat p-SMAD2/3 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc) and rabbit p-SMAD1/5/8 (1:500 dilution, Cell Signaling). Quantification was performed using Odyssey LI-COR software.

Ring-barrier migration assay

Ring-barrier based migration assays were performed as previously described.[20, 21] For BEL-7404 and its sh-SMAD4 cells, $4x10^5$ cells were seeded, while for Huh7 and its sh-SMAD4 cells, $3x10^5$ cells were seeded in the ring in DMEM+ 10% FBS+ 1% P/S. After 24 h, the migration barrier was removed and the cells were washed twice followed by the addition of fresh medium.

Cell migration was monitored with time-lapse microscopy on Axiovert 100 M inverted microscopes, equipped with AxioCam MRC digital cameras (Carl Zeiss B.V., Sliedrecht, NL). The total distance covered by the cells in 24 h was termed 'total distance of migration' (µm), while directional cell movement to the cell-free center of the coverslip was termed 'effective distance of migration' (µm). Migration efficiency (%) was determined as the percentage of the effective migrated distance over the total migrated distance. Migration velocity was calculated as the total distance migrated as a function of time (µm/h). All cell tracking measurements were conducted using AxioVision 4.9.1 software. P-values were calculated

using the two-tailed Mann-Whitney test. Track diagram images were processed in Adobe Illustrator CS6 (Adobe Systems Inc, San Jose, CA).

HCC xenograft tumor in nude mice

HCC xenograft tumor model in nude mice was established as previously described.[22] Ten mice for each cell line (BEL-7404 and Huh7), aged 6-8 weeks, were subcutaneously engrafted with 1 million control (CTR) and SMAD4 knockdown cells into the lower left or right flank, respectively. Tumor initiation in the mice was monitored. At the end of experiment, mice were sacrificed and tumors were harvested and weighed. The use of animals was approved by the Animal Care and Ethics Committee at Hangzhou Normal University, Hangzhou, China.

Statistical analysis

Statistical analysis was performed by using Chi Square test, nonparametric Mann–Whitney test, cox regression analysis and Kaplan Meier survival analysis in IBM SPSS Statistical 21. T-test was also used using GraphPad InStat software (GraphPad Software Inc., San Diego, USA). P-values <0.05 were considered as statistically significant.

Results

Drastic elevation of nuclear SMAD4 expression in the tumors of sub-population of HCC patients

The paucity of data surrounding the functionality of SMAD4 in HCC prompted us to analyze SMAD4 expression and activation in a panel of resected HCC from 140 individual patients and compare the results to adjacent non-transformed tissue. In these patients, nuclear SMAD4 protein (Fig. 1A) was taken as measure of SMAD4 signaling activity, as it is generally assumed that this fraction of the SMAD4 pool represents the transcriptionally active form of the protein. The staining was scored by two independent investigators with a Kappa test of 0.773, suggesting that there was an excellent agreement in scoring between the two investigators. The levels of SMAD4 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites (Fig. 1A & 1B). Strikingly, nuclear SMAD4 levels were considerably higher in human HCC tissue as compared to normal adjacent liver tissue (n =140, $P < 0.01$) (Fig. 1A & 1C). Subsequent

subgroup analysis according to the nuclear SMAD4 score in the tumor showed that there was no difference of SMAD4 levels between tumor and adjacent tissue in patients displaying low to moderate nuclear SMAD4 scores (n = 97, data not shown); whereas a drastic elevation was observed in tumor compared with adjacent tissue in the high SMAD4 expression group, (3.47 ± 0.45 *vs* 2.27 ± 0.92, mean ± s.e.m, n = 43, $P < 0.001$) (Fig. 1C).

Fig. 1

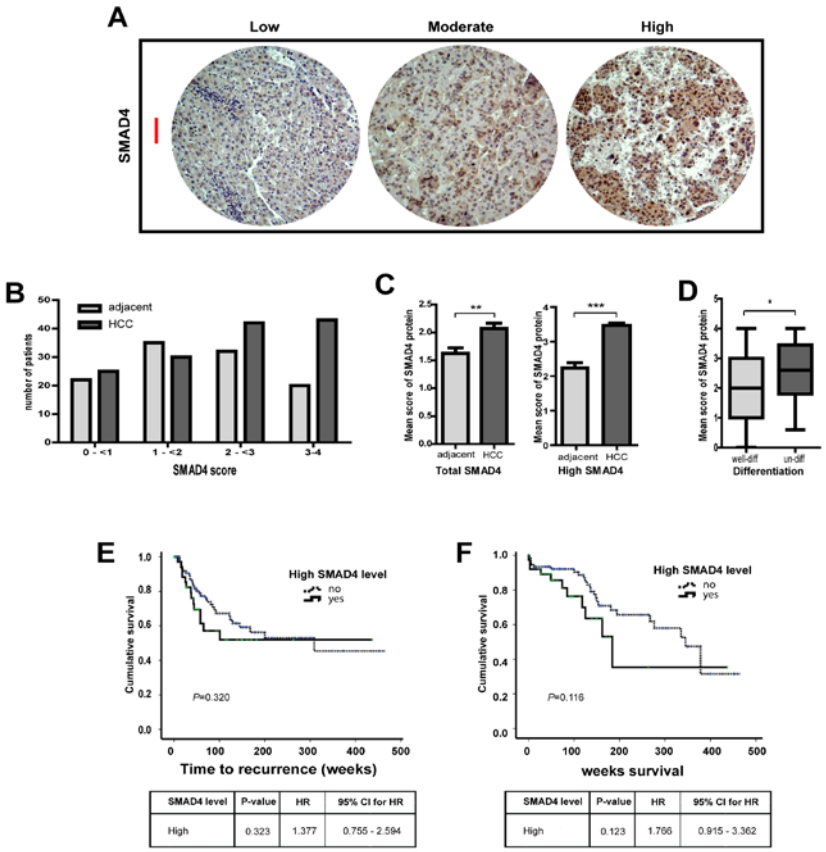


Fig. 1. Strong elevation of nuclear SMAD4 expression in the tumors of sub-population of HCC patients. (A) The levels of SMAD4 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (B) The distribution of SMAD4 score among HCC patients. (C) Overall, SMAD4 expression levels were significantly higher in human HCC tissues compared with normal adjacent liver tissues. Error bars represents mean ± s.e.m from n = 140, paired *t*-test, $^{**}P < 0.01$. A significant increase was also observed in tumors compared with adjacent tissues in the high grade patients (n = 43, paired *t*-test, $^{***}P < 0.001$). (D) Nuclear SMAD4 level was significantly higher in undifferentiated tumor than in well differentiated tumor of HCC. From cox regression analysis (n = 130 analyzable patients), high SMAD4 level in surgical resected HCC tumor tend to have higher risk of fast recurrence (hazard ratio; HR = 1.377) (E) and higher risk of poor survival (HR = 1.766) (F). Kaplan Meier analysis (n = 130) also indicated a trend of faster disease recurrence (E) and lower cumulative survival (F), although not statistically significant.

Analysis focusing on clinical behavior of the cancer (Supplementary Table 1) revealed that high levels of nuclear SMAD4 were not significantly associated with tumor size (n = 98 analyzable patients), number of tumor lesions (n = 129 analyzable patients) and vascular invasion (n = 78 analyzable patients), but significantly associated with higher levels of alpha-fetoprotein (AFP) pre-resection (n = 135 analyzable patients, $P < 0.01$). Serum AFP has been suggested as an independent indicator for HCC prognosis and patients with high AFP levels have been reported to have shorter survival.[24] In addition, SMAD4 is significantly associated with fibrosis ($P < 0.01$) (Supplementary Fig. 1). Liver fibrosis is in turn strongly correlated with HCC development.[25] Furthermore, nuclear SMAD4 level was significantly higher in undifferentiated tumor than in well differentiated tumor of HCC (2.53 ± 0.23 *vs* 1.94 ± 0.11, mean ± s.e.m, n = 127, $P < 0.05$) (Fig. 1D). Interestingly, SMAD4 is also significantly correlated with the positivity of SALL4, an oncofetal protein (Supplementary Fig. 2). It was recently identified as a marker of a subtype of HCC with progenitor-like features. It is associated with a poor prognosis and is a potential target for treatment.[26, 27]

Importantly though, apparently high SMAD4 positivity in surgically resected HCC (n = 130 analyzable patients) tend to have higher risk of fast recurrence (Hazard Ratio (HR) = 1.377, 95% CI: 0.755-2.594) and higher risk of poor survival (HR = 1.766, 95% CI: 0.915-3.362) (Fig. 1E & 1F). Kaplan Meier analysis (n = 130 analyzable patients) also indicated a trend of shorter time to recurrence and lower cumulative survival in high SMAD4 level patients, although not statistically significant (Fig. 1E & 1F). We interpreted that higher nuclear SMAD4 levels may be associated with more aggressive types of tumors in HCC patients.

Silencing of SMAD4 expression reduced colony formation in human hepatoma cell lines

In order to obtain an insight into the mechanisms possibly mediating the negative relation between SMAD4 signaling and HCC clinical behavior, we employed lentiviral RNAi vectors expressing shRNA (sh-SMAD4) to stably knockdown SMAD4 expression in human HCC cell lines and subsequently characterized the cellular consequences thereof. Supplementary figure 3 showed the efficacy of gene silencing using this strategy. A vector expressing shRNA targeting GFP served as control (CTR). The success of this approach was confirmed by western blot and probing for SMAD4 protein (Fig. 2A), which showed almost absence of the

protein in the knockdown cell lines, whereas the control cell lines remain SMAD4 proficient. Using immunofluorescent staining, it has confirmed the efficiency of SMAD4 knockdown in both Huh7 and BEL-7404 cell lines. But BEL-7404 cells have much stronger nuclear SMAD4 level than Huh7 cells (Fig. 2B), suggesting that SMAD4 is more active in BEL-7404 cells.

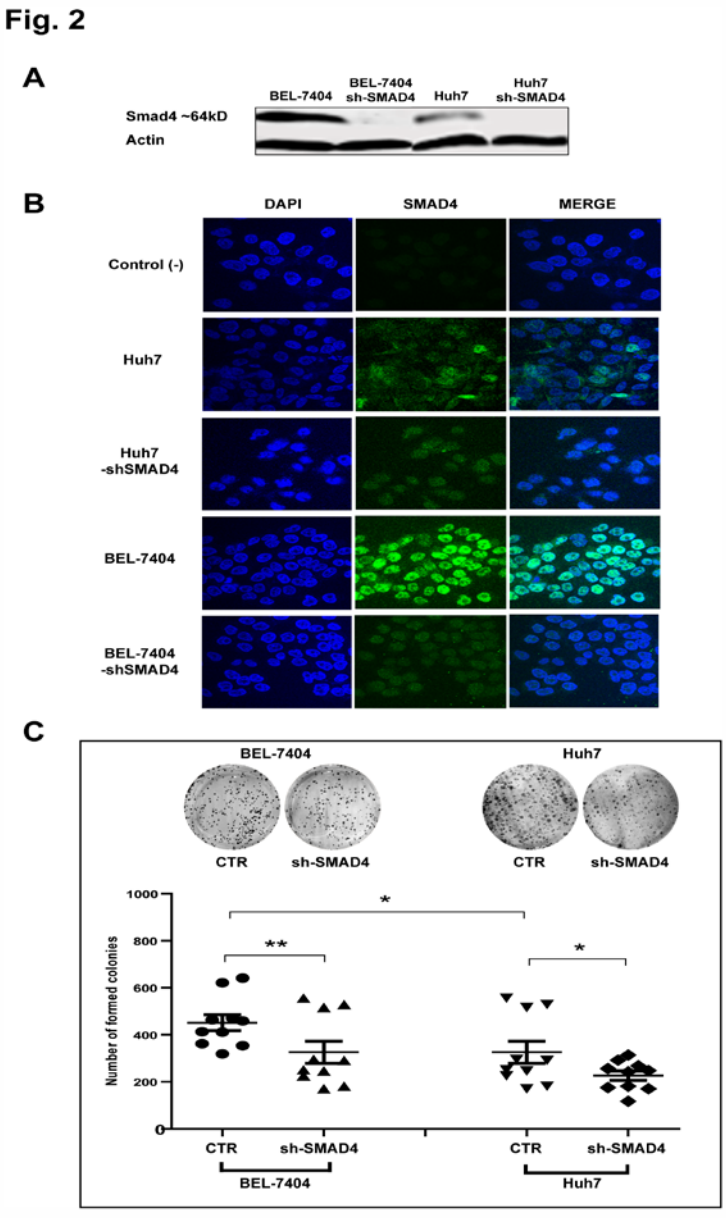


Fig. 2. Decreased efficiency of colony formation in SMAD4 knockdown HCC cells. (A) Successful knockdown of SMAD4 in BEL-7404 and Huh7 cell lines was first confirmed on protein levels by western blot. (B) Immunofluorescent staining confirmed the efficacy of SMAD4 knockdown. BEL-7404 cells have much stronger nuclear SMAD4 level compared to Huh7 cells. (C). BEL-7404 cells are significantly more efficient in forming colony than Huh7 cells. A significant decrease of the numbers of formed colonies was observed in BEL-7404 cells with SMAD4 knockdown (sh-SMAD4), compared with mock knockdown (CTR). Error bars represent mean ± s.d, n = 10, *t*-test, $^{**}P < 0.01$. Similar results were observed in Huh7 cells (n = 10), *t*-test, $^{*}P < 0.05$.

Colony formation assay is a robust tool to evaluate the ability of a single cell to support proliferation. Employing this assay, we observed that BEL-7404 cells are more efficient in colony formation than Huh7 cells, which is consistent with their higher nuclear SMAD4 levels (Fig. 2C). Furthermore, a significant decrease of the numbers of formed colonies was detected in BEL-7404 cells with SMAD4 knockdown compared with the mock cells (CTR *vs* sh-SMAD4: 565.7 ± 117.4 *vs*. 451.5 ± 107.2 colonies/1000 cells, mean ± s.d, n = 10, $P < 0.01$) (Fig. 2C). Similar results were observed in Huh7 cells (CTR *vs* sh-SMAD4: 326 ± 149 *vs* 226.3 ± 62.5 colonies/1000 cells, mean ± s.d, n = 10, $P < 0.05$) (Fig. 2C). Thus, in contrast to most other cell types where SMAD4 expression is associated with reduced cancer growth, SMAD4 expression supports proliferation of HCC cells.

Knockdown of SMAD4 attenuated the ability of HCC cell migration

Cell migration is a fundamental function underlying cellular processes including invasion or metastasis of cancer cells. We thus investigated the role of SMAD4 in migration of HCC cells using a ring-barrier system. Silencing of SMAD4 expression resulted in attenuated migratory capacity towards the cell free area both in BEL-7404 and Huh7 cells. In BEL-7404 cells with SMAD4 knockdown, significant reduction was observed in total migration (CTR *vs* sh-SMAD4: 97 ± 28.4 *vs* 77 ± 21.2 µm, mean ± s.d, n = 30, $P < 0.001$), effective migration (CTR *vs* sh-SMAD4: 46.8 ± 12.2 *vs* 33.7 ± 11.6 µm, mean ± s.d, n = 30, $P < 0.001$), and migration velocity (CTR *vs* sh-SMAD4: 4.0 ± 1.2 *vs* 3.2 ± 0.9 µm/hour, mean ± s.d, n = 30, $P < 0.001$) (Fig. 3A).

In Huh7 cells with SMAD4 knockdown, quantification revealed a significant reduction in total migration (CTR *vs* sh-SMAD4: 174.1 ± 54.3 µm *vs* 128.7 ± 42.1 µm, mean ± s.d, n = 30, $P < 0.01$), effective migration (CTR *vs* sh-SMAD4: 109.1 ± 33.2 µm *vs* 55.4 ± 22.4 µm, mean ± s.d, n = 30, $P < 0.001$), migration efficiency (CTR *vs* sh-SMAD4: 63.60 ± 9.60% *vs* 43.95 ± 16.62% mean ± s.d, n = 30, $P < 0.0001$) and migration velocity (CTR *vs* sh-SMAD4: 7.3 ± 2.3 *vs* 5.4 ± 1.8 µm/hour, mean ± s.d, n = 30, $P < 0.001$) (Fig. 3B). These results indicate that SMAD4 in HCC cells supports migration and in conjunction with the colony formation data support the notion of a non-canonical pro-oncogenic function of SMAD4 in HCC.

Fig. 3

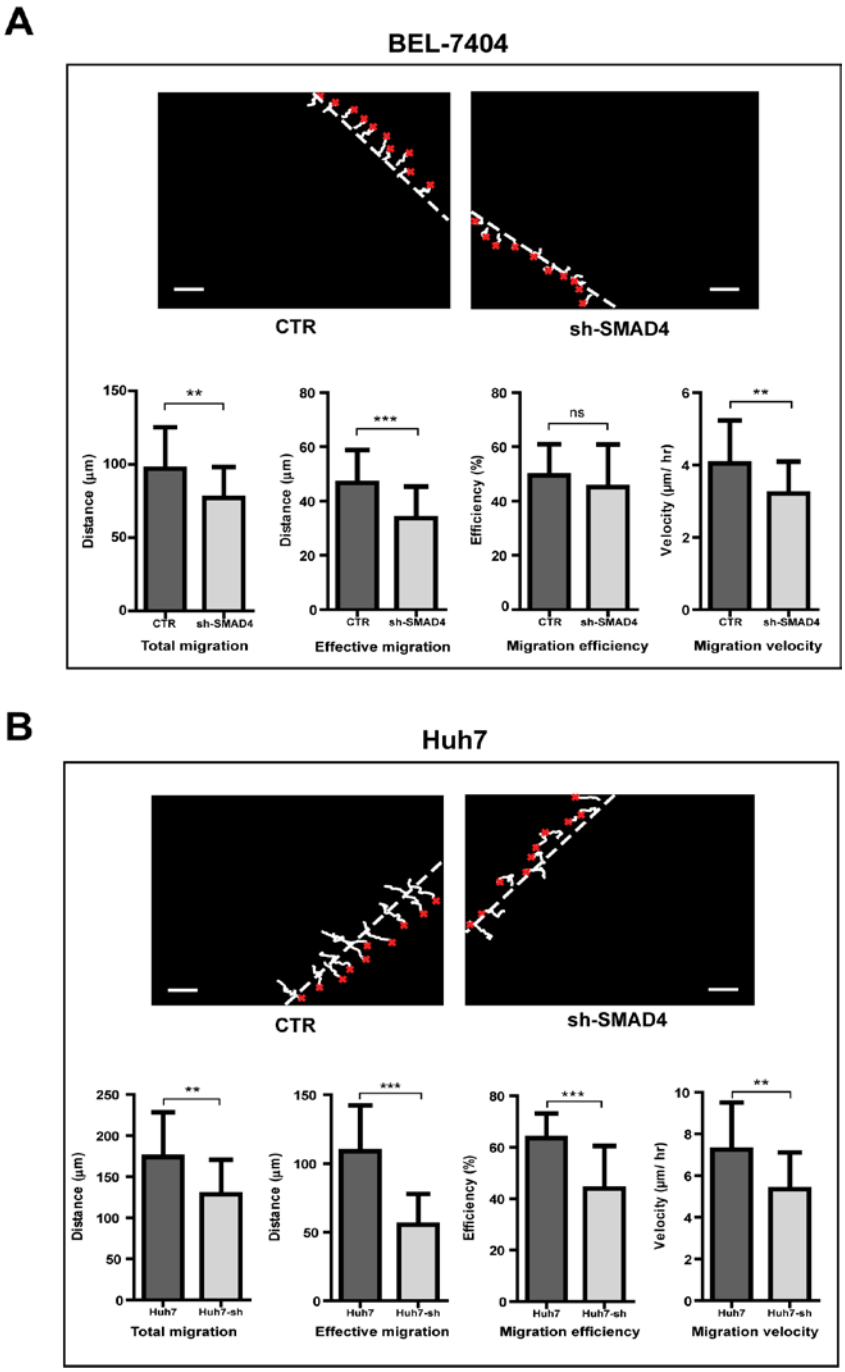


Fig. 3. Silencing of SMAD4 inhibited HCC cell migration. (A) In BEL-7404 cells with SMAD4 knockdown, significant reduction was observed in total migration, effective migration and migration velocity in 24 hours whereas (B) in Huh7 cells with SMAD4 knockdown, quantification revealed a significant reduce in total migration, effective migration, migration efficiency and migration velocity in 24 hours. Error bars represent mean ± s.d from n = 30, Mann Whitney test, **P* < 0.05, ***P* < 0.01, *** *P* < 0.001, ns: not significant. Scale bar, 100 µm.

Silencing of SMAD4 limited hepatoma initiation and growth in mice

To finally ensure the tumor promoting effects of SMAD4, we evaluated the impact of SMAD4 loss on tumor initiation and growth in nude mice. One million CTR and SMAD4 knockdown cells were subcutaneously injected into the left or right side of the mice, respectively.

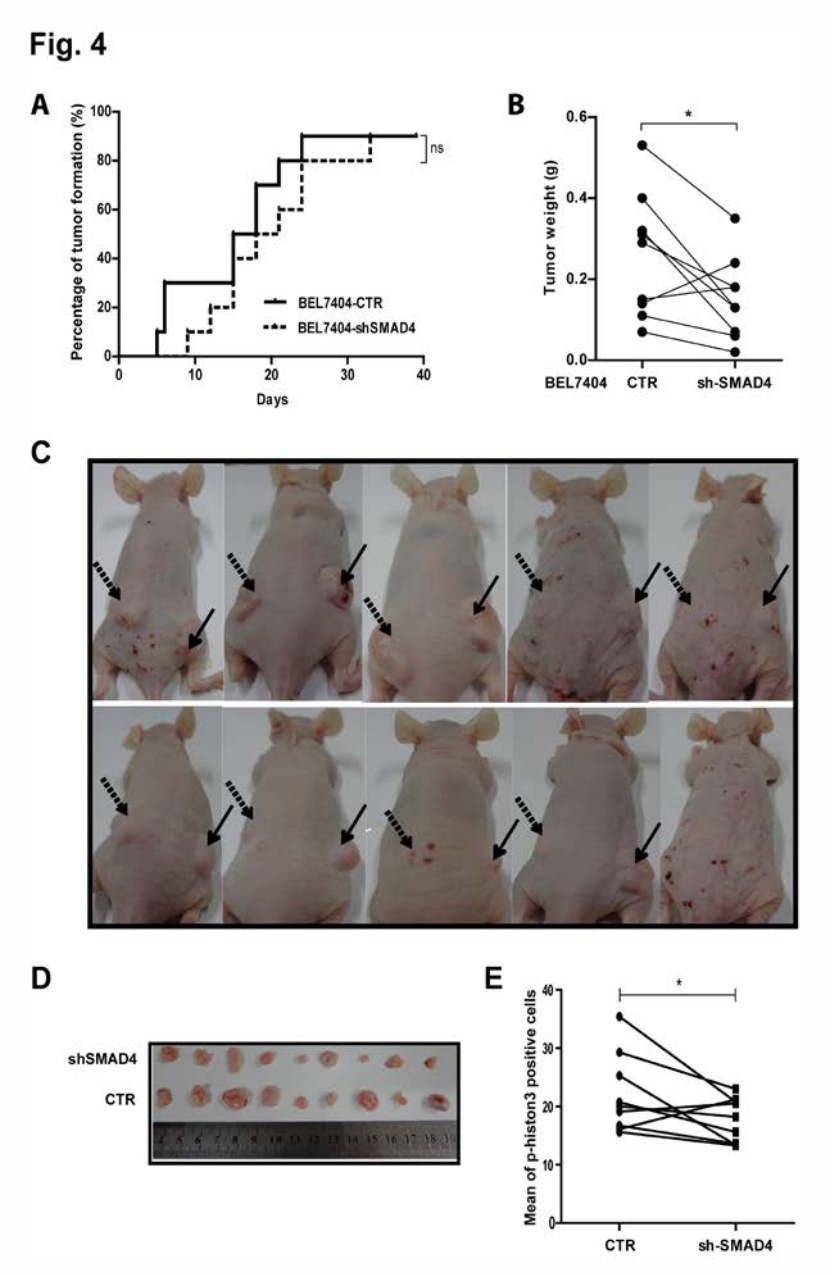


Fig. 4. Knockdown of SMAD4 inhibited growth of BEL-7404 cell formed tumors in nude mice. (A) No significant difference regarding the time required for tumor initiation between control cells and SMAD4 knockdown BEL-7404 cells. ns: not significant. (B) The weight of formed tumor in SMAD4 knockdown cells was significantly lower than in control cells (n = 10), paired *t*-test, $*P < 0.05$ (C). The solid arrows indicate the tumors formed by control cells and the dashed arrows indicate SMAD4 knockdown BEL-7404 cells formed tumor. (D) The appearance of formed tumors in centimeter length. (E) Immunohistochemistry staining for a proliferation marker p-histone H3 demonstrated a significant reduction of proliferating cells in tumors formed by SMAD4 knockdown cells (n = 9, paired *t*-test, $*P < 0.05$).

As shown in figure 4A, there was no significant difference regarding the time required for tumor initiation between CTR and SMAD4 knockdown BEL-7404 cells. However, the weight of formed tumor in SMAD4 knockdown cells was significantly lower than those formed by CTR cells (CTR vs sh-SMAD4: 0.25 ± 0.05g vs 0.15 ± 0.03g, mean ± s.e.m, n = 10, P < 0.05) (Fig. 4B, 4C and 4D). Immunohistochemistry staining for a proliferation marker p-histone H3 in these tumors confirmed a significant reduction of proliferating cells in tumors formed by SMAD4 knockdown BEL-7404 cells (n = 9, *P* < 0.05) (Fig. 4E).

Fig. 5

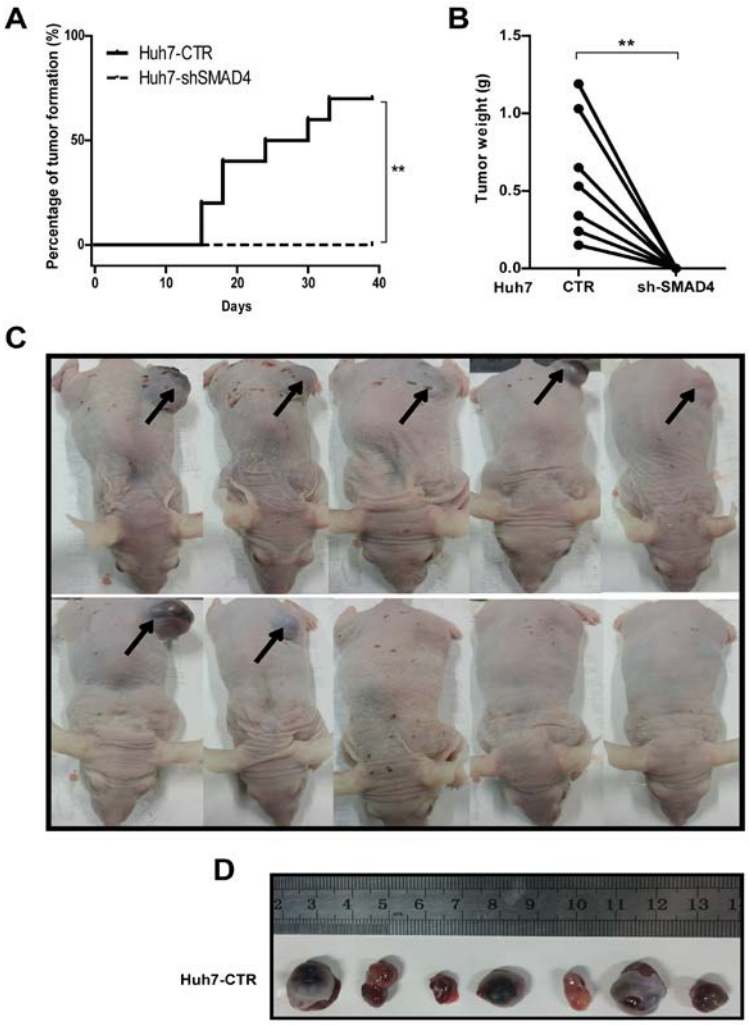


Fig. 5. Knockdown of SMAD4 in Huh7 cells failed to initiate tumor in nude mice. (A) Knockdown of SMAD in Huh7 significantly abolished the tumor formation, whereas 7 out of 10 mice in the control group formed tumors (paired *t*-test, ***P* < 0.01) (B) The weight of formed tumor. (C) The solid arrows indicate Huh7 in control group formed tumors in mice. (D) The appearance of formed tumors in centimeter length.

Most impressively, knockdown of SMAD in Huh7 cells resulted in complete abolishment of tumor formation, whereas 7 out of 10 mice in the CTR group formed tumor (weight: 0.59 ± 0.15g, mean ± s.e.m, n = 7) (Fig. 5). Collectively, these results are in line with the outcomes of our *in vitro* experimentation and the observation that high SMAD4 expression in human HCC tissue is associated with worse prognoses firmly demonstrates that SMAD4 exerts a tumor promoting role in HCC.

Simultaneous elevation of SMAD4 and phosphorylated SMAD2/3 is significantly associated with poor patient outcome

Upon binding of the cognate ligands to the TGFB receptor, phosphorylated SMAD2/3 (p-SMAD2/3) binds to SMAD4 to form heteromeric complex, translocate to the nucleus and activate TGFB signaling.[28] We thus performed immunohistochemistry staining of p-SMAD2/3 in the TMA that was used for SMAD4 staining (n = 140). The levels of p-SMAD2/3 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites (Fig. 6A).

The patient groups (low, moderate or high) is categorized according to expression levels in the tumors. Although no significant difference overall (n = 140), p-SMAD2/3 levels were significantly lower in HCC tissue as compared to normal adjacent liver tissue in patients with low to moderate scores (n = 86, $P < 0.001$, data not shown); whereas it is significantly higher in the tumor of in patients with high scores (n = 54, $P < 0.001$) (Fig. 6B). No statistically significant relation with p-SMAD2/3 levels was observed regarding to the size (n = 98), the differentiation stage (n = 127) or the number of tumor foci (n = 129) (Supplementary Table 2). However, high p-SMAD2/3 expression in tumor is significantly associated with high recurrence rate (n = 47, $P < 0.05$) and patient death rate (n = 44, $P < 0.05$) (Supplementary Table 2). Moreover, cox regression analysis (n =130) indicated that patients with high level of p-SMAD2/3 may have higher risk of fast recurrence (HR = 1.649, 95% CI: 1.008-3.174) and higher risk to poor survival (HR = 1.633, 95% CI: 0.856-3.116) (Fig. 6C & 6D). Kaplan Meier analysis (n = 130) also revealed a tendency of shorter time to recurrence and a trend to less cumulative survival in patients with high levels of p-SMAD2/3 in the tumor (Fig. 6C & 6D).

Fig. 6

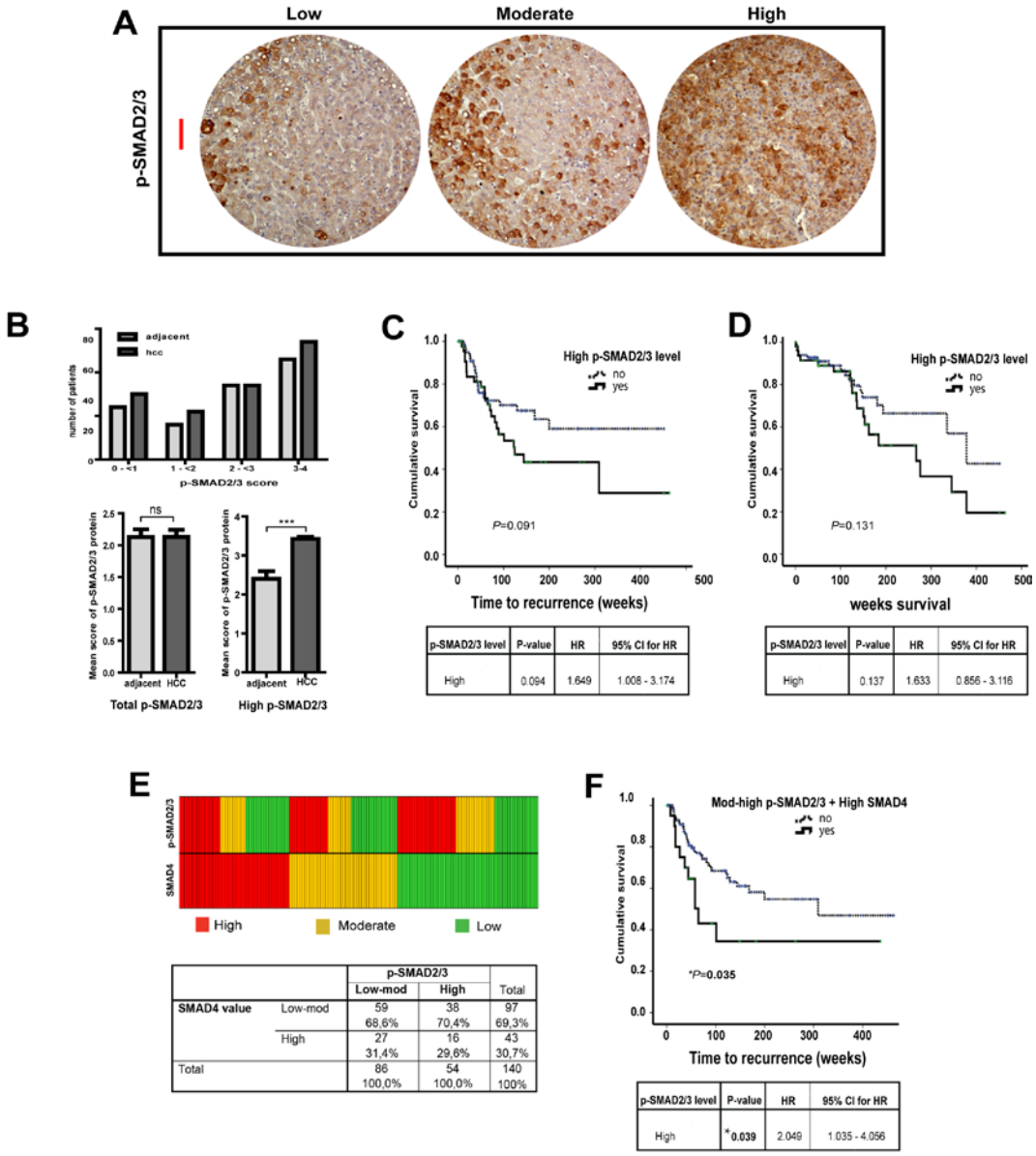


Fig. 6. Simultaneous elevation of p-SMAD2/3 and SMAD4 is significantly associated with poor clinical outcome in HCC patients. (A) The levels of p-SMAD2/3 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (B) There were more patients with higher p-SMAD2/3 score both in tumor and adjacent sites. No significant overall difference of p-SMAD2/3 expression between HCC tissue and normal adjacent liver tissue. Nevertheless, in the high grade patients group, p-SMAD2/3 expression was significantly higher in HCC tissues compared to adjacent sites (n = 54). Error bars represents mean ± s.e.m, paired *t*-test, *** *P*<0.001. From cox regression analysis (n = 130), high levels of p-SMAD2/3 tend to have higher risk of fast recurrence (HR = 1.649) (C) and tend to have higher risk of poor survival (HR = 1.633) (D). Kaplan Meier analysis (n = 130) confirmed a trend of shorter time to disease recurrence (C) and also a trend of less cumulative survival (D). (E) 22 out of 140 patients have simultaneously sufficient levels of both p-SMAD2/3 (n = 16 high; n = 6 moderate levels) and SMAD4. (F) These patients have significantly poor clinical outcome as shown by both cox regression and Kaplan Meier analysis. * $P < 0.05$.

As a phosphorylated protein, moderate levels of p-SMAD2/3 would be expected to be already sufficient to trigger the downstream signaling transduction in the presence of SMAD4. A sub-population (n = 22) HCC patients have a simultaneous elevation of SMAD4 and p-SMAD2/3, which represents as a hallmark for the activation of the downstream signaling of TGFB (Fig. 6E). Cox regression analysis revealed that these patients have extremely higher risk of fast recurrence (progression) (HR = 2.049, 95% CI: 1.035-4.056) (Fig. 6E). Kaplan Meier analysis also confirmed that these patients are significantly faster to disease recurrence ($P < 0.05$) (Fig. 6E). These results indicate that SMAD4 together with p-SMAD2/3 exerts a tumor promoting function in HCC patients.

An anti-tumor signaling mediated by phosphorylated SMAD1/5/8 and SMAD4 is inactivated in majority of HCC patients

Upon binding of BMP ligands, phosphorylated SMAD1/5/8 (p-SMAD1/5/8) binds to SMAD4 to form heteromeric complex, translocate to the nucleus and activate BMP signaling.[28] Although the exact role of BMP signaling in cancer is highly context-dependent, a recent study demonstrated that BMP4, a BMP ligand, inhibited the tumorigenic capacity of HCC cells.[29] We further examined the effects of BMP4 on HCC cells. In Huh7 cells, BMP4 significantly reduces colony formation ability of Huh7 cells and knockdown of SMAD4 attenuated the effects of BMP4. The efficiency of colony formation was reduced by BMP4 treatment in CTR cells by 47.02 ± 6.5% but only by 25.3 ± 6.4% in SMAD4 knockdown Huh7 cells (mean ± s.d, n = 4, $P < 0.01$) (Fig. 7A). Consistently, adding BMP inhibitor Noggin appears to increase the efficiency of colony formation in CTR cells (124.5 ± 19.1%, mean ± s.d, n = 4) but has much less effect (109.6 ± 9.5%, mean ± s.d, n = 4) in SMAD4 knockdown Huh7 cells (Fig. 7B). Thus BMP4 significantly reduced the colony formation ability of hepatoma cells, which was consistent with previous reports in other cancer,[30-32] and knockdown of SMAD4 attenuated the effects of BMP4. Western blot analysis showed the effects of BMP4 and Noggin on the protein levels of SMAD4, p-SMAD2/3 and p-SMAD1/5/8.

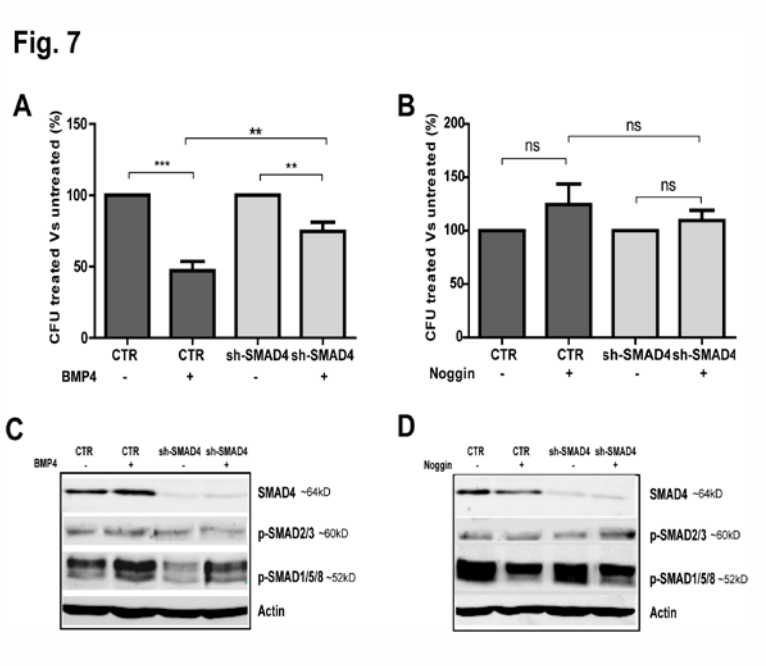
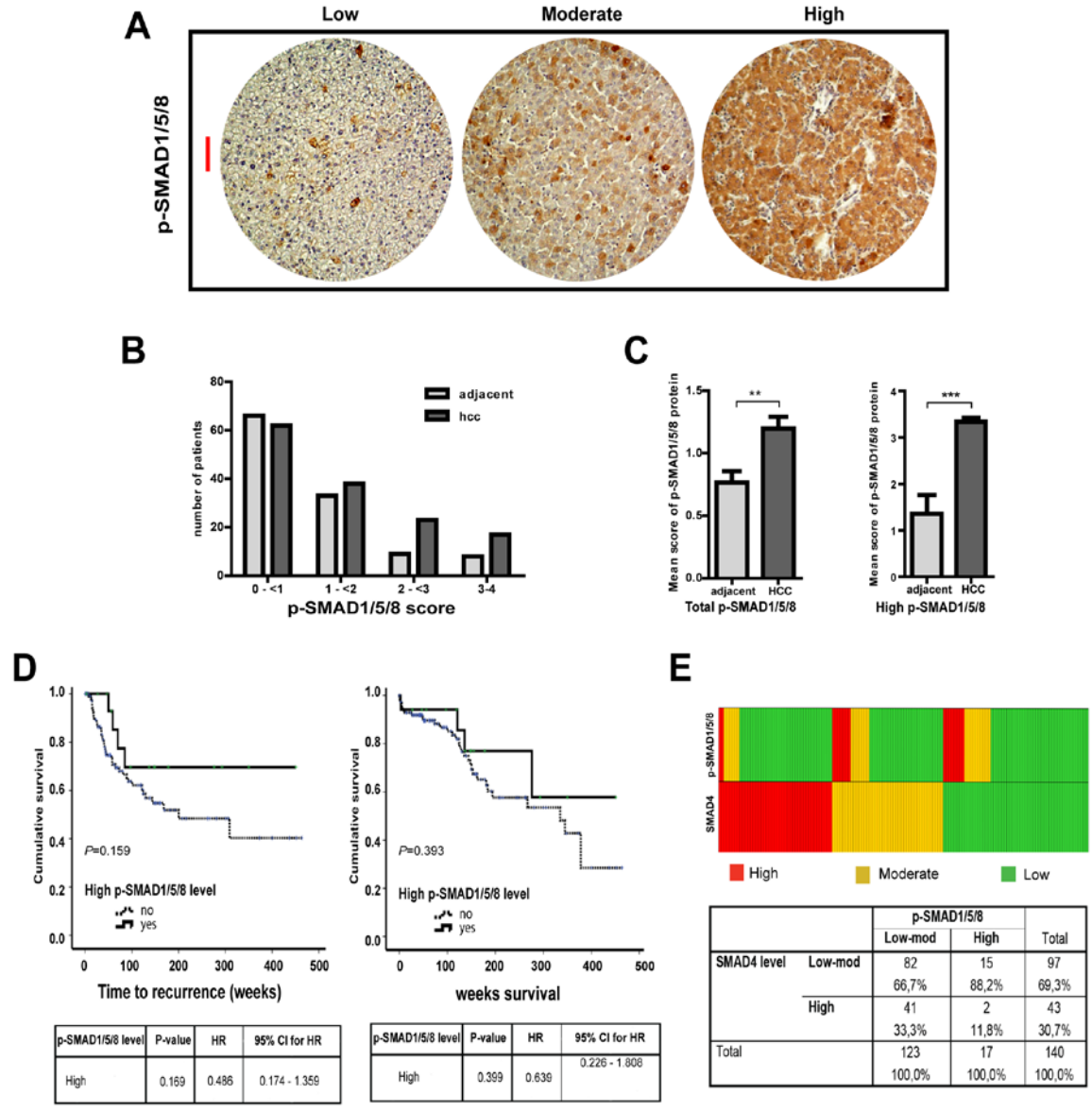


Fig. 7. BMP4 significantly reduced the colony formation ability of HCC cells and its ability was attenuated by silencing SMAD4. (A) The efficiency of decreasing colony formation by BMP4 treatment was significantly reduced in Huh7 cells with SMAD4 knockdown. Error bars represent mean ± s.d from n = 4, paired *t*-test, $**P < 0.01$. (B) Although the difference was not statistically significant, adding BMP inhibitor Noggin appeared to increase the efficiency of colony formation in control cells and to less extend in SMAD4 knockdown Huh7 cells. Error bars represent mean ± s.d from n = 4, paired *t*-test, ns: not significant. (C) Protein levels of SMAD4, phospho-SMAD2/3 and phospho-SMAD1/5/8 after BMP4 treatment and (D) protein levels of SMAD4, phospho-SMAD2/3 and phospho-SMAD1/5/8 after Noggin treatment.

This was in broad agreement with the efficacy of the experimental strategy but also suggested the existence of SMAD4-dependent feedback loops on BMP signaling elements (Fig. 7C & 7D). Our results confirm that activation of BMP signaling, which involves both SMAD4 and p-SMAD1/5/8, exerts anti-HCC effects.

Next, we further explored the role of this pathway in our HCC cohort. Immunohistochemistry staining of p-SMAD1/5/8 was performed in the TMA (n = 140), and was scored and categorized as described for SMAD4 and p-SMAD2/3 (Fig. 8A & B). Although p-SMAD1/5/8 is significantly higher in the tumor tissue compared to adjacent liver tissue (Fig. 8C), only a small subset of patients have high levels of p-SMAD1/5/8 in the tumor (17 out of 140, see Supplementary Table 3).

Fig. 8



		p-SMAD1/5/8		
		Low-mod	High	Total
SMAD4 level	Low-mod	82	15	97
		66,7%	88,2%	69,3%
	High	41	2	43
		33,3%	11,8%	30,7%
Total		123	17	140
		100,0%	100,0%	100,0%

Fig. 8. The anti-tumor signaling mediated by p-SMAD1/5/8 and SMAD is inactivated in most of the HCC patients. (A) The levels of p-SMAD1/5/8 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (B) Different to SMAD4 or p-SMAD2/3, there were less patients with high p-SMAD1/5/8 score both in tumor and adjacent sites, although (C) overall p-SMAD1/5/8 expression was significantly higher in HCC tissue compared to adjacent liver tissue. The p-SMAD1/5/8 levels were also significantly higher in HCC tissue compared to adjacent tissue in the high grade (n = 17) group. Error bars represents mean ± s.e.m, paired *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$. (D) From cox regression analysis (n = 130), patients with high level of p-SMAD15/8 tend to have less risk of fast recurrence (HR = 0.486) and less risk to poor survival (HR = 0.639). Kaplan Meier analysis (n = 130) showed similar trends. (E). However, there are only 8 patients have simultaneously sufficient levels of both SMAD4 and p-SMAD1/5/8 (n = 2 high; n = 6 moderate levels), suggesting that this signaling is inactivated in most of the HCC patients.

No significant relation was observed regarding to the size (n = 98) and the number of tumor foci (n = 129) (Supplementary Table 3). Interestingly, a Bonferroni-corrected clinical parameter analysis revealed a negative correlation between tumor p-SMAD1/5/8 level and age (Supplementary Table 3). Patients with high levels of p-SMAD1/5/8 appear to have lower risk of fast recurrence (HR = 0.486, 95% CI: 0.171-1.359) and lower risk to poor survival (HR = 0.639, 95% CI: 0.226-1.808) (Fig. 8D). Kaplan Meier analysis also revealed a trend of longer time to recurrence and higher cumulative survival in these patients (Fig. 8D).

As a phosphorylated protein, p-SMAD1/5/8 could sensitively control the downstream signaling transduction. Since the anti-tumor function of this signaling requires both SMAD4 and p-SMAD1/5/8, we further categorized the expression levels of both proteins in the same patients. As shown in figure 8E, there are only 8 patients having simultaneously sufficient levels of both SMAD4 and p-SMAD1/5/8 (n = 2 high; n = 6 moderate levels). These results suggest that SMAD4 and p-SMAD1/5/8 mediated anti-tumor signaling is inactivated in majority of our HCC patients.

Discussion

In this study, we reported a drastic elevation of nuclear SMAD4 expression in tumors of subset of HCC patients. High expression of SMAD4 was further demonstrated to be functionally important for hepatoma formation and progression. Importantly, simultaneous elevation of SMAD4 and p-SMAD2/3 in sub-population of HCC patients significantly associated with poor outcome after surgery. Although SMAD4 coupled with p-SMAD1/5/8 can also mediate an anti-tumor effect, this signaling however is silent in majority of our HCC patients. Thus, we conclude that high SMAD4 expression has been screwed towards a tumor promoting signaling in HCC (Fig. 9). This is unexpected in view of the dogma that SMAD4 is a potent tumor suppressor.

SMAD4 was initially described in pancreatic cancer, named DPC4 (deleted in pancreatic carcinoma, locus 4), and appears critical in pancreatic cancer progression.[33, 34] *SMAD4* loss occurs in 40–50% of colon cancers,[35] which is associated with metastasis, advanced disease and reduced survival. Similarly, its loss in cholangiocarcinoma[36] or prostate cancer[8] is also related to more progressive disease. In head-and-neck cancer and squamous cell carcinoma of esophageal, skin or mammary glands, however, loss of *SMAD4* promotes both tumor initiation and disease progression.[37] The tumor suppressor function of SMAD4 is often closely linked to its capacity to mediate TGFB and BMP signals. However, we question whether activation or silencing of TGFB/BMP downstream components, including SMADs, is always ligand dependent in cancer? Because in xenografts of human hepatoma cell lines in mice, which are thus unlikely to encounter their (human) ligands, we observed that high expression of SMAD4 is even required for tumor formation and growth. In contrast to our observation, a previous study has reported a lower protein level of SMAD4 in HCC tissue compared with adjacent liver tissue in an Asian cohort.[38] A possible explanation could be that the etiologies of HCC may influence the expression of SMAD4. In Asia, viral hepatitis is the main cause of HCC; whereas only less than 30% of patients in our European cohort have viral hepatitis history. In addition, technical differences, including the source of antibody and the protocol of immunohistochemical staining, may also result in discrepancy. In this study, we have used a robust staining protocol for SMAD4 (see Methods section) that was optimized and established in our previous studies.[39, 40]

The essential role of TGFB/BMP signaling in cancer is certainly well-documented, whereas its exact functions are also context dependent.[41] TGFB1 was also well-recognized for its dual role in carcinogenesis.[42] It acts as a tumor suppressor in early stages of hepatocarcinogenesis by inducing apoptosis[43] and at a later stage, however, liver tumor cells often become resistant to its pro-apoptotic effect, and produce large amounts of TGFB themselves.[44] Using genomic approaches, previous studies have established an early and a late TGFB signatures that can discriminate distinct subgroups of HCC. Late TGFB gene signature displays more invasive phenotype and increased tumor recurrence.[45] This signature also apply in HCC cell lines and Huh7 was identified to have early TGFB signature[45] and is associated with response to TGFB cytostatic and cytotoxic effects (Supplementary Fig.

4). This inhibitory effect of TGFB is SMAD4 dependent (Supplementary Fig. 4). In our HCC cohort, a subset of patients have simultaneous elevation of SMAD4 and p-SMAD2/3 (Supplementary Table 4), indicating the activation of TGFB downstream signaling. These patients however have a significant worse outcome after surgery, confirming an tumor promoting function of TGFB signaling in these HCC patients. These results suggest that our HCC patients probably have a late TGFB gene signature, although we do not have the right format of patient materials stored for experimental examination of this signature.

Several distinct BMP ligands were reported to act together to promote the migratory and invasive potential of cancer cells,[46] including in HCC.[47] In contrast, a recent study demonstrated that BMP4 induced differentiation of HCC cancer stem cells and inhibited their tumorigenic capacity.[29] Our *in vitro* study indicated activating BMP signaling by adding BMP4 ligand in HCC was able to effectively suppress colony formation of HCC cells, which was consistent with previous reports in other cancer.[30-32] However, silencing of *SMAD4* gene attenuated this effect, confirming that these anti-oncogenic actions require basal levels of SMAD4. Despite an anti-tumor effects of BMP pathway, this signaling however is silent in majority of our HCC patients, by losing the key components, either SMAD4 or p-SMAD1/5/8, or both of them. The obvious implication of this observation is that HCC cells should prove exquisitely sensitive to stimulation with BMP ligands mediating such signaling. In conjunction with the recent FDA approval of BMP2 and BMP7 as treatment for certain bone pathologies.[48] However, we have to be cautious that there are also studies reporting pro-oncogenic roles of BMP ligands in particular settings. For instance, BMP7 and BMP9 have been shown to have tumor promoting functions in some experimental cancer (including HCC) models.[47, 49, 50] Nevertheless, our results call for further study exploiting this Achilles' heel of HCC.

In summary, this study reports a significant elevation of nuclear SMAD4 expression in patient HCC tumors. High SMAD4 expression has been screwed towards tumor promoting effects due to simultaneous elevation of p-SMAD2/3 in subset of patients. SMAD4 can also mediate an anti-tumor signaling by coupling p-SMAD1/5/8, this complex however is absent in majority of patients due to lack of either SMAD4 or p-SMAD1/5/8, or both of them. These

results have certainly shed new light on the molecular biology of HCC and more importantly SMADs-based molecules may have potential as outcome predictors for patient stratification.

Fig. 9

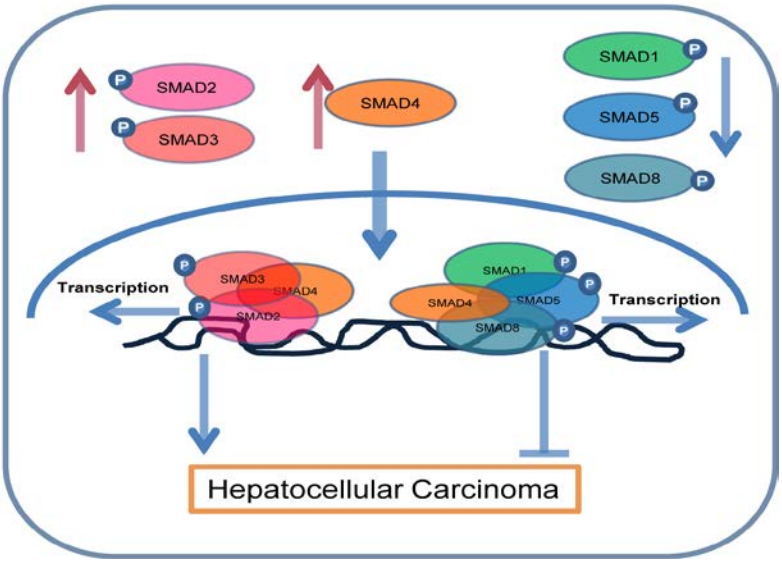


Fig. 9. The model of action of SMADs in HCC. SMAD4 and p-SMAD2/3 are elevated in subset of patients that mediate a tumor promoting effect. Although SMAD4 together with p-SMAD1/5/8 can also exert an anti-tumor effect, this complex however is absent in majority patients, due to missing of either SMAD4 or p-SMAD1/5/8, or both of them.

SUPPLEMENTARY MATERIAL

Supplementary Figure 1

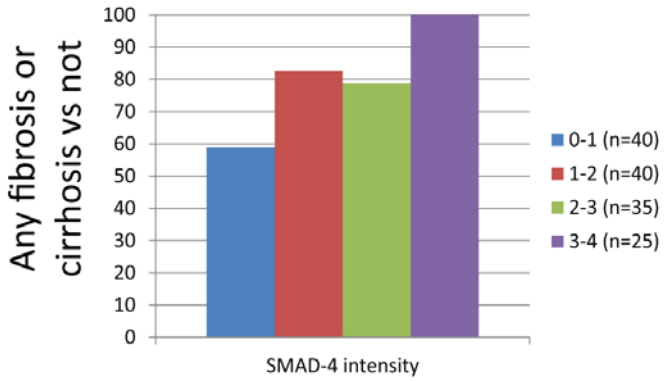


Fig. S1. SMAD4 is significantly associated with liver fibrosis. Score 0 for 0-10% positive staining, score 1 for 10-30% positive staining, score 2 for 30-70% positive staining, score 3 for >70% positive staining, and score 4 for >70% positive staining. 0-1 represents any score from 0 to (including) 1; 1-2 represents any score higher than 1 and up to 2; 2-3 represents any score higher than 2 and up to 3; 3-4 represents any score higher than 3 and up to 4. $P < 0.01$; Chi-Square test.

Supplementary Figure 2

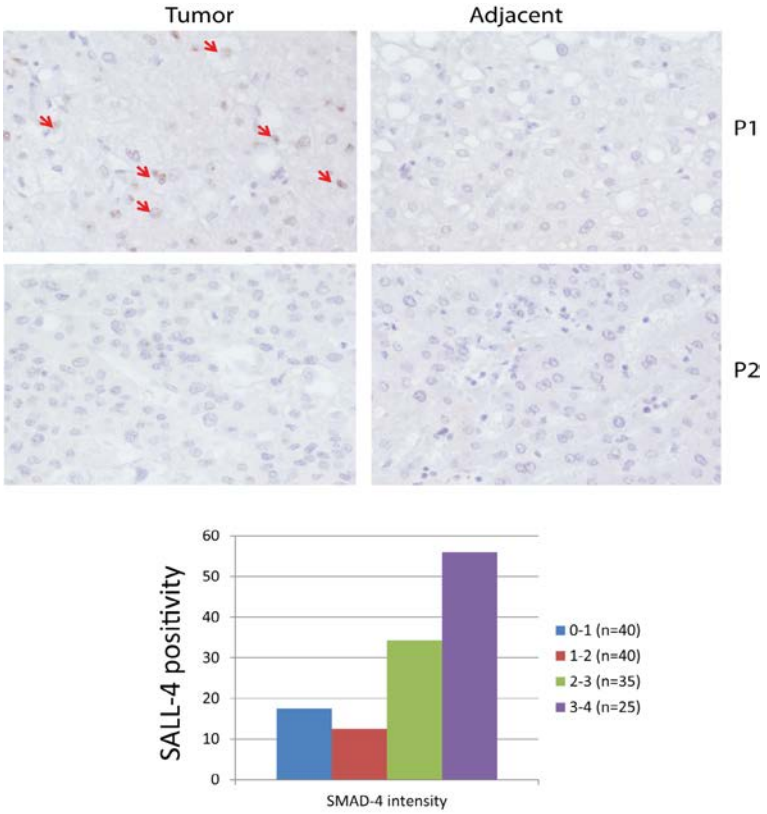


Fig. S2. SMAD4 is significantly correlated with the positivity of SALL4, an oncofetal protein. SALL4 is negative in adjacent liver tissue, but is positive in the HCC tumors of sub-population of patients. P1 represents a patient with positive SALL4 in the tumor and P2 represents a patient with negative SALL4 in the tumor. $P < 0.01$; Chi-Square test.

Supplementary Figure 3

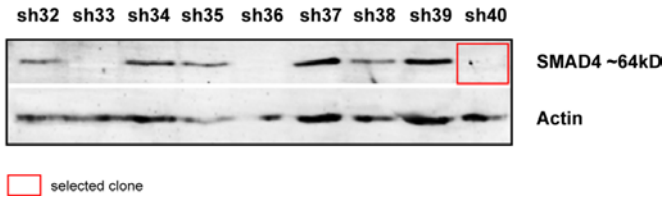


Fig. S3. Selection of optimal lentiviral shRNA vectors for targeting SMAD4. Western blotting was used to evaluated the efficacy of SMAD4 knockdown in Huh7 cells. Sh40 was selected for follow-up experimentation.

Supplementary Figure 4

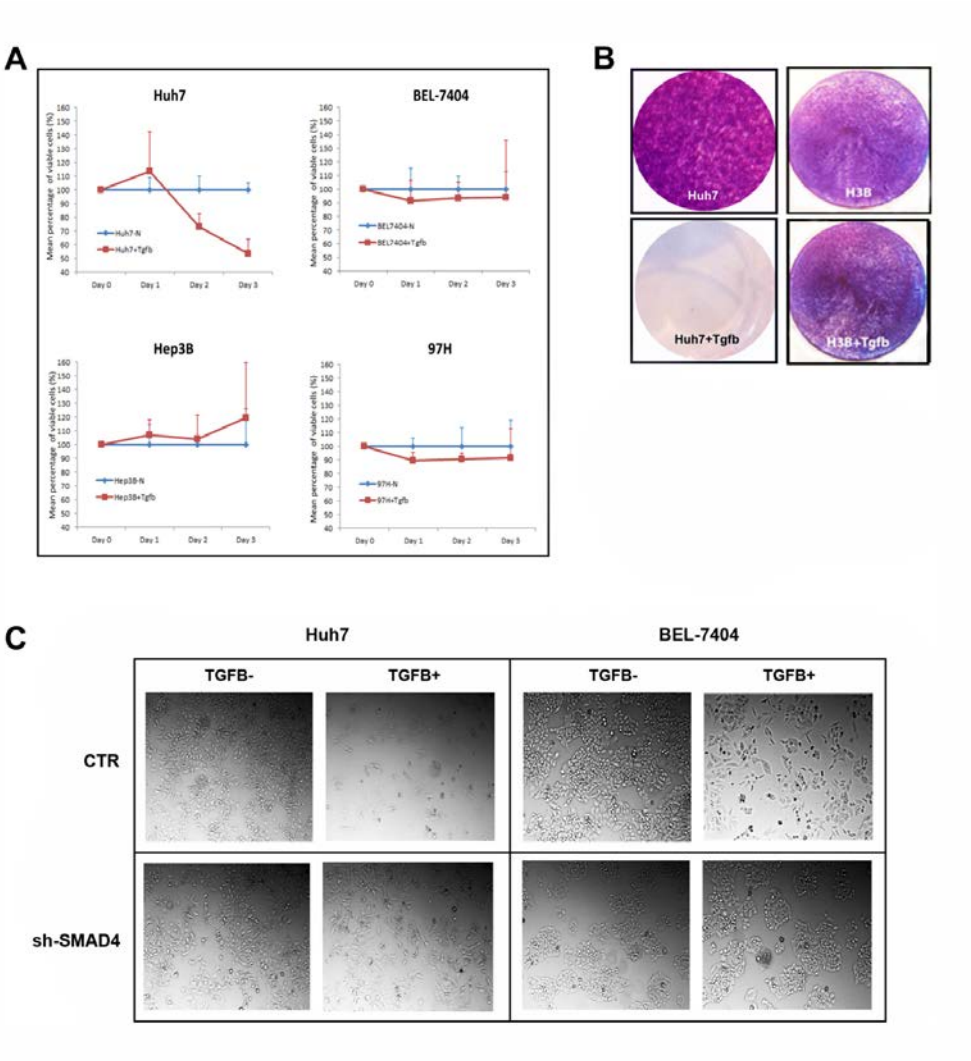


Fig. S4. The diverse effects of TGF-b on different HCC cell lines. (A) The effects on HCC cell proliferation/viability was determined by MTT assay. Treatment with TGF-b (5 ng/ml) resulted in potent inhibitory effects on Huh7 cells, but only minor effect on BEL-7404 and 97H cells, even slightly promoted growth of Hep3B cells. Data presented as Mean ± SD, n = 3 independent experiments with 7 replicates in total. (B) One week treatment of TGF-b (2 ng/ml) confirmed the differential effects on Huh7 and Hep3B cells. (C). TGF-b (5 ng/ml, for 5 days) treatment inhibited cell growth but also altered their morphology. Knockdown of SMAD4 attenuated the inhibitory effects of TGF-b.

Supplementary Table 1 Patient characteristics according to SMAD4 expression level.

No	Characteristics	SMAD4 expression		Total patients	*P*-value[a]
		Low-mod	High		
1	Age	60.70 ± 15.62	59.88 ± 12.61	140/140	0.763
2	Sex (% male)	68/97 (70.1%)	29/43 (67.4%)	97/140	0.753
3	Recurrence	32/97 (33,0%)	15/43 (34.9%)	47/140	0.827
4	Death	30/97 (30,9%)	14/43 (32.6%)	44/140	0.848
5	Size of tumor	6.88 ± 0.63	6.55 ± 0.99	98/140	0.777
6	Number of lesions	1.56 ± 0.11	1.39 ± 0.17	129/140	0.413
7	Vascular invasion	54/85 (63.5%)	24/36 (66.7%)	78/140	0.742
8	AFP before resection*	5.00	16.00	135/140	0.005

* *P* value < 0.01

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table 2 Patient characteristics according to p-SMAD2/3 expression level.

No	Characteristics	p-SMAD2/3 expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age	58.71 ± 15.76	63.22 ± 12.56	140/140	0.077
2	Sex (% male)	57/86 (66.3%)	40/54 (74.1%)	97/140	0.330
3	Recurrence*	23/86 (26.7%)	24/54 (44.4%)	47/140	0.031
4	Death*	21/86 (24.4%)	23/54 (42.6%)	44/140	0.024
5	Size of tumor	6.68 ± 5.38	6.97 ± 5.13	98/140	0.796
6	Number of lesions	1.44 ± 1.00	1.6 ± 1.20	129/140	0.376
7	Vascular invasion	51/74 (68.9%)	27/47 (57.4%)	78/140	0.199
8	AFP before resection	10.00	7.00	135/140	0.077

* *P* value < 0.05

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test [a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test

Supplementary Table 3 Patient characteristics according to p-SMAD1/5/8 expression level.

No	Characteristics	p-SMAD1/5/8 expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age***	62.24 ± 11.96	47.53 ± 24.29	140/140	0.000
2	Sex (% male)	88/123 (71.5%)	9/17 (52.9%)	97/140	0.119
3	Recurrence	43/123 (35,0%)	4/17 (23.5%)	47/140	0.350
4	Death	40/123 (32.5%)	4/17 (23.5%)	44/140	0.454
5	Size of tumor	6.70 ± 5.14	7.45 ± 6.38	98/140	0.657
6	Number of lesions	1.50 ± 1.07	1.41 ± 1.23	129/140	0.708
7	Vascular invasion	70/105 (66.7%)	78/121 (64.5%)	78/140	0.194
8	AFP before resection	8.00	7.50	135/140	0.664

****P* value < 0.0001

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table 4 Patient characteristics according to high SMAD4 expression and moderate-high p-SMAD2/3 expression level.

No	Characteristics	High SMAD4 + Mod-high p-SMAD2/3 expression level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	61.04 ± 15.45	57.85 ± 10.82	140/140	0.319
2	Sex (% male)	80/114 (70.2%)	17/26 (65.4%)	97/140	0.633
3	Recurrence	35/114 (30.7%)	12/26 (46.2%)	47/140	0.132
4	Death	34/114 (29.8%)	10/26 (38.5%)	44/140	0.392
5	Size of tumor	6.85 ± 5.23	6.50 ± 5.53	98/140	0.800
6	Number of lesions	1.52 ± 1.08	1.44 ± 1.12	129/140	0.744
7	Vascular invasion	67/100 (67%)	11/21 (52.4%)	78/140	0.203
8	AFP before resection	6.50	14.00	135/140	0.136

* *P* value < 0.05

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

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Chapter 6

Does Hepatitis Virus Infection Interact with Bone Morphogenetic Protein Signaling in the Development of Hepatocellular Carcinoma ?

Pratika Y. Hernanda[1,2#], Xinying Zhou[1#], Lei Xu[1#], Kostandinos Sideras[1], Jaap Kwekkeboom[1], Maikel P. Peppelenbosch[1], Qiuwei Pan[1]

[1]Department of Gastroenterology and Hepatology, Erasmus MC Cancer Institute, Erasmus University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, The Netherlands. [2]Laboratory of Medical Genetics, Biomolecular Research Centre, Wijaya Kusuma University, Surabaya, Indonesia

In preparation

Abstract

Aim. To investigate whether hepatitis virus infection interacts with bone morphogenetic protein (BMP) signaling in the process of hepatocellular carcinogenesis

Methods. A tissue microarray (TMA) of 140 paraffin-embedded surgically resected hepatocellular carcinoma (HCC) patient specimens were used for immunohistochemical staining for p-SMAD1/5/8 and SMAD4. Hepatitis C and B cell culture models were used for *in vitro* studies.

Results. P-SMAD1/5/8 and SMAD4 protein were expressed at a higher level in HCC as compared to the normal adjacent area. There is a negative association between the apparent activation of BMP signaling in cancer cells and a history of prior hepatitis in patients with HCC. Patients with prior hepatitis exhibit significantly lower p-SMAD1/5/8 expression ($P<0.01$), although SMAD4 expression in HCC is higher in patient with a history of viral hepatitis. The association between activation of BMP signaling and a prior viral hepatis is remarkable in that from clinical parameters, we found only chirrosis to be significantly associated with history of hepatitis in our cohort and no significant relation was observed between prior viral hepatitis and patient's characteristics such as age, sex, tumor size and the number of tumor foci. In a cell culture models, hepatitis B or Hepatitis C infection did not activate BMP signaling, and treatment of cells with a BMP ligand or inhibitor did not affect hepatitis C virus (HCV) infection.

Conclussion. Although downregulation of BMP signaling is a striking characteristic in HCC patients with a prior history of viral hepatitis, viral infection of liver cells itself does not majorly involve BMP signaling. Hence, the difference in activation of elements involved in BMP signaling seen in HCC patients more likely represents altered interaction between the cancer cells and the surrounding stroma in viral hepatitis-associated HCC rather as cancer cell intrinsic properties.

Introduction

Hepatocellular carcinoma (HCC) is the major manifestation of primary liver malignancy and the fifth most prevalent neoplasm and the third most frequent cause of cancer-related death. HCC is characterized by a remarkable failure of conventional treatments, making better prevention and treatment modalities of paramoubt importance. Most cases of HCC are found in patients with cirrhosis caused by chronic hepatitis B or C virus infection. The unique etiology as well as the distinct environment that the liver holds may govern a differential signaling network compared to other cancers, but is still largely understood. Most likely, however, corruption of existing signaling mechanisms involved in cell fate specification in normal liver development play an important role. Unfortunately the nature of signals involved remains only partly understood and its likely that as yet unidentified morphogenetic signals are important here.

A possible mediator of aberrant morphogenetic signalling in liver pathology are the Bone morphogenetic proteins (BMPs). BMPs are a group of morphogens that belong to the Transforming Growth Factor beta (TGF-beta) superfamily of developmentally active signals. BMPs act in physiology through an autocrine and paracrine mechanisms, binding to cell surface receptors and triggering a sequence of downstream events. Initially, BMPs were only identified by their ability to induce the formation of bone and cartilage and controlled release from BMP family members has now become an accepted clinical mode aiding for instance fusion of vertebral bodies to prevent neurologic trauma or for the treatment of tibial non-union where a bone graft has failed [1]. However, BMPs are now emerged to be pivotal morphogenic signals, specifying cell fate in a myriad of cell types and in addition a plethora of other biological functions in physiology and pathophysiology [2]. Deregulation of BMP pathway is often associated with developmental defects and/or diseases, in particular cancer of tissues derived of endodermal origen [3].

A possible functionality of BMP in the liver and its associated pathology has been less well established. A recent study, however, described that human liver sinusoid endothelial cells (LSEC) can promote hepatitis C virus (HCV) replication within hepatocytes via secretion of BMP4 [4,5]. In contrast, an earlier study reported that the BMP family member BMP7 can inhibit HCV replication [6]. In addition, overexpression of soluble BMP inhibitors was decribed

in HCV-associated HCC tumors [7]. Thus although the data in the literature is not consistent, it appears that BMPs are involved in the biology of viral infection of hepatocytes and the associated cancers, prompting further research. Especially given the potential importance of the biological and clinical implications that establishing a role for BMP signalling in viral hepatitis and its associated cancer might have, we attempted to further investigate the role of BMP signalling in HCV infection in the context of HCC. Our results reveal that specifically hepatitis virus infection-associated cancers are characterised by a downregulation of BMP signalling, probably through processes that are established after viral infection has occurred and thus relate to the oncological process rather as to the viral infection *per se.* We thus propose that exogenous application of BMPs may become a treatment option for specifically hepatitis-related cancers.

Methods

Tissue microarray (TMA)

To make the tissue microarray, paraffin-embedded surgically resected HCC patient tissues (n = 140, obtained between 2004 to 2013) were collected from the pathology department of Erasmus Medical Centre (Erasmus MC) Rotterdam and processed through previously-described routine procedures [8]. The use of patient materials was approved by the medical ethical committee of the Erasmus MC (Medisch Ethische Toetsings Commissie Erasmus MC).[9]

Immunohistochemistry

Paraffin embedded liver tumor tissue in tissue microarray (TMA) slides was deparaffinized in xylene, rehydrated in graded alcohols, and rinsed once in phosphate-buffered saline (PBS) plus Tween 0.05%. For antigen retrieval, slides were boiled in Tris/EDTA pH 9.0 for 30 min (for the anti-SMAD4 antibody) and in Citrate acid pH 6.0 for 10 min (for the anti-p-SMAD1/5/8 antibody) ; 3% H2O2 was used to block endogenous peroxidase for 10 min at room temperature. The slides were incubated in a 5% milk blocking solution followed by overnight incubation with a mouse anti-SMAD4 antibody (1:100 dilution, Santa Cruz Biotechnology, Inc) or a rabbit anti-p-SMAD1/5/8 (1:500 dilution, Cell Signaling), followed by

an appropriate secondary antibody and then counterstained with haematoxylin. As negative control, the primary antibody was replaced by 5% milk blocking solution; the positive controls were taken from other slides that had successfully been stained before. The SMAD4 scoring was based on the nuclear staining and the p-SMAD1/5/8 scoring were based on cytoplasm and/or nuclear staining. The following scores were applied: score 0 for 0-10% positive staining, score 1 for 10-30% positive staining, score 2 for 30-70% positive staining, score 3 for >70% positive staining, and score 4 for >70% positive staining + high intensity. The scores were categorized from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4). The scorings were done by two investigators and the difference of scoring was determined through a Kappa test.

Hepatitis B and C cell culture models

Huh7-ET replicon assay system was based on Huh7 cells containing a subgenomic HCV bicistronic replicon (I389/ns3-3v/LucUbiNeo-ET) [10]. Stable luciferase expressing cells were generated by transducing naïve Huh7 cells with a lentiviral vector expressing the firefly luciferase gene (LV-PGK-Luc). Transduced cells were expanded for at least 10 days before use in experiments. As an infectious HCV model, Huh7.5.1 cells harboring the full-length JFH1-derived genome were used [11].Hepatitis B virus (HBV) particles were produced in the HepG2.2.15 cell line [12].

Measurement of luciferase activity

The HCV permissive Huh7 cells were transduced with LV-PGK-Luc and were plated in 96-well multiplates. Recombinant human BMP4 protein (100µg/ml, Merck Millipore) and recombinant human noggin (50µg/ml, R&D System) were used to treat cells, respectively, as appropriate. After 24, 48 and 72 hrs of culture, t luciferase activity was measured. For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 minutes at 37℃. Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Quantitative RT-PCR

RNA was isolated using a Machery-Nagel NucleoSpin RNA II kit (Bioké, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using an iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). HCV IRES and GAPDH cDNA of was amplified for 40 cycles and quantified by real-time PCR (MJ Research Opticon, Hercules, CA, USA) using SYBRGreen according to the manufacturer's instructions. GAPDH was used as a reference gene to normalize gene expression.

Western blotting

Subconfluent cells were lysed in Laemmli sample buffer containing 0.1 M dithiothreitol and incubated for 5 min at 96°C. Immunoblotting was performed using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE) as described previously [11]. Antibodies used were mouse SMAD4 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc) and rabbit p-SMAD1/5/8 (1:500 dilution, Cell Signaling). Quantification was performed using Odyssey LI-COR software.

Statistical analysis

Statistical analysis was performed by using Chi Square test in IBM SPSS Statistical 21. Mann-Whitney or T-test was also used using GraphPad InStat software (GraphPad Software Inc., San Diego, USA). P-values <0.05 were considered as statistically significant.

Results

A history of prior hepatitis is associated with deregulation of BMP signalling in HCC

Following engament of the BMP receptor complex with its cognate ligands, further BMP signalling involves the of phosphorylated SMAD1/5/8 (p-SMAD1/5/8) to SMAD4 and the thus-formed heteromeric complex supsequently translocates to the nucleus to provoke specific genetic responses.[2] Hence, we explored the activation of BMP signalling using p-

SMAD1/5/8 levels and nuclear localization of SMAD4 as a read-out. For this purpose immunohistochemical staining was performed of a paraffin-embedded tissue microarray (TMA) (*n*=140). The staining was scored by two independent investigators and the Kappa test for SMAD4 scoring in TMA was 0.773, which was deemed acceptable.

Tabel 1. Clinical parameters of the HCC patients investigated in this study

No	Characteristics	Hepatitis history		Patients	*P*-value[a]
		No	Yes		
1	Age	60.73 ± 2.32	58.55 ± 1.69	140/140	0.093
2	Sex (% male)	61/95 (64.2%)	36/45 (80.0%)	97/140	0.059
3	Size of tumor	7.15 ± 0.73	5.48 ± 0.76	98/140	0.128
4	Number of lesions	1.29 ± 0.07	1.27 ± 0.25	129/140	0.346
5	Differentiation*			127/140	0.043
	Good	33/94 (35.1%)	8/33 (24.2%)	41/127	
	Moderate	50/94 (53.2%)	15/33 (45.5%)	65/127	
	Bad	11/94 (11.7%)	10/33 (30.3%)	21/127	
6	Cirrhosis**	37/95 (38.9%)	30/45 (66.7%)	67/140	0.002
7	High-mod SMAD4	53/95 (55.8%)	32/45 (71.1%)	85/140	0.083
8	High-mod p-SMAD1/5/8**	34/95 (35.8%)	6/45 (13.3%)	40/140	0.006
9	High-mod SMAD4 + p-SMAD1/5/8	18/95 (18.9%)	4/45 (8.9%)	22/140	0.127

*$P<0.05$, **$P<0.01$

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test

Interestingly, nuclear SMAD4 protein levels were considerably higher in the HCC tumor as compared to the normal adjacent area (Fig. 1a). Analysis of the relation between nuclear SMAD4 levels and clinical parameters of the patients involved revealed that nuclear SMAD4 levels correlate well with a history of prior hepatitis (P<0.05) (Fig. 1b), but not with other clinical parameters. We thus concluded that activation of common component of signalling within the TGF-beta superfamily of patients is observed in HCC but that this effect is restricted to subgroup of patients that develop this disease as a consequence of a prior hepatitis.

Fig. 1

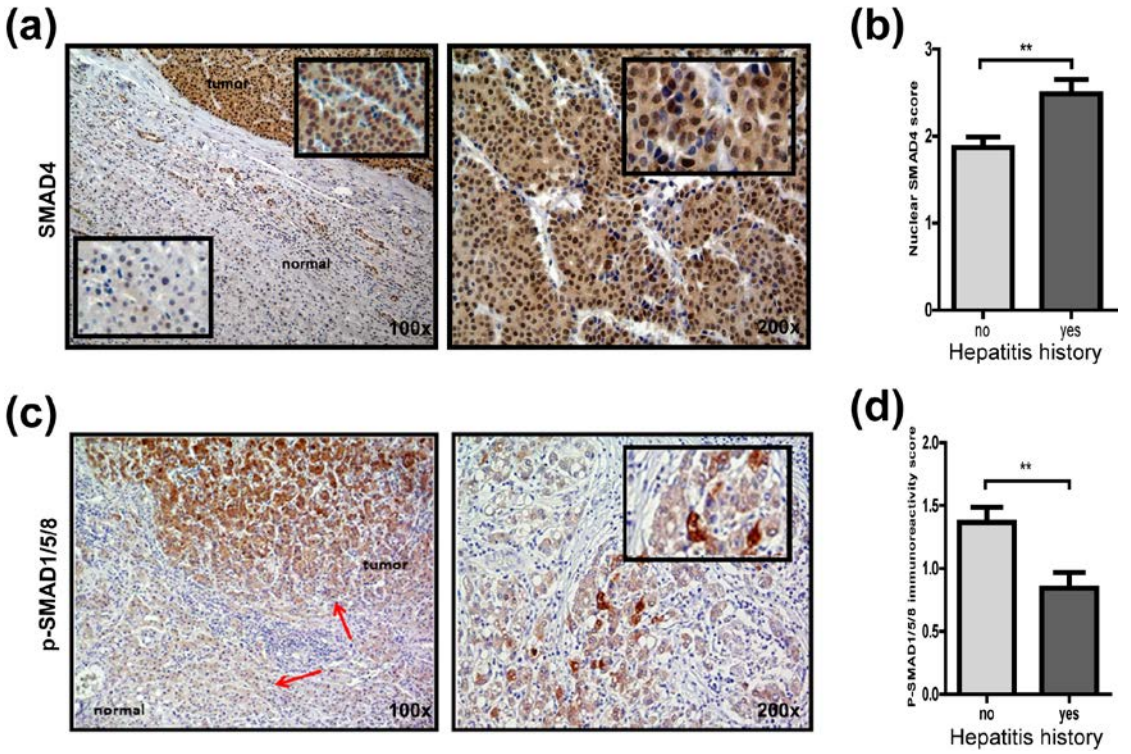

Figure 1. Dysregulation of BMP signaling components in HCC tumors of patients with hepatitis history. (a) An increase of SMAD4 expression in HCC tumor area compared to the normal adjacent area. (b) An increased of SMAD4 expression was associated with hepatitis history. (c) p-SMAD1/5/8 was predominantly expressed in the HCC tumor compared to the normal adjacent area and (d) patients with hepatitis history had significantly lower p-SMAD1/5/8 expression than those without hepatitis history.

A similar analysis was performed with regard to p-SMAD1/5/8 immunoreactivity in these patients. Mirroring the results obtained with nuclear SMAD4 staining, p-SMAD1/5/8 protein was predominantly present in the HCC tumor when compared to the normal adjacent area (Fig. 1c) and patients with a history of hepatitis had significantly lower p-SMAD1/5/8 expression than those without hepatitis history (Fig. 1d and Table 1). A positive correlation between cirrhosis and prior hepatitis was also detected (Table 1) however, no significant relation was observed between p-SMAD1/5/8 immunoreactivity and patient's characteristics such as age, sex, tumor size (n = 98 analyzable patients) and the number of tumor foci (n = 129 analyzable patients) (Table 1). There were only 22 out of 140 HCC patients (15.7%, Table 1) which displayed both moderate to high level of SMAD4 and p-

SMAD1/5/8 expression. Since the activation of BMP signaling is associated with both SMAD1/5/8 phosphorylation and nuclear localization of SMAD4, a possible interpretation of our results isthat in HCC patients, and especially those with a history of prior hepatitis canonical BMP signaling is inactivated since only a small subset of patients have tumors with evident nuclear SMAD4 combined with a p-SMAD1/5/8 signal (4 out of 45 HCC patients with a history of hepatitis) (Tabel 1).

BMP signaling does not affect hepatitis viral infection

The apparent negative association between SMAD1/5/8 phosphorylation or nuclear SMAD4 and HCC linked to a prior hepatitis might suggest that either BMP signaling is somehow involved in hepatitis virus infection or that down regulation of BMP signaling is important for progression to HCC once infection has been established. To distinguish between these possibilities, we further investigated whether HBV or HCV infection directly affects tBMP signaling. To this end, immortalized hepatocyte cell models were infected with HBV or HCV *in vitro.* However, no clear effect on either SMAD4 or p-SMAD1/5/8 immunoreactivity was observed upon infection determined by western blot (Fig. 2a), suggesting that viral infection does not directly interact with BMP signaling. Consistently, treatment with human recombinant BMP4 did not show any effect either on luciferase activity of a HCV replicon [10] (Fig. 2b) or on cellular viral RNA in aof HCV infectious model [11] (Fig. 2c) employing either 1, 10 & 100 ng/ml of the recombinant protein. Parallel performed western blotting analysis for p-SMAD1/5/8 (Fig. 2c) confirmed the biological activity of BMP4 used in our experimentation and demonstrated a capacity of our model to respond to the morphogen. Conversely, Noggin, a protein that binds and inactivates members of the TGF-beta superfamily signaling proteins including BMP4 [2] did not affect HCV infection in both models (Fig. 2d and 2e), but was proficient in inhibiting BMP4-provoked smad1/5/8 phosphorylation (Fig. 2e). Thus, it appears that BMP signaling activation is not implicated in hepatitis virus infection, at least in cell culture models.

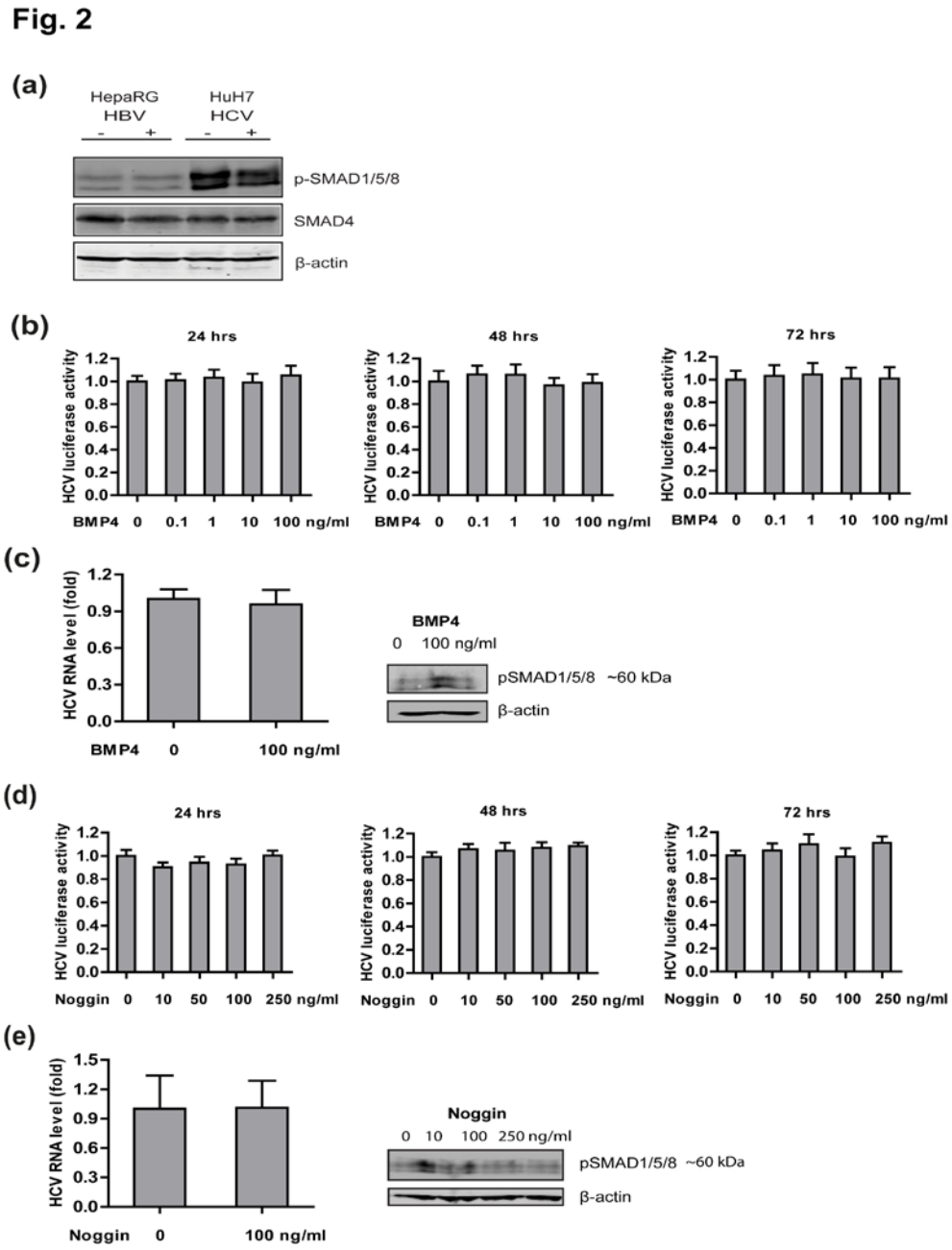

Figure 2. The effects of modulating BMP signalling on HCV infection in Huh7-based cell culture models. (a) Western blot analysis showed HCV infection could downregulate p-SMAD1/5/8 expression (b) HCV subgenomic replicon containing luciferase reporter gene was treated with human recombinant BMP4 at indicated concentrations. HCV luciferase was measured at 24, 48 and 72 hrs. No significant difference was observed (mean±SD, n=8). (c) Treatment of BMP4 did not affect cellular HCV RNA in the JFH-1 derived infectious model (mean±SD, n=4). Western blot confirmed the activation of SMAD1/5/8 phosphorylation, the downstream events of BMP signaling. (d) Treatment of noggin, the inhibitor of BMPs, also did not affect HCV infection in either subgenomic replicon (mean±SD, n=6) or (e) infectious model (mean±SD, n=3). The activity of noggin was confirmed by showing inhibition of SMAD1/5/8 phosphorylation.

Discussion

In this study, we report a specific dysregulation of BMP signaling in tumors of HCC patients with a history of hepatis. As we did not detect any effect of BMP signaling in the hepatitis virus infection process *per se*, the most straightforward interpretation of our results is that downregulation of BMP signaling a process occurring specifically in the progression towards hepatititis-associated liver cancer. Thus our results call for further research in the potential usefulness of BMP signaling-activating medication in this subgroup of patients. A possibility here would be treatment with the relatively side-devoid statins, which have been associated with increased BMP signaling in colon cancer through demethylating the BMP2 promoter, activating BMP signalling, inducing differentiation of CRC cells, and reducing 'stemness' [13]. If liver cells, like colon cells respond to statin treatment by the activation of BMP signaling, statins could be a useful addition to treatment regimens in hepatitis-associated HCC.

The literature on the role of BMP signaling in hepatitis virus infection is confusing. A recent study described that LSEC can promote HCV replication within hepatocytes via secretion of BMP4 [4,5] and that this effect can be mimicked by commercially available BMP4 in cell culture models. We could not confirm this effect in our experimental set up, using the same concentration of exogeneously applied BMP4. In contrast, an earlier study reported that the BMP family member BMP7 can inhibit HCV replication. By inhibiting BMP signalling with Noggin, we also did not observe an effect on HCV infectionWe found that BMP signaling was not able to modulate HCV infection [4]. Thus it is still a question whether BMP4 is the key factor that contributes to the proviral effects of LSEC, but the current study does not support this concept and other not virus infection-related mechanisms linking BMP signalling to the carcinogenesis of hepatitis-associated HCC are more likely to be responsible for the negative association between this disease and BMP signalling observed in the present study.

Despite a decrease of p-SMAD1/5/8 in hepatitis associated HCC tumors, we also observed an increase of SMAD4 expression. However, this increase in SMAD4 may not be related to activation of BMP signaling, since SMAD4 can also mediate signalling through the TGFB pathway [14]. HBV-encoded oncoproteins have been associated with an increase in SMAD4 nuclear translocation and amplification of TGFB signaling [15], but further work is

necessary to address the importance of TGFB signalling in the context of viral hepatitis-associated HCC.

In summary, this study reports downregulation of BMP signaling components in patient HCC tumors, restricted to those patients with a history of prior hepatitis , but did not uncover evidence that BMP signaling can directly modulate hepatitis virus infection, suggesting that down regulation of BMP signaling is especially important in the progression to full blown cancer following the establishment of hepatitis. Defining the exact mechanism involved may yield useful novel therapeutic avenues for dealing with specifically this subpopulation of HCC patients. .

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Chapter 7

IMPDH2-Targeted Constraint of Hepatocellular Carcinoma by Mycophenolic Acid in Experimental Models and in Patients

Kan Chen[1, 2#], **Pratika Y. Hernanda**[1,3#], Juan Li[1#], Angela S. W. Tjon[1#], Kostandinos Sideras[1], Xiangdong Kong[2], Dave Sprengers[1], Luc J. W. van der Laan[4], Jaap Kwekkeboom[1], Kwan Man[5], Herold J. Metselaar[1], Maikel P. Peppelenbosch[1], Qiuwei Pan[1]

[1]Department of Gastroenterology and Hepatology, Erasmus MC Cancer Institute, Erasmus University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, The Netherlands. [2]Bio-X Center, College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou, China. [3]Laboratory of Medical Genetics, Biomolecular Research Centre, Wijaya Kusuma University, Surabaya, Indonesia [4]Department of Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands. [5]Department of Surgery, Hong Kong University, Hong Kong, China

#Contributed equally

Submitted

ABSTRACT

BACKGROUND AND AIMS: Disease recurrence is a major challenge for the ultimate success of liver transplantation (LT) as treatment for hepatocellular carcinoma (HCC). The use of immunosuppressants is an important risk factor for HCC recurrence; whereas such impact could differ drastically among different types of immunosuppressants. This study investigated the effects and mechanism-of-action of mycophenolic acid (MPA) in experimental HCC models and in liver transplanted HCC patients. **METHODS**: Five HCC cell lines and nude mice with partial immunodeficiency were used. The association of mycophenolate mofetil (MMF), the pro-drug of MPA, with HCC recurrence was retrospectively analyzed in a LT cohort. **RESULTS**: With clinically achievable concentrations, MPA effectively inhibited cell proliferation and single cell colony formation. It can does-dependently trigger cell apoptosis and arrest HCC cells in G0/G1 phase. Supplementation of exogenous guanosine nucleotide partially restored the inhibitory effects of MPA on HCC cells. Ectopic over-expression of IMPDH2, the target of MPA, that lacking the binding sites of MPA but retaining its enzyme activity resulted in complete resistance to MPA. In nude mice subcutaneously engrafted with HCC cells, MPA significantly delayed tumor formation and constrained tumor growth. Immunohistochemical staining in harvested tumor tissues confirmed the cell cycling arresting and apoptosis triggering effects of MPA in mice. Most importantly, the use of MMF has a strong association with reduced disease recurrence and improved survival in liver transplanted HCC patients. **CONCLUSIONS**: By targeting IMPDH2, MPA can specifically counteract HCC in experimental models. In liver transplanted HCC patients, the use of MMF is strongly associated with reduced disease recurrence and improved survival. These results warrant prospective clinical trials for further investigation.

INTRODUCTION

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide.[1] Surgical resection or liver transplantation (LT) are the only potentially curative treatment options. LT is particularly attractive because of the resection of the tumor along with the replacement of the diseased liver that is at risk for development of new malignant lesions. Moreover, liver transplantation cures the underlying liver disease and related other complications. However, tumor recurrence is a common challenge for the ultimate success of both surgical resection and LT.[2] An unique risk factor strongly associated with recurrence in LT patients is the universal use of immunosuppressants after transplantation, which is to prevent graft rejection.[3-5]

Besides a general impairment of the immunosurveillance system, different types of immunosuppressants however could have distinct mechanisms that are independent of the host immunity to affect the process of malignancy.[5-8] Current research is mainly focusing on the mammalian target of rapamycin (mTOR) inhibitors, including rapamycin (sirolimus) and everolimus. They are thought to be the only class of immunosuppressive agents that may reduce HCC recurrence, as having been reported by some retrospective or meta-analysis studies.[9-13] However, these studies do not provide firm evidence to conclude the superiority of mTOR inhibitors on HCC recurrence.[14] In particular, prospective studies supporting this notion are still lacking, and higher rejection rates were reported for monotherapy of sirolimus or everolimus in liver transplanted HCC patients.[15, 16] Yet, HCC is a rather heterogeneous malignancy with multiple etiologies.[17, 18] It is unlikely that one immunosuppression protocol fits all cases. Therefore, the impact of other immunosuppressants also deserve to be carefully investigated, in order to define appropriate immunosuppressive regimens for management of HCC recurrence after LT.

Mycophenolic acid (MPA) and its prodrug, mycophenolate mofetil (MMF), are currently widely used for prevention of allograft rejection because of lacking nephrotoxicity.[19] These drugs act through depletion of guanine nucleotide pools by inhibition of inosine monophosphate dehydrogenase (IMPDH), in particular the isoform 2 (IMPDH2).[20] This results in blockage of *de novo* guanine nucleotide synthesis and inhibition of lymphocyte

proliferation.[19] Interestingly, MPA has been reported to be able to inhibit cancer cell proliferation and induce caspase/ mitochondrial-dependent apoptosis in several experimental models of human solid tumors and hematological malignancies.[21-24] A large prospectively conducted observational cohort study observed a tendency toward a lower risk of malignancy in MMF versus non-MMF treated renal transplanted patients.[25] However, this class of immunosuppressants has not been extensively studied in the setting of HCC recurrence after LT. This consideration inspired us to explore the effects and mechanism-of-action of MPA in experimental HCC models and in HCC-related LT patients.

Patients, Materials and Methods

Patient Information

A LT database established by our previous study[4] was used for retrospective analysis of the effect of MMF use on HCC recurrence. This cohort included patients transplanted between October 1986 to December 2007 at the Erasmus MC University Medical Centre Rotterdam, The Netherlands. 44 out of 385 LT patients were identified as HCC-related LT and thus subjected to the analysis.

Patient Tissue

Tissue samples from individuals who were eligible for surgical resection of HCC were collected. The use of patient materials was approved by the medical ethical committee of Erasmus MC (Medisch Ethische Toetsings Commissie Erasmus MC). [26]

Reagents

Stocks of MPA and guanosine (AMRESCO LLC, USA) was dissolved in dimehtyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO), Antibodies against IMPDH2, p-Histone3 and Cleaved caspase3 were purchased from Abcam, MILLIPORE and Cell Signaling, respectively.

Cell Culture

HCC cell lines, including BEL7404, Hep3B, SMCC7721, PLC/PFR/5 and Huh7, were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies), supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone Technologies), 100 units/mL of penicillin and 100 μg/mL of streptomycin. All the cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2.

MTT Assay

Cells were seeded in 96-well plate, at a concentration of 6×10^3 cells/well in 100 μL medium. All of the cells were incubated overnight to attach to the bottom of the wells, and then treated with serials dilutions MPA (1, 5, 10, 15, 20, 25 and 30 μg/mL). Cell viability was analyzed by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) (Sigma-Aldrich, St Louis, MO) and 150 μL DMSO. Absorbance was determined using Enzyme mark instrument at the wavelength of 490 nm.

Colony Formation Assay

Cells were harvested and resuspended in culture medium, then counted and plated in 6-well plates (for BEL7404 cell, 500 cells/well, and for Hep3B cell, 1000 cells/well). Formed colonies were fixed by 70% ethanol and counterstained with haematoxylin & eosin after two weeks. Colony numbers were counted and their sizes were measured by microscope.

Analysis of Cell Cycle

Cells (5×10^5/well) were plated in six-well plates and incubated overnight to attach the wells, then serials concentrations of MPA were added. Control group was added with equal volume of PBS. After 48 h, control and treated cells were trypsinized and washed with PBS and then fixed in cold 70% ethanol overnight at 4℃. The cells were washed twice with PBS and incubated with 20 μg/mL RNaseA at 37℃ for 30 min, and then with 50 μg/mL propidium iodide (PI) at 4℃ for 30 min. The samples were analyzed immediately by Flow cytometric (FACS) (Aria™, BD Biosciences). Cell cycle was analyzed by ModFit LT 3.0 software.

Analysis of Cell Apoptosis

Cell apoptosis analysis were performed by staining cells with AnnexinV-FITC and PI. Cells (5×10^5/well) were seeded in six-well plates and incubated at 37℃ in 5% CO_2 for overnight, then serials dilutions of MPA were added, control group was added with equal volume of PBS. After 24, 48 and 72 h, all of the cells were trypsinized and resuspended in Annexin-binding buffer, then stained with Alexa Fluor 488 AnnexinV and PI, and incubated at room temperature for 15 min. Detection of apoptosis was performed by FACS.

Xenograft Assays in Nude Mice

HCC xenograft tumor model in nude mice was performed in accordance with current prescribed guidelines and under a protocol approved by the Institutional Animal Care and Use Committee of Hangzhou Normal University, China.[27] Mice were all female and 4-6 weeks of age at the time of inoculation, and were subcutaneously inoculated with $5X10^6$ of BEL7404 cells. After 20 hours, mice were divided into 3 groups and were treated with different doses of MPA or placebo (240 mg/kg body weight, n = 10; 60 mg/kg body weight, n = 11 and PBS, n = 10).[28] MPA was injected via the intraperitoneal (IP) route for 20 consecutive days. Tumor formation was monitored. The size of tumors were measured by vernier caliper every 3 days. $Volume_{(tumor)}=0.5\times length\times width^2$. At day 30 post-engraftment, mice were sacrificed and tumors were harvested and imaged. Tumor tissue were fixed with 4% paraformaldehyde and embedded in paraffin for histology evaluation or immunohistochemistry.

Immunohistochemistry

Paraffin embedded tumor tissue slides were deparaffined in xylene, rehydrated in graded alcohols, and rinsed once in PBS plus Tween 0.05%. Slides were boiled in citrate acid buffer pH6.0 for 10 min for antigen retrieval; 3% H2O2 was used to block endogenous peroxidase for 20 min at room temperature. The slides were incubated in 5% milk blocking solution followed by overnight incubation with rabbit monoclonal antibody against IMPDH2, rabbit polyclonal antibody against p-Histone H3 and rabbit polyclonal antibody against cleaved caspase 3 with concentration of 1:500, 1:1000, 1:300 and then counterstained with

haematoxylin. As negative control, the primary antibody was replaced by PBS; the positive controls were taken from other slides that had successfully stained before. IMPDH2, phospho-Histone H3 and Cleaved staining was scored by two independent observers.

The number of cell mitosis was counted in 10 high-power fields (x400). Median number of mitosis for each 10 field was calculated for each sample of different groups. The percentage of cleaved caspase 3 positive cells was also counted in each of 10 high-power fields (x400). The average number of every 10 percentages was taken as the value of each sample. Three categories was used to evaluate the percentage of positive apoptosis: <10% mild; 10%-50% moderate; >50% high. And the intensity of IMPDH2 staining was presented by categories: + weak; ++ moderate; +++ strong.

Statistical Analysis

Statistical analysis was performed by using the paired nonparametric test, the unpaired nonparametric Mann–Whitney test or paired T-test using GraphPad InStat software. Chi Square test, cox regression analysis and Kaplan Meier survival analysis in IBM SPSS. P-values <0.05 were considered statistically significant.

Results

MPA Inhibited Cell Proliferation and Colony Unit Formation in HCC Cell Lines

In order to investigate whether MPA may directly affect cellular physiology of HCC cells, the effects of this drug on cell proliferation and single cell colony unit formation (CFU) were evaluated in different cell line models of this disease. MPA does- and time-dependently inhibited cell proliferation in all 5 HCC cell lines with clinically achievable concentrations[29] (Figure 1). In apparent agreement, MPA profoundly inhibited the number and size of colonies formed in the CFU assay. It appears that relatively low concentration (1 µg/ml) of MPA has already impeded colony formation, whereas higher concentrations (2-5 µg/ml) are not compatible with colony formation at all (Figure 2). In BEL7040 cells, 322 ± 27 colonies/500 cells were formed in untreated but only 148 ± 27 colonies were formed in 1 µg/ml MPA treated groups (mean ± SEM, n = 6, P < 0.01) (Figure 2*A*). Accordingly, the size of

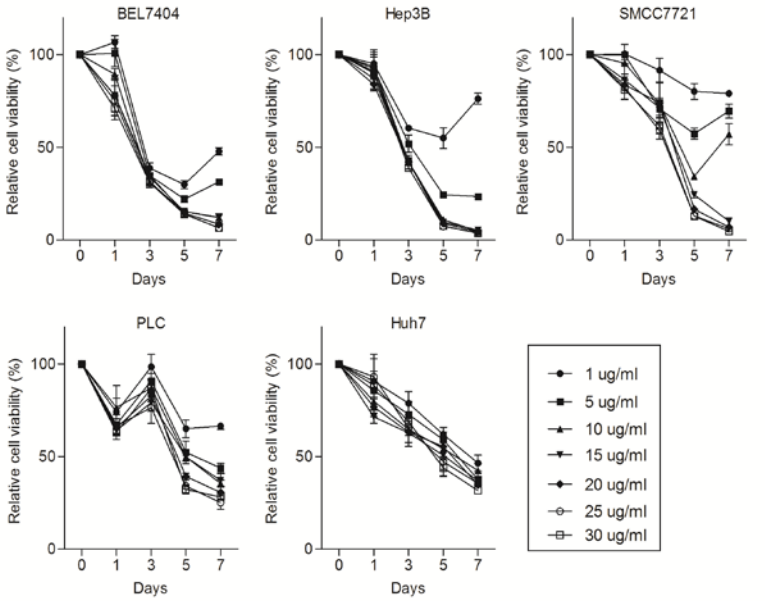


Figure 1. MPA inhibited cell proliferation in HCC cell lines. With clinically achievable concentrations, MPA potently inhibited cell proliferation in all 5 HCC cell lines determined by the MTT assay (Mean ± SD, n = 5).

CFU was significantly smaller in MPA treated compared to untreated groups (96 ± 5 pixels Vs 278 ± 8 pixels, mean ± SEM, n = 30, P < 0.01) (Figure 2*B*). Similar results were also observed in Hep3B cells (Figure 2*C* and 2*D*). We concluded that MPA strongly interferes with HCC expansion *in vitro* and experiments were initiated to explore the cell kinetic basis of this effect.

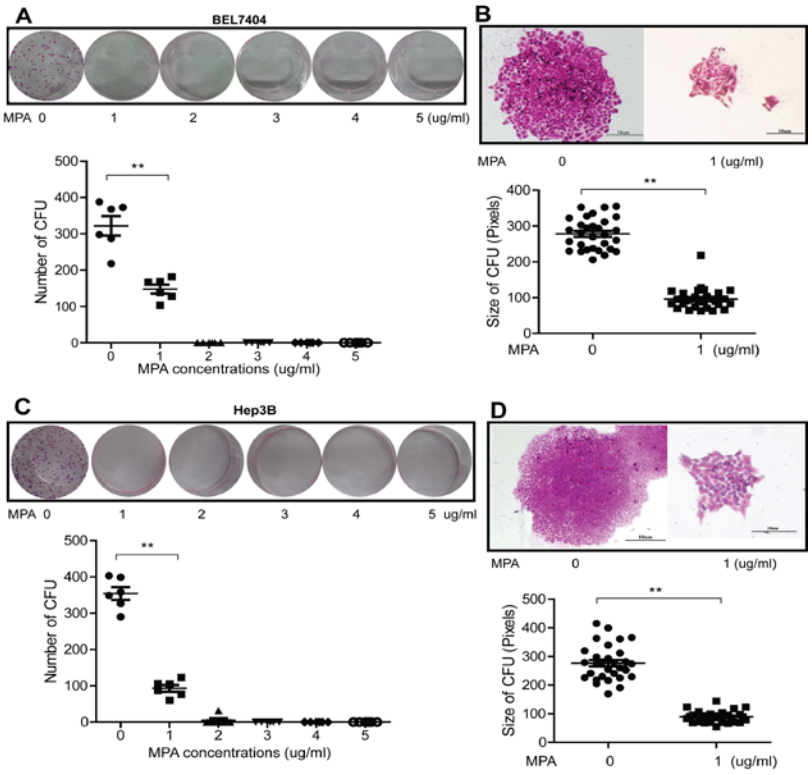


Figure 2. MPA inhibited single cell colony formation. MPA inhibited the ability of colony formation in BEL7404 cell line (the number (A) and the size (B) of CFU) and in Hep3B cell line (the number (C) and the size (D) of CFU) (Mean ± SEM, n = 6 or 30, respectively, **P < 0.01).

MPA Arrested Cell Cycling and Induced Cell Apoptosis

To further understand how MPA acts on HCC cell growth, assays for quantifying cell cycling and apoptosis were performed. Treatment of MPA does-dependently increased the proportion of HCC cells in the G0/G1-phase and concomitantly decreased the proportion of cells in the S-phase and the G2/M-phase of both BEL7404 (Figure 3*A*) and Hep3B (Figure 3*B*) cell models of HCC. In addition, MPA dose-dependently triggered early and late cell apoptosis (Figure 3*C*, 3*D* and 3*E*). These data suggested that MPA inhibit HCC cell growth by arresting cell cycle and inducing apoptosis.

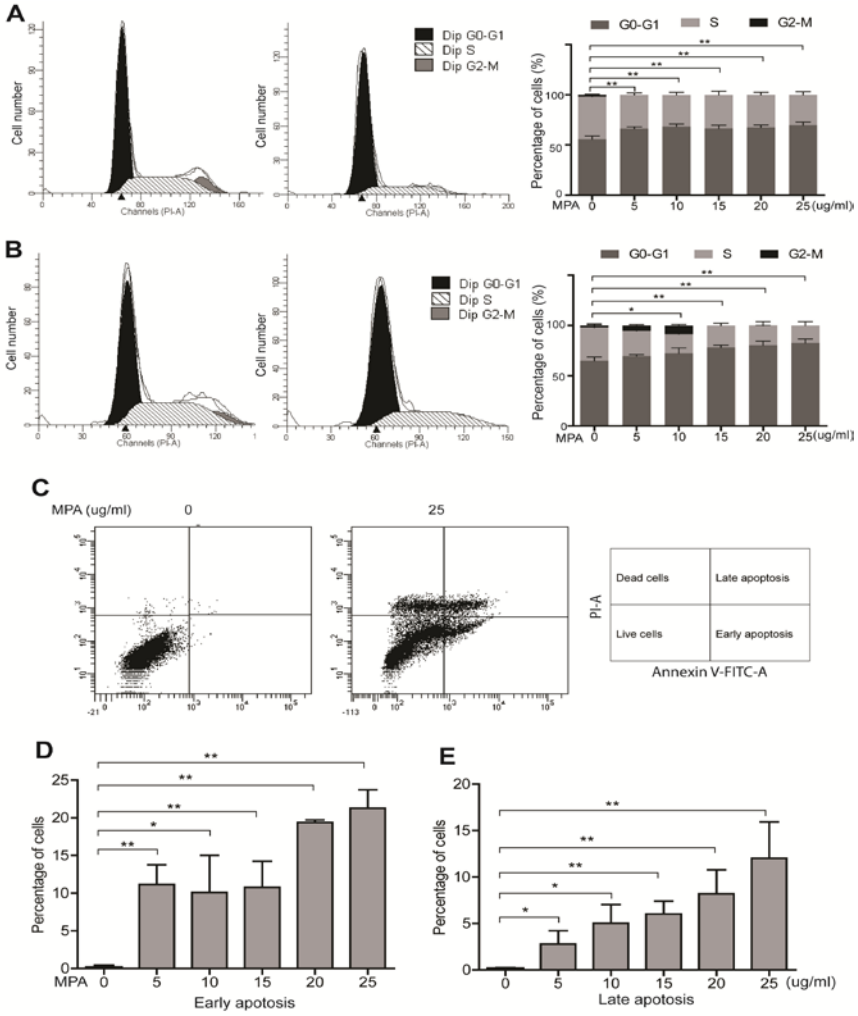


Figure 3. MPA arrested cell cycling and triggered cell apoptosis. BEL7404 cells (A) and Hep3B cells (B) were arrested in the G0/G1 phase by MPA treatment (Mean ± SD, n = 3. *P < 0.05; **P < 0.01); (C) Flow cytometric analysis of cell apoptosis. (D) MPA significantly enhanced both early and late apoptosis at 5-25 ug/ml concentrations (Mean ± SD, n = 3, *P < 0.05; **P < 0.01).

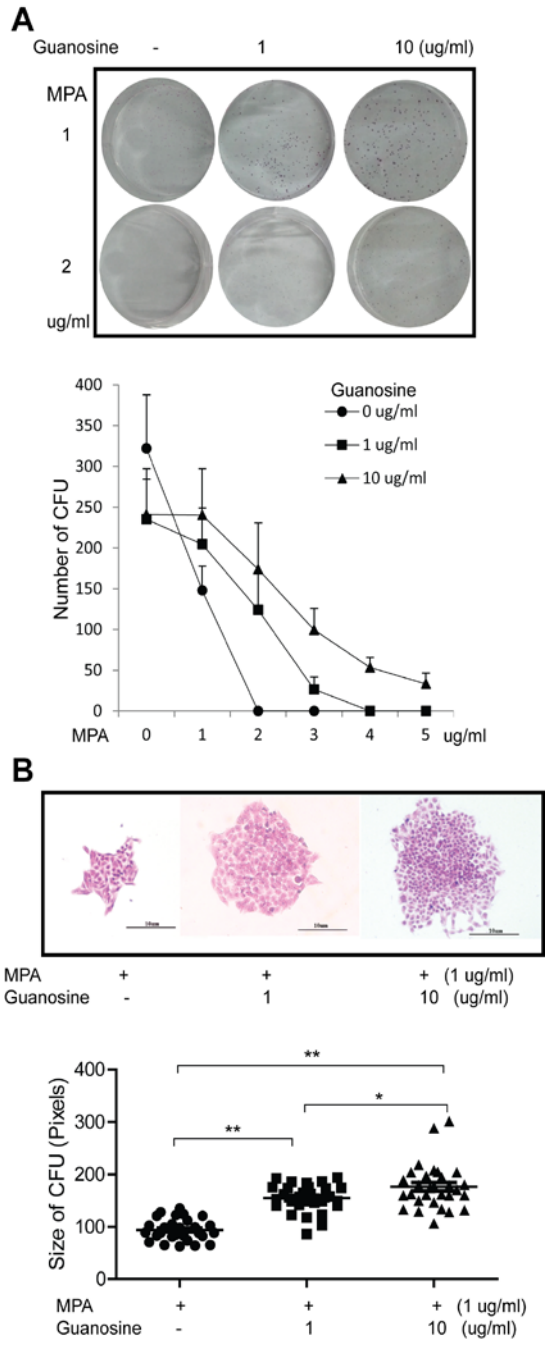


Figure 4. Anti-proliferative effects of MPA were partially restored by supplementation of exogenous guanosine. The number (A) and the size (B) of CFU were partially restored by supplementation of exogenous guanosine after treatment of MPA in BEL7404 cell line (Mean ± SD or SEM, n = 6 or 30, respectively, *P < 0.05; **P < 0.01).

Exogenous Nucleotide Supplementation Partially Restored the Anti-growth Effect of MPA

Depletion of intracellular nucleotide pool is the key immunosuppressive mechanism employed by MPA to inhibit lymphocytes proliferation.[30] Supplementation of exogenous guanosine nucleotide indeed partially counteracted the anti-proliferation effects of MPA on HCC cells. This is marked at low doses of MPA (1 and 2 µg/ml), but high doses of MPA out-compete exogenous guanosine nucleotides (Figure 4*A*). This effect was also observed when colony formation by HCC cell line models was studied (Figure 4*B*). These results promoted further investigation on the molecular mechanisms.

IMPDH2 Is a Key Mediator of MPA Affecting HCC Cell Growth

MPA can effectively bind to IMPDH, leading to the inhibition of the enzymatic activity of *de novo* nucleotide biosynthesis. Although there are two isoforms, IMPDH2 exhibits a 5-fold higher sensitivity to inhibition by MPA as compared to the type I isoform (IMPDH1).[20] IMPDH2 is often up-regulated in cancer.[31] Immunohistochemical staining of our patient HCC tissues showed remarkable high expression of IMPDH2 at protein level (Figure

S1), suggesting that this enzyme constitutes a relevant target for MPA in HCC.

To further investigate the functional role of IMPDH2, we used a lentiviral vector expressing an experimentally mutated IMPDH2 (LV-mutIMPDH2) fused with GFP.[32, 33] The product of this construct has normal IMP hydrogenase activity but lacks the binding site for MPA. Transduction of this vector resulted in successful expression of this mutated allele in HCC cells (Figure 5*A*). In BEL7404 cells, forced expression of this mutated IMPDH2 coincided with resistance of these cells with respect to MPA effects on cell proliferation as determined by MTT assay (Figure 5*B*). In CFU assay, forced expression provoked a non-competitive resistance to MPA effects on colony size and number (Figure 5*C* and 5*D*). Furthermore, expression of the mutated IMDH2 allele prevented MPA-induced apoptosis (Figure 5*E* and 5*F*). These results demonstrated a key role of IMPDH2 in mediating the effects of MPA on HCC cellular physiology.

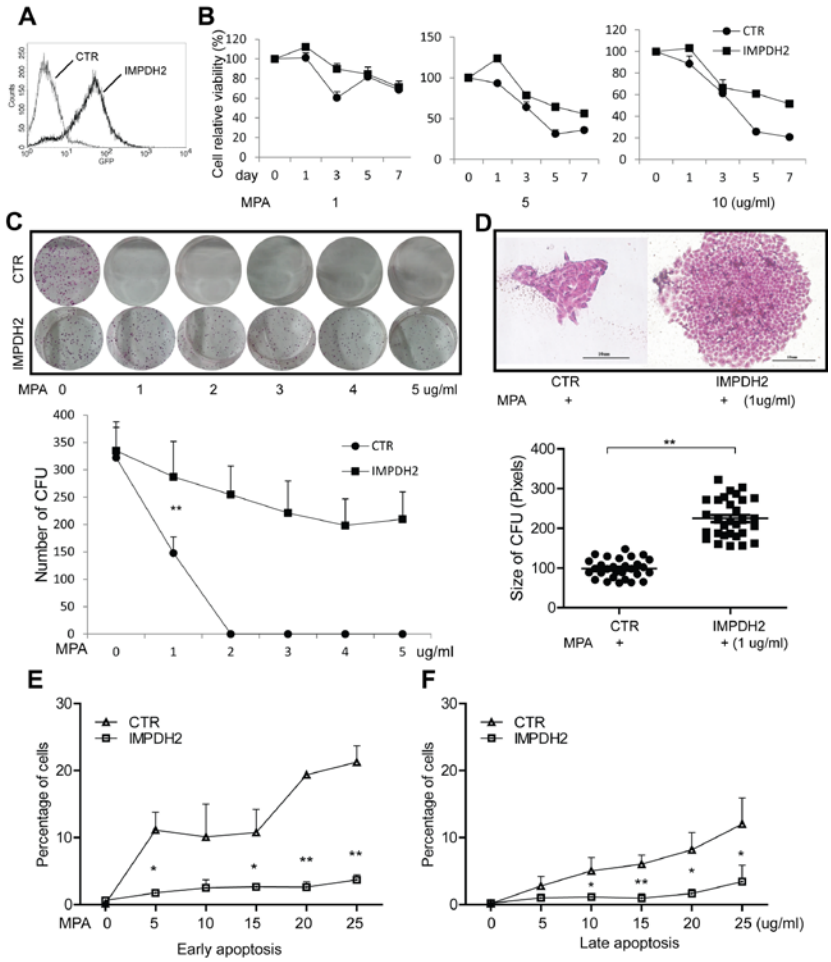
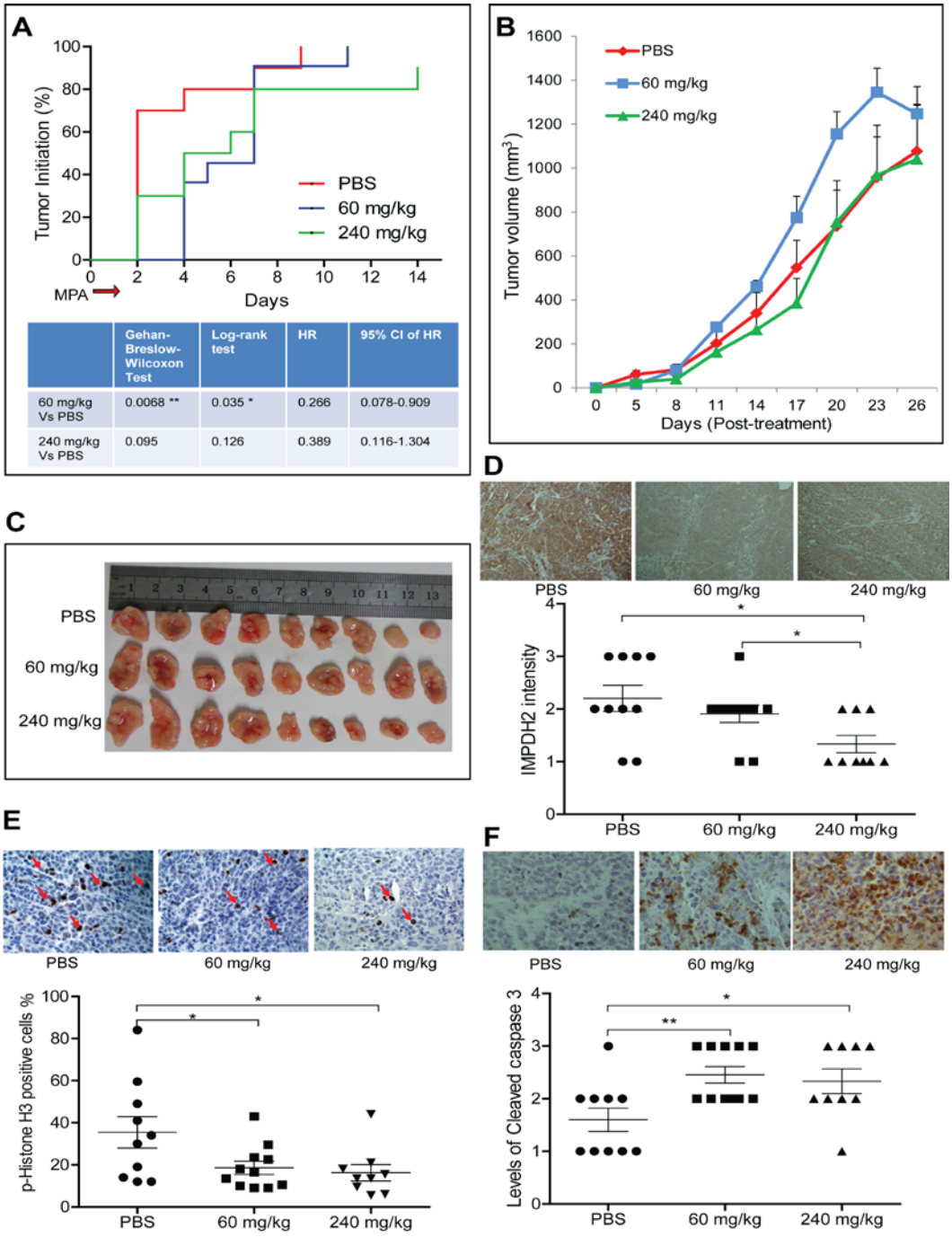


Figure 5. Anti-proliferative effects of MPA were almost completely restored by ectopic over-expression of IMPDH2. (A) An experimentally mutated IMPDH2 fused to GFP reporter was expressed by a lentiviral vector. It has normal IMP hydrogenase activity but lacks the binding site for MPA. (B) Ectopic expression of IMPDH2 restored the inhibitory effects of MPA on cell viability. The number (C) (Mean ± SD, n = 6, **P<0.01).and the size (D) (Mean ± SEM, n = 30, **P<0.01) of CFU were largely restored in IMPDH2 over-expressed BEL7404 cells. The percentage of early apoptosis (E) and late apoptosis (F) induced by MPA were significantly reduced in IMPDH2 over-expressing cells (Mean ± SD, n = 3. *P < 0.05; **P < 0.01).



	Gehan-Breslow-Wilcoxon Test	Log-rank test	HR	95% CI of HR
60 mg/kg Vs PBS	0.0068 **	0.035 *	0.266	0.078-0.909
240 mg/kg Vs PBS	0.095	0.126	0.389	0.116-1.304

Figure 6. MPA delayed tumor initiation, inhibited HCC cell proliferation and induced cell apoptosis in mice. (A) MPA treatment significantly delayed tumor initiation of BEL7404 cells in nude mice. (B) Low dose (60 mg/Kg body weight) of MPA slightly promoted tumor growth, but high dose (240 mg/Kg body weight) constrained tumor growth, compared with PBS treated mice. (C) The appearance of formed tumors. (D) Immunohistochemical staining of harvested tumor tissue section revealed a significant down-regulation of IMPDH2 protein levels by treatment of MPA. Accordingly, treatment of MPA significantly reduced the percentage of p-histone H3 positive (proliferating) cells (E) and significantly increased cleaved caspase 3 positive (apoptotic) cells (F). (Mean ± SEM, PBS, n = 10; 60 mg/kg, n = 11; 240 mg/kg, n = 9, *P<0.05; **P<0.01).

MPA Delayed Tumor Initiation, Inhibited HCC Cell Proliferation and Induced Cell Apoptosis in Mice

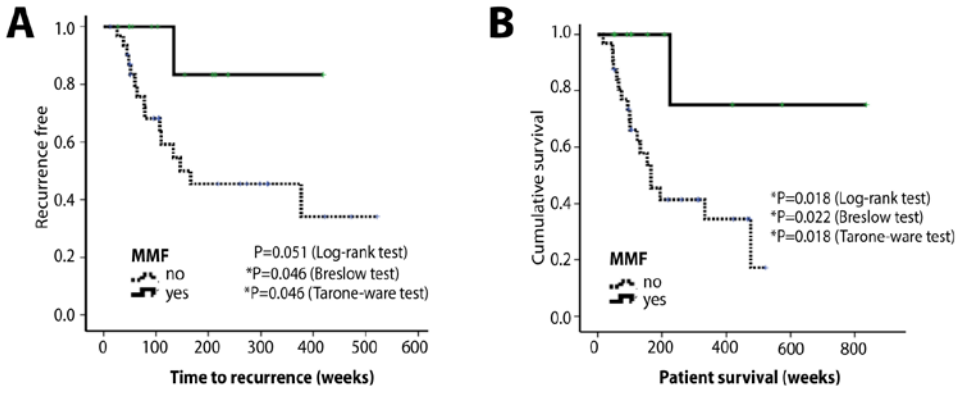
Insight into the effects of MPA on HCC *in vivo* was obtained in experiments in which nude mice were used for subcutaneous engraftment of BEL7404 cell line (5×10^6 cells). 20 hours after inoculation, mice were IP injected with MPA or vehicle for 20 consecutive days. In this xenograft model of experimental HCC, treatment of MPA (60 mg/kg body weight) significantly ($P < 0.05$) delayed tumor initiation (Figure 6*A*). In 240 mg/kg body weight of MPA treated group, one mouse was failed to form tumor; whereas other mice all successfully formed tumors. Tumor growth was followed by measuring the volume every 3 days. Treatment of 60 mg/kg body weight of MPA slightly increased the tumor volume (conceivable due to immunosuppression); whereas 240 mg/kg body weight of MPA constrained tumor growth (Figure 6*B* and 6*C*).

Immunohistochemical staining of tumors harvested from these mice has demonstrated a significant down-regulation ($P < 0.05$) of IMPDH2 at protein levels by treatment of MPA (Figure 6*D*). MPA significantly inhibited tumor cell proliferation, as shown by significant reduction ($P < 0.05$) of proliferative cells assessed by the percentage of p-histone H3 positive cells (Figure 6*E*). Furthermore, MPA treatment coincides with a substantial tumor cell apoptosis, as shown by a significant increase in the percentage of cleaved caspase 3 positive cells (Figure 6*F*). These results showed that MPA can significantly delay tumor initiation, specifically inhibit tumor cell proliferation and effectively trigger HCC cell apoptosis in mice; whereas its immunosuppressive property can potentially mask this specific anti-tumor effect.

MMF Use Is Strongly Associated with Reduced HCC Recurrence and Improved Survival in Liver Transplant Patients

Confirming the anti-tumor effect of MPA in patients is of foremost importance in managing immunosuppressive medication in HCC patients after LT. In fact, the transplantation setting is more fair for assessing this specific anti-tumor effect, since all the patients are under immunosuppressive condition. Retrospective analysis was performed in a LT cohort that was used for our previous study.[4] 44 out of 385 patients were identified as HCC-related liver

transplantation and thus included in this analysis. 12 patients were treated with immunosuppressive regimens containing MMF (Table S1); whereas 32 patients were treated with immunosuppressive regimens that do not contain MMF. As shown in Table 1, there is no significant difference regarding to patient characteristics, including age and sex. Importantly, There are also no significant differences regarding to known prognostic factors of HCC recurrence after LT,[31] including the size of tumor, the number of lesions, tumor differentiation stage, vascular invasion and the level of AFP before transplantation (Table S2).



C

MMF use	P-value	HR	95% CI for HR
yes	0.086	0.169	0.022-1.284

D

MMF use	P-value	HR	95% CI for HR
yes	*0.046	0.128	0.017-0.967

Figure 7. MMF use in patient is associated with better clinical outcome in HCC transplanted patients. (A) Kaplan Meier analysis (n = 44) revealed that patients using MMF is significantly slower to HCC recurrence ($P < 0.05$) (A) and with better survival (B). Consistently, cox regression analysis showed that patients using MMF have a lower risk of fast recurrence (progression) (C) and lower risk of poor survival (D). HR: Hazard Ratio; *$P < 0.05$.

However, only 1 out of 12 patients (8.3%) in the MMF group developed recurrence; whereas 15 out of 32 patients (46.9%) in the control group developed recurrence during follow up. Thus, the use of MMF was significantly associated with lower recurrence rates ($P < 0.05$) and higher survival rates ($P < 0.01$) (Table S2). Kaplan Meier analysis confirmed that patients using MMF have significantly slower HCC recurrence ($P < 0.05$) (Figure 7*A*) associated with better survival ($P < 0.05$) (Figure 7*B*). Consistently, cox regression analysis

revealed that patients using MMF have a lower risk of fast recurrence (progression) (HR = 0.169, 95% CI: 0.022-1.284) (Figure 7*C*) and lower risk of poor survival (HR = 0.128, 95% CI: 0.017-0.967) (Figure 7*D*). These results indicate that MMF use is associated with reduced HCC recurrence and improved survival in liver transplant patients.

Discussion

Although it is suspected that immunosuppressive medication following LT facilitate HCC recurrence, the issue of how specific immunosuppressive drugs affect the disease process is only poorly understood.[35] Obviously, a regimen that can perform its immunosuppressive function necessary for preventing graft rejection but that concomitantly exerts anti-tumor effects against HCC should be the preferential clinical choice in this particular setting. In this respect, mTOR inhibitors attract attention as they are potent renal function-sparing immunosuppressants but potentially also have anti-proliferative activity on tumor cells.[9-13] However, only approximately 50 % of all HCC exhibits activation of mTOR downstream signaling elements and retrospective or meta-analysis based studies do not provide firm evidence to conclude that mTOR inhibitors exert beneficial effects with regard to HCC recurrence.[12, 36, 37] Indeed, both experimental and clinical evidence suggests that tumors bearing different genetic mutations can respond differentially to mTOR inhibitors.[38, 39] Given the heterogeneity of HCC,[17] other immunosuppressive regimens also deserve careful attention. In this study, we demonstrated a cancer-constraint effect of MPA through IMPDH2 in experimental HCC models. We further provided clinical evidence that MMF is associated with reduced disease recurrence and improved survival in HCC-related liver transplant patients.

Mechanistically, the effect of MPA on HCC appears through inhibition of its canonical target, IMPDH. Such enzymes catalyze the rate-limiting step in *de novo* guanine nucleotide biosynthesis. The enzymatic activity of IMPDH is composed of two separate isoenzymes, type 1 and 2, encoded by the genes IMPDH1 and IMPDH2.[40] IMPDH2, which exhibits a 5-fold higher sensitivity to MPA than IMPDH1, is up-regulated in proliferating cells,[41] including in various types of tumors as well as well in HCC as we observed in this study. Ectopic expression of the experimentally-mutated IMPDH2 that retained enzymatic activity but

lacked the binding site of MPA largely restored the anti-HCC effects of MPA. Surprisingly, supplementation of guanosine restored to a much lesser extent, the inhibitory effects of MPA. Interestingly, a recent study in Drosophila has demonstrated a double role of IMPDH. It can also act as a transcription factor to either enable or restrict cell proliferation.[42] MPA might also act in HCC as a transcription factor, but obviously further experimentation is necessary to address this possibility.

A potential anti-malignant effect of MPA in patients has been evaluated in clinical settings other than LT. A phase 1 clinical trial in patients with advanced multiple myeloma showed a positive correlation between clinical responses and depletion of the intracellular deoxyguanosine triphosphate levels by MMF.[43] However, another clinical study in pancreatic cancer failed to show any beneficial effects, thus these effects may be cancer type specific or can be masked by the immunosuppressive properties of MPA.[44] Our data support that MPA has potent inhibitory effects on HCC growth *in vitro* and delays tumor initiation in mice. However, in mice, low dose of MPA slightly stimulated tumor growth (conceivable by suppressing anti-tumor immunity) but high dose constrained tumor growth again. More importantly, a clear inhibition of tumor cell proliferation and induction of HCC cell apoptosis were observed in MPA treated mice. These results indicate an immunosuppressive and an anti-tumor action simultaneously occurring *in vivo*. Thus, the transplantation setting is perfect for investigating its specific antitumor effect, since all the patients are under immunosuppression. Indeed, a tendency toward a lower risk of malignancy in MMF-treated renal transplant patients versus non–MMF-treated renal transplant patients has been reported in a large, prospectively conducted, observational cohort study.[25]

Excitingly, after performing a retrospective analysis in our LT cohort, we found a strong association between MMF use and reduced HCC recurrence and improved patients survival. Importantly, there are no significant differences regarding to patient characteristics and no significance of some essential prognostic factors of HCC recurrence[34] between these two groups. Given the single center and retrospective nature, other clinical and preferentially randomized studies are needed to confirm our findings.

In summary, this study demonstrated that clinically relevant concentrations of MPA are capable in constraining HCC cell growth in experimental models. We further provided

clinical evidence that MMF is associated with reduced HCC recurrence and improved survival in liver transplant patients. Confirming these experimental findings and retrospective clinical observations by prospective randomized trials could lead to better management of immunosuppressive medication for HCC patients after liver transplantation.

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SUPPLEMENTARY MATERIAL

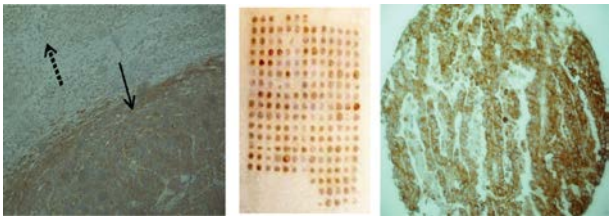

Figure S1. Protein expression of IMPDH2 in patient HCC tumors.
As shown by immunohistochemical staining (left panel), IMPDH2 is highly expressed in patient HCC tumor (solid arrow) compared with adjacent tissue (dashed arrow). To further evaluate IMPDH2 expression in more patients, a tissue microarray (TMA, middle panel) containing surgical resection of HCC tissues were used (12 cases have both normal and tumor tissue and 4 cases have tumor tissue only). The use of patient materials was approved by the medical ethical committee of Erasmus MC. (Medisch Ethische Toetsings Commissie Erasmus MC) The high expression level of IMPDH2 was confirmed in this cohort (right panel).

Table S1. Clinical informations on patients that use MMF

No	Age (yrs)	LTx date	Recurrence date	Start date MMF	End date MMF	MMF Period (weeks)	AFP before transplantation
1	58	22-May-1992	-	26-Jan-98	21-Feb-08	525	N/D
2	50	18-Jan-1998	-	26-Sep-02	04-Feb-11	436	116
3	55	18-May-2006	-	24-May-06	04-Jul-13	371	15
4	53	28-Jul-2007	-	13-Aug-07	12-Jun-13	304	6
5	60	21-Dec-2005	-	25-Jan-06	07-May-09	171	94
6	69	21-May-2000	-	19-Dec-05	22-Sep-08	144	15
7	63	05-Sep-2007	-	11-Sep-07	15-Jun-10	144	19
8	65	20-Nov-2004	19-Jun-2007	15-Sep-05	17-Mar-08	130	101
9	58	01-Jan-2007	-	29-Jan-07	21-Jan-10	155	9
10	24	09-Feb-2005	-	02-Mar-05	20-Apr-06	59	1
11	65	23-Aug-2007	-	23-Aug-07	21-Sep-07	4	2
12	56	22-Jan-2007	-	26-Jan-07	15-Oct-07	37	200

Note: - no recurrence; N/D not determined.

Table S2 Patient characteristics according to MMF use.

No	Characteristics	MMF use		*P*-value[a]
		No (%/Median)	**Yes** (%/Median)	
1	Age	56.50	58.00	0.383
2	Sex (% male)	23/32 (71.9%)	10/12 (83.3%)	0.698
3	Recurrence*	15/32 (46.9%)	1/12 (8.3%)	0.032*
4	Death**	18/32 (56.2%)	1/12 (8.3%)	0.006**
5	Size of tumor	2 cm	2 cm	0.890
6	Number of lesions	2	2	0.808
7	Differentiation - Good - Moderate -Bad	 9/31 (29.0%) 22/31 (71.0%)	 3/11 (27.3%) 8/11 (72.7%)	1.000
9	Vaso - invasion	9/30 (30%)	1/11 (9.1%0	0.238
10	AFP before resection	35	15	0.408
11	Prednisone use after LTx[b]	26/31 (83.9%)	10/12 (83.3%)	0.644
12	Follow up time	130 weeks	114.5 weeks	0.663

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Mann Whitney test

[b]LTx = Liver transplantation, given around 3 months following LTx

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Chapter 8

Summary and Discussion

The only potentially curative treatment options for hepatocellular carcinoma (HCC) are surgical resection and, for patients who are not eligible for resection, liver transplantation (LT), although the majority of HCC patients, in fact, are not eligible for either resection or transplantation. However, disease recurrence is the main challenge to the success of these treatments and thus further studies are urgently called for. The present thesis tries to make a contribution here.

Molecular targeted therapy against HCC provides only temporary clinical efficacy. For patients with advanced disease, which represent the majority of patients at diagnosis, the only major option is sorafenib (Nexavar), an oral multi-kinase inhibitor, which increases patient survival by approximately 3 months[1]. I reasoned that, further understanding of the molecular biology and pathogenesis of HCC would provide clues for optimizing current therapies and developing novel theraupetic approaches providing enhanced survival benefit for these patients and this train of thought has been the guiding motif of the work described in this thesis.

Pivotal role of mesenchymal stromal cells in the microenvironment of liver cancer

Remodeling of the liver microenvironment is a hallmark in the pathogenesis of liver cancer[2]. Co-evolution of tumor cells with their microenvironment during tumorigenesis suggests that tumor-stroma crosstalk may likely influence the phenotype of tumor cells and may provide a selective pressure for tumor initiation, progression and metastasis[3]. In addition, the liver provides a distinct immunological environment and the ultimate effects of this environment on cancer progression may differ in the liver as compared to other organs[4]. Looking at the pathogenesis of HCC in which the major risk factors include

infection with HBV or HCV[5], carcinogenesis might develop in particular when chronic infection with HBV or HCV provokes activation of regulatory feed back loops that include the repeated recruitment of MSCs to the liver. In turn, secreted factors by these cells can suppress immune system and promote tumor growth. A detailed literature investigation that supports my view in this is provided in this thesis (**Chapter 2**). However, despite a significant body of scientific literature on this subject it is still not clear whether MSCs in liver tumor are recruited locally (thus true liver MSCs) or that they derive from the circulation and represent cells that were originally released from the BM (BM MSCs). Indeed, both sources might act in conjunction. In response to injury or infection, MSCs can be released from BM into the blood circulation and migrate towards the injured sites to promote tissue regeneration[6]. High circulating levels of BM originated cells, such as endothelial progenitor cells, have been observed in HCC patients, which can conceivably subsequently home into the tumor and promote tumor growth [7]. In addition, in experimental rodents intravenous infusion of MSCs results in the accumulation of these MSCs into the lung, the spleen and the liver which already have high endogenous levels of MSCs. Especially enriched with MSCs become orthotopic HCC tumors, when compared to lung and spleen, indicating that liver tumors are able to recruit MSCs with high efficiency [8]. Together, the case for MSCs as an important factor contributing to the progression of HCC seems compelling.

Indeed, even although the role of MSCs in the tumor microenvironment has dualistic characteristics (in general the attenuation of the inflammatory process will counteract neoplastic transformation), I provide good experinmental evidence that apparently in liver cancer and especially in HCC the tumor promoting role is more prominent than the tumor suppressive role (**Chapter 3**). Their existence in the tumor environment of liver cancer was initially suggested through studies showing that MSCs are enriched in human liver tumor compartment and could exert trophic effects on tumor cells. Genome-wide gene expression array and pathway analysis confirmed the up-regulation of cell growth and proliferation-related processes and down-regulation of cell death-related pathways by treatment of MSC-conditioned medium (CM) in Huh7 cells. Similar results I found also with respect to the effect of MSCs in CRC metastasis to the liver (**Chapter 4**). Trophic factors produced by MSCs can favor the growth of metastasic CRC cells. This may help to understand the role of MSCs in

favoring CRC liver metastasis. Thus a picture emerges that especially in the liver the MSCs shape the immunological and growth factor landscape to favour neoplastic processes.

MSCs have been extensively investigated in clinical trials to treat various diseases, including for treating cancer (NCT02068794; NCT02079324; NCT01983709). Although MSCs have not been used for treating liver cancer yet (to our knowledge), over 30 trials have been registered at ClinicalTrials.gov for treating various liver diseases. Such studies almost unavoidably involve patients who are positive for hepatitis B or C virus infection, or liver transplant recipients. These infections are, however, important drivers of cirrhosis and HCC [9]. In addition, HCC is an important indication for liver transplantation and liver transplant patients also have increased incidence of de novo cancer. Thus, I call for caution of using MSCs to treat liver cancer or even premalignant liver diseases. In fact, tumor MSCs may represent as anticancer targets, given their potent immunosuppressive and tumor promoting properties, therefore targeting tumor MSCs in HCC may represent a new avenue for therapeutic intervention. Antibodies recognizing tumor MSC-specific epitopes provide an obvious opportunity here and I feel that in view of the results presented in this thesis studies aimed at identifying such epitopes are urgently needed. Lacking progress here, are the technical difficulties associated with such studies

Pivotal role of SMAD4 in the tumor microenvironment of liver cancer

The unique etiology of HCC in conjunction with the distinct environment that the liver provides with regard to the growth of oncogenenic processes suggests that differential signaling network compared to other cancers may exist in HCC as compared to other cancers. Such specific signaling networks may allow rational design of novel therapeutic avenues to combat disease. This thesis provides evidence that this may indeed be the case. SMAD signaling appears important in the tumor microenvironment of liver cancer. SMAD4 was originally identified as a candidate tumor suppressor gene and SMAD4 loss or inactivation is associated with several types of cancers. However, a drastic elevation of nuclear SMAD4 levels was observed in patient HCC tumors (**Chapter 5**). High SMAD4 expression converts signaling through this protein towards tumor promoting effects, probably due to the simultaneous elevation of p-SMAD2/3 in a subset of HCC patients. This indicates a role for TGFB signaling as a tumor promoting factor in HCC. In apparent

agreement, at a later stage in disease progression, liver tumor cells produce large amounts of TGFB[10]. HBV encoded oncoprotein pX, which increases Smad4 nuclear translocation and amplifies TGFB signaling[11], is one of possible mechanisms to explain the why in liver cancer SMAD4 has different behavior as compared to other cancers where SMAD4 predominantly acts as a tumor suppressor. This is an unexpected result in a view of the dogma that SMAD4 is a potent tumor suppressor and thus these results have certainly shed a new light on the molecular biology of HCC. The challenge now is to translate this finding into novel therapeutic approaches.

Importantly in support of my interpretation of the role of SMAD signaling in liver cancer is that my findings do not necessarily contradict to previous findings in other tumors types. A possible explanation for the tumor type specificity of the role for SMAD signaling I detected, is that SMAD4 in liver cancer is highly associated with the presence of other SMAD components. SMAD4 can also mediate anti-tumor signaling by coupling to SMAD1/5/8-dependent BMP signaling. Intriguingly, this complex is absent in the majority of HCC patients.

In addition, downregulation of BMP signaling components in patient HCC tumors was associated with hepatitis history (**Chapter 6**). I did not find evidence that BMP signaling can directly modulate HCV infection, and thus the effect of BMP signaling appears connected to carcinogenis *per se*, rather as in the initiation of oncological disease. Others reported an increase in a set of genes of cognate BMP inhibitors (GPC3, GREM1, FSTL3, and FST) in HCC tumor samples[12], which might be the reason why BMP signaling is mostly inactivated in HCC, but this awaits further study. Furthermore, the exact mechanism as to how hepatitis infection interacts with BMP signaling needs to be further explored. Most importantly, SMAD molecules may have potential as outcome predictors for patient stratification and are targets for therapeutic development in personalized medicine.

Mycophenolic acid represents a potential favoured immunosuppressant for HCC patients after liver transplantation

Liver transplantation is an effective treatment for HCC. However disease recurrence following LT remains a major challenge. The use of immunosuppressants following LT is known to be an important risk factor for HCC recurrence. Immunosuppressants are widely

used after LT to reduce the risk of graft rejection. Beside a general impairment of the immunosurveillance system, different types of immunosuppressants could have other distinct mechanisms that are independent of the host immunity to affect the process of malignancy [13-16]. Current research is exclusively focusing on the mammalian target of rapamycin (mTOR) inhibitors, including rapamycin (sirolimus) and everolimus. They are thought to be the only class of immunosuppressive agents that may reduce HCC recurrence reported by some retrospective or meta-analysis studies [17-20]. However, these studies do not provide firm evidence to conclude the superiority of mTOR inhibitors on HCC recurrence [21].

Our study demonstrated a specific effect of immunosuppressant mycophenolic acid (MPA) in limiting HCC cell growth by targeting IMPDH2 in experimental models. More importantly, we found that MPA is strongly associated with reduced disease recurrence in liver transplanted HCC patients and with significantly improved patient survival (**Chapter 7**). Although this is a single center, retrospective study with relatively small number of patients, the potent protective effects on HCC recurrence that we have observed for sure warrant prospective clinical trials for further validation.

Not only on tumor cells, MPA can also effectively modulate the tumor microenvironment. MPA has been shown to potently inhibit endothelial cell and fibroblast proliferation, invasion/migration[22]. Moreover, genomic data analysis provide a molecular basis for the anti-angiogenic and anti-fibrotic effects of MPA [22]. Interestingly, MPA can inhibit MSCs proliferation and affect their multilineage differentiation at therapeutic doses [23]. These results suggest that inhibition of tumor MSCs by MPA may also occur in patients and contribute to its anti-tumor activity.

Although MPA will likely not be used as anti-cancer treatment alone, dissemination of its anti-HCC effects and mechanism promises to be particular helpful for the optimal management of HCC patients after LT especially to reduce the risk of HCC recurrence.

Perspectives

- Despite the fact that MSCs have mainly a tumor promoting role in liver cancer, they also have tumor suppressive actions which are described in chapter 2. Therefore, further laboratory and clinical investigations are warranted to devise strategies for making MSCs more effective with regard to their anti-tumor action. If successful, MSCs could become a preventive therapy for chronic hepatitis leads to liver cancer since MSCs are recruited continuously in the setting of chronic infection.

- Discovery of the tumor promoting function of SMAD4 in HCC provides another novel avenue of combating liver cancer, especially in the context of viral hepatitis that is associated with increased SMAD4 levels. The hepatitis B encoded oncogene, which provokes nuclear SMAD4 accumulation provides another target for novel therapy for HCC prevention.

- MPA represents a promising option of immunosuppressive therapy for HCC patients after LT, because of tis anti-HCC function. Thus, prospectively designed trials are warranted to further validate our findings.

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Publiekssamenvatting in het Nederlands

De lever is een belangrijk en veelzijdig orgaan. Met een massa van anderhalve kilogram en 20% van het zuurstofverbruik is de lever het actiefste, na de huid het zwaarste en na de hersenen wellicht ook het veelzijdigste orgaan. De lever is uniek onder de andere organen, in die zin dat een beschadigde lever weer regenereert. Anders ligt het wanneer het uiterst fijn opgebouwde leverweefsel verwoest wordt en overwoekerd wordt door bindweefsel. Zo'n verschrompeling van de lever (cirrose) kan zeer veel verschillende oorzaken hebben, waaronder alcoholisme en virale hepatitis (waaronder met name hepatitis B en C), en vormt vaak het voorstadium van leverkanker. De eerste symptomen verschijnen vaak pas laat. Een vol gevoel in de leverstreek, vermoeidheid, slechte eetlust , geelzucht en gewichtsverlies. Binnen afzienbare tijd, als de tumor gegroeid is, kan bij palpatie de tumor gevoeld worden. Patiënten kunnen ook klagen over pijn in de bovenbuik. De prognose is, net als bij alle kankers, afhankelijk van de stadium van de ziekte. Bij een primaire levertumor zowel als bij uitzaaiingen zijn de vooruitzichten echter over het algemeen niet goed. In dit proefschrift heb ik een bijdrage proberen te geven aan beter begrip en behandeling van leverkanker. Hoofdstuk 1 beschrijft de exacte kaders en motivaties die mijn promotieonderzoek hebben gevormd.

Hiertoe voer ik in hoofdstuk 2 een literatuurstudie uit naar een celtype in de omgeving van de kankercel, de mesenchymale stamcel. Deze cellen zijn biologische erg actief en werken met name ontstekingsremmend. Interessant is, is dat mesenchymale stamcellen specifiek lijken op te hopen in en rond levertumoren. Omdat mesenchymale stamcellen buiten het lichaam gekweekt en gemanipuleerd kunnen worden is het dus denkbaar om ze in het laboratorium eerst te voorzien van anti-kanker genen en vervolgens terug te geven aan de patiënt. Uit mijn literatuur studie blijkt echter dat mesenchymale stamcellen van nature het kankerproces ondersteunen en ik roep dus op tot voorzichtigheid in het doen van klinische testen met deze cellen. Deze bezorgdheid werd helaas bewaarheid in hoofdstuk 3 en hoofdstuk 4 waar ik experimenteel het effect van mesenchymale stamcellen op primaire leverkanker alsook op in de lever groeiende uitzaaiingen van dikke darm kanker onderzocht door ze te transplanteren naar muizen waar ik experimenteel kanker in veroorzaak. Het blijkt dat de kankers sneller groeien. Ik moet dus concluderen dat

mesenchymale stamcellen in het geheel niet geschikt zijn om kanker mee te behandelen. In hoofdstuk 8 betoog ik echter wel dat de stamcellen zelf geschikt zijn als doelwit van antikankerbehandeling. Antilichamen bijvoorbeeld die specifiek mesenchymale stamcellen neutraliseren zouden een rol kunnen spelen bij de behandeling van leverkanker.

In hoofdstuk 5 richt ik mijn aandacht op de leverkankercel zelf. Hierbij ontdek ik dat leverkankercellen andere eigenschappen hebben in vergelijking tot kankercellen elders uit het lichaam. Waar zogenaamde SMAD signalen normaliter een belangrijke rol spelen bij het voorkomen van kanker, blijken deze zelfde SMAD signalen in de lever juist te leiden tot stimulatie van het oncologisch proces. Met deze kennis kan gepoogd worden therapie te ontwikkelen die juist gericht op het remmen van SMAD signalen en op deze wijze de ziekte te bestrijden. In hoofdstuk 6 diep ik deze materie nog verder uit en kijk ik met name hoe deze signalen zich verhouden tot de (vermoedelijke) oorzaak van de kanker (virale hepatitis versus andere oorzaken, zoals overmatig alcoholgebruik).

In het laatste experimentele hoofdstuk, hoofdstuk 7, richt ik mij op wat er gebeurd na de behandeling. Leverkanker wordt vaak bestreden door chirurgische verwijdering van lever, en transplantatie van een donorlever. Door de verschillen in immuunmoleculen tussen de donorlever en die aanwezig in de patiënt, wil het lichaam de nieuwe lever afstoten. Om dit te voorkomen wordt immunosuppressie gebruikt. Er zijn vele soorten immunosuppressie en er wordt al lang vermoed dat de keuze van het immunosuppressief de kans op het terugkomen van de kanker kan beinvloeden. Zowel door biostatistische analyse van patiëntendossiers alsook gebruik van diermodellen en celcultuurstudies laat ik zien dat gebruik van het het immuunsuppressief mycofenolzuur veel betere resultaten oplevert, met betrekking tot de groei van leverkanker, in vergelijking tot andere medicatie. Het lijkt dus aanbevelingswaardig om juist deze medicatie te gebruiken na transplantatie van levers bij patienten met leverkanker. Deze resultaten worden nog een uitvoerig tegen het licht gehouden in hoofdstuk 8. Uiteindelijk heeft mijn proefschrift dan ook nieuw inzichten opgeleverd zowel wat betreft de moleculaire mechanismen in de kankercel, haar interactie met omgeving van de kanker cel alsook de interactie van de kankercel met medicatie die gebruikt wordt bij behandeling.

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Curriculum Vitae

Pratika Yuhyi Hernanda was born in October 22, 1979, in Lumajang, East Java province, Indonesia. She grew up and attended elementary, middle and high school at her hometown, Surabaya, East Java, Indonesia.

In 1997, she started her study of medicine at the Medical Faculty of Airlangga University, Surabaya Indonesia. Soon after her graduation in 2004, she worked at Wijaya Kusuma University, faculty of Medicine, as a lecturer and also researcher in the laboratory of medical genetics and doing research in the field of medical genetics, especially on Down syndrome and thalassemia, under supervision of Prof. S.M Soeatmadji. Then she took a study leave to enroll in the master programme on Genetic Epidemiology, at the department of Epidemiology, Erasmus Medical Centre Rotterdam, The Netherlands in 2007, which required 1 year of full time study. She subsequently went back to Indonesia and continued her work as a lecturer and researcher at the same institution, and she retains a formal position thereup till now. Highly motivated to succeed, in 2010 she did laboratory training abroad to do research on thalassemia, at one of the best hemoglobinopathy centres in the world, the Hemoglobinopathy laboratory of Leiden University Medical Centre (LUMC), The Netherlands. Those experiences are truly indicative of her motivation for success.

In 2012, she started her PhD programme at the department of Gastroenterology and Hepatology, Erasmus Medical Center Rotterdam, The Netherlands, to carry out research on liver cancer. Under supervision of the remarkable professor Prof. Maikel .P Peppelenbosch and the sharp-minded, young-talented researcher Dr. Qiuwei Pan, she focused on the role of mesenchymal stromal cells and SMADs family in liver cancer.

PhD Portfolio

Name PhD Student	Pratika Yuhyi Hernanda
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	March 2012 - October 2014
Promotors	Prof.dr. M.P Peppelenbosch
Copromotor	Dr. Q. Pan

General Courses

- Partek transcriptomics- NGS and microarray analysis courses
- Analysis of microarray gene expression data using R/BioC and web tools course
- Adobe Photoshop CS5 workshop
- EMBL-EBI Roadshow
- Basic and translational oncology course
- Grant proposal writing workshop
- Basic SPSS course

Oral Presentations at National Conferences

- 2014, Nederlandse Vereniging voor Gastroenterologie (NVGE) Voorjaarcongres 2014. Veldhoven, The Netherlands.
- 2013, Nederlandse Vereniging voor Gastroenterologie (NVGE) Voorjaarcongres 2013. Veldhoven, The Netherlands.

Poster Presentations at International / National Conferences

- 2014, The 49th Annual Meeting of the European Association for the Study of the Liver (EASL), London, United Kingdom
- 2014, The 19th Annual Molmed Day 2014, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands.
- 2013, The 48th Annual Meeting of the European Association for the Study of the Liver (EASL), Amsterdam, The Netherlands
- 2013, The 19th Annual Molmed Day 2013, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands

Academic Awards

Best Abstract Awards:

- 2013, Nederlandse Vereniging voor Gastroenterologie (NVGE) Voorjaarcongres 2014. Veldhoven, The Netherlands (€500)

Young Investigator Travel Awards:

- 2013, The 48th Annual Meeting of the European Association for the Study of the Liver (EASL), Amsterdam, The Netherlands

Publications

International (refereed) journals

1. **PY. Hernanda**, A. Pedroza-Gonzalez, D Sprengers, M.P. Peppelenbosch, Q. Pan. Multipotent *Mesenchymal stromal cells in liver cancer : implications for tumor biology and therapy* (Resubmitted with revisions to BBA, Reviews on Cancer)

2. **P.Y. Hernanda**[#], Kan Chen[#], A.M. Das[#], K. Sideras[#], S.J.A. Bots, Liudmila L. Kodach, R.A. de Man, J.N.M Ijzermans, H.L.A. Janssen, D. Sprengers, M.J. Bruno, H. Metselaar, T.L.M. ten Hagen, J.Kwekkeboom, M.P. Peppelenbosch, Q. Pan. *SMAD4 acts as a tumor promoter in hepatocellular carcinoma*. (Oncogen, conditionally accepted).

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6. **P.Y Hernanda**, L. Tursilowati, S.G.J Arkesteijn, I.D.G Ugrasena, M.C.S Larasati, S.M Soeatmadji, P.C Giordano, and C.L Harteveld. *Towards a prevention program for beta thalassemia. The molecular spectrum in East Java, Indonesia*. Hemoglobin. 2012, (36) 1:1-6.

National journals and research reports

1. S. L Utami, **P.Y Hernanda**, R.D Wulandari. *Aplikasi ARMS Amplification Refractory Mutation System) Untuk Identifikasi Jenis Mutasi Dan Skrining Thalassemia Beta Studi Pada Penderita Dan Keluarga Inti (nuclear Famili)*. Indonesian Science and Technology Digital Library. Rep. 2012. 13/1450.

2. **P.Y Hernanda**, Arwin Munawariko, S. M Soeatmadji. *Cytogenetic Analysis of Mozaic Turner Syndrome*. Journal of Indonesian Medical Association. 2011, (61) 8.

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5. **P.Y Hernanda**. *A Genomic Study of Determinants of Successful Treatment in Hypertension*, Thesis of Master of Science in Genetic Epidemiology, Erasmus Medical Centre, The Netherlands, 2008.

6. **P.Y Hernanda**, Marmi. *Insidensi Sindroma Down Di Pendidikan Sekolah Luar Biasa Alpa Kumara Wardhana I Dan II Surabaya*. Indonesian Science and Technology Digital Library. Rep. 2006. 10/0492.