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**Original Paper**

# Bone morphogenetic protein 4 is induced in hepatocellular carcinoma by hypoxia and promotes tumour progression

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## Abstract

**Striking similarities exist between molecular mechanisms driving embryonic liver development and progression of hepatocellular carcinoma (HCC). Bone morphogenetic proteins (BMPs), particularly BMP4, have been proposed to regulate embryonic hepatic development. BMP expression has been observed in neoplasia but the expression and biological role of BMP4 in human HCC are unknown. We found increased *BMP4* mRNA and protein in HCC cell lines and tissue samples compared to primary human hepatocytes and corresponding non-tumourous tissue. Hypoxia further induced BMP4 expression in HCC cells, which was abolished by transfection of a dominant negative form of HIF-1alpha (dnHIF-1alpha). However, gel shift assays revealed only minor binding activity in nuclear extracts from (hypoxic) HCC cells to a putative hypoxia-response element in the *BMP4* promoter. Sequence analysis of the *BMP4* promoter revealed two Ets-1 binding sites, and Ets-1 activity was increased in HCC cells under hypoxic conditions. Transfection of dnHIF-1alpha completely abrogated hypoxia-induced Ets-1 activity as well as BMP4 expression. Overexpression of Ets-1 markedly enhanced *BMP4* promoter activity, while antisense Ets-1 almost completely abolished basal as well as hypoxia-induced BMP4 expression. These data demonstrate that Ets-1 activity contributes to baseline expression of the *BMP4* gene and is the predominant mediator of the HIF-dependent BMP4 induction under hypoxic conditions. To determine the functional relevance of BMP4 expression, HCC cell lines were treated with antisense BMP4 constructs or siRNA against BMP4. BMP4 suppression resulted in a strong reduction of the migratory and invasive potential and anchorage-independent growth. Furthermore, tube formation assays indicated that BMP4 expressed by HCC cells promotes vasculogenesis. Our findings demonstrate that BMP4 is increased in HCC and promotes HCC progression. Therefore, BMP4 expression may have clinical relevance, and interfering with BMP4 signalling appears as an attractive therapeutic target for this highly aggressive tumour.**



**Keywords: hepatocellular carcinoma; bone morphogenetic protein 4; transcriptional regulation; invasion; ets-1; hypoxia**

## Introduction

Bone morphogenetic proteins (BMPs) have been linked to several aspects of embryonic development including regulation of cell proliferation, differentiation, chemotaxis, the establishment of the basic embryonic body plan, and morphogenesis of organs [1]. Like other members of the transforming growth factor-beta (TGF-beta) superfamily, BMPs elicit their cellular effects via specific membrane receptors. Activated BMP type I receptor phosphorylates receptor-regulated SMAD1/5/8 proteins, which can then assemble into heteromeric complexes with SMAD4, translocate into the nucleus, and regulate the transcription of target genes [2].

In clear contrast to normal cells, carcinoma cells derived from several organs have been shown to express BMPs, and several groups including our own have reported that aberrations in BMP signalling play an important role in the pathogenesis of human cancer [3–8]. However, there is growing evidence that BMPs may play important roles as tumour suppressors

as well. One of the most evident examples is BMP4. On the one hand, BMP4 has been shown to positively regulate proliferation, resistance to apoptosis, migration or invasion of carcinoma cells derived from several organs such as the breast [9], ovary [10], pancreas [11,12], melanoma [3], and colon [13]. In addition, induction of angiogenesis by endothelial cells in the tumour environment was demonstrated [14]. On the other hand, BMP4 inhibits tumourigenesis in glioblastomas [15], lung adenocarcinoma [16], and myeloma cells [17].

Primary hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third most common cause of cancer-related deaths in the world. Striking similarities have been observed between molecular mechanisms driving embryonic liver development and HCC progression [18,19]. BMP signalling has been proposed to regulate embryonic hepatic development, and BMP4 in particular has been shown to be critically involved in the process of liver bud formation, the proliferative outgrowth from the ventral foregut endoderm, and the subsequent invasion of primitive hepatic cells into the septum transversum mesenchyme [20–22]. However, BMP expression levels have not been analysed in HCC, and despite their significant morphogenetic activities during embryogenesis, the biological role of BMPs in human HCC has not been investigated. As noted above, BMP4 has been studied in several cancers, with contradictory results [10,11,14,15,23,24]. In HCC, information on BMP4 is missing so far. We therefore focused our attention on BMP4 regulation, expression, and function in human HCC, and investigated the biological role of BMP4 signalling in hepatocarcinogenesis.

## Materials and methods

### Cells and cell culture

The human HCC cell lines HepG2 (ATCC HB-8065), Hep3B (ATCC HB-8064), and PLC (ATCC CRL-8024) were cultured as previously described [25]. Primary human hepatocytes (PHHs) were isolated and cultured as previously described [26]. Human liver tissue for cell isolation was obtained according to the guidelines of the charitable state-controlled foundation HTCR with the patient's informed consent. Hypoxia was induced by incubation with 100 μM DP (2,2′-dipyridyl; Sigma Deisenhofen, Germany) or exposure to 0.5% $O_2$ for the indicated periods of time.

### Human HCC tissue

Corresponding primary HCC tissue and non-neoplastic liver tissue were obtained from HCC patients undergoing surgical resection. In all cases, HCC had developed in cirrhotic livers but at the time of liver resection, patients presented in a compensated state (child A cirrhosis). Further clinicopathological patient characteristics are summarized in Table 1. Tumourous as well as non-tumourous tissue specimens were collected from vital tissue and processed without delay, eg immediately snap-frozen and stored at −80 °C.

### Immunohistochemistry

Paraffin-embedded preparations of normal liver and HCC were screened for BMP4 protein expression by the avidin–biotin complex (ABC) method (DAKO-LSAB2-Kit, DAKO, Hamburg, Germany) as described in detail previously [3].

### RNA isolation and reverse transcription

Total cellular RNA was isolated from cultured cells and tissue samples using the RNeasy kit (Qiagen, Hilden, Germany) and cDNAs were generated as previously described [3].

### Analysis of BMP4 expression by quantitative PCR

*BMP4* and *CD31* mRNA expression was analysed by quantitative real-time PCR (qRT-PCR) on a Lightcycler (Roche, Mannheim, Germany) as described in refs 3 and 14, using primers specified for qRT-PCR. All experiments were repeated at least thrice.

### Transfection experiments

*BMP4* promoter reporter constructs were generated by cloning a human gene fragment of 625 bp (including the putative hypoxia-responsive element (HRE) and the Ets-1 binding sites, see Figure 2B) into the reporter gene plasmid pGL3 basic (Invitrogen, Karlsruhe, Germany); the Ets-Luc construct has been previously described [27]. Cells ($2 \times 10^5$ per well) were seeded into six-well plates and transfected with 0.5 μg of reporter constructs using lipofectamine plus (Invitrogen). Co-transfections were performed using a dominant negative hypoxia-inducible factor (HIF)-1alpha construct, which contained the bHLH (basic-helix-loop-helix) and PAS (Per-ARNT-Sim) domains, but no transactivation domain [28], or a sense and antisense Ets-1 construct in pcDNA3 [27], respectively. Hypoxia was induced by incubation with 100 μM DP for 24 h. Subsequently, cells were lysed and the luciferase activity was measured. To normalize for transfection efficiency, 0.2 μg of a pRL-TK plasmid (Promega, Mannheim, Germany) was co-transfected and renilla luciferase activity measured by a luminometric assay (Promega).

Two different siRNAs (small interference RNAs) against BMP4 (Qiagen) and an antisense BMP4 expression plasmid [3] were transfected using HiPerfect (Qiagen) or lipofectamine plus. The transfection efficiency of siRNA in Hep3B and PLC cells was approximately 90% (89.4–91.7%), as measured by fluorescence-activated cell sorting analysis applying Alexa Fluor 488-labelled control siRNA (Qiagen). All transfection experiments were repeated at least three times.

### Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from the cultured cells by the method of Dignam *et al* [29]. Double-stranded oligonucleotides comprising the HRE of the human *phosphoglycerate kinase 1* gene [with or without inactivation of the binding site for the hypoxia-inducible factor (HIF)] or the putative HRE of the *BMP4* gene were used (HRE for: 5′-GGA TCT GTG AGA CGT GCG GCT TCC GTT T; HRE rev: 5′-AAA CGG AAG CCG CAC GTC TCA CAG ATC C; BMP4-HRE for: 5′-CCT CCG CAC GTG GTC CCC AGG TGA GCC; BMP-HRE rev: 5′-GGC TCA CCT GGG GAC CAC GTG CGG AGG). The BMP4-HRE oligonucleotide corresponded to a region from −172 to −204 upstream to the transcription start of the *BMP4* promoter. The oligonucleotides were end-labelled with T4-polynucleotide kinase (Roche) and [$\gamma$-$^{32}$P]ATP (Amersham, GE Healthcare, Munich, Germany). Band shifts were performed by incubating 5 μg of the nuclear extract in the binding assay mix [1 μg/μl poly(dI-dC)·(dI-dC), 10% glycerol, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 50 mM KCl, 20 mM HEPES–KOH (pH 7.9), 0.1% NP-40] with the DNA probe before separation on a 4% non-denaturing polyacrylamide gel at 250 V for 1.5 h. As a control for binding of the HIF complex *in vitro* transcribed and translated (IVTT), HIF-1alpha and HIF-1beta protein was used.

### Enzyme-linked immunosorbent assay (ELISA)

For protein preparation, 500 000 cells were washed in 1× PBS and lysed in 120 μl of RIPA buffer (Roche). The protein concentration was determined using the BCA™ protein assay kit (Pierce, Rockford, IL, USA). The amount of BMP4 protein was determined using a DuoSet® BMP4 ELISA (R&D Systems) according to the manufacturer's instructions. The BMP4 concentration was calculated based on the total protein amount.

### Migration and invasion assay

Migration and invasion assays were performed as previously described [27]. Briefly, migration was assessed in Boyden chambers containing polycarbonate filters with an 8 μm pore size (Costar, Bodenheim, Germany) coated with gelatin. The lower compartment was filled with fibroblast-conditioned medium used as a chemoattractant, and the filter was placed above. Transfected HCC cells were harvested by trypsinization, resuspended in DMEM without FCS, and 800 μl of the cell suspension at a density of $2 \times 10^5$ cells/ml was placed in the upper compartment of the chambers. After incubation at 37 °C for 4 h, the filters were removed and the cells were fixed, stained, and counted. For invasion assays, the filters were coated with a commercially available reconstituted basement membrane (Matrigel, diluted 1 : 3 in $H_2O$; Becton Dickinson, Heidelberg, Germany). Each condition was assayed in triplicate and assays were repeated at least twice.

### Anchorage-independent growth assay

To study anchorage-independent growth, we performed colony formation assays as described in detail previously [30]. HCC cells were transfected with antisense BMP4 (asBMP4) or siRNA against BMP4, and subsequently colony formation was observed in comparison to control transfected cells. After an incubation period of 10 days, the colonies were photographed and the size of the colonies was measured. For each condition, the diameters of ten colonies were determined and the assays were repeated at least twice.

### Matrigel tube formation assay

Tube formation assays were performed as previously described in detail [14]. Briefly, human microvascular endothelial cells (HMECs) were treated with supernatants of HCC cells transfected with asBMP4 expression constructs or siRNA against BMP4, as well as control transfected cells. The tube formation of each condition was documented by five photographs taken with five-fold magnification. Furthermore, four pictures were taken with 2.5-fold magnification to quantify the number of tubes formed. Each experiment was repeated at least twice.

### Statistical analysis

Statistical analyses were performed using SPSS version 10.0 (SPSS, Chicago, IL, USA) and GraphPad Prism Software (GraphPad Software, Inc, San Diego, USA). Results are expressed as mean ± SEM (range) or per cent. Comparison between groups was made using the Student's paired $t$-test. A $p$ value of less than 0.05 was considered significant. Contingency table analysis and the two-sided Fisher's exact test were used to study the statistical association between clinicopathological and immunohistochemical variables.

## Results

### BMP4 expression in hepatocellular carcinoma

Initially, we analysed the expression of BMP4 in three HCC cell lines (PLC, Hep3B and HepG2) in comparison to primary human hepatocytes (PHHs). Quantitative RT-PCR analysis revealed marked up-regulation of BMP4 expression in all three HCC cell lines (Figure 1A). To further address BMP4 expression in HCC *in vivo*, we examined a panel of 39 paired tissue specimens obtained from patients with HCC. BMP4 was found to be up-regulated in 24 (62%) HCC tissues compared with surrounding non-tumourous liver tissue of the same donor (Figure 1B).

For descriptive data analysis, clinicopathological characteristics were compared in patients with and without increased BMP4 expression in HCC (Table 1). It was noteworthy that *BMP4* mRNA expression was significantly associated with higher tumour stage

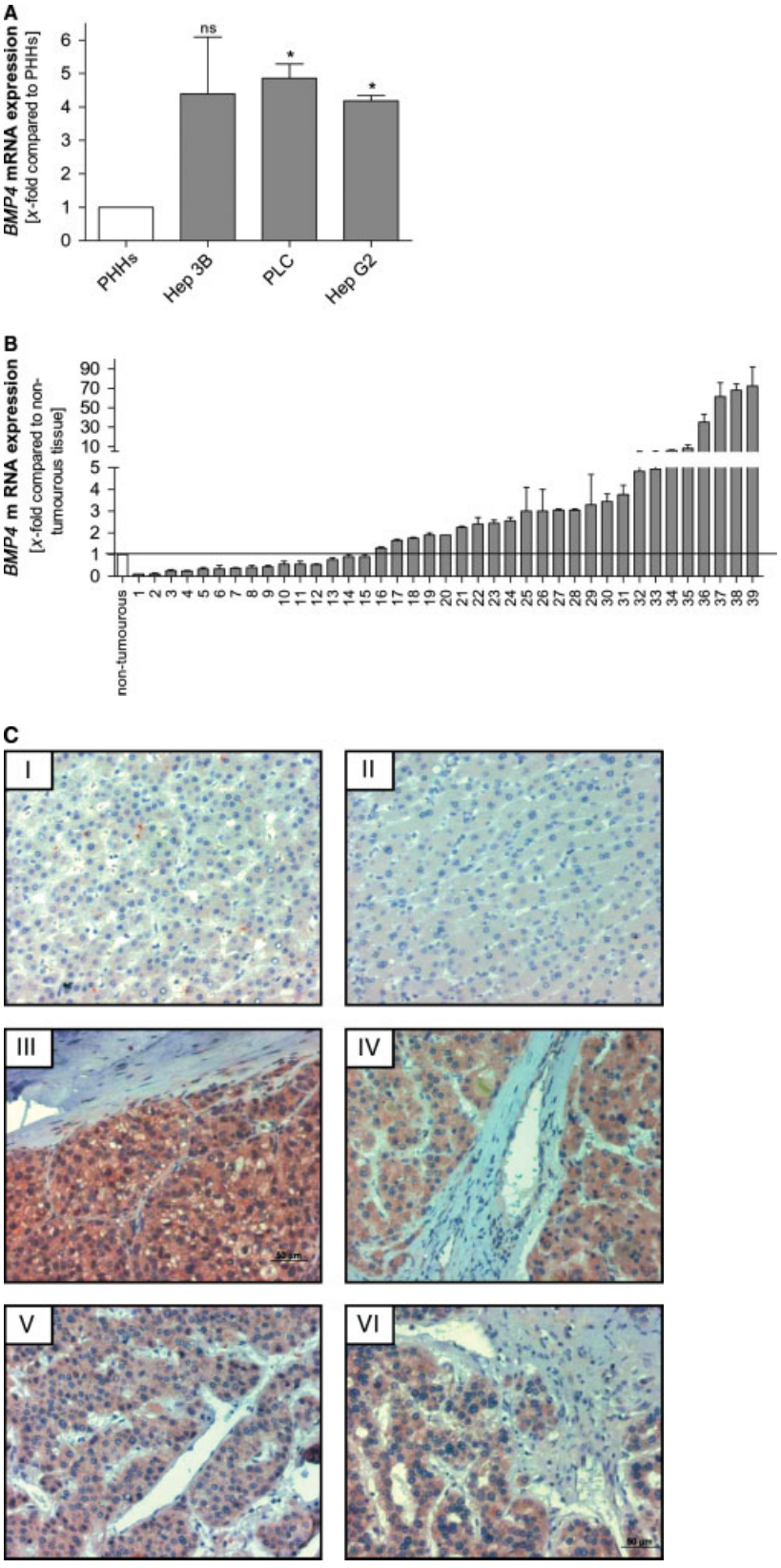


**Figure 1.** Expression of BMP4 in HCC. (A) Expression of *BMP4* mRNA was analysed by qRT-PCR in the HCC cell lines PLC, Hep3B, and HepG2, and in primary human hepatocytes (PHHs). HCC cell lines showed strong induction in BMP4 expression compared with PHHs (*$p < 0.05$ compared with PHHs; PHHs = 1). (B) *BMP4* mRNA expression in human HCC tissue specimens of 39 patients relative to corresponding non-tumourous tissue samples as determined by quantitative RT-PCR (non-tumourous tissue = 1). BMP4 expression was enhanced in 24 HCC tissues (62%) compared with surrounding non-tumourous liver tissue. (C) BMP4 protein expression was analysed by immunohistochemistry in normal liver and five HCC tissues. Strong expression of BMP4 was confirmed in epithelial cells of HCC tissues as shown in representative pictures (original magnification 200×) of two normal liver tissues (I + II) and four HCC tissues (III–VI)

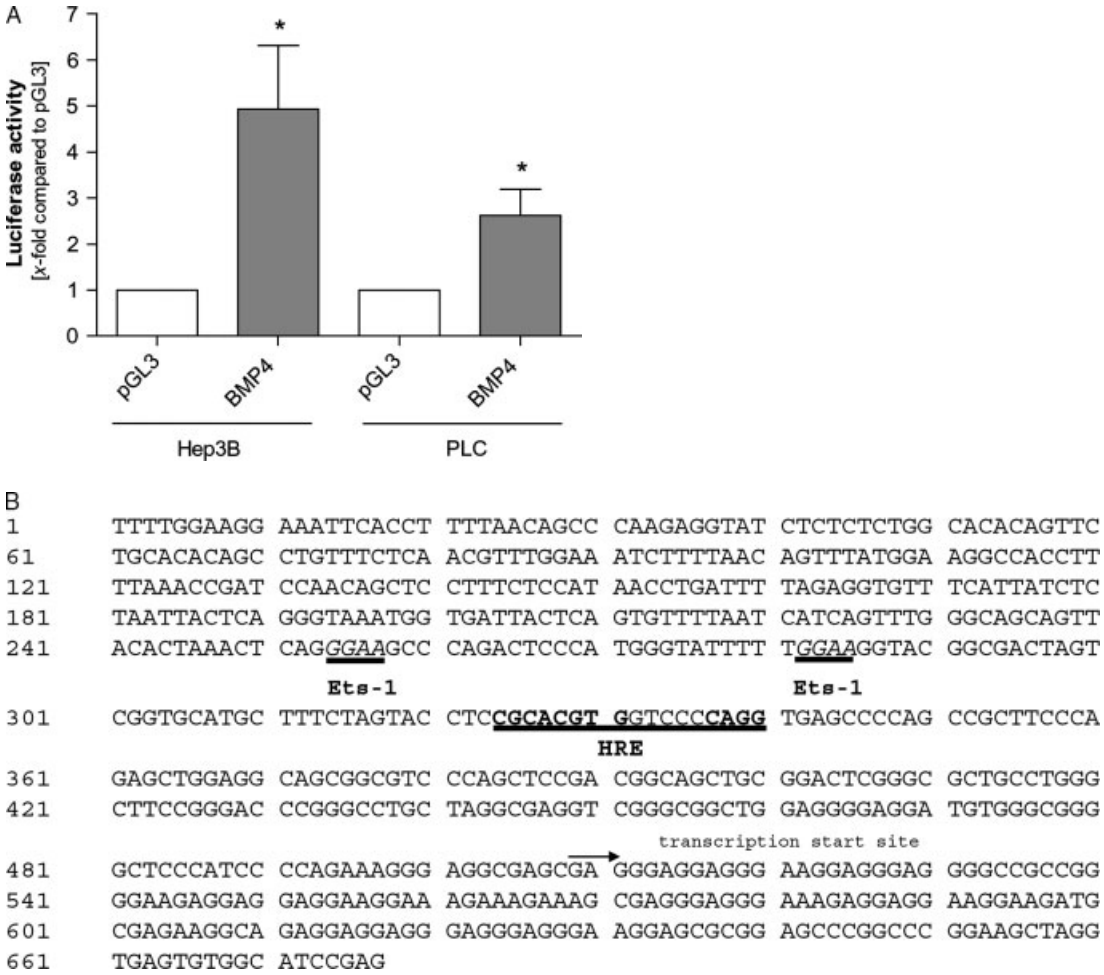


**Figure 2.** *BMP4* promoter activity in HCC. (A) Activity of the sub-cloned human *BMP4* promoter was assessed using reporter gene assays. Strong activity was observed compared with pGL3 as control transfection in PLC and Hep3B cell lines. (*$p < 0.05$ compared with pGL3; pGL3 = 1; data are expressed as mean ± SEM; the experiment was repeated three times.) (B) Analysis of the human *BMP4* promoter sequence revealed binding sites for hypoxia-inducible factor (HRE — in bold) and Ets-1 (in italics)

($p = 0.033$). No correlation was found between BMP4 expression and tumour grading and size, patient's age or gender, serum levels of transaminases or bilirubin, and the aetiology of the underlying liver disease.

To assess BMP4 expression in HCC *in situ*, we performed immunohistochemical staining for BMP4 in the normal and tumourous tissue of five patients. Representative immunohistochemical sections are presented in Figure 1C. In accordance with the mRNA data, immunohistochemistry revealed a strong cytoplasmic immuno-signal in epithelial cells of all HCC specimens, while the BMP4 immuno-signal was undetectable in normal hepatic tissues.

## Hypoxia-induced BMP4 expression is not directly regulated by HIF-1alpha

To analyse the mechanisms of regulation of *BMP4* gene expression in HCC cells, we subcloned the promoter regions of the human *BMP4* gene into a reporter vector. Transcriptional activity of the *BMP4* promoter was assessed in two different HCC cell lines to ensure that the activation of the promoter constructs was consistent between these cell lines. As shown in Figure 2A, the *BMP4* promoter was highly active in both Hep3B and PLC cell lines compared with cells transfected with the control vector.

Sequence analysis of the *BMP4* promoter region revealed the presence of putative binding sites for several transcription factors, including Ets-1 and HIF (Figure 2B). We therefore hypothesized that BMP4 expression is regulated by hypoxia.

In line with this hypothesis, activation of HIF by incubation with 100 μM DP (2,2′-dipyridyl) (Figure 3A) or in 0.5% $O_2$ (data not shown) induced *BMP4* promoter activity in both Hep3B and PLC cells. Co-transfection of HCC cells with a dominant negative form of HIF-1alpha abolished the DP-induced *BMP4* promoter activity (Figure 3A).

Stabilization of HIF-1alpha by DP treatment has been described before and confirmed by western blot (ref 28 and data not shown). To verify direct binding of HIF-1alpha to the putative HRE in the *BMP4* promoter, gel shift assays were performed. These assays revealed binding of *in vitro* translated HIF-1 complex to the HRE derived from the *BMP4* promoter. However, nuclear extracts from Hep3B or PLC cells showed only minor binding activity (Figure 3B), and extracts from HCC cells grown under hypoxic

**Table 1.** Comparison of clinicopathological characteristics in 39 HCC patients with or without increased *BMP4* mRNA expression in tumourous tissue in relation to non-tumourous liver tissue

| Variable | | Increased *BMP4* mRNA expression in HCC | | *p* |
|---|---|---|---|---|
| | | No | Yes | |
| | *n* | | | |
| Gender | | | | 1.000* |
| Female | 10 | 4 | 6 | |
| Male | 29 | 11 | 18 | |
| Tumour stage | | | | **0.033*** |
| pT1 | 7 | 6 | 1 | |
| pT2 | 14 | 4 | 10 | |
| pT3 | 16 | 5 | 11 | |
| pT4 | 2 | 0 | 2 | |
| Histological grade | | | | 0.338* |
| G1 | 10 | 5 | 5 | |
| G2 | 26 | 8 | 18 | |
| G3 | 3 | 2 | 1 | |
| Underlying liver disease | | | | 0.070* |
| Alcohol | 20 | 8 | 12 | |
| Viral hepatitis† | 5 | 4 | 1 | |
| Others/unknown | 14 | 3 | 11 | |
| Age at diagnosis (years)‡ | | 58 ± 3 | 63 ± 3 | 0.150§ |
| Tumour size (cm)‡ | | 6.3 ± 1.2 | 7.1 ± 1.0 | 0.953§ |
| Serum ALT levels (IU/ml)‡ | | 88 ± 26 | 82 ± 16 | 0.400§ |
| Serum bilirubin levels (mg/dl)‡ | | 1.4 ± 0.3 | 1.3 ± 0.3 | 0.545§ |

* Categorized parameters were compared using Fisher's exact test.
† Chronic hepatitis B or hepatitis C infection.
‡ Mean ± SEM.
§ Numeric values were compared using the Student's *t*-test (two-sided); bold face represents $p < 0.05$.
ALT = alanine aminotransferase.

conditions did not reveal enhanced binding activity (data not shown).

Next, qRT-PCR was applied to analyse *BMP4* mRNA expression in HCC cells during the course of hypoxia. Two and six hours after incubation with DP, BMP4 expression did not differ from control Hep3B cells. However, 24 h after DP stimulation, a significant enhancement of *BMP4* mRNA expression was observed. After 48 h, the strongest induction of BMP4 expression was noticed (Figure 3C). PHH cells revealed a similar BMP4 expression pattern following DP treatment (data not shown). These results were confirmed on the protein level in HCC cells, where significantly increased BMP4 levels were observed 48 h after HIF activation (Figure 3D).

Although binding of the *in vitro* translated HIF-1 complex to the *BMP4* promoter HRE was observed in gel shift assays, these findings rather suggested an indirect effect of HIF-1 on BMP4 expression.

## Ets-1 regulates BMP4 expression in HCC cells under normoxic and hypoxic conditions

In addition to the HRE, we identified two Ets-1 binding sites in the *BMP4* promoter (Figure 2b). We have previously shown that Ets-1 is involved in the regulation of BMP expression in melanoma [3]. Interestingly, the transcriptional regulator Ets-1 is known to be regulated by hypoxia [31]. Together, these findings pointed to the hypothesis that Ets-1 activation is responsible for hypoxia-induced BMP4 expression in HCC. To follow up with this hypothesis, we confirmed at first that Ets-1 is active in Hep3B and PLC cells compared with cells transfected with the empty control vector (Figure 4A). We then demonstrated that Ets-1 activity is increased in HCC cells after stimulation with the hypoxia mimetic DP (Figure 4A). Co-transfection of a dominant negative HIF-1alpha expression vector (dnHIF-1alpha) completely abrogated DP-induced Ets-1 activity in Hep3B and PLC cells.

Next, we analysed the effect of Ets-1 overexpression and inhibition on *BMP4* promoter activity in PLC cells (Figure 4B). To verify overexpression of Ets-1, we performed Ets-1 qRT-PCR for the transfected cells and found *Ets-1* mRNA levels to be increased about 100-fold in comparison with control vector-transfected cells (data not shown). Overexpression of Ets-1 markedly enhanced *BMP4* promoter activity under control conditions, and this increase was not further augmented by DP treatment. In contrast, transfection of PLC cells with antisense Ets-1 expression plasmids strongly reduced *BMP4* promoter activity under control conditions and almost completely abolished DP-induced promoter activity. Similar results were obtained analysing Hep3B cells (data not shown).

Taken together, these data demonstrate that endogenous Ets-1 activity contributes to baseline expression of the *BMP4* gene under normoxic conditions and is the predominant mediator of the HIF-dependent induction of BMP4 expression after DP stimulation in HCC cells.

## Effects of inhibition of BMP4 expression on migration and invasion of HCC cells

To gain insight into the functional role of increased BMP4 expression in HCC, we studied the effects of siRNA directed against BMP4 and antisense BMP4 on HCC cells. We confirmed the inhibitory effect of *BMP4* siRNA and antisense BMP4 on *BMP4* mRNA levels expressed in HCC cells (Figure 5A).

We investigated the effect of BMP4 knock-down on the invasive potential of Hep3B and PLC cells *in vitro*. Boyden chamber assays revealed that cell migration of HCC cells treated with *BMP4* siRNA was significantly inhibited compared with cells treated with control siRNA (Figure 5B). Furthermore, down-regulation of BMP4 by antisense transfection significantly inhibited the invasive potential of both Hep3B and PLC cells *in vitro* (Figure 5C). In addition, we analysed cell proliferation but found no significant difference to control transfected cells (data not shown). Next, to examine anchorage-independent growth, we performed colony formation assays. Cell colonies derived from asBMP4 or siRNA transfected HCC cells grown in anchorage-independent conditions showed significantly reduced number and size, respectively, compared with control transfected cells (Figure 5D).

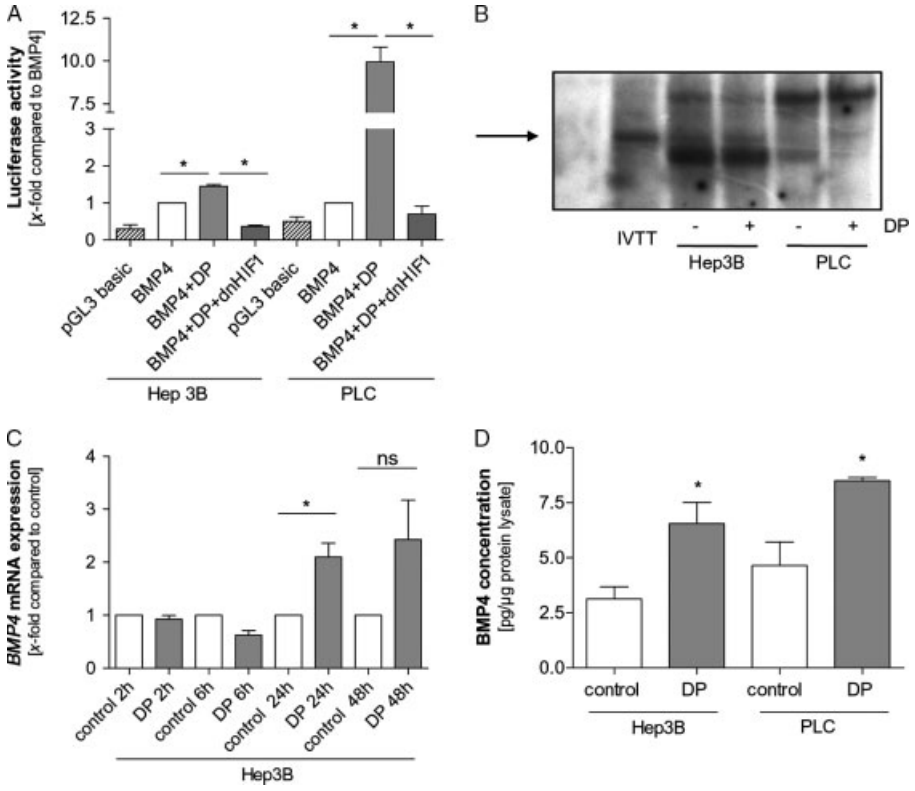


**Figure 3.** Effect of hypoxia on the *BMP4* promoter. (A) Activity of the *BMP4* promoter was enhanced by a hypoxia mimetic DP (2,2′-dipyridyl). Up-regulation of promoter activity by DP was suppressed by co-transfection of dominant negative HIF-1alpha (dnHIF1). ($^*p < 0.05$; BMP4 = 1.) (B) EMSA experiments using the HRE of the *BMP4* promoter revealed binding of *in vitro* translated HIF (IVTT). In contrast, nuclear extracts of HCC cells showed only minor binding activity (with or without DP treatment). (C) Analysis of *BMP4* mRNA expression in Hep3B cells at different time points after incubation with the hypoxia mimetic DP showed enhanced expression of BMP4 after 24 h and 48 h compared to normoxic control conditions (control = 1; $^*p < 0.05$). (D) BMP4 protein concentration was measured by ELISA, revealing increased BMP4 levels 48 h after induction of hypoxia by DP ($^*p < 0.05$ compared with control). Each experiment was repeated at least three times

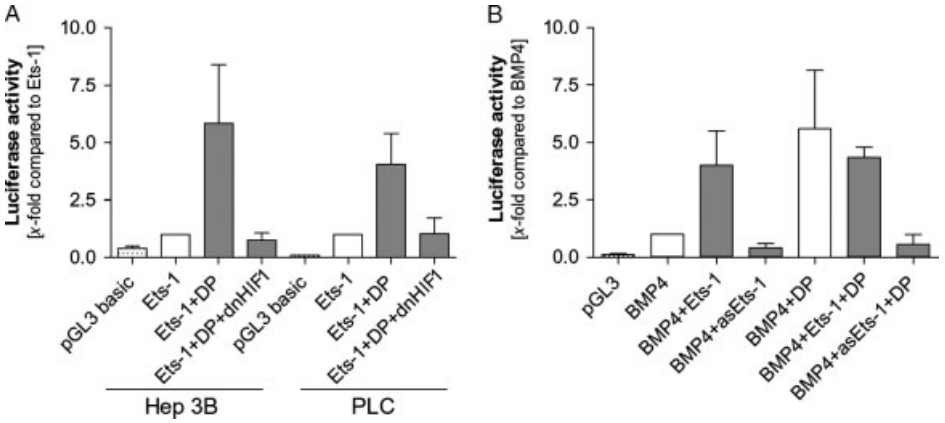


**Figure 4.** Role of Ets-1 in hypoxia-induced activation of the *BMP4* promoter. (A) Ets-1 activity was induced by DP treatment and reduced after co-transfection of a dominant negative form of HIF1alpha (dnHIF1) in PLC and Hep3B cells (unstimulated Ets-1 activity was set at 1). (B) *BMP4* promoter activity was enhanced by overexpression of Ets-1 in PLC cells, whereas the effect of DP treatment on *BMP4* promoter activity could be mimicked by Ets-1. Down-regulation of Ets-1 expression by co-transfection of an Ets-1 antisense construct (asEts-1) resulted in inhibition of *BMP4* promoter activation under normoxic and hypoxic conditions ($^*p < 0.05$; BMP4 = 1). Each experiment was repeated at least three times

## Effects of inhibition of BMP4 expression on vascularization

Recently, an effect of BMP4 on angiogenesis was observed [14]. We therefore performed tube formation assays analysing human microvascular endothelial cells (HMECs) in the presence of supernatants of asBMP4 or *BMP4* siRNA transfected cells, as well as control transfected PLC cells. Both treatments significantly inhibited tube formation (Figures 6A and B). Similar results were obtained analysing Hep3B cells (data not shown). Furthermore, we determined *CD31*

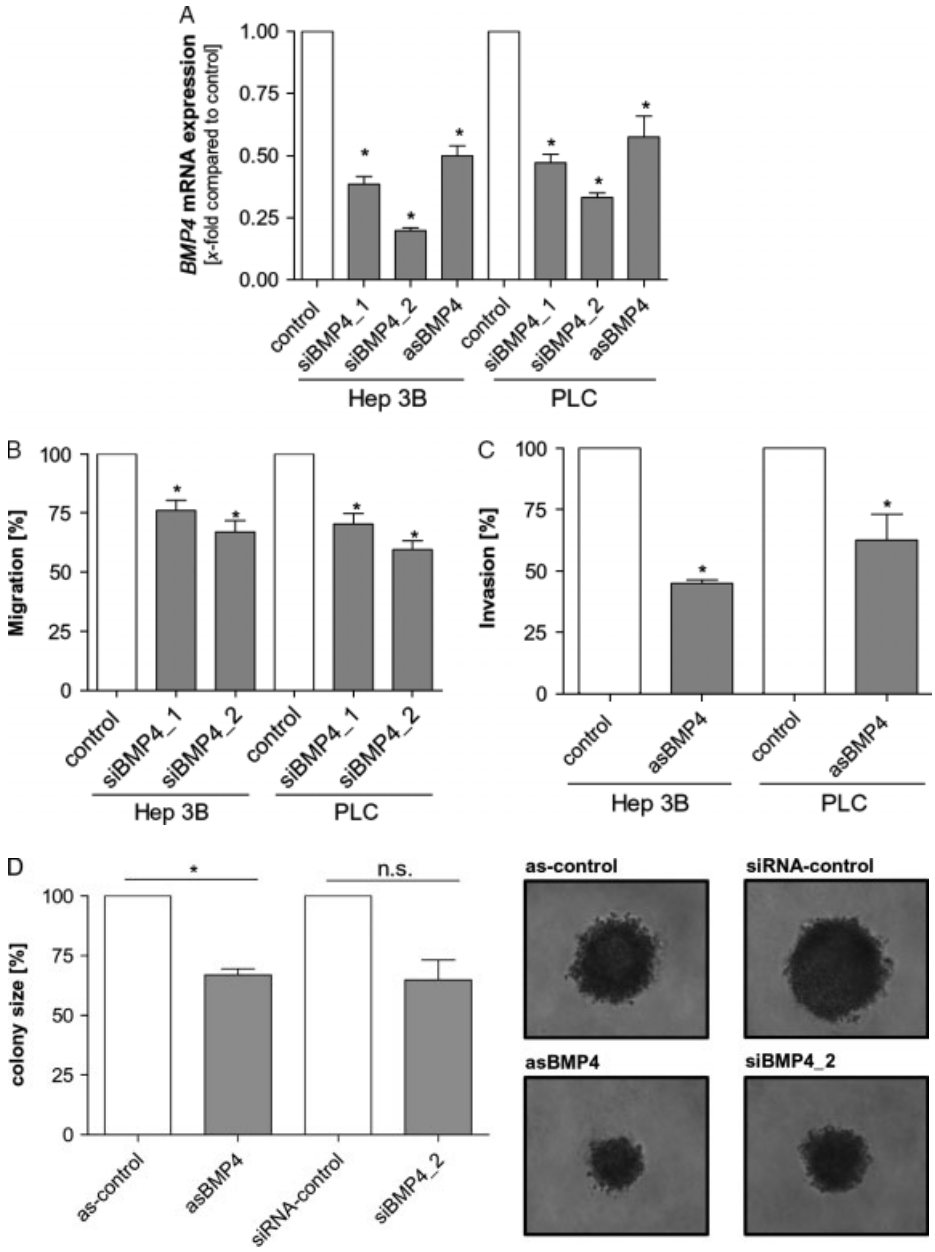


**Figure 5.** Functional role of BMPs in HCC. (A) Quantitative RT-PCR revealed down-regulation of *BMP4* mRNA expression after treatment with two different siRNAs against BMP4 (siBMP4_1 and siBMP4_2) or asBMP4 transfection, respectively (control = 1). (B) Migration assays of siRNA-treated HCC cells and (C) invasion assays of asBMP4 transfected HCC cells were performed with the Boyden chamber model. Transfection of siRNA and asBMP4 revealed a significant reduction of the migratory and invasive potential compared with control transfected cells ($^*p < 0.05$ compared with control; control = 100%). (D) Colony-forming assays revealed inhibitory effects of asBMP4 and *BMP4* siRNA on the number and size of the growing cell colonies compared with control transfected PLC cells. Measurement of the diameters of the colonies confirmed the result ($^*p < 0.05$ compared with control; control = 100%). All experiments were repeated at least twice

mRNA expression in the same 39 HCC specimens that had been assessed for *BMP4* mRNA expression (Figure 1B). Here, no correlation between *BMP4* and *CD31* mRNA expression was observed (data not shown). We speculate that by *in situ* analyses at a defined time point, it is not possible to verify the effect of BMP4 on the highly dynamic process of tumour angiogenesis *in vivo*. Presumably, BMP4 levels rise in situations of hypoxia, but CD31 levels are still normal, eg no increased vascularization. In contrast, increased CD31 expression as a marker of completed neovascularization may be associated with relatively normal tissue oxygenation, and thus lower BMP4 levels.

## Discussion

BMPs are multifunctional proteins that regulate the fate of different cell types, including mesenchymal and endothelial cells. BMPs are important regulators of cell development and differentiation of various organs, and further, are involved in tumour formation and progression. However, until now, the role of

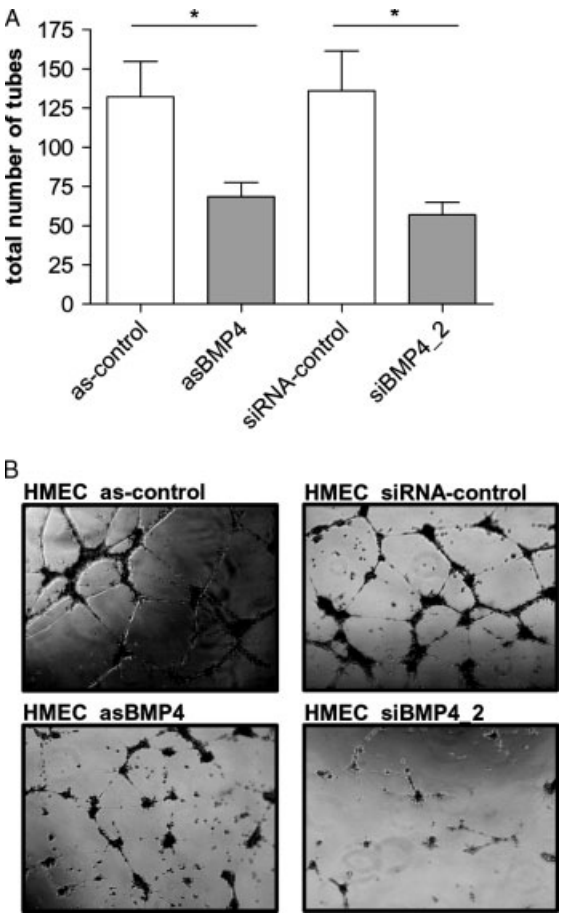


**Figure 6.** Effects of BMP4 expression by HCC cells on tube formation of endothelial cells. (A) Quantification of total tube number revealed strong inhibition of tube formation in human microvascular endothelial cells (HMECs) treated with supernatants of PLC cells transfected with asBMP4 or *BMP4* siRNA compared with control transfected PLC cells ($^*p < 0.05$). (B) Tube formation assay revealed poor tube structures of HMECs seeded onto matrigel after incubation with supernatants of asBMP4 or *BMP4* siRNA transfected PLC cells. In contrast, HMECs treated with supernatants of control transfected PLC cells showed strong formation of tubes (five-fold magnification)

BMPs in hepatocellular carcinomas (HCCs) has not been addressed. Here, we report that BMP4 is highly expressed in HCC cell lines compared with PHHs. RNA analysis and immunohistochemistry confirmed strong BMP4 expression in most HCC tissues compared with normal liver. Promoter analysis revealed a conserved binding site for hypoxia-inducible factor (HIF) in the *BMP4* promoter. As hypoxia is a central regulator of gene expression in cancer including HCC, and overexpression of HIF-1alpha has been reported as an early and frequent event in hepatocarcinogenesis [32,33], we focused on analysing the regulation of BMP4 by hypoxia.

Luciferase reporter assays with the *BMP4* promoter construct demonstrated positive regulation by the hypoxia mimetic DP that was attenuated or abolished by inhibition of HIF activity. However, although we demonstrated binding of the *in vitro* translated HIF-1 complex to the *BMP4* promoter, further experiments suggested an indirect regulation of *BMP4* promoter activity and gene expression by HIF via Ets-1 activation. Thus, hypoxia-induced *BMP4* promoter activation could be completely abolished by transfection of antisense Ets-1. Recently, we have shown that Ets-1 is overexpressed and active in melanoma [27]. Here, we provide strong evidence that Ets-1 is involved in the regulation of the endogenous *BMP4* gene and further accounts for hypoxia-induced BMP4 expression in HCC. Still, we want to emphasize that additional mechanisms such as post-transcriptional regulation may contribute to BMP4 up-regulation in HCC [34,35], but this hypothesis is an issue for further investigations.

BMPs have been shown to stimulate the migration of non-cancerous human cells [36–39]. Since migration is important for the ability of a tumour to invade and metastasize, we examined whether the endogenous expression of BMP4 affects the tumourigenicity of HCC cells *in vitro*. It was noteworthy that down-regulation of BMP4 activity induced a strong reduction of the migratory and invasive potential, as well as anchorage-independent growth. Moreover, we observed a significant association between BMP4 up-regulation in HCC and tumour staging, which further suggests that BMP4 plays an important role in HCC progression. Additionally, impairment of BMP4 in HCC cells inhibits their stimulatory effect on tube formation of human microvascular endothelial cells, indicating that BMP4 also promotes tumour vasculogenesis. In agreement with our results, recent studies in malignant melanoma described a role for BMP4 in the promotion of tumour metastasis and angiogenesis [3,14]. Together, these findings indicate a prominent role for BMP4 in different pathophysiologically relevant tumourigenic processes.

In summary, our studies show that BMP4 is strongly expressed in HCC and suggest that BMP4 expression promotes HCC progression. Future studies will most likely define an even broader role of BMP4 and other BMPs in postnatal tissues including cancers. Consequently, further studies are needed to define in more detail the autocrine effects of BMPs on hepatocarcinogenesis. Based on the current knowledge, BMP4 appears as a prognostic marker and as an attractive molecular target for the therapy of this highly aggressive tumour.

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