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# GDF11 contributes to hepatic HAMP inhibition through SMURF1-mediated BMP-SMAD signalling suppression

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## Summary

Hepcidin (HAMP) synthesis is suppressed by erythropoiesis to increase iron availability for red blood cell production. This effect is thought to result from factors secreted by erythroid precursors. Growth differentiation factor 11 (GDF11) expression was recently shown to increase in erythroid cells of β-thalassaemia, and decrease with improvement in anaemia. Whether GDF11 regulates hepatic HAMP production has never been experimentally studied. Here, we explore GDF11 function during erythropoiesis-triggered HAMP suppression. Our results confirm that exogenous erythropoietin significantly increases *Gdf11* as well as *Erfe* (erythroferrone) expression, and *Gdf11* is also increased, albeit at a lower degree than *Erfe*, in phlebotomized wild type and β-thalassaemic mice. *GDF11* is expressed predominantly in erythroid burst forming unit- and erythroid colony-forming unit-cells during erythropoiesis. Exogeneous GDF11 administration

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Contributions

Z. F. and Z. Z. designed the experiments, collected and analysed the data, and drafted the manuscript. H. Z, Y. P., J. L., H. L., L. L. and S. X. performed research. J. L., Q. W., B. F., K. W. and L. Z. provided technical or logistical support. Y. G. analysed the data and edited the paper. J. L. and H. C. designed the experiments, interpreted the data, and drafted and revised the manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1**. *Gdf11*, *Hamp* and *Erfe* expression in $Hbb^{th3/+}$ β-thalassemia intermedia mice.

**Figure S2**. *Gdf11* expression shows a more sensitive response to erythropoietic stimulation in Ter119 negative cells than in Ter119 positive cells.

**Figure S3**. GDF11 declines BMP-SMAD target genes expression.

**Figure S4**. Changes of TMPRSS6 protein levels in rGDF11-treated cells.

**Figure S5**. *Smurf1* expression in mouse liver after phlebotomy or rGDF11 injection.

**Figure S6**. *SMURF1* overexpression has no influence on BMP-SMAD signaling and *HAMP* expression.

**Figure S7**. Proposed working model of GDF11 on HAMP regulation.

**Table SI**. qRT-PCR primers.

results in HAMP suppression *in vivo* and *in vitro*. Furthermore, exogenous GDF11 decreases BMP-SMAD signalling, enhances SMAD ubiquitin regulatory factor 1 (SMURF1) expression and induces ERK1/2 (MAPK3/1) signalling. ERK1/2 signalling activation is required for GDF11 or SMURF1-mediated suppression in BMP-SMAD signalling and HAMP expression. This research newly characterizes GDF11 in erythropoiesis-mediated HAMP suppression, in addition to ERFE.

**Keywords**

iron; erythropoiesis; hepcidin/HAMP; GDF11; erythroid factor

Hepcidin (HAMP) is a peptide hormone that is mainly produced by hepatocytes and plays a key role in iron homeostasis. HAMP works by binding to its target, ferroportin (FPN), the only known iron exporter (Nemeth et al, 2004). HAMP-FPN binding prevents the conformational transition and iron export of FPN, initiating the cellular internalization and degradation of FPN (Aschemeyer et al, 2018). FPN is mostly abundantly expressed in macrophages, duodenal enterocytes, hepatocytes, the placenta, erythroblasts and mature red cells (Donovan et al, 2000; Wallace, 2016; Zhang et al, 2018). HAMP deficiency results in increased cell-surface FPN and more iron released into plasma while excess HAMP decreases cell-surface FPN, leading to cellular iron retention.

Although the essential negative role of HAMP in iron homeostasis is well known, the regulation of HAMP synthesis has not been fully elucidated. HAMP is increased by iron and inflammation and suppressed by erythropoiesis (Nemeth & Ganz, 2006). During erythropoietic stimulation, such as in conditions associated with anaemia, hypoxia or during erythropoietin (EPO) administration, HAMP expression is decreased, resulting in increased iron availability for erythropoiesis. This effect is thought to result from factors secreted by erythroid precursors (Shenoy et al, 2014; Wang et al, 2017a). Several potential candidate ‘erythroid factors’ have been identified. Growth differentiation factor 15 (GDF15) and twisted gastrulation 1 (TWSG1) were once thought to be the candidates in β-thalassaemic humans and mice, respectively (Tanno et al, 2007; Tanno et al, 2009). However, these two factors are possibly pathological but unlikely to be physiological erythroid regulators of HAMP (Casanovas et al, 2013; Kautz et al, 2014; Ganz, 2019).

Erythroferrone (ERFE) is a recently identified physiological erythroid regulator. Haemorrhage and EPO administration can induce ERFE production in erythroblasts from mice and human (Kautz et al, 2014; Ganz et al, 2017). It has been confirmed that ERFE acts directly on hepatocytes to suppress HAMP production by sequestration of BMP6 (Kautz et al, 2014; Arezes et al, 2018). Strengthened BMP-SMAD signalling via TMPRSS6 inhibition blunts ERFE-mediated HAMP suppression (Nai et al, 2016). ERFE also contributes to pathological HAMP suppression in β-thalassaemia. Serum ERFE levels are very high in both un-transfused and transfused β-thalassaemic patients, and decreased by blood transfusion (Ganz et al, 2017). However, loss of ERFE does not fully rescue HAMP suppression and liver iron load in adult β-thalassaemic mice (Kautz et al, 2015). These observations may suggest that other erythroid factors besides ERFE contribute to the erythropoiesis-dependent HAMP suppression.

GDF11, also known as bone morphogenetic protein 11 (BMP11), belongs to the transforming growth factor-β (TGF-β) super-family and has been extensively investigated for its roles in development, aging and disease (Jamaiyar et al, 2017; Zhang et al, 2017). Interestingly, *Gdf11* mRNA was highly expressed in erythroid progenitors of β-thalassaemic mice (Dussiot et al, 2014). In β-thalassaemic mice treated with activin receptor ligand, improvement of anaemia was accompanied by increased *Hamp* and decreased *Gdf11* expression (Dussiot et al, 2014). However, whether and/or how GDF11 regulates HAMP expression has never been studied. To explore GDF11 effects on HAMP synthesis, we performed experiments with commercial recombinant human GDF11 (rGDF11) *in vivo* and *in vitro*. We confirm that GDF11 expression is increased as ERFE in bone marrow and spleen from erythropoiesis-stimulated mice. GDF11 causes increased hepatic SMURF1 expression and ERK1/2 signalling, resulting in decreased BMP-SMAD signalling and HAMP expression. These finding demonstrate that, in addition to ERFE, GDF11 is involved in HAMP regulation by erythropoiesis, providing new insights into its role in iron homeostasis.

## Materials and methods

### Cell culture and treatments

The human liver-origin cells Huh7 (JCRB 0403) and HepG2 (ATCC CCL-243) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco) at 37°C in 5% $CO_2$. Mouse primary hepatocytes were obtained from C57BL/6 (wild-type; WT) mice by two-step liver perfusion. In brief, the mouse livers were perfused by collagenase IV (Sigma, St. Louis, MO, USA) and 250 000 cells/well were plated on collagen-coated 6-well plates in DMEM supplemented with 5% FBS, and cultured as previously described (Chen et al, 2016). For experiments using rGDF11, Peprotech [Rocky Hill, NJ, USA] or R&D Systems [Minneapolis, MN, USA]), hepatocytes were incubated with 5% FBS and increasing doses of rGDF11 for 24 h. The specific MEK/ERK1/2 inhibitor, U0126 (Promega, Madison, WI, USA), was applied 2–2·5 h prior to cell harvest, and the proteasome inhibitor MG132 (Sigma) was added 4–6 h before cell harvest. Human $CD34^+$ haematopoietic progenitor cells were purified, erythroid differentiation-induced, and cells collected on culture days 0, 4, 6, 8, 10, 12 and 14, as previously reported (Sun et al, 2015). Primary human erythroid burst forming unit (BFU-E) and erythroid colony-forming unit (CFU-E) cells were isolated using flow cytometry as previously reported (Li et al, 2014). Human erythroblasts at each distinct stage of terminal erythropoiesis were sorted from EPO-induced $CD34^+$ cells with a combination of cell surface markers for glycophorin A, band 3, and a4-integrin (Hu et al, 2013; An et al, 2014).

### Mice

Wild-type C57BL/6 (WT; SiLaiKeJingDa, Changsha, China) and $Hbb^{th3/+}$ β-thalassaemia intermedia (C57BL/6 background; the Jackson Laboratory, Bar Harbor, ME, USA) mice were maintained on an iron-balanced diet containing 200 ppm iron within the laboratory animal facility at Central South University (Changsha, China). The experimental protocols were approved by the Animal Research Committee of Hunan Province. WT mice (6–8 weeks old) were phlebotomized by retro-orbital puncture (500 μl) to stimulate erythropoiesis

and analysed at 0, 4, 12 or 48 h post-phlebotomy. Mice were injected intraperitoneally with a single dose of rGDF11 (0·1 mg/kg body weight; Peprotech) or saline solution and analysed 15 h post-injection. Mouse serum iron concentrations were assessed using the serum iron assay kit (Jiancheng, Nanjing, China). For chronic EPO treatment, 6-week-old mice were injected intraperitoneally with either 200 units (u) recombinant mouse EPO (Biolegend, San Diego, CA, USA) or sterile saline 3 times per week for 2 weeks, and analysed 15 h after the final injection, as previously described (Coffey et al, 2018). For acute EPO treatment, 8-week-old mice were injected with a single dose of 200u recombinant mouse EPO or sterile saline 15 h prior to sacrifice. Mouse $Ter119^+$ and $Ter119^-$ cells were isolated from bone marrow and spleen using anti-mouse Ter119 particles (Becton Dickinson, San Diego, CA, USA) according to the manufacturer's instructions, and confirmed to have a purity of more than 90% using fluorescein isothiocyanate-conjugated anti-mouse Ter119 antibody (Thermo Fisher Scientific, Shanghai, China) by flow cytometry analysis.

### Plasmid construct and cell transduction

SMURF1-shRNA (5′-GGTTACACCACATCATGAA-3′) and NC-shRNA (5′-TGCGTTGCTAGTACCAAC-3′) plasmids were constructed and confirmed by at the Beijing Institute of Lifeomics, Beijing, China) (Xie et al, 2014). Recombinant over-expression plasmid of *SMURF1* was constructed using vector plasmid pCMV-tag2B, and confirmed by DNA sequencing. Plasmids were transfected to cells using lipofectamine Imafect (Yimeiang, Beijing, China) and then selected by puromycin (1 μg/ml).

### RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Shanghai, China). cDNA was synthesized by Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR was performed using SYBR Green qPCR Master Mixes (Takara, China) and the primers listed in Table SI. *HAMP*, *ERFE*, *SMURF1*, *GDF11*, *ID1*, *ID2* and *ATOH8* mRNA levels were normalized to mRNA of the reference genes, *GAPDH* or *ACTB*. Results were presented as fold changes relative to the control, and shown as mean ± standard deviation (SD).

### Western blot analyses

Cultured cells were lysed by radioimmunprecipitation assay buffer (Thermo Fisher, USA) in the presence of protease inhibitor or PhosStop cocktail (Roche, Shanghai, China). Mouse livers were homogenized and total protein was extracted using T-PER Tissue Protein Extraction (Thermo Scientific) supplemented with protease and phosphatase inhibitor cocktail (Sigma). Proteins were quantified using BCA protein assay (Thermo Fisher), separated by 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Berkeley, CA, USA). The membranes were incubated in 5% (w/v) skim milk or bovine serum albumin dissolved in TBST (tris-buffered saline and Polysorbate 20) for 1 h at room temperature to block the non-specific binding location, and incubated overnight at 4°C with the following primary antibodies: SMURF1 (1:1000; Cell Signaling, Shanghai, China), phospho-SMAD1/5/9 (1:1000; Cell Signaling), SMAD1 (1:1000; Cell Signaling), phospho-ERK1/2 (1:1000; Santa Cruz Biotechnology, Santa Cruz,

CA, USA), ERK1/2 (1:1000; Santa Cruz), TMPRSS6 (1:1000; Sangon Biotech, Shanghai, China), GAPDH (1:5000; Cell Signaling), and ACTB (1:5000; Thermo Scientific). Protein blots were visualized by chemiluminescence using the ChemiDoc XRS+ imaging system with Image Lab software (Bio-Rad), and band intensity was quantified using the Gel-Pro software (Medica Cybernetics Inc., Bethesda, MD, USA).

### Statistical analysis

All data are shown as mean ± SD and statistically analysed by SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Significant differences between the groups were determined using the Student unpaired *t*-test, and considered statistically significant at $P < 0{\cdot}05$.

## Results

### *Induction of* GDF11 *expression by erythropoiesis*

GDF11 was reported to be the only increased TGF-β super-family member in erythroblasts of β-thalassaemia intermedia ($Hbb^{th1/th1}$) mice (Dussiot et al, 2014). To evaluate *Gdf11* mRNA after stimulating erythropoiesis, we examined *Gdf11* expression in the bone marrow and spleen after phlebotomy. Six-week-old C57BL/6 female mice were phlebotomized (500 μl blood drawn) and tissues were collected at baseline and 4, 12 and 48 h after. Total RNA was extracted and then evaluated for gene expression by qRT-PCR. Data demonstrated that *Gdf11* mRNA levels were greatly increased between 4 and 12 h and recovered within 48 h of phlebotomy both in bone narrow and spleen (Fig 1A, 1). Liver *Hamp* mRNA levels were maximally suppressed within 12 h with evidence of partial recovery at 48 h post-phlebotomy (Fig 1C). Increased *Gdf11* and decreased *Hamp* expression were also observed in $Hbb^{th3/+}$ β-thalassaemia intermedia mice (Figure S1A–C). In an addition, *Erfe* expression changed in similar patterns, as previously reported (Kautz et al, 2014; Kautz et al, 2015), and was increased much more than *Gdf11* in phlebotomized WT and β-thalassaemic mice (Fig 1D and Figure S1D). To examine the response of GDF11 expression to EPO, wild type mice were acutely or chronically injected with EPO. Both acute and chronic EPO treatment successfully decreased liver *Hamp* expression (Fig 2A) and increased *Gdf11* (Fig 2B) and *Erfe* expression (Fig 2C) in bone marrow and spleen. This indicated that EPO administration seems to have similar or stronger induction effects on *Gdf11* than *Erfe*.

We next tested erythroid-specific expression of *GDF11*. Through deep RNA sequencing of sorted EPO-induced human cord blood $CD34^+$ cells (An et al, 2014), *GDF11* expression increased at BFU-E and CFU-E stages compared to $CD34^+$ cells and decreased at pro-erythroblast stage and throughout terminal erythroid differentiation (Fig 3A). In addition, EPO-induced human $CD34^+$ cells were also placed in culture and evaluated at baseline and after 4, 6, 8, 10, 12 and 14 days. qRT-PCR results demonstrate similar changes to deep RNA sequencing, with *GDF11* mRNA expression increased at the earliest time points and then progressively suppressed to day 12 (Fig 3B). To further identify the specific expression of *Gdf11* in erythroid cells, we isolated $Ter119^+$ and $Ter119^-$ cells from mouse bone marrow and spleen. *Gdf11* expression in $Ter119^-$ cells was more sensitive to erythropoietic stimulation than in $Ter119^+$ cells (Figure S2). $Ter119^-$ cells contain higher levels of *Gdf11* mRNA than $Ter119^+$ cells under all conditions. Although the $Ter119^-$ negative fraction also

included non-erythroid cells, these observations may indirectly confirm the above findings in human cells, as BFU-E and CFU-E cells are Ter119/glycophorin A (GPA) negative (Stumpf et al, 2006; Flygare et al, 2011; Li et al, 2014). Our findings are similar to previous reports of erythroid precursors from mouse fetal liver cells cultured *ex vivo*, demonstrating the highest *Gdf11* mRNA expression at the earliest stage and progressively declining thereafter (Suragani et al, 2014a). These findings indicate GDF11 is an erythroid factor and is produced by early erythroid precursors.

### GDF11 directly suppresses HAMP production through BMP-SMAD signalling

To elucidate whether the secreted GDF11 protein directly suppresses HAMP expression, male C57BL/6 mice were injected intraperitoneally with rGDF11 (0·1 mg/kg) and sacrificed 15 h later. Livers and sera were collected for qRT-PCR evaluation of *Hamp* expression and serum iron concentration, respectively. *Hamp* mRNA expression was significantly decreased (Fig 4A) and serum iron concentrations increased (Fig 4B) in GDF11-treated mice relative to saline-injected controls. To assess a potential direct effect of GDF11 on *Hamp* expression, we evaluated hepatocytes 24 h after exposure to escalating doses of GDF11 *in vitro*. We demonstrated that that addition of rGDF11 results in a dose-dependent decrease in *Hamp* mRNA expression in mouse primary hepatocytes (Fig 4C) and two human hepatocyte cell lines: Huh7 (Fig 4D) and HepG2 (Fig 4E). It should be noted that, due to the unavailability of specific GDF11 antibodies that can discriminate GDF11 and GDF8, endogenous GDF11 proteins were not evaluated. Although the amino acid sequences of GDF11 and GDF8 show 90% identity, qRT-PCR results indicate that *Gdf8* mRNA levels are not increased by erythropoietic stimulation (data not shown). Taken together, these findings demonstrate the direct ability of GDF11 to inhibit HAMP expression as part of it is an important erythroid factor, and can directly act on the liver.

The BMP-SMAD signalling pathway is a major regulator of HAMP transcription, and has been demonstrated to be involved in erythropoiesis-mediated HAMP suppression. Exogenous EPO injection reduces SMAD5 phosphorylation and BMP-SMAD target genes expression in WT mice (Nai et al, 2016). Recently, sequestration of several BMPs, including BMP6, was experimentally proposed as the mechanism for ERFE regulation of HAMP (Arezes et al, 2018). Thus, to assess whether GDF11 attenuates BMP-SMAD signalling to suppress HAMP expression, we evaluated BMP-SMAD signalling activation and target genes expression by Western Blot and qRT-PCR, respectively, in mouse primary hepatocytes as well as inHuh7 and HepG2 cells. Both phosphorylated SMAD1/5/9 and total SMAD1 protein levels were found to decrease in a dose-dependent manner in response to GDF11 (Fig 4C–E). Consistently, mRNA levels of BMP-SMAD target genes (e.g. *ID1*, *ID2* and *ATOH8*) progressively decreased with increasing GDF11 concentrations in different cells (Figure S3). These results confirm the inhibitory effect of GDF11 on BMP-SMAD signalling with the most unequivocal effects of rGDF11 on BMP-SMAD signalling in Huh7 cells compared to HepG2 cells or mouse primary hepatocytes. Based on this observation, we chose Huh7 cells for our subsequent experiments.

## Ubiquitin Proteasome System is involved in GDF11-mediated BMP-SMAD signalling suppression

To further explore the mechanisms of GDF11-mediated suppression of BMP-SMAD signalling, we evaluated TMPRSS6 protein levels. TMPRSS6, known as matriptase-2, cleaves the BMP co-receptor haemojuvelin (HJV) to attenuate the BMP-SMAD pathway activation and reduce HAMP expression (Silvestri et al, 2008). Treatment with GDF11 did not increase TMPRSS6 protein concentration in the liver (Figure S4). In addition to TMPRSS6, the ubiquitin proteasome system (UPS) has also been reportedly involved in the suppression of SMAD signalling (Sapkota et al, 2007; Alarcon et al, 2009). We thus treated Huh7 cells with increasing doses of MG132, a proteasome inhibitor, for 5 h. We found that both *HAMP* mRNA expression and phosphorylated SMAD1/5/9 concentration increased in a dose-dependent manner in Huh7 cells after MG132 treatment (Fig 5A). To check the effect of combining GDF11 with MG132, we treated Huh7 cells with GDF11 (5 ng/ml) for 24 h, and MG132 (5 μmol/l) for 5 h prior to cell harvest. Compared to the treatment with GDF11 alone, the combination with both MG132 and GDF11 balances the decreases in *HAMP* expression and BMP-SMAD signalling (Fig 5B). Similar results were observed in HepG2 cells (data not shown). Our results demonstrate that GDF11-triggered inhibition of BMP-SMAD signalling and HAMP expression is rescued by MG132, suggesting that UPS may play an important role in GDF11-mediated HAMP suppression.

## GDF11 inhibits BMP-SMAD signalling through SMURF1

To further assess the role of ubiquitination in HAMP expression, we characterized SMURF1, the SMAD specific E3 ubiquitin ligase, in our system. SMURF1 exerts biological function via targeting SMADs for ubiquitination and degradation (Iyengar, 2017). We evaluated liver *Smurf1* expression after phlebotomy in mice. Interestingly, the time course of *Smurf1* expression (Figure S5A) was inversely correlated to *Hamp* expression (Fig 1C). Liver *Smurf1* mRNA expression was maximally induced at 12 h and remained elevated 48 h after phlebotomy. Liver *Smurf1* mRNA expression was also induced by rGDF11 injection (Figure S5B). Lastly, *SMURF1* mRNA and SMURF1 protein levels increased in rGDF11-treated Huh7 cells (Fig 6A). Similar results were also obtained in HepG2 cells (data not shown).

To clarify SMURF1 function in HAMP regulation, we performed *SMURF1* knockdown experiments using a confirmed *SMURF1*-specific shRNA (Xie et al, 2014) in Huh7 cells. *SMURF1* expression is effectively suppressed in SMURF1 shRNA experiments, resulting in increased *HAMP* expression and BMP-SMAD signalling (Fig 6B). In addition, SMURF1 shRNA plasmid transfected (for 5 h) cells subsequently treated with rGDF11 (for 24 h) recovered SMURF1 protein concentration to levels of control cells and restored *HAMP* mRNA expression and SMAD signalling to that of controls (Fig 6C).

## *ERK1/2 signalling is necessary for GDF11 or SMURF1-mediated* HAMP *regulation*

Although *SMURF1* knockdown increases HAMP expression in hepatocytes, *SMURF1* overexpression does not inhibit *HAMP* expression because neither BMP-SMAD signalling nor *HAMP* expression was obviously changed in Huh7 cells transfected with *SMURF1*-overexpressing plasmids compared to the untreated (Figure S6). We hypothesized that

SMURF1-mediated HAMP suppression was dependent on ERK1/2 signalling. ERK1/2 signalling results in the phosphorylation of the SMAD linker domain for polyubiquitination by SMURF1, and degradation (Sapkota et al, 2007), and we have previously demonstrated activation of ERK1/2 signalling in hepatocytes from β-thalassaemic mice, a mouse model in which HAMP regulation is dominated by erythropoiesis (Chen et al, 2016). Thus, the evaluation of ERK1/2 signalling demonstrated that ERK1/2 phosphorylation is increased by GDF11 in a dose-dependent manner (Fig 7A), but not affected by *SMURF1* overexpression (Figure S6C).To further explore whether ERK signalling is required for GDF11-triggered BMP-suppression of SMAD signalling, we treated Huh7 cells with GDF11 with or without U0126, a MEK/ERK1/2 inhibitor (Fig 7B, 7). Data showed that GDF11 induced SMURF1, unaffected by U0126. U0126 successfully depressed activation of ERK1/2 signalling in the treatments with or without addition of GDF11. Furthermore, the combination of these agents failed to restrain *HAMP* synthesis compared with the treatment with GDF11 alone. These findings indicated that ERK1/2 signalling activation is indispensable for GDF11 or SMURF1-mediated HAMP repression.

## Discussion

In this study, we first evaluated the role of GDF11 in erythropoiesis-mediated HAMP suppression and demonstrated that *GDF11* is predominantly expressed in early erythroid progenitors (e.g. BFU-E and CFU-E). Acute or chronic erythropoietic stimulation induces both *Gdf11* and *Erfe* expression in the bone marrow and spleen. GDF11-treated wild type mice exhibited increased serum iron and decreased liver *Hamp* mRNA expression. *In vitro* experiments using two human hepatocyte cell lines and mouse primary hepatocytes demonstrated a dose-dependent decrease in *HAMP* expression in response to rGDF11. Interestingly, we observed a predominant increase of *Erfe* over *Gdf11* expression in phlebotomized and β-thalassaemic mice, but not in EPO-injected mice. *Erfe* knockout mice showed only a minimal suppression of *Hamp* after phlebotomy (Kautz et al, 2014), compared to modest *Hamp* inhibition after acute EPO injection (Arezes et al, 2018), and failed to block *Hamp* suppression following chronic EPO treatment (Coffey et al, 2018). These observations could indicate that GDF11 plays an important complementary role to ERFE, especially in HAMP inhibition caused by EPO-induced erythropoiesis.

GDF11 plays intensive roles in development, aging and disease (McPherron et al, 1999; Nakashima et al, 2003; Yokoe et al, 2007; Augusciak-Duma & Sieron, 2008; Ding et al, 2013; Loffredo et al, 2013; Kaiser, 2014; Katsimpardi et al, 2014; Alvarez et al, 2016; Jamaiyar et al, 2017; Zhang et al, 2017), and was recently identified as a regulator of erythropoiesis, which maintains the survival of erythroid progenitors but inhibits further differentiation (Carrancio et al, 2014; Suragani et al, 2014a). The beneficial effects of GDF11 inhibition were previously reported to be involved in activin receptor ligand traps on terminal erythropoietic differentiation in the mouse models of myelodysplastic syndrome and β-thalassaemia (Dussiot et al, 2014; Suragani et al, 2014a; Suragani et al, 2014b). However, this role has been questioned because conditional deletion of *Gdf11* did not ameliorate erythropoiesis in β-thalassaemia or prevent the activity of the trap-ligand RAP-536 (Guerra et al, 2018). This discrepancy could be explained by non-specific synthesis of GDF11 from other tissues. *Gdf11* is expressed in multiple tissues, including

haematopoietic tissues (Zhang et al, 2017), and because of to the unavailability of specific GDF11 antibodies, it has not been validated whether circulating GDF11 is effectively decreased in these conditional knockout mice.

In GDF11-mediated HAMP suppression, we confirm the expected inhibition of the BMP-SMAD pathway. BMP-SMAD signalling is necessary for erythropoiesis-mediated HAMP suppression. In *Tmprss6* knockout mouse, the BMP-SMAD pathway is enhanced, and EPO injection failed to suppress *Hamp* expression (Nai et al, 2016). The erythroid factor ERFE has recently been proposed to interact with BMP6 to inhibit BMP-SMAD signalling and HAMP expression (Nai et al, 2016; Wang et al, 2017b; Arezes et al, 2018). Here, we demonstrate that the UPS is required for GDF11-mediated suppression of SMAD signalling in hepatocytes because the proteasome inhibitor MG132 rescues HAMP expression in GDF11-treated cells. Furthermore, GDF11 treatment increases SMURF1 expression both *in vivo* and *in vitro*. SMURF1 is known to play a role in the UPS by modulating TGF-β and BMP signalling in osteoblast differentiation, embryonic development, epithelial-mesenchymal transition and cancer (Zhu et al, 1999; Ozdamar et al, 2005; Yamashita et al, 2005; Sanchez & Barnett, 2012; Cao & Zhang, 2013). Our current work extends the role of SMURF1 to HAMP regulation in hepatocytes.

We demonstrate that SMURF1 inhibition increases BMP-SMAD signalling and HAMP expression while SMURF1 overexpression does not result in the suppression of SMAD signalling and HAMP expression. GDF11 treatment enhances ERK1/2 signalling while the MEK/ERK1/2 inhibitor U0126 blocks the effect of GDF11 on HAMP expression. ERK1/2 signalling is involved in SMURF1 and TGF family function (Fuentealba et al, 2007; Sapkota et al, 2007; Alarcon et al, 2009). Given that therapeutic strategies using HAMP activation have been shown to improve ineffective erythropoiesis and reverse anaemia in β-thalassaemia (Gardenghi et al, 2010; Li et al, 2010; Guo et al, 2013; Suragani et al, 2014b; Schmidt et al, 2015; Casu et al, 2016; Chen et al, 2016), SMURF1 inhibition or ERK1/2 block could be a new therapeutic strategy in iron disorders and related haematological diseases.

In summary, we newly characterized GDF11 during erythropoiesis-triggered HAMP suppression, in addition to ERFE (Figure S7). We propose that stimulated erythropoiesis induces GDF11 expression in erythroid progenitors and secretion into the circulation to act on hepatocytes. Hepatocytes then enhance SMURF1 expression and ERK1/2 signalling, contributing to downregulated BMP-SMAD signalling and depressed HAMP expression by ERFE, in turn increasing iron availability for new red blood cell production. Nevertheless, because of the broad expression of GDF11 in various tissues and unavailability of specific antibodies or antagonist, it is still challenging to fully demonstrate the value of endogenous GDF11 in erythropoiesis-mediated HAMP inhibition *in vivo*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowlegements

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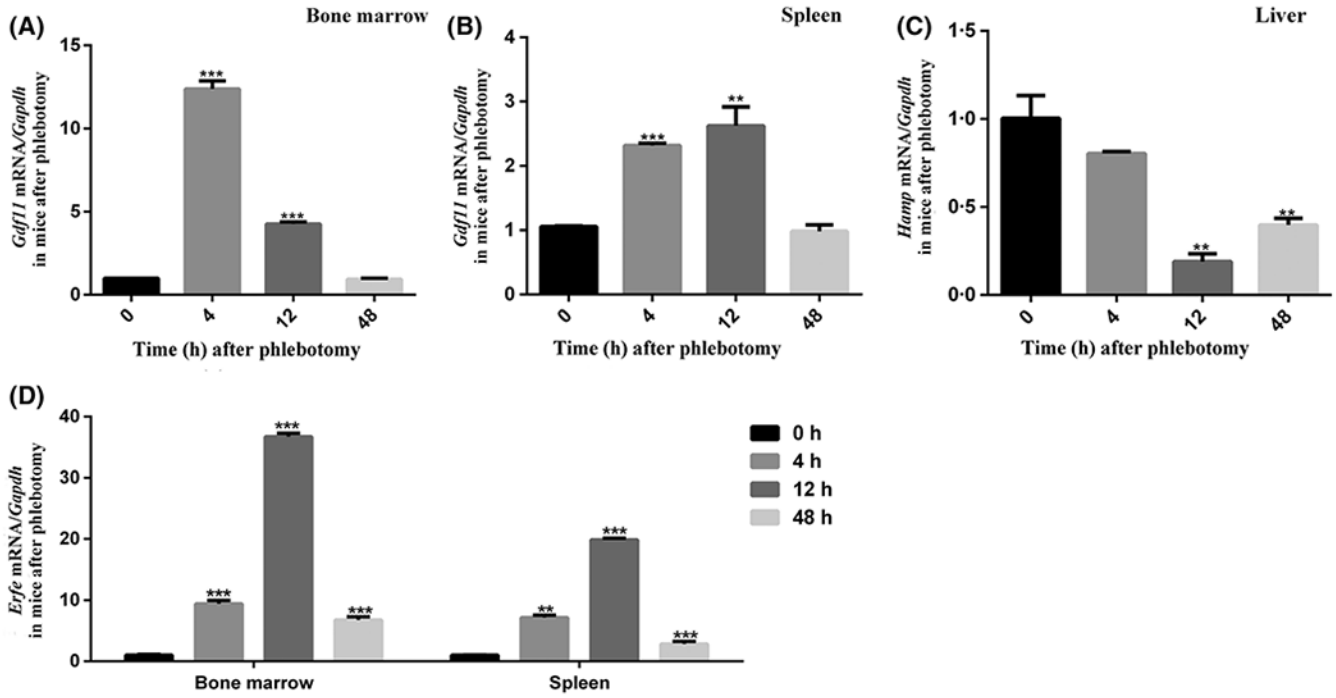


**Fig 1.**

Time course of *Gdf11*, *Erfe* and *Hamp* expression after phlebotomy. Six-week-old C57BL/6 female mice were phlebotomized by blood drawn 500 μl ($n$ = 4-6 mice per group). mRNA levels at different time point after bleeding were measured by qRT-PCR for (A) bone narrow *Gdf11*, (B) spleen *Gdf11*, (C) liver *Hamp*, and (D) *Erfe* in the bone marrow and spleen. *Gapdh* was used as the housekeeping gene. Results were normalized to the control mice. ***$P$ < 0·001, **$P$ < 0·01, *$P$ < 0·05 compared with levels in the control mice ($n$ = 6–8 per group).

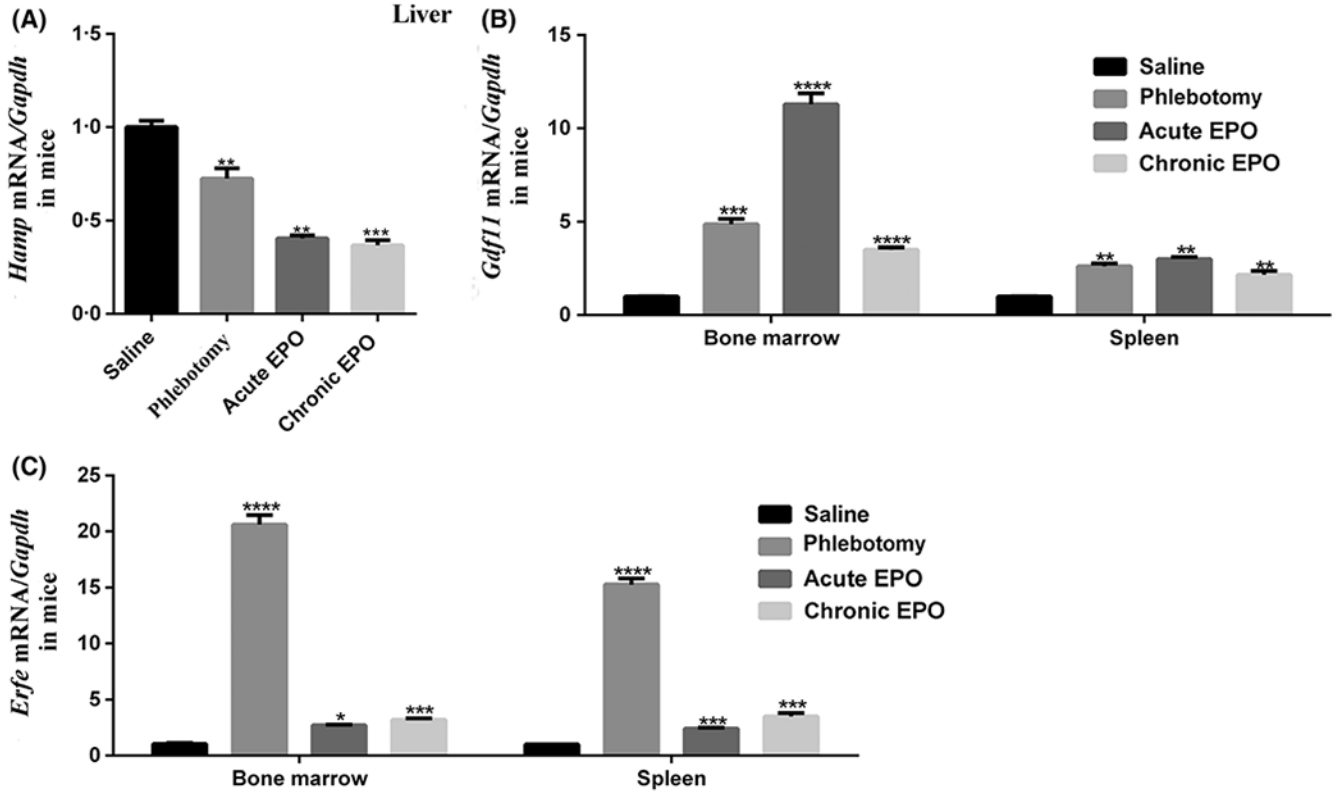


**Fig 2.**

Changes of *Gdf11*, *Erfe* and *Hamp* expression after EPO administration. Wild-type C57BL/6 male mice were phlebotomized by blood draw (500 μl) or treated with acute or chronic erythropoietin (EPO) administration, and sacrificed for tissue collection 15 h after treatment or last EPO administration. mRNA levels of (A) *Hamp*, (B) *Gdf11* and (C) *Erfe* in various tissues were measured by qRT-PCR, using *Gapdh* as the housekeeping gene, and normalized to the levels in control mice. ****$P < 0{\cdot}0001$, ***$P < 0{\cdot}001$, **$P < 0{\cdot}01$, *$P < 0{\cdot}05$ compared with levels in the saline injected group ($n = 6$–8 per group).

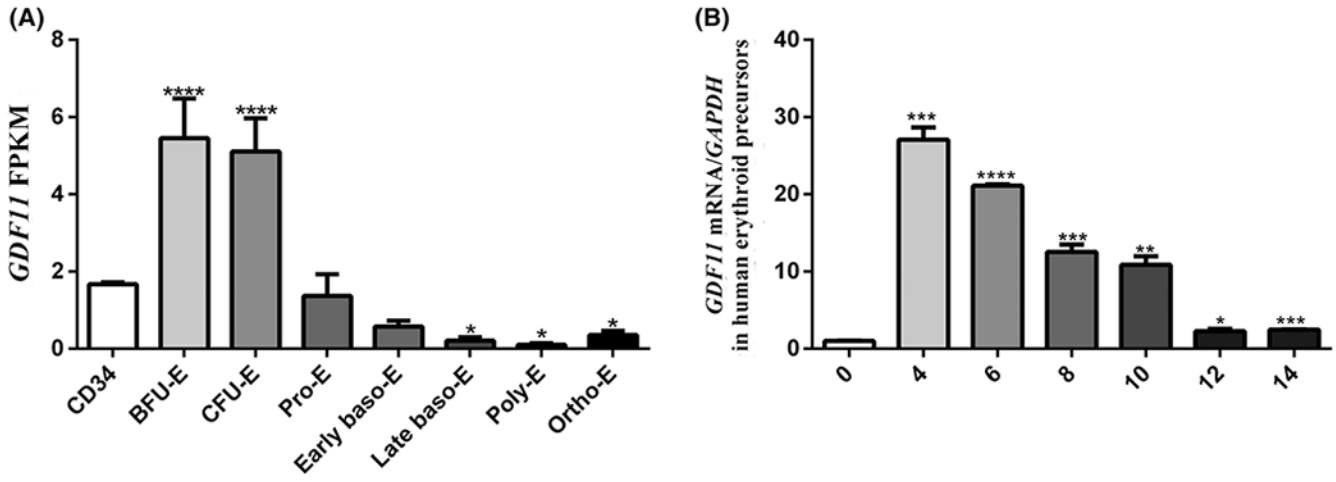


**Fig 3.**

Identification of *GDF11* expression specifity in erythroid cells. (A) *GDF11* mRNA expression was assessed by deep RNA sequencing at each distinct stage of human erythropoiesis. BFU-E, erythroid burst-forming unit; CFU-E, erythroid colony-forming unit; pro-E, pro-erythroblast; baso-E, basophilic erythroblast; ortho-E, orthochromatic erythroblast; poly-E, polychromatic erythroblast. (B) *GDF11* mRNA levels relative to *GAPDH* mRNA were analysed by qRT-PCR during EPO-induced human $CD34^+$ cells on culture days 0, 4, 6, 8, 10, 12 and 14. ****$P < 0{\cdot}0001$, ***$P < 0{\cdot}001$, **$P < 0{\cdot}01$, *$P < 0{\cdot}05$ compared with $CD34^+$ cells or the starting time point. Data represent 3 independent experiments. FPKM: Fragments per kilobase of transcript per million mapped reads.

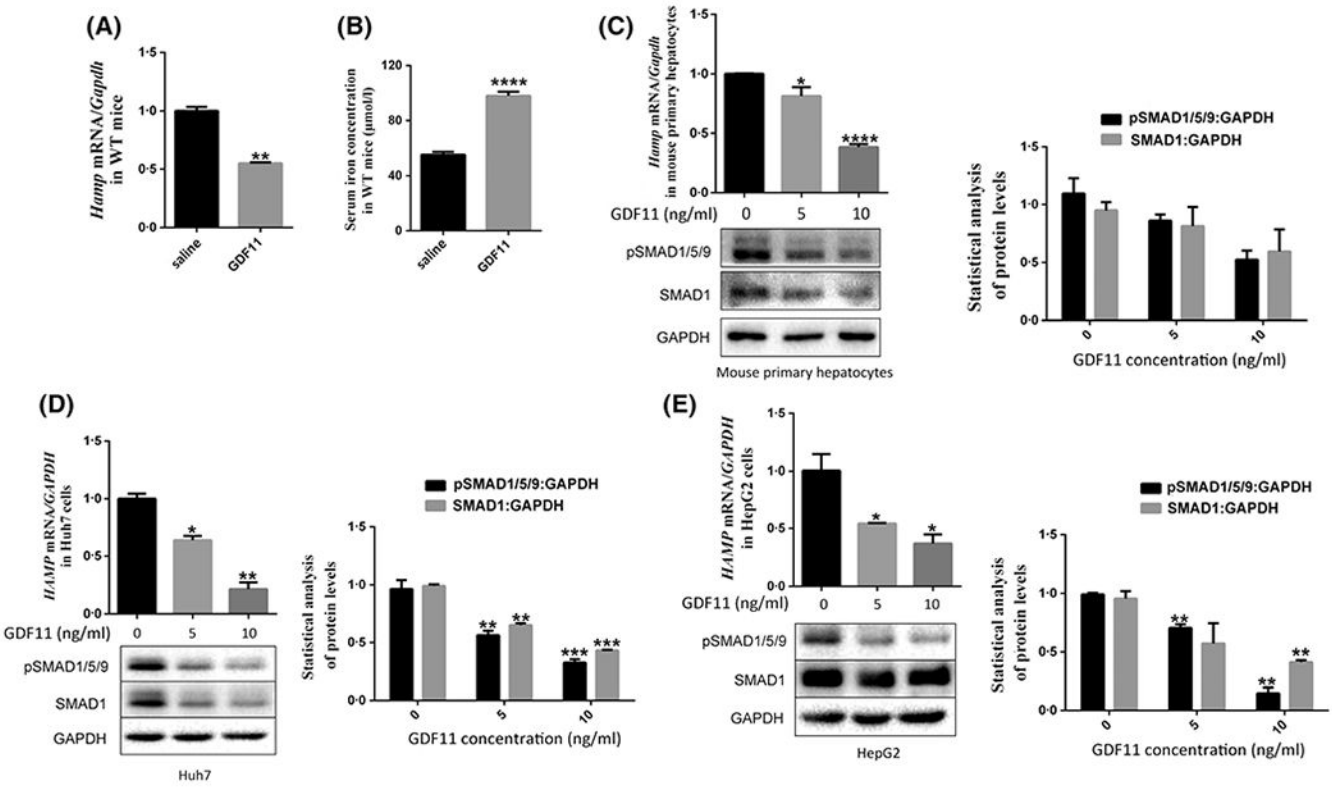


**Fig 4.**

GDF11 induces direct HAMP suppression through BMP-SMAD signaling. Male C57BL/6 mice (wild-type, WT) were injected intraperitoneally with either recombinant GDF11 (rGDF11, 0·1 μg/g body weight) or saline solution and analysed for (A) liver *Hamp* mRNA levels and (B) serum iron concentrations 15 h later ($n = 6$ per group). Mouse primary hepatocytes (C), and the human hepatocarcinoma cell lines,Huh7 (D) and HepG2 (E), were treated with rGDF11 (0, 5 or 10 ng/ml) for 24 h. Levels of *HAMP* mRNA were measured by qRT-PCR and normalized to control. Protein levels of pSMAD1/5/9, SMAD1 were detected by Western Blot, and quantified. *GAPDH* was used as the housekeeping gene. Experiments were repeated independently 4–6 times. ****$P < 0·0001$, ***$P < 0·001$, **$P < 0·01$, *$P < 0·05$ compared with levels in the control.

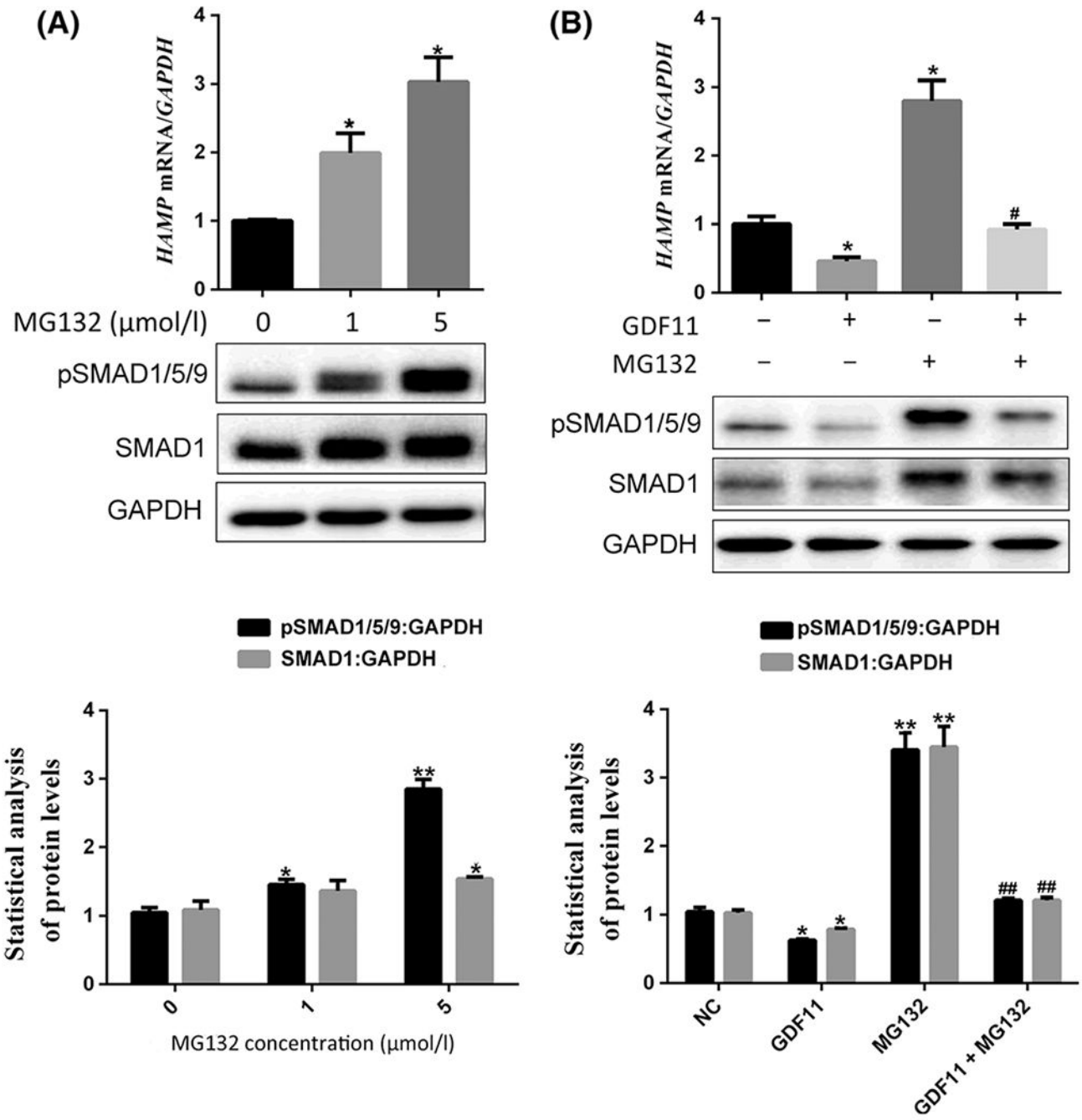


**Fig 5.**

GDF11-mediated HAMP and BMP-SMAD signalling suppression is restored by the proteasome inhibitor MG132. (A) Dose-response activation of *HAMP* expression and BMP-SMAD signalling with presence of MG132 (0, 1 or 5 μmol/l) for 4 h in Huh7 cells. (B) Rescuing effects of MG132 on *HAMP* expression and BMP-SMAD signalling in Huh7 cells. Cells were pre-treated by recombinant GDF11 (rGDF11, 5 ng/ml) for 24 h, and then co-treated with or without MG132 (5 μmol/l) 4 h before cell harvest. *HAMP* mRNA levels were measured by qRT-PCR and normalized to control. Protein levels of pSMAD1/5/9 and

SMAD1 were detected by Western Blot, and quantified. *GAPDH* was used as the housekeeping gene. Experiments were repeated independently for 4 times. $^{**}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$ compared with levels in the control. $^{\#\#}P < 0{\cdot}01$, $^{\#}P < 0{\cdot}05$ compared with levels in the treatment with GDF11 alone.

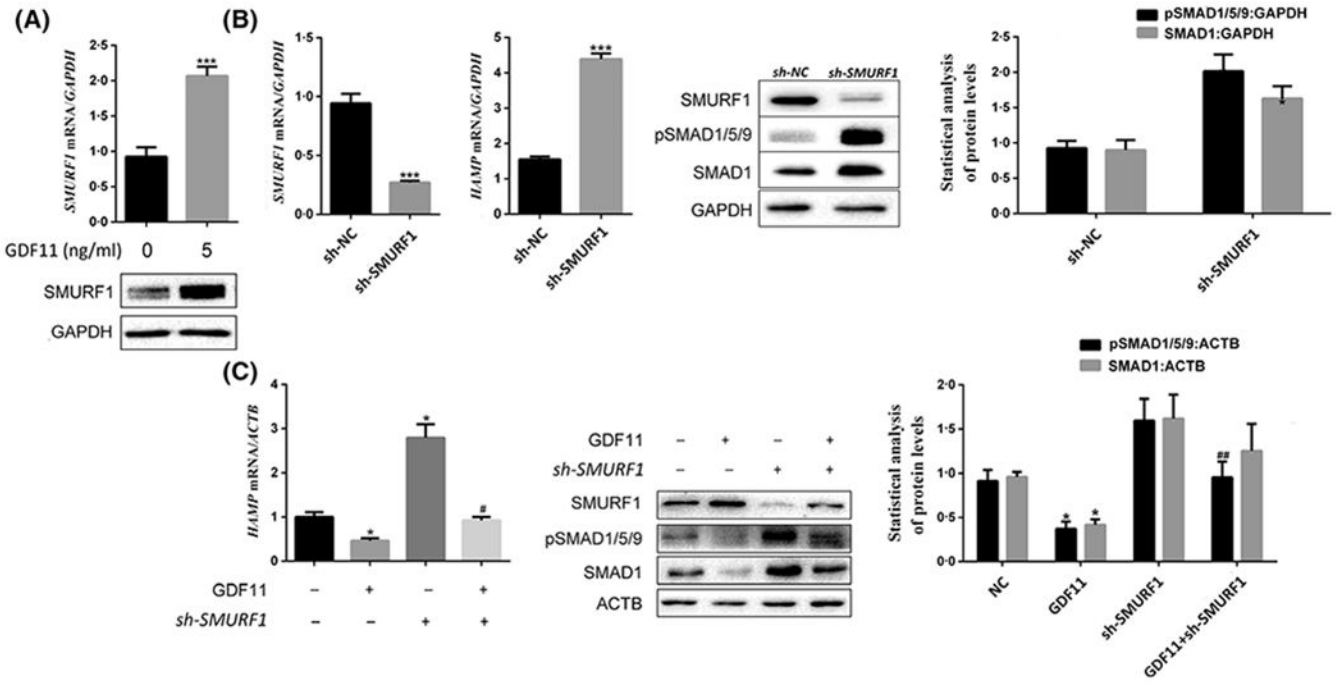


**Fig 6.**

GDF11 inhibits HAMP expression and BMP-SMAD signalling through SMURF1. (A) Increased *SMURF1* mRNA and SMURF1 protein levels following recombinant GDF11 (rGDF11) treatment. Huh7 cells were treated with or without rGDF11 (5 ng/ml) for 24 h. (B) *SMURF1* knockdown resulted in increased *HAMP* expression and SMAD1/5/9 phosphorylation. (C) Rescuing effects of *SMURF1* knockdown on *HAMP* expression and BMP-SMAD signalling. To knockdown *SMURF1*, cells were transfected with SMURF-specific shRNA for 48 h. Levels of *SMURF1* and *HAMP* mRNA were measured by qRT-PCR and normalized to the control, statistically presented as mean ± SD. Protein levels of SMURF1, pSMAD1/5/9, SMAD1 were detected by Western Blot, and quantified. *GAPDH* or *ACTB* were used as the housekeeping gene. Experiments were repeated independently for 6 times. ***$P < 0{\cdot}001$, **$P < 0{\cdot}01$, *$P < 0{\cdot}05$ compared with levels in the control. ##$P < 0{\cdot}01$, #$P < 0{\cdot}05$ compared with levels in the treatment with GDF11 alone.

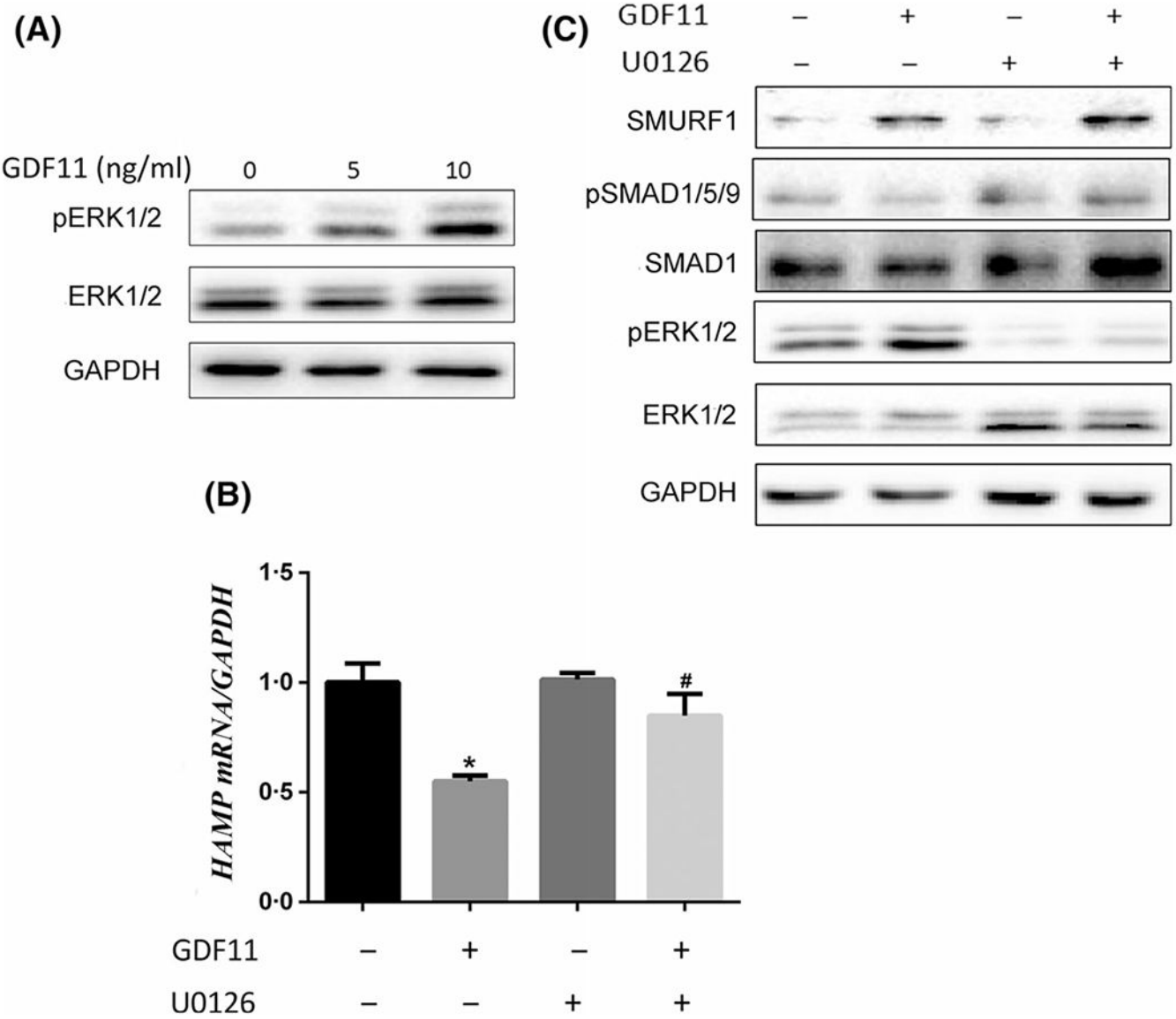


**Fig 7.**

ERK1/2 activation is required for GDF11-dependent suppression of hepcidin expression. (A) Increased activation of ERK1/2 signalling after treatment with GDF11. Huh7 cells were treated by recombinant GDF11 (rGDF11, 0, 5 or 10 ng/ml) for 24 h, and analysed by Western Blot. (B) qRT-PCR analysis of *HAMP* mRNA changes in the combination treatments. (C) Western Blot analysis of SMURF1, BMP-SMADs and ERK1/2 pathways in the combination treatments. Huh7 cells were treated with or without rGDF11 (5 ng/ml) for 24h, and then co-treated with or without the MEK/ERK inhibitor, U0126, at 2h before cell harvest. *$P < 0{\cdot}05$ compared with levels in the control. #$P < 0{\cdot}05$ compared with levels in the treatment with GDF11 alone.