Krüppel-like factor 4 is involved in cell scattering induced by hepatocyte growth factor

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Summary

Hepatocyte growth factor/scatter factor (HGF) is unique by inducing epithelial cell scattering, a cellular event pivotal to HGF-mediated invasive-growth response essential for embryonic development and metastasis. Krüppel-like factor 4 (KLF4) is a multifunctional zinc-finger transcription factor involved in cell proliferation, differentiation and self-renewal. We herein present the first evidence for the functional connection between KLF4 and HGF-induced cell scattering. In particular, we found that KLF4 was upregulated by HGF in two independent epithelial cell types, HepG2 and MDCK, whereas KLF4 knockdown inhibited HGF-induced E-cadherin suppression and cell scattering. Moreover, enforced nuclear KLF4 expression alone was sufficient to upregulate KLF4, downregulate E-cadherin and trigger scattering. Chromatin immunoprecipitation (ChIP) analysis further revealed that KLF4 induced suppression of E-cadherin transcription by directly binding to the E-cadherin promoter. Additionally, we proved that HGF-induced upregulation of KLF4 transcription and cell scattering require activation of the MEK/ERK signaling pathway and the induction of early growth response 1 (EGR-1). At the mechanistic level, ChIP analysis validated a direct binding of EGR-1 to the KLF4 promoter to induce KLF4 transcription; in turn, EGR-1-induced KLF4 binds to its own promoter, thus creating a positive feedback mechanism to sustain KLF4 expression and the resultant cell scattering. We conclude that KLF4 upregulation by HGF represents a novel mechanism mediating HGF-induced cell scattering and perhaps other associated events such as cell migration and invasion.

Key words: HGF, KLF4, EGR-1, E-cadherin, Cell scattering, EMT

Introduction

Hepatocyte growth factor/scatter factor (HGF) is a multifunctional protein capable of inducing mitogenic, motogenic, morphogenic and survival effects through binding and consequent activation of its cognate receptor, c-MET (Birchmeier et al., 2003). Signaling of HGF is transmitted via c-MET into cells through both RAS–MAPK- and PI3K–AKT-mediated signaling pathways, each accounting for the mitogenic and anti-apoptotic effect of HGF, respectively (Chan et al., 2002; Liang and Chen, 2001). Importantly, HGF is distinct among other migration-inducing growth factors in its ability to induce epithelial cells undergoing ‘invasive growth’, a genetic program to initiate a variety of cellular features required for cell migration and invasion (Boccaccio and Comoglio, 2006). These include the loss of epithelial cell-to-cell junctions and the acquisition of the motile and invasive mesenchymal features, consistent with the principal characteristics of epithelial–mesenchymal transition (EMT) (Kalluri and Weinberg, 2009). There has been mounting evidence in recent years of the central role of EMT in promoting carcinoma invasion and metastasis (Yang and Weinberg, 2008; Voulgaris and Pintzas, 2009) and the acquisition of stem cell traits by carcinoma cells (Polyak and Weinberg, 2009). Consistent with its EMT-inducing effect, deregulated HGF/c-MET signaling is closely associated with increased aggressiveness in a broad range of human cancer, making the HGF/c-MET axis an attractive target for cancer therapeutics (Gherardi et al., 2012). In multiple epithelial cell lines, especially in Madin-Darby canine kidney (MDCK) cells, HGF is sufficient to induce cell scattering, a cellular process reminiscent of EMT in the aspects of disrupting intercellular contacts, primarily E-cadherin-mediated adhesion, and acquiring a fibroblast-like shape with increased cell motility and invasiveness (Thiery, 2002). For that reason, HGF-induced cell scattering is an invaluable cellular system to study EMT as well as to identify key molecules responsible for cell migration and invasion (Chen, 2005; Chen and Chen, 2009).

Krüppel-like factor 4 (KLF4) is a C2H2 zinc-finger transcription factor that preferentially binds to CACCC elements and/or GC-rich sequences in the promoter of its target genes (McConnell and Yang, 2010). Accumulating evidence has started to unravel the pleiotropic actions of KLF4 in various physiological and pathological cellular processes, including proliferation, differentiation, apoptosis, inflammation, and maintenance of stemness (McConnell and Yang, 2010; Takahashi and Yamanaka, 2006; Yu et al., 2011). Intriguingly, it is often difficult to define the biological roles of KLF4 due to its cell context-dependent mode of action (Lin et al., 2011). One striking example is that KLF4 acts as an oncogene in certain types of human cancer whereas it functions as a tumor suppressor in others (Bureau et al., 2009), ostensibly depending on the functional status of p21Cip1/Waf1 in the cells of interest (Rowland et al., 2005).
Even though our knowledge about the molecular mechanisms underlying HGF-induced cell scattering/invasion is increasing, little was known regarding the transcription factors responsible for this process. A recent study by Grotegut et al. identified SNAIL, an EMT inducer, as required for HGF-induced scattered response, highlighting the close connection between cell scattering and EMT in response to HGF (Grotegut et al., 2006). It is also noteworthy that a positive connection between KLF4 and EMT as well as the integral role of KLF4 in cell migration and invasion were recently established in breast cancer cells (Yu et al., 2011). These findings therefore raise the question as to the possible involvement of KLF4 in HGF-induced cell scattering, migration and invasion, which has never been investigated previously.

In this report, we present the first evidence supporting the involvement of KLF4 upregulation by HGF in HGF-induced cell scattering. Mechanistic insights regarding how HGF upregulates KLF4 and how KLF4 downregulates E-cadherin are also elucidated. Our findings therefore facilitate a better molecular understanding of the scattered response and in general the malignant progression induced by HGF.

**Results**

**HGF upregulates KLF4**

To study the possible involvement of KLF4 in HGF-induced cell scattering, an *in vitro* cell-scattering model using the human hepatocellular carcinoma cell line HepG2 was established (Grotegut et al., 2006). Briefly, HepG2 cells were allowed to grow as tight colonies and then treated with HGF to induce scattering. A colony is judged as ‘scattered’ when half of the cells have lost contact with their neighbors and exhibited a fibroblast-like morphology (Chen and Chen, 2009). As shown in Fig. 1A, an obvious scattering of HepG2 cells was induced following 48 h treatment with HGF (50 ng/ml). With this cell-scattering model in hand, we next addressed the effect of HGF on KLF4. To this end, HepG2 cells were treated with a graded dosage (0–80 ng/ml) of HGF for 24 h, followed by immunoblot analysis for KLF4 expression. It is apparent that HGF treatment led to a dose-dependent increase in KLF4 protein levels (Fig. 1B). To further understand HGF-induced kinetic change of KLF4 expression, HepG2 cells were stimulated with 50 ng/ml of HGF for 96 h, and the levels of KLF4 protein at indicated time points were determined by immunoblotting. As shown in Fig. 1C, a time-dependent increase in KLF4 levels was observed until 24 h after HGF treatment, followed by a gradual decline thereafter. In parallel with the kinetics of HGF-induced KLF4 protein expression, quantitative real-time RT-PCR analysis revealed a clear increase in KLF4 mRNA levels starting at 6 h and reaching the maximal level of induction at 24 h following HGF treatment, where KLF4 expression was induced by 4.13±0.12-fold compared with the untreated control (*P*<0.01; Fig. 1D). Remarkably, a 1.52±0.09-fold KLF4 induction was observed even after 96 h treatment with HGF (*P*<0.05; Fig. 1D). Altogether, these results for the first time established a promoting effect of HGF on KLF4 expression at both the mRNA and protein levels.

**KLF4 is involved in HGF-induced cell scattering**

We went on to define the physiological significance of KLF4 upregulation in HGF-induced cell scattering. To address this issue, we established HepG2 cells stably expressing KLF4-targeting short hairpin RNA (shRNA) to neutralize HGF-induced increase of KLF4. As shown in Fig. 2A, depletion of endogenous KLF4 protein was clearly verified in HepG2 stable clones expressing either of the two individual KLF4 shRNAs (i.e. shKLF4#1 or shKLF4#2). Notably, KLF4 depletion was sustained even after HGF treatment, in contrast to the evident increase of KLF4 levels in stable clones expressing a firefly luciferase-targeting shRNA (shLuc; Fig. 2B). We next explored the role of KLF4 in E-cadherin expression, given suppression of E-cadherin and the consequent loss of E-cadherin-mediated intercellular adhesion is a prerequisite to initiate cell scattering (Thiery, 2002). Not surprisingly, HGF lowered E-cadherin levels in shLuc control cells as previously reported (Grotegut et al., 2006); nevertheless, E-cadherin expression was rather resistant to HGF-induced reduction when KLF4 is depleted (Fig. 2B). These results suggest KLF4 as a negative regulator of E-cadherin expression. We further examined the effect of KLF4 on *E-cadherin* mRNA expression by quantitative real-time RT-PCR analysis. Consistent with its action to downregulate E-cadherin, HGF treatment lowered the extent of *E-cadherin* mRNA expression in shLuc stable clones. Specifically, the levels of *E-cadherin* mRNA were reduced to ~41%, 25% and 17% of that in untreated cells after HGF treatment for 24 h, 48 h and 96 h, respectively (Fig. 2C). In contrast, KLF4 depletion led to a 2.23±0.05-fold increase in the basal expression of *E-cadherin* mRNA as compared with that of shLuc control cells, illustrating the inhibitory role of KLF4 in E-cadherin expression (Fig. 2C). It is also noteworthy that *E-cadherin* mRNA levels remained relatively constant in KLF4-depleted cells even after HGF treatment (Fig. 2C). These results together highlight the notion...
that HGF-induced E-cadherin suppression depends on KLF4. We next aimed to probe the functional consequence of KLF4-mediated E-cadherin suppression on HGF-induced cell scattering. As shown in Fig. 2D, an evident cell scattering was induced in shLuc control cells treated with 50 ng/ml of HGF for 48 h, whereas the majority of KLF4-depleted cells remained attached and were refractory to scattering. In particular, 32.63 ± 5.16% of shLuc control cells were scattered following 96 h treatment with HGF, while the levels of scattering were respectively lowered to 14.88 ± 2.66% or 13 ± 3.19% in cells stably expressing shKLF4#1 or shKLF4#2 (P < 0.01; Fig. 2E). Importantly, both E-cadherin downregulation and cell scattering caused by KLF4 depletion were rescued in KLF4-depleted cells when KLF4 expression was restored (supplementary material Fig. S1). Taken together, it is evident that KLF4 upregulation contributes to HGF-induced cell scattering.

Fig. 2. KLF4 is required for HGF-induced cell scattering in HepG2 and MDCK cells. (A) Validation of KLF4 depletion. HepG2 cells stably expressing either of KLF4-specific shRNAs (shKLF4#1 or shKLF4#2) were subject to immunoblotting for KLF4 expression. β-tubulin was used as the loading control. (B) KLF4 depletion inhibits HGF-induced repression of E-cadherin. HepG2 cells stably expressing control shRNA (shLuc; left) or shKLF4#1 (right) were treated with HGF (50 ng/ml) for 0, 6, 24, 48 h and then the protein levels of KLF4 and E-cadherin (E-cad) were analyzed by immunoblotting. β-tubulin was used as the loading control. (C) KLF4 depletion leads to an increase in the basal expression of E-cadherin mRNA but also abolishes HGF-induced E-cadherin suppression. shLuc- or shKLF4#1-expressing HepG2 stable clones were treated with HGF (50 ng/ml) for 0, 24, 48, 96 h. Quantitative real-time RT-PCR was then performed to determine E-cadherin (E-cad) mRNA levels, which are expressed as fold (means ± s.e.m.) induction relative to that without HGF treatment. *P < 0.05. **P < 0.01. (D) KLF4 depletion inhibits HGF-induced cell scattering in HepG2 cells. shLuc- or shKLF4#1-expressing HepG2 stable clones were treated with HGF (50 ng/ml) for 48 h, followed by light microscopy analysis to evaluate the extent of cell scattering. Scale bar: 50 μm. (E) Quantification of the numbers of colonies undergoing cell scattering as shown in D at the indicated time points. A colony was judged as ‘scattered’ when half of the cells had lost contact with their neighbors and exhibited a fibroblast-like phenotype. Values (means ± s.e.m.) were calculated from three independent experiments (n = 100). **P < 0.01. (F) KLF4 depletion in MDCK cells. Stable clones of MDCK cells expressing shLuc or shKLF4 were stimulated with or without HGF (50 ng/ml) for 24 h. KLF4 protein levels were then analyzed by immunoblotting. β-tubulin was used as the loading control. (G) KLF4 depletion confers MDCK cells resistance to HGF-induced E-cadherin suppression. shLuc- or shKLF4-expressing MDCK stable clones were treated with HGF (50 ng/ml) for 6 h and the level of E-cadherin (E-cad) on the plasma membrane was evaluated by immunofluorescence microscopy using anti-E-cadherin antibody (green). The plasma membrane was revealed by staining with fluorophore-conjugated wheat germ agglutinin (WGA). (H) KLF4 depletion lowers the level of HGF-induced scattering of MDCK cells. At various time intervals after HGF stimulation, the percentage of scattered colonies in all counted colonies was determined. **P < 0.01.
To rule out whether the promoting role of KLF4 in HGF-induced scattered response is only restricted to HepG2 cells, we also performed similar experiments in MDCK cells, a canine kidney epithelial cell line known as a cardinal model for HGF-induced cell scattering (Chen, 2005). As shown in Fig. 2F, HGF upregulated KLF4 in MDCK cells stably expressing shLuc, whereas KLF4 induction was attenuated in shKLF4-expressing cells treated with HGF. Immunofluorescence analysis further revealed that HGF treatment led to a marked loss of E-cadherin signals and the induction of cell scattering in shLuc-expressing cells. In contrast, both of these events were clearly ablated in cells with KLF4 depletion (Fig. 2G). Particularly, the levels of HGF-induced scattering was reduced from 57.42±6.98% in shLuc control cells to 32.88±8.74% in KLF4-depleted cells (P<0.01; Fig. 2H). In conclusion, the fact that consistent results were observed in both HepG2 and MDCK cells underpins the notion that KLF4 appears to be one of the molecular components responsible for HGF-induced E-cadherin suppression and cell scattering.

**Enforced nuclear KLF4 expression is sufficient to trigger cell scattering and potentiates the HGF-induced scattering response**

We next asked whether KLF4 alone is sufficient to replace HGF to trigger E-cadherin suppression and cell scattering. To address this issue, we established HepG2 stable clones expressing a conditional, 4-hydroxytamoxifen (4-OHT)-inducible KLF4 fused at the C-terminus with the mouse estrogen receptor fragment carrying a 4-OHT-responsive element (KLF4–ER) (Foster et al., 2005). It appears that the KLF4–ER fusion protein dispersed evenly in the cytoplasm without 4-OHT induction (Fig. 3A, upper panel). However, addition of 4-OHT led to the translocation of KLF4–ER to the nucleus, where it ostensibly regulates the transcription of its target genes (Fig. 3A, lower panel). In line with this, nuclear KLF4–ER expression appeared to reduce E-cadherin protein levels as effectively as that lowered by HGF treatment (Fig. 3B). Intriguingly, we noticed that 4-OHT treatment alone led to an increase in KLF expression comparable to that after HGF stimulation, suggesting the possibility of KLF4 self-regulation (Fig. 3B). At the cellular level, HepG2 KLF4–ER stable clones without 4-OHT induction remained in their typical cobblestone-like epithelial morphology with tight intercellular contacts. In contrast, 4-OHT-induced stable clones apparently lost cell-to-cell attachment and showed elongated and spindle-shaped morphology, which was reminiscent of the scattered phenotype induced by HGF (Fig. 3C). It is noteworthy that nuclear KLF4–ER expression alone upregulated the level of scattered cells (from 1.67±1.25% in control cells to 20.00±3.82% at 96 h after 4-OHT induction, Fig. 3. Ectopic nuclear expression of KLF4 is sufficient to induce cell scattering independently of HGF. (A) Nuclear translocation of KLF4–ER following 4-OHT induction. HepG2 clones stably expressing a conditional wild-type KLF4 protein fused with 4-OHT-responsive mouse estrogen receptor (Flag–wtKLF4–ER) were treated without or with 4-OHT (1 µM). Nuclear translocation of KLF4–ER is induced upon 4-OHT treatment, as revealed by the localization of KLF4–ER (detected by the anti-FLAG antibody, green) in the nucleus (revealed by DAPI staining, blue). Scale bar: 5 µm. (B) Nuclear KLF4 expression downregulates E-cadherin without HGF treatment. HepG2 cells stably infected with FLAG–wtKLF4–ER were treated without or with HGF (50 ng/ml) for 24 h in the absence or presence of 4-OHT (1 µM). The protein levels of E-cadherin (E-cad) and KLF4 were then determined by immunoblotting. The numbers below the KLF4 immunoblot indicate the density of KLF4 immunoblotting signals, which were quantified using an ImageJ algorithm and expressed as the ratio of the signal density without co-treatment of HGF and 4-OHT. β-tubulin was used as the loading control. (C) Nuclear expression of KLF4 is sufficient to induce HepG2 cell scattering without HGF treatment. HepG2 FLAG–wtKLF4–ER stable clones were treated without or with HGF (50 ng/ml) for 48 h in the absence of presence of 4-OHT (1 µM). Cell scattering was then observed by light microscopy. Scale bar: 50 µm. (D) Quantification of the colonies shown in C undergoing cell scattering at the indicated time points. A colony was judged as ‘scattered’ when half of the cells had lost contact with their neighbors and exhibited a fibroblast-like phenotype. Values (means ± s.e.m.) were calculated from three independent experiments (n=100). *P<0.05. **P<0.01.
KLF4 directly suppresses E-cadherin transcription upon HGF treatment

The mechanism underlying how KLF4 downregulates E-cadherin was further investigated. In particular, we asked whether KLF4 represses the transcription of the E-cadherin gene, given KLF4 depletion led to a twofold increase in the basal level of E-cadherin mRNA (Fig. 2C). To address this, we generated an E-cadherin promoter luciferase reporter construct (Fig. 4A, pEDcad-Luc), which carries the (−397/+44) human E-cadherin proximal promoter fragment potentially carrying several KLF4 binding sites (Yori et al., 2010). HepG2 cells without (shLuc) or with KLF4 depletion (shKLF4) were then transiently transfected with pEDcad-Luc, followed by 24-h treatment with HGF (50 ng/ml) and determination of luciferase activity at indicated time points to evaluate the E-cadherin promoter activity. We found that E-cadherin promoter activity was markedly reduced in shLuc-infected cells upon HGF treatment (reduction to 0.41±0.01 and 0.30±0.02-fold of the untreated control after HGF treatment for 6 h and 24 h, respectively; P<0.01). In contrast, in KLF4-depleted cells the basal activity of the E-cadherin promoter was enhanced nearly by twofold and remained relatively constant after HGF stimulation (Fig. 4A). These results therefore indicated that KLF4 downregulates the E-cadherin promoter activity. To further elucidate whether KLF4 directly regulates the E-cadherin promoter, chromatin immunoprecipitation (ChIP) analysis was performed using the primers flanking the putative KLF4 response elements (−170→+10) (Yori et al., 2010). A clear binding of KLF4 to the E-cadherin promoter was observed as early as 6 h following HGF treatment (Fig. 4B), indicating that KLF4 represses E-cadherin transcription through direct binding to the E-cadherin promoter.

In addition to directly regulating the E-cadherin promoter activity, KLF4 is also likely repressing E-cadherin transcription through the action of other E-cadherin transcriptional repressors. A previous report by Grotegut et al. indicated that SNAIL, a well-known E-cadherin transcriptional repressor, is rapidly upregulated by HGF for E-cadherin downregulation and scattering induction in HepG2 cells (Grotegut et al., 2006). Using the same cellular system, we also observed that SNAIL was upregulated by HGF with faster kinetics than that of KLF4 (Fig. 4C, left panel), and that SNAIL knockdown evidently inhibited HGF-induced E-cadherin suppression and cell scattering (supplementary material Fig. S2). Accordingly, we

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P<0.05, \text{ but the capacity was not as potent as that induced by HGF (36.00±5.35%, } P<0.05; \text{ Fig. 3D). In addition to promoting cell scattering, nuclear KLF4-ER expression further enhanced HGF-induced scattered response (from 36.00±5.35% after 96 h treatment with HGF alone to 75.33±6.18% when co-treated with HGF and 4-OHT, } P<0.01; \text{ Fig. 3D). Taken together, these results indicate that although KLF4 alone is not sufficient to trigger full-scale cell scattering, it greatly facilitates HGF-induced scattered phenotype.}
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asked whether KLF4 downregulates E-cadherin through the action of SNAIL. To test this possibility, HepG2 cells without (shLuc) or with KLF4 depletion (shKLF4) were treated with HGF (50 ng/ml) for 24 h, and the protein levels of SNAIL at indicated time points were then evaluated by immunoblotting. As shown in Fig. 4C, we found that both the kinetics and the level of SNAIL upregulation induced by HGF were comparable irrespective of KLF4 depletion. Similarly, SNAIL mRNA expression levels were increased by HGF in KLF4-depleted cells as effectively as that in the shLuc control (Fig. 4D), indicating that SNAIL upregulation by HGF is not subject to the control of KLF4. This notion and the faster kinetics of SNAIL induction by HGF than that of KLF4 together suggest that SNAIL does not appear to mediate the inhibitory effect of KLF4 on E-cadherin transcription. To sum up, we conclude that following HGF stimulation, KLF4 downregulates E-cadherin by transcriptional suppression through its direct action on the E-cadherin promoter.

HGF-induced KLF4 upregulation is independent of p53

With the role of KLF4 in HGF-induced suppression of E-cadherin and cell scattering firmly established, we then aimed to elucidate the mechanism whereby HGF upregulates KLF4. Given KLF4 has been reported as a transcriptional target of p53 (Yoon et al., 2003), we first examined the role of p53 in HGF-induced KLF4 upregulation in HepG2 cells, which is known to express functional p53. To this end, we generated stable p53-depleted HepG2 clones by the use of a p53-targeting shRNA (shp53). Depletion of endogenous p53 was clearly validated by immunoblotting (Fig. 5A, middle panel). These p53-depleted clones were then treated with increasing doses of HGF (0–80 ng/ml) for 24 h or with 50 ng/ml of HGF for 96 h to evaluate the levels of KLF4 expression or cell scattering, respectively. As shown in Fig. 5A, it is apparent that KLF4 protein levels were dose-dependently upregulated by HGF irrespective of p53 depletion. Importantly, the extent of HGF-induced scattering of p53-depleted clones was comparable to that of shLuc control cells, consistent with p53-independent KLF4 upregulation upon HGF treatment (Fig. 5B). Overall, these results clearly establish the dispensable role of p53 in HGF-induced KLF4 upregulation and cell scattering.

HGF induces the activation of the MEK/ERK signaling to upregulate KLF4

The upstream signaling pathway mediating HGF-induced KLF4 upregulation was further investigated. The MEK/ERK signaling is one of the downstream pathways following the engagement of the HGF/c-MET axis (Gherardi et al., 2012), and, importantly, is required for HGF-induced cell scattering (Grotegut et al., 2006; Liang and Chen, 2001). We therefore hypothesized that HGF induces MEK/ERK activation to upregulate KLF4, which in turn triggers cell scattering. To test this, HepG2 cells were treated with 50 ng/ml of HGF for 24 h, and the levels of KLF4 and ERK activation, revealed by a dual phosphorylation of ERK at threonine 202 and tyrosine 204 (Thr202/Tyr204), were determined by immunoblotting. We observed that Thr202/Tyr204 phosphorylation of ERK was rapidly induced upon HGF treatment, which peaked at 1 h, declined afterwards, and was sustained even after 24 h of treatment (Fig. 6A). In contrast, the increase of KLF4 levels was not evident until HGF treatment for 6 h and continued to increase thereafter, suggesting ERK activation is an upstream event of KLF4 upregulation upon HGF stimulation. Importantly, functional blockade of ERK activation by U0126, a MEK1/2-specific inhibitor, nearly abolished the promoting effect of HGF on the expression of KLF4 protein (Fig. 6B) and mRNA (Fig. 6C,D). In agreement with the decrease in KLF4 expression, HGF-induced cell scattering was abrogated upon U0126 treatment (Fig. 6E). It is also noteworthy that pharmacological inhibition of other HGF-initiated signaling pathways such as JNK, PI3K and STAT3 (Gherardi et al., 2012) all showed limited effect on HGF-induced KLF4 expression (supplementary material Fig. S3). Altogether, these lines of evidence support the notion that activation of the MEK/ERK signaling is fundamental for HGF-induced KLF4 upregulation to promote cell scattering.

The KLF4 gene is a transcriptional target of EGR-1 and KLF4 itself upon HGF treatment

Our previous observation of nearly fourfold induction in KLF4 mRNA levels after 24 h of HGF treatment (Fig. 1D, Fig. 6D) implicated a possible transcriptional control of the KLF4 gene by HGF. To examine this possibility, HepG2 cells were transiently transfected with a KLF4 promoter reporter construct, in which a human genomic fragment encompassing 2568 bases upstream of the KLF4 translational start site was used to drive the expression of firefly luciferase gene (Fig. 7A, P1). It is noteworthy that HGF treatment for 24 h led to a 2.85±0.01-fold of induction in the KLF4 promoter activity as compared with the untreated control (P<0.01), indicating the KLF4 gene is indeed a transcriptional target of HGF-induced signaling. Serial deletions from the 5’-end of the P1 reporter construct identified the region between −2568 and −1375 bases was likely the minimal KLF4 promoter responsible for the action of HGF, since deletion of this region nearly abolished HGF-induced activation (Fig. 7A).
Role of KLF4 in HGF-induced cell scattering

Fig. 6. MEK/ERK activation is required for HGF-induced KLF4 expression and cell scattering. (A) ERK activation precedes KLF4 induction upon HGF treatment. HepG2 cells were exposed to HGF (50 ng/ml) for 24 h and the levels of KLF4 and Thr202/Tyr204-phosphorylated (activated) ERK at the indicated time points were determined by immunoblotting. β-tubulin was used as the loading control. (B) Blockade of MEK/ERK activation prevents HGF-induced increase in KLF4 protein expression. HepG2 cells were stimulated with HGF (50 ng/ml) in the absence of presence of the MEK1/2-specific inhibitor U0126 (5 μM), followed by immunoblotting for the levels of KLF4 and phosphorylated/activated ERK. β-tubulin was used as the loading control. (C, D) MEK/ERK inhibition abolishes the promoting effect of HGF on KLF4 mRNA expression. HepG2 cells were stimulated with HGF (50 ng/ml) for 24 h without or with U0126 co-treatment. The levels of KLF4 mRNA were then determined by regular RT-PCR (C) or quantitative real-time RT-PCR (D) analyses. s-actin mRNA was used as a loading control for regular RT-PCR analysis. KLF4 mRNA levels determined by quantitative real-time RT-PCR are expressed as fold induction (means ± s.e.m.) compared with that of drug-untreated controls. **P < 0.01. (E) HGF-induced cell scattering requires MEK/ERK activation. The status of cell scattering of HepG2 cells was observed by light microscopy after 48 h of HGF stimulation without or with U0126 co-treatment (scale bar: 25 μm).

To further elucidate the molecular basis controlling KLF4 gene transcription, analysis of the minimal KLF4 promoter sequence was performed using the algorithm in the TESS website (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and led to the identification of putative cis-acting response elements for a number of transcription factors, including EGR-1, ELK-1, AP-1 and KLF4 itself (Fig. 7B). Aside from KLF4 revealed in this study, accumulating evidence has indicated the involvement of EGR-1, ELK-1 and AP-1 in MAPK-mediated biological processes (Gaggioli et al., 2005; Lopez-Bergami et al., 2010; Yoshida et al., 2008), and, particularly, EGR-1 has been identified as an essential component mediating HGF-induced SNAIL expression in an MAPK-dependent manner (Groteteg et al., 2006). For these reasons, we asked whether KLF4 promoter activity is subject to the regulation by these transcription factors. To answer this question, a number of reporter constructs with distinct combinations of the response element (RE) for the aforementioned transcription factors were generated, as depicted in Fig. 7C. It is apparent that HGF induced a time-dependent increase in the activity of the KLF4 promoter (p–2183 to −1885) as early as 6 h after HGF treatment, followed by a decline to the basal level at 24 h (Fig. 7C, second from left, black bars). It is noteworthy that the KLF4 promoter carrying the EGR-1 RE alone (p–2183 to −2083) demonstrated a similar kinetic response to HGF treatment as that of p–2183 to −1885 (Fig. 7C, second from left, white bars), indicating that EGR-1 is responsible for the early response of the KLF4 promoter to HGF stimulation. In line with the effect of EGR-1, deletion of the EGR-1 RE alone led to a delay in the activation of the KLF4 promoter (p–2083 to −1476) until 24 h following HGF treatment (Fig. 7C, second from right, black bars). Intriguingly, the promoter containing only the KLF4 RE (p–1905 to −1476) responded to HGF stimulation in a manner comparable to that of p–2083 to −1476 (Fig. 7C, second from right, white bars), highlighting the role of KLF4 in the later response to HGF treatment. Actually, the notion of KLF4 self-regulation was consistent with our previous finding that nuclear KLF4 expression alone upregulated KLF4 (Fig. 3B), likely resulting from transcriptional activity (supplementary material Fig. S4). Taken together, these results indicated that both EGR-1 and KLF4 are necessary for full activation of the KLF4 promoter in response to HGF stimulation.

Given MEK/ERK activation is required for HGF to increase KLF4 mRNA expression (Fig. 6C, D), the role of the MEK–ERK signaling in HGF-induced KLF4 promoter activation was investigated. To this end, HepG2 cells were transiently transfected with the KLF4 reporter plasmids P1 (containing both REs of EGR-1 and KLF4), p–2083 to −1476 (devoid of EGR-1 RE) or p–2183 to −1885 (lacking KLF4 RE) and were subject to HGF stimulation for 24 h with co-treatment of the MEK inhibitor U0126. It is apparent that HGF induced a marked increase in the activity of all promoters examined, whereas this promoting effect of HGF was nearly abolished when ERK activation was blocked by U0126 (Fig. 7D). Altogether, these results indicated that HGF-induced KLF4 promoter activation depends on MEK/ERK activation.

We next performed ChIP analyses to elucidate whether EGR-1 and KLF4 mediate HGF-induced KLF4 transactivation through direct binding to the KLF4 promoter. As shown in Fig. 7E, an increased binding of endogenous EGR-1 to the KLF4 promoter was observed at 6 h after HGF stimulation and then the binding declined to the basal level at 24 h (upper panel). On the other hand, endogenous KLF4 barely bound to the KLF4 promoter without HGF stimulation, while an evident binding activity started after 6 h treatment with HGF and the binding sustained even at 24 h (lower panel). Overall, these lines of evidence confirmed that the KLF4 gene is a direct transcriptional target of both EGR-1 and KLF4 itself upon HGF stimulation, and further revealed a positive feedback loop mechanism to sustain the expression of KLF4.

EGR-1 is required for HGF-induced KLF4 upregulation and cell scattering

Given EGR-1 appears to bind and transactivate the human KLF4 promoter upon HGF stimulation (Fig. 7C, E), the role of EGR-1 in HGF-induced KLF4 transcription and cell scattering was...
further characterized. Immunoblot analysis revealed an increase of EGR-1 protein levels in HepG2 cells treated with HGF (50 ng/ml), starting as early as 1 h of HGF treatment, peaked at 6 h and nearly returned to the basal level at 24 h (Fig. 8A). Apparently, induction of EGR-1 by HGF preceded that of KLF4, which was not evident until 6 h after HGF treatment (Fig. 8A). This finding coincided with our previous observations that EGR-1 mediated the early response to HGF stimulation (Fig. 7C,E). To further define the effect of EGR-1, we established HepG2 clones stably expressing the zinc-finger DNA-binding domain of Egr-1 (Egr1Znf) (Chapman and Perkins, 2000) fused to the 4-OHT-responsive mouse estrogen receptor (ER). Upon 4-OHT

Fig. 7. Activation of the KLF4 promoter by EGR-1 and KLF4 upon HGF treatment. (A) Identification of the minimal KLF4 promoter region susceptible to HGF-induced activation. KLF4 promoter reporter constructs carrying the human genomic sequence of the KLF4 gene from −2568 (P1), −1374 (P2), −1045 (P3), and −891 (P4) to +183 are shown. The translational start site of the KLF4 coding region is denoted as +1. The shaded box represents the first exon of the KLF4 gene. These DNA fragments were used to drive the expression of the firefly luciferase gene (Luc). HepG2 cells were transiently transfected with these KLF4 promoter reporter constructs for 24 h, followed by treatment with HGF (50 ng/ml) for 24 h and luciferase activity assayed thereafter. The activity of the KLF4 promoter is expressed as fold induction (means ± s.e.m.) compared with that of HGF-untreated controls. *P<0.05, **P<0.01. (B) Putative binding sequence of transcription factors EGR-1, ELK-1, AP1 (c-JUN/c-FOS) and KLF4 were identified on the minimal KLF4 promoter. The predicted cis-acting binding sites of EGR-1, ELK-1, AP-1 or KLF4 are boxed. (C) EGR-1 and KLF4 mediate the early and late responses to HGF treatment, respectively. Various KLF4 promoter reporter constructs with different combinations of binding sites of EGR-1, ELK-1, AP-1 or KLF4 are depicted below. These reporter constructs were then transiently transfected into HepG2 cells to evaluate their activity after HGF stimulation. (D) HGF-induced activation of the KLF4 promoter requires MEK/ERK activity. HepG2 cells were transiently transfected with various KLF4 promoter reporter constructs (P1, p−2083−−1476 or p−2183−−1885) and subjected to HGF stimulation in the absence or presence of the MEK1/2-specific inhibitor U0126 (5 µM), followed by determination of luciferase activity. *P<0.05, **P<0.01. (E) Direct binding of EGR-1 and KLF4 to the KLF4 promoter. ChIP analysis was performed to reveal the direct binding of EGR-1 and KLF4 to the KLF4 promoter. HepG2 cells were stably transfected with HGF (50 ng/ml) for 0, 6 and 24 h, followed by incubation with IgG, anti-EGR-1 or anti-KLF4 antibodies to pull down protein–DNA complexes. Subsequent PCR analysis was performed using primers complementary to the KLF4 promoter region flanking the EGR-1 (−2200−−2077) and KLF4 (−1579−−1457) binding sites. PCR products were analyzed by agarose gel electrophoresis.
treatment, the Egr1Znf–ER fusion protein was translocated to the nucleus (Fig. 8B), where it ostensibly exerts a dominant-negative action by competing with endogenous EGR-1 for binding to the response elements on their target genes, thus leading to functional blockade of EGR-1-mediated gene transcription. It is interesting to note that, although HGF increased the protein levels of KLF4 while suppressing that of E-cadherin, both of these actions of HGF were clearly reversed when nuclear Egr1Znf–ER expression was induced by 4-OHT (Fig. 8C). Similarly, HGF-induced KLF4 promoter activation was nearly abrogated in HepG2 cells where EGR-1 is functionally blocked, illustrating the requirement of EGR-1 for HGF-induced KLF4 transcription (Fig. 8D). In line with its inhibitory effect on KLF4 expression, nuclear Egr1 Znf–ER clearly lowered the extent of HGF-induced HepG2 scattering than that without 4-OHT treatment (Fig. 8E). Specifically, 4-OHT stimulation reduced the level of scattered cells to 24.67±3.3% in contrast to 36.67±4.11% in 4-OHT-untreated cells (P<0.05; Fig. 8F). In summary, we conclude that EGR-1 is an essential mediator of HGF to induce KLF4 upregulation, E-cadherin suppression and cell scattering.

Discussion

Induction of epithelial cell scattering is a unique feature of HGF and is fundamental for HGF/c-MET signaling-elicited invasive growth, which is pivotal to drive cancer cell invasion and metastasis (Boccaccio and Comoglio, 2006). Not surprisingly, aberrant HGF/c-MET signaling is associated with increasing aggressiveness in a broad range of human cancer and thus making the HGF/c-MET axis a promising target for cancer therapeutics (Gherardi et al., 2012). We herein present compelling evidence to support KLF4, a zinc-finger transcription factor known to regulate cell proliferation and induce somatic cell reprogramming, as a novel molecular mediator of HGF to induce cell scattering. In particular, we demonstrated that HGF upregulates KLF4 expression in two independent epithelial cellular models HepG2 and MDCK (Figs 1, 2). Notably, knockdown of KLF4 in these cell lines effectively lowered the extents of HGF-induced E-cadherin suppression and cell scattering (Fig. 2). Moreover, enforced nuclear KLF4 expression was sufficient to downregulate E-cadherin and trigger cell scattering, but also capable to further potentiate HGF’s scattered effect (Fig. 3). To our best knowledge, the functional connection between KLF4 and HGF-induced cell scattering established in this study has never been reported previously.

It is noteworthy that epithelial cell scattering share cellular features reminiscent of cells undergoing EMT, including the loss of predominantly E-cadherin-mediated epithelial cell-to-cell adhesion and the acquisition of a motile mesenchymal phenotype (Chen, 2005). Given its promoting effect on HGF-induced cell scattering, KLF4 is likely acting as an EMT inducer. One molecular hallmark of cells undergoing EMT is ‘cadherin switching’, in which the expression level of N-cadherin, a
mesenchymal cadherin, is increased with a concomitant decrease in that of E-cadherin, an epithelial cadherin (Hazan et al., 2004; Maeda et al., 2005). Indeed, we found that HGF-treated HepG2 cells displayed such ‘cadherin switching’, and, importantly, such process was abolished when KLF4 was depleted (supplementary material Fig. S5). These results suggest that HGF treatment triggers EMT in HepG2 cells and KLF4 is required for this process. This notion is however inconsistent with that reported by Grotegut et al., in which SNAIL, a well-recognized EMT inducer, is upregulated by HGF but no upregulation of mesenchymal markers was observed (Grotegut et al., 2006). The disparity between Grotegut et al. (Grotegut et al., 2006) and ours with regard to HGF-induced EMT in HepG2 cells is likely due to the higher dosage of HGF (50 ng/ml) used in our study compared with that in Grotegut et al. (10 ng/ml) (Grotegut et al., 2006). Alternatively, even though HepG2 cells were the cellular model used in both studies, a possible genetic heterogeneity present in the seemingly identical cell line could lead to dissimilar observations. Thus, whether HGF induces EMT in HepG2 cells and whether KLF4 functions as an EMT inducer in this process remain as open questions and warrant further investigation.

Data presented here indicate that KLF4 is a transcriptional repressor of E-cadherin, likely through its direct binding to the E-cadherin promoter (Fig. 4A,B), and that KLF4-induced suppression of E-cadherin ostensibly mediates the scattering of HepG2 and MDCK cells upon HGF stimulation (Figs 2, 3). Intriguingly, using mammary epithelial cell line MCF-10A and breast carcinoma cell line MDA-MB-231 as cellular models, Yori et al. (Yori et al., 2010) have identified KLF4 as a positive regulator of E-cadherin transcription through direct activation of the E-cadherin promoter. The obvious contradiction in the findings between Yori et al. and ours is currently unknown (Yori et al., 2010). It is noteworthy that the general biological function of KLF4 is often hard to define, mostly resulting from its cell context-dependent nature of action (Lin et al., 2011). For instance, the role of KLF4 as an oncogene or a tumor suppressor largely depends on the functional status of p21 (Refinement) in the cells examined (Rowland et al., 2005). Additionally, KLF4 is responsible for TGF-β1-induced differentiation of vascular smooth muscle cells (Li et al., 2010), whereas it is a negative regulator of the prostatic EMT triggered by TGF-β1 (Liu et al., 2012). More strikingly, an opposite conclusion was described even in the same cell line MDA-MB-231 regarding the role of KLF4 in cell migration and invasion (Yori et al., 2010; Yu et al., 2011). At the molecular level, the cell context-dependent nature of the action of KLF4 is likely attributed to the repertoire of transcriptional co-activators or co-repressors expressed in the cells-of-interest or the genetic heterogeneity of the apparently identical cell line (Evans et al., 2007; Evans et al., 2010; Yoshiida et al., 2008; Yu et al., 2011). Thus, characterization of the roles of KLF4 in additional cellular/animal model systems, the transcriptional co-activators/co-repressors recruiting by KLF4 as well as the transcriptional targets of KLF4 is warranted to gain a better understanding of the action of KLF4 in physiological or pathological processes.

Our results also noted that KLF4 depletion inhibited but not abolished HGF-induced scattered response (Fig. 2E). Similarly, the level of cell scattering elicited by 4-OHT-induced KLF4 alone was less potent than that induced by HGF (Fig. 3D). These observations suggest the involvement of additional HGF-induced effectors in stimulating a complete scattered response. Along this line, SNAIL is a potential candidate, given its upregulation by HGF and its essential role in HGF-elicted scattering of HepG2 cells has been reported (Grotegut et al., 2006) and were also recapitulated in our experimental systems (supplementary material Fig. S2). Interestingly, the observations that SNAIL upregulation by HGF is not subject to KLF4-mediated regulation and also shows faster kinetics than that of KLF4 (Fig. 4C) together challenge the role of SNAIL acting as a mediator downstream of the action of KLF4. Instead, these two HGF-induced effectors are likely acting in parallel to trigger E-cadherin suppression and cell scattering. Queries for the possible involvement of additional KLF4-induced E-cadherin transcriptional repressors in HGF-elicted scattered response are currently undergoing in our laboratory.

Despite an increase in our knowledge about KLF4-mediated pleiotropic functions, little was known as to the physiological/pathological stimuli, the upstream signaling and the molecular mechanisms that regulate the expression of KLF4. Our discovery therefore adds HGF as a novel member of the few stimuli reported to regulate KLF4 expression (Chen and Tseng, 2005; Chen et al., 2005, Feinberg et al., 2005; Li et al., 2010; Liu et al., 2012). Additionally, it is noteworthy that we identified KLF4 as its own transcriptional activator (Fig. 3B, Fig. 7C,D; supplementary material Fig. S4). To our best knowledge, the notion of KLF4 self-regulation is novel regarding human KLF4 expression. Also, previous studies have indicated a reciprocal regulation between KLF4 and the tumor suppressor p53. Specifically, p53 transcriptionally upregulates KLF4 following DNA damage (Yoon et al., 2003) while KLF4 acts as a transcriptional repressor of p53 (Rowland et al., 2005). Interestingly, although we found that p53 is dispensable in HGF-induced KLF4 upregulation, p53 knockdown led to an increase in KLF4’s basal expression (Fig. 5). This finding proposes p53 as a transcriptional repressor instead as an activator of KLF4, at least in our experimental model, and this question should be addressed in the future.

The evidence reported here indicates HGF-induced KLF4 expression and consequent cell scattering depend on the activation of the MEK/ERK signaling pathway and subsequent induction of EGR-1 expression (Figs 6, 7, 8). These findings are in line with the report by Grotegut et al. and thus highlight the central role of the MEK–ERK–EGR-1 pathway to mediate HGF-induced cell scattering (Grotegut et al., 2006). Notably, we further reveals that KLF4 is a direct transcriptional target of EGR-1 and KLF4 itself (Fig. 7). This notion is underpinned by the evidence from ChIP analysis indicating a direct binding of EGR-1 and KLF4 to the KLF4 promoter upon HGF stimulation (Fig. 7D), along with the abolishment of HGF-induced KLF4 promoter activation when EGR-1 activity is blocked by its dominant-negative mutant (Fig. 8D). Furthermore, both the kinetics of HGF-induced binding of EGR-1 and KLF4 to the KLF4 promoter (Fig. 7C) and that of HGF-elicted induction of EGR-1 and KLF4 (Fig. 8A) clearly point out that EGR-1 induction by HGF is an upstream event to upregulate KLF4 transcription. It is also noteworthy that EGR-1 is essential for SNAIL upregulation after HGF treatment (Grotegut et al., 2006). Given SNAIL upregulation induced by HGF proceeds irrespective of KLF4 depletion (Fig. 4C,D), it is likely that KLF4 and SNAIL act in parallel pathways. Based upon these findings, an order of events in HGF-induced cell scattering is delineated
Role of KLF4 in HGF-induced cell scattering

**Materials and Methods**

**Chemicals and antibodies.** Recombinant human HGF was purchased from Invitrogen. U0126, 4-OHT and puromycin were obtained from Sigma-Aldrich. The primary antibody against human KLF4 was from Invitrogen, and those against E-cadherin and N-cadherin were from BD Biosciences. Both anti-FLAG and anti-β-tubulin antibodies were from Sigma-Aldrich. Polyclonal antibodies detecting SNAIL, total ERK and Thr202/Tyr204-phosphorylated ERK were from Cell Signaling Technology. The anti-EGR1 antibody was purchased from Santa Cruz Biotechnology.

**Cell culture.** HepG2 (ATCC no. HB-8065) and MDCK (ATCC no. CCL-34) cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

**Scattering assay.** HepG2 and MDCK cells were allowed to grow as distinct colonies by seeding at the density of 5 × 10³/60-mm dish. The majority of colonies consist of around 20–40 cells/colony 24–48 h after seeding. Colonies were then replaced by fresh medium supplemented with 10% FBS and 50 ng/ml of HGF. If necessary, U0126 (5 μM) or 4-OHT (1 μM) were added to cells 1 h before HGF stimulation. The effect of HGF on cell scattering was evaluated at indicated time points until 48 h and photographed under a phase contrast microscope at 200–400× magnification. A colony would be regarded as ‘scattered’ when half of the cells in a colony had lost contact with their neighboring cells and exhibited a fibroblast-like morphology. In total, 100 colonies were counted to quantify the extent of scattering (Chen and Chen, 2009).

**Immunoblotting.** Whole cell lysates preparation, immunoblotting and chemiluminescence detection were performed as described previously (Ho et al., 2009). In each immunoblotting experiment, the level of human β-tubulin was used as the control for equal loading.

**RT-PCR and quantitative real-time RT-PCR.** Total RNA extraction, 1st strand cDNA preparation and SYBR-green-based real-time PCR were performed according to our previous report (Ho et al., 2009). The mRNA expression levels of genes of interest were normalized to that of TBP. Primers used to detect the mRNA expression of genes of interest are listed in supplementary material Table S1.

**Immunofluorescence.** HepG2 and MDCK cells were cultured on chamber slides and treated with HGF (50 ng/ml) for 48 h and 6 h, respectively. Cells were then fixed with 4% paraformaldehyde for 10 min and incubated in PBS. Samples were blocked with 10% skim milk for 1 h at room temperature and stained with primary antibodies for 1 h at 37°C, followed by incubation with FITC-conjugated secondary antibodies.

**Dual-luciferase reporter assays.** The human E-cadherin proximal promoter fragment covering the region −397~+44 (with the transcriptional start site denoted as +1) were PCR-amplified according to our previous report (Rowland et al., 2005) and denoted as shKLF4#1. The shRNA targeting sequence no. 2 was inserted into the retroviral vector pMKO, a generous gift from Dr Alan Y. L. Lee (National Health Research Institute, Taiwan), and denoted as shKLF4#2. The target sequence of canine shKLF4 differs from that of shKLF4#2 by a single nucleotide and thus share 95% identity (supplementary material Fig. S6). Additionally, the pLKO.1-shLuc plasmid carrying a shRNA targeting luciferase (clone ID: TRCN0000005316; target sequence: 5'-CTTGGAAATGTCGTCGTGTTT-3') and the pMKO carrying the same shRNA sequence were used as negative controls.

**Construction of pBabe.puro-based expression plasmids.** A cDNA fragment encoding the wild-type human KLF4 fused with the murine estrogen receptor mutant specifically responsive to 4-OHT induction was PCR-amplified from the plasmid pBpuro-KLF4-ER, a generous gift of Professor Mike Ruppert (West Virginia University School of Medicine, USA) (Foster et al., 2005). The PCR-amplified fragment was then subcloned into a modified pBabe.puro vector allowing to place an FLAG epitope (DYKDDDDK) at the N-terminus, thus enabling a FLAG-tagged KLF4-ER fusion protein (FLAG-wtKLF4-ER). The cDNA containing the zinc-finger DNA-binding domain of murine Egr-1 (Egr-1 ZnF) was kindly provided by Professor Neil D. Perkins (Newcastle University Medical School, UK) (Chapman and Perkins, 2000). This cDNA fragment was PCR-amplified and then replaced the KLF4 fragment of the FLAG-wtKLF4-ER plasmid, thus generating a plasmid expressing FLAG-tagged Egr-1 ZnF-ER fusion protein (FLAG–Egr1ZnF-ER).

**pLKO.1-derived lentiviral and pBabe.puro-derived retroviral particle production and infection.** HIEK-293T cells (7 × 10⁶) were transiently transfected for 24 h by jetPEI™ transfection reagent (Polyplus, USA) with 2.5 μg of pLKO.1-shLuc, pLKO.1-shKLF4s, pMKO-shLuc, pMKO-KLF4#2 or pBabe.puro-based plasmids along with the plasmids expressing gag-pol and VSV-G proteins required for the package of viral particles. Viral particles released into the fresh culture medium replaced at 24 h and 48 h following initial transfection were harvested by centrifugation and the supernatant containing viral particles was collected. To perform viral infection, HepG2 cells (1 × 10⁴) were incubated for 48 h with viral particles-enriched medium supplemented with 8 μg/ml of polybrene (Sigma-Aldrich, USA) to promote infection efficiency. Subsequently, cells with stable infection were selected for 48~72 h in medium supplemented with puromycin (2 μg/ml). Validated of KLF4 depletion or ectopic expression of genes of interest was performed by immunoblotting analysis.

**Chromatin immunoprecipitation.** In brief, HepG2 cells (1 × 10⁴) were treated with HGF for 6, 24 and 48 h, followed by crosslinking fixation in 1% formaldehyde. The fixation was quenched by addition of glycine to a final concentration of 200 mM, and the fixed chromatin complexes were then sonicated to an average size of 250 base-pair long using MISONIX Sonicator 3000. The sonicated protein–DNA complexes were subject to immunoprecipitation using 2 μg of antibodies against KLF4 or EGR-1. The immunoprecipitated DNA was recovered by a PCR purification kit (Qiagen,}

(Fig. 8G): HGF treatment initiates MEK/ERK activation to induce the expression of EGR-1, whose binding to the KLF4 and SNAIL promoters leads to transcriptional upregulation of KLF4 and SNAIL, respectively. In turn, KLF4 and SNAIL act in concert to suppress the transcription of E-cadherin for cell scattering induction. Moreover, the induced KLF4 also binds to its own promoter, thus establishing a positive feedback loop to sustain KLF4 expression and cell scattering.

In conclusion, we herein present the first evidence for the involvement of KLF4 in HGF-induced cell scattering as well as the underlying mechanistic basis. Considering the critical role of cell-scattering induction in HGF-induced invasive growth as well as the causal linkage between aberrant HGF/c-MET signaling and the aggressive nature in a broad range of human cancer, the findings reported here would provide a novel insight into the molecular mechanisms underlying cancer progression and facilitate the development of novel cancer therapeutics targeting this critical process.
Germany), and the amount of target DNA was detected by PCR using the primers complementary to the sequence flanking the response elements of KLF4 on the E-cadherin promoter (between −170 and +10) (Yori et al., 2010), or that of EGR-1 (between −2200 and −2077) and KLF4 (between −1579 and −1457) on the KLF4 promoter.

Statistical analysis

All data were expressed as a means ± s.e.m. from at least three independent experiments. Differences between groups were examined for statistical significance using Student’s t-test. A P-value lower than 0.05 was regarded as statistically significant.

Acknowledgements

The authors are very grateful to Prof. Hong-Chen Chen (National Chung Hsing University, Taiwan) for inspiring the concept of this study. Additionally, we are indebted to Prof. J. Mike Ruppert (West Virginia University School of Medicine, USA), Prof. Neil D. Perkins (Newcastle University Medical School, UK) and Dr Alan Yuel-Luen Lee (National Health Research Institute, Taiwan) for providing the plasmids pBpuro-KLF4-ER, pcDNA3 Egr1 (ZnF) and pMKO, respectively. The technical assistance from Mr Cong-Hao Lai and the scientific editing by Dr Wan-Lai Chang and Mr Peter Fan are greatly appreciated.

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Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.10891/-/DC1

References


Fig. S1. Re-expression of KLF4 restores E-cadherin suppression and cell scattering defective in KLF4-depleted cells. (A) Validation of KLF4 re-expression. KLF4-depleted HepG2 cells (shKLF4) were infected with the pBabe.neo vector expressing FLAG-tagged KLF4 (FLAG-KLF4), followed by G418 selection for FLAG-KLF4 stable clones. Enforced expression of FLAG-KLF4 in shKLF4 stable clones is validated by immunoblotting for both FLAG and KLF4. β-tubulin was used as the loading control. (B) KLF4 re-expression corrects the defects in E-cadherin suppression in KLF4-depleted cells. HepG2 cells expressing shLuc, shKLF4 or shKLF4 with FLAG-KLF4 were treated without or with HGF (50 ng/ml) for 24 h, and the levels of E-cadherin and KLF4 were determined by immunoblotting afterwards. β-tubulin was used as the loading control. (C) KLF4 re-expression rescues HGF-induced scattering defective in KLF4-depleted cells. HepG2-shLuc, -shKLF4 or -shKLF4 with FLAG-KLF4 stable clones were treated without or with HGF (50 ng/ml) for 4 days, and the levels of scattered cells were determined at indicated time points. A colony is judged as ‘scattered’ when half of the cells have lost contact with their neighbors and exhibited a fibroblast-like phenotype. Values (means ± SEM) are calculated from three independent experiments (n=100). *P<0.01.
Fig. S2. Involvement of SNAIL in HGF-induced cell scattering. (A) SNAIL knockdown. HepG2 cells were infected with lentiviral vectors carrying shRNAs targeting firefly luciferase or SNAIL (target sequence 5’-AAAggCCTTCAACTgCAAATA-3’) (Grotegut et al., 2006). Knockdown of SNAIL expression was then validated by immunoblotting. β-tubulin was used as the loading control. E-cad: E-cadherin. (B) SNAIL knockdown inhibits HGF-induced cell scattering. HepG2 cells carrying shLuc or shSNAIL were subjected to a 4-day treatment with HGF (50 ng/ml), and the extent of HGF-induced cell scattering was then evaluated at indicated time points. A colony is judged as ‘scattered’ when half of the cells have lost contact with their neighbors and exhibited a fibroblast-like phenotype. Values (means ± SEM) are calculated from three independent experiments (n=100). **P<0.01.

Fig. S3. The MEK/ERK signaling pathway is predominantly responsible for mediating HGF-induced KLF4 upregulation. HepG2 cells (1×10⁵) were pre-treated with U0126 (MEK inhibitor; 5 μM), SP600125 (JNK inhibitor; 5 μM), Wortmannin (PI3K inhibitor; 1 mM) or WP1066 (STAT3 inhibitor; 2 μM) for 1 h. Cells were then treated with HGF (50 ng/ml) for 24 h, followed by evaluating the levels of KLF4 protein (A) or mRNA (B) using immunoblotting or quantitative real-time RT-PCR, respectively. (A) The numbers below the KLF4 immunoblot indicate the density of KLF4 immunoblotting signals, which are quantified using ImageJ algorithm and are expressed as the ratio to the signal density of the drug-untreated control. Human β-tubulin was used as the loading control. (B) The levels of KLF4 mRNA are represented as fold induction compared with that without HGF treatment and expressed as means ± SEM. The difference between the KLF4 mRNA levels treated with HGF treatment alone and that with co-treatment of HGF and U0126 is significant. Similarly, a statistically significant difference is observed between HGF-induced KLF4 mRNA expression co-treated with U0126 and those with co-treatments of SP600125, Wortmannin or WP1066. However, the difference between the KLF4 mRNA levels of HGF treatment alone and those treated with HGF and SP600125, HGF and Wortmannin, or HGF and WP1066 are not statistically significant. **P<0.01.
Fig. S4. Self-regulation of KLF4 expression. (A) Nuclear KLF4 expression alone increases the levels of KLF4 mRNA expression. HepG2 cells were stably infected with pBabe-FLAG-KLF4-ERTM allowing for 4-OHT-inducible nuclear translocation of the KLF4-ER fusion protein. The stable clones were then treated with increasing doses of 4-OHT for 24 h, followed by quantitative real-time RT-PCR analysis for the levels of KLF4 mRNA expression. KLF4 mRNA levels are depicted as fold of induction compared with that without 4-OHT treatment and expressed as means ± SEM. **P<0.01. (B) Nuclear KLF4 expression alone activates the KLF4 promoter. HepG2-KLF4-ER stable clones were transiently transfected with the KLF4 promoter (P1) reporter plasmid, followed by 24 h-treatment with increasing doses of 4-OHT. Luciferase activity were determined thereafter to evaluate the activity of the KLF4 promoter, which is depicted as fold of induction compared with that of the 4-OHT-untreated control and expressed as means ± SEM. **P<0.01. 4-OHT: 4-hydroxytamoxifen.

Fig. S5. KLF4 depletion ablates HGF-induced ‘cadherin switching’, a cardinal hallmark of cells undergoing EMT. HepG2 cells stably expressing control shRNA (shLuc) or shKLF4#1 were treated with HGF (50 ng/ml) for 0, 6, 24, 48 h and then the protein levels of KLF4, E-cadherin (E-cad) and N-cadherin (N-cad) were determined by immunoblotting. Human β-tubulin was used as the loading control.
Fig. S6. Sequence alignment between the region of the human and canine *KLF4* mRNA targeted by their respective shRNAs. The target sequences of shKLF4s are shown in red. The one-nucleotide difference between the shRNAs targeting human and canine KLF4 (shKLF4) is shown in blue.

Table S1. Primers used in this study

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