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Up-Regulation of Krüppel-Like Factor 5 in Pancreatic Cancer Is Promoted by Interleukin-1β Signaling and Hypoxia-Inducible Factor-1α

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Abstract

Krüppel-like factor 5 (KLF5) is a transcription factor involved in cell transformation, proliferation, and carcinogenesis that can be up-regulated by *RAS* mutations. However, controversy persists as to whether it functions as a tumor suppressor or as an oncogene. Because *KRAS* is frequently mutated in pancreatic cancer, we investigated the regulation of KLF5 in this cancer entity. Our results show that KLF5 is overexpressed in pancreatic cancer cells and exceeds KLF5 expression of *KRAS*-mutated colon cancer cells. Surprisingly, inhibition of B-Raf/C-Raf or MAPK/Erk did not reduce KLF5 levels, suggesting that KLF5 expression is not promoted by KRAS-Raf-MEK-Erk signaling in pancreatic cancer. This finding is in striking contrast to reports on MEK-Erk–mediated KLF5 induction in colon cancer cells. Moreover, KLF5 expression levels neither correlated with the mutational status of *KRAS* nor with MEK phosphorylation in pancreatic cancer cells. Importantly, KLF5 was significantly up-regulated by interleukin (IL)-1β or hypoxia. The IL-1 β–mediated induction of KLF5 was diminished by blocking the p38 pathway. In addition, blocking IL-1R reduced the constitutive KLF5 expression, suggesting an autocrine activation loop. Moreover, KLF5 coimmunoprecipitated with hypoxia-inducible factor-1α (HIF-1α) and HIF-1α$_{siRNA}$ reduced constitutive KLF5. Similarly, KLF5$_{siRNA}$ reduced the expression of the HIF-1α target gene *GLUT-1*. Furthermore, KLF5 expression was significantly elevated by high cell density, by anchorage-independent cell growth, and in tumor spheroids. Down-regulation of KLF5 by RNAi reduced the expression of the target genes, survivin, and platelet-derived growth factor-A. In conclusion, overexpression of KLF5 in human pancreatic cancer cells is not mediated by KRAS/Raf/MAPK/Erk signaling, but involves the IL-1β/IL-1R system, p38, and the transcription factor HIF-1α. (Mol Cancer Res 2009;7(8):1390–8)

Introduction

Pancreatic cancer represents a highly aggressive cancer entity that requires the development of novel targeted therapy concepts to improve overall outcome. However, the identification of valid molecular targets remains a great challenge. Recently, Krüppel-like factor 5 (KLF5), a zinc finger transcription factor, has been shown to be involved in tumor progression, cell transformation, and angiogenesis (1-4). This transcription factor is also known as an intestinal-enriched Krüppel-like factor and is predominantly present in the proliferating crypt epithelial cells of the intestine (5). Nevertheless, controversy persists as to whether this transcription factor functions as a tumor suppressor or as an oncogene because clinical studies have shown that KLF5 overexpression is either associated with a poor prognosis in patients with breast cancer (6) or correlates with improved survival in patients with gastric cancer (7). Moreover, a recent experimental study showed that restoring KLF5 expression in prostate cancer cells reduces tumor growth in preclinical tumor models (8), and one study additionally suggested a metastasis-suppressive action of KLF5 in TE2 esophageal carcinoma cells (9). Hence, the biological function of KLF5 elicits some relevant discrepancy among various cancer entities, requiring additional efforts for defining the role of KLF5 in certain malignancies.

In view of this fact, the expression and regulation of KLF5 in pancreatic cancer has not been addressed to date. This is of particular interest, as recent studies showed that oncogenic *HRAS* may induce KLF5 overexpression through a mitogen-activated protein kinase (MAPK) signaling cascade, involving extracellular signal-regulated kinase (ERK) and early growth response gene 1 (4, 10). Because pancreatic cancers frequently harbor activating *KRAS* mutations (11), which consecutively activate MEK/MAPK/Erk signaling (12), we hypothesized that KLF5 could be up-regulated in this cancer entity. In favor of this hypothesis are results from a recently published study demonstrating that *KRAS* activation promotes KLF5 up-regulation in human colon cancer (HCT116) cells (13). Moreover, because

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hypoxia-mediated and cytokine-mediated activation of the oncogenic transcription factor hypoxia-inducible factor-1α (HIF-1α) is frequently encountered in pancreatic tumors, we also hypothesized that the interleukin (IL)-1β system and/or HIF-1α may be involved in the regulation of KLF5 expression (14-18). We therefore sought to further define the expression and regulation of KLF5 in human pancreatic cancer cells to expand our knowledge on this particular transcription factor and to potentially reveal a novel molecular therapeutic target.

Results

Expression of KLF5 in Pancreatic Cancer

To provide evidence that KLF5 is expressed in pancreatic cancers, various human pancreatic cancer cell lines were analyzed for the expression of KLF5. Because recent studies showed a substantial overexpression of KLF5 in *KRAS*-mutated HCT116 colon cancer cells, we used this particular colon cancer cell line as a positive control (13). Interestingly, our results show that, at a similar cell density, KLF5 is overexpressed in pancreatic cancer cell lines and, furthermore, exceeds the KLF5 expression levels of HCT116 colon cancer cells, suggesting that *KRAS* may play a pivotal role in mediating KLF5 in pancreatic cancer (Fig. 1A and B). Due to the fact that high expression of KLF5 is also observed in breast cancer, we additionally compared KLF5 expression to MCF-7 breast cancer cells and found similar results in terms of a higher expression by pancreatic cancer cells (data not shown; refs. 6, 19). Importantly, we found that phosphorylation of MEK did not strongly correlate with KLF5 levels, suggesting that the MEK pathway might not be the critical determinant for KLF5 up-regulation in pancreatic cancer cells (Fig. 1B).

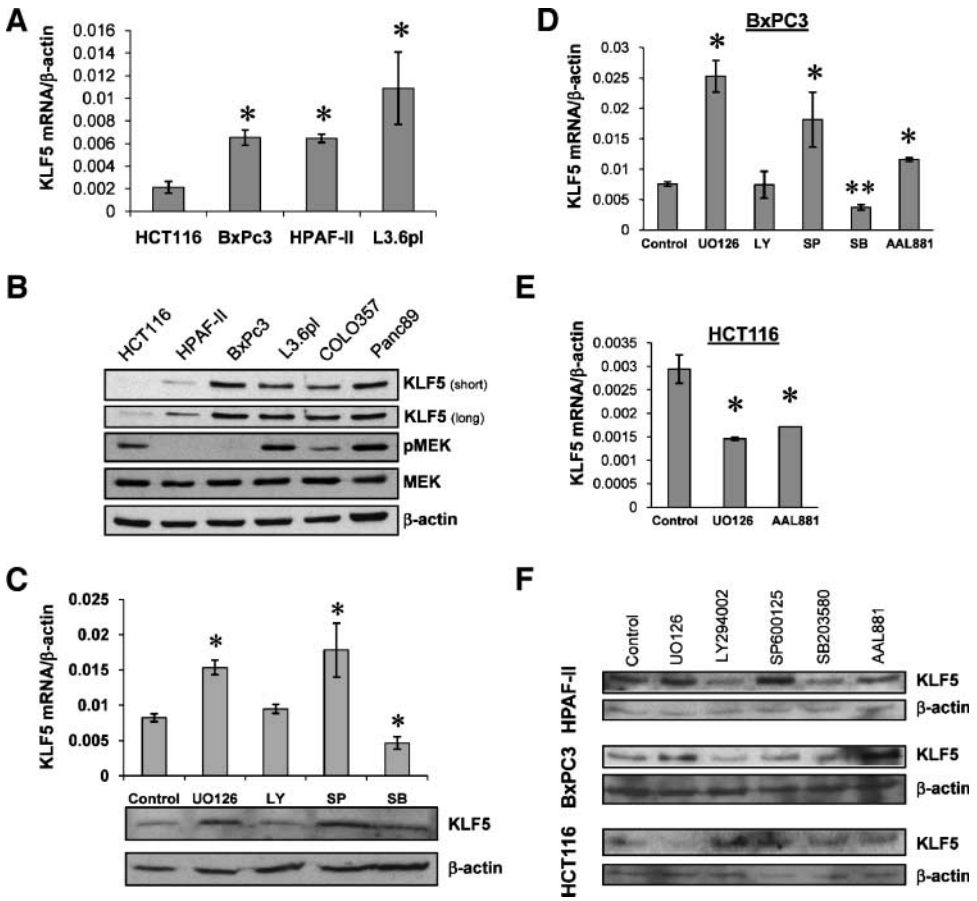

FIGURE 1. Expression and regulation of KLF5 in pancreatic cancer. Expression of KLF5 in *KRAS*-mutated pancreatic cancer cells (*COLO357, HPAF-II*, and *L3.6pl*) and *KRAS* wild-type cells (*BxPC3* and *Panc89*) were investigated by real-time PCR and Western blotting (at 50% cell density). **A.** KLF5 mRNA expression was markedly higher in pancreatic cancer cells compared with colon cancer cells (HCT116) harboring a *KRAS* mutation, which served as a positive control (*, $P < 0.05$; compared with HCT116). **B.** Western blot analysis for KLF5 expression and MEK phosphorylation in cancer cells. **C.** Signaling inhibitors to Erk1/2 (*UO126*), PI3K/Akt (*LY294002*), SAPK (*SP600125*), and p38 (*SB203580*) were used to identify pathways involved in KLF5 regulation. Changes in KLF5 expression (*HPAF-II*) were determined after 24 h of incubation with inhibitors by real-time PCR (*top*) and by Western blotting (*bottom;* *, $P <$ 0.01, versus control). **D.** Similarly, in BxPC3 cells, regulation of KLF5 mRNA expression was additionally investigated by using a Raf kinase inhibitor (*NVP-AAL881*; 20 h; *, $P < 0.01$; **, $P < 0.05$, versus control). **E.** In HCT116 cells, blocking either MAPK/Erk or Raf kinase significantly lowered KLF5 expression (*, $P < 0.01$). **F.** Western blot analysis for KLF5 expression in pancreatic (*HPAF-II* and *BxPC3*) and colon cancer cells (*HCT116*) upon inhibition of signaling pathways (20 h) Again, inhibition of MAPK/Erk (*UO126*) did not lower KLF5 in pancreatic cancer cells but reduced KLF5 in HCT116 cells. Columns, mean; bars, SEM.

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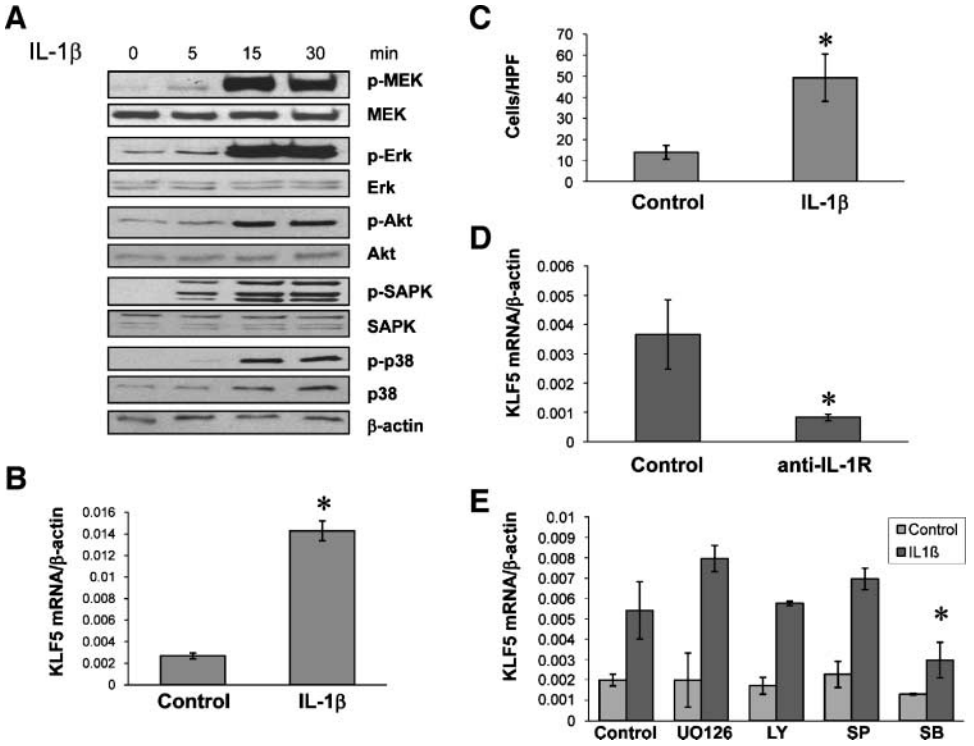

FIGURE 2. Effect of interleukin-1β on KLF5 expression. **A.** Western blot analysis of IL-1β (10 ng/mL)–mediated signaling in HPAF-II pancreatic cancer cells. Stimulation with IL-1β activated multiple signaling pathways, including Erk, Akt, SAPK, and p38. **B.** The effect of IL-1β (10 ng/mL; 24 h) on KLF5 mRNA expression (*HPAF-II*) was determined by real-time PCR, demonstrating an inducible KLF5 expression in pancreatic cancer cells (*, $P < 0.01$). **C.** The effect of IL-1β (10 ng/mL) on cancer cell migration (*HPAF-II*) was determined in modified Boyden chambers after 48 h (*, $P < 0.01$). IL-1β increased pancreatic cancer cell motility. **D.** Treatment of BxPC3 cells with a function-blocking anti–IL-1R antibody (10 μg/mL, 72 h) diminished constitutive KLF5 mRNA expression as determined by real-time PCR (*, $P < 0.05$). **E.** Signaling inhibitors to Erk1/2 (*UO126*), PI3K/Akt (*LY*), SAPK (*SP*), and p38 (*SB*) were used to identify pathways involved in IL-1β–mediated KLF5 up-regulation. Changes in KLF5 expression (*HPAF-II*) were determined after 24 h by real-time PCR (*, $P < 0.01$, versus control + IL-1β). Experiments were done in triplicate. Columns, mean; bars, SEM.

Identification of Signaling Pathways Involved in KLF5 Regulation

A connection between *KRAS* and KLF5 expression was recently proven in *KRAS*-inducible colon cancer cells, showing that activation of *KRAS* up-regulates KLF5. In that particular study, the effect of KLF5 induction was inhibited by blocking MAPK/Erk using UO126 (13). We therefore investigated whether signaling inhibition could alter KLF5 expression, expecting that blocking MAPK/Erk would reduce KLF5. Surprisingly, we found that in pancreatic cancer, inhibition of Erk1/2 did not lower KLF5 expression (Fig. 1C). Rather, it seems that inhibition of MAPK/Erk, and also stress-activated protein kinase (SAPK), increased the constitutive KLF5 expression, whereas blocking the p38 pathway slightly diminished a basal KLF5 expression (Fig. 1C and D). The inhibition of phosphoinositide 3-kinase (PI3K)/Akt had no substantial effect on KLF5 expression. In contrast, in colon cancer cells (HCT116), blocking Erk1/2 clearly decreased KLF5 (Fig. 1E and F; ref. 13). Because Raf kinases represent important downstream signaling components of *KRAS*, we additionally investigated whether inhibition of Raf by a small-molecule inhibitor against B-Raf/C-Raf (NVP-AAL881) would alter KLF5 expression in pancreatic cancer cells and colon cancer cells (20). Similarly, in contrast to HCT116 colon cancer cells, inhibition of Raf did not decrease KLF5 in pancreatic cancer cell lines (Fig. 1E and F). Our observations were confirmed in pancreatic cancer cells harboring an activating *KRAS* mutation (HPAF-II and L3.6pl), as well as in cells that lack such *KRAS* mutation (BxPC3; ref. 21). Hence, we propose that *KRAS* signaling does not promote or sustain the KLF5 expression observed in pancreatic cancer and conclude from these experiments that *KRAS* is not the driving force for KLF5 overexpression in this malignancy.

Effect of IL-1β on KLF5 Expression in Pancreatic Cancer Cells

We hypothesized that IL-1β could alter KLF5 expression in pancreatic cancer cells, as this cytokine is relevant for pancreatic cancer progression due to its complex implication in cancer growth (22) and angiogenesis (23-25). IL-1β has been reported to activate multiple signaling pathways, thus promoting cell proliferation, up-regulation of vascular endothelial growth factor, cell survival, and metastasis (16, 23, 26). Interestingly, stimulation of pancreatic cancer cells with IL-1β activated multiple signaling pathways, including Akt, MEK/Erk1/2, SAPK, and p38 (Fig. 2A). Moreover, this stimulation with IL-1β resulted in a significant increase in KLF5 mRNA expression in pancreatic cancer cells (Fig. 2B). Notably, the IL-1β–mediated signaling was also accompanied by a significant increase in cancer cell motility (Fig. 2C). In addition, we identified an IL-1β–triggered KLF5 autocrine loop in pancreatic cancer, as inhibition of the IL-1 receptor (IL-1R) with a function-blocking

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IL-1R antagonist had a significant lowering effect on constitutive KLF5 mRNA expression (Fig. 2D). We therefore speculate that this mechanism may account in part for a *KRAS*-independent up-regulation of KLF5 in pancreatic cancer.

We next sought to identify specific signaling pathways involved in an IL-1β–mediated KLF5 up-regulation. Again, inhibition of MAPK/Erk led to an increase in basal and IL-1β–mediated KLF5 expression, providing further evidence that the MEK/MAPK/Erk pathway is not promoting the overexpression of KLF5 in pancreatic cancer (Fig. 2E). In contrast, blocking p38 significantly lowered the response to IL-1β, suggesting that this pathway is involved in an inducible KLF5 up-regulation.

Effect of Hypoxia and HIF-1α on KLF5 Expression in Pancreatic Cancer

Interestingly, the stimulation of cancer cells with IL-1β also led to an increase in nuclear HIF-1α protein in pancreatic cancer cells, which could imply that the HIF-1α pathway is involved in IL-1β–mediated KLF5 up-regulation (Fig. 3A). We therefore investigated whether hypoxia (1% O_2) or chemical hypoxia using desferroxamine could induce KLF5. The results show that both hypoxia and desferroxamine lead to a substantial time-dependent up-regulation of KLF5, supporting the hypothesis that HIF-1α could mediate KLF5 overexpression (Fig. 3B and C). To identify a potential direct interaction of HIF-1α with KLF5, a feature that is described for the HIF-1 transcription factor (27), we performed an immunoprecipitation assay and found that KLF5 indeed coimmunoprecipitates with HIF-1α and vice versa (Fig. 3D and E). This interaction of transcription factors occurred independently of stimulation by hypoxia (desferroxamine). However, stimulation with IL-1β increased the amount of HIF-1α protein that coimmunoprecipitated with KLF5 (Fig. 3E). Western blotting confirmed that either hypoxia or IL-1β induced KLF5 in this experiment (data not shown). To determine the functional consequence of HIF-1α on KLF5, we used transient RNAi for suppressing HIF-1α expression in pancreatic cancer cells (HPAF-II). Down-regulation of HIF-1α by RNAi markedly diminished KLF5 expression (Fig. 3F). To detect a potential functional dependence of HIF-1α on KLF5, we used RNAi to down-regulate KLF5. This, in turn, significantly diminished the expression of the HIF-1α target gene *GLUT-1* (Fig. 3G). HIF-1α protein levels (constitutive

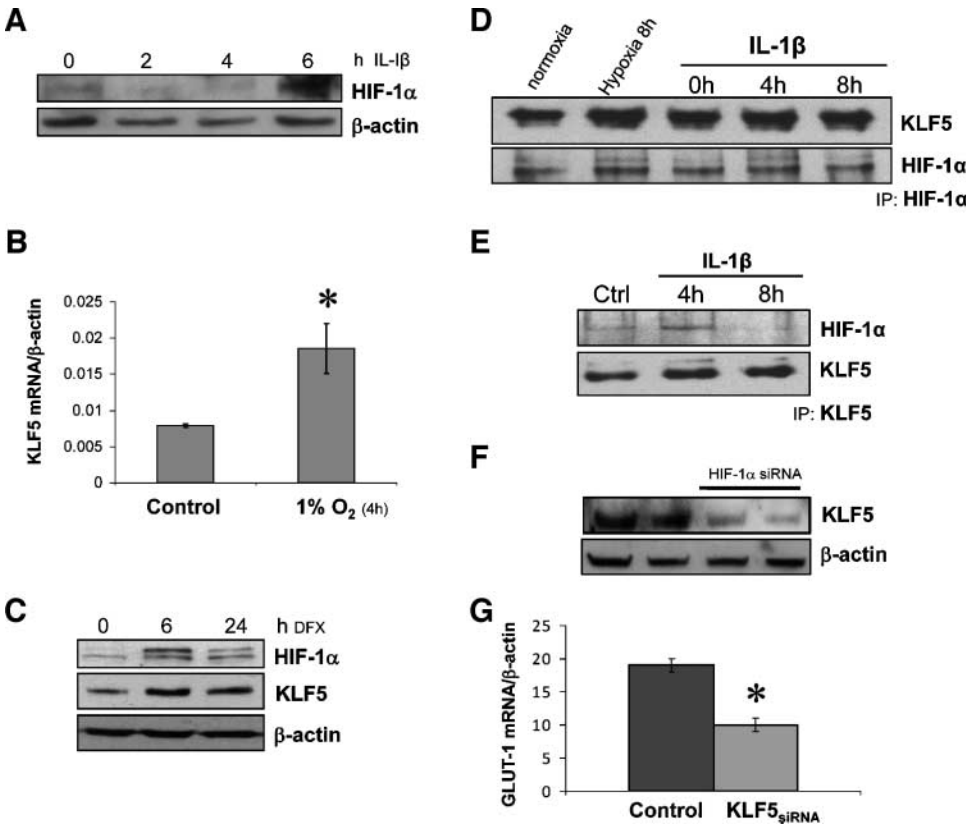

FIGURE 3. Role of HIF-1α in KLF5 regulation in pancreatic cancer. **A.** Western blot analysis for HIF-1α protein was done to determine the effects of IL-1β. Results show a time-dependent stimulation of cancer cells with IL-1β (10 ng/mL) and a consecutive increase in nuclear HIF-1α protein (*HPAF-II*). **B.** The effect of hypoxia on KLF5 expression was investigated. Cells were incubated under either hypoxic (1% O_2) or nonhypoxic conditions (20% O_2), and real-time PCR was done thereafter to determine the effect on KLF5 mRNA expression (*, $P < 0.05$). **C.** Similarly, chemical hypoxia with desferroxamine (*DFX*; 100 μmol/L) time-dependently up-regulated KLF5 protein levels in BxPC3 cells as determined by Western blotting. **D.** A potential interaction of KLF5 and HIF-1α was investigated by immunoprecipitation (*IP*) and Western blotting. Results show that KLF5 coimmunoprecipitates with HIF-1α (*IP: HIF-1α*), independent of stimulation. **E.** HIF-1α protein coimmunoprecipitated with KLF5 (*IP: KLF5*) and stimulation with IL-1β increased the amount of HIF-1α that complexed with KLF5. **F.** Effect of HIF-1α down-regulation on KLF5 expression. Transient RNAi of HIF-1α (stealth siRNA) was done. Blocking HIF-1α lowered KLF5 expression (48 h) under constitutive conditions. **G.** Similarly, inhibition of KLF5 by transient RNAi (48 h) diminished the expression of the HIF-1α target gene *GLUT-1* in BxPC3 pancreatic cancer cells (*, $P < 0.05$). Columns, mean; bars, SEM.

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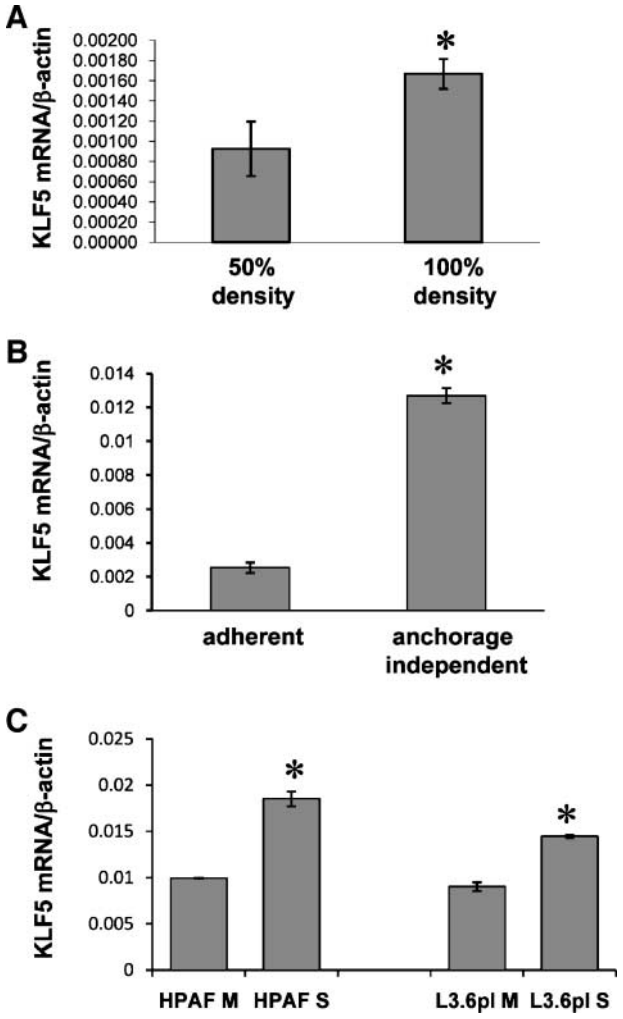

FIGURE 4. Effect of cell density on KLF5 expression. **A.** Effects of cell density on KLF5 expression. Cells (*HPAF-II*) were grown to the indicated monolayer cell confluence and real-time PCR for KLF5 mRNA expression was done (*, $P < 0.01$; $n = 3$/group). **B.** Viable anchorage-independently growing cells were collected from culture medium supernatants and the remaining adherent cells were isolated for RNA extraction to determine KLF5 mRNA by PCR. Anchorage-independently growing cells elicited a significantly higher KLF5 mRNA expression (*, $P < 0.01$). **C.** Pancreatic cancer cells (*HPAF-II* and *L3.6pl*) were grown either in monolayer (*M*; confluence) or as tumor cell spheroids (*S*) over 3 d. Changes in KLF5 mRNA expression were evaluated by real-time PCR (*, $P < 0.05$). Columns, mean; bars, SEM.

protein) were not affected (data not shown). This finding is of particular relevance to pancreatic cancer because HIF-1α upregulation is frequently detected and we have previously shown that HIF-1α is constitutively active in these cells (17). Results from these experiments suggest that, in addition to the IL-1β/IL1R system, the transcription factor HIF-1α plays an important role in mediating KLF5 overexpression in pancreatic cancer cells through a direct interaction with KLF5.

Effect of Cell Density and Spheroid Growth on KLF5 Expression

Interestingly, a connection between KLF5 expression and activation of the integrin-linked kinase was recently shown in a keratinocyte model, suggesting that cell contact could influence KLF5 expression, thus mediating cell migration and invasiveness (28). We therefore investigated whether cell density modulates KLF5 expression in pancreatic cancer cells. We found that high cell density led to a significant up-regulation of KLF5 expression in cancer cells (Fig. 4A). Moreover, vital anchorage-independent growing cells showed a marked increase in KLF5 expression, as compared with attached monolayer tumor cells, which may imply that KLF5 could be involved in the process of metastasis and cell survival (Fig. 4B). To test this hypothesis, we aimed to mimic metastatic growth conditions *in vitro* by growing cancer cells as tumor spheroids, a model requiring the activation of survival pathways involved in enabling growth at a metastatic organ site (29). Importantly, we found that KLF5 expression was significantly higher in "metastatic" cells (i.e., tumor cell spheroids) as compared with monolayer cultures (Fig. 4C). The results from these experiments suggest that KLF5 is complexly regulated in pancreatic cancer cells, which also involves cell contact.

Effect of Transient KLF5 Knockdown on Target Gene Expression

To further assess whether inhibition of KLF5 has an effect on relevant target genes in pancreatic cancer cells, we used transient RNAi to diminish KLF5 expression in tumor cells (Fig. 5A). Our results show that KLF5 inhibition markedly decreased the expression of platelet-derived growth factor-A (PDGF-A) and survivin (Fig. 5B and C), which are identified target genes of KLF5 (3, 30). Because both PDGF-A and survivin have been implicated in promoting pancreatic cancer growth, blocking KLF5 is a novel consideration for new molecular treatment strategies (31-33).

Discussion

Our study shows that KLF5 is overexpressed in human pancreatic cancer cells. Importantly, unlike in colon cancer, the up-regulation of KLF5 in pancreatic cancer is not promoted via the KRAS/Raf/MAPK/Erk pathway. Instead, we identified the IL-1β/IL-1R system and the transcription factor HIF-1α, which additionally complexes with KLF5, as mediators of KLF5 induction in this cancer entity. In addition, cell density and spheroid growth substantially modulated KLF5 expression in our experiments, suggesting that KLF5 could be involved in the process of cell adhesion and pancreatic cancer metastasis.

The involvement of KRAS/MAPK/Erk signaling in the up-regulation of KLF5 has recently been elegantly shown in a model with *KRAS*-inducible colon cancer cells (13). In that model, specific inhibition of MAPK/Erk using Erk1/2 signaling inhibitors robustly abrogated *KRAS*-mediated KLF5 induction, whereas other pathways were not involved in this process. However, in our experiments, we detected a strong expression of KLF5 in human pancreatic cancer cell lines, regardless of whether cells harbored *KRAS* mutation or wild-type *KRAS*. In addition, KLF5 was detectable in human pancreatic cancer specimens; however, due to the lack of suitable antibodies for staining analyses, these data were not integrated into our study (data not shown). Interestingly, the KLF5 expression levels in pancreatic cancer cell lines exceeded that of *KRAS*-mutated colon cancer cells (and breast cancer cells), but blocking either the

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MAPK/Erk or the Raf/MAPK pathway did not lower this KLF5 expression. In addition, we could not see a convincing correlation between constitutive MEK phosphorylation and KLF5 expression, suggesting that neither the KRAS/Raf/MAPK nor the MEK/MAPK/Erk pathway represent the driving force for KLF5 overexpression in pancreatic cancer cells. This finding is also in contrast with reports showing that the transcription factor KLF5, as a downstream mediator of EGFR signaling per se, might up-regulate MEK activation in cells (i.e., squamous epithelial cells; ref. 9). Nevertheless, we identified the MAPK/p38 signaling intermediate as a positive mediator in this regard, as inhibition of this component reduced KLF5 expression and diminished the IL-1β/IL-1R–induced KLF5 up-regulation. The up-regulatory influence of IL-1β/IL-1R on KLF5 has not been reported to date and, importantly, we additionally identified an IL-1/IL-1R autocrine activation loop because inhibition of IL-1R lowered constitutive KLF5 expression. This lowering effect was modest but statistically significant. However, we speculate that the effects of a potent IL-1R inhibitor would have been stronger, and thus we propose that such an autocrine activation of IL-1R could, in part, be responsible for sustaining KLF5 overexpression in pancreatic cancer.

Moreover, we hypothesized that hypoxia and consecutive activation of HIF-1α, which typically is detectable in pancreatic cancers (17, 34, 35), could modulate KLF5 expression. Indeed, we found that both hypoxia and IL-1β stimulation lead to HIF-1α activation followed by a robust up-regulation of KLF5, hence providing evidence that HIF-1α could be involved in mediating KLF5 overexpression in that cancer entity. Interestingly, KLF5 protein was not only up-regulated upon HIF-1α induction but also complexed with this particular transcription factor, as determined by coimmunoprecipitation. Furthermore, the stimulation of cancer cells with IL-1β (4 hours) led to an increased amount of HIF-1α/KLF5 protein complex that immunoprecipitated. This novel finding suggests a potential functional interaction of HIF-1α with KLF5, a feature that has also been shown for other proteins and transcription factors in terms of binding to HIF-1α, such as STAT3, Ref-1/APE, and Hsp90 (27, 36). The involvement of HIF-1α in the regulation of KLF5 was further supported by results from our experiments using transient RNAi for down-regulating either HIF-1α or KLF5 in cancer cells. This approach led to a substantial reduction of KLF5 protein or the HIF-1α target gene *GLUT-1*, respectively, in transfected pancreatic cancer cells. This finding has not been reported to date. We therefore conclude that KLF5 represents an important transcription factor in pancreatic cancer growth, as it associates with HIF-1α and also in part modulates HIF-1α activity.

Besides investigating the effects of IL-1β and hypoxia on KLF5, we also determined whether cell density could modulate the expression of this particular transcription factor in pancreatic cancer cells, as cell contact is known to affect the activation and expression of certain transcription factors in a multifactorial way (37, 38). Importantly, the implication of KLF5 in mediating anchorage-independent cancer cell growth has recently been elegantly shown by Nandan and colleagues, a process that was promoted via *KRAS* activation (13). However, the opposite effect in terms of up-regulating KLF5 by cell density or anchorage-independent growth has not yet been described. We found that both cell density and anchorage-independent cell growth significantly affected the expression of KLF5 in various cancer cell lines, suggesting that KLF5 is indeed regulated in a multifactorial way (39). In addition, in our experiments, in which we aimed to mimic metastatic growth conditions *in vitro* by growing cancer cells as tumor spheroids, a model requiring activation of survival pathways, we detected a strong overexpression of KLF5 in metastatic tumors (29). We speculate that based on the reports by Nandan et al., KLF5 could potentially represent a mechanism for enabling the process of cancer metastasis (4, 10). However, one must be careful in interpreting the role of KLF5 in this setting because multiple factors are being up-regulated in tumor spheroids, as recently shown in a study using colon cancer cells (39). Interestingly, one such factor up-regulated in tumor spheroids is the HIF-1α target gene

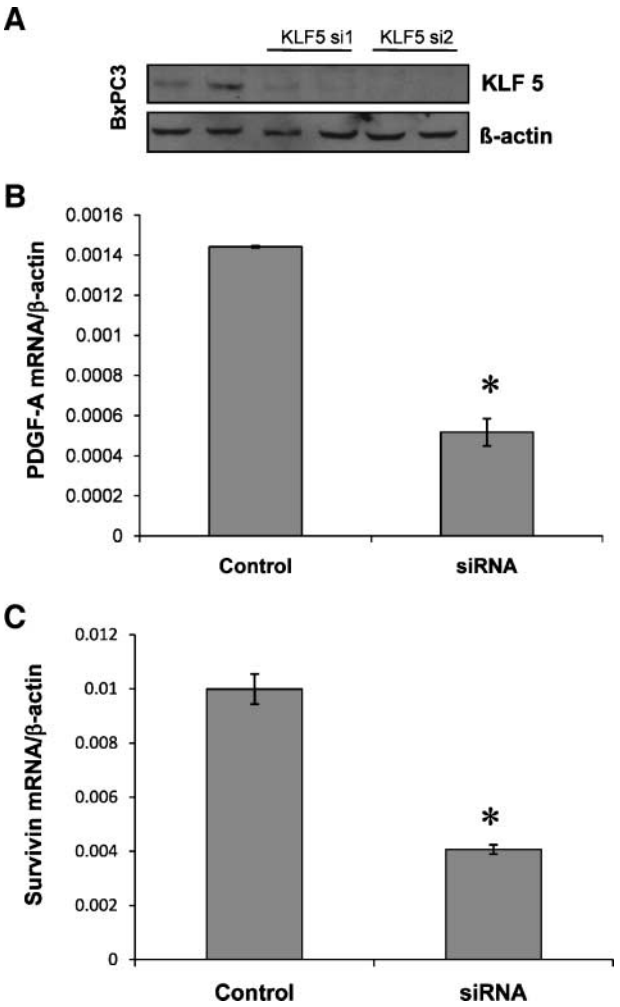

FIGURE 5. Effects of KLF5 knockdown on pancreatic cancer cells. **A.** KLF5 siRNA (stealth siRNA) was transiently transfected into BxPC3 pancreatic cancer cells. Both siRNA constructs markedly diminished KLF5 protein after 48 h as determined by Western blotting. **B.** Analysis of PDGF-A mRNA expression. Transient KLF5 inhibition by RNAi (48 h) in BxPC3 cells led to a significant reduction in PDGF-A expression (*, $P < 0.01$). **C.** Similarly, survivin mRNA expression was markedly reduced upon transient KLF5 inhibition (48 h; *BxPC3 cells*; *, $P < 0.01$). Columns, mean; bars, SEM.

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GLUT-1 (40). Nevertheless, consistent with our theory, we also found that increases in KLF5 were accompanied by a substantial up-regulation of survivin mRNA in spheroids (Supplementary Fig. S1), which is a known target gene of KLF5 (30). Together, these results imply that KLF5 may serve as a promising target for cancer therapy, as KLF5 may not only regulate cell proliferation and survival but may also define the metastatic behavior of pancreatic cancer cells.

In summary, our study is the first to show that KLF5 overexpression in pancreatic cancer involves the IL-1β/IL-1R system, p38, and cell density. Moreover, KLF5 overexpression is mediated in a *KRAS*-Raf-MEK–independent manner. This is in sharp contrast to a reported *KRAS*-mediated or MEK-mediated up-regulation of KLF5 in colonic malignancies (9, 13). Furthermore, we identified HIF-1α as an important mediator of KLF5 expression in pancreatic cancer cells and, interestingly, HIF-1α activity is in turn modulated by KLF5. Results from this study suggest that KLF5 might serve as an attractive target for therapy of pancreatic cancer because of its multifactorial regulation and its implication in cell survival, proliferation, and, potentially, metastasis. In light of recent advances in targeting the transcription factor KLF5 by using small molecules, further investigations in models of pancreatic cancer are warranted (41).

Materials and Methods

Cell Culture and Reagents

The human pancreatic cancer cell line HPAF-II and the HCT116 (*KRAS* mutated) colon cancer cell line were purchased from the American Type Culture Collection, whereas L3.6pl, COLO357, and BxPC3 cells were kindly provided by Dr. I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX), and the cell lines Panc89 and COLO357 were kindly provided by Dr. H. Kalthoff (University of Kiel, Kiel, Germany). According to the literature, *KRAS* is mutated in HPAF-II, L3.6pl, and COLO357 cells, whereas Panc89 and BxPC3 cells harbor wild-type *KRAS* (21, 42). Cells were cultured in DMEM (Life Technologies) supplemented with 15% FCS and maintained in 5% CO_2 at 37°C. The following signaling inhibitors to the indicated signaling intermediate were used: MEK/Erk (UO126; Cell Signaling Technologies), PI3K/Akt (LY294002; Sigma), SAPK (SP600125), and p38 (SB203580) were purchased from Calbiochem (EMD Chemicals). The Raf/VEGFR2 inhibitor NVP-AAL881 was kindly provided by Novartis and dissolved in DMSO (20). Recombinant human IL-1β was purchased from R&D Systems, and the IL-1R inhibitor was from Calbiochem. Hypoxic conditions were achieved by using a modular incubator chamber (1% O_2; Billups-Rothenberg) or by exposing cells to desferroxamine (Sigma Aldrich).

Real-time PCR for KLF5, Survivin, and PDGF-A Expression

The changes in expression of KLF5 and the effects of KLF5 RNAi were determined by real-time PCR. For this purpose, total RNA was isolated using the Trizol reagent (Invitrogen) and subsequently purified by ethanol precipitation. For each RNA sample, a 1-μg aliquot was reverse transcribed into cDNA using the Superscript II Kit (Qiagen). Selected primer pairs for PCR were as follows: KLF5 (forward, 5′-CCACCACCCTGCCAGTTAAC; reverse, 5′-TAAACTTTTGTGCAACCAGGGTA), survivin (forward, 5′-GGACCACCGCATCTCTACAT; reverse, 5′-GACAGAAAG GAAAGCGCAAC), PDGF-A (forward, 5′-ACACGAGCAGTGTCAAGTGC; reverse, 5′-TCTGGTTGG-CTGCTTTAGGT), GLUT-1 (forward, 5′-AACTCTTCAGCCA-GGGTCCAC; reverse, 5′-CACAGTGAAGATGATGAAGAC), and β-actin (forward, 5′-AGAGGGAAATCGTGCGTGAC; reverse, 5′-CAATAGTGATGACCTGGCCGT). Primers were optimized for $MgCl_2$ and annealing, and PCR products were confirmed by gel electrophoresis. Real-time PCR was performed using the LightCycler system and Roche Fast-Start LightCycler-Master Hybridisation Probes master mix (Roche Diagnostics). Transient knockdown (24 h) of KLF5 or HIF-1α was achieved by using commercially available stealth RNAi (Invitrogen; KLF5 stealth RNA sequences used: no. 1, GCAGCUCCAGAG-GUGAACAAUAUUU; AAAUAUUGUUCACCUCUGGAG-CUGC; no. 2, GGACACUCUUAAUGUUUCUAUGUCA; UGACAUAGAAACAUUAAGAGUGUCC; no. 3, CAU-CAAACAAGAACUUCCUACACCA; UGGUGUAGGAA-GUUCUUGUUUGAUG) and Lipofectamine (Invitrogen; ref. 43). For experiments using stealth RNAi, sole administration of Lipofectamine to the culture medium served as a control. In addition, results were confirmed by transient transfection using a shRNA KLF5 expression plasmid (5′-TCCATCC-TATGCTGCTACAAT; SuperArray), with shRNA to luciferase (5′-GGAATCTCATTCGATGCATAC) serving as a control.

Western Blot Analysis for KLF5 Expression and Activated Signaling Pathways

Experiments were done in triplicate at a cell density of 60% to 70%. Unless otherwise indicated, cells were incubated in serum-reduced conditions (1% FCS) for 24 h before stimulation with IL-1β (10 ng/mL), chemical hypoxia (desferroxamine, 100 μmol/L), or signaling inhibitors (24 h treatment), respectively. Whole cell lysates were prepared, as described elsewhere (17). Protein samples (50 μg) were subjected to Western blotting on a denaturating 10% SDS-PAGE. Membranes were sequentially probed with antibodies to indicate signaling intermediates. Antibodies to phosphorylated MEK, MEK, phosphorylated Akt^{Ser473}, Akt, phosphorylated $Erk^{Thr202/Tyr204}$, Erk, phosphorylated SAPK, SAPK, phosphorylated p38, and p38 were purchased from Cell Signaling Technologies, and antibodies to β-actin and anti–HIF-1α were obtained from Santa Cruz Biotechnologies. The anti-KLF5 antibody was obtained from R&D Systems. For coimmunoprecipitation analyses, 800 μg of protein samples were incubated with their respective antibody (1 μg) in radioimmunoprecipitation assay buffer and the presence of agarose beads (A/G plus, Santa Cruz Biotechnologies) overnight at 4°C, followed by precipitation and Western blotting as described (17).

Cell Density and Tumor Cell Spheroid Assays

The effects of cell density on KLF5 expression were investigated by growing cells to either 50% or 100% confluence in a large volume of full serum medium for reducing the effects of differences in oxygen consumption. In addition, the medium was replaced every 24 h. Anchorage-independently growing cells were harvested from supernatants of exponentially growing cancer cell cultures; a fraction was screened

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for viability by trypan blue and the remaining cells were subjected to RNA extraction and real-time PCR. Three-dimensional pancreatic tumor spheroids were generated using the liquid overlay culture technique. In brief, 5×10^3 suspended cells from exponentially growing tumor cell monolayers were cultured on 1% solid agarose in 96-well plates. After 3 d of culture, cells formed tight aggregates (44). Control cells (monolayer) were grown to confluence for comparing KLF5 levels to spheroids.

Statistical Analysis

Statistical analyses were done using SigmaStat (version 3.0). The two-sided Student's *t* test was applied for analysis of *in vitro* data. All results are expressed as the mean ± SEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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