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KLF6 transcription factor protects hepatocellular carcinoma-derived cells from apoptosis

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Hepatocellular carcinoma (HCC) is a major public health concern because of the absence of early diagnosis and effective treatments. Efficient diagnosis modalities and therapies to treat HCC are needed. Kruppel-like factor (KLF) family members, such as KLF6, are involved in cell proliferation and differentiation. KLF6 is inactivated in solid tumors, which may contribute to pathogenesis. However, KLF6 status in HCC is controversial. Thus, we undertook the characterization of KLF6 expression and function in HCC and HCC-derived cell lines. We found that HCC, HepG2 and HuH7 cells expressed KLF6 messenger ribonucleic acid and protein. Next, using RNA interference, we demonstrated that inhibiting KLF6 expression *in vitro* strongly impaired cell proliferation-induced G_1-phase arrest, inhibited cyclin-dependent kinase 4 and cyclin D1 expression, and subsequent retinoblastoma phosphorylation. Finally, KLF6 silencing caused p53 upregulation and inhibited Bcl-xL expression, to induce cell death by apoptosis. Taken together, these data demonstrated that KLF6 is essential for HCC-derived cells to evade apoptosis.
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Hepatocellular carcinoma (HCC) is a major public health concern because of the absence of early diagnosis and effective treatments. It is the most common primary malignancy of the liver and the fourth most common cancer worldwide with an incidence of one million new cases per year. HCC represents the third cause of death by cancer worldwide, with an increasing incidence because of the hepatitis C virus pandemic.[1] Three main curative therapeutic options are currently used for HCC treatment, that is, hepatic resection, percutaneous destruction of the tumor (radiofrequency) and orthotopic liver transplantation.[2] However, these treatments are limited to patients with relatively restricted hepatocarcinoma. The bulk of patients cannot benefit from curative therapeutic options because of large tumor size or underlying liver disease. In consequence, there is an urgent need for new diagnosis modalities and therapies to treat HCC.

Kruppel like factor (KLF) family members share a three C2H2 zinc-finger DNA binding domain, and are involved in cell proliferation and differentiation control in normal as well as in pathological situations.[3] KLF6 is a transcription factor that can both activate and repress genes that are involved in cell-cycle regulation. Among them, many upregulated genes are inhibitors of proliferation,[4] whereas genes that promote cell proliferation are repressed.[5,6] However, some studies do present KLF6 as a transcription factor involved in regulating genes to stimulate cell proliferation.[7,8] KLF6 has been recently demonstrated to be deregulated in multiple cancers by loss of heterozygosity (LOH),[4,9–12] inactivating somatic mutation[4,13–15] and transcriptional silencing by promoter hypermethylation.[16] Accordingly, KLF6 expression has been shown to mediate growth inhibition when ectopically expressed in prostate,[4] non-small lung[9] and colorectal[11] cancer cells through the inhibition of a number of key oncogenic signalling pathways, and to revert the tumorigenic phenotype in glioblastoma cell lines in culture and *in vivo*.[10] These observations, taken together, indicate that KLF6 might act as a tumor suppressor.

In HCC, KLF6 was first shown to be frequently inactivated, and that its inactivation may contribute to pathogenesis in a significant number of tumors. Indeed, LOH and/or inactivating somatic mutations were detected in 49% of the tumors.[17] However, KLF6 status in HCC has recently been largely debated, with contradictory results. No somatic mutations were found in 71 freshly isolated HCC samples,[18] and no difference in KLF6 expression was seen between HCC and adjacent normal liver.[19] To clarify such discrepancy, we undertook the characterization of (i) the expression profile of KLF6 in HCC-derived cell lines, and (ii) the potential role of KLF6 in HCC-derived cell proliferation. We first confirmed that HCC samples and HCC-derived cells, HepG2 and HuH7, expressed KLF6 to detectable levels compared with normal liver. Next, using RNA interference, we demonstrated that specific inhibition of KLF6 expression in HepG2 and HuH7 cells strongly impaired cell proliferation. Furthermore, depleting HCC-derived cells in KLF6 inhibited cyclin-dependent kinase (CDK)4 and cyclin D1 expression, and subsequent retinoblastoma (Rb) phosphorylation. Finally, KLF6 silencing caused B-cell lymphoma protein (Bcl)-xL inhibition and

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Abbreviations: KLF, Kruppel-like factor; HCC, hepatocellular carcinoma; LOH, loss of heterozygosity; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; RT-PCR, reverse transcription-polymerase chain reaction; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; Bcl, B-cell lymphoma protein; TGF, transforming growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid; Rb, retinoblastoma protein; PARP, poly ADP-ribose polymerase; EDTA, ethylene diamine tetraacetic acid; NaF, sodium fluoride; PBS, phosphate-buffered saline
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upregulated p53, to induce cell death by apoptosis of HepG2 and HuH7 cells. Taken together, these data demonstrated that KLF6, previously identified as a tumor suppressor gene, is in fact essential for HCC-derived cells to evade apoptosis, *in vitro*.

Results

KLF6 expression in HCC-derived cells. We first look for previously reported inactivating mutations in KLF6 sequence in HCC samples. As shown in Figure 1a, P149S, P166S and T179I mutations were not present in DNA extracted from 10 HCC samples (Figure 1a). In addition, no additional mutations were found when KLF6 exon 2 was sequenced (Figure 1b). We next established the baseline level of KLF6 expression in two well-characterized HCC-derived cell lines, HepG2 and HuH7, and immortalized hepatocytes (IHH). We found that KLF6 messenger ribonucleic acid (mRNA) expression was higher in HCC and HCC-derived cells, compared with control hepatocytes, as ascertained by real-time reverse transcription-polymerase chain reaction (RT-PCR) (Figure 1c). By Western blotting, densitometric analysis of the KLF6 protein showed an average increase in KLF6 protein expression of 1.8$\pm$0.35-fold (HepG2 and HuH7 cells *versus* IHH cells) and 15.85$\pm$0.63-fold (HCC *versus* normal liver) (Figure 1d). Finally, confocal microscopy analysis of HepG2 cells confirmed nuclear as well as diffuse cytoplasmic staining for KLF6 in HCC-derived cells (Figure 1e). Overall, these results demonstrated that KLF6 is readily detectable in HCC-derived cells.

Targeted inhibition of KLF6 expression in HCC-derived cells using RNA interference. We next used RNA interference to assess the function of KLF6 in HCC-derived cells. HepG2 and HuH7 cells were transiently transfected with specific KLF6 small interfering RNA (siRNA)[5] or with

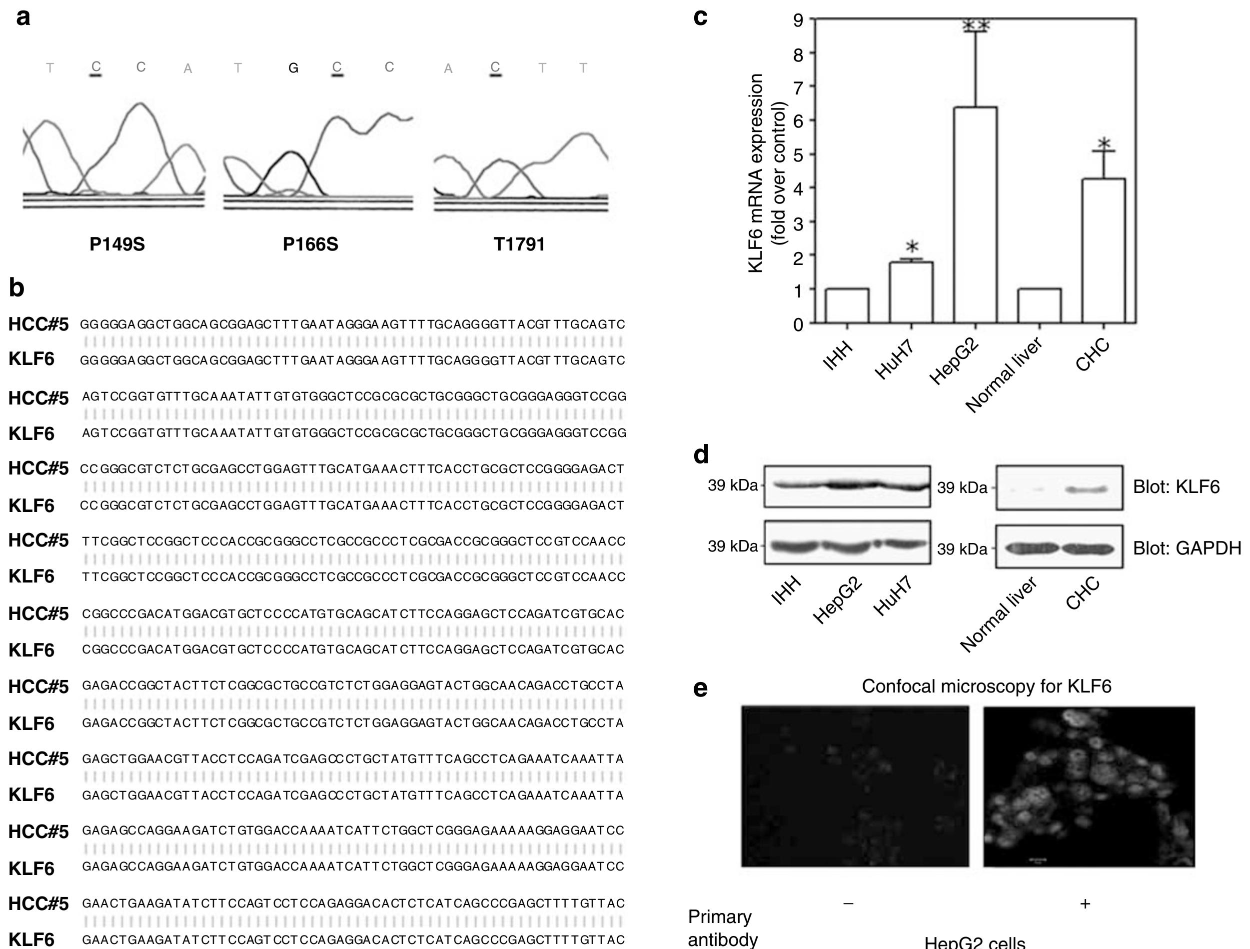

Figure 1 KLF6 transcription factor is expressed in HCC and HCC-derived cell lines. (**a**) KLF6 exon 2 was sequenced in DNA originating from clinical samples as described in Materials and Methods for P149S, P166S and T179I mutations (underlined base), or compared with wild-type sequence (**b**). Results are representative of duplicate analysis performed on 10 different HCC-derived DNA samples. (**c**). Total RNA was collected from clinical samples and HCC-derived cell lines as described in Materials and Methods. KLF6 mRNA was quantified by quantitative RT-PCR. Results are expressed as KLF6 mRNA expression corrected by 18S expression (*$P<0.05$, **$P<0.01$). Analysis of KLF6 expression in IHH, HuH7 and HepG2 cells was performed in triplicate. For HCC, result is mean of 10 samples. (**d**) Proteins were extracted and purified from clinical samples and derived cell lines, before Western blotting for KLF6 as described in Materials and Methods. To ensure an equal loading of the lanes, filters were stripped and reprobed with anti-GAPDH antibodies. Result is representative of five independent experiments. (**e**) HepG2 cells were plated in four-chamber glass slides and immunostained for KLF6 as described in Materials and Methods. KLF6 immunoreactivity was detected using a Carl Zeis' confocal microscope. Result is representative of five independent experiments

control siRNA targeting luciferase. At 72 h post-transfection, expression of KLF6 mRNA was measured by real-time RT-PCR. Using RNA interference, we observed a marked reduction in KLF6 mRNA level in both HepG2 and HuH7 cells (−75±12 and −49±3%, respectively, Figure 2a). By immunofluorescence, we further confirmed efficient inhibition of KLF6 protein expression in HCC-derived cells using siRNA (Figure 2b). Lastly, KLF6 silencing in HepG2 and HuH7 cells was quantified by Western blotting (−89±23 and −86±17%, respectively, Figure 2c).

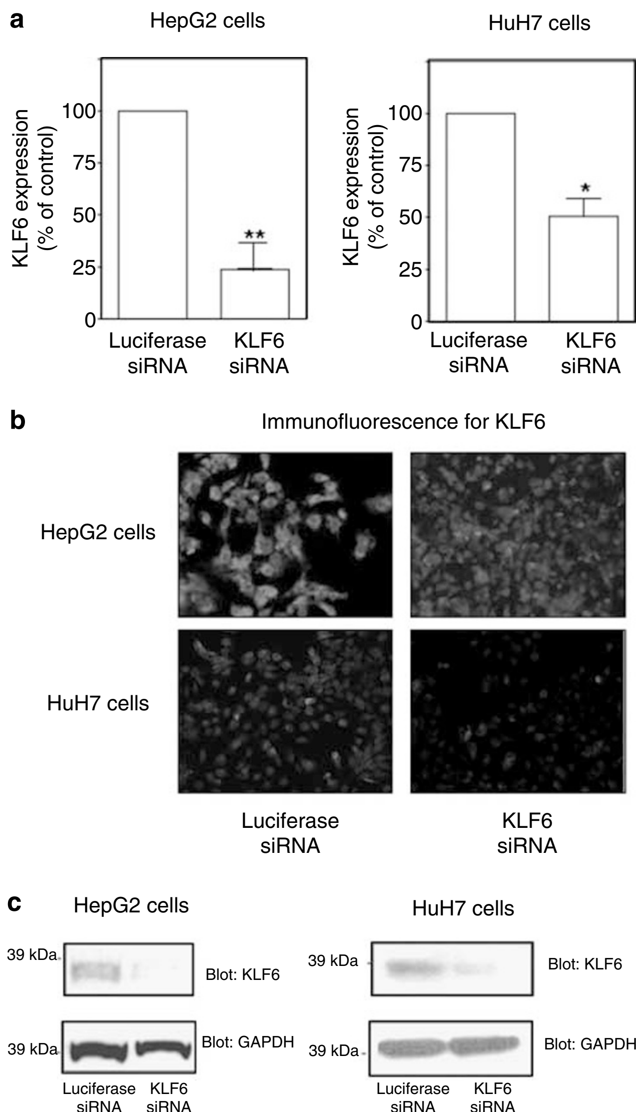

Figure 2 Efficient silencing of KLF6 expression in HCC-derived cells using RNA interference. HepG2 and HuH7 cells were transfected with siRNA targeting KLF6 as described in Materials and Methods. Control cells were transfected with siRNA targeting luciferase. (**a**) KLF6 mRNA expression was quantified by quantitative RT-PCR as described before. Results are represented as mean±S.E.M. of five independent experiments (*$P<0.05$, **$P<0.01$). KLF6 protein expression was assayed by immunostaining (**b**) and Western blotting (**c**). To ensure an equal loading of the lanes, filters were stripped and reprobed with anti-GAPDH antibodies. Results are representative of three independent experiments

KLF6 depletion of HCC-derived cells strongly affects cell proliferation. We next examined the effect of targeted downregulation of KLF6 by RNA interference-mediated gene silencing on growth of HCC-derived cell lines. First, we established the growth parameters of HepG2 and HuH7 cells (Figure 3a). Next, HepG2 and HuH7 cells were transiently transfected with siRNA targeting KLF6, and subsequently cultured for 72 h in the presence of 10% FCS. As a control, cells were transfected with either a siRNA targeting luciferase, or the proto-oncogene c-myc. We found that proliferation rates were drastically affected in HepG2 and HuH7 cells following KLF6 depletion. Cell proliferation decreased by almost 50±9 and 68±3% in HepG2 and HuH7 cells, respectively (Figure 3b). As a control, c-myc depletion of HepG2 cells resulted in 62±11% inhibition of cell proliferation (Figure 3b). Interestingly, siRNA targeting KLF6 were ineffective in altering cell proliferation, when transfected in IHH cells (Figure 3c). In addition, KLF6 transfection resulted in IHH cell proliferation, compared with untransfected cells. Taken together, these results clearly indicate that KLF6 expression is essential to HCC-derived cells proliferation.

G_1 block induced by KLF6 silencing. To determine the phase of the cell cycle at which KLF6 exerts its growth-inhibition effect, exponentially growing HuH7 cells were treated with KLF6 siRNA for 72 h and analyzed by flow cytometry (Figure 4a). Depletion of KLF6 led to a 29.9±4.9% increase of HuH7 cells in G_1 phase, with a concomitant decrease of cells in S phase of the cell cycle (−14.45±5.2%) (Figure 4b).

To differentiate between KLF6 depletion-induced G_1 arrest and early S-phase arrest, HuH7 cells were first transfected with KLF6 siRNA or mock-transfected for 24 h, then synchronized in early S phase with aphidicolin. Twenty-four hours later, aphidicolin was removed, and cells were further incubated with nocodazole for 18 h. As shown in Figure 4c, all the cells, depleted or not in KLF6, went through S phase and stopped at the G_2–M peak in the presence of nocodazole. Thus, KLF6 inhibition has no effect on the S-phase progression once HuH7 cells passed the G_1–S phase transition, where KLF6 might act. Again, KLF6 depletion resulted in increase of HuH7 cells in G_1 (+31±1.5%, Figure 4d), indicating that KLF6 also induced G_1 arrest in the second cell cycle after aphidicolin addition. Collectively, these results demonstrated that KLF6 depletion induced G1-phase arrest of HCC-derived cells, without arresting the S-phase progression.

Inhibition of G_1-specific cell cycle proteins following KLF6 depletion. To correlate the consequence of KLF6 inhibition on cell-cycle progression with molecular effectors at the restriction point, HuH7 and HepG2 cells were transiently transfected with KLF6 siRNA, or control transfected. Among the most important substrates of the G_1-specific CDKs is Rb, which binds to the E2F transcription factor when underphosphorylated (pRb). Rb phosphorylation (ppRb) by cyclin D-dependant CDKs is critical for the G_1- to S-phase transition. By Western blotting, we tested the effect of KLF6 depletion on Rb phosphorylation. We found that

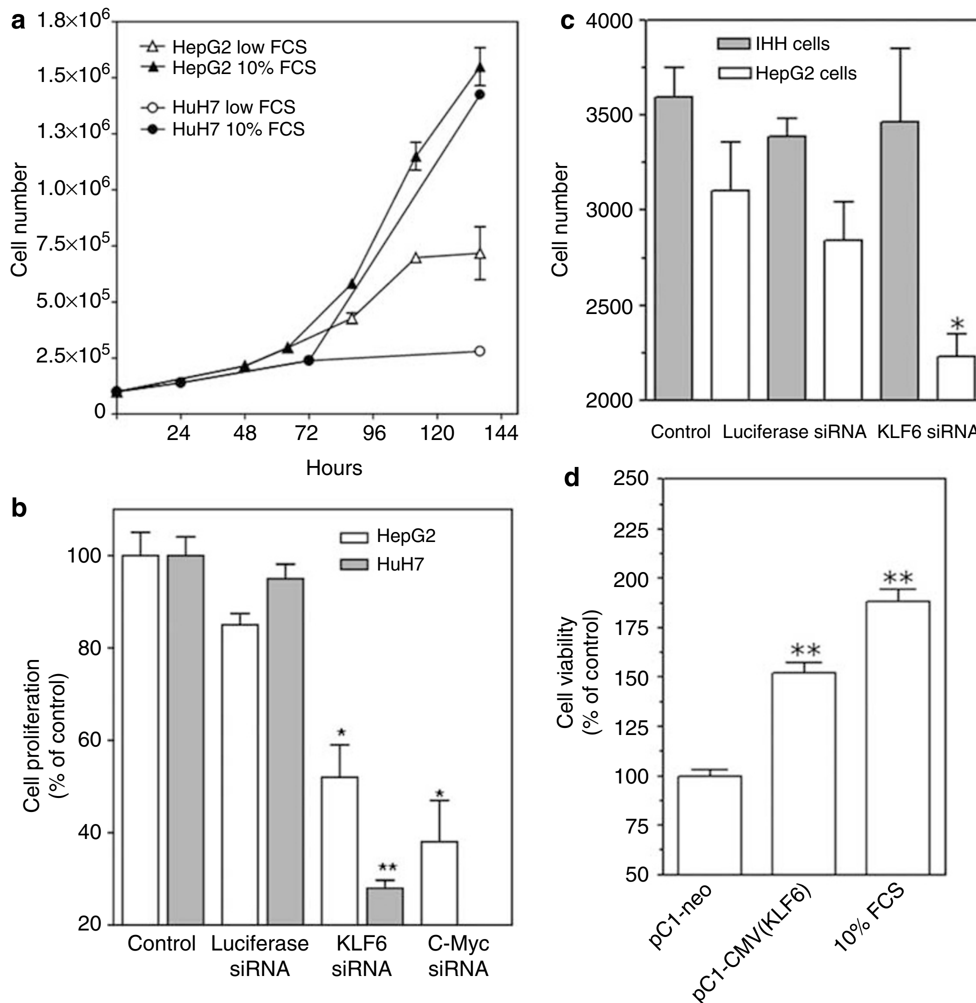

Figure 3 Depletion of KLF6 in HCC-derived cells inhibits cell proliferation. (**a**) 1×10^5 HuH7 and HepG2 cells were cultured for up to 5 days in 35-mm dishes in either low (0.5%) or 10% FCS-containing medium. Cell proliferation was ascertained by cell counting at the time indicated. Results are mean of three independent experiments. (**b**) 1×10^5 HuH7 and HepG2 cells were platted in 35-mm dishes. Forty-eight hours latter, cells were transfected or not with KLF6 siRNA. As a control, cells were transfected with either Luciferase (negative) or c-myc (positive) control siRNAs. Cell proliferation was measured 72 h following siARN transfection by cell counting. Results are represented as mean $\pm$ S.E.M. of three independent experiments ($*P<0.05$, $**P<0.01$). (**c**) 1×10^5 IHH and HepG2 cells were platted in 35-mm dishes. Forty-eight hours latter, cells were transfected or not with KLF6 siRNA. As a control, cells were transfected with luciferase control siRNAs. Cell proliferation was measured 72 h following siRNA transfection by cell counting. Results are represented as mean $\pm$ S.E.M. of three independent experiments ($*P<0.05$). (**d**) 1×10^4 IHH cells were transfected with Amaxa nucleofector II, then deprived in serum-free medium for 16 h. Forty-eight hours later, cell viability was measured at 490 nm using CellTiter 96 AQueous One Solution Cell Proliferation Assay. Results are represented as mean $\pm$ S.E.M. of three independent experiments ($**P<0.01$)

KLF6 silencing induces a marked decrease in ppRb levels in HuH7 cells (-73 ± 6%), to virtual disappearance of ppRb in treated HepG2 cells (Figure 5). CDK4 and cyclin D1, which are critical for pRb phosphorylation, also decreased following KLF6 depletion in both cell lines tested (Figure 5). On the contrary, CDK1, CyclinA and CyclinE, which are not involved in the G_1–S transition, remained relatively unchanged (data not shown) (Supplementary Figure). Altogether, these results demonstrated that KLF6 depletion strongly impaired the expression or phosphorylation patterns of key proteins regulating the G_1–S-phase transition.

KLF6 is essential for HCC-derived cells protection from apoptosis. To further explore KLF6 depletion-induced G_1 arrest of the cell cycle, HuH7 cells were transfected with KLF6 siRNA or mock-transfected for 24 h, then synchronized with aphidicolin. Twenty-four hours later, aphidicolin was removed, and cells were further incubated in fresh medium for 48 h. KLF6–siRNA-treated cells remained in G_1 compared with mock-transfected cells, with a sub-G_1 peak emerging, suggesting that KLF6 depletion may induce apoptosis in HCC-derived cells following G_1-phase arrest (Figure 6a).

To confirm that KLF6 expression inhibition induces apoptosis in HCC-derived cells, gel for DNA fragmentation were performed, as well as Western blotting for key proteins implicated in apoptosis. As shown in Figure 6b, transfection of HuH7 cells with siRNA targeting KLF6 induced low-molecular weight DNA fragmentation, compared with control-transfected cells. In addition, we found that KLF6 depletion resulted

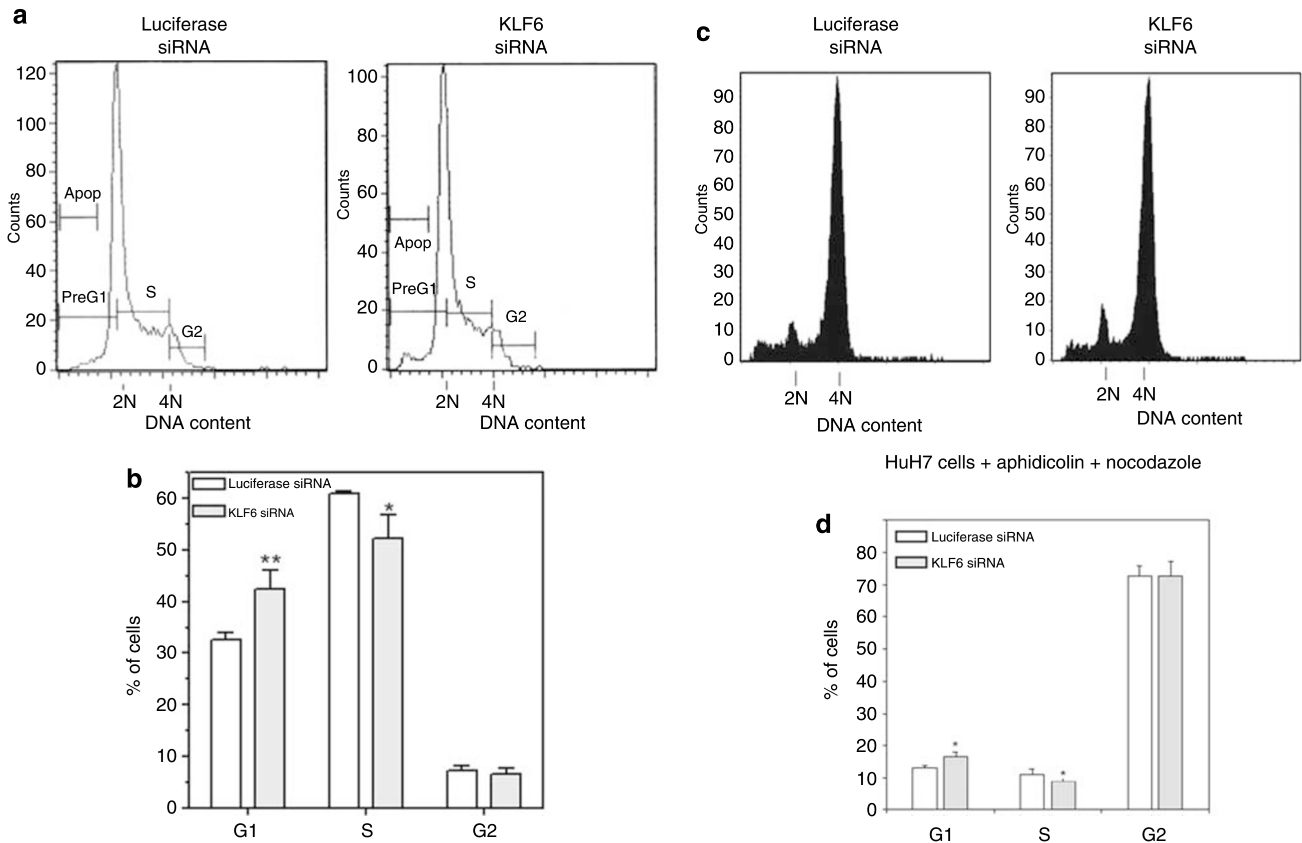

Figure 4 Invalidation of KLF6 induces G_1-phase arrest in HCC-derived cell line. (**a**) 25×10^5 HuH7 cells were platted in 60-mm dishes before transfection with KLF6 siRNA for 48 h as described in Materials and Methods. As a control, cells were transfected with siRNA targeting luciferase. Result is representative of three independent experiments (apop: apoptosis) (**b**). Quantification of cell-cycle analysis using ModFit software. Results are represented as mean $\pm$ S.E.M. of three independent experiments (**P*<0.05, ***P*<0.01). (**c**) HCC-derived cells were first synchronized in early S phase with 1 μmol/l aphidicolin for 24 h before transfection with KLF6 siRNA for 24 h as described in Materials and Methods. As a control, cells were transfected with siRNA targeting luciferase. After aphidicolin removal, cultures were further incubated or not with nocodazole (0.2 μg/ml) for 18 h. The DNA content distribution histograms were analyzed by fluorescence-activated cell sorter (FACS) analysis. (**d**) Quantification of cell-cycle analysis using ModFit software. Results are represented as mean $\pm$ S.E.M. of three independent experiments (**P*<0.05)

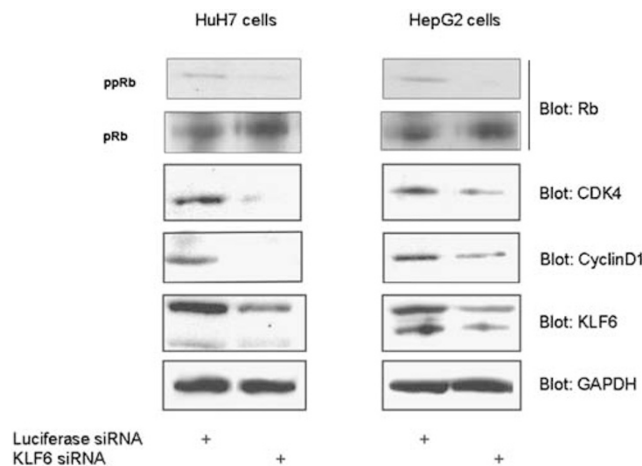

Figure 5 Effect of KLF6 silencing on Rb phosphorylation and levels of cyclin D1 and CDK4. 100×10^5 HuH7 and HepG2 cells were platted in 100-mm dishes before transfection with KLF6 siRNA as described in Materials and Methods. As a control, cells were transfected with siRNA targeting luciferase. Forty-eight hours later, cellular proteins were purified and Western blotting was performed for Rb phosphorylation, CDK4, cyclin D1 and KLF6 expression. To ensure an equal loading of the lanes, filters were stripped and reprobed with anti-GAPDH antibodies. Results are representative of at least three independent experiments

in caspase-3 activation, followed by poly ADP-ribose polymerase (PARP) cleavage (Figure 6c).

The p53/p21^{CIP1} pathway plays a critical role in regulating the G_1–S transition in response to a variety of cellular stresses. We found that inhibiting KLF6 expression in wild-type p53-containing HepG2 cells induced p53 expression (Figure 7a). By contrast, p53 levels were much higher in HuH7 cells than in HepG2 cells, as expected for p53-mutant cells, and remained unchanged following transfection of siRNA targeting KLF6 (data not shown). Moreover, p21^{CIP1} was not induced in HepG2 cells following KLF6 depletion, and remained undetectable in HuH7 cells under the same condition. These results show that p53 activation can also contribute to KLF6 silencing-induced G_1 arrest in p53-competent cells.

We next investigated the expression of Bcl-xL, which possess an antiapoptotic function similar to that of Bcl-2. Bcl-xL expressed in hepatoma cells plays an integral role in suppressing p53-mediated apoptosis when they are exposed to proapoptotic conditions.[20] We found that depleting KLF6 in both HuH7 and HepG2 cells strongly inhibited Bcl-xL expression, when Bcl-2 expression remained relatively unchanged, as shown in Figure 7b. Taken together,

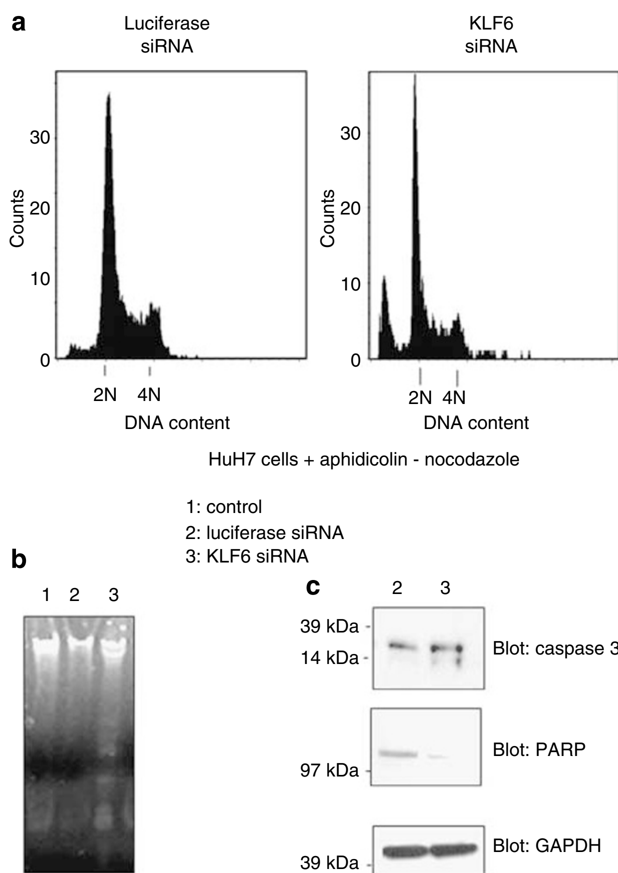

Figure 6 KLF6 depletion induces cell death by apoptosis in HCC-derived cells. HCC-derived cells were first synchronized in early S phase with 1 μmol/l aphidicolin for 24 h before transfection with KLF6 siRNA as described in Materials and Methods. As a control, cells were transfected with siRNA targeting luciferase. After aphidicolin removal, cultures were further incubated for 48 h before subsequent manipulation. (**a**) The DNA content distribution histograms were analyzed by FACS analysis. The different phases of the cell cycle were determined with ModFit DNA analysis software. Data shown are representative of three independent experiments. (**b**) DNA fragmentation was ascertained by DNA electrophoresis as described in Materials and Methods. Results are representative of three independent experiments. (**c**) Caspase-3 and PARP cleavage was illustrated by Western blotting. Data shown are representative of at least three independent experiments

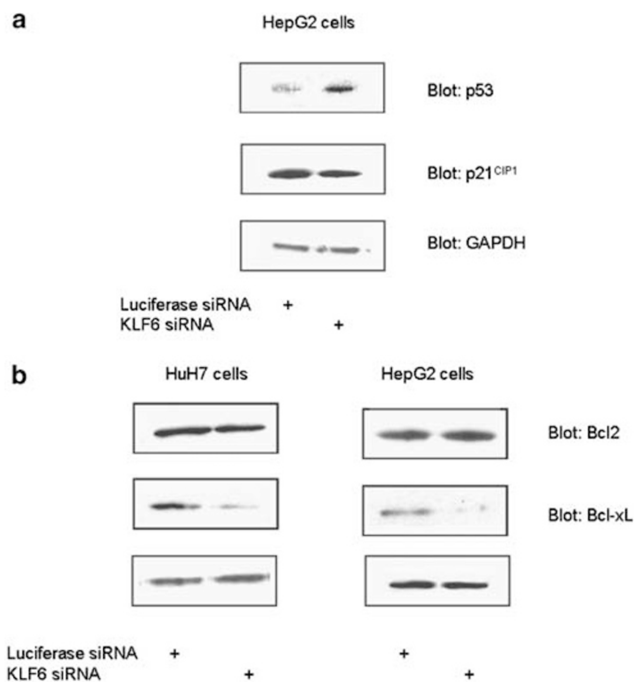

Figure 7 KLF6 invalidation activates p53 and inhibits Bcl-xL expression in HCC-derived cell lines depletion. 100×10^5 HuH7 and HepG2 cells were platted in 100-mm dishes before transfection with KLF6 siRNA as described in Materials and Methods. As a control, cells were transfected with siRNA targeting luciferase. Forty-eight hours later, cellular proteins were purified and Western blotting was performed for p53 and $p21^{CIP1}$ (**a**), and Bcl2 and Bcl-xL (**b**). To ensure an equal loading of the lanes, filters were stripped and reprobed with anti-GAPDH antibodies. Results are representative of three independent experiments

these results demonstrate that KLF6 protects HCC-derived cells from programmed cell death by regulating the expression of both antiapoptotic (Bcl-xL) and proapoptotic proteins (p53).

Discussion

HCC, one of the most common cancers in the world, develops from transformed hepatocytes during the course of chronic liver diseases.[21] When transformed hepatocytes expand *in vivo*, they encounter various microenvironmental stresses, such as hypoxia, decreased growth factor and lack of nutrient supply that may activate apoptosis.[22] Resistance to apoptosis may provide a selective advantage for growth and progression of HCC, and contributes to the development of resistance to cancer therapy.

Established genetic events in HCC include loss of tumor suppressor gene function through a combination of genetic and epigenetic events, including allelic losses, mutation or promoter methylation.[23] Amplification and/or mutation of oncogenes such as *K-Ras* and *c-myc* have also been described, as has cyclin overexpression.[24] Altogether, these studies depict many genetic alterations associated with the development and/or progression of HCC. KLF6, a ubiquitously expressed zinc-finger transcription factor,[3] has been identified as a tumor suppressor gene in prostate,[4] gastric,[25] colon[11] and nasopharyngeal[26] cancers, as well as astrocytic gliomas.[14] Moreover, aberrant KLF6 mRNA levels have been described in lung[9] and esophageal cancers,[16] the latter also being associated with promoter methylation. In addition, frequent inactivation of KLF6 has been reported in HCC.[17] However, the extent of KLF6 loss in major human cancers has been recently subjected to passionate debate. Numerous studies now report the absence of somatic mutation nor KLF6 inactivation in colorectal carcinoma,[27] in glioblastomas,[28] in prostate cancer,[29,30] in meningiomas,[28,31] in astrocytic tumors,[32] and, finally, in HCC.[18] Here, we confirmed that KLF6 is not mutated, and expressed in HCC samples, and in HCC-derived cell lines. Disagreement in KLF6 mutation frequency between the studies of Kremer-Tal *et al.*,[17] Boyault *et al.*[18] and the present study remain to be elucidated. It is not likely to be due to a failure in our mutation screening method, as its sensitivity has been previously validated in a larger

series of tumor DNA samples originating from pancreatic cancer by the identification of *KRAS*, *P16* and *DPC4* gene mutations.[33] As suggested by Lievre *et al*.,[27] these discrepancies could reflect the procedure used for the samples' preservation in the study by Kremer-Tal *et al*.,[17] that might increase the risk for false-positive mutated KLF6, compared with the fresh frozen tissue approach that we used here. Other studies found high frequencies of mutations in ovarian and lung cancer that have finally been proved to be artifact because of formalin-fixation.[34]

Many studies depict KLF6 as a tumor suppressor gene, when overexpressed in various cell lines, in which its endogenous expression is apparently lost.[4,5,9,11,17] The finding that KLF6 transcription factor is expressed in HCC samples and derived cell lines prompted us to study KLF6 effect on HCC-derived cell proliferation control. To better understand the biological functions of KLF6 in a cellular context, we used RNA interference in HCC-derived cells. We first confirmed that siRNA targeting KLF6 is efficient in reducing KLF6 mRNA and protein expression in both HepG2 and HuH7 cells. Next, we measured HepG2 and HuH7 cell proliferation following specific KLF6 depletion. An siRNA targeting luciferase was used as a control. Surprisingly, both HCC-derived cell lines were highly sensitive to KLF6 inhibition. By 3 days following treatment, cell proliferation was strongly decreased in both HepG2 and HuH7 cells. As this was surprising in view of the cytostatic action of KLF6 in most cellular settings, we have used this finding as a starting point to elucidate the molecular pathways by which KLF6 stimulates HCC-derived cell proliferation.

Cell cycle is regulated by a series of checkpoints monitoring genomic integrity and ensuring that DNA replication proceeds in a coordinated manner. Different combination of CDK and cyclin subunits operate at checkpoint controls during cell cycle to integrate mitogenic and antiproliferative signals. In the present study, we demonstrate that KLF6 depletion induces G1-phase arrest, followed by apoptosis in HCC-derived HuH7 cells. Similar results were found in p53/wild-type HepG2 cells. Abnormality of G1–S transition regulators, and more specifically of the Rb pathway, has been recognized as significant factors in the development of human cancers. For instance, Rb proteins bind to the E2F family of transcription factors to ensure that they remain inactive during mitosis and G0.[35] Activity of the Rb proteins is modulated by sequential phosphorylation by CDK4/6–cyclin-D and CDK2–cyclin-E complexes.[35] Hyperphosphorylated Rb proteins release the molecules that bind to their hypophosphorylated isoforms, allowing them to carry out their specific tasks in cell-cycle progression. Consequently, modulation of CDKs is a major target for tumor prevention and therapy. Here, we demonstrated that KLF6 inhibition downregulates the hyperphosphorylated form of Rb, probably by modulating CDK4 and cyclin D1 expression. The nature of KLF6-mediated control of CDK expression remains to be established. When overexpressed in colon carcinoma-derived cells, KLF6 interacts with cyclin D1, to disrupt cyclin D1/CDK4 complexes, to redistribute $p21^{CIP1}$ to CDK2, which promotes G_1 cell-cycle arrest.[5] However, low concentration of KLF6 rather promotes cyclin D1/CDK4 complexes in the same cells.[5] Thus, results obtained here and previously reported data are definitely not mutually exclusive. One can argue that KLF6 expressed in HCC-derived cells, as a transcription factor, may transcriptionaly modulate CDKs expression. On the other hand, KLF6 may prevent the degradation of these critical proteins. Studies are currently undergoing to clarify these hypotheses.

We next analyzed the expression levels of cell-cycle-related proteins known to have a key role in cell proliferation control. Members of the Cip/Kip family ($p21^{CIP1}$, $p27^{Kip1}$ and $p57^{Kip2}$) bind to CDK complexes and inhibit their activities. Although the tumor suppressor p53 is not a cyclin-dependent kinase inhibitor (CDKI), it has been implicated in a variety of cellular processes, including induction of G1 arrest and apoptosis by transactivating a number of downstream genes.[36] Remarkably, KLF6 had different effects on the CDKI $p21^{CIP1}$ and p53 in p53-competent HCC-derived cells. First, KLF6 downexpression did not modify $p21^{CIP1}$. Second, KLF6 silencing strongly induced p53 levels in p53/wild-type HepG2 cells. This occurred independently of $p19^{ARF}$, as the levels of this p53 regulator remained unaffected in the presence of siRNA targeting KLF6 (data not shown). These results indicate that the increased level of p53 protein as a transcriptional activator did not mediate p21 expression in p53/wild-type HepG2 cells. This raises the possibility that suppression of p53 represents another crucial function of KLF6, at least in HCC cells that coexpress both proteins.

Mitochondrial release of cytochrome *c* plays a central role in triggering apoptosis. Enforced expression of antiapoptotic Bcl-2 family protein has been shown to suppress apoptosis in response to a wide variety of stimuli such as activation of mitogenic oncogenes, hypoxia withdrawal of trophic factors and loss of adherence (for a review see Cory and Adams[37]). In particular, dysregulated expression of Bcl-xL, but not Bax, Bad, nor Bid, plays an essential role in the regulation of apoptosis in HCC.[20] We confirmed in the present study that Bcl-xL is expressed in both wild-type and p53-negative HCC-derived cell lines. Interestingly, downregulating KLF6 in HepG2 and HuH7 cells strongly impaired Bcl-xL protein expression. Because Bcl-xL is a well-characterized apoptosis antagonist, these data straighten the conclusion that KLF6 may play an essential role in protecting HCC-derived cells from apoptosis.

Taken together, the duality of KLF6 function is highly similar to that of transforming growth factor-β (TGF-β), KLF4 and oncostatin-M. TGF-β acts cytostatically in early-stage cancers, whereas it promotes tumorigenicity of malignant lesions, which is conceivably related to changes in its genetic context during tumor progression.[38] Depending on the 'cellular content', both KLF4, a member of the KLF family, and oncostatin-M function either as inhibitors or promoters of cell proliferation.[39,40] In addition, both can suppress p53 transcription, again supporting an essential role in such settings. Together with the results describing KLF6 as a tumor suppressor gene, our observation creates a paradox and might indicate that not only loss, but also enforced expression of KLF6 may contribute to tumorigenesis. This phenomenon is still not understood, and might be attributed to 'cell-type' specificity. Further studies are needed to identify such cellular factors, for KLF6 to switch from a growth-inhibiting tumor suppressor to a growth-promoting oncogene. Finally, even though we demonstrated that inhibition of KLF6 expression

can drive cultured HCC-derived cells into apoptosis, inactivation of KLF6 will not be desirable in cellular setting where KLF6 acts as a tumor suppressor. As a consequence, we are currently devising a targeted gene therapy approach to specifically disrupt KLF6 expression in HCC-derived cells.

Taken together, the data presented here demonstrated that KLF6 is expressed in HCC samples as well as in HCC-derived cells. KLF6 silencing by RNA interference induced a dramatic inhibition of HCC-derived cell proliferation, by the inhibition of Rb phosphorylation and Bcl-xL expression, to induce cell growth arrest in G1 phase and cell death by apoptosis. Studies are currently undergoing to ask whether KLF6 might be of potential interest for the targeted therapy of HCC.

Materials and Methods

Tissue samples. Surgical specimens were obtained from explanted livers. Cancerous and non-cancerous tissues were enucleated separately from the tumorous and non-tumorous parts of the liver. Histopathological diagnoses of HCC ($n=10$) were made following surgery. Written informed consent for tissue collection and further analyses were obtained for all patients before surgery. Samples were snap-frozen in liquid nitrogen for subsequent RNA extraction.

Cell lines and cultures. Immortalized IHH cells were used as normal hepatocytes. HuH7 cells (derived from HCC) and HepG2 cells (derived from hepatoblastoma) were obtained from LGC Promochem in partnership with ATCC (LGC Promochem, Molsheim, France). Cells were cultured in DMEM medium containing 4.5 g/l glucose, 10% FCS supplemented with fungizone, antibiotics, L-glutamine and antimycoplasma reagent (Plasmocin™, InvivoGen, Toulouse, France) and kept at 37°C and 5% CO_2.

siRNA production and transfection. siRNA used in this study was generated using the Silencer siRNA construction kit (Ambion Europe Ltd, Huntingdon, UK). The target sequence for KLF6 exon 2 was 5′-ggagaaaagccttacagat-3′, as described previously.[5] siRNA targeting luciferase was purchased from Euromedex (Souffelheim, France). Target sequence was the following: 5′-cgtacgcggaauacttcga-3′, corresponding to positions 153–171 of cloning vector pGL2-control from Promega (Charbonnières, France). siRNA (100 nM) per 1×10^6 cells were transfected using Jet-SI transfection reagent from Polyplus transfection (Illkirch, France), following the manufacturer's instructions. Under these conditions, 95% of the cells were efficiently transfected using a CY3-conjugated siRNA, as ascertained by fluorescent microscopy (data not shown).

Plasmid transfection. pC1neo-KLF6 plasmid encoding for human KLF6 was a kind gift of Dr Scott L Friedman (Division of Liver Diseases, Department of Medicine, Mount Sinai, School of Medicine, New York, NY, USA). 1×10^4 IHH cells were transfected with Amaxa nucleofector II (amaxa GmbH, Cologne, Germany), using solution T and program T-028. Under these conditions, cell transfection efficiency was $35\pm7\%$. Cells were deprived for 16 h in serum-free medium. Forty-eight hours later, cell viability was measured at 490 nm using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Charbonnières, France).

Flow cytometry analysis of DNA content. 2.5×10^5 cells were seeded in 60-mm dishes in complete medium. Twenty-four hours later, cells were transfected or not with KLF6 siRNA, and synchronized or not in phase S with 1 μM aphidicolin. Twenty-four hours later, aphidicolin was removed, and cells were incubated or not with 0.2 μg/ml nocodazole for another 18 h. Control cells were transfected with an siRNA targeting luciferase. Cells were then incubated in phosphate-buffered saline (PBS) supplemented with 1 mM ethylene diamine tetraacetic acid (EDTA) at 37°C for 10 min, and collected by centrifugation at $130\times g$ for 5 min. DNA content was analyzed by propidium iodide staining (DakoCytomation France SAS, Trappes, France) using Cell quest software on a FACSCalibur Station (Becton Dickinson, Sunnyvale, CA, USA). Cell-cycle distributions were calculated on DNAplots by ModFit LT software (Verity Software House Inc., Topsham, ME, USA).

Real-time quantitative RT-PCR. Cell line and HCC-derived RNA samples were collected and extracted using the Rneasy Mini Kit (Qiagen SA France, Courtaboeuf, France). RNA was treated with DNAse (Invitrogen Inc., Carlsbad, CA, USA). For RT-PCR analyses, 1 μg of total RNA was reverse-transcribed using Superscript III RNase H^- reverse transcriptase and random hexamers (Invitrogen Inc., Carlsbad, CA, USA). Quantitative real-time PCR was performed in duplicate using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA) and primers spanning KLF6' splicing site, as described previously.[15] KLF6 expression level were normalized to 18S ribosomal RNA levels (forward primer: aaa cgg cta cca cat cca ag, reverse primer: cct cca atg gat cct cgt ta).

Western blot analysis. Tissues and cell lines were solubilized in lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM sodium fluoride, 2 mM sodium orthovanadate, pH 7.4) containing 0.1% NP-40, 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonylfluoride, 20 μg/ml aprotinin and 20 μM leupeptin. Cell lysates were gently agitated for 20 min at 4°C and centrifuged at $12\,000\times g$ for 10 min. This extraction procedure was determined to efficiently extract both nuclear and cytoplasmic proteins by analysis of histone H1 and fibronectine, respectively. For immunoblotting, 50 μg of soluble proteins were resolved through 4–20% sodium dodecyl sulfate-polyacrylamide gradient gels (Invitrogen, Carlsbad, CA, USA), and transferred to a nitrocellulose membrane. After room temperature blocking for 1 h, blots were incubated with either antibodies to KLF6 (1 : 250) (CliniSciences Inc., Montrouge, France). The following antibodies were from Santa Cruz Biotechnology Inc.: CDK4 (1 : 500, sc-260), Cyclin D1 (1 : 500, sc-124), p21 (1 : 1000, sc-817), CyclinA (1 : 250, sc-239), CyclinE (1 : 250, sc-247), Bcl-xL (1 : 500, sc-8392), Bcl2 (1 : 500, sc-492), p16 (1 : 500, sc-1207), caspase-3 (1 : 250, sc-7148), PARP (1 : 250, sc-8007), p53 (1 : 250, sc-98). Rb antibodies were from Becton Dickinson (1 : 500, 554 136). Secondary antibodies of goat anti-mouse and goat anti-rabbit HRP-conjugated antibodies (1 : 10 000, Perbio Science, Erembogdegem-Aalist, Belgium) were added, and blots were incubated for 1 h at room temperature. Immunoreactive proteins were visualized using ECL immunodetection system (Supersignal West pico, Perbio Science, Erembogdegem-Aalist, Belgium). To ensure an equal protein loading of the various lanes, Western blots were reprobed using either monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or anti-β-tubulin antibodies (1 : 1000, Santa Cruz Biotechnology Inc. sc-24778 and sc55529, respectively). For Rb immunoblotting, proteins were resolved on 6% Tris-tricine gels (Invitrogen Inc., Carlsbad, CA, USA).

Immunostaining for KLF6 expression. To detect human KLF6 protein, 50×10^3 HepG2 or HuH7 cells were plated in complete medium in four-well glass slides (Lab-Tek II chamber slide, Nalge Nunc Internatinal, Naperville, IL, USA). Two days later, medium was removed, and cells were washed twice with PBS before fixation in 4% PFA pH 7.4 for 8 min at room temperature, and permeabilization in 0.1% NP-40 diluted in PBS for 10 min. Slides were treated 10 min with Dako block reagent (Dakocytomation, Glostrup, DK). Slides were incubated overnight at 4°C with rabbit anti-KLF6 antibodies (1 : 250, Santa Cruz Biotechnology Inc., sc-7158), followed by Cy3-goat-anti-rabbit IgG (1 : 300, Jackson Immunoresearch Europe, Cambridgeshire, UK) for 1 h at room temperature. Antibody incubations were performed in PBS supplemented with 1% gelatine. Cell nucleuses were counterstained with DAPI. Resulting immunostaining was observed with a Zeiss LSM 510 confocal microscope (Carl Zeiss France SAS, Le Pecq, France).

DNA fragmentation assay. 2.5×10^5 cells were seeded in 60-mm dishes in complete medium. Twenty-four hours later, cells were transfected or not with KLF6 siRNA. Seventy-two hours later, cells were collected in PBS supplemented with 1 mM EDTA at 37°C for 10 min, spun down and resuspended with 0.5 ml PBS. Lysis buffer (55 μl) (625 mM EDTA, 100 mM TrisCl pH 8.0, 1% Triton X-100 diluted in PBS) was added for 20 min on ice (4°C). Samples were centrifuged at $12\,000\times g$ for 30 min and 4°C. DNA was extracted with phenol–chloroform and precipitated in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. Following centrifugation and air drying, total DNA was resuspended in 30 μl of deionized water-RNase solution. Samples were heated in gel loading buffer for 30 min at 37°C, before electrophoresis in acrylamide gels.

Cell growth assay. HepG2 and HuH7 cells were cultured in 35-mm dishes at 50×10^3 cells/ml (2 ml per dish) in complete medium. After an overnight attachment phase, cells were transfected or not in the presence of serum with siRNA targeting KLF6. Control cells were transfected with an siRNA targeting luciferase. As a positive

control, HepG2 cells were transfected with an siRNA targeting c-myc. Cell growth was measured after 72 h of culture by cell counting using Coulter counter model ZM.

Statistical analysis. Results are expressed as mean $\pm$ S.E. Student's *t*-test was used to compare data. $P < 0.05$ was considered significant.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)