In vitro c-met inhibition by antisense RNA and plasmid-based RNAi down-modulates migration and invasion of hepatocellular carcinoma cells

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Abstract. The receptor tyrosine kinase c-met is over-expressed in several types of human tumours. In hepatocellular carcinoma (HCC), its expression is inversely correlated to patient survival. To determine the role of c-met in the malignant properties of HCC cells, we tested the effectiveness of two ablative strategies to down-modulate c-met expression in SKHep1C3, an HCC-derived cell line, i.e. stable expression of antisense RNA c-met and RNA interference. A plasmid coding a 965-nt fragment complementary to 5' portion of c-met mRNA was constructed for the antisense strategy. RNA interference methodology was applied for transient silencing, achieved by small interfering RNAs, and for stable silencing using an RNA polymerase III promoter carrying plasmid coding small hairpin RNAs (shRNAs) that targeted c-met. The transfected cells showed consistently lower levels of c-met mRNA and protein. The results showed that the antisense and RNAi sequences chosen to target c-met mRNA reduced c-met expression efficiently and inhibited malignant properties of SKHep1C3 cells. These data indicate that c-met is an essential factor in the processes of migration and invasion of hepatocarcinoma cells; and c-met down-regulation may be included in a therapeutic strategy for HCC in experimental animal models.

Introduction

Hepatocellular carcinoma (HCC) is an aggressive malignancy with a poor prognosis; its incidence is increasing, mainly due to a rising incidence of infection by hepatitis B/C viruses (1). Orthotopic liver transplantation and/or surgical resection are still the best therapeutic strategies, but tumour recurrence is still the major cause of death (2). c-met is a receptor tyrosine kinase (RTK) located on chromosome 7q31, the gene consists of 21 exons and 20 introns. The 170-kDa correspondent precursor protein is further cleaved to form an α-chain of 50 kDa linked by disulfide bonds with a 145-kDa ß-chain that has an intracellular tyrosine kinase (TK) domain (3,4). The ligand for c-met has been identified as hepatocyte growth factor (HGF) also called scatter factor (SF) (5). The HGF/SF binding to c-met activates its kinase activity and initiates several cellular responses, such as tissue regeneration, angiogenesis, proliferation and migration. The HGF/c-met system is involved in the physiological invasive growth in embryogenesis; it works in a paracrine fashion during organogenesis (i.e., in the development of the placenta, of the liver), given that HGF and c-met are mainly produced by mesenchymal and epithelial cells respectively. In tumour cells, c-met overexpression leads to an invasive program generating events of tumour progression and metastasis; and the most frequent c-met disregulation occurs in the absence of mutations or autocrine HGF production. This type of c-met-overexpression has been reported in carcinomas of the ovary, breast, pancreas, prostate, stomach and in colorectal cancer (6,7). Further c-met germline and somatic mutations (mainly in the TK portion and therefore activating RTK) have been found in patients with hereditary papillary renal, familial gastric cancer and in childhood HCC also supporting a causative role for c-met of a transforming event which may lead to certain types of tumours (8-10). For all these reasons, targeting the HGF/c-Met system is being evaluated as a therapeutic approach for several types of tumours. Preclinical data suggest that c-met can be inhibited with antisense/small interfering RNA (siRNA), peptides/antagonists of HGF, small-molecule tyrosine kinase inhibitors, and also antibodies directed against c-Met or HGF. Very little is known on targeting the HGF/c-met system in HCC. On c-met disregulation, it has been reported that c-met is overexpressed in HCC tumour tissues compared with normal ones (11-13); our previous data showed that c-met mRNA overexpression in HCC is inversely related to patient survival, is associated with HGF mRNA down-modulation, and it correlates with the expression

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of urokinase-type plasminogen activator (uPA) (14). This is a serine-protease that plays an important role in proteolytic cascades that govern the extra-cellular matrix (ECM) turnover, in migration and/or proliferation of several types of tumour and normal cells (15-17), including hepatocytes in the process of liver regeneration (18).

The aim of this study was therefore to down-modulate c-met expression in HCC derived cells *in vitro*, in SKHep1C3 cells (at high levels of c-met expression with no c-met mutation) and to examine the biological effects of c-met silencing. To knock-down c-met expression, two strategies were chosen, the stable expression of antisense RNA (AS RNA) (19,20), and RNAi technology which employs as effector molecules short double strand small interfering RNAs of 19-25 nt (21-25). A plasmid vector expressing small hairpin RNAs (shRNAs) against c-met under the control of the human U6 promoter was developed; and using these two strategies (AS RNA and shRNA), the specific down-regulation of c-met expression was studied in relationship with its effects on migration, invasion and proliferation of SKHep1C3 cells.

Materials and methods

Cell lines. SKHep1Clone3 (SKHep1C3) (26), selected from human hepatocellular carcinoma derived cells (SKHep1: ATCC HTB52), and SKHep1C3 transfected cells were maintained in Earle's MEM and supplemented with 10% heat-inactivated bovine serum (FBS) (Invitrogen, USA) at 37˚C in a 5% CO_2 incubator. SKHep1C3 cells (not carrying c-met mutations at 14-21 exons, not shown) do produce high levels of c-met without expressing hepatocyte growth factor mRNA.

Both SKHep1C3 transfected and non-transfected cells that were ~80% confluent were used for most experiments. AB5 (17) human dermal fibroblasts were maintained in Earle's MEM and supplemented with 10% bovine serum (FBS).

siRNA design and synthesis. siRNAs targeting c-met mRNA were obtained from Ambion (Austin, TX, USA). The siRNA target finder and the design tool provided by Ambion were used to select the siRNA sequences. Twenty-one nucleotide RNA oligonucleotides called siRNA c-met_2 (targeting c-met mRNA at nt 412-430; NCBI access no. NM_000245) and siRNA c-met_5 (targeting c-met mRNA at nt 427-445) forming a 19-bp duplex with 2-nucleotide 3' overhangs were used. siRNA duplexes were transiently transfected into SKHep1C3 cells at concentration of 100 nM using Dotap reagent according to the manufacturer's instruction (Roche Diagnostic, Basel, Switzerland).

Preparation of the vectors. The human EF6 promoter plasmid pEF6/V5-His Topo vector (Invitrogen, Carlsbad, CA, USA) was used for stable antisense technology. Briefly, a fragment of 965 bp cDNA from SKHep1C3 cells corresponding to nucleotides 169-1133 of c-met mRNA was amplified using the synthetic primers c-met S (5'-CGAAAGATAAACCTCT CATAATGA-3') and c-met rev (5'-ATTAAACACTTCCTT CTTTGTGG-3'). The 965-bp c-met fragment was directly ligated into the plasmid pEF6/V5-His Topo (containing the blasticidine-selectable marker gene). The construct was cloned into chemically competent *E. coli* and the antisense orientation was verified by automatic sequencing.

Small interfering oligonucleotides specific for c-met from 412 to 430 (NCBI access no. NM_000245) bases (GATCC GGAAGACCTTCAGAAGGTTCTCAAGAGAAACCTTCT GAAGGTCTTCCTCTTTTTTGGAAA) were synthesized and annealed at 90˚C for 3 min and cooled to 37˚C for 1 h.

The p*Silencer*™ 2.1-U6 hygro plasmid vector that expresses shRNAs for c-met under the control of an RNA polymerase III human U6 promoter was constructed by inserting pairs of the annealed DNA oligonucleotides specific for c-met described above at *Hin*dIII and *Bam*HI sites. A p*Silencer* 2.1-U6 vector with a sequence that does not target any gene was used to develop the control vector. After transformation in chemically competent *E. coli* and plasmid DNA purifications, all inserted sequences were confirmed by automatic DNA sequencing.

Transfection. SKHep1C3 cells, were grown to 60-80% confluency (in 6-cm diameter Petri dishes) and then transfected with 10 μg of pEF6/V5-His Topo vector coding antisense c-met using Dotap Liposomal transfection reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Transient transfection with siRNA c-met_2 and siRNA c-met_5 (at concentration of 100 nM) was performed in a 24-well costar plate using Dotap liposomal reagent, in order to choose the best sequence to clone into a plasmid for stable expression of the shRNA c-met. Transfection of SKHep1C3 cells with p*Silencer* 2.1-U6 plasmid coding shRNAs against c-met was performed in a 6-well costar plate. Briefly, 2.5 μg of plasmid was used for each well. The cells were then cultured until stable resistants were grown and further analysis carried out.

Transfected cells were selected for blasticidine resistance (6 μg/ml) and hygromicin (200 μg/ml) (pEF6: cells transfected with a mock vector; AS c-met: cells transfected with antisense c-met construct; pH C3: cells transfected with shRNAs control plasmid; pH shRNA c-met C3: cells transfected with shRNAs targeting c-met).

Genomic PCR and RT-PCR. Genomic DNA from parental and transfected cells was obtained with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). To verify transfection with an antisense construct, we amplified 50 ng of DNA from SKHep1C3, pEF6 and AS c-met cells with a pair of pEF6/V5-His Topo specific primers, T7 (5'-taatacgactataggg-3') and BGH (5'-tagaaggcacagtcgagg-3') besides the insertion region of insert. The size of PCR products was 264 bp for mock plasmid and 1229 bp for plasmid with antisense expressing insert.

Total RNA of transfected SKHep1C3 was extracted, quantified and reverse-transcribed. We first verified the expression of the antisense constructs of 1229 bp by the T7, BGH primers set. The following RT-PCR was performed to determine the percentage of c-met mRNA inhibition: 1 μg of total RNA was reverse transcribed using random hexamers and the amplification reaction was performed essentially as previously described (27). The quantification of the ribosomal

protein L7, and GAPDH was also performed using specific primers: L7 up: 5'-gaggtatcaatggagtgagcc-3' and L7 down: 5'-actctccatgcagatgatgcc-3'; GAPDH 1DIR: 5'-aagaagatgcggc tgactgtcgagccacat-3' and GAPDH 2 REV: 5'-tctcatggttcacacc catgacgaacacatg-3'.

Endogenous c-met was detected by PCR analysis using specific primers: MET-1 5'-ctagacacatttcaattggt-3' (nt 2247-2266); MET-2 antisense: 5'-tgttgcagggaaggagtggt-3' (nt 2591-2610). The conditions used in RT-PCR avoided PCR plateau values being reached for any of the mRNAs studied. A comparative PCR method was used to determine the % of c-met inhibition, together with an image analysis system (Gel-Pro Analyzer 3.1) capable of scanning the PCR amplified products directly from the images of the agarose gel bands, thus obtaining a relative quantification of the c-met products compared to the c-met expression level in SKHep1C3 untransfected cells. The relative values expressed in pixels as integrated optical density (IOD) were normalized compared to the correspondent level of the housekeeping gene and reported as arbitrary units (27-29).

Western blot analysis. Cell extracts (CE) from cultures of SKHep1C3 and transfected cells were collected by adding 0.05% SDS and equal amounts of total proteins were analysed by Western blotting. After determining the protein concentration using the Bradford-assay, constant amounts of CE proteins were loaded in SDS-PAGE, under reducing conditions, on a three layer polyacrylamide gel, containing different acrylamide concentrations at 4, 6 and 12%. Gels were blotted on nitrocellulose membranes and immunoreacted with rabbit anti-human c-met antibodies (Sigma-Aldrich) (1:1,000 in 0.3% BSA); phosphospecific rabbit anti-c-met (pY1003) (1:1,000 in 0.3% BSA) and alkaline phosphatase-conjugated anti-rabbit IgG (1:7,500 vol/vol). The same amounts of proteins were loaded on an 8% polyacrylamide gel. The separated proteins were electroblotted onto a nitrocellulose membrane, then immunoblotted using mouse monoclonal antibodies anti-GAPDH (1:300 in 1% BSA) and alkaline phosphatase-conjugated anti-mouse IgG, rabbit polyclonal antibodies anti-PKR (PKR: Protein Kinase R), anti-phospo-PKR (Thr 446/451), anti-eIF2α (eIF2α: α subunit of eukaryotic initiation factor 2), anti-phospho-eIF2α (Ser51) (1:1,000 in 5% BSA) (Cell Signaling Technology, MA, USA) and alkaline phosphatase-conjugated anti-rabbit IgG. The positive immunoreaction was detected with nitroblue tetrazolium and bromochloroindolyl phosphate (Promega, WI, USA). The bands corresponding to c-met (170 and 145 kDa) and GAPDH were scanned using a digital system (Gel-Pro Analyzer 3.1) and the values of the integrated optical density (IOD) expressed in pixels. To quantify the c-met protein expression levels the bands corresponding to c-met precursor and ß-chain forms were directly scanned. The expression percentage were the sum of the IOD of the bands correspondent to the precursor and the ß-chain forms of c-met protein. The amount of c-met protein in SKHep1C3 untransfected cells were considered as 100% (29).

Proliferation assay. The tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES, Promega) were used to perform a growth curve to assess the effect of c-met inhibition on cell proliferation. Cells were plated in triplicate in 96-well plates at a density of $2x10^3$ cells/well in cultured medium. Then, every 24 h for 3 days, 20 μl of sterile MTS dye was added and the plates were incubated at 37 ˚C. After 4-h spectrometric absorbance at 490 nm was measured using a microplate reader. The absorbance values at 490 nm was directly proportional to the number of living cells in the culture. Each group contained 3 wells.

Table I. Comparison of inhibition obtained by RNAi and antisense RNA technologies.

	% of inhibition				
	c-met mRNA	c-met protein	Migration	Invasion	Proliferation
AS RNA c-met	42	50	47	47	0
siRNA c-met_2	67[a]	76[a]	nd	nd	nd
siRNA c-met_5	22[a]	67[a]	nd	nd	nd
shRNA c-met	69	54/50[b]	61	41	37[a]

[a]Maximum inhibition obtained. [b]p-met. nd, not detected.

Invasion and migration assays. Invasion and motility assays were performed in a 24-well transwell chamber (Costar, Bodenheim, Germany). The 8-μm pore inserts were coated with 15 μg of Matrigel (Becton Dickinson Labware, Bedford, MA, USA). Cells were added to coated filters ($2x10^5$ cells/filter) in 100 μl of serum-free medium in triplicate wells. In the lower compartments of the chambers, 600 μl of AB5 human fibroblast-serum free-CM was used as a chemo-attractant.

After 24 h at 37˚C in a 5% CO_2 incubator the Matrigel coating on the upper surface of the filter was wiped off using a cotton swab. Cells that migrated to the filters were fixed, stained with Hema-3, photographed and counted.

The motility assay was conducted in a similar fashion without Matrigel coating. Cells ($1x10^5$/filter) were loaded on transwell polycarbonate membrane inserts in triplicate wells. The plates were incubated for 24 h at 37˚C in a 5% CO_2 incubator. The cells migrating to the lower compartment of the chambers were trypsinized and counted. Each sample was tested in triplicate.

Statistical analysis. Each experiment was carried out twice. Histograms represent mean values, bars indicate SEM. The statistical significance of the results was determined using t-test comparing pH shRNA c-met C3 or AS c-met cells with SKHep1C3 untransfected cells. Data were considered to be significant when $p \leq 0.05$.

Results

Inhibition of c-met by stable expression of antisense sequences. SKHep1C3 cells were transfected with a vector

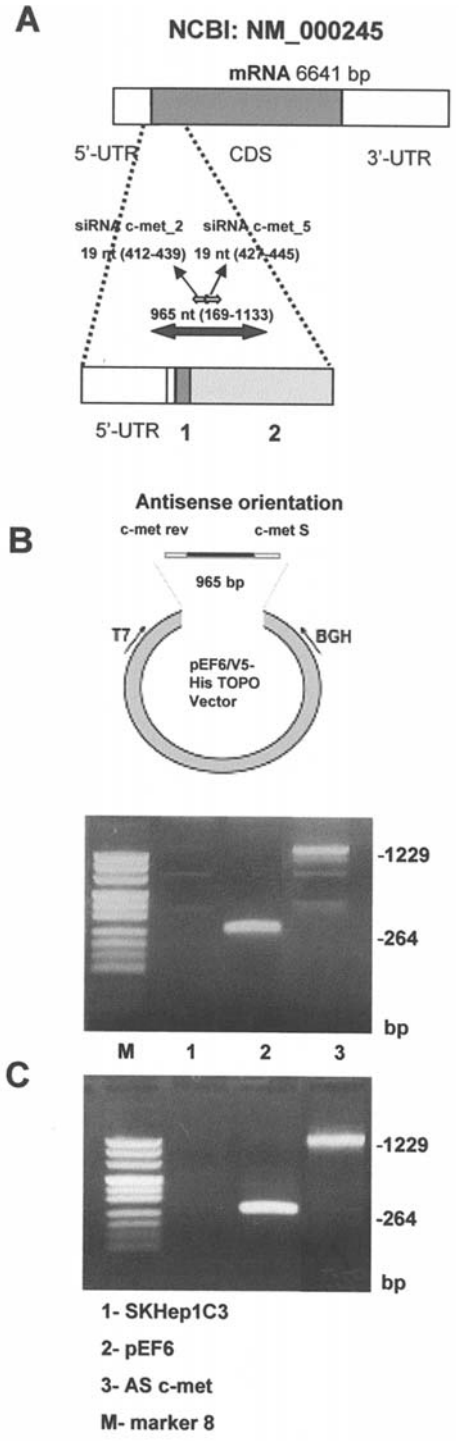

Figure 1. A, The target sequence positions for antisense and RNAi technologies against c-met mRNA are shown. B, The vector sequence is present into the genome of transfected cells. PCR analysis with plasmid-specific primers (T7, BGH) of genomic DNA from AS c-met and pEF6 cells reveals a specific band of 1229 bp (lane 3) and 264 bp (lane 2), respectively. C, RT-PCR analysis of the expression vector in an antisense orientation in non-transfected and transfected cells. Lanes 1-3, RT-PCR products obtained by amplifying cDNA from SKHep1C3 and transfected cells with the expression vector (pEF6; 264 bp) and with the anti-sense construct (AS c-met; 1229 bp) using T7 and BGH primers.

coding a fragment of 965 nt complementary to 5' portion of c-met mRNA (Fig. 1A). The presence of plasmid DNA in the genome of transfected cells was verified by PCR using primer pairs flanking the insert region. The pEF6 control cells showed a specific band of 264 bp, while the antisense transfected cells showed a band of 1229 bp due to the anti-sense sequence (Fig. 1B). Similarly the expression of antisense constructs by RT-PCR into transfected cells was also verified (Fig. 1C). The level of endogenous c-met mRNA was inhibited by 42% compared with control cells (Fig. 2A). The Western blot analysis, showed that in AS c-met cells, c-met expression decreased of about 50% compared to untransfected and pEF6 cells. The analysis performed under reducing conditions showed a decrease of both precursor and ß-chain forms of endogenous c-met (170 and 145 kDa respectively) (Fig. 2B).

No changes were found in the level of GAPDH expression as shown by the intensity levels of the bands ($M_r \cong 36$ kDa) (Fig. 2B). The silencing of c-met appeared to be specific since the PKR/eIF2α pathways were not activated as shown in Fig. 2C by Western blot analysis of PKR, phospho-PKR, eIF2α and phospho-eIF2α. The PKR, P-PKR and eIF2α levels were comparable in all samples and P- eIF2α forms were not expressed.

In order to determine whether the down-modulation of c-met affected malignant properties of the transfected cells, their migration, invasion and proliferation capabilities were analyzed. The migration assay evidenced a reduction in migratory capability of about 47% ($p<0.05$; Fig. 3A) in the AS c-met cells. These cells also showed a 47% reduction in invasion capability (Fig. 3B), but did not exhibit any difference on proliferation as compared with control cells (Fig. 3C).

Transient inhibition of c-met by siRNAs. To down-modulate c-met by RNAi, two siRNAs sequences were transiently transfected in SKHep1C3 cells. The sequences chosen according to recommendations for siRNAs design and the prediction of favourable target regions were called: siRNA c-met_2 and siRNA c-met_5 (Fig. 1A). The level of c-met mRNA was tested by RT-PCR after 48 and 72 h after the transfection. The siRNA c-met_2 transfected cells showed at 72 h an 82% inhibition of c-met mRNA compared to controls (Fig. 4A) while the sequence siRNA c-met_5 did not affect the expression level of c-met mRNA. The levels of L7 mRNA (Fig. 4A) were comparable in all the samples examined. On the contrary, the level of c-met proteins was inhibited at comparable levels by siRNA c-met_2 and siRNA c-met_5 as detected by Western blot analysis at 48 h (76 and 67% respectively) and at 72 h (60% for siRNA c-met_5 and 48% for siRNA c-met_2) (Fig. 4B). The protein levels of GAPDH did not change at any of these times (Fig. 4B). Based on these results the siRNA c-met_2 sequence was used for stable transfection experiments.

Inhibition of c-met by stable expression of shRNAs. SKHep1C3 were stable transfected with plasmid vector that expressed shRNAs for c-met under the control of an RNA polymerase III human U6 promoter. The plasmid coded shRNAs targeting nt 412-430 of c-met mRNA (NCBI access no. NM_000245), correspondent to siRNA c-met_2 used in transient transfection studies. The down-modulation of the c-met target gene was examined at mRNA and protein levels (Fig. 5A and B). A 69% reduction of c-met mRNA was detected by RT-PCR in pH shRNA c-met C3 transfected

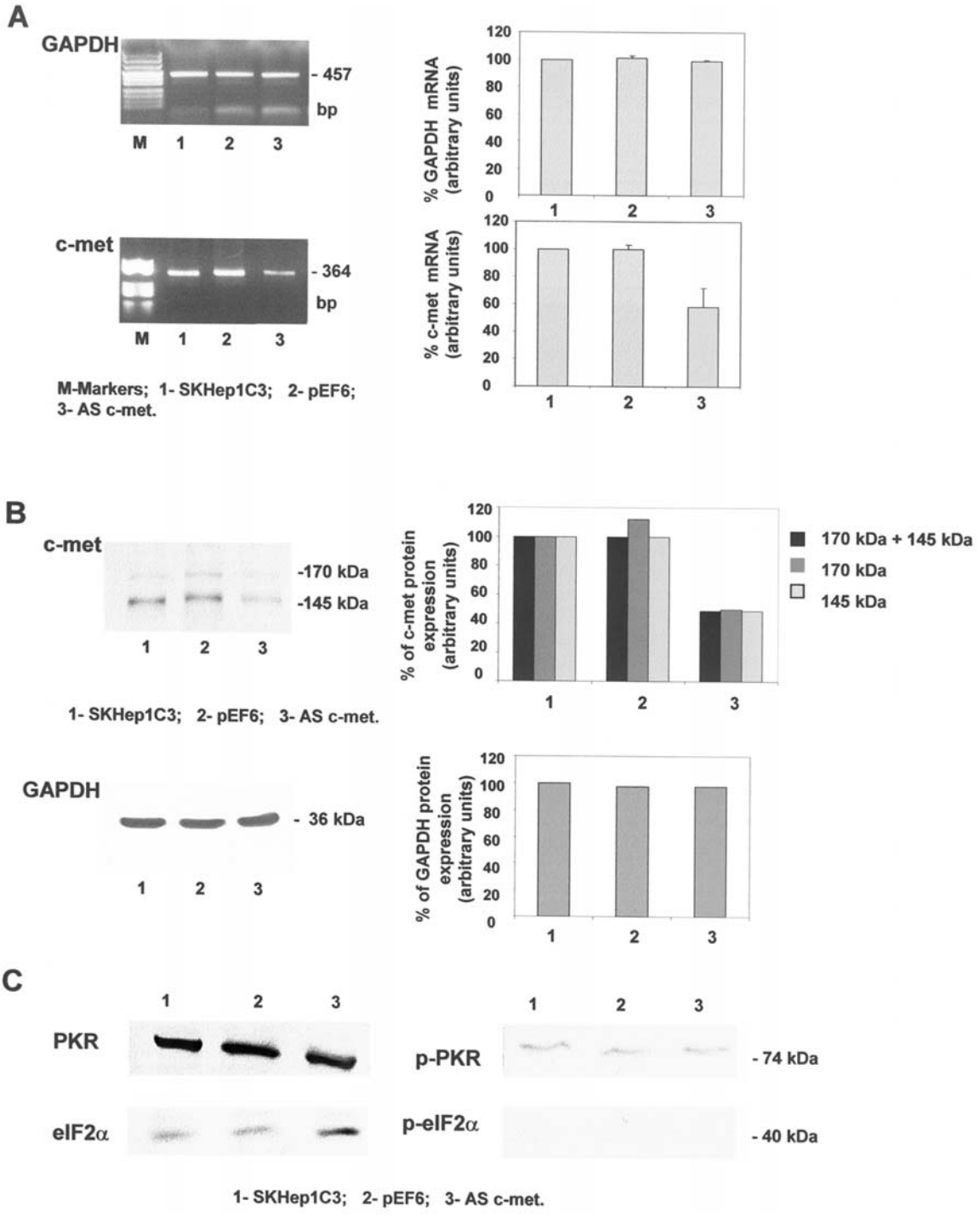

Figure 2. A, RT-PCR detection of c-met and GAPDH in SKHep1C3 and transfected cells (pEF6 and AS c-met). The amount of c-met and GAPDH (364 and 457 bp) was evaluated using a digital Gel-Pro Analyzer 3.1, and the relative percentages of c-met mRNA values were calculated as reported in Materials and methods. c-met expression level of AS c-met cells (lane 3) was 58% (lane 1): showed a c-met mRNA inhibition of 42% compared to SKHep1C3 cells. B, Western blot analysis of cellular extracts of SKHep1C3 transfected cells (pEF6 and AS c-met) and untransfected cells, to determinate the % of c-met and GAPDH protein levels (as reported in Materials and methods). In AS c-met transfected cells there was a c-met total protein inhibition of about 50%, in particular c-met precursor of 170 kDa was inhibited by 50%, while ß-subunit, recognised by polyclonal antibody, was inhibited by 51%. C, Detection of PKR, phospho-PKR, eIF2α, phospho-eIF2α, in cellular extracts of SKHep1C3, pEF6 and AS c-met cells, verified by Western blotting. The PKR/eIF2α pathway was not activated in the transfected cells.

cells compared to untransfected cells; no changes were found for L7 mRNA (Fig. 5A).

The Western blot analysis showed a 54 and 50% inhibition of c-met and p-met (Y1003) protein respectively in pH shRNA c-met C3 cells as compared to controls (Fig. 5B). The GAPDH protein levels did not change in the three cell lines analysed (Fig. 5C). The malignant properties of the cells were reduced in pH shRNA c-met C3 as compared to parental and scrambled control. The proliferation inhibition of the pH shRNA c-met C3 cells was 37% ($p<0.05$), 26 and 26% ($p<0.05$), respectively at 24, 48 and 72 h after seeding (Fig. 6C). The migration and invasion properties of the pH shRNA c-met C3 cells

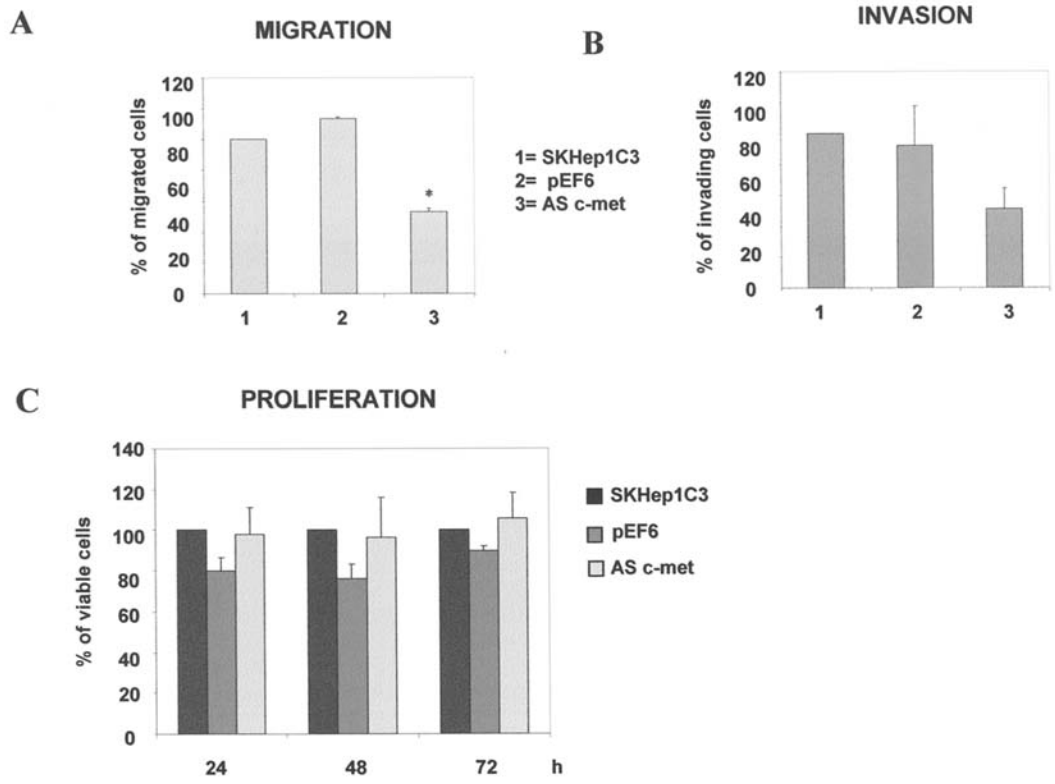

Figure 3. A, Cellular migration of stable transfected (pEF6 and AS c-met) and untransfected SKHep1C3 cells. Cellular migration was evaluated using a 24-well transwell chamber with 8-μm pore inserts. The values are the average % of migrated cells; *P≤0.05, a significant difference between AS c-met and and SKHep1C3 cells. The % of AS c-met cells migrated at T=24 h was 53%, i.e. an inhibition of 47%. B, Cellular invasion of stable transfected (pEF6 and AS c-met) and untransfected SKHep1C3 cells. The % of AS c-met invading cells at T=24 h is 53%, i.e. an inhibition of 47%. C, Proliferation assay of parental, vector and c-met antisense transfected cells. The percentage of proliferating cells at T=24, 48 and 72 h after seeding was calculated as reported in Materials and methods.

were affected by 61 ($p<0.05$) and 41% ($p<0.05$), respectively (Fig. 6A and B) suggesting a relevant role of c-met in the migration and invasion of SKHep1C3 cells.

Discussion

In this study the silencing of c-met expression was investigated in HCC-derived cells by plasmid vectors expressing c-met AS RNA or shRNA. Non-viral vectors were chosen given that some gene strategies for HCC therapy, which are part of phase I or II clinical trials, utilize plasmid vectors (30). The two new constructs developed in this study (AS RNA and pH shRNA c-met) with sequences coding c-met AS RNA and shRNA, not selected before, once transfected in SKHep1C3 cells, gave a comparable reduction of c-met protein, as well as inhibitory effects on migration and invasion, thus indicating an essential role of c-met in these functions of HCC cells. A 37% decrease of cell proliferation was observed in pH shRNA c-met C3 cells, but not in AS c-met transfected cells, although the level of c-met protein knock-down (50%) was comparable to that obtained by RNAi (54%). Therefore, it seems that in SKHep1C3 cells c-met inhibition is essential for the migratory and invasive abilities and less so for proliferation. This could be explained given that c-met, once activated, recruits several adaptors for signal transduction needed to initiate the complex process of invasive growth (31). Indeed specific biological responses are associated to defined pathways [i.e. PI(3)K alone is sufficient for cell motility, Ras pathway is both sufficient and necessary for cell proliferation] and the response can also be determined by the duration of c-met signaling (32). AS knock down of c-met, unlike RNAi, may not affect the complex biological pathway involved in cell proliferation control.

Concerning c-met abrogation, both c-met ablative strategies used in the present study led to a specific c-met knock-down; and in AS c-met transfected cells the lack of activation of PKR forms and the absence of phospho-eIF2α (tested by immunoblotting analysis) excluded a non-specific inhibition of translation via the PKR pathway (33). For the action mechanisms of gene silencing (25,34), the data obtained provide evidence for a contribution of an mRNA degradation pathway in the case of transient and stable c-met silencing by siRNA c-met_2 (c-met mRNA inhibition was 67 and 69% respectively); while siRNA c-met_5 probably induced target gene silencing mainly by translation impairment (c-met mRNA inhibition was only 22%).

In addition data reported on the decreased aggressiveness of HCC derived cells (i.e. inhibition of migration, invasion and proliferation) following a 50% c-met protein inhibition, are consistent with other reports in the literature. Indeed, a reduction of only 30% of c-met expression may be sufficient to decrease the malignant properties of tumour cells *in vitro* and to decrease also the ability to form tumours in nude mice (35). Further a comparable proliferation inhibition (38%) was

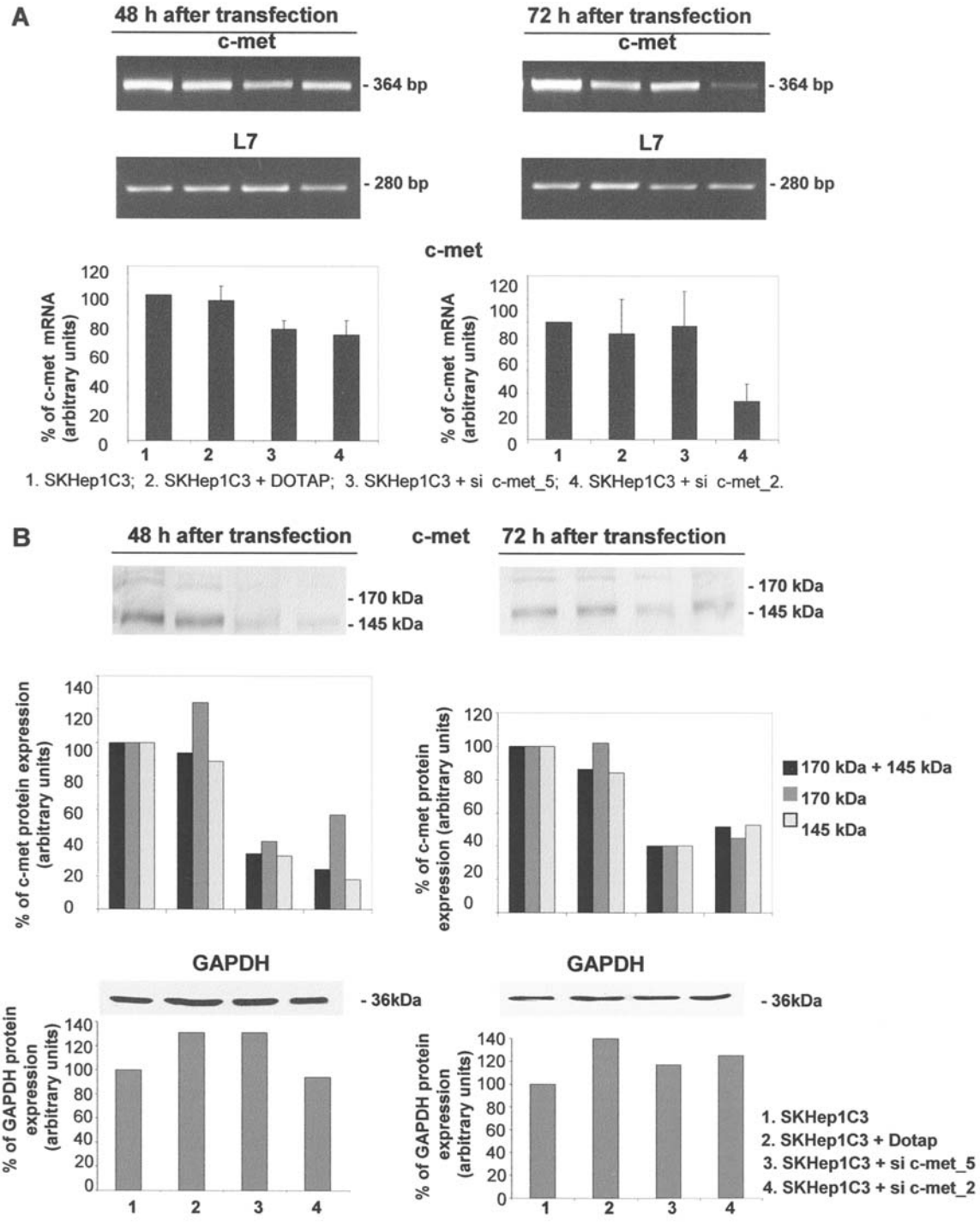

Figure 4. A, RT-PCR analysis of endogenous L7 house-keeping gene and c-met after 48 and 72 h from SKHep1C3 cells transfection with siRNA c-met_5 and siRNA c-met_2. The amount of c-met and L7 RT-PCR products (364 and 280 bp respectively) were evaluated using a digital image analysis system (Gel-Pro Analyzer 3.1) and the relative percentages of c-met mRNA values were calculated as reported in Materials and methods. SKHep1C3 siRNA c-met_2 transfected cells expressed 33% c-met RNA level at 72 h after transfection, i.e. an inhibition of 67% c-met mRNA. B, Western blotting conducted on cellular extracts to evaluate c-met and GAPDH protein levels in siRNA c-met_5 e siRNA c-met_2 SKHep1C3 transfected cells after 48 and 72 h from transfection. In siRNA c-met_2 transfected cells the inhibition of c-met at 48 h was 76%, in particular the 145 kDa ß-subunit was inhibited by 82%.

obtained with a 90% c-met ablation (by AdH1-siRNA/met in MHCC97-L cells; 36) indicating that even very high inhibition of c-met expression could impair proliferation to a certain extent in HCC cells (36) as well as tumour growth in nude mice by 61%.

In conclusion we have shown that the inhibition of endogenous c-met using AS RNA and shRNA technology reduces the motility, invasion and proliferation of HCC cells and c-met may be a potential therapeutic target for HCC in experimental animal models. The AS RNA results provide new support for HCC as well as the shRNA plasmid expression vector which may be an alternative *in vitro* strategy to abrogate c-met and to perform functional studies on the role of c-met in HCC and other tumour cells. Overexpression

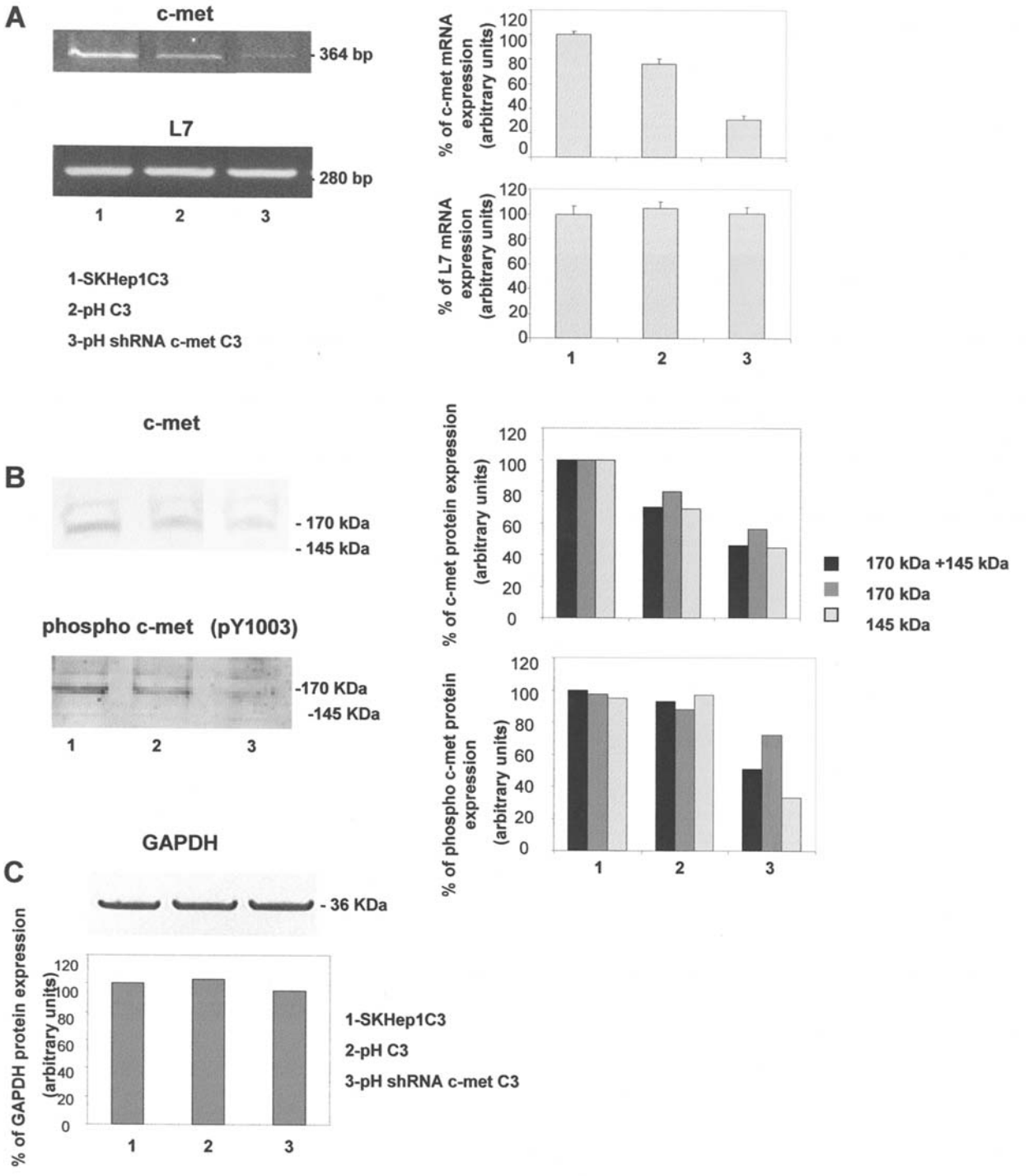

Figure 5. A, RT-PCR detection of c-met and L7 in SKHep1C3 and transfected cells (pHC3 and pH shRNA c-met C3). c-met expression level of pH shRNA c-met C3 (lane 3) was 31%: showing a c-met mRNA inhibition of 69% compared to SKHep1C3 cells. B, Western blot analysis of cellular extracts from SKHep1C3 transfected and untransfected cells. In pH shRNA c-met C3 there was a c-met total protein inhibition of about 54% and a p-met (Y1003) inhibition of about 50%. C, The three cell lines showed comparable amounts of GAPDH protein.

of c-met is a hallmark of human HCC pathogenesis (11-14) and causes the development and maintenance of HCC in transgenic mice overexpressing human c-met (37). Further attention should be focused on c-met targeting in HCC by ablative approaches, by c-met targeting drugs (like tyrosine kinase inhibitors) and by antibodies. As a novel strategy to control the aggressiveness of HCC cells it would be of interest to design a multi-target gene approach considering uPA RNAi, known to greatly decrease the malignant behavior of several types of cancer cells (38), *in vitro*, as well as HCC cells (27), and human xenografts in nude mice (29,38). The biological rationale of this design relies on the fact that u-PA and c-met are unfavorable prognostic factors for HCC and they might cooperate in the acquisition of malignant phenotype of cancer cells (14,39). Furthermore, since c-met may be a target for radio-sensitization (40,41), the cell

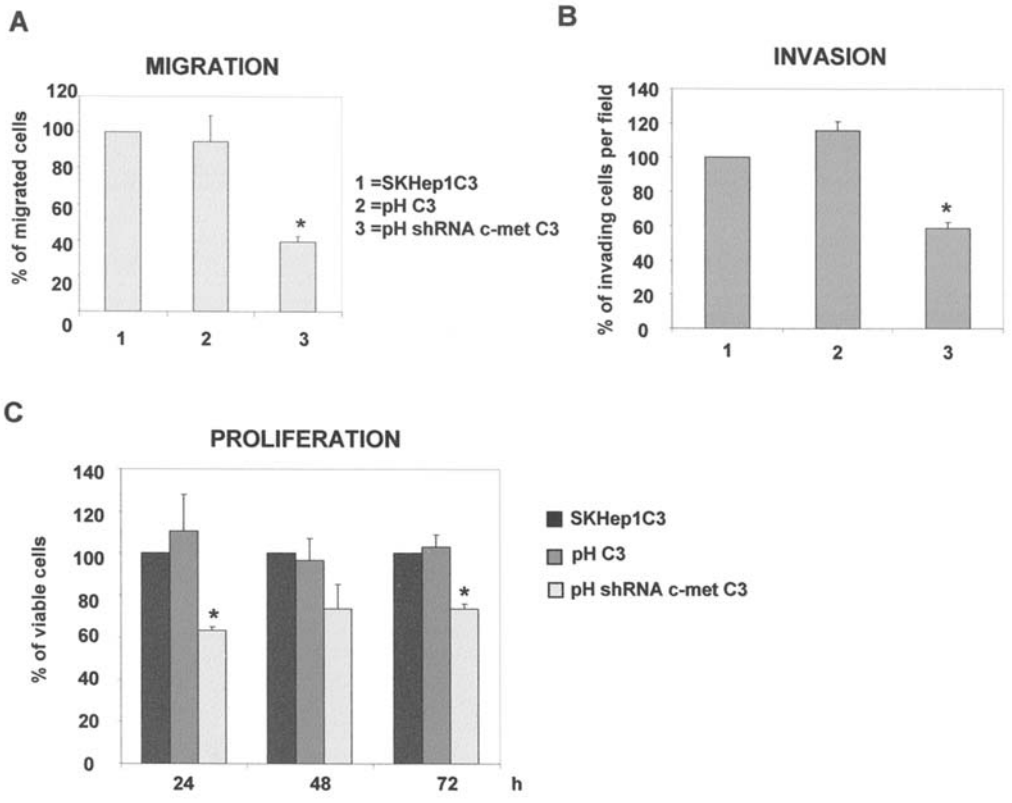

Figure 6. A, Cellular migration of stable transfected (pH C3 and pH shRNA c-met C3) and untransfected SKHep1C3 cells. Cellular migration was evaluated using 24-well transwell chamber with 8-μm pore inserts. The values were the average % of migrated cells; *P≤0.05, a significant difference between pH shRNA c-met C3 and and SKHep1C3 cells. The % of pH shRNA c-met C3 cells migrated at T=24 h is 39%, i.e. an inhibition of 61%. B, Cellular invasion of stable transfected and untransfected SKHep1C3 cells. The % of invading pH shRNA c-met C3 cells at T=24 h was 59%, i.e. an inhibition of 41% (p<0.05). C, Proliferation assay of transfected and untransfected cells. The percentage of inhibition of proliferative capability of pH shRNA c-met C3 cells were 37, 26 and 26%, respectively at T=24, 48 and 72 h after seeding.

experimental models set up in our study could be used to verify whether c-met ablation influences the radio-resistance of HCC cells.

Finally our findings agree with the concept that down-modulation of c-met may effectively reduce the malignant properties of HCC derived cells. These results could help to design and test one/two target shRNA expressing plasmid constructs as anti-invasive therapeutic strategies *in vitro* and *in vivo* in experimental animal models, also combined with conventional chemo- and radio-therapies.

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