Roles of heterogeneous nuclear ribonucleoproteins A and B in cell proliferation

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Summary

Overexpression of heterogeneous nuclear ribonucleoproteins (hnRNPs) A2 and B1 has been observed in a variety of tumour types, however, it is unknown whether this dysregulation is a consequence of, or a driving force for, unregulated cell proliferation. We have shown that the levels of hnRNPs A1, A2 and B1, but not A3, are modulated during the cell cycle of Colo16 squamous carcinoma cells and HaCaT immortalized keratinocytes, suggesting that A1, A2 and B1 are needed at particular cell cycle stages. However, the levels of hnRNP A1, A2 and B1 mRNAs were constant, indicating that regulation of protein levels was controlled at the level of translation. RNAi suppression of hnRNP A1 or A3 alone did not affect the proliferation of Colo16 cells but the proliferation rate was significantly reduced when both were suppressed simultaneously, or when either was suppressed together with hnRNP A2. Reducing hnRNP A2 expression in Colo16 and HaCaT cells by RNAi led to a non-apoptotic-related decrease in cell proliferation, reinforcing the view that this protein is required for cell proliferation. Suppression of hnRNP A2 in Colo16 cells was associated with increased p21 levels but p53 levels remained unchanged. In addition, expression of BRCA1 was downregulated, at both mRNA and protein levels. The observed effects of hnRNP A2 and its isoforms on cell proliferation and their correlation with BRCA1 and p21 expression suggest that these hnRNP proteins play a role in cell proliferation.

Key words: hnRNP, Cell cycle dependence, p53, BRCA1, p21

Introduction

Heterogeneous nuclear ribonucleoprotein particle proteins (hnRNPs) are abundant, multi-tasking proteins that play a central role in RNA metabolism. They are involved in packaging nascent hnRNA, alternative RNA splicing, mRNA export from the nucleus, and cytoplasmic trafficking, stability and translation (Dreyfuss et al., 2002; Krecic and Swanson, 1999; Shyu and Wilkinson, 2000; Weighardt et al., 1996). They also have an as yet poorly defined role in telomere maintenance.

More than 20 hnRNPs, many of which are characterised by possession of RNA-recognition motifs (RRMs), have been identified. The hnRNPs A/B are the major components of the 40S particles that package hnRNA. This packaging was originally envisaged to mimic that of histones in nucleosomes but this analogy appears to be inappropriate as the levels of the core proteins are not stoichiometric: they differ markedly between cell types, and their association with RNA is dependent on the nucleic acid sequence (Dreyfuss et al., 1993). However, given the role of these core particles in hnRNA packaging one might anticipate a correlation between the abundance of their proteins and the transcriptional activity in the cell. This is borne out experimentally: there is a marked difference in the concentration of hnRNP A1 between resting or slowly dividing cells and rapidly dividing cells. In the latter, hnRNPs A1 and A2 have been proposed to be present at similar levels. But hnRNP A1, which is abundant in a range of human, hamster and mouse proliferating cells, is present at markedly lower levels in confluent or resting cells, whereas hnRNP A2 is less affected (Celis et al., 1986; LeStourgeon, 1978).

The levels of hnRNPs A/B differ not only between proliferating and resting cells: some also fluctuate during the cell cycle (Leser and Martin, 1987; Minoo et al., 1989). In HeLa cells hnRNPs A2 and B1 are synthesized in the G1 phase, and their levels fall in G2 and M phases, with hnRNP B1 protein level falling more markedly (Kamma et al., 2001). The relative and absolute levels of these two proteins also differ markedly between tissues, both being particularly abundant in rat brain, testis, lung, spleen and ovary (Kamma et al., 1999; Ma et al., 2002).

Many genes show a correlation between strong expression in proliferating cancer cells and the fluctuations in the protein level across the cell cycle (Dreyfuss et al., 1993; Whitfield et al., 2002). The levels of hnRNP A/B proteins are of particular interest as it has been suggested that upregulation of some members of this protein family is associated, as either a cause or consequence, with cellular proliferation and cancer. The hnRNP A2 and its longer B1 isoform are expressed at an early stage in a variety of tumours and have been proposed as early markers for cancer, especially lung cancer (Fielding et al., 1999; Mulshine et al., 2002; Pino et al., 2003; Sueoka et al., 1999; Whitfield et al., 2002; Zhou et al., 1996) and possibly breast cancer (Zhou et al., 2001a). The upregulation of hnRNP A2/B1 in cancer parallels its expression in lung development, supporting the view that it is an oncodevelopmental protein (Montuenga et al., 1998). Levels of hnRNP A1 are also

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elevated in some cancers, including oligodendrogliomas (Xu et al., 2001).

We report here studies of hnRNP A1, A2 and A3 expression in a range of normal and transformed cells. Until recently, the published components of hnRNP core particles excluded hnRNP A3 even though it is very closely related to hnRNPs A1 and A2 (Percipalle et al., 2002; Rappsilber et al., 2002). The tandem RRMs of hnRNP A3 have a higher sequence identity than hnRNP A2 with hnRNP A1, and its glycine-rich region more closely matches hnRNP A2 than hnRNP A1 (Ma et al., 2002). Moreover, hnRNP A3 binding to single-stranded oligoribonucleotides parallels that of hnRNP A2, reinforcing the close relationship between these two paralogues. The levels of these three proteins, however, varied markedly between cell types. The expression of hnRNP A2 and to a lesser extent A1, but not their mRNAs, fluctuated during the cell cycle, peaking during S phase, declining in G2 and M phases, and being restored in late G1. By contrast, hnRNP A3 levels remained constant. In general, hnRNPs A2/B1 are overexpressed in various epithelial cancer cells when compared with normal cells, but some cancers showed reduced expression of hnRNP B1 and overexpression of hnRNP A3. Suppression of hnRNP A2, but not A1 or A3, with shRNA treatment lowered the growth rate of HaCaT and Colo16 cells, whereas simultaneous downregulation of any two of the three genes had a more marked effect on cell growth. Suppression of the hnRNP A2/B1 gene did not affect the level of p53, which has been shown to be dysregulated in most cancer cells, but was accompanied by downregulation of BRCA1 and upregulation of p21, proteins that are closely linked to cell growth.

Materials and Methods

Cell culture

Colo16 cells were grown in RPMI 1640 medium (Gibco, Mount Waverley, Australia) supplemented with 10% fetal bovine serum (FBS, Gibco). HaCaT and HeLa cells were grown in DMEM (Gibco) supplemented with 10% FBS. SCC25 cells were grown in DMEM-F12 (Gibco) supplemented with 10% FBS and 0.4 μg/ml hydrocortisol (Sigma, Sydney, Australia). A549 cells were grown in Ham's F12K medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine and 1.5 g/l sodium bicarbonate. MCF10a cells were grown in DMEM-F12 supplemented with 10% horse serum (Invitrogen, Mount Waverley, Australia), 10 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisol and 20 ng/ml epidermal growth factor (EGF, Sigma). MCF7 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS, Gibco), 1 mM sodium pyruvate, 10 μg/ml insulin and 0.1 mM non-essential amino acids. T-47D cells were grown in RPMI 1640 medium supplemented with 10% FBS, 10 μg/ml insulin, 1 mM sodium pyruvate, and 2 mM L-glutamine. RPMEI cells were grown in keratinocyte-SFM (Invitrogen) supplemented with 10% FCS. LNCAP cells were grown in RPMI 1640 medium supplemented with 10% FCS. Penicillin and streptomycin (Gibco) were used in all the cultures at 100 U/ml and 10 μg/ml gentamicin (Gibco) was used in SCC25 and Colo16 cell cultures.

Antibodies

Rabbit polyclonal antibodies against peptides unique to hnRNPs A1, A2, B1 and A3 were raised in our laboratory. The synthetic peptides, SKSESPKEPEQLC for A1, GGNFGFGDSRGC for A2, VKPPPGRPQPDSC for A3 and KTLETVPLERKKC for B1 were conjugated to diphtheria toxin before injection into rabbits. The A1 antibody binds A1 and the minor $A1^B$ isoform (the latter not visible on Fig. 1B); the A2 antibody detects A2 and its B1 isoform and the A3 antibody recognizes all four isoforms, which are unresolved on the short gels used in these experiments. In immunofluorescence studies, a mouse monoclonal antibody against α-tubulin (Sigma; used at 1:100 dilution) was used to counterstain the cell cytoskeleton, together with antibodies against hnRNPs A1, A2, B1 or A3. The secondary antibodies included FITC-conjugated goat anti-rabbit IgG (Sigma; 1:50 dilution) and Cy3-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA; 1:300 dilution). For western blotting, mouse monoclonal antibodies were used to detect BRCA1 (Oncogene, Boston, MA; 1:500 dilution), p53 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000 dilution), p21 (Oncogene; 1:2000 dilution), and GAPDH (Chemicon International, Boronia, Australia; 1:50,000 dilution). The secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma; 1:5000 dilution) and HRP-conjugated rabbit anti-mouse IgG (Sigma; 1:5000 dilution).

Synchronization of Colo16 and HaCaT cells

A double thymidine block was used to synchronize cells for cell cycle studies. Briefly, Colo16 or HaCaT cells were plated onto 24-well plates and cultured overnight. After 16 hours, cell growth was arrested at the G1-S phase transition by two subsequent 2 mM thymidine (Sigma) blocks for 17 hours, separated by a period of 10 hours without thymidine. Cells were sampled once an hour after being released from the second thymidine arrest to determine when S, G2, M and G1 phases occurred by flow cytometry on a FACScalibur system (BD Biosciences, Palo Alto, CA).

Immunocytochemistry

Cells cultured on coverslips were collected at S, G2, M and G1 phases, washed twice in chilled phosphate-buffered saline (PBS, pH 7.0), fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature and rinsed twice with cold PBS prior to immediate staining or storage at 4°C. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, rinsed twice with PBS, then incubated in 50 mM ammonium chloride in PBS for 10 minutes before washing again with PBS. Following a 30-minute block in 0.2% bovine serum albumin (BSA) in PBS, cells were incubated in the primary antibody diluted in PBS containing 0.2% BSA for 1 hour at 37°C and then washed four times with PBS. The cells were incubated with secondary antibodies diluted in PBS containing 0.2% BSA for 30 minutes in the dark. Finally, coverslips were mounted with Vectashield containing 4′,6-diamidino-2-phenylindole hydrochloride (DAPI) (Vector Laboratories, Burlingame, CA) before being visualized with BioRad Radiance 2000MP confocal and multiphoton microscope (BioRad, Hercules, CA).

Western blotting

Cells were lysed in a total protein extraction buffer (Hoek et al., 1998). Equal amounts of protein (20 μg/lane for detection of hnRNPs A1, A2 and A3, and 30 μg/lane for B1) were separated on SDS/12% polyacrylamide gels (Gradipore, Sydney, Australia) and transferred onto polyvinylidene difluoride membranes (Millipore, Sydney, Australia). The membrane was then incubated overnight at 4°C in a blocking buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, 0.1% Tween-20 and 1% skimmed milk powder). The membrane was probed with antibodies against hnRNP A/B proteins in the blocking buffer for 1 hour at room temperature, washed four times with the blocking buffer mixed with 0.5% BSA, incubated in blocking buffer containing an HRP-conjugated goat anti-mouse antibody for 45 minutes and washed again. The target proteins were detected with the

ECL-PLUS western blotting detection system (Amersham Biosciences, Buckinghamshire, UK) and recorded on X-ray film. The membrane was then stripped in 62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol and 2% SDS in a 60°C oven, followed by a thorough wash with 0.1% Tween-20 in PBS. GAPDH on the stripped membrane was detected using a mouse anti-GAPDH monoclonal antibody. Band intensities were assessed using an imaging densitometer to determine the relative abundance of the proteins, using GAPDH as a loading control. To detect BRCA1, 100 μg/lane of total protein was separated on an SDS/5% polyacrylamide gel. Western blotting for BRCA1 was performed using a mouse anti-BRCA1 monoclonal antibody buffered with 5% skimmed milk powder in PBS containing 0.15% Tween-20. The mouse monoclonal antibody against p53 and p21 was diluted in 5% skimmed milk powder/PBS and 5% BSA, respectively.

Northern blotting

Total RNA was isolated using TRIzol reagent (Invitrogen), separated on 1.2% agarose/formaldehyde gels and transferred onto nylon membranes (Amersham Biosciences). The probes for *BRCA1* and *p21* were labelled by random-primer synthesis with Klenow DNA polymerase in the presence of [^{32}P]dCTP. A p21 cDNA fragment from 1653 to 1856 bases downstream of the starting codon, and the first 1 kb of the coding region of BRCA1 mRNA were used as templates. Hybridization was performed in Rapid-hyb buffer (Amersham Biosciences) and the signals were visualized by autoradiography. Signals of the mRNAs of interest were normalized to the levels of β-actin mRNA.

Real-time PCR

Total RNA isolated by TRIzol reagent was reverse-transcribed to produce cDNA using Superscript III (Invitrogen). mRNAs of interest were quantified by SYBR green real-time PCR on an ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using β-actin mRNA as a normalization control. The specific primers (Proligo, Lismore, Australia or GeneWorks, Adelaide, Australia) were: hnRNP A1 forward, 5′-GGAGAAGCCATTGTCTTCGGA-3′ and reverse, 5′-GCATAGGATGTGCCAACAATCA-3′; hnRNP A2 forward, 5′-GAGTCCGCGATGGAGAGAGA-3′ and reverse, 5′-GATCCCTCATTACCACACAGTCTGT-3′; hnRNP A3 forward, 5′-CATAGAAGTTATGGAAGACAGGCAGAG-3′ and reverse, 5′-GGCCTTTTTCACTTCACAATTATGC-3′; hnRNP B1 forward, 5′-GGAGAAAACTTTAGAAACTGTTCCTTTG-3′ and reverse, 5′-GCTTTCCCCATTGTTCGTAGTAGT-3′; β-actin forward, 5′-CGTTACACCCTTTCTTGACAAAACC-3′ and reverse, 5′-GCTGTCACCTTCACCGTTCCA-3′; p21 forward, 5′-TAGCAGCGGAACAAGGAGTCA-3′ and reverse, 5′-GCCAGTATGTTACAGGAGCTGGAA-3′.

shRNA and transfection

The selected shRNA sequences targeting hnRNP A1, A2 and A3 mRNAs were 5′-AGCAAGAGATGGCTAGTGC-3′, 5′-CGTGCTGTAGCAAGAGAGG-3′ and 5′-AGAGAGCTGTTTCTAGAGA-3′, respectively. BLAST alignment showed no sequence identity with other transcripts of human genes. An oligonucleotide (5′-CGTACGCGGAATACTTCGA-3′) targeting firefly luciferase mRNA was used as a negative control. The synthesized oligonucleotides (GeneWorks) were annealed and inserted into pSUPER plasmids following established protocols (Brummelkamp et al., 2002). For transfection, exponentially growing cells were seeded into a six-well plate at a density of 360,000 cells/well and cultured for 20 hours before the medium was replaced with OptiMEM with 5% FBS. shRNA plasmids (3.0 μg) were mixed with 7.5 μl Lipofectamine 2000 (Invitrogen) and incubated at room temperature for 20 minutes before being applied to cells. The medium was changed back to RPMI 1640 with 10% FBS 6 hours after transfection, and cells were cultured for another 66 hours before harvesting, unless otherwise specified.

MTT proliferation assay

Cells were trypsinized 48 hours after transfection, counted and re-plated into 96-well plates at 1500 cells/well (Time 0 started at this point). After being re-plated for 3 hours, cells were sampled at 6-hour intervals to perform an MTT proliferation assay. For each time point, six wells were used for each treatment; 10 μl methylthiazoletetrazolium (MTT; Sigma; 5 mg/ml in PBS) was added to three and an equal volume of PBS to the other three as a control. The cells were cultured in the presence or absence of MTT for another 3 hours and lysed in an SDS-HCl solubilization buffer (10% SDS in 0.01 M HCl) for 18 hours at 37°C before measuring the absorbance at 570 nm. The experiment was repeated three times for each cell line.

TUNEL assay

Cell apoptosis was determined with an in situ cell death detection kit (Roche Diagnostics Australia, Castle Hill, Australia) following the manufacturer's protocol.

Results

Expression of hnRNPs A1, A2 and B1, but not A3, is modulated during the cell cycle

hnRNPs A2 and B1 are more abundant in G1 and S phases, than in G2 and M of HeLa cells (Kamma et al., 2001). Considering the high sequence identity among hnRNPs A1, A2 and A3 and their isoforms, it was anticipated that other members of this subfamily could also be expressed in a cell-cycle-dependent manner. Therefore, we compared the levels of hnRNPs A1, A2, B1 and A3 in Colo16, a squamous cancer cell line, at different cell cycle stages. An immortalized keratinocyte cell line, HaCaT was also studied to determine whether the two cell lines share a similar hnRNP A/B expression profile.

Lysates were prepared from synchronously growing Colo16 and HaCaT cells harvested at intervals after being released from a double thymidine block. The progression through the cell cycle was determined by flow cytometry. The S, G2, M and G1 phases of Colo16 cells started at 0, 7, 8 and 10 hours post-synchronization, respectively, whereas HaCaT cells entered G2, M, and G1 phases 1 hour later than Colo16 cells (Fig. 1A). Proteins were isolated from cell lysates, separated by SDS/polyacrylamide gel electrophoresis and immunoblotted using antibodies raised against peptides from hnRNPs A1, A2, B1 and A3. The specificities of these antibodies were confirmed by western blotting (Fig. 1B). hnRNPs A1, A2 and B1 all manifested cell cycle-specific changes though their profiles differed (Fig. 1C). The amount of hnRNP A1 was relatively stable during most of the cell cycle except for a clear decrease in early G1 phases for both cell lines, whereas hnRNP B1 expression dropped markedly during the short period from G2 to M phases, remained low in early G1 and slowly increased from late G1. In contrast to a previous observation in HeLa cells (Kamma et al., 2001), a dramatic change of hnRNP A2 expression was detected in both cell lines, though Colo16 cells expressed the lowest level of hnRNP

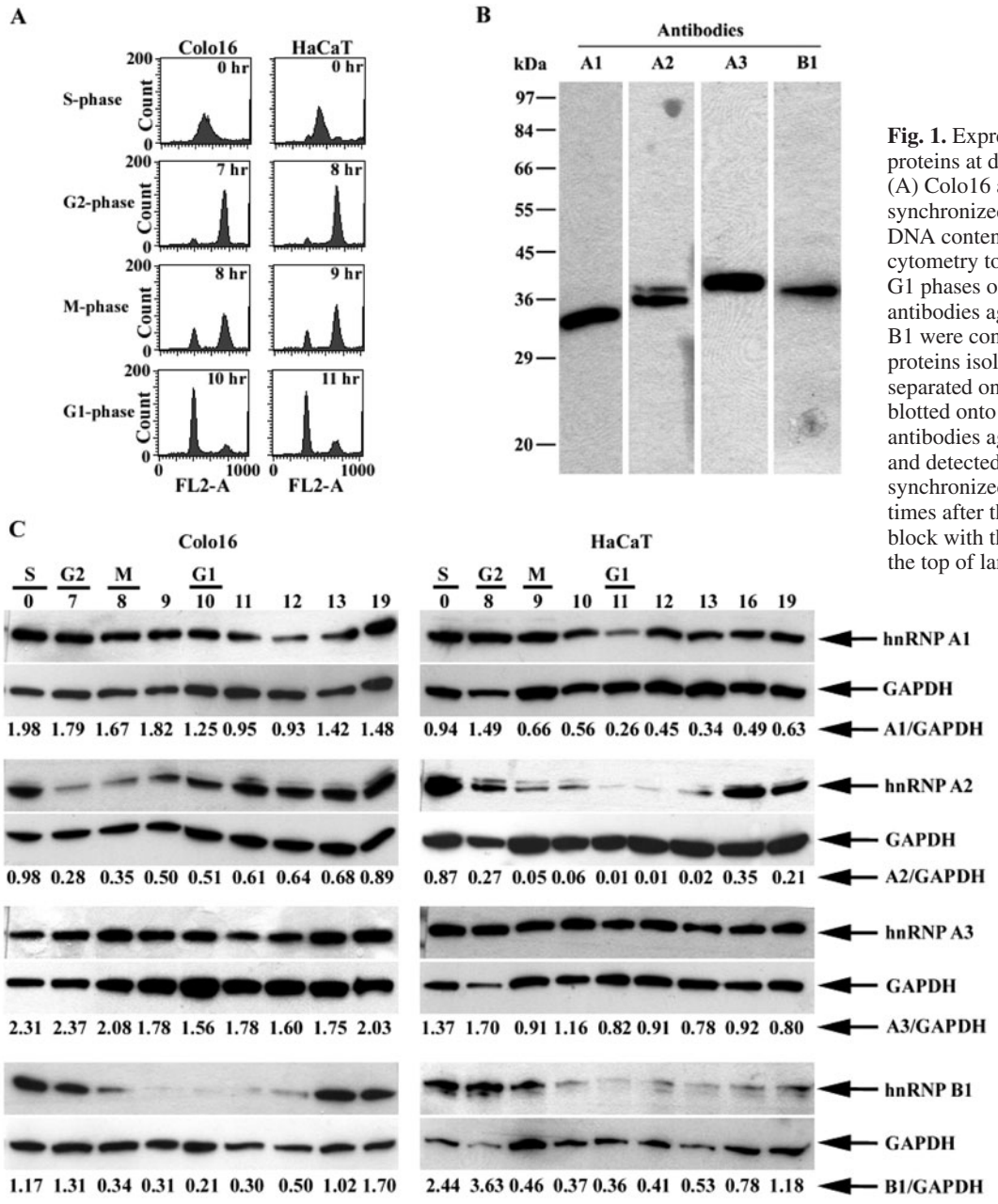

Fig. 1. Expression of main hnRNP A/B proteins at different cell cycle stages. (A) Colo16 and HaCaT cells were synchronized by double thymidine block and DNA contents were analysed by flow cytometry to determine when S, G2, M and G1 phases occur. (B) Specificities of antibodies against hnRNPs A1, A2, A3 and B1 were confirmed by western blotting. Total proteins isolated from HaCaT cells were separated on SDS/12% polyacrylamide gels, blotted onto PVDF membrane, probed with antibodies against hnRNP A1, A2, A3, or B1 and detected with ECL-Plus. (C) The synchronized cells were collected at different times after the release of second thymidine block with the sampling times indicated at the top of lanes. For Colo16 cells, S, G2, M and G1 phase started at 0, 7, 8 and 10 hours after synchronization, respectively, whereas HaCaT cells entered G2, M and G1 phases 1 hour later. Levels of hnRNP A/B proteins were determined by western blotting following the same protocol as above. The membranes were then stripped, and re-probed with a mouse anti-GAPDH antibody to ensure a same loading ratio of samples. Band intensities were measured by densitometry to assess the relative abundance of the target protein against GAPDH, which was indicated under the lanes. The experiments were performed in duplicate.

A2 in G2 phase, and HaCaT cells in G1. No change was detected in the hnRNP A3 protein level at different cell division stages (Fig. 1C).

In view of the significant fluctuation of hnRNP A2 and B1 proteins in different cell cycle stages, we also investigated the localization of these proteins in the nucleus and cytoplasm. Colo16 cells collected at S, G2, M and G1 phases were immunostained with the antibody that recognizes hnRNPs A2 and B1. The predominantly nuclear localization for both proteins was not significantly different in S, G2 and G1 phases (Fig. 2). During M phase, the proteins were almost uniformly distributed in the cell body as a result of collapse of the nuclear envelope. Interestingly, hnRNPs A2 and B1 were found at lower levels around the chromosomes than other parts of M phase cells (Fig. 2).

The levels of hnRNP A1, A2 and A3 mRNAs are stable during the cell cycle

Our western blots revealed significant changes of hnRNP A1, A2 and B1 proteins during the cell cycle. Thus, we reasoned that mRNAs encoding these proteins might vary with cell cycle stage. In order to test this possibility, real-time PCR was performed to quantify the abundance of hnRNP A1, A2, B1 and A3 mRNAs in Colo16 and HaCaT cells harvested at S, G2, M and G1 phases, using β-actin cDNA as a normalization control. No apparent changes were detected (Fig. 3) for hnRNP A1, A2 and B1 mRNA in both cell lines even though their protein levels changed markedly (Fig. 1C). The hnRNP A3 mRNA level was also stable in Colo16 cells in correlation with its protein, but was significantly higher in HaCaT cells at G1 phase compared with S phase.

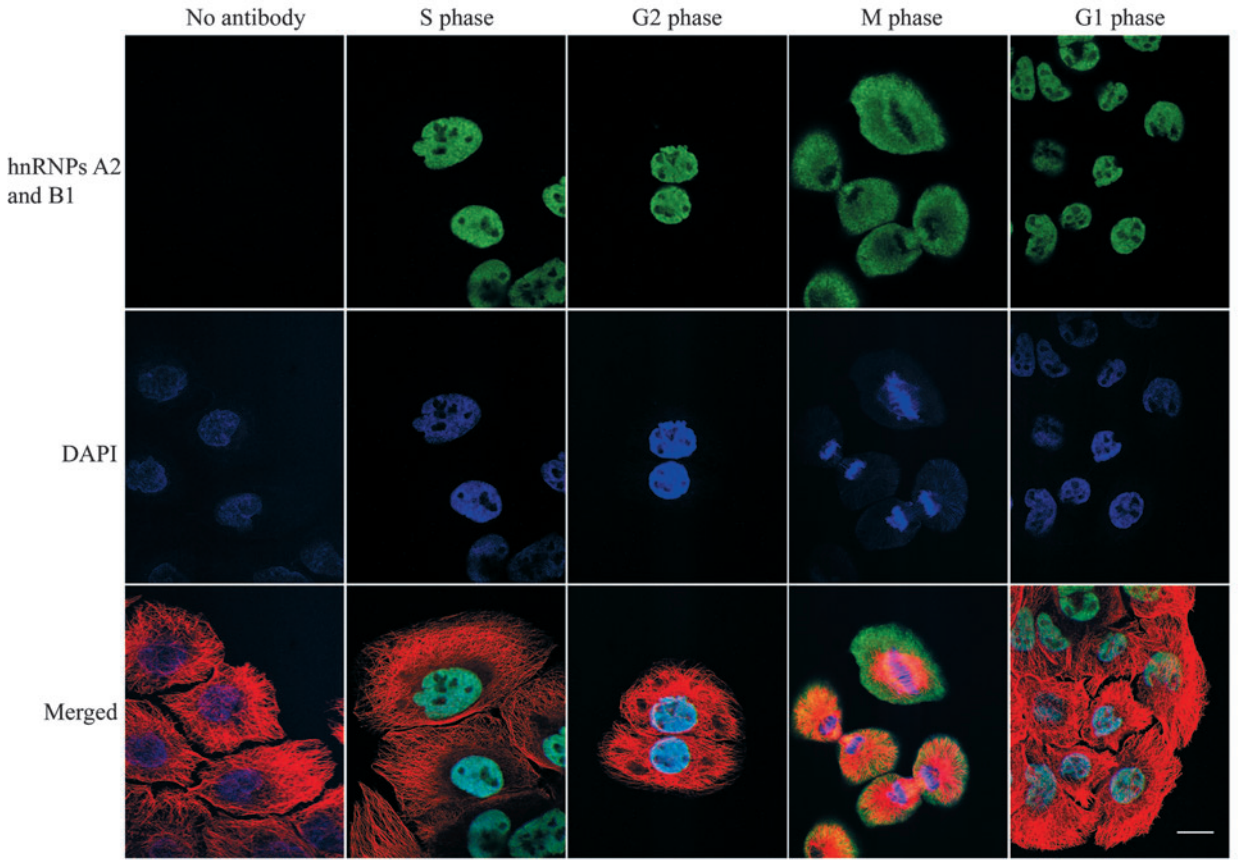

Fig. 2. Distribution of hnRNPs A2 and B1 in Colo16 cells at different cell cycle stages. Cells collected at S, G2, M and G1 phases were immunostained with a rabbit hnRNP A2 antibody, which also recognizes the B1 isoform, and visualized using a FITC-conjugated donkey secondary antibody. A monoclonal α-tubulin antibody raised in mouse was used to counterstain microtubules with a Cy3-conjugated goat secondary antibody (red in merged images). DAPI was used to stain DNA (blue). Bar, 10 μm.

Expression of hnRNPs A1, A2, B1 and A3 in cancer and normal cells

The levels of hnRNPs A2 and B1, or B1 alone in some cases, are elevated in lung, breast cancers and lymphomas (Sueoka et al., 1999; Sueoka et al., 2001; Tani et al., 2002; Zhou et al., 2001a). Nevertheless, controversy still exists on whether hnRNP B1 or both A2 and B1 are overexpressed in these tissues. We therefore assessed the amounts of hnRNP A/B proteins by western blotting in normal keratinocytes, and in cancer or immortalized cell lines. The expression patterns of hnRNPs A1, A2, B1 and A3 differed considerably between various cell lines, though all were epithelial in origin (Fig. 4). The breast cancer cell line, T-47D, expressed higher levels of all of the detected proteins compared with the immortalized cells, MCF10a, whereas another breast cell line, MCF7, showed only a higher hnRNP B1 level. Upregulation of hnRNP A2 or B1, or both, was detected in all cancer cell lines compared to normal keratinocytes or immortalized cells, whereas A1 was less abundant in MCF10a, MCF7 and two prostate cell lines. Higher levels of hnRNP A3 were found in Colo16, A549 and T-47D cells. Significantly higher levels of hnRNP B1, which is overexpressed in a variety of cancers (Sueoka et al., 1999; Sueoka et al., 2001; Tani et al., 2002), were detected in Colo16, A549, T-47D and RPMEI cells.

Quantification of mRNA levels of hnRNPs A/B by real-time PCR showed increases of 121- and 80-fold in hnRNP A2 and B1 mRNAs in Colo16 cells, respectively, compared to the cultured keratinocytes isolated from normal foreskin (Fig. 5). The increase of mRNA was consistent with the significant increase of A2 and B1 proteins in these cells (Fig. 4). The level of hnRNP A2 mRNA in HeLa cells also correlated well with western blotting analyses, which showed overexpression of hnRNP A2 protein. A moderate increase of hnRNP A3 mRNA in Colo16 and HeLa cells compared with primary keratinocytes confirmed our western blot findings for hnRNP A3 (Figs 4, 5). Intriguingly, the level of hnRNP A1 mRNA in Colo16 cells was 18 times greater than that in keratinocytes, whereas the amount of A1 protein was comparable in both. Except for *hnRNP A3*, both cell lines examined manifested very high gene transcription levels compared with the primary keratinocytes. The protein concentrations were also elevated, but not to the same extent. By contrast, the difference between A3 mRNA levels in cancer cells and keratinocytes was small and correlated well with the protein concentrations.

Suppression of hnRNP A2 expression slows cell proliferation

The upregulation of hnRNPs A1 and A2/B1 in proliferating

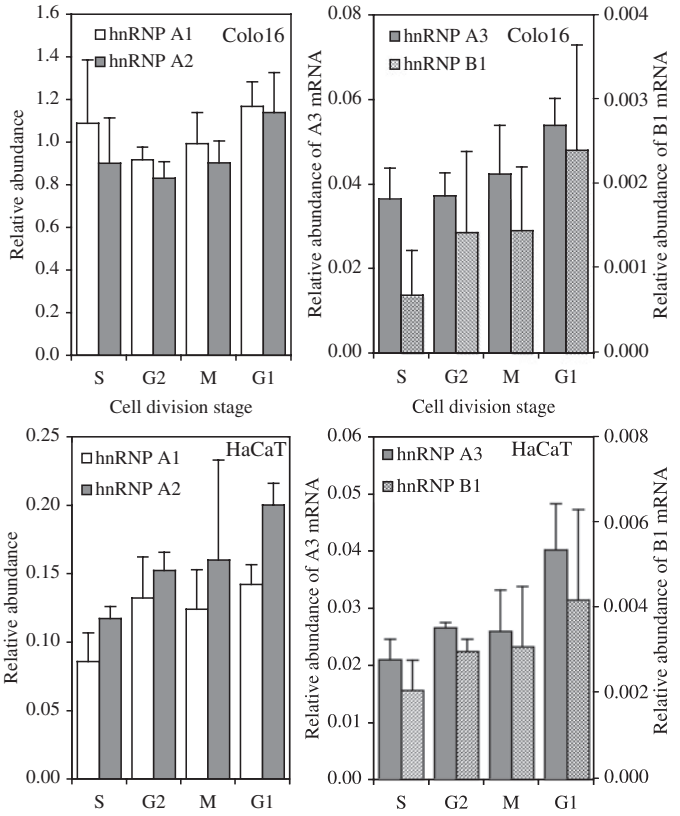

Fig. 3. Real-time PCR revealed stable levels of hnRNP A/B mRNAs during the cell cycle processes. Cells at S, G2, M and G1 phases were collected at 0, 7, 8 and 10 hours after synchronization for Colo16 cells, and 0, 8, 9 and 11 hours for HaCaT cells. The relative abundance of hnRNP A1, A2, B1 and A3 mRNAs was determined using β-actin as a control. The means±s.e.m. were calculated from six replicates. Data were processed by ANOVA.

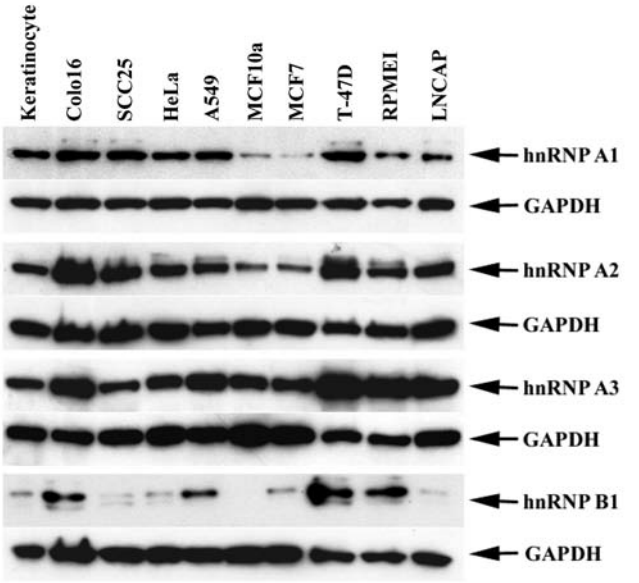

Fig. 4. Expression of hnRNP A1, A2, B1 and A3 in different cells. Western analysis of total protein extracts with rabbit polyclonal antibodies raised against the A1, A2, B1 and A3 peptides, using a mouse monoclonal GAPDH antibody as a loading control. The keratinocytes were isolated from normal foreskin. Colo16 and SCC25 are squamous cancer cell lines originated from tumours on foreleg and tongue, respectively. HeLa is a cervical cancer cell line and A549 was cloned from lung cancer. MCF10a is an immortalized non-tumorigenic breast cell line, and MCF7 and T-47D are two breast cancer cell lines. RPMEI is an immortalized prostate cell line and LNCAP was cloned from human prostate cancer.

cells and cancer suggests potential roles for these proteins in cell growth. Binding of hnRNPs A1 and A2 to telomere sequences and the established role of A1 in telomere biogenesis (Dallaire et al., 2000; Erlitzki and Fry, 1997; LaBranche et al., 1998) present further evidence for their involvement in cell replication control. We therefore hypothesized that suppressing expression of hnRNPs A1 and A2/B1 would affect cell proliferation. To investigate the effect of suppression of hnRNP A/B proteins on cell proliferation, RNA interference-induced gene suppression was used in Colo16 cells. Synthesized oligonucleotides consisting of a 19-nucleotide sense A1, A2, or A3 sequence and its reverse complement with a 9-nucleotide spacer between them was inserted downstream of the pSUPER vector H1-RNA promoter. These plasmids generated hairpin shRNAs in vivo. A sequence from firefly luciferase (pS-Luc), which has no sequence identity to any part of human genome according to BLAST alignment, was used as a negative control. Western blotting showed substantial decreases of hnRNP A1, A2 and A3 levels in cells transfected with pS-A1, pS-A2 and pS-A3, compared with those transfected with pS and pS-Luc (Fig. 6). When one of these hnRNPs was suppressed by RNAi, the other two were not affected, establishing the specificity of the action of the shRNAs (Fig. 6).

The effect of suppression of hnRNP A1, A2 and A3 was first assessed by cell counting. Colo16 cells were transfected with pS-A1, pS-A2, pS-A3 or a combination of two of them, and the cell number was compared with cells transfected with pS and pS-Luc after 72 hours post-transfection. Reducing hnRNPs A1 or A3 did not affect cell growth whereas suppression of A2 isoforms caused a small but statistically significant reduction in cell number (Fig. 7). Simultaneous suppression of any two of the hnRNPs slowed cell proliferation, with the combination of pS-A1 and pS-A2 affecting cell growth most significantly (Fig. 7). Secondly, an MTT proliferation assay, in which expression of hnRNP A2 and its alternative splicing isoforms was suppressed, was performed to support the above data. Colo16 and HaCaT cells were transfected as above, and after 48 hours, the cells were re-plated into 96-well plates. Between 3 and 45 hours after re-plating, cells were sampled at 6-hour intervals for MTT proliferation assays. A decrease in the proliferation rate of cells treated with pS-A2 was observed, whereas pS and pS-Luc had no impact (Fig. 8 and Table 1).

The slower proliferation of the pS-A2-treated Colo16 cells was unlikely to have resulted from apoptosis because the TUNEL assay did not show significant increases in apoptotic cells (data not shown). Flow cytometry also revealed similar percentages of apoptotic cells in the treatments with pS, pS-Luc and pS-A2 (data not shown).

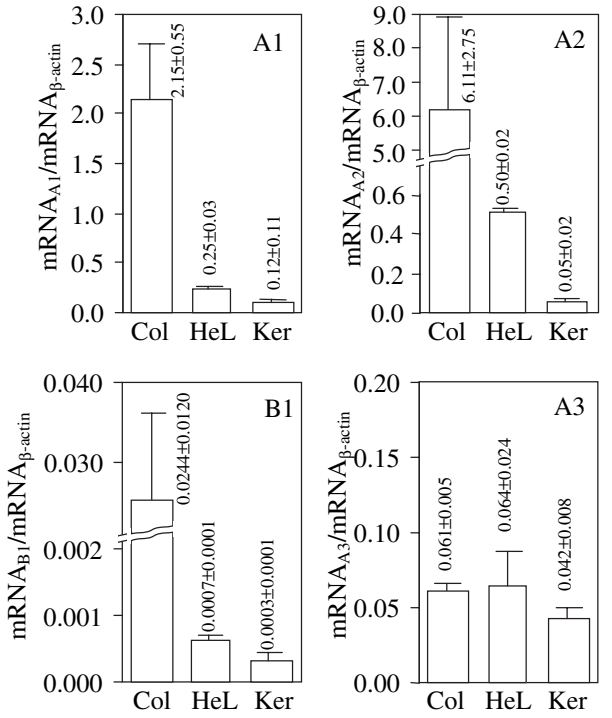

Fig. 5. hnRNP A/B mRNA levels in different cells. Real-time PCR was carried out to quantify the amount of A1, A2, B1 and A3 mRNAs in the selected cell lines using β-actin as a control. The relative abundance of individual mRNA, shown above the columns, was calculated as the ratio between the targeted and β-actin mRNAs. Means±s.e.m. of three experiments, each with two replicates, were calculated for each type of cell. Col, Colo16; HeL, HeLa; Ker, keratinocytes.

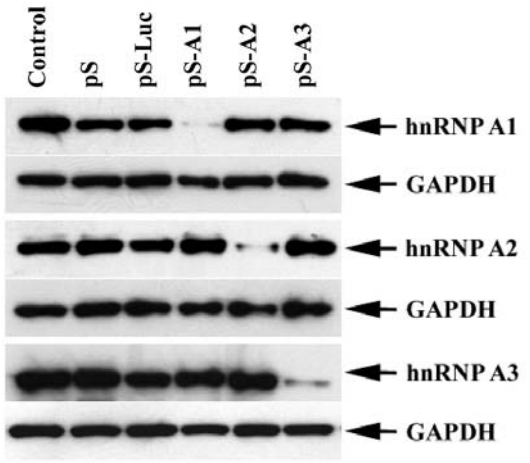

Fig. 6. shRNA suppression of hnRNPs A1, A2 and A3 in Colo16 cells. Colo16 cells were seeded in six-well plates (360,000 cells/well) and transfected 20 hours later. Control samples were treated with lipofectamine only. 72 hours after transfection, cells were collected and lysed. Total proteins were then separated on SDS-PAGE, transferred onto PVDF membrane and probed with antibodies against A1, A2 or A3 using GAPDH as a loading control.

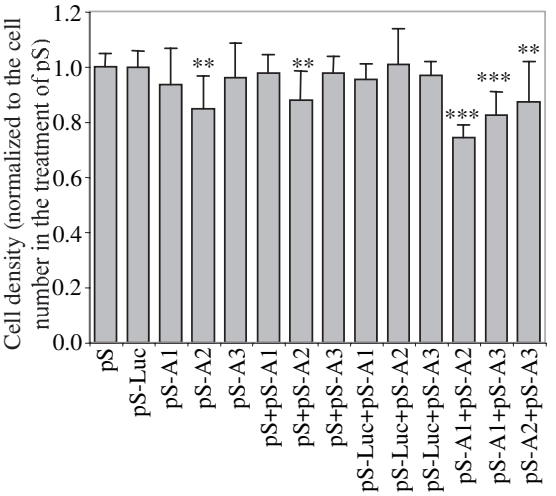

Fig. 7. Impact of shRNAs against hnRNPs A1, A2 and A3 on cell growth. Colo16 cells were seeded into six-well plates (360,000 cells/well) 20 hours before transfection. The number of cells in each well was counted 72 hours after transfection. Values are means±s.e.m. of eight replicates. Significance differences $^{**}P<0.05$ and $^{***}P<0.001$ were found using Student's *t*-test compared to counts in cells transfected with empty vector.

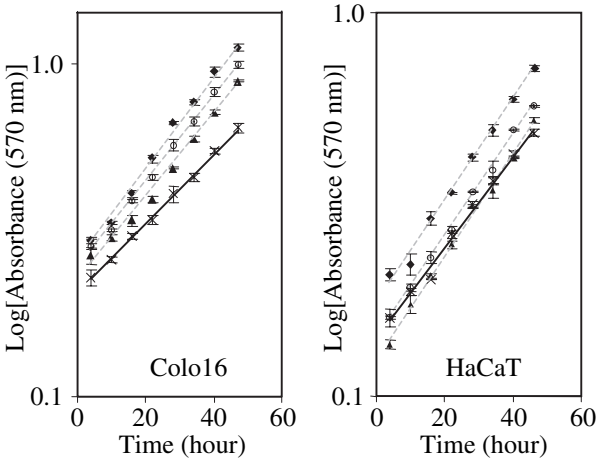

Fig. 8. Suppression of hnRNP A2 alone slows cell growth. Colo16 cells were collected 48 hours after transfection, counted and re-plated into 96-well plates (1500 cells/well, six replicates for each treatment). From 3 hours after re-plating, cells were sampled at 6-hour intervals to perform MTT proliferation assays. The absorbance at 570 nm was then logarithmically plotted against the time after re-plating. The experiment was repeated three times. ◆, lipofectamine; ○, pS; ▲, pS-Luc; x, pS-A2.

Table 1. Slopes of cell growth curves

Treatment	Colo16	HaCaT
Lipofectamine	0.0312±0.0016	0.0308±0.0002
pS	0.0301±0.0008	0.0297±0.0016
pS-Luc	0.0287±0.0008	0.0307±0.0011
pS-A2	0.0242±0.0005	0.0275±0.0016

Significance was $P<0.001$ for comparison of pS-A2 and lipofectamine-treated Colo16 cells, and $P<0.05$ for comparison of pS-A2 and lipofectamine-treated HaCaT cells, as assessed by ANOVA analysis.

hnRNP A2 suppression inhibits expression of BRCA1 but induces p21

The cell growth retardation caused by suppression of hnRNP A2 isoform expression led us to explore the effect of these proteins on several known cell cycle regulation factors. We chose p21 because it not only controls the cell cycle progression through its inhibitory binding to cyclin/cyclin-dependent kinase complexes, the driving force for cell cycle progression, but also inhibits DNA synthesis and proliferating cell nuclear antigen (PCNA) activity (Pan et al., 1995; Waga et al., 1997), which is pivotal for nucleic acid metabolism (Kelman, 1997). Cell cycle checkpoints are regulated through p21 by p53, which activates cell apoptosis in the presence of irreparable DNA damage, to inhibit tumour development (el-Deiry et al., 1993). BRCA1 can also regulate p21 expression by binding to the p21 promoter region (Somasundaram et al., 1997), and loss of BRCA1 function is associated with cell cycle defects and p21 induction in the mouse (Hakem et al., 1996) and p21 overexpression in human tumours (Sourvinos and Spandidos, 1998). We compared the expression levels of p53, BRCA1 and p21, which are known to be involved in cell growth regulation, in Colo16 cells before and after pS-A2 transfection. Western blotting showed that the p53 level was maintained (Fig. 9A) but BRCA1 decreased markedly (Fig. 9B) in Colo16 cells when hnRNP A2 isoforms were suppressed. Analysis of *BRCA1* gene expression by northern blotting confirmed that this suppression resulted from the deficiency of hnRNP A2 family proteins (Fig. 9C). On the other hand, expression of the cyclin-dependent kinase inhibitor p21, increased significantly at both protein and mRNA levels (Fig. 10) following suppression of hnRNP A2. These results suggest a linkage between the functions of hnRNP A2 isoforms in cell proliferation and the expression of cell growth regulators such as BRCA1 and p21, which are known to control the growth of various types of cells (el-Deiry et al., 1993; Zhang et al., 1995).

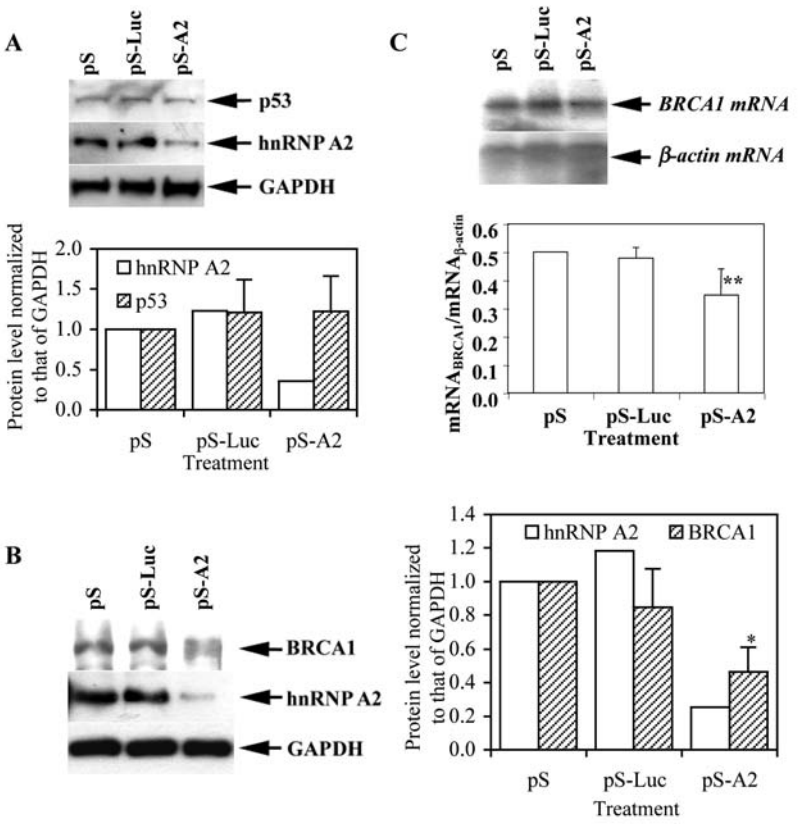

Fig. 9. Effect of A2 suppression by RNAi on the expression of p53 and BRCA1. (A) Western blotting for p53 after treating Colo16 cells with pS, pS-Luc and pS-A2. Total protein isolated from cells collected 72 hours post-transfection was separated on SDS/12% polyacrylamide gel, transferred onto PVDF and probed with a monoclonal mouse antibody against p53. The band intensities of p53 and hnRNP A2 shown in the graph were normalized to that of GAPDH. The p53 data are the means of four replicates. (B) Western blot analysis of BRCA1 after treating Colo16 with pS, pS-Luc and pS-A2. Total proteins isolated from cells harvested 72 hours post-transfection were separated on SDS/5% polyacrylamide gel, transferred onto PVDF and probed with a monoclonal mouse anti-BRCA1 antibody. Levels of BRCA1 protein, averaged from three replicates were calculated as the ratio of BRCA1 to GAPDH protein. A significant difference (*P<0.05) in protein levels in the pS-A2 transformed cells was observed compared to levels in control cells. (C) Northern blot analysis was carried out to determine the amount of BRCA1 mRNA in Colo16 cells collected 72 hours after transfection with pS, pS-Luc or pS-A2. The results were quantified by densitometry and the relative amount of BRCA1 was calculated using β-actin as a control. Results are the means±s.e.m. of four experiments. A significant difference (**P<0.05) in BRCA1 level was observed in pS-A2 transfected cells compared to levels in control cells.

Discussion

A number of studies suggest that hnRNP A2/B1 proteins are overexpressed in a variety of cancers and may be useful markers for screening for breast, pancreatic, non-small cell lung cancers and oral squamous cell carcinoma (Goto et al., 1999; Sueoka et al., 1999; Sueoka et al., 2001; Tockman et al., 1997; Wu et al., 2003; Yan-Sanders et al., 2002; Zhou et al., 2001a; Zhou et al., 1996; Zhou et al., 2001b). A recent study showed that these proteins are also potentially new broad targets in cancer therapeutics as cancer cells with simultaneously lowered levels of hnRNPs A1 and A2 underwent apoptosis (Patry et al., 2003). This was attributed to the failure of the telomere capping process at low concentrations of these hnRNPs. Here we have presented further evidence that hnRNP A/B proteins, especially A2 and B1, play important roles in cell proliferation.

The cancer cell lines showed widely varying levels of the hnRNP A/B proteins. Although these cell lines were all epithelial in origin, they displayed different expression patterns for hnRNPs A2 and B1 (Fig. 4). Two cell lines, Colo16 and T-47D, expressed high levels of both hnRNPs A2 and B1. In accord with previous immunohistochemistry studies (Sueoka et al., 1999; Sueoka et al., 2001), lung cancer cells overexpressed hnRNP B1 rather than A2 (Fig. 4). On the other hand, hnRNP B1 was reduced in the prostate cancer cell line, LNCAP, but not in an immortalized prostate epithelial cell-line RPMEI (Fig. 4). This result, which parallels that observed

in adult T-cell lymphoma/leukaemia (Tani et al., 2002), suggests that increased hnRNP B1 expression is not a universal phenomenon in different kinds of cancer. Overexpression of hnRNP B1 may be useful for the diagnosis of lung cancer as previously suggested (Hamasaki et al., 2001; Sueoka et al., 1999; Sueoka et al., 2001), but is clearly inappropriate for other cancer types. In addition we observed overexpression of hnRNP A3 in three out of seven cancer cell lines.

Proliferation-dependent expression of hnRNP A1 in different cell types at both the protein (Celis et al., 1986; LeStourgeon, 1978; Loeb et al., 1976; Martin et al., 1979) and mRNA levels (Biamonti et al., 1993; Buvoli et al., 1988) has been reported. Levels of hnRNP A2 mRNA also increase in proliferating fibroblasts compared to the quiescent state (Biamonti et al., 1993; Martin et al., 1979). The low levels of hnRNPs A1 and A2 in slowly dividing cells led to the proposition that they may play a role during cell division and growth. Our observation of significant fluctuations in hnRNP A1, A2 and B1 expression during the cell cycle (Fig. 1C) presents further evidence that these proteins are specifically required at certain stages of the cell cycle. However, the levels of hnRNP A1, A2, B1 and A3 gene transcripts did not change significantly during cell division, except for that of *hnRNP A3* in the G1-phase of HaCaT cells, suggesting that the modulation of their protein expression occurred at the post-transcriptional level, through changes in the rate of translation or protein degradation. This is the first demonstration of post-transcriptional regulation of these genes during the cell cycle.

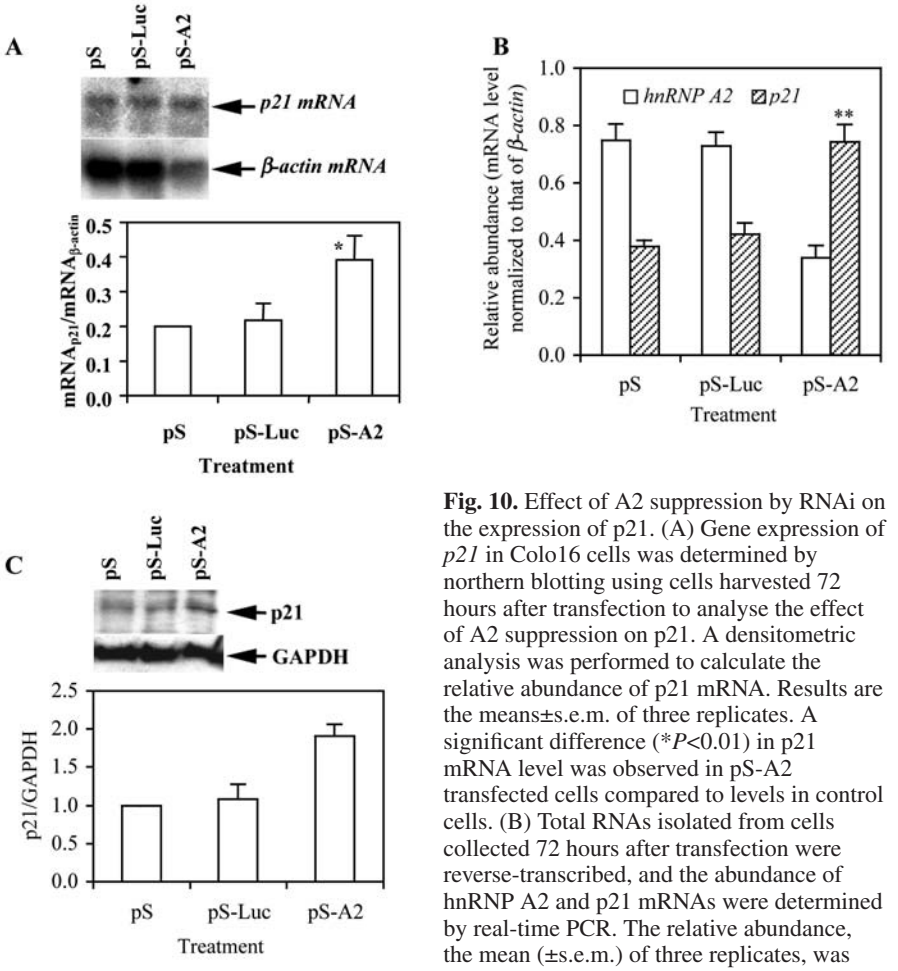

Fig. 10. Effect of A2 suppression by RNAi on the expression of p21. (A) Gene expression of *p21* in Colo16 cells was determined by northern blotting using cells harvested 72 hours after transfection to analyse the effect of A2 suppression on p21. A densitometric analysis was performed to calculate the relative abundance of p21 mRNA. Results are the means±s.e.m. of three replicates. A significant difference (*P<0.01) in p21 mRNA level was observed in pS-A2 transfected cells compared to levels in control cells. (B) Total RNAs isolated from cells collected 72 hours after transfection were reverse-transcribed, and the abundance of hnRNP A2 and p21 mRNAs were determined by real-time PCR. The relative abundance, the mean (±s.e.m.) of three replicates, was calculated as the ratio between A2 or p21 mRNA and β-actin. A significant difference (**P<0.05) in relative abundance of p21 mRNA was observed in pS-A2 transfected cells compared with levels in control cells. (C) Western blot analysis of p21 after suppressing hnRNP A2. Total proteins isolated from cells harvested 72 hours post-transfection were separated on SDS/12% polyacrylamide gel, transferred onto PVDF and probed with a monoclonal mouse anti-p21 antibody. The mean levels (±s.e.m.) of p21 shown in the graph were taken from duplicate experiments.

The sequence similarity of hnRNP A3 to A1 and A2 (Good et al., 1993; Ma et al., 2002) raised the question of whether A3 is similarly modulated to the latter two during the cell cycle. In *Xenopus*, hnRNP A3 mRNA was most abundant in the ovary and late embryonic stages in parallel with A1 and A2, suggesting A3 may contribute to rapid cell proliferation in embryogenesis (Good et al., 1993). In addition, an in vitro overlay assay showed that hnRNP A3 bound to protein kinase C (Rosenberger et al., 2002), which is involved in the control of cell division and differentiation (Clemens et al., 1992; Fishman et al., 1998; Schwantke et al., 1985; Watters and Parsons, 1999), suggesting a role for A3 during certain stages of cell division. Our experiments, however, showed no apparent change in either mRNA or protein level of hnRNP A3, and its function during the cell cycle remains to be elucidated.

The effects of downregulation of hnRNPs A1, A2, B1 and A3 provided further evidence that these proteins play important roles in cell growth. Specific shRNA suppression of hnRNP A1 or A3 had no effect, suggesting that neither is essential for cell growth. Our results for hnRNP A1 are consistent with previous findings in the mouse erythroleukaemic CB3 cell line (Ben-David et al., 1992) and in HeLa and HCT116 cells (Patry et al., 2003). However, suppressing expression of hnRNPs A1 and A3 at the same time did affect cell growth, suggesting that these two proteins may functionally compensate for each other. This result is in accord with the high sequence identity of these two proteins (Ma et al., 2002).

Colo16 and HaCaT cells with lowered levels of hnRNP A2 isoforms were found to grow at a slower rate, though they can still proliferate (Figs 7 and 8), in contrast to the results of Chabot and co-workers who found that suppressing hnRNP A2 by siRNA duplexes had no effect on the proliferative capacity of HeLa and HCT116 cells (Patry et al., 2003). This difference may be due to the variable performance of siRNAs in different cell lines. In this study, Colo16 cells showed a suppression efficiency of 70-95% (based on western blotting results) for

hnRNP A2 shRNA, depending on the degree of cell confluency before transfection. Cells with a simultaneous reduction in A1 and A2 exhibited more significant growth retardation compared to those lacking A2 alone. Similar synergistic effects with hnRNPs A1 and A2 have been observed in HeLa and HCT116 cells (Patry et al., 2003). However, there was no such effect when shRNAs against hnRNP A2 and A3 were used together: the growth rate was comparable to that of cells treated with A2 shRNA alone.

Simultaneous suppression of hnRNPs A1 and A2 in HeLa cells results in apoptosis through their effect on the telomere capping process (Patry et al., 2003). Earlier proteomic studies also suggested that hnRNPs A1 and A2/B1 are involved in the αCD95-induced apoptosis in Jurkat T cells because of the translocation, cleavage and dephosphorylation of these proteins during apoptosis (Brockstedt et al., 1998; Hermann et al., 2001; Thiede et al., 2002). In our study, apoptosis was not detected by either the TUNEL assay or flow cytometry analyses. Colo16 cells deficient in A1/A2, A1/A3, A2/A3 or A2 alone were able to proliferate, but at a lowered rate, and no truncated forms of these proteins were detected in our western blots, suggesting a mechanism differing from that observed in Jurkat T cells (Brockstedt et al., 1998; Thiede et al., 2001; Thiede et al., 2002). The mechanism behind the growth retardation observed in A2-suppressed Colo16 cells is unknown. As hnRNPs A/B proteins play a major role in splicing, trafficking and export of mRNA out of nucleus, their suppression may impede the mRNA processing and trafficking, causing a reduction in cell growth rates.

In our study we have examined the effects of the hnRNP A2 proteins on a group of cell growth regulation factors, including p53, p21 and BRCA1, which have been implicated in the uncontrolled proliferation of tumour cells (Hakem et al., 1996; Perez-Roger et al., 1999; Ullrich et al., 1992). The changes in expression of p21 and BRCA1 in cells with reduced levels of hnRNP A2 are consistent with the observed reduction in proliferation rate of Colo16 cells, and also correlated with the well-established negative roles that two proteins play in cell growth (Hakem et al., 1996; Vermeulen et al., 2003). Although the hnRNP proteins may affect cell proliferation through BRCA1 and p21, there are multiple pathways regulating cell growth, and it will be of interest to see how widespread the effects of hnRNPs are.

In summary, our results demonstrate that hnRNP A/B proteins, especially A2 and B1, are dysregulated in a wide range of epithelial cancer cells. Their expression and that of hnRNP A1, are cell cycle-dependent, whereas the hnRNP A3 level is constant during cell division. We have also presented evidence that hnRNPs A2 and B1 play an important role in cellular proliferation, in accord with their effects on expression of p21 and BRCA1. Defining the mechanisms behind these interactions will aid understanding of the functions of hnRNP A2/B1 during cell growth as well as their dysregulation in some cancers.

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