RNA interference (RNAi) is a process through which double-stranded RNA (dsRNA) is cleaved into small interfering RNAs (siRNAs) that trigger RNA degradation in a sequence-specific manner (Tomari & Zamore, 2005). RNAi is evolutionarily conserved in eukaryotes (Dykxhoorn et al., 2004). One important biological function of RNAi in plants and invertebrate animals is to mobilize a defence response against invading nucleic acids such as viruses and transposons (McManus, 2004), but it remains to be fully understood as to whether RNAi fulfils a similar protective role in mammals. For productive replication of their genomes, plant and insect viruses have developed various strategies to combat RNAi, such as the employment of viruses-encoded suppressors of RNAi (Ding et al., 2004; Voinnet, 2005). Recent studies have expanded the list of viral RNAi suppressors to proteins and RNAs encoded by mammalian viruses. As such, adenovirus VA1 and VA2 RNAs (Lu & Cullen, 2004; Andersson et al., 2005), La Crosse virus NSs protein (Soldan et al., 2005), Nomamura virus B2 protein (Sullivan & Ganem, 2005) and human immunodeficiency virus type 1 Tat protein (Bennasser et al., 2005) have been demonstrated to suppress RNAi in mammalian cells.

The influenza A virus NS1 protein is critically involved in virus–cell interaction (Krug et al., 2003). NS1 is known to counteract the interferon response at several steps, to interfere with post-transcriptional processing and/or translation of mRNA, and to modulate NF-κB and JNK signalling as well as apoptosis (Nemeroff et al., 1998; Wang et al., 2000; Schultz-Cherry et al., 2001; Ludwig et al., 2002; Salvatore et al., 2002; Zhirnov et al., 2002). Three independent groups have provided evidence that NS1 inhibits RNAi in plants and Drosophila (Bucher et al., 2004; Delgadillo et al., 2004; Li et al., 2004). This is actually the first protein encoded by a human virus that has been shown to suppress RNAi in plant and insect cells, probably through direct binding to dsRNA and siRNA (Li et al., 2004). These and other findings suggest the conservation of an RNAi-mediated antiviral response in mammals. However, for NS1 to act as an RNAi suppressor, it should be able to shut off RNAi in mammalian cells. In addition, if NS1 could suppress RNAi efficiently in human cells, it might also be necessary to reconsider the strategies of using RNAi to inhibit influenza virus replication (Ge et al., 2004; Zhou et al., 2004). To date, the experimental evidence for NS1 inhibition of RNAi in human cells is not available. In light of this, in the present study we set out to investigate this issue in cultured human cells.

First, we expressed influenza virus NS1 in HeLa cells. A cDNA of NS1 gene from influenza virus strain A/WSN/33 (a kind gift from Zhiping Ye, CBER/FDA, Maryland, USA) was subcloned into pcDNA3 expression vector and V5-tagged NS1 protein was expressed in cultured HeLa cells as verified by Western blot analysis (Fig. 1a) and immunofluorescence microscopy (Fig. 1b). Transiently expressed NS1 appeared as a single discrete band of correct size (~ 30 kDa) in the immunoblot (Fig. 1a, lane 2 compared with V5-tagged β-galactosidase detected in lane 1). Consistent with previous reports (Li et al., 1998; Ludwig et al., 2002), NS1 was predominantly found in the nucleus [Fig. 1b, see panel (i) for NS1 staining and compare with nuclear morphology revealed in panel (ii)]. Next, we tested whether NS1 might impinge on the interferon pathway. To this end, we used a luciferase reporter plasmid under the control of interferon-stimulated response element (ISRE). ISRE is directly responsive to interferon signalling (Stark et al., 1998) and it contains binding sites for transcription factor interferon regulatory factor 3, which is inhibited by NS1 (Talon et al., 2000). Indeed, we observed that NS1 was capable of suppressing the dsRNA-induced activation of ISRE in a dose-dependent manner (Fig. 1c, bars 3 and 4 compared with bar 2). Thus, NS1 acts as an antagonist of interferon in our experimental setting.
To determine whether NS1 inhibits RNAi in mammalian cells, we employed an siRNA targeting firefly luciferase (siFF) and an expression vector (kindly provided by Greg Hannon from Cold Spring Harbour Laboratory, New York, USA) for a small hairpin RNA (shRNA) against firefly luciferase (shFF). Both siFF and shFF have been shown previously to be capable of downregulating the expression of luciferase reporter effectively (Paddison et al., 2002; Zhou et al., 2002). Surprisingly, when we compared the shFF- and siFF-mediated silencing effect on the luciferase reporter, we were unable to observe a noticeable difference between NS1-non-expressing and NS1-expressing HeLa cells (Fig. 1d, compare bar 5 to 2 and bar 6 to 3). Because NS1 plasmid and shFF/siFF were simultaneously transfected into the cells, there was a concern as to whether RNAi might take effect before sufficient amounts of NS1 could be expressed. To address this issue, we transfected NS1 plasmid 24 h before the introduction of shFF/siFF. However, we could not see an effect of NS1 on shFF- or siFF-mediated gene silencing (Fig. 1d, compare bar 8 to 2 and bar 9 to 3). Because a V5-tagged NS1 was used and there is a possibility that the V5 tag might render NS1 inactive, we also compared the behaviours of untagged and V5-tagged NS1 in the inhibition of shFF or siFF. In this experiment, we used the lowest concentrations of shFF (5 ng) and siFF (10 nM), under which an inhibition of luciferase expression could still be
**Fig. 1.** Influence of NS1 on RNAi in cultured mammalian cells. (a) Expression of NS1. Plasmids expressing V5-β-galactosidase (β-Gal) and V5-tagged NS1 were transiently transfected into HeLa cells. Immunoblotting was performed with mouse anti-V5 (Invitrogen). The arrow points to the NS1 band. (b) Subcellular localization of NS1. HeLa cells transiently expressing V5-tagged NS1 were stained with anti-V5 (i). Nuclear morphology was visualized with propidium iodide (PI; ii). Bar, 20 μm. (c) Suppression of ISRE activity by NS1. HeLa cells were transfected with pISRE-Luc (Clontech) alone (bars 1 and 2) or with pISRE-Luc plus 1–2 μg NS1 plasmid (bars 3 and 4). Some cells were stimulated with 50 μg poly I:C ml⁻¹ for 6 h (bars 2–4). The ISRE-dependent luciferase activity was calculated by normalizing to Renilla luciferase activity. Values shown are means ± SD from three separate transfections. (d) Influence of NS1 on RNAi. HeLa cells were transfected with pGL3-control (Promega) alone (Ctrl; bars 1, 4 and 7), pGL3-control plus shFF vector (100 ng; bars 2, 5 and 8) or plus siFF (80 nM; bars 3, 6 and 9). Cells were also transfected with NS1 plasmid either simultaneously (bars 4–6) or 24 h before transfection with shFF/siFF (bars 7–9). (e) Influence of untagged NS1 on RNAi. HeLa cells were transfected with pGL3-control (Ctrl; bars 1 and 5), pGL3-control plus shFF (5 ng; bars 2–4) or plus siFF (10 nm; bars 6–8). Cells were also co-transfected with 0.5 μg empty vector (bars 2 and 6), expression plasmid for untagged NS1 (bars 3 and 7) or for V5-tagged NS1 (bars 4 and 8). (f) Dose dependence of shRNA-induced gene silencing. HeLa cells were transfected with pRLCMV plasmid (Promega) plus different amounts (1–100 ng) of an expression vector for shRL (5’-TAGGAATTATAATGCTTAT-3’), targeting 1778–1796 nt. Renilla luciferase activity was normalized to firefly luciferase activity. (g) Time dependence of shRNA-induced gene silencing. HeLa cells were transfected with pRLCMV alone (Ctrl; black bars), pRLCMV plus 5 ng shRL (shRL; grey bars), pRLCMV plus 5 ng shRL plus 300 ng VA1 vector (shRL+VA1; hatched bars), or pRLCMV plus 5 ng shRL plus 300 ng NS1 vector (shRL+NS1; white bars). The VA1 gene of Ad5 was PCR-amplified from pAdEasy-1 (Stratagene).

**Fig. 2.** Influence of NS1 on silencing of GFP and MAD2B genes. (a) Microscopic analysis of GFP expression. HeLa cells were co-transfected with pEGFP (Clontech) plus NS1 plasmid alone [1 μg; panels (i–iii)], plus 80 nM siGFP [panels (iv–vi)] or plus NS1 plasmid and siGFP [panels (vii–ix)]. Cells were stained with either anti-V5 [panels (i) and (vii)] for NS1 or PI [panel (v)] for nucleus. Bar, 20 μm. (b) Western blot analysis of GFP expression in HeLa cells. (c) Western blot analysis of MAD2B expression. CNE1 cells were stably transfected with expression vector for shMAD2B (5’-GATGCAGCTTTACGTGGAAGA-3’, targeting 705–725 nt). CNE1 cells with (lanes 4–6) or without (lanes 1–3) shMAD2B were transfected with NS1 plasmid. Mouse anti-MAD2B and anti-β-actin were from BD Transduction and Zymed, respectively. (d) Comparison between NS1 and adenovirus VA1 in the silencing of MAD2B. CNE1 cells with (lanes 5–8) or without (lanes 1–4) shMAD2B were either mock-transfected or transfected with the indicated amounts of NS1 or VA1 plasmid. MAD2B was detected by Western blot analysis. Mouse anti-β-tubulin was from Sigma.
observed, but neither untagged NS1 nor V5-tagged NS1 had any effect on shFF/siFF (Fig. 1e, bars 3 and 4 compared to bar 2, bars 7 and 8 compared to bar 6).

The above experiments were conducted with shFF/siFF. To test the effect with another reporter gene, we used an shRNA that specifically targets Renilla luciferase (shRL). We first determined the dose dependence of the shRL-mediated silencing effect (Fig. 1f). Using the lowest concentration of shRL (i.e. 5 ng) required for an observable inhibition, we compared the effects of adenovirus VA1 RNA and influenza virus NS1 protein on Renilla luciferase expression in a time course (Fig. 1g). In this setting, VA1 can efficiently inhibit shRL-mediated gene silencing within 24–48 h after transfection. These results are consistent with previous findings (Lu & Cullen, 2004; Andersson et al., 2005). In contrast, under the same conditions NS1 could not rescue the effect of shRL at any time point (Fig. 1g). Hence, NS1 had no influence on RNAi-mediated silencing in cultured mammalian cells.

Next, we assessed the influence of NS1 with a third reporter gene using confocal microscopy. When NS1 and green fluorescent protein (GFP) were co-expressed in HeLa cells, we could not detect any enhancement or reduction of the GFP fluorescent signal [Fig. 2a, panels (i)–(iii)]. Notably, both in the absence and the presence of NS1, an siRNA targeting GFP (siGFP), which has previously been shown to be effective (Chiu & Rana, 2002; Bennasser et al., 2005), was able to reduce the GFP signal to an undetectable level [Fig. 2a, panels (iv)–(ix)]. Western blot analysis confirmed the silencing of GFP expression at different concentrations and in the presence of NS1 (Fig. 2b, lanes 2–5, compared with lanes 1 and 6). In agreement with our results from luciferase assays (Fig. 1d), NS1 did not suppress siRNA-mediated silencing of GFP expression in HeLa cells.

To determine whether NS1 might have an effect on RNAi-mediated silencing of cell-endogenous genes, we made use of a CNE1 nasopharyngeal carcinoma cell line in which an shRNA against MAD2B (shMAD2B) is stably expressed. MAD2B is a paralogue of mitotic checkpoint protein MAD2 and is also homologous to regulatory subunit Rev7p of yeast DNA lesion bypass polymerase ζ (Cheung et al., 2006). When we transfected NS1 plasmid into CNE1 cells stably expressing shMAD2B, the downregulation of MAD2B was not rescued (Fig. 2c, lanes 5 and 6 compared with lane 4). In contrast, the expression of adenovirus VA1 RNA in these cells led to the restoration of MAD2B expression (Fig. 2d, lane 8 compared with lanes 6 and 4). Consistent with our results obtained with exogenously introduced luciferase and GFP reporter genes, the expression of NS1 did not counteract shRNA-dependent silencing of cell-endogenous MAD2B gene.

Finally, we constructed a HEK293 cell line that stably expressed NS1. The expression of NS1 in this cell line designated 293NS1 compared with its parental HEK293 cells was confirmed by Western blot analysis (Fig. 3a, compare lane 2 with 1). The ability of NS1 to bind to dsRNA in vitro was also verified by ribonucleoprotein immunoprecipitation (RIP) assay (Fig. 3b). Consistent with previous demonstration of the in vitro siRNA- and dsRNA-binding activity of NS1 from the same strain (Li et al., 2004), we observed that NS1 expressed in the 293NS1 cells was able to form a stable protein–RNA complex with dsRNA (Fig. 3b, lanes 5 and 6 compared with lanes 2–4). Moreover, the expression of NS1 in 293NS1 cells led to a substantial reduction of dsRNA-induced activation of ISRE (Fig. 3c, compare bar 7 with 3 and bar 8 with 4). In this experiment, we over-expressed Toll-like receptor 3 (TLR3) in some groups of the cells to facilitate the stimulatory effect of dsRNA (Fig. 3c, bars 2, 4, 6 and 8). The roles of TLR3 in dsRNA-induced activation of ISRE have been well documented (Alexopoulos et al., 2001; Sen & Sarkar, 2005). Our data indicated that NS1 stably expressed in 293NS1 cells was fully competent for the inhibition of interferon signalling. In contrast, NS1 failed to block shFF- or siFF-dependent silencing of luciferase expression in 293NS1 cells (Fig. 3d, compare bars 4–6 to bars 1–3). Thus, all lines of evidence (Figs 1–3) consistently support
the notion that NS1 from A/WSN/33 strain did not suppress RNAi in mammalian cells. We noted that NS1 proteins from different strains of *Influenza A virus* varied in their subcellular localization and in their ability to inhibit interferon β signalling (Hayman et al., 2006). It will be of great interest to see whether NS1 from other strains might also exhibit differential activity in the suppression of RNAi.

Our findings that NS1 protein from influenza virus A/WSN/33 did not suppress RNAi in mammals are surprising because it does antagonize RNAi in plants and *Drosophila* (Bucher et al., 2004; Delgadillo et al., 2004; Li et al., 2004). In addition, virus-encoded RNAi suppressors have recently been identified in several human viruses (Lu & Cullen, 2004; Bennasser et al., 2005; Sullivan & Ganem, 2005). While it remains to be understood whether RNAi is part of an innate antiviral immunity in mammals, our results could be explained either by the difference in RNAi mechanisms in different species or by the different properties of the NS1 protein in different systems. To date, systemic RNAi based on cell-to-cell spreading of silencing effect, which is common in plants and nematodes (Feinberg & Hunter, 2003), has not been demonstrated in mammals. In addition, two homologues of Dicer are functional in *Drosophila*, but in humans, only a single Dicer homologue has been identified (Gregory et al., 2005). Further investigations are required to elucidate whether these differences could account for our observation that influenza A virus NS1 does not suppress RNAi in mammals.

**Acknowledgements**

We thank Z. Ye for providing a cDNA clone of the NS gene of influenza virus strain A/WSN/33; G. J. Hannon for providing shRNA expression vector; H. W. Cheung for providing shRNA against MAD2B; E. Y. W. Choy for providing an expression plasmid for adenovirus VA1; K. L. Siu for technical help with confocal microscopy; and K. T. Chin, Y. P. Ching, E. Y. W. Choy and A. C. S. Chun for critical reading of the manuscript. This work was supported by the Innovation and Technology Fund (grant ITS/112/02) from the Innovation and Technology Commission of Hong Kong and by the Run Run Shaw Research and Teaching Endowment Fund from the University of Hong Kong. D.-Y. J. is a Leukemia and Lymphoma Society Scholar.

**References**


