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Human microRNA-24 modulates highly pathogenic avian-origin H5N1 influenza A virus infection in A549 cells by targeting secretory pathway furin

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A common critical cellular event that many human enveloped viruses share is the requirement for proteolytic cleavage of the viral glycoprotein by furin in the host secretory pathway. For example, the furin-dependent proteolytic activation of highly pathogenic (HP) influenza A (infA) H5 and H7 haemagglutinin precursor (HA0) subtypes is critical for yielding fusion-competent infectious virions. In this study, we hypothesized that viral hijacking of the furin pathway by HP infA viruses to permit cleavage of HA0 could represent a novel molecular mechanism controlling the dynamic production of fusion-competent infectious virus particles during the viral life cycle. We explored the biological role of a newly identified furin-directed human microRNA, miR-24, in this process as a potential post-transcriptional regulator of the furin-mediated activation of HA0 and production of fusion-competent virions in the host secretory pathway. We report that miR-24 and furin are differentially expressed in human A549 cells infected with HP avian-origin infA H5N1. Using miR-24 mimics, we demonstrated a robust decrease in both furin mRNA levels and intracellular furin activity in A549 cells. Importantly, pretreatment of A549 cells with miR-24 mimicked these results: a robust decrease of H5N1 infectious virions and a complete block of H5N1 virus spread that was not observed in A549 cells infected with low-pathogenicity swine-origin infA H1N1 virus. Our results suggest that viral-specific downregulation of furin-directed microRNAs such as miR-24 during the life cycle of HP infA viruses may represent a novel regulatory mechanism that governs furin-mediated proteolytic activation of HA0 glycoproteins and production of infectious virions.

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INTRODUCTION

MicroRNAs (miRNAs) are small endogenous, non-coding, highly conserved RNAs that are considered a powerful tool for regulating gene expression through the RNA interference pathway (Ambros, 2004; Bartel, 2004). Deregulation of miRNA expression can profoundly change gene expression in the cell and has been associated with many human pathologies (Zimmerman & Wu, 2011).

For human RNA viruses, modulation of host miRNAs can influence viral pathogenesis (Gottwein & Cullen, 2008). During hepatitis C virus (HCV) infection, a liver-specific miRNA, miR-122, increases the accumulation and translation of HCV RNA by binding to the 5′ UTR of the virus genome (Henke *et al.*, 2008; Jangra *et al.*, 2010; Jopling *et al.*, 2005, 2008). During human immunodeficiency virus type-1 (HIV-1) infection, cellular miRNAs expressed in resting $CD4^+$ T-lymphocytes were shown to target the 3′ UTR of almost all HIV-1 mRNA, reducing viral protein production and possibly contributing to HIV-1 latency (Huang *et al.*, 2007). It has recently been demonstrated that miR-141 is induced upon human enterovirus 71 (EV71) infection and targets eIF4E, a key element in regulating cap-dependent translation initiation (Ho *et al.*, 2011). The same study found that antagonizing miR-141 during EV71 infection dramatically reduced virus production, suggesting that expression of this cellular miRNA is important for the virus life cycle (Ho *et al.*, 2011).

For influenza A virus (infA), limited information exists on the role of cellular miRNAs during infection. Microarray analysis of mouse miRNA expression during infection with the reconstructed 1918 pandemic influenza virus and a seasonal H1N1 virus (Tx/91) established that the miRNAome was modulated during infA infection (Li *et al.*, 2010). In our previous study, we profiled human miRNA expression during infection with the low-pathogenicity (LP) swine-origin 2009 pandemic H1N1 infA and the highly pathogenic (HP) avian-origin H7N7 infA (Loveday *et al.*, 2012). Our studies demonstrated temporal and strain-specific

regulation of miRNAs during infA infection, especially for the HP H7N7 virus. Interestingly our array data showed that infection of human A549 cells with HP H7N7 results in the downregulation of the miR-23 cluster at 4, 8 and 24 h post-infection (p.i.) (Loveday *et al.*, 2012).

The miR-23 cluster of miRNAs consists of two highly conserved paralogue primary miRNA transcripts composed of three miRNAs: miR-23a/b, miR-27a/b and miR-24 (Chhabra *et al.*, 2010). The miR-23b–27b–24-1 cluster is localized on chromosome 9q22, while the miR-23a–27a–24-2 cluster is localized on chromosome 19p13. The mature sequences of both miR-23a and 23b and miR-27a and 27b differ by only one nucleotide, while the miR-24-1 and miR-24-2 mature sequences are identical. MiR-24 is implicated in a number of human diseases, especially cancer, and has been shown to play a role in proliferation, cell cycle arrest, apoptosis and differentiation; it has a number of experimentally validated mRNA targets (Chhabra *et al.*, 2010). Most recently, miR-24 has been shown to regulate the transforming growth factor (TGF)-β pathway by targeting different mRNA targets, including the proprotein convertase (PC) furin (Dogar *et al.*, 2011; Luna *et al.*, 2011).

During HP infA virus infection, furin plays an important role in the virus life cycle (Horimoto *et al.*, 1994; Kawaoka & Webster, 1988; Stieneke-Gröber *et al.*, 1992; Walker *et al.*, 1994). The furin-mediated endoproteolytic cleavage of the HA precursor (HA0) into two disulfide-bonded subunits, HA1 and HA2, is necessary for infectivity as it exposes the fusion peptide (FP), the hydrophobic amino terminus of HA2 (Skehel & Wiley, 2000). The FP facilitates fusion between the late endosomal membrane and virus envelope when the cleaved HA is exposed to the low pH environment of the endosome (Colman & Lawrence, 2003). The HP H5- and H7-infA virus subtypes contain a multibasic R-X-R/K-R HA0 cleavage sequence that LP infA viruses lack; furin cleaves at this site intracellularly before the assembly of progeny virions (Kawaoka & Webster, 1988).

In this study we investigated the regulation of miR-24 expression in A549 cells during HP H5N1 infA virus infection. In addition, we investigated the role of miR-24 as a potential post-transcriptional regulator of the furin-mediated activation of HA0 that occurs within the host secretory pathway, which is necessary for the production of fusion-competent virions. We hypothesize that viral hijacking of the furin pathway by HP infA to promote cleavage of HA0 during the viral life cycle could represent a novel molecular mechanism controlling the dynamic production of fusion-competent infectious virions.

We observed the downregulation of miR-24 expression with a concomitant upregulation of furin mRNA during the H5N1 viral life cycle. Transfection of A549 cells with exogenous synthetic miR-24 mimics results in a strong decrease in both furin mRNA level and intracellular furin activity. Importantly, pretreatment of A549 cells with miR-24 mimics results in a robust decrease in production of H5N1 infectious virions and a complete block of H5N1 virus spread that was not observed during infection with the LP swine-origin infA H1N1 virus. These results suggest that virus-specific regulation of furin-directed microRNAs such as miR-24 during the life cycle of HP infA viruses may represent a novel regulatory mechanism that governs furin-mediated proteolytic activation of HA0 glycoproteins and production of infectious virions.

RESULTS AND DISCUSSION

Downregulation of miR-24 with a concomitant upregulation of furin mRNA during the HP H5N1 viral life cycle

In our previous study of microRNA expression during infA infection, the miR-23 cluster was significantly downregulated during infection with the HP avian-origin H7N7 strain only (Loveday *et al.*, 2012). The miR-23 cluster was not deregulated during infection with the LP 2009 H1N1 pandemic strain, suggesting that deregulation of the miR-23 cluster is a strain-specific effect. MiR-24, a member of the miR-23 cluster, is a well-conserved microRNA found in several vertebrates and is ubiquitously expressed in multiple tissues (Bang *et al.*, 2012; Chhabra *et al.*, 2010). Validated targets include $p14^{ARF}$ in retinoblastoma cell lines, the aryl hydrocarbon receptor nuclear translocator in liver cell lines, stimulator of interferon genes (STING) in rats, the histone variant H2AX in $CD8^+CD28^-$ T-cells along with E2F2, MYC and a number of other cell cycle genes associated with proliferation, cell cycle arrest, apoptosis and differentiation (Brunner *et al.*, 2012; Chhabra *et al.*, 2010; Huang *et al.*, 2012; Lal *et al.*, 2009; Oda *et al.*, 2012; Srivastava *et al.*, 2011; To *et al.*, 2012). Recent research has demonstrated that miR-24 plays a role in the feedback loop associated with TGF-β processing in HeLa and human trabecular meshwork cell cultures by targeting the proprotein convertase furin (Dogar *et al.*, 2011; Luna *et al.*, 2011). With furin and furin-like proteases playing a significant role in the processing of the HA precursor HA0 during HP infA infections, we hypothesized that downregulation of miR-24 by HP infA would allow for increased expression of furin and lead to enhanced HA0 processing into the HA1 and HA2 subunits (Fig. 1a). We focused on the role of miR-24 during H5N1 infection due to the pandemic potential of this virus and the optimal furin cleavage sequence R-X-K/R-R between the HA1 and HA2 subunits (Basak *et al.*, 2001; Kawaoka & Webster, 1988; Seidah, 2011) (Fig. 1a). After inoculating A549 cells with H5N1 at an multiplicity of infection (m.o.i.) of 0.0001, we confirmed infection using quantitative real-time PCR (qRT-PCR) and titration of the culture supernatant at 24, 48 and 72 h p.i. (data not shown). A low m.o.i. allows for multiple rounds of replication while minimizing excessive cell death; this more closely reflects clinical viral loads, allowing us to evaluate the effects of miR-24 mimics on H5N1 viral replication, infectious viral production and viral spread in host cells (Ng *et al.*, 2005).

To assess any changes in miR-24 or furin expression in H5N1-infected A549 cells, total RNA was isolated at

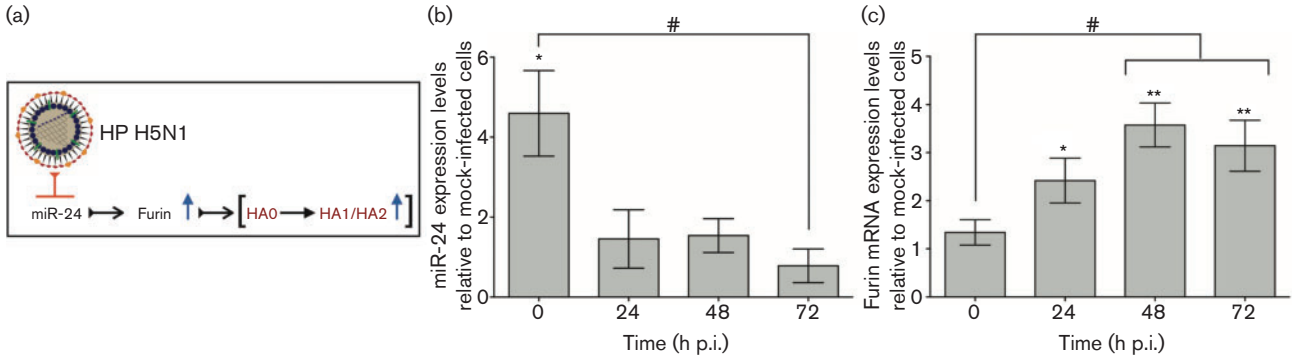

Fig. 1. MiR-24 and furin RNA expression are inversely correlated during highly pathogenic avian-origin influenza A H5N1 infection. (a) Model of proposed regulation of miR-24 and furin during H5N1 infection. H5N1 modulation of miR-24 results in an increase in furin expression leading to an increase in HA processing. (b) A549 cells were infected with A/Ck/Vietnam/14/2005/H5N1. Cells were harvested at multiple intervals (0, 24, 48 and 72 h) post-infection (p.i.). MiR-24 expression was assessed by qRT-PCR compared with mock-infected controls collected at 72 h p.i. (c) Furin mRNA expression was assessed by qRT-PCR at the same time points compared with mock-infected controls collected at 72 h p.i. Data points represent the mean ± SEM of two experiments. Significance between infected and mock-infected samples is indicated by *, while significance between specific time points is indicated by #. Significance is based on two-tailed Student's *t*-test. (*or #, *P* value <0.05; **, *P* value <0.01).

multiple time points (0, 24, 48 and 72 h p.i.) and analysed for miR-24 and furin expression using qRT-PCR. RNA expression was determined using the $\Delta\Delta C_t$ method and normalized to mock-infected cells for H5N1 samples. MiR-24 expression was significantly increased following viral adsorption (0 h p.i.) when compared to mock-infected cells (indicated by *; Fig. 1b). This robust virus-mediated induction of miR-24 was abolished by 24 h p.i., and there was a significant change in miR-24 expression by 72 h p.i. compared with the 0 h time point (indicated by #; Fig. 1b). Furthermore, furin mRNA expression in H5N1-infected A549 cells increased significantly at 24, 48 and 72 h p.i. compared with mock-infected cells (indicated by *) and the expression of furin was significantly increased at 48 and 72 h p.i. compared with the 0 h time point (indicated by #; Fig. 1c).

These results demonstrated a temporal induction of miR-24 and furin that is specifically initiated during viral adsorption and which changes up to 72 h p.i. in A549 cells. The rapid and temporal induction of miR-24 following viral adsorption observed in Fig. 1b could be triggered by the virus-mediated induction of NF-κB signalling pathways, inducing the expression of miR-23b–27b–24 cluster members in A549 cells (Xu *et al.*, 2012; Zhou *et al.*, 2010). The molecular mechanism(s) and player(s) involved in the abolishment of miR-24 induction during viral infection in A549 cells remain to be determined. It is interesting to note that previous research demonstrated an important role for infA NS1 in the regulation of host cell responses triggered by virus infection, including the prevention of NF-κB activation (Geiss *et al.*, 2002; Wang *et al.*, 2000). Our results also demonstrate that a decrease in miR-24 expression is concomitant with increased furin mRNA levels at 72 h p.i. with H5N1. To date, no other reports have indicated that furin expression is upregulated during H5N1 infA infection.

MiR-24 overexpression reduces furin mRNA and its enzymic activity in human A549 cells

The human *fur* gene, which codes for furin, is predicted to contain four miR-24 binding sites in its 3′ UTR based on the analysis of three different databases in the mirnabodymap database (http://www.mirnabodymap.org/). To verify that miR-24 can reduce furin expression in A549 cells, miR-24 mimics were transfected into A549 cells at concentrations of 30, 60, 120 and 240 nM. As per previous studies, in which miR-24 mimics were transfected into various cell lines at concentrations ranging from 5 to 100 nM, we examined a wide range of concentrations to investigate the biological effects of miR-24 mimics in A549 cells (Brunner *et al.*, 2012; Dogar *et al.*, 2011; Fiedler *et al.*, 2011). Luciferase reporter constructs bearing various full-length 3′ UTRs were cotransfected with miR-24 or control (neg-miR) mimics to establish if miR-24 contributes to regulation of furin in A549 cells. Luciferase activity from a luciferase reporter containing three perfect miR-24 binding sites was significantly reduced (>80 %) compared with the reporter only, indicating that the miR-24 mimics function in A549 cells and that neg-miR mimics do not target miR-24 binding sites (Fig. 2a). In the luciferase constructs containing full-length furin 3′ UTR, luciferase activity was significantly reduced by (~50 %) at 60, 120 and 240 nM (Fig. 2b) following transfection with miR-24 mimics; luciferase activity in constructs without 3′ UTR modifications were not affected (Fig. 2c). Our data show similar levels of reduced luciferase activity with the furin 3′ UTR

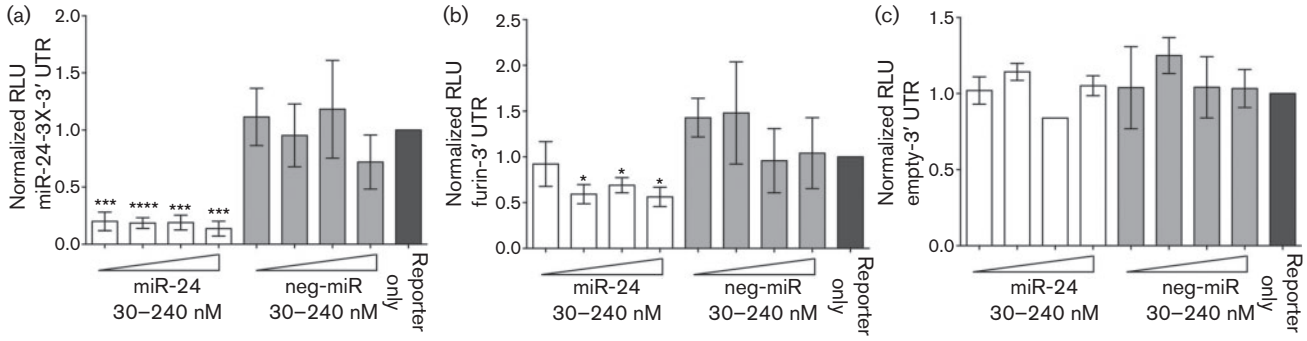

Fig. 2. MiR-24 targets furin in A549 cells. (a–c) 3′ UTR luciferase assays with miR-24 and neg-miR mimics (30, 60, 120, and 240 nM). (a) Cells transfected with miR-24 or neg-miR mimics and a luciferase expression plasmid with three repeated miR-24 binding sites in the 3′ UTR (miR-243X-3′ UTR). (b) Cells transfected with miR-24 or neg-miR mimics and a luciferase expression plasmid containing the furin 3′ UTR (miR-24-3X-3′ UTR). (c) Cells transfected with miR-24 or neg-miR mimics and an empty 3′ UTR reporter plasmid. Luciferase activity was measured 24 h post transfection (p.t.). Relative luminescence units (RLU) are displayed as the mean of triplicate transfections as a proportion of reporter only, error bars represent SEM. Significance is based on two-tailed Student's *t*-test. (*, *P* value <0.05; ***, *P* value <0.001; ****, *P* value <0.0001).

constructs as seen in HeLa cells transfected with 45 nM of a miR-24 mimic (Dogar *et al.*, 2011).

To confirm that miR-24 expression was maintained for at least 72 h post-transfection (p.t.), total RNA was isolated for analysis by qRT-PCR. Twenty micromolar decanoyl-Arg-Val-Lys-Arg-CH_2Cl (FI, furin inhibitor), a potent peptide-based irreversible inhibitor of furin-like enzymes (Jean *et al.*, 1995, 1998), was used as a positive control for inhibition of furin enzymic activity. Levels of miR-24 increased over 100-fold p.t. at 60, 120 and 240 nM relative to untreated cells and were maintained up to 72 h p.t. (Fig. 3a). No increase of miR-24 was observed following transfection with the neg-miRs or FI. The increase in levels of the miR-24 mimic was reflected by a significant decrease in furin mRNA expression by 72 h p.t., further confirming that furin mRNA is targeted by miR-24 in A549 cells (Fig. 3b). While furin mRNA expression was reduced in A549 cells, reflecting the published data for HeLa cells, a large number of miR-24 targets are only regulated at the translational level, with no effect being observed on levels of mRNA expression (Guo *et al.*, 2012; Hatziapostolou *et al.*, 2011; Lal *et al.*, 2008; Oda *et al.*, 2012). We therefore used a fluorescence-based assay for detecting host cell furin enzymic activity (Bourne & Grainger, 2011; Jean *et al.*, 1995, 1998; Richer *et al.*, 2004). At 48 h p.t., furin enzyme activity was reduced in cells treated with 120 nM of the miR-24 mimic or 20 μM FI compared to cells treated with

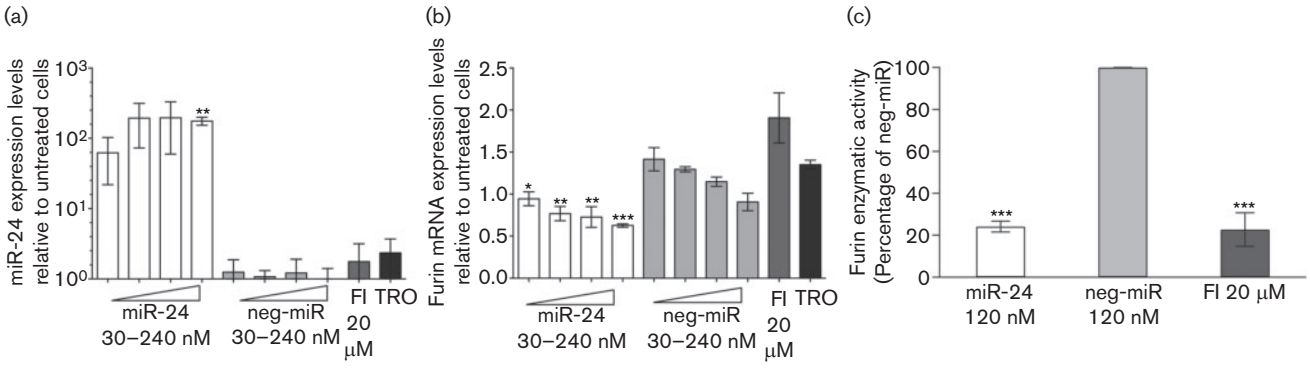

Fig. 3. Overexpression of miR-24 reduces furin mRNA expression and enzymic activity. (a, b) A549 cells were transfected with a miR-24 mimic or neg-miR mimic (neg-miR) at 30–240 nM, treated with the FI at 20 μM, or treated with the transfection reagent only (TRO). RNA levels of miR-24 (a) or furin (b) were assessed at 72 h p.t. Data points represent the mean ± SEM of three experiments. Significance is based on two-tailed Student's *t*-test. (c) Percentage furin activities associated with A549 cells treated with miR-24 mimics (120 nM) or FI (20 μM) 48 h p.t. The values were calculated by normalizing the initial reaction rates of pERTKR-MCA hydrolysis by cellular furin measured in A549 cells treated with miR-24 mimics or FI to the initial reaction rate measured in A549 cells transfected with neg-miR mimic. (*, *P* value <0.05; **, *P* value <0.01; ***, *P* value <0.001).

120 nM of neg-miR (Fig. 3c). Our data therefore confirmed that miR-24 targets furin in A549 cells, resulting in a decrease in furin mRNA expression and enzymic activity.

Treatment with exogenous miR-24 reduces HP avian-origin infA H5N1 infectious virus release and virus spread in human A549 cells

With evidence that miR-24 is downregulated during HP H5N1 infection at multiple time points, we next determined whether miR-24 could reduce furin expression during H5N1 infection, thereby limiting furin-mediated HA0 activation and infectivity of the virus. We began by transfecting A549 cells with the miR-24 or neg-miR mimics at 30, 60, 120 and 240 nM for 24 h before infecting with H5N1 at an m.o.i. of 0.0001. Upon infection, cells in select wells (with no previous treatment) were treated with 20 μM FI. At 24 and 48 h p.i., cells and supernatant were harvested. Using qRT-PCR to quantify furin expression from infected cells, A549 cells to which exogenous miR-24 was added demonstrated a significant reduction in furin mRNA at 24 h p.i. compared with untreated, infected cells (48 h p.t.) (Fig. 4a). Reduction in furin mRNA expression by the neg-miR at the highest concentrations may be a result of off-target effects although this did not translate into a significant reduction in infectious virus release or virus spread. By 48 h p.i., furin mRNA expression remained lower than controls although not significantly (Fig. 4b). Since FI acts as a catalytic-site directed inhibitor of furin-like enzymes (Jean *et al.*, 1995, 1998), no reduction in furin mRNA levels was expected or observed following treatment with FI at both 24 (Fig. 4a) and 48 h p.i. (Fig. 4b).

In addition to furin mRNA expression, cells harvested following treatment with the miR-24 or neg-miR mimics, as described previously, were assayed for viral RNA by qRT-PCR. Interestingly, there was no reduction in the amount of viral RNA produced at 24 or 48 h p.i. (Fig. 4c). These results indicate that high levels of miR-24 do not affect viral replication. To determine the release of infectious virus, plaque assays using supernatant collected at 24 and 48 h p.i. were performed using Madin–Darby canine kidney (MDCK) cells (Fig. 4d). A two-log significant decrease in infectious virus was observed at 24 h p.i. following treatment with FI. Treatment with the miR-24 mimic led to a one-log significant decrease in infectious virus release. Treatment with the transfection reagent by itself resulted in no significant reduction of infectious virus production. By 48 h p.i., the effect from the exogenously added miR-24 mimic was abrogated, and no significant reduction was observed from any treatment (Fig. 4d).

In addition to pre-treating A549 cells with the miR-24 and neg-miR mimics, we evaluated the effect of miR-24 on infected cells post-infection. A549 cells were infected with H5N1 at an m.o.i. of 0.0001 and were transfected at 24 h p.i. with 240 nM miR-24 or neg-miR mimics or treated with 20 μM FI. Cells and supernatant were harvested at 24 h p.i. to establish a baseline for infection and again at 48 and 72 h p.i., corresponding to 0, 24 and 48 h p.t., respectively. Viral RNA was quantified by qRT-PCR and similarly to previous results, there was no effect on virus replication (Fig. 5a). Supernatant was titrated by plaque assays on MDCK cells, and no reduction in infectious virus was observed at 48 and 72 h p.i., even following treatment with the FI that resulted in a two-log drop in infectious virus release when added at the time of virus inoculation (Fig. 5b). We hypothesize that an increase in furin expression following infection is at levels that cannot be reduced sufficiently by overexpression of the miR-24 mimic or by addition of the FI to reduce HA cleavage and impair the release of infectious virions. Additionally, an altered intracellular microenvironment upon infection may also play a role in the reduced effect of miR-24 and the FI post-infection. For example, the intracellular localization of furin molecules may be altered in response to physiological stress, and this phenotype could also play a role in the reduced effectiveness of the FI and/or miR-24 (Arsenault *et al.*, 2012).

To look at virus spread, A549 cells were transfected with the various concentrations of miR-24 or neg-miR mimics for 24 h before infection with H5N1 at an m.o.i. of 0.0001 (Fig. 6a). Immediately following infection, cells in select wells were treated with 20 μM FI. At 24 h p.i., the cells were fixed and probed for expression of the viral nucleoprotein (NP) (Fig. 6a). Virus spread from individually infected cells was observed as a comet-like formation of NP staining (Fig. 6b). Treatment with FI completely blocked any virus spread and only individually infected cells were visible. Compared to the neg-miR mimic, miR-24 completely blocked virus spread in a dose-dependent manner with full inhibition of virus spread at 240 nM as only individually infected cells were visible, similar to the wells treated with FI. Interestingly, the impact of miR-24 on H5N1 infectivity appeared to be greater in the virus-spread assay when the cell culture was pretreated with miR-24 mimics, unlike the plaque assay where virus recovered from the supernatant of miR-24 treated cells was assayed on naive cells. This could indicate that miR-24 affects more than just HA0 cleavage and activation, and it may leave neighbouring cells non-permissive to maintaining virus infection or virus spread.

In summary, we have shown that treating A549 cells with exogenous miR-24 prior to infection reduces infectious virus production and spread, and we have demonstrated that this impact is concomitant with reduced furin expression. Importantly, our data demonstrate that high levels of cellular miR-24 negatively impact HP H5N1 virus infection.

MiR-24 does not affect LP swine-origin infA H1N1 virus infection

To further investigate the role of miR-24 during infA infection, the effects of exogenous miR-24 on the 2009 pandemic H1N1 virus strain were assessed. Unlike the HP H5N1 virus, LP H1N1 does not require furin for HA0 cleavage. As previously described, A549 cells were transfected

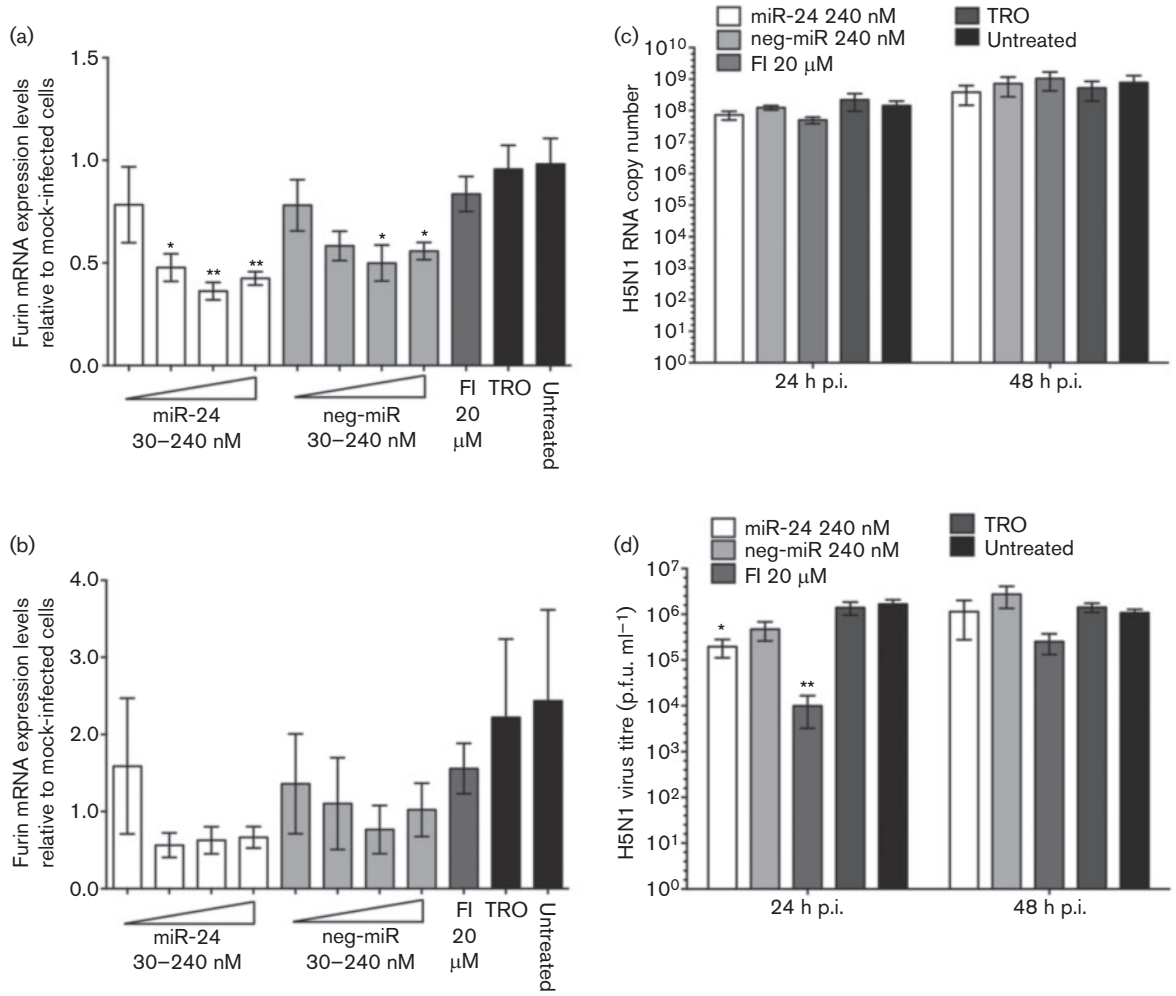

Fig. 4. Overexpression of miR-24 during highly pathogenic avian-origin influenza A H5N1 infection reduces furin mRNA expression and infectious virus released. (a, b) qRT-PCR analysis of furin mRNA expression at (a) 24 and (b) 48 h p.i. and treatment with miR-24 mimic, neg-miR mimic, FI, transfection reagent only (TRO), or normal infection (no additional treatment, Untreated) compared with mock-infected cells. Data points represent the mean ± SEM of five experiments. Significance is based on one-way ANOVA with Bonferroni's post-test. (c. d) A549 cells were transfected with 240 nM miR-24 mimic, 240 nM neg-miR mimic, or TRO before infecting with H5N1 24 h later. Upon infection select wells were treated with 20 μM FI. Cells and supernatant were harvested at 24 and 48 h p.i. Total RNA was assessed for viral RNA (c) while supernatant was titrated by plaque assay on MDCK cells (d). Data points represent the mean ± SEM of five experiments. Significance is based on one-way ANOVA with Bonferroni's post-test. (*, P value <0.05; **, P value <0.01).

with 240 nM of miR-24 or neg-miR mimics. At 24 h p.i., the cells were then infected with the LP 2009 H1N1 infA virus at an m.o.i. of 0.0001. Select wells were treated with 20 μM FI, and cells and culture supernatant were harvested at 24 and 48 h p.i. Viral RNA was quantified by qRT-PCR and, as with H5N1, no effect was seen on H1N1 virus replication (Fig. 7a). Supernatant from 24 and 48 h p.i. was titrated by plaque assay on MDCK cells (Fig. 7b). At 24 and 48 h p.i., no significant reduction in infectious virus was observed, suggesting that miR-24 acts in a strain-specific manner and is most likely affecting HA0 cleavage by downregulating furin. Since furin is not required for HA0 cleavage of LP infA viruses, miR-24 modulation of furin would not be expected to result in inhibition of HA0 cleavage of LP infA viruses (Foucault *et al.*, 2011; Kido *et al.*, 2012).

In conclusion, we showed a viral-specific upregulation of furin mRNA in A549 cells during the HP H5N1 infA life cycle that was not observed in A549 cells infected with LP swine-origin infA H1N1 virus. The viral-associated deregulation of furin expression inversely correlated with the expression of miR-24, a novel regulator of furin activity in A549 cells. Using miR-24 mimics, we demonstrated that the expression levels of miR-24 and furin in A549 cells are two important molecular determinants of HP H5N1 infectious virion production and virus spread in host cells. Since many human enveloped viruses such as HP H5N1

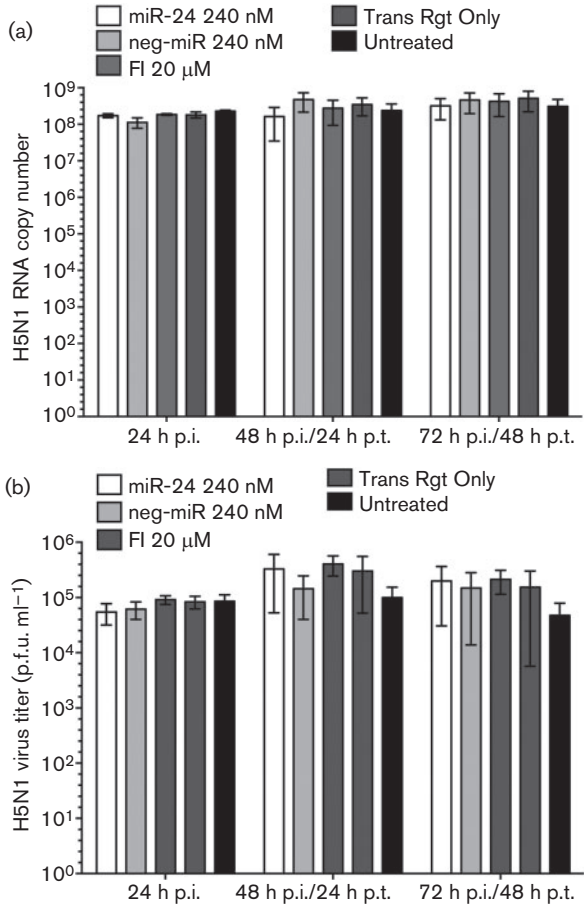

Fig. 5. Treatment with miR-24 following H5N1 infection does not reduce viral titres. A549 cells were infected with H5N1 at an m.o.i. of 0.0001 for 24 h before transfecting with 240 nM of miR-24 mimic, neg-miR mimic or transfection reagent only (Trans Rgt Only) or treating with 20 μM of FI. Cells and supernatant were harvested at 24 h p.i. and at 24 and 48 h p.t. (48 and 72 h p.i., respectively). (a) Viral RNA was assessed by qRT-PCR and no changes were observed between the different treatments compared to normally infected cells. (b) Supernatant was titrated by plaque assay on MDCK cells and again no significant difference was observed with any of the various treatments. Data points represent the mean ± SEM of three experiments.

share an absolute requirement for a selective proteolytic cleavage of the viral spike glycoprotein by furin to yield fusion-competent infectious virions, our results identifying human miR-24 as an important regulator of furin activity and HP H5N1 viral spread provide new insights into host–virus interactions. Furthermore, these results open up potential new therapeutic avenues for developing a novel class of broad-spectrum indirect-acting antivirals.

METHODS

Cell lines and viruses. All *in vitro* experiments were performed under the guidance of the National Centre for Foreign Animal Disease (NCFAD) in a negative pressure high efficiency particulate air-filtered enhanced Biosafety level 3 laboratory with the use of a powered air-purifying respirator, according to Biomedical Microbiological and Biomedical Laboratory procedures [Canadian Food Inspection Agency (CFIA), Laboratory Safety Guidelines]. Influenza viruses A/Mexico/InDRE4487/2009 (H1N1) and A/Chicken/Vietnam/14/2005 (H5N1) were propagated on MDCK cells in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10 % heat-inactivated FBS, 1 % glutamax and 1 % penicillin/streptomycin (P/S; Gibco). Viruses were titrated on MDCK cells to determine p.f.u. ml^{-1}. The human type II pulmonary epithelial cell line A549 was used for all subsequent infections, and cells were propagated in F-12 medium supplemented with 1 % P/S and 10 % heat-inactivated FBS. A549 cells were infected with the H1N1 and H5N1 viruses at an m.o.i. of 0.0001. In contrast to highly pathogenic avian-origin infA H5N1 virus, infection with low-pathogenicity swine-origin infA H1N1 virus was performed in the presence of 1 μg (L-tosylamido-2-phenyl)ethyl chloro-methyl ketone-treated (TPCK) trypsin ml^{-1} (Weingartl *et al.*, 2010). Cell supernatant and RNA were collected at up to 72 h p.i. Zero hour time points correspond to samples collected immediately after the 1 h virus incubation with additional time points numbered with regard to the end of virus incubation. Mock-infected A549 cells were propagated for each experiment with samples collected at the 72 h time point.

RNA isolation. Total RNA was isolated using TRIzol (Invitrogen Life Technologies) following the instructions of the supplier. The concentration of total isolated RNA was measured by using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific).

qTR-PCR. Real-time (RT)-PCR was carried out on total RNA collected from each time point to determine the amount of viral RNA present. Viral RNA levels were evaluated for viral matrix gene transcript levels in duplicate on the Stratagene Mx3005P PCR multiplex quantitative PCR instrument (Agilent Technologies) using the Quantitect Probe RT-PCR kit (Qiagen). Matrix gene forward and reverse primers and probe as described previously were used in the HP infA H5N1 experiments (Spackman *et al.*, 2002). A modified version of this assay (Weingartl *et al.*, 2010) was used to quantify viral RNA levels in LP infA H1N1 experiments. Samples from each time point were analysed in duplicate. Data are represented as the mean ± SEM.

qRT-PCR was used to validate mRNA and miRNA expression changes using the Stratagene Mx3005P real-time PCR system (Agilent Technologies). The mRNA reverse transcription reactions were performed using a High Capacity cDNA RT kit (Applied Biosystems), according to the manufacturer's instructions. For each reaction, 200 ng total RNA was used. Using the Stratagene Brilliant III qPCR master mix, qRT-PCR was used to determine furin mRNA expression using TaqMan gene expression assays (catalogue number 4331182; Applied Biosystems), and data were normalized to β-actin (IDT) expression using the $2^{-\Delta\Delta C_t}$ method. Primer sequences for β-actin assay-probe, ACTCCATGCCCAGGAAGGAAGGC: primer 1, GCCC-TGAGGCACTCTTCC; primer 2, GGATGTCCACGTCACACTTC. Data are represented as the mean ± SEM. MiRNA reverse transcription reactions were performed using the Universal cDNA Synthesis kit and RNA Spike-In kit (Exiqon). For each reaction, 20 ng total RNA was used and all samples were spiked with a synthetic miR-39-3p of *Caenorhabditis elegans* (cel-miR-39-3p) as a standard for qPCR control. miRCURY LNA PCR primer sets (Exiqon) for miR-24-3p and miR-39-3p were used in conjunction with the SYBR Green master mix (Qiagen) for qRT-PCR to determine miR-24 expression levels. Data were normalized to miR-39-3p expression using the $2^{-\Delta\Delta C_t}$ method; data are represented as the mean ± SEM.

Transfections. A549 cells were seeded at 1×10^5 cells per well in 24-well plates and transfected with miR-24-3p miRNA or All star negative control siRNA-AF 555 (neg-miR) mimics (Qiagen) at 30, 60,

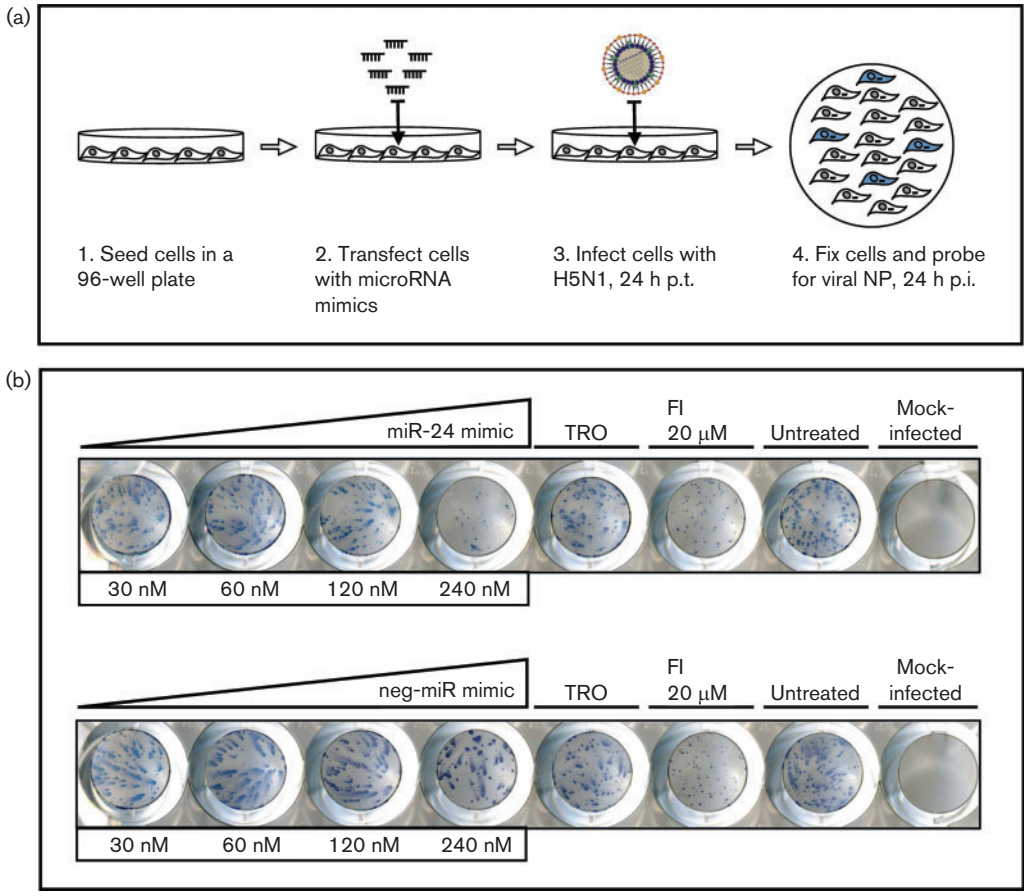

Fig. 6. Overexpression of miR-24 reduces spread of highly pathogenic avian-origin influenza A H5N1 infectious virus. (a) Model of virus spread assay in which A549 cells are transfected with miR-24 mimics or neg-miR mimics at 30–240 nM for 24 h before infecting with H5N1 at an m.o.i. of 0.0001. At 24 h p.i. the cells were fixed and probed for the viral NP protein via immunostaining to assess virus spread. (b) Virus spread assay following transfection with the miR-24 or neg-miR mimics at various concentrations (30 nM–240 nM). Controls included FI, transfection reagent only (TRO) and untreated cells. Individual infected cells are visible in the FI- (20 μM) and 240 nM miR-24-treated cells, whereas virus spread in the form of a comet like appearance is readily apparent in the other treated cells.

120 or 240 nM using X-tremeGENE siRNA transfection reagent (Roche). Following transfection, cells were incubated for 24 h before infection with HP H5N1 or LP H1N1 infA virus. Select wells were left untreated until infection and were then treated with 20 μM FI (decanoyl-Arg-Val-Lys-Arg-CH_2Cl; Calbiochem) post-virus adsorption (Jean *et al.*, 1995, 1998). Supernatant from the infected cells was collected at indicated time points, and viral yield was determined by plaque assay (see below).

Spread assays and plaque assays. A549 cells were seeded at 3×10^4 cells per well in 96-well plates and transfected with the miR-24 or neg-miR mimic. Cells were incubated for 24 h before infection with HP H5N1 at an m.o.i. of 0.0001. Twenty micomolar FI was added to appropriate wells following virus adsorption. Normal infected wells were untreated until infected with the virus, and mock-infected wells received no treatment or virus. The cells were then incubated for an additional 24 h before removing media and fixing with 10 % buffered formalin. The cells were permeabilized with acetone at 37 °C for 1 h before blocking for 1 h at 37 °C. The permeabilized cells were then incubated with an anti-NP monoclonal antibody (1 : 1000; H5: F26NP-9-2-1; CFIA) for 1 h at room temperature. A secondary horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz) was used at 1 : 2000, and virus spread was visualized following staining with True Blue peroxidase substrate (Mandel Scientific). Plaque assays for HP H5N1 were performed on confluent MDCK cells and stained as described above for viral NP expression at 3 days p.i. Plaque assays for LP H1N1 were performed on confluent MDCK cells and were fixed and stained with crystal violet at 5 days p.i. All plaque assays were done in FBS-free DMEM supplemented with 1 % P/S. H1N1 plaque assay medium was supplemented with 2 μg TPCK trypsin ml^{-1}. A 3 % CM-cellulose overlay was diluted to 1.5 % with 2x modified Eagle's medium (MEM) and supplemented with 0.3 % BSA and 1 % P/S. H1N1-infected cells were overlaid with 2 μg TPCK trypsin ml^{-1}. All cells were fixed with 10 % buffered formalin.

Furin enzymic assay. The furin enzymic assay was performed as described previously using the pyroGlu-Arg-Thr-Lys-Arg-4-methyl-coumaryl-7-amide (pERTKR-MCA; Bachem) fluorogenic substrate (100 μM) (Bourne & Grainger, 2011; Jean *et al.*, 1995, 1998). Briefly, A549 cells were seeded at 1×10^5 cells per well and transfected with the miR-24 or neg-miR mimic. At the same point, 20 μM FI was

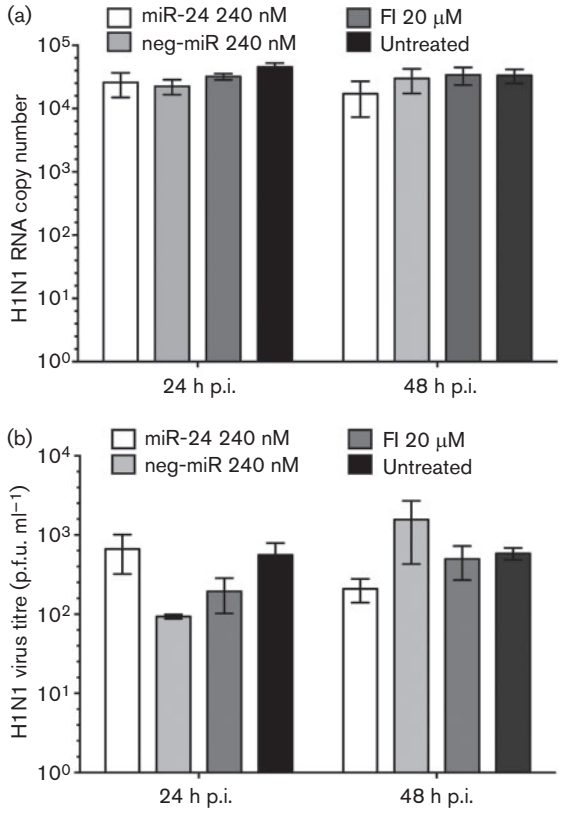

Fig. 7. Overexpression of miR-24 does not affect low-pathogenicity swine-origin influenza A H1N1 virus. A549 cells were transfected with 240 nM miR-24 mimic or neg-miR mimic for 24 h before infecting with H1N1 at an m.o.i. of 0.0001. Upon infection, select wells were treated with 20 μM FI. Cells and supernatant were harvested at 24 and 48 h p.i. (a) Viral RNA levels were assessed by qRT-PCR at 24 and 48 h p.i. (b) Supernatant was titrated by plaque assay on MDCK cells at 24 and 48 h p.i. Data points represent the mean ± SEM of three experiments. Significance is based on one-way ANOVA with Bonferroni's post-test.

added to select wells. At 24, 48 and 72 h p.t., cell lysates were harvested and whole-cell lysates were assayed for furin-like enzyme activity using a SpectraMax Gemini XS spectrofluorometer (Richer *et al.*, 2004).

Luciferase assay. Firefly luciferase reporter genes containing repeat miR-24 binding sites or full-length human 3′ UTR from furin were used (S209837; SwitchGear Genomics). For luciferase assays, A549 cells were grown in black 96-well plates; 50 ng plasmid DNA was transfected per well along with miR-24 or neg-miR mimics at various concentrations using DharmaFECT Duo reagents according to the manufacturer's instructions (T-2010; Thermo Scientific). After 24 h, cells were treated with LightSwitch luciferase assay reagents according to the manufacturer's instructions (LS100; SwitchGear Genomics). Luminescence signals were measured on a Varioskan Flash Multimode microtitre plate reader (Thermo Scientific).

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