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TITLE PAGE

Syk mediates BEAS-2B cell migration and proliferation, and HRV-induced expression of VEGF and IL-8

Xiaomin Wang, Mirek Mychajlowycz, Christine Lau, Carlos Gutierrez, Jeremy A. Scott and Chung-Wai Chow

From the Division of Respirology and Multi-Organ Transplantation Programme, University Health Network, Department of Medicine (XW, MM, CL, CG, CWC), and the Division of Occupational and Environmental Health, Dalla Lana School of Public Health, Faculty of Medicine (JAS), University of Toronto, Toronto, Ontario, Canada (XW, MM, CL, CG, JAS, CWC)

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RUNNING TITLE PAGE

Running Title: Syk regulates airway epithelial cell proliferation

Corresponding Author: Chung-Wai Chow,

University Health Network, University of Toronto

585 University Avenue, NCSB 11C-1185

Toronto, ON, Canada M5G 2N2

Tel: 416-340-3512 Fax: 416-340-3109

Email: cw.chow@utoronto.ca

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List of abbreviations: Syk: spleen tyrosine kinase; HRV: human rhinovirus; BEAS-2B: human bronchial epithelial cell line, NHBE: normal human bronchial epithelial cells; ICAM-1: intercellular adhesion molecule; MAP: mitogen activated protein; IL-8: interleukin-8; VEGF: vascular endothelial growth factor; TGF: transforming growth factor; EGF: epidermal growth factor; ANOVA: analysis of variance; VCAM: vascular cell adhesion molecule

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ABSTRACT

Syk is an immunoregulatory tyrosine kinase that was originally identified in leukocytes. It is a key regulator of innate immunity as well as hematopoietic cell differentiation and proliferation. A role for Syk in regulating normal cellular functions in non-hematopoietic cells is increasingly recognized. We have previously shown robust Syk expression in airway epithelium where it regulates the early inflammatory response to human rhinovirus (HRV) infections, and HRV cell entry by clathrin-mediated endocytosis. To test the hypothesis that Syk plays a role in modulating airway epithelial cell proliferation, migration and production of VEGF and IL-8, we studied the BEAS-2B human bronchial epithelial cell line and primary human airway epithelia from normal and asthmatic donors using Syk-specific pharmacologic inhibitors and siRNA. Using an *in vitro* 'wounding' model, we demonstrated significant impairment of 'wound' closure following treatment with the Syk inhibitors, R406 and Bay61-3606, over-expression of the kinase-inactive Syk^{K396R} mutant, and Syk knock-down by siRNA. HRV infection also impaired wound healing, an effect that was partly Syk-dependent as wound healing was further impaired when HRV infection occurred in the presence of Syk inhibition. Further investigation of potential regulatory mechanisms revealed that inhibition of Syk suppressed HRV-induced vascular endothelial growth factor (VEGF) expression while promoting activation of caspase-3, a mediator of epithelial cell apoptosis. Together, these results indicate that Syk plays a role in promoting epithelial cell proliferation and migration, while mitigating the effects of apoptosis.

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INTRODUCTION

Syk is an important immune-regulatory protein tyrosine kinase that has been well described in leukocytes to regulate multiple aspects of innate immunity. These include regulation of myeloid differentiation and proliferation, as well as modulation of immunoreceptor signaling pathways that govern humoral, allergic and cytotoxic immunity (Mocsai et al., 2010). A functional role for Syk is increasingly being recognized in non-hematopoietic cells. Our laboratory has shown Syk to be robustly expressed in airway epithelium where it signals downstream of intercellular adhesion molecule (ICAM)-1, the major receptor for human rhinovirus (HRV), the most common cause of acute infections in humans. In the human bronchial epithelial cell line (BEAS-2B) and normal human bronchial epithelial cells (NHBE), we have previously demonstrated recruitment and activation of Syk within minutes of HRV-ICAM-1 binding (Wang et al., 2006). Syk then activates two signaling pathways: the ERK1/2 and p38 mitogen activation protein (MAP) kinase pathway that lead to expression of the neutrophil chemokine, IL-8 (Wang et al., 2006), and the phosphotidylinosital-3 kinase (PI3K) pathway that mediates HRV cell entry by clathrin-mediated endocytosis (Lau et al., 2008).

Ulanova et al. also observed a role for Syk in mediating the airway epithelial cell inflammatory response: engagement of β_1 integrin led to Syk activation, Syk-dependent induction of IL-6 expression and enhanced surface expression of ICAM-1 (Ulanova et al., 2005) while tumour necrosis factor (TNF)-α stimulation resulted in Syk-dependent nitric oxide expression and induction of iNOS (Ulanova et al., 2006). These observations, together with ours in the context of HRV infection, clearly indicate a role for Syk in the regulation of the airway epithelial cell inflammatory response.

HRV has been reported to induce cytotoxicity and impair wound healing in an *in vitro* model of tissue injury using BEAS-2B cells (Bossios et al., 2005). Other studies have shown HRV to induce airway epithelial cell apoptosis by activation of caspase 3 and caspase 9 (Deszcz et al., 2005; Wark et al., 2005). The role of Syk in these cellular events following HRV infection is not known, although Syk has been shown to be an important regulator of cell survival and proliferation in different cell types. For example, in addition to its critical role in the differentiation of hematopoietic cells to mature B and γδ T lymphocytes, the normal and appropriate activity of Syk appear to be important for B cell homeostasis. Tonic activation of Syk has been found in several common B cell lymphoma subtypes (Gobessi et al., 2009), and is thought to be responsible for abnormal proliferation and survival of the lymphomatous cells.

There is also evidence that Syk regulates proliferation and migration in non-hematopoietic cells. Indeed, the fatal intrauterine hemorrhage observed in homozygous Syk knock-out mice is a result of aberrant development of the blood and lymphatic vessels due to abnormal endothelial cell proliferation and migration (Yanagi et al., 2001; Abtahian et al., 2003). Syk also regulates breast epithelial cell proliferation, migration and differentiation: studies in human ductal cell carcinomas reveal that loss of Syk correlated with increased aggressiveness and metastases of the tumours (Coopman et al., 2000). Subsequent *in vitro* studies have shown that reconstitution of Syk expression abrogated the abnormal cell proliferation observed in a cancerous breast epithelial cell line (Moroni et al., 2004). Therefore, we tested the hypothesis that Syk regulates *airway* epithelial cell proliferation and migration, and plays a role in mitgating the effects of HRV-induced epithelial cell damage and apoptosis.

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MATERIAL AND METHODS

Cell lines and Primary Cells

BEAS-2B cells, a human bronchial epithelial cell line (a generous gift from Dr. Curtis Harris, NCI, Bethesda), were cultured in Clonetics bronchial epithelial growth medium (BEGM; Cambrex Bio Science, Walkersville MD) at 37°C, 5% CO_2 in a humidified environment as previously described (Lau et al., 2008).

Primary airway epithelial cells obtained from normal, non-smoking donors and asthmatics were purchased from MatTek Corporation (Ashland, MA). The clinical history of the donors was provided by MatTek Corporation. The cells were equilibrated in Air 100-MM media (MatTek Corp.) for 16-18 h upon arrival, in an air-liquid interface in a humidified 5% CO_2 incubator prior to experimentation.

Antibodies, Inhibitors, HRV16

Antibodies were purchased from the following sources: mouse monoclonal Syk 4D10 from Santa Cruz (Santa Cruz, CA); rabbit polyclonal phospho-Akt (Thr308), rabbit polyclonal Akt, rabbit polyclonal phospho-p38 and total p38, rabbit polyclonal full length caspase 3 and rabbit polyclonal cleaved-caspase 3 antibodies from Cell Signaling Technology (Danvers, MA). The HRP-labelled anti-mouse and anti-rabbit secondary antibodies were from Jackson ImmunoResearch Laboratory (West Grove, PA). HRV16 was propagated in WI-38 cells (both from ATCC, Manassas, VA), harvested by repeated freeze-thaw cycles at -80°C, purified and stored at -80°C in BEBM in aliquots containing approximately $10^{4.5}$ $TCID_{50}$, as assessed using a microtiter plate assay (Lau et al., 2008).

The Syk selective inhibitors R406 and BAY61-3606 (Yamamoto et al., 2003; Matsubara et al., 2006a; Matsubara et al., 2006b; Li et al., 2009; Ruzza et al., 2009; Riccaboni et al., 2010)

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were a gift of Boehringer Ingelheim AG (Biberach am Riss, Germany). NVP-QAB-205 was a gift from GSK Inc. (Mississauga, ON, Canada)(MacGlashan et al., 2008). While the compounds have been extensively evaluated *in vitro* and *in vivo* (Matsubara et al., 2006a; Matsubara et al., 2006b; Riccaboni et al., 2010), these studies have all been performed in the context of leukocyte signaling. Therefore, we evaluated the efficacy in airway epithelial cells by evaluating known Syk-mediated cellular events following HRV infection in BEAS-2B cells (Supplemental Figure 1): R406 Bay61-3606, and NVP-QAB-205 impaired HRV-induced p38 MAP kinase phosphorylation and PI3 kinase activation to similar degrees as had been previously reported by our group when using genetic means to knock-down Syk activity (Wang et al., 2006; Lau et al., 2008).

Transfection, plasmids and siRNA

BEAS-2B cells were transfected using the Amaxa Nucleofector system according to the manufacturer's instructions using 4 x 10^6 cells and 2 µg of plasmid DNA or 2 x 10^6 cells with 0.75 µg Control *SMART*pool® siRNA reagent or Syk *SMART*pool® siRNA (Upstate, Charlottesville, VA). The cells were plated in normal culture medium following transfection and cultured at 37°C in 5% CO_2 for 36-48 h prior to use for the experiments. Sham-transfected cells underwent the same procedure in the absence of siRNA.

The plasmids expressing human Syk mutants were generated from the pcDNA3 plasmid containing amino-terminal-hemaglutinen (HA) tagged wild type (WT) human Syk using the Quikchange® Site Directed Mutagenesis Kit (Stratagene, #200518, La Jolla, CA). The Syk cDNAs were excised from pcDNA3 as a 1.553 kb *Bam*HI fragment and subcloned into the *Bgl*II and BamHI sites of pEGFP-N2 (Clontech, Mountain View, CA). All mutants were verified by sequencing and Western blot analysis to ensure expression of the protein of appropriate size and

immunoreactivity prior to use for experimentation, and have already been validated in BEAS-2B cells (Lau et al., 2008). We have previously shown that transfection of BEAS-2B cells with Syk *SMART*pool® siRNA (Upstate) specifically knock-down Syk expression and did not affect expression of other HRV signaling pathway molecules, such as ICAM-1 or ezrin (Wang et al., 2006; Lau et al., 2008).

Scratch "Wound" Test and HRV 16 Stimulation Experiments

BEAS-2B cells were grown to confluence on 6-well plates in normal culture medium. 'Wounding' was perform by scoring the cell monolayer using a sterile P100 pipette tip. Two 'scratches' were made per well. Detached cells were removed by washing with prewarmed normal culture medium, and the cells were returned to normal culture conditions for 24 h. For the HRV stimulation experiments, purified HRV16 stock preparations were diluted 1/10 in BEBM and subsequently applied to the confluent BEAS-2B cells. Cells were incubated at 37°C for 1 h. Medium containing HRV16 was aspirated and fresh normal culture medium was added back to the cells. Where indicated, 'wounding' was performed at this time point. The cells were returned to incubation at 37°C for 24 h and harvested for immunoblotting experiments or fluorescence microscopy (described below). Non-stimulated cells were treated in the same manner in BEBM without HRV16. For the pharmacological studies, Syk inhibition was performed by pre-incubating the cells with 0.5-10 μM R406 or BAY61-3606 dissolved in 1M DMSO for 1 h prior to the HRV16 inoculation or 'wounding'. One molar DMSO alone was used as control. Presence of R406 and BAY61-3606 was maintained throughout the duration (24 h) of the experiment. In the experiments with the primary airway epithelial cells, HRV16 inoculation was performed on the apical surface using 200 μl of medium.

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Transmission and Fluorescence Microscopy and Quantification of Wound Repair

Following the induction of 'wounding' using a sterile P100 pipette, BEAS-2B were imaged immediately (0 h) and at 24 h after wounding using a Nikon eclipse TE 200 microscope (Nikon Canada, Mississauga, ON, Canada) and 10X objective using the Simple PCI software (Hamamatsu Corporation, Sewickley, PA, USA). The images were saved as .tiff files and exported into Canvas X. We measured the size of the wound at 0 and 24 h at time of image acquisition using a size bar calibrated for the 10X objective. For each image, 3 measurements across the opposing edges of the wound were recorded. The mean of these measurements (counted as an n of 1) was expressed as a percentage of the initial measurement at 0 h. At least 4 separate experiments were performed for each of the experimental conditions studied.

SDS-PAGE, Western Blot Analysis and ELISA.

We have previously described the methods used for harvesting of cell lysates and measuring protein concentrations (Wang et al., 2006; Lau et al., 2008). For Western analysis of whole cell lysates, 30 μg of protein were loaded per lane and separated by SDS-PAGE using 7.5-10% polyacrylamide gel, transferred to nitrocellulose membrane and immunoblotted with primary and secondary antibodies as previously described (Wang et al., 2006). Quantitation of IL-8, VEGF, TGF-beta and EGF expression was performed by ELISA according to the manufacturer's instructions. The kits for IL-8 (Hu IL-8/NAP-1), VEGF (Hu VEGF), EGF (Hu EGF) and TGF-beta (Muti-species TGF-β1) were purchased from Medicorp Inc. (Montreal, PQ).

Statistical Analysis

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Analysis of variance (ANOVA) was used for factorial analysis. Post hoc tests were conducted when ANOVA reached $p<0.05$, using Tukey's adjustment method. The statistical analysis program Prism 4.0c was used for analysis (GraphPad Software).

RESULTS

Syk mediates cell proliferation and wound closure following injury.

The role of Syk in airway epithelial cell proliferation was assessed using the human BEAS-2B cell line following transfection with Syk-siRNA to knock-down Syk expression. Equal numbers of cells were plated immediately following transfection with Syk- or Control-siRNA, and the cells were returned to normal culture conditions for an additional 72 h. At this time, total cell counts were determined following trypsinization in an automated cell counter (Coulter systems). As shown in Figure 1A and B, total cell numbers were consistently lower in BEAS-2B cells that were transfected with Syk-siRNA compared with Control-siRNA ($p<0.05$, n=4/group). Western blot analysis of the whole cell lysates revealed effective Syk knock-down at 72 h following transfection (Figure 1B, lower right corner). Proliferation of sham-transfected cells was similar to Control-siRNA transfected BEAS-2B cells (data not shown).

Next, we used an *in vitro* model of tissue injury to evaluate the role of Syk in wound repair. Confluent monolayers of BEAS-2B cells grown on 6-well plates were wounded as described in *Material and Methods*. Wound repair/cell migration was then assessed at 24 h post-injury. To evaluate the role of Syk, we used three different approaches: 1) inhibition with the Syk-selective inhibitors, R406 and Bay61-3606, 2) Syk knock-down with siRNA, and 3) over-expression of the dominant-negative kinase-inactive Syk^{K396R} mutant. As shown in Figure 1C, control BEAS-2B cells and those treated with DMSO (vehicle control,) exhibit almost complete closure of the wound at 24 h after wound induction. In contrast, wound closure was impaired in

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cells treated with the Syk selective inhibitors, R406 (0.5 - 1 μM) and Bay61-3606 (1 – 5 μM). Both R406 and Bay61-3606 significantly attenuated wound repair at 24 h (Table 1, No HRV, *$p<0.05$, n=4/group). Although the higher concentrations of the inhibitors appeared to impair wound healing to a greater degree, this effect was not statistically significant.

We also employed complementary techniques to assess the role of Syk activity in this *in vitro* model of wound repair to minimize any ambiguity about the role of Syk in this cellular function. As shown in Table 1 (No HRV), knock-down of Syk using siRNA also impaired wound healing compared with sham-transfected and Control-siRNA transfected cells (**$p<0.05$, n=4/group); no differences were noted between the Sham- and Control-siRNA transfected cells. Expression of the kinase inactive Syk^{K396R} and the dual SH2 domain mutant $Syk^{R46,201A}$ mutant also impaired wound healing compared with Sham- and WT-Syk transfected cells (***$p<0.05$, n=4/group). As integrity of the SH2 domains are critical for Syk activation and function (Lau et al., 2011), impaired wound healing in cells over-expressing $Syk^{R46,201A}$ provides additional evidence in support of a role for Syk in wound healing following injury.

HRV infection impairs wound repair, and is further impaired by Syk inhibition

HRV has been reported to induce cytotoxicity and impair wound healing (Bossios et al., 2005) although a role for Syk in this process has not been described. Therefore, we evaluated the role of Syk on wound healing in BEAS-2B cells following HRV infection (Figure 2). While non-infected Control and DMSO-treated cells exhibited almost complete wound closure at 24 h, the presence of HRV infection significantly impaired closure in both the Control and DMSO-treated cells (Table 1; $p<0.05$, n=4/group). The same phenomenon was observed in cells undergoing Sham, Control-siRNA and WT-Syk transfection; i.e., wound closure was almost 100% at 24 h in

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the non-infected cells, but decreased by one-third in the HRV infected cells (Table 1; $p < 0.05$ when compared with No HRV cells, n=4).

Syk inhibition in the setting of HRV infection further impaired wound healing regardless of the method employed to knock-down Syk activity (Figure 2). In HRV-infected cells, treatment with R406 and Bay61-3606 significantly reduced wound healing to a greater extent than non-infected cells (Table 1; $p<0.05$, n=4/group). Similarly, Syk knock-down by siRNA, and over-expression of the kinase-inactive Syk^{K396R} or $Syk^{R46,201A}$ mutants had a greater effect on delaying wound healing in the presence of HRV infection when compared to cells treated with comparable conditions but not infected with HRV (Table 1; $p<0.05$, n=4/group).

Syk appears to protect from caspase-3 induced apoptosis following HRV infection

While not as cytotoxic as other respiratory viruses such as respiratory syncytivial virus (RSV), HRV has been reported to induce airway epithelial cytotoxicity and apoptosis by activation of caspase-3 (Deszcz et al., 2005; Wark et al., 2005). To evaluate the role of Syk in HRV-induced activation of caspase-3, we used Western blot analysis to detect presence of the 17 kDa and 19 kDa caspase 3 cleavage products. As shown in Figure 3, HRV16 infection alone resulted in activation of caspase-3, with emergence of the 19 kDa cleavage product being most apparent at 3 h following HRV inoculation in Sham and Control-siRNA transfected cells. On the other hand, transfection with Syk-siRNA (Figure 3A, far right 5 panels) enhanced caspase 3 activation at all time points evaluated. Note that Syk expression was decreased only in cells transfected with Syk-siRNA. Similar observations were made in BEAS-2B cells treated with 1 and 5 μM Bay61-3606 (Figure 3B)

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HRV-induced expression of vascular endothelial growth factor (VEGF) and IL-8 is mediated by Syk

VEGF is known to be induced by HRV infections and has been implicated as a mediator of airway remodeling in asthma (Leigh et al., 2008). Therefore, we assessed the role of Syk in the regulation of HRV-induced VEGF expression. We observed significantly increased VEGF expression at 3 h and 9 h following HRV infection in control and DMSO-treated BEAS-2B cells (Figure 4A, $p < 0.001$, n=3/group). Inhibition of Syk with 10 μM R406 and Bay61-3606 significantly decreased VEGF expression at both time points ($p<0.05$, n=3/group). We had previously shown HRV-induced IL-8 expression to be attenuated when Syk expression was knock-downed by siRNA (Wang et al., 2006). Therefore, we evaluated the effects of R406 and Bay61-3606 on IL-8 expression, and observed that both inhibitors significanty decreased IL-8 expression at 3 h and 9 h post HRV infection (Figure 4B, $p < 0.05$). Subsequent studies using 1 and 2 μM of R406 and Bay61-3606 yielded similar observations with no obvious dose-dependence (data not shown). Taken together, it appears that Syk mediates the induction of VEGF expression, and is also responsible for maintaining EGF expression following HRV infection.

Syk mediates VEGF and IL-8 expression in primary normal and asthmatic airway epithelia

To further validate the role of Syk in airway epithelial cell function, we assessed the effects of R406 and Bay61-3606 on VEGF and IL-8 secretion by primary airway epithelium obtained from donors with and without asthma. As shown in Figure 5A, we observed high basal VEGF expression in both non-asthmatic and asthmatic primary airway epithelial cells (p=n.s., n=3/group). R406 and Bay61-3606 exhibited no significant effect on basal VEGF expression in either the non-asthmatic or asthmatic epithelium (n=3/group). Increased VEGF expression was

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observed following HRV inoculation, which was decreased to varying degrees in cells treated with inhibitors; with R406-treated cells showing significant differences (*$p<0.05$, n=3).

We also measured IL-8 expression in the primary airway epithelia (Figure 5B). Basal IL-8 expression was significantly higher in the non-asthmatic epithelial cells compared with the asthmatic epithelial cells ($p<0.05$, n=3/group). Treatment with the Syk inhibitors did not affect basal IL-8 expression in either the asthmatic or non asthmatic epithelium. HRV induced IL-8 expression in both non-asthmatic and asthmatic epithelial cells to similar levels at 9 h following inoculation, despite differences in basal expression. In both asthmatic and non-asthmatic epithelia, treatment with the R406 and Bay61-3606 attenuated expression of IL-8 compared with Control and DMSO treated cells (*$p<0.05$, respectively; n=3/group). This was most apparent at 9 h following HRV inoculation.

We also evaluated a third generation Syk inhibitor, NVP-QAB-205, a purine derivative with an IC_{50} of 10 nM that is similar to Bay61-3606, an imidazopyrimidine analog, but with greater potency than R406, an imidazopyrimidine analog that has an IC_{50} of 40-160 nM (Li et al., 2009; Ruzza et al., 2009; Riccaboni et al., 2010). NVP-QAB-205 significantly attenuated HRV-induced expression of VEGF and IL-8, and similar to R406 and Bay61-3606, had no effect on EGF expression (Figure 5C and D, *$p<0.05$ compared to control and DMSO-treated cells, same time point, n =3).

DISCUSSION (1428 words)

Our observations in an *in vitro* model using BEAS-2B cells clearly reveal a role for Syk in wound repair following injury and HRV infection. The effect of HRV is, in part, independent of Syk as infection alone was sufficient to significantly reduce wound repair with evidence of an

additive effect when infection occurred in the presence of Syk inhibition. In primary cells, treatment with R406 and NVP-QAB-205 significantly attenuated VEGF expression at 3h and 9h following HRV infection, reducing expression levels below that at baseline, suggesting a role for Syk in mediating basal VEGF expression. In the BEAS-2B cell line, basal VEGF expression was negligible, and treatment with Syk inhibitors only partially reduced VEGF expression at 3h and 9h post HRV infection. This disparity is most likely due to inherent differences between cell lines vs. primary cells. Indeed, while the BEAS-2B cell line is extensively used as a surrogate of airway epithelial cells, they were derived by transforming human bronchial epithelial cells with an adenovirus12-simian virus 40 (SV40) construct (Reddel et al., 1988). In the process of immortalization, they have lost some of the characteristics of airway epithelium, such as formation of tight junctions. Moreover, the primary cells were grown at an air-liquid interface to allow for epithelial cell differentiation and maturation whereas BEAS-2B cells were grown in submerged culture, another difference that contribute to the observed difference in the BEAS-2B and primary airway epithelial cell responses (Figure 4 and 5).

Differences between the BEAS-2B and the primary cells were most notable in the epidermal growth factor (EGF) response to HRV infection (Supplemental Figure 2). EGF and EGF receptor signaling have been shown to promote chemotaxis and wound repair in scrape-wounded airway epithelial cell monolayers (Puddicombe et al., 2000). Whereas HRV infection suppressed EGF expression in BEAS-2B cells, it induced expression in normal primary airway epithelia; in the asthmatic primary airway epithelia, basal levels were already high, and HRV infection did not induced further augmentation of EGF production. Under no condition was EGF expression Syk-dependent as treatment with R406, Bay61-3606 and NVP-QAB-205 had no effect when compared to Control and DMSO-treated cells.

While previous studies have reported Syk to regulate cell proliferation and migration during malignant transformation of breast ductal epithelial cells (Coopman et al., 2000; Moroni et al., 2004), our observations suggest that Syk is important in maintaining the *homeostatic* integrity of the epithelium in response to mechanical injury, and following infection with a common virus, such as HRV.

Syk as a regulator of cell proliferation and differentiation is well described in hematopoietic cells. Studies in chimeric mice that were Syk-deficient in the hematopoietic progenitor lineages revealed Syk to be critical for the expansion and differentiation of B and γδ T-lymphocytes. Normal Syk function is critical for maintaining B cell homeostasis; dys-regulated Syk activation results in aberrant proliferation and the development of several common types of B cell lymphomas and chronic lymphocytic leukemia (Gobessi et al., 2009; Young et al., 2009). Increased B cell survival has been attributed to tonic Syk activation, leading to sustained Akt activation, up-regulation of anti-apoptotic proteins such as Mcl-1 and increased resistance to apoptosis (Longo et al., 2008; Gobessi et al., 2009). Conversely, inhibition of Syk with R406 induced apoptosis, correlating with decreased ERK and Akt signaling, and Mcl-1 expression (Gobessi et al., 2009). In addition to B cells, Syk promotes survival of natural killer cells in response to osmotic stress and ultra-violet irradiation (Jiang et al., 2002), and eosinophils in response to cytokine-induced apoptosis (Yousefi et al., 1996).

Our observations indicate Syk to have a positive role in promoting airway epithelial cell migration proliferation and survival: wound closure requires both cell migration and proliferation. A similar role of Syk had been shown in vascular endothelium. The fatal bleeding diathesis of the Syk-deficient mouse *in utero* result from abnormal vascular endothelial cell development, with reduced cell numbers and aberrant morphogenesis (Yanagi et al., 2001;

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Abtahian et al., 2003). Studies with human umbilical vein endothelial cells revealed that Syk was required for cell proliferation and migration: over-expression of a dominant-negative Syk decreased cell growth and impaired wound healing (using the *in vitro* wound assay) when compared with WT-Syk (Inatome et al., 2001). Similar observations have been made in rat aortic smooth muscle cells, where inhibition of Syk pharmacologically or with Syk-siRNA decreased cell proliferation and migration in response to mitogens such as platelet-derived growth factor-BB (Lee et al., 2007), soluble vascular cell adhesion molecule-1 (Lee et al., 2008) and angiotensin II (Mugabe et al., 2010). Syk also regulates cell migration in myeloid cells: in the neutrophilic HL-60 cell line, Syk mediates cell migration by regulating the formation of the leading edge following β2 integrin engagement, and regulates activation and recruitment of PI3K-δ to the leading edge (Schymeinsky et al., 2005; Schymeinsky et al., 2007). CD95 ligand-mediated recruitment of myeloid cells to sites of injury is also mediated by Syk recruitment and activation (Letellier et al., 2010).

Previously reports of Syk as a promoter of cell survival, proliferation and migration in *epithelial cells* are controversial. Studies in breast ductal cell carcinomas revealed that progressive loss of Syk expression in breast epithelial cells was associated with increasing malignancy and worse prognosis (Coopman et al., 2000). Studies to-date have suggested that Syk suppresses abnormal proliferation of the cancerous cells by regulating centrosome function and transcription as well as the urokinase-type plasminogen activator via the PI3K/NFκB pathway, rather than by regulation of apoptosis (Coopman et al., 2000; Mahabeleshwar and Kundu, 2003; Zyss et al., 2005). On the other hand, a report in breast epithelial cell lines of various cancerous origins found the presence of Syk, rather than its absence, to have a protective role in apoptosis (Zhou and Geahlen, 2009). Adding to the controversy is a report in melanoma cells identifying Syk as a tumour suppressor that induces senescence-like growth arrest (Bailet et al., 2009). It is

possible that the role of Syk in cell survival and apoptosis is cell-type and/or stimulus-specific. Our observations using siRNA indicate that Syk mitigates the cytotoxicity of HRV infections by attenuating caspase-3 activation is further supported by similar observations using Bay 61-3606 to inhibit Syk activity.

It should be noted, however, that the effects of HRV infection on airway epithelia also remain controversial. Some investigators reported no appreciable airway epithelial cytotoxicity or apoptosis following HRV infection despite evidence of disruption of tight junction and Zo-1 localization leading to increased paracellular permeability, bacterial adhesion and transmigration (Sajjan et al., 2008). Others demonstrated HRV serotypes to have differential cytotoxicity with HRV7 being the most, and HRV16, the least cytotoxic (Bossios et al., 2005; Wark et al., 2009). We observed peak caspase-3 activation at 3 h post inoculation; induction of apoptosis at this early time point following HRV infection may be a protective mechanism to limit the extent of infection, as propagation of the virus depends on intracellular replication which typically occurs 8-12 h following inoculation. Recent studies have suggested intrinsic differences between the airway epithelia of asthmatic and non-asthmatic individuals (Freishtat et al., 2011; Hackett et al., 2011); HRV infections in asthmatic patients are prolonged and are more severe when compared to those in non-asthmatics (Corne et al., 2002; Message et al., 2008). A recent study found the asthmatic airway epithelia to be more susceptible to HRV-induced apoptosis and cell lysis, and to have a blunted anti-viral interferon, β and λ, response (Contoli et al., 2005; Wark et al., 2005), mechanisms that favour enhanced infections in asthmatics. However, these and our own studies comparing normal and asthmatic airway epithelial responses are not adequately powered, or in fact designed, to address the question of whether intrinsic differences in susceptibility underlie the differences observed. As such, these limitations must be acknowledged.

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We had previously reported Syk to be an important early modulator of HRV signaling by regulating the replication-independent activation of the p38 and PI3 kinase signaling pathways leading to IL-8 expression, and mediates viral cell entry by clathrin-mediated endocytosis (Wang et al., 2006; Lau et al., 2008). In the current manuscript, we have expanded on these observations in primary airway epithelia using three novel Syk-specific inhibitors: our observations reveal that HRV-induced expression of VEGF and IL-8 are attenuated in the presence of Syk inhibitors at 3 h and 9 h post-infection. These time points precede viral replication and corroborate earlier studies made in BEAS-2B and human bronchial epithelial (HBE) cells where VEGF expression was first detected at 8 h post inoculation (Leigh et al., 2008). Our studies using the pharmacological inhibitors also indicate Syk to be important for promoting airway epithelial cell growth and proliferation, and for modulating activation of caspase-3 in response to HRV infection, observations that were confirmed in parallel studies in which Syk activity was down-regulated by Syk-siRNA and by over-expression of dominant-negative Syk mutants. Taken together, our studies suggest that novel Syk inhibitors may be potential therapeutic agents that can modulate some of the inflammatory sequelae of HRV infections.

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AUTHOR CONTRIBUTIONS

Participated in research design: X.W., C.L., J.A.S., C.W.C.

Conducted experiments: X.W., M.M., C.L.

Contributed to new reagents or analytic tools: n/a

Performed data analysis: X.W., M.M., C.G., C.W.C.

Wrote or contributed to the writing of the manuscript: C.L., J.A.S, C.W.C.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Syk promotes BEAS-2B cell proliferation and wound healing

A: Cell densities at 72 h were higher for the Control-siRNA cells when compared to Syk-siRNA transfected cells regardless of the initial plating densities. **B (left panel)**: The total number of cells was lower in the Syk-siRNA transfected cells in all three groups (*$p<0.05$, n=4). **B (right panel)**: Western blot analysis indicated decreased Syk protein at 72 h in the Syk-siRNA transfected cells. **C**. Treatment with R406 and Bay61-3606 impaired wound healing at 24 h when compared to Control or DMSO-treated cells. Representative of 4 independent experiments.

Figure 2: HRV infection in the presence of Syk inhibition further impairs wound healing

In comparison to cells that were not infected with HRV (**A-C),** HRV infection (**D-F**) impaired wound healing under basal conditions ('Control' and 'Sham'- transfected cells). Wound healing was further impaired when HRV infection occurred in the setting of Syk inhibition by pharmacologic (5μM R406, Bay61-3606, NVP-QAB-205) (**D**) or molecular means (**E,F**). Measurement of the wound at 0 and 24 h is shown. Representative of 4 independent experiments.

Figure 3: Syk attenuates caspase-3 activation in airway epithelial cells

A: *Top panel*: Transfection with Syk-siRNA effectively knocked down Syk expression. *Bottom panel*: HRV induces cleavage of caspase 3 at 3 h in all three experimental groups of cells. Syk-siRNA induces caspase 3 activation in the absence of HRV (C and 0 h) with further enhancement following HRV infection at 1 and 3 h. **B**: Preincubation with Bay 61-3606 (1-5μm) or DMSO prior to inoculation with HRV16, induced caspase-3 cleavage in the absence of HRV, with further induction in the presence of HRV infection. Representative of 3 separate experiments.

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Figure 4. Syk regulates BEAS-2B cell expression of VEGF and IL-8

HRV infection significantly induced expression of VEGF (**A**) and IL-8 (**B**) at 3 h and 9 h (*$p<0.05$, n=3). Inhibition of Syk with R406 and Bay 61-3606 significantly decreased expression of both VEGF and IL-8 at 3 h and 9 h when compared with control and DMSO treated cells (**$p<0.05$, n=3).

Figure 5: Syk regulates primary airway epithelial cell expression of IL-8 and VEGF in response to HRV infection

A, C: In both asthmatic and non-asthmatic epithelia, there is a trend for increased VEGF expression at 3 h and 9 h post HRV infection. Treatment with R406, Bay61-3606 and NVP-QAB-205 decreased VEGF expression when compared to Control and DMSO-treated cells (*$p<0.05$, n=3). **B, D**: IL-8 expression was induced by HRV infection, most notably at 9 h post inoculation (*$p<0.5$ compared to time 0, n=3). R406, Bay61-3606 and NVP-QAB-205 attenuated the IL-8 expression in both asthmatic and non-asthmatic epithelium at both time points when compared to the Control and DMSO-treated cells at 3 h and 9 h, respectively (**$p <0.05$, n=3). The asthmatic airway epithelial cells exhibited lower basal levels of VEGF and IL-8 expression when compared to Non-asthmatic epithelial cells.

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TABLE

Table 1: Wound healing is impaired by HRV infection, and is further impaired in the absence of Syk activity.

In the absence of HRV infection (No HRV column), Syk inhibition by pharmacologic means, knock-down by Syk-siRNA or overexpression of the kinase-inactive Syk^{K396R} or SH2-domain $Syk^{R46,201A}$ mutants significantly impaired wound healing at 24 h. In the presence of HRV infection alone, wound healing was significantly impaired. HRV infection in presence of Syk inhibition or knock-down further impaired wound healing (*, **, *** $p<0.05$ to Control and DMSO-treated cells, Sham- and Control-siRNA transfected cells, and Sham- and WT-Syk transfected cells, respectively; n=4 /group; †$p<0.05$ when compared to No HRV cells, similar conditions, n=4).

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TABLE 1: Percentage Wound Healing at 24 h

Syk Blockade	Treatment	No HRV	HRV
Pharmacologic	Control	99.5±0.5	73.8±4.1†
	DMSO	95.2±3.2	76.9±5.9†
	R406 (0.5 uM)	21.1±3.6*	10.0±2.8†
	R406 (1 uM)	10.5±2.1*	1.3±1.2†
	Bay61-3606 (1 uM)	56.8±4.0*	31.2±4.5†
	Bay61-3606 (5 uM)	49.9±4.5*	33.5±4.7†
siRNA	Sham Transfection	97.7±1.2	53.5±7.1†
	Control siRNA	96.1±1.3	54.9±6.8†
	Syk siRNA	40.0±5.8**	29.0±2.9†
Mutant Syk	Sham Transfection	97.3±2.7	68.6±9.2†
	WT-Syk	100±0	68.4±7.8†
	Syk-K396R	72.7±6.4	52.9±5.4†
	Syk-R46,201A	51.4±5.9***	31.8±3.4†

Figure 1

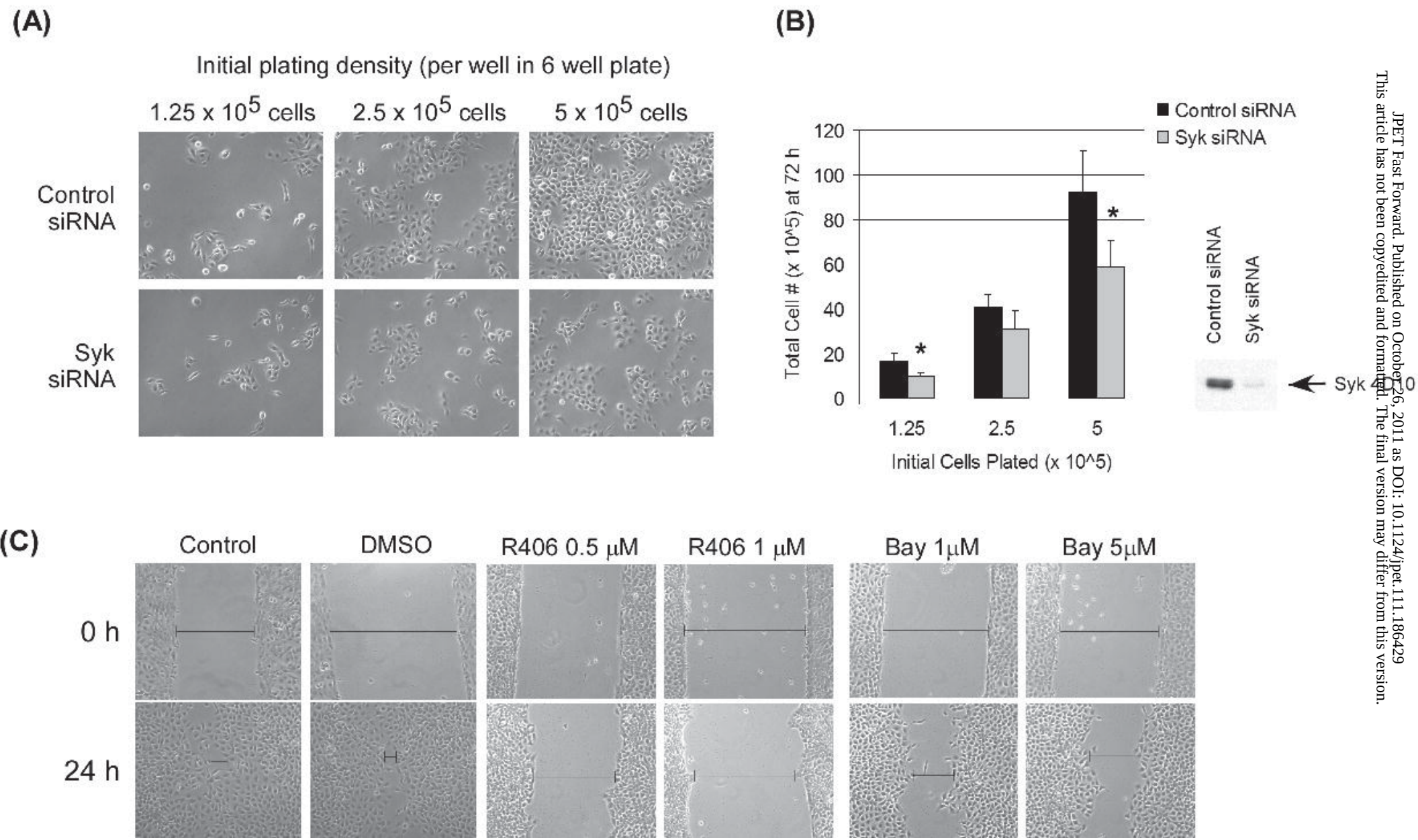

Figure 2

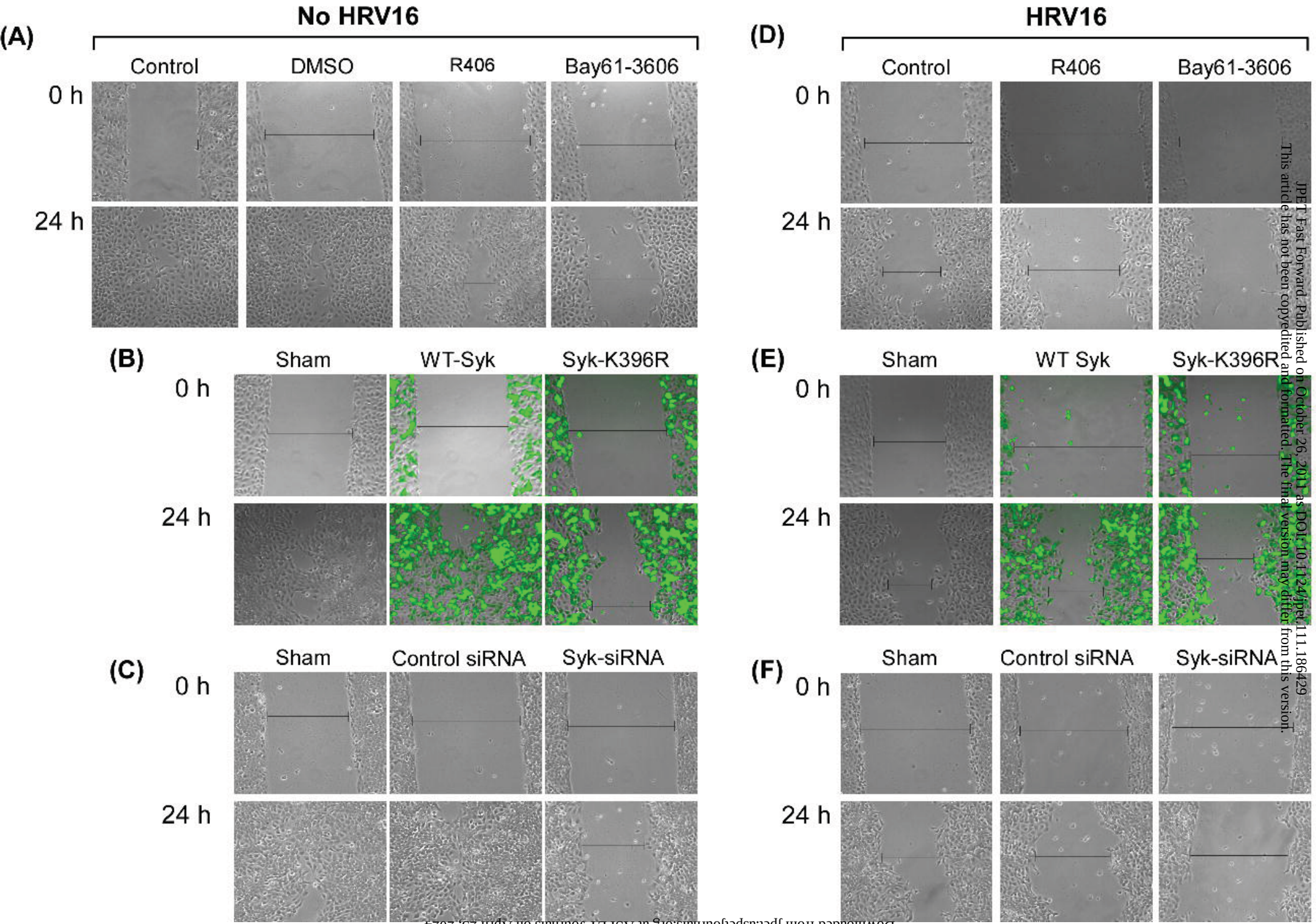

Figure 3

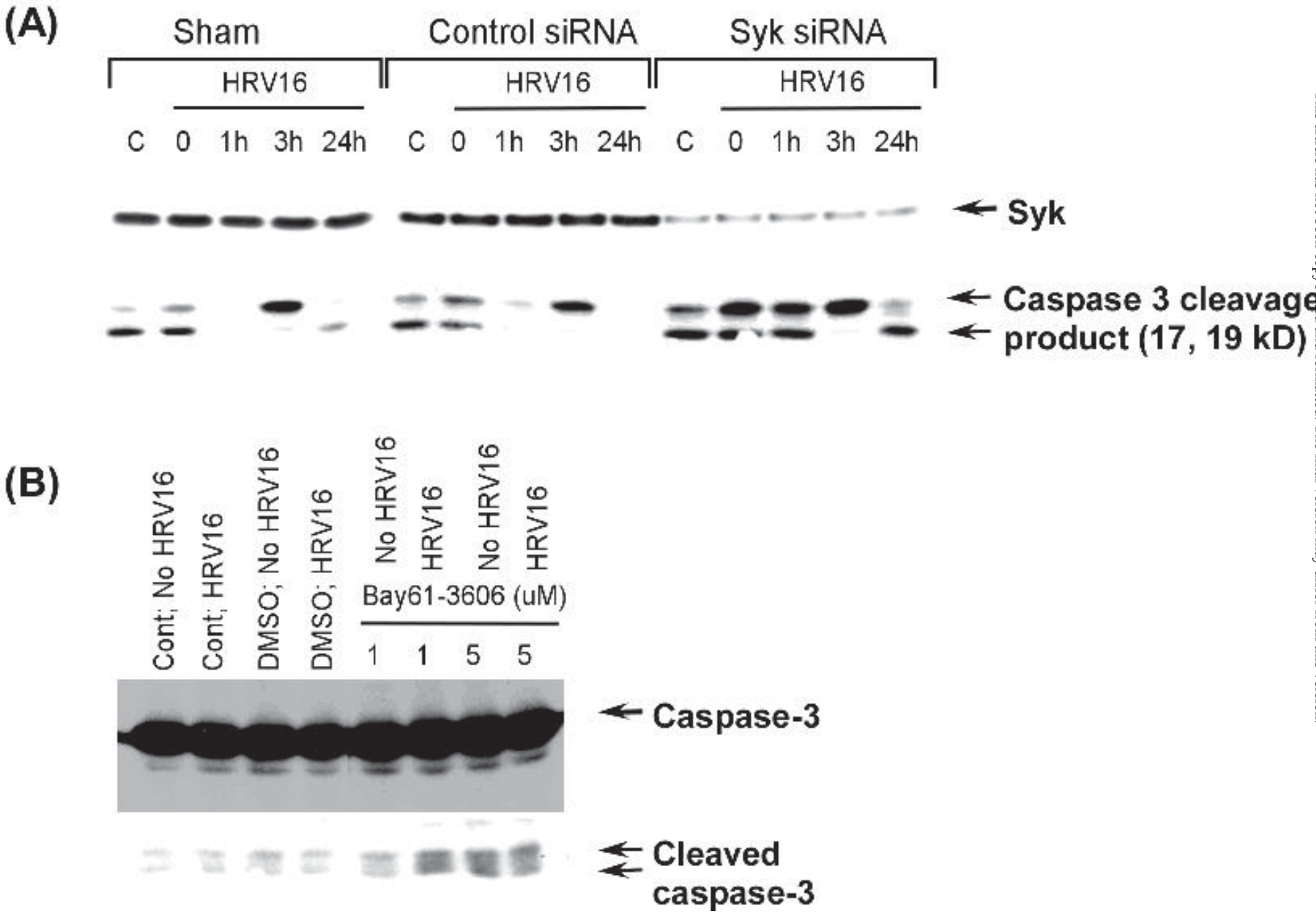

Figure 4

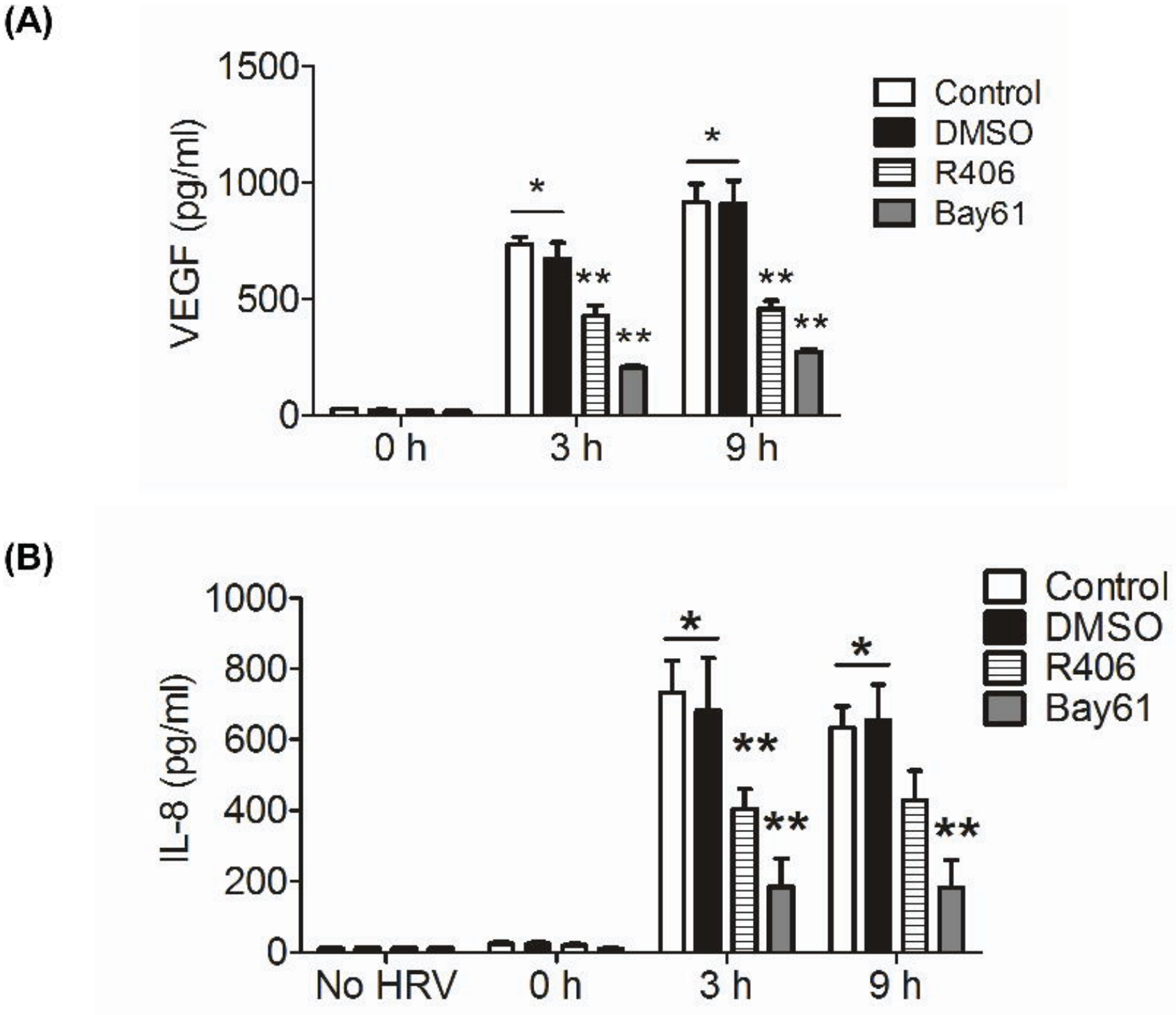

Figure 5