Kilham rat virus-induced type 1 diabetes involves beta cell infection and intra-islet JAK–STAT activation prior to insulitis

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Introduction

It is hypothesized that viruses play a key role in triggering type 1 diabetes (T1D) in genetically susceptible individuals (Fujinami et al., 2006; Jun and Yoon, 2001, 2003; Laitinen et al., 2014), but how they lead to T1D is not yet clear. Recent studies support the hypothesis that the process of islet destruction involves beta cell infection and intra-islet innate immune activation prior to insulitis. KRV induces genes involved in type I and type II interferon pathways in islet cell lines in vitro and in islets from day-5-infected animals in vivo via mechanisms that do not involve insulitis, beta cell apoptosis, or impaired insulin expression. Immunohistochemistry studies indicated that KRV protein is expressed in beta cells 5 days following infection. KRV induces the phosphorylation of Janus Kinase 1/2 (JAK1/2) and signal transducer and activator of transcription 1 (STAT-1) in islet cells via a mechanism that could involve TLR9 and NF-κB pathways. These data demonstrate for the first time that KRV-induced islet destruction is associated with beta cell infection and intra-islet innate immune upregulation early in the disease process.

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pathway in beta cells on day 5 post-infection. We hypothesize that the mechanism of virus-induced islet destruction involves beta cell infection and innate immune upregulation early in the disease process.

**Results**

**KRV induces proinflammatory gene expression in islet cells in vitro and in vivo**

Recent in vitro studies suggested that human T1D involves the induction of proinflammatory responses in pancreatic islets (Sarkar et al., 2012; Schulte et al., 2012). We tested the hypothesis that KRV-induced T1D in the LEW1.WR1 rat involves proinflammatory gene expression in islet cells. To do so, we employed two experimental systems. First, we measured the level of transcripts for proinflammatory genes in the INS-1 and RIN-14B islet cell lines which produce insulin and somatostatin, respectively (Moore et al., 2011), following incubation in the presence or absence of KRV. We also analyzed proinflammatory gene expression in islets isolated from naïve uninfected (n = 3 to 7) versus day-5-infected rats (n = 5 to 7). Data presented in Fig. 1A indicate that KRV induces the expression of transcripts for IRF-7, a transcription factor involved in type I interferon signaling (Honda et al., 2005), in both INS-1 and RIN-14B cell lines (p < 0.007 and p < 0.002, respectively). KRV also induced the expression of transcripts for CXCL-10 in RIN-14B (p < 0.002) but not in INS-1 cells. Consistent with these observations, data presented in Fig. 1B further demonstrate that there was a significant increase in the level of transcripts for IFN-γ (p = 0.04), CXCL-10 (p = 0.02), CXCL-11 (p = 0.004), IRF-7 (p = 0.001), IFN-α (p = 0.02), STAT-1 (p = 0.04), and JAK-2 (p = 0.003) in islets 5 days following infection compared to uninfected rats. Fig. 1C further indicates that islets from days 3, 5, and 8 post-infection are insulitis-free. Taken together, these data imply that infection with KRV results in proinflammatory gene activation in islets on day 5 post-infection via a mechanism that is not associated with insulitis.

**KRV infects islet cell lines in vitro and beta cells in vivo**

Previous data implied that virus-induced T1D does not involve beta cell infection (Brown et al., 1993); however, the observation that KRV upregulates proinflammatory gene expression in islets in vitro and in vivo prompted us to revisit the issue of KRV infectivity in islet cells. To that end, we tested the ability of KRV to infect islet cells in vitro and in vivo. To assess in vitro infectivity, the INS-1 and RIN-14B cell lines were cultured in the presence or absence of KRV and the expression of virus transcripts was assessed with quantitative RT-PCR. The data presented in Fig. 2A indicate that transcripts for KRV are detectable in both the INS-1 and RIN-14B cell lines following a 4 h incubation in the presence of KRV but not medium only (n = 3–7, p < 0.001). Fig. 2A further shows that transcripts for KRV VP2 are readily detectable in pancreatic islets from 5-day-infected animals but not naïve uninfected rats (p = 0.001 versus uninfected).

We next assessed the expression of KRV protein in islet cells from day-5-infected (n = 5 to 7) versus naïve uninfected (n = 3 to 7) rats. We used spleens from infected and uninfected rats as positive and negative controls, respectively. A polyclonal rabbit antibody against the C terminus of KRV VP1/VP2 was used to detect KRV expression in beta cells. Because immunofluorescence was not...
Fig. 2. KRV expression in islet cells. RNA was extracted from INS-1 and RIN-14B islet cell lines cultured in the presence or absence of KRV for 4 h or islets purified from 5-day-infected and uninfected LEW1.WR1 rats as indicated (A). The expression level of KRV VP2 transcripts was assessed using quantitative RT-PCR. The results are expressed as the mRNA expression of the gene of interest relative to the expression of \( \beta \)-actin. Statistical analyses were performed using the Mann–Whitney \( U \) test. Immunofluorescence staining was performed on spleens (B) and pancreata (C) from 5-day-infected and uninfected rats. Spleen sections were stained for KRV (green) and counterstained with DAPI (blue). Sections of pancreata were stained for insulin (red) and KRV (brown). Images were obtained using a \( \times 20 \) objective. Colocalization is visualized on the merge images. The data are representative of images obtained from three individual rats.
able to detect viral expression in the pancreas, we used immunofluorescence combined with immunohistochemistry for virus detection and co-localization analyses. The data presented in Fig. 2B demonstrate reactivity against KRV in sections of spleens from 5-day-infected but not uninfected rats. The data shown in Fig. 2C further demonstrate that the reactivity against KRV could be seen in insulin producing beta cells from 5-day-infected but not control uninfected animals. Collectively, these findings suggest that virus-induced T1D may involve direct infection of beta cells with KRV 5 days after infection.

**Fig. 3.** Beta cell function. Pancreata and sera were removed from infected and uninfected animals on different time points following virus inoculation as indicated. Beta cell apoptosis and insulin expression were evaluated in paraffin sections of pancreata using immunofluorescence by staining for insulin (red) and TUNEL (green) (A). The level of insulin in the serum was assessed by ELISA (B). The results shown are representative of at least three independent experiments.

**Fig. 4.** KRV induces pSTAT-1 expression in islet cells in vitro. Islet cell lines (A) or primary islets isolated from naïve rats (B) were cultured in the presence or absence of KRV, TLR agonists or type I and type II interferons as indicated. The cells were lysed and the lysate was subjected to Western blotting for pSTAT-1 and total STAT-1 protein detection. Densitometry performed on the bands shown in Panel B demonstrated a 1.4-fold increase in the level of pSTAT-1 in islets cultured in the presence of KRV compared with the control. No differences were observed in the expression level of STAT-1. Panel C: RIN-14B cells were cultured with or without KRV in the presence or absence of serial dilutions of the indicated TLR and NF-κB inhibitors. The cells were lysed and subjected to Western blotting for the expression of the pSTAT-1 and STAT-1. The results are representative of at least three independent experiments. iCpG, inhibitory CpG; CQ, chloroquine; PDTC, pyrrolidine dithiocarbamate.
Infection with KRV does not lead to impaired insulin expression in islets or peripheral blood on day 5 post-infection

We next tested whether expression of KRV in pancreatic beta cells involves beta cell apoptosis or abnormal insulin expression. Immunohistochemistry data indicate that like control rats, beta cells from days 3, 5 and 8 exhibit minimal apoptosis (Fig. 3A). Furthermore, similar insulin expression was observed in beta cells from day-5-infected versus the uninfected control (Fig. 3A). Consistent with these observations, Fig. 3B demonstrates that the level of insulin in the serum from day-5-infected rats was similar to that of the control (n=5/group). These data imply that infection of islets with KRV does not result in altered insulin expression on day 5 following virus inoculation.

KRV induces pSTAT-1 pathway in rat islet cell lines and islets in vivo

Because we observed that infection with KRV induces transcripts for IFN-α, IFN-γ, and STAT-1 in islet cells on day 5 post-infection (Fig. 1B), and since STAT-1 plays a major role in mediating type I and type II interferon biological effects (Platanias, 2005) and was earlier implicated in islet destruction (Rasschaert et al., 2005), we tested the possibility that KRV activates the STAT-1 to undergo phosphorylation in islet cells. To do so, we cultured islet cell lines or primary islets from naive rats in the presence or absence of KRV and analyzed the expression of pSTAT-1 (n=3). We used purified TLR ligands and type I and type II interferons that induce STAT signaling as a positive control. Fig. 4A shows that consistent with the notion that KRV is a rat-specific virus, it induced the expression of pSTAT-1 in the rat INS-1 and RIN-14B islet cell lines, but not mouse MIN-6 cells. Moreover, unlike KRV, ligands of TLR3, TLR7/8, and TLR9 were unable to induce pSTAT-1 expression in RIN-14B and INS-1 cell lines. Only Poly (I:C) induced pSTAT-1 in mouse MIN-6 cells. Finally, all three islet cell lines included in these studies responded to type I and type II interferons by upregulating pSTAT-1 expression.

Next, we assessed the level of pSTAT-1 and total STAT-1 in primary islets cultured in the presence or absence of KRV. Fig. 4B indicates that KRV induced a modest 1.4-fold increase in the level of pSTAT-1 compared to the control (n=3). No difference in the level of the total STAT-1 protein was observed in islets incubated in the presence of KRV versus the uninfected control.

Given that islets express TLR9 (Wen et al., 2004) and are activated following TLR ligation (Rasschaert et al., 2005), we examined the hypothesis that KRV induces STAT signaling in islet cells via the TLR9 and NF-κB pathways. To this end, RIN-14B cells

![Image](https://via.placeholder.com/150)

**Fig. 5.** KRV-induced pSTAT-1 in islets in vivo. Immunofluorescence images of islets from 5-day-infected and uninfected LEW1.WR1 rats. Sections were stained with antibodies against pSTAT-1, STAT-1, or the isotype control (red) and insulin (green) and counterstained with DAPI (blue) as indicated. Images were obtained using a ×40 objective. Colocalization is visualized on the merge images. The data are representative of images obtained from three individual rats.
were cultured in the presence or absence of KRV and the respective inhibitors of TLR9 and NF-κB, iCpG and PDTC \((n = 3)\). These pathways have previously been implicated by us in the development of T1D in the BioBreeding Diabetes Resistant (BBDR) rat (Zipris et al., 2007). We also used chloroquine, a blocker of endosomal acidification, to determine whether acidification of endosomes is required for the induction of activated STAT-1. Intracellular TLRs such as TLR3, 7, 8, and 9 are expressed in endosomal compartments, thus allowing interactions with TLR ligands such as viral DNA and RNA occurring in a pH-dependent manner (de Bouteiller et al., 2005). Fig. 4C indicates that both iCpG and PDTC suppressed the KRV-induced expression of pSTAT-1 in a dose-dependent fashion. The data further indicate that CQ markedly inhibited the virus-induced STAT-1 activation. Similar observations were made in INS-1 cells (data not shown).

Together, the data imply that KRV-induced STAT-1 activation in islet cells may be dependent on TLR9 and NF-κB pathways and on endosomal acidification.

**KRV induces pSTAT-1 expression in beta cells in vivo**

The observation that KRV induces pSTAT-1 in islets in vitro led us to hypothesize that infection with KRV results in STAT-1 activation in beta cells in vivo. To test this possibility, rats were left untreated or were infected with KRV \((n = 3\) per group). Pancreata were removed on day 5 post-infection and immunofluorescence was used for pSTAT-1, STAT-1 and insulin detection. The data presented in Fig. 5 suggest that the expression level of pSTAT-1 in islets from uninfected rats is minimal. In contrast, islets from day-5-infected animals had higher expression levels of pSTAT-1 that was localized in insulin producing cells and was expressed in both the cytoplasm and nucleus. Almost no staining was observed in sections incubated in the presence of the isotype control. These findings suggest that infection with KRV induces STAT-1 activation in beta cells on day 5 post-infection.

**KRV induces JAK1/2 signaling in beta cells**

Because the induction of STAT-1 is dependent on JAK1/2 activation (Platanias, 2005), we examined the hypothesis that infection with KRV leads to JAK1/2 phosphorylation in beta cells \((n = 3\) per group). The data presented in Fig. 6 demonstrate an increase in the level of pJAK-1/2 in insulin producing cells from 5-day-infected versus uninfected controls. Moreover, the data suggest increased expression of JAK1/2 in the exocrine tissue surrounding the islets from infected versus uninfected rats. These findings could imply that infection with KRV induces the JAK-1/2 pathway in islets early after infection.

**Discussion**

How virus infections trigger T1D is not yet clear. It is hypothesized that viruses induce T1D via mechanisms associated with beta cell infection and immune upregulation; however most of the data supporting this concept come from in vitro studies performed in beta cell cultures (reviewed in Ref. Eizirik et al. (2009)). In this study, we elucidated early mechanisms involved in the development of virus-induced T1D. We present for the first time in vivo evidence of a link between beta cell infection and virus-induced intra-islet immune activation and the development of the disease. Furthermore, we show that beta cell infection on day 5 post-infection is not associated with insulitis, apoptosis, or abnormal insulin expression in the pancreas or peripheral blood.

The hypothesis that infection with KRV leads to virus expression in beta cells early in the disease course is supported by three lines of evidence. We found that KRV transcripts can be detected in
islet cell lines cultured in the presence of the virus. Furthermore, infection of rats with KRV results in the expression of KRV transcripts and protein in islets or beta cells from 5-day-infected rats. Our data implying that viral proteins are detectable in beta cells are in contrast to previous data demonstrating that KRV is not expressed in insulin producing cells on day 5 following infection (Brown et al., 1993). This discrepancy is probably due the higher detection sensitivity of the RT-PCR and immunohistochemistry methodologies we have used compared to in situ hybridization employed in the earlier study (Brown et al., 1993).

Our in vitro and in vivo findings suggesting that KRV activates the JAK–STAT pathway in beta cells are reminiscent of our earlier data that this virus induces a robust STAT-1 activation in lymphoid cells in vitro and in vivo (Ref. Nair et al. (2008) and data not shown). Furthermore, the fact that transcripts for type I and type II interferons are expressed in islets from infected rats (this manuscript) could provide a mechanistic explanation for the upregulation of JAK–STAT upregulation. Engagement of the IFN-α and IFN-γ receptors activates JAK-1 and TYK-2 and JAK-1 and JAK-2, respectively (Leonard and O’Shea, 1998). This leads to the recruitment and phosphorylation of STAT proteins, their dimerization and nuclear translocation resulting in the synthesis of proinflammatory cytokines and chemokines involved in anti-viral responses (Platanias, 2005; Ivashkiv and Donlin, 2014). The data showing that KRV infects beta cells and induces intra islet immune mechanisms on day 5 post-virus inoculation may explain a previous report that endoplasmic reticulum (ER) stress pathways are upregulated in beta cells from KRV-infected BBDR rats on day 4 post-infection (Yang et al., 2013). Indeed, virus infection has previously been linked with the upregulation of ER stress pathways (Saeed et al., 2011).

The data indicating that virus-induced T1D involves beta cell infection and inflammation is consistent with the hypothesized role of pancreatic viruses such as enteroviruses in triggering T1D in humans (Schulte et al., 2012). It was recently demonstrated that Coxsackie B enterovirus (CVB) viral protein-1 is expressed in insulin-producing beta cells from newly diagnosed patients with T1D compared with control individuals (Richardson et al., 2009). Similar to our data, previous studies indicated that infection of human beta cells with CVB3 in vitro can trigger the expression of genes associated with type I and type II interferon signaling (Schulte et al., 2012).

The presence of transcripts for CXCL-10 in KRV-infected islet cells in vitro and in islets in vivo supports the hypothesis that disease mechanisms could be linked with the upregulation of CXCL-10 in the microenvironment of islets prior to insulitis. These data are consistent with earlier reports that islets from patients with T1D co-express enterovirus-capsid protein, interferon-γ, CXCL10 and other chemokines (Roep et al., 2010; Tanaka et al., 2009), and CXCL10 is the dominant chemokine expressed in vivo in islets from humans and animal models (Sarkar et al., 2012). CXCL-10 is a major chemoattractant for activated T-cells and macrophages and can be produced and released by beta cells following infection (Frigerio et al., 2002). It is conceivable that the expression of CXCL-10 in islet cells from infected rats may be involved in recruiting immune cells to the site of inflammation. Although it was demonstrated that beta cells can express proinflammatory cytokines and chemokines such as type I and type II interferons and CXCL-10 (Schulte et al., 2012; Uno et al., 2010), our studies do not identify the cell subsets expressing these immune mediators. We were unable to detect cytokine and chemokine proteins in islets from infected rats probably due to their low expression level. We are therefore unable to exclude the possibility that cells other than islets are involved in the KRV-induced proinflammatory response. In addition to beta cells, islets contain other types of endocrine cells as well as various types of immune cells such as dendritic cells and macrophages.

Infection with KRV results in the induction of IRF-7, a major transcription factor involved in the synthesis of type I IFNs (Honda et al., 2005) in islet cell lines in vitro and in vivo (this manuscript). Additionally, the amount of transcripts for IRF-7 significantly correlated with the levels of KRV on day 5 post-infection (data not shown), implying a direct relationship between virus infection and type I IFN induction. The finding that TLR9 and NF-κB inhibitors block the STAT-1 activation in islet cell lines in vitro is in accordance with our previous observation demonstrating that the upregulation of innate immunity in spleen cells induced by KRV is dependent on TLR9 and NF-κB pathways (Zipris et al., 2007). Beta cells express TLR9 (Wen et al. (2004) and our unpublished observations) and it can thus be assumed that the KRV-induced proinflammatory signature in islet cells might be mediated by TLR9. Based on these considerations, one can envision a model in which TLR9 ligation by KRV in the periphery or islets may lead to the expression of transcription factors involved in the production and signaling of type I and type II interferons as well as other proinflammatory cytokines and chemokines in islets and/or immune cells recruited to the islets, culminating in inflammation and islet destruction. The presence of type I and type II interferons in the microenvironment of beta cells has been shown to be detrimental for beta cells (Doguson et al., 2008).

In conclusion, we present in vivo evidence linking islet infection and innate immune activation early in the disease process with mechanisms of KRV-induced islet destruction in the LEW1. WR1 rat model. Identifying immune pathways associated with the early course of T1D may facilitate the development of new means to prevent islet destruction.

Materials and methods

Animals, viruses and cell lines

Specific pathogen-free LEW1.WR1 rats were obtained from BRM Inc. (Worcester, MA) and were bred and housed in a specific pathogen-free facility and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Colorado Denver.

KRV and NRK cells were obtained from stocks maintained in our laboratories (Guberski et al., 1991). KRV was propagated in NRK cells, grown in Dulbecco’s minimal essential medium (DMEM) and quantified using plaque assays, as was previously described (Zipris et al., 2003). The pancreatic cell lines INS-1, RIN-14B, and MIN-6 were kindly provided by Dr. John Hutton (Barbara Davis Center for Childhood Diabetes, University of Colorado Denver).

KRV infection, tissue removal, and serum insulin measurements

Rats at 21 days of age were injected i.p. with 1×10^7 PFU of KRV as previously described (Guberski et al., 1991). The animals were monitored for insulitis and T1D for 40 days following infection or were sacrificed on day 5 post-infection. Pancreata or purified islets were isolated from uninfected or KRV-treated rats at different time points following virus inoculation. Spleens and sera were collected from uninfected or day-5-infected animals. Serum insulin levels were quantituated using an ELISA kit from Alpco Diagnostics (Salem, NH).
Histological analysis and fluorescence microscopy of pancreata and spleens

Pancreatic tissue was fixed for 24 h in 10% neutral-buffered formalin, embedded in paraffin, cut (5–6 μm), and mounted on microscope slides. The tissue was stained with hematoxylin and eosin.

For insulin and apoptosis detection, pancreata were embedded in Tissue-Tek OCT (Miles, Elkhart, IN), snap frozen and stored at −80 °C until use. Cryostat sections (5 μm) were mounted on slides. Sections were labeled with a monoclonal antibody against mouse insulin (Clone K36AC10, Sigma-Aldrich) followed by Texas Red® dye-conjugated AffiniPure donkey anti-mouse IgG (H+L) (Jackson Immunoresearch, West Grove, PA). For the detection of apoptosis and nucleus staining, we used a Tunel staining kit (Roche Applied Science, Indianapolis, IN) and 4,6-Diamidine-2'-phenylindole dihydrochloride (DAPI, from Sigma-Aldrich), respectively. For a positive control, sections were incubated in the presence of DNase I (Sigma-Aldrich) to induce DNA strand breaks, prior to the labeling procedure. For a negative control, sections were incubated with label solution only.

For staining KRV or JAK/STAT staining, pancreata or spleens were removed from control uninfected or 5-day-infected rats. Five-micrometer sections of formalin-fixed, paraffin-embedded tissues were mounted on slides, deparaffinized in xylene, and then rehydrated with distilled H₂O through graded alcohols. Antigen retrieval was enhanced by microwaving the slides in citrate buffer (pH 6). For KRV staining, pancreas and spleen sections were incubated with rabbit polyclonal antibody against the C-terminus portion of KRV VP1/VP2 (Biosis, Woburn, MA) followed by incubation with donkey anti-rabbit IgG (H&L)-Alexa fluor 488 (Jackson Immunoresearch, West Grove, PA). For detecting KRV in the pancreas, sections were incubated with HRP-conjugated goat anti-rabbit IgG (H&L) (Dako, Carpinteria, CA) and bound HRP was localized with diaminobenzidine tetrachloride (Dako). For pJAK1 (Tyr 1022), pJAK2 (Tyr 1007/Tyr 1008), STAT-1, and pSTAT-1 (Tyr 701) detection, we used rabbit polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), followed by Cy3-conjugated donkey anti-rabbit (Jackson Immunoresearch). Anti-human α-synuclein from Santa Cruz was used as an isotype control. For the insulin staining in this part of the study we used guinea pig anti-mouse/rat/human insulin (Abcam, Cambridge, MA) followed by FITC-conjugated donkey anti-guinea pig (Jackson Immunoresearch). Sections from pancreata were also stained with mouse against human insulin mAb followed by incubation with donkey anti-mouse IgG (H+L) conjugated to Cy3 (Jackson Immunoresearch). DAPI (Sigma-Aldrich) was added to the slides prior to mounting with cover slips.

Tissue sections were analyzed using an Olympus BX51-TRF Microscope BX51 (Olympus, Center Valley, PA) equipped with a Penguin Pro-150 ES Camera system (Pixera, Los Gatos, CA).

Iset isolation and TLR activation and blockade

Pancreatic islets were isolated from KRV-infected and control uninfected BBDR rats by digestion with collagenase and purification by Histopaque (both from Sigma-Aldrich) as described previously (Nicolls et al., 2002). The islets were free of acinar tissue. Activation of primary islets was performed by incubating 800 islets per 1 ml in the presence or absence of 2 × 10⁶ PFU/ml of KRV. Islet cell lines were activated by incubating 4 × 10⁶ cells per ml in the presence or absence of 2 × 10⁶ PFU/ml of KRV, or purified TLR agonists with or without TLR inhibitors for 4 h as previously described (Zipris et al., 2005). For TLR activation we used 50 μg/ml of poly (I:C), 1 μg/ml for CpG DNA (Life Technologies), or R848 (Axxora, San Diego, CA). TLR blockade was induced with chloroquine and pyrrolidine dithiocarbamate (PDTC) from Sigma-Aldrich and inhibitory CpG (ClpG) from Life Technologies. For interferon-induced islet cell activation, we used rIFN-α and rIFN-β from PBL (Piscataway, NJ) and rIFN-γ from PeproTech (Rocky Hill, NJ).

RNA extraction, cDNA synthesis, and quantitative RT-PCR

RNA extraction, cDNA synthesis and quantitative RT-PCR were performed as previously described (Wolter et al., 2009). The primers used were synthesized by Integrated DNA Technologies (Coraville, IA) and their sequences have been previously published (Wolter et al., 2009).

Western blot analyses

Pancreatic islets, RIN-14B, INS-1, and MIN-6 islet cell lines were lysed and subjected to Western blotting as previously described (Zipris et al., 2005). The antibodies used were as follows: anti-pSTAT-1 (clone ST1P-11A5 and mouse IgG2a) from Life Technologies (Carlsbad, CA) and anti-STAT-1 (clone 42/Stat1 and mouse IgG2b) purchased from BD Biosciences (San Jose, CA), and a rabbit polyclonal anti-beta-actin purchased from Abcam (Cambridge, MA).

Statistical analysis

Comparisons between two experimental groups were performed using the Mann–Whitney U test.

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