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Upregulation of miR-221 and -222 in response to increased extracellular signal-regulated kinases 1/2 activity exacerbates neointimal hyperplasia in diabetes mellitus

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Abstract

Background and aims—Diabetes is associated with accelerated arterial intimal thickening that contributes to the increased cardiovascular disease seen in this population. In healthy arteries, intimal thickening is inhibited by elevated levels of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$, and intimal thickening is promoted by activation of the mammalian Target of Rapamycin to promote degradation of $p27^{Kip1}$ protein. Recently, we reported that two microRNAs, miR-221 and -222, which promote intimal thickening via down-regulation of mRNA encoding $p27^{Kip1}$, are elevated in the arteries of diabetic patients. To determine if these miRNAs are critical to the increased intimal thickening under diabetic conditions, we examined the regulation of $p27^{Kip1}$ in a mouse model of diabetes.

Methods—Comparisons of $p27^{Kip1}$ signaling in NONcNZO10 mice fed a diabetogenic *versus* control diet were performed using immunochemistry and real-time PCR.

Results—Vascular smooth muscle cells and arteries of diabetic mice exhibited decreased levels of $p27^{Kip1}$ that derived from destabilization of $p27^{Kip1}$ mRNA in an extracellular signal response kinase-1/2 (ERK-1/2) dependent manner. The activity of ERK-1/2 is increased in the arteries of diabetic mice and promotes an increase in miR-221 and -222. Inhibition of miR-221 and -222 restores normal levels of $p27^{Kip1}$ mRNA and protein in the arteries of diabetic mice and reduces intimal thickening following wire injury.

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Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

D.J.L. and S.CM. performed animal care, sample collection, assays and data analysis T.C.W. designed the study, interpreted the results, performed animal care and sample collection, and edited the manuscript. All authors contributed to the final version of the manuscript. T.C.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conclusions—These data suggest diabetes is accompanied by increases in arterial miR-221 and -222 expression that promotes intimal thickening. Inhibition of the increased miR-221 and -222 may be efficacious in the prevention of the cardiovascular complications of diabetes.

Keywords

Diabetes Mellitus; Intimal thickening; miR-221; miR-222; $p27^{Kip1}$

1. Introduction

Diabetes mellitus is accompanied by an increase in intimal thickening, a central component of cardiovascular diseases, including atherosclerosis and restenosis following percutaneous transluminal coronary angioplasty[1–4]. Intimal thickening consists of the proliferation and migration of vascular smooth muscle cells (VSMCs) in the intimal layer of an artery. Intimal thickening results from activation of multiple signal transduction pathways in response to mitogenic stimulation of VSMCs. Activation of one of these, the mammalian Target of Rapamycin (mTOR) pathway, promotes the degradation of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$. Elevated $p27^{Kip1}$ protein, as seen in healthy arteries and quiescent VSMCs, blocks intimal thickening through inhibition of VSMC proliferation and migration that occur in response to mitogenic stimulation[5–8]. Inhibition of mTOR with the macrolide antibiotic rapamycin or its analogs is highly effective at preventing VSMC proliferation and migration[9–11]. In diabetic patients, the use of mTOR inhibitors is less effective at preventing in-stent restenosis[12].

Recently, we reported that two microRNAs that promote intimal thickening, miR-221 and -222, are elevated in the arteries of patients with diabetes[13, 14]. miR-221 and -222 accelerate intimal thickening by promoting the degradation of mRNA encoding $p27^{Kip1}$, in VSMCs[15, 16]. Loss of $p27^{Kip1}$ expression is associated with increased VSMC proliferation and migration and resistance to rapamycin[9, 11, 17–19]. Whether this increase in miR-221 and -222 in response to diabetes promotes an increase in intimal thickening and resistance to rapamycin remains to be seen.

We and others have also reported that changes in insulin signaling associated with diabetes result in an increase in the activation of the ERK-1/2 pathway[17, 20]. Prolonged activation of this pathway promotes increased expression of miR-221 and -222[21]. Thus, the presence of diabetes may lead to a switch in the regulation of $p27^{Kip1}$ from mTOR regulation at the protein level to ERK-1/2 regulation at the mRNA level. We hypothesize that this loss of $p27^{Kip1}$ through increased miR-221 and -222 promotes the increase in intimal thickening seen in diabetic patients.

Here we report that, in a mouse model of type 2 diabetes, neointimal hyperplasia following femoral wire injury is increased in a miR-221 and -222 dependent manner. This is derived from the down-regulation of $p27^{Kip1}$ in an mTOR independent manner. The dysregulation of mTOR and $p27^{Kip1}$ occurs in response to increased activation of the ERK-1/2 pathway that results in an increase in miR-221 and -222 that destabilizes $p27^{Kip1}$ mRNA. These data provide a molecular basis for the increased neointimal hyperplasia in diabetic subjects.

2. Materials and methods

2.1. Mouse model of type 2 diabetes

The NONcNZO10/LtJ mouse, a recombinant cogenic mouse model of type 2 diabetes, develops an obesity associated form of diabetes when fed a moderate fat (MF) diet of 11% fat (LabDiet, 5K20) for 12 weeks[22–24]. NONcNZO10 mice weaned onto 11% fat diet and aged 12 weeks to induce diabetes served as our diabetic model (DM). Our non-diabetic control mice (ND) were NONcNZO10/LtJ mice maintained on a 4% fat diet (LabDiet, 5K54). The diabetic state of all mice was confirmed using fed blood glucose measurement with the DM group having greater than 350 mg/dL. All procedures were performed with approval of the Institutional Animal Care and Use Committee.

2.2. Cell culture

Murine VSMCs were isolated from the abdominal aortae of DM (DM-VSMCs) and ND (ND-VMSCs) mice as previously described[17]. Greater than 90% purity of the VSMC cultures was confirmed by positive staining for α-smooth muscle actin. VSMCs were sub-cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Inc. Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS, Invitrogen, Inc.) at 37°C in a humidified 5% CO_2 atmosphere with the media being replaced every ~48 h. All tissue culture experiments were conducted using a minimum of 3 independent isolates from ND and DM mice.

2.3. Cell proliferation

The percent of proliferating VSMCs was seeded at ~50% confluence and incubated in DMEM supplemented with 0.5% FBS for 24 h, followed by incubation in DMEM supplemented with 20% FBS for 24 h. During the final 5 h, treatment medium was supplemented with 10 μM BrdU. Following fixation and permeabilization, cells were treated with 300 μg/mL DNAse in phosphate buffered saline for 1 h at 37°C. Cells were stained with an anti-BrdU antibody conjugated to fluorescein isothiocyanate (FITC, BD Pharmingen) at 1:50 dilution and 4',6-diamidino-2-phenylindole (DAPI). The percentage of total cells (DAPI) staining positive for BrdU was calculated for four high power fields and a minimum of 100 cells for each condition.

2.4. Cell migration

Cell migration was measured as previously described.[10] VSMCs at less than 50% confluence were pretreated with rapamycin in normal growth media for up to 48 h prior to seeding onto cell inserts. VSMCs (2×10^5) were seeded into the cell inserts and incubated for 4 h over DMEM containing chemoattractant (PDGF, 10 ng/mL) or 0.1% bovine serum albumin (BSA). For comparison of migration rates, data are expressed as the mean of four high power fields of triplicate samples normalized to the number of cells migrating toward media without chemoattractant.

2.5. Real-time PCR

Total RNA was isolated from VSMCs and arterial tissues using the miRNeasy Plus Mini kit (Qiagen, Inc., Valencia, CA). For measurement of mRNA, 10 ng of total RNA from each sample was analyzed to determine the relative amounts of mRNA encoding $p27^{Kip1}$ and hypoxanthine phosphoribosyltransferase 1 (hprt) using QuantiTect SYBR Green one-step RT-PCR (Qiagen) and QuantiTect primer assays (Supplemental Table 1). For miRNA measurement, the Qiagen miRScript II RT Kit was used to prepare cDNA, which was then used as a template in the miScript SYBR Green PCR Kit reaction according to manufacturer's instructions. The expression of mRNAs and miRNAs was normalized to the expression of hprt and U6, respectively, for each sample, and the fold difference between samples was calculated using the $2^{-\Delta\Delta Ct}$ method. For measurement of the RNA half-life, VSMCs were treated with actinomycin D (5 ng/mL), stimulated with insulin (10 nM). Prior to measuring $p27^{Kip1}$ half-life, VSMCs were treated with either the ERK-1/2 pathway inhibitor, PD98059 (20 μM), siRNA targeting ERK-2, control oligonucleotide (Ambion), or vehicle. The levels of $p27^{Kip1}$ and hprt were determined as described above and the half-life was calculated as $t_{1/2} = \ln(2)/k$ where k is the first order rate of decay constant.

2.6. Protein measurements

Phosphorylation of ERK-1/2 at threonine 202 and tyrosine 204 (p-ERK-1/2) and total ERK-1/2 and $p27^{Kip1}$ were measured using DuoSet IC ELISA kits (R&D Systems, Inc) according to manufacturer's instructions. Western blots were prepared as previously described[11] and probed with antibodies purchased from BD Biosciences ($p27^{Kip1}$), Cell Signaling Technology (Danvers, MA, β-actin, p-Akt (Ser473), total Akt,), and Bethyl Laboratories (Montgomery, TX, 4E-BP1). All primary antibodies were used at a 1:1000 dilution except for the β-actin which was used at 1:2000. Blots were incubated with primary antibody overnight at 4°C with the exceptio n of $p27^{Kip1}$, which was incubated for 1 h at 25°C. Blots were incubated with secondary a ntibodies (Vector Laboratories, Inc., Burlingame, CA) for 1 h at 25°C. Phosphorylation of insulin receptor substrate-1 (IRS-1) at serine 307 residue and $p70^{S6kinase}$ at tyrosine 421 and serine 424 residues was measured using the AKT pathway 7-Plex Panel (ThermoFisher, Inc.) and the Bio-Plex 200 Suspension Array System (Bio-Rad, Inc.) according the manufacturer's instructions.

2.7. Mouse femoral wire injury model

The mouse femoral artery wire injury model of Roque et al. was used, with minor modifications[25]. Mice were injured via three passes of a 0.009" diameter angioplasty guidewire. Antisense to miR-221 and -222 (2'OMe-miR-222) was administered locally via a perivascular 30% Pluronic F-127 gel containing 1% HiPerfect transfection reagent (Qiagen) and 90 μg of either control oligonucleotide or 2'OMe-miR-222. Inhibition of the ERK-1/2 pathway was achieved via daily intraperitoneal injections of U0126 (7.5 mg/kg/d, Tocris Bioscience). U0126 was used in these studies based on previous studies demonstrating its efficacy in similar *in vivo* models[26]. Femoral arteries were harvested at 14 days post-injury. Tissues were fixed in formalin and stained with Mason's Trichrome stain. The area of the intima, media, and lumen of each artery was measured using Adobe Photoshop.

2.8. Statistics

All data are expressed as the mean ± standard deviation with the exception of Figure 5 B and C where the data are reported as mean ± standard error of the mean for clarity of the figure. For the mRNA half-life studies, ANCOVA was used to test for statistical differences with time treated as a covariate. Student's *t*-test was used to compare the means of two samples. Statistical analysis between multiple groups was performed using one way ANOVA, and Tukey's HSD test was used to compare the individual mean values. A p-value < 0.05 was considered significant.

3. Results

3.1. Diabetes induces an increase in miR-221 and -222 that reduces $p27^{Kip1}$ protein levels and increases intimal hyperplasia

Our goal in this study is to investigate the impact of diabetes induced increased miR-221 and -222, we observed in patients on intimal thickening in the vasculature. To do this, we chose a murine model of type 2 diabetes, the NONcNZO10. This mouse develops an obesity associated hyperglycemia and insulin resistance at 12–14 weeks of age, when fed a moderately elevated fat diet (11–12%). In our hands, DM mice developed a significantly higher body weight (37.7 ± 2.8 g *vs.* 30.0 ± 5.9 g, $p < 0.05$), mean fed blood glucose (406 ± 76 mg/dL *vs.* 241 ± 31 mg/dL, $p < 0.05$), and fasting blood glucose (156.2 ± 7.8 mg/dL *vs.* 90.7 ± 12.0 mg/dL, $p < 0.05$). First, we confirmed that arteries of these mice exhibit elevated miR-221 and -222 and that we can restore normal miR-221 and -222 levels using perivascular administration of an antisense inhibitor of both miR-221 and -222 (2'-OMe-miR-222). We measured miR-221 and -222 and their targets in the femoral arteries of ND and DM mice treated perivascularly with either 2'-OMe-miR-222 or control oligo. DM mice exhibited increased miR-221 and -222 as well as reduced levels of $p27^{Kip1}$ mRNA in uninjured femoral arteries, and treatment with 2'OMe-miR-222 restored normal levels of miR-221 and -222 and their targets (Fig. 1A and B). Similarly, $p27^{Kip1}$ protein levels are restored by 2'OMe-miR-222 treatment (Fig. 1C). Finally, treatment with 2'OMe-miR-222 inhibited the elevated level of neointimal hyperplasia following wire injury (Fig. 2). These data demonstrate that increased miR-221 and -222 in the arteries promote an increase in neointimal hyperplasia.

3.2 VSMCs isolated from diabetic mice exhibit increased rates of proliferation and migration and a relative resistance to rapamycin

VSMCs were isolated from DM and ND mice to directly measure the effect of diabetes on VSMC proliferation and migration. Insulin resistance of DM-VSMCs was confirmed by a significant decrease in Akt phosphorylation in response to insulin stimulation compared to ND-VSMCs (Fig. 3A). Phosphorylation of insulin receptor substrate-1 (IRS-1) at serine 307 residue, and serine 421 and serine 424 residues of $p70^{S6kinase}$, both of which are associated with obesity related insulin resistance, is increased in basal DM-VSMCs (Fig. 3B).[27–29] IRS-1 contains multiple phosphorylation sites and phosphorylation of these sites is complex under insulin resistant conditions.[30] However, taken together, these data suggest the diabetic phenotype is maintained *in vitro*. The DM-VSMCs exhibited increased cell proliferation in response to serum stimulation and migration toward platelet derived growth factor compared

to ND-VSMCs (Fig. 3C and D). As expected, inhibition of the mTOR pathway with the macrolide antibiotic, rapamycin and its analogs, is highly effective at blocking VSMC proliferation and migration under normal conditions. The DM-VSMCs, however, exhibited a relative resistance to the effects of rapamycin treatment on VSMC proliferation and migration, suggesting a change in the mechanisms controlling VSMC proliferation and migration.

3.3. $p27^{Kip1}$ protein and mRNA are reduced in the diabetic mice and VSMCs

As resistance to the anti-proliferative and anti-migratory effects of rapamycin is associated with loss of $p27^{Kip1}$ expression[9, 18], we measured the ability of rapamycin treatment to restore $p27^{Kip1}$ levels. Rapamycin treatment was effective at inhibiting mTOR activity, as evidenced by a reduction in the phosphorylation of 4E-BP1, a downstream target of the mTOR complex 1 pathway, in the injured arteries of both DM and ND mice (Fig. 4A). In contrast, the injured arteries of DM mice did not exhibit an increase in $p27^{Kip1}$ protein in response to rapamycin treatment (Fig 4A and B). Similarly, $p27^{Kip1}$ protein levels are elevated in ND-VSMCs in the presence of rapamycin, but are maintained low in DM-VSMCs (Fig 4C and D). Previous reports have linked resistance to rapamycin to a dysregulation of mTOR and $p27^{Kip1}$ [9, 31]. To determine if $p27^{Kip1}$ expression was reduced in DM mice in the absence of injury, we compared $p27^{Kip1}$ protein in the uninjured abdominal aortae of DM and ND mice. $p27^{Kip1}$ protein and mRNA levels are reduced in the aortae of DM mice compared to ND mice (Fig 4E and F). This latter point suggests that $p27^{Kip1}$ is repressed at the mRNA level under diabetic conditions in an mTOR-independent manner.

3.4. $p27^{Kip1}$ mRNA is decreased through increased extracellular signal response kinase 1/2 activation in DM mice

Our laboratory and others have demonstrated an increase in activation of the ERK-1/2 pathway in the arteries of diabetic animal models and patients[17, 20]. Similarly, ERK-1/2 phosphorylation is significantly increased in DM mice compared to ND mice (Fig. 5A). Our previous work has shown that under insulin resistant conditions, the half-life of $p27^{Kip1}$ mRNA is reduced in an ERK-1/2 dependent manner[17]. The half-life of $p27^{Kip1}$ mRNA in DM-VSMCs following insulin stimulation was approximately half of that of ND-VSMCs (Fig. 5B and C). Treatment with an ERK pathway inhibitor (PD98059) or siRNA targeting ERK2 increased the half-life of $p27^{Kip1}$ mRNA in DM-VSMCs to levels similar to those of controls. Treatment of DM-VSMCs, but not ND-VMSCs, with the ERK pathway inhibitor (U0126) abolished the increase in miR-221 and -222 (Fig. 5F) and both U0126 and PD98059 reduced the elevation of miR-221 and -222 in arteries of DM mice *ex vivo* (Supplementary Fig. 1). Finally, we confirmed that $p27^{Kip1}$ was repressed in an ERK-1/2 dependent manner *in vivo*, by measuring $p27^{Kip1}$ mRNA and protein in the aortae of ND and DM mice treated with the ERK-1/2 pathway inhibitor, U0126. $p27^{Kip1}$ mRNA and protein were reduced in the DM aortae and U0126 treatment restored normal levels (Fig. 5D and E).

4. Discussion

Diabetics are at greater risk of cardiovascular disease and complications of cardiovascular interventions[32–36]. Our previous work has suggested that the response of VSMCs to

mitogenic stimuli may be augmented in the setting of diabetes[17, 37]. Additionally, we have reported that miR-221 and -222 are elevated in both healthy and atherosclerotic arteries of diabetic patients.[13, 14] This study was conducted to elucidate the role of miR-221 and -222 in the regulation of VSMC cell proliferation and migration under diabetic conditions that serve to promote intimal hyperplasia. We demonstrate that intimal hyperplasia in response to wire injury is increased in response to elevated arterial miR-221 and -222 levels reducing $p27^{Kip1}$ expression. This increase in miR-221 and -222 occurs in response to elevated ERK-1/2 activity and produces a dysregulation of $p27^{Kip1}$ and mTOR.

Previous studies linked dysregulation of mTOR and $p27^{Kip1}$ to a relative resistance to rapamycin[9, 11, 31]. BC3H1 cells, an immortalized myogenic cell line, develop resistance to rapamycin characterized by normal inhibition of p70S6Kinase and 4E-BP1 phosphorylation, but lack of increase in $p27^{Kip1}$ in response to rapamycin treatment after repeated passaging in the presence of high doses of rapamycin (>100 nM)[9]. VSMCs and endothelial cells lacking $p27^{Kip1}$ exhibit a relative resistance to rapamycin's anti-migratory effects[7, 10]. VSMCs obtained from diabetic subjects and VSMCs lacking the insulin receptor both exhibit the increased proliferation and migration coupled with a dysregulation of mTOR and $p27^{Kip1}$ [17, 37]. The present study demonstrates that increased arterial expression of miR-221 and -222 levels induces a similar dysregulation of mTOR and $p27^{Kip1}$.

Our studies in two different *in vitro* models of diabetes, VSMCs lacking the insulin receptor and VSMCs obtained from diabetic subjects, link an increase in ERK-1/2 activation to a reduction in $p27^{Kip1}$ mRNA[17, 37]. Jonas *et al.* have previously demonstrated that ERK-1/2 activity is increased in the arteries of diabetic animal models[20]. Despite clear evidence that ERK-1/2 is activated following arterial injury[38], the efficacy of ERK-1/2 inhibitors for prevention of intimal hyperplasia is unclear. An initial report using the synthetic MEK1 inhibitor, PD98059, found no effect of ERK-1/2 pathway inhibition on intimal hyperplasia[39]. In contrast, administration of an adenovirus encoding a dominant negative mutant of p44 ERK did reduce intimal hyperplasia[40]. Here, we link the increased ERK-1/2 in the arteries of diabetic animal models to increased miR-221 and -222 expression and intimal hyperplasia.

As described above, increased miR-221 and -222 expression promotes intimal thickening in VSMCs through a reduction in p27Kip1 mRNA levels. Additionally, elevated miR-221 and -222 inhibit endothelial cell proliferation and migration through down-regulation of a second target, c-Kit.[15, 16] miR-222 has also been reported to down-regulate signal transducer and activator of transcription 5A signaling in endothelial cells, reducing inflammation driven neovascularization.[41] Thus, the impact of elevated miR-221 and -222 on an artery may extend beyond the effects on VSMCs described here.

For these studies, we chose the NONcNZO10/LtJ (RCS10) mouse, a recombinant cogenic mouse model of type 2 diabetes[23]. Compared with monogenic strains that develop diabetes based on the manipulation of a single gene, this strain develops an obesity associated form of diabetes due to multiple genetic mutations, making it more representative of the human disease. This strain develops a visceral obesity, insulin resistance in the skeletal muscle, liver, and heart, and hyperglycemia when fed a moderate fat (MF) diet of 10–11% fat as

opposed to a low fat (LF, 4%) diet[22, 23]. It has also been used as tool in studying diabetic nephropathy and impaired wound healing[42, 43]. Importantly for our studies, the diabetic NONcNZO10 mouse exhibits increased arterial miR-221 and -222 expression as seen in type 2 diabetic patients.[14] This increase was also seen in the New Zealand Obese mouse following feeding of a high fat diet to induce a type 2 diabetic state (unpublished data). Here, we demonstrate that diabetic NONcNZO10 mice recapitulate the increased intimal thickening seen in the diabetic population. As such, this study extends the use of this model into the field of the cardiovascular complications of diabetes.

Changes in the regulation of intimal thickening have the potential to greatly impact the progression of cardiovascular complications of diabetes. Diffuse intimal thickening provides a supportive environment for lipid deposition in the artery wall, initiating plaque formation[44, 45]. Pathologic intimal thickening occurs as part of an artery's response to the inflammation of plaque formation[46]. Type 2 diabetes is accompanied by an increase in carotid intimal-medial thickness[47, 48]. The data presented here suggest that increased ERK-1/2 activity in the arteries of diabetic subjects promotes intimal thickening via increased miR-221 and -222 expression under diabetic conditions. Thus, inhibition of this pathway may be efficacious in reducing the cardiovascular complications of diabetes. It should be noted, however, that a previous report from our laboratory demonstrates a decrease in miR-221 and -222 in carotid plaques acutely following stroke, suggesting a potential role in maintaining plaque stability.[13] Thus, such a therapy should aim to restore normal expression of these miRNAs rather than completely block their activity. For example, while the internal mammary artery of type 2 diabetic patients exhibits elevated miR-221 and -222 expression, we found that type 2 diabetic patients on metformin had similar expression levels as that of non-diabetic patients.[14] Such normalization of miR-221 and -222 expression may prevent the increase in intimal thickening in response to diabetes while avoiding effects on plaque vulnerability.

In conclusion, we observed an increase in intimal hyperplasia in the NONcNZO10 type 2 diabetic mouse that was dependent on elevated expression of miR-221 and -222. The increased miR-221 and -222 expression is linked to an increase in ERK-1/2 activity and results in destabilization of $p27^{Kip1}$ mRNA. Loss of $p27^{Kip1}$ protein expression results in a relative resistance to the effects of inhibition of mTOR on VSMC proliferation and migration. Additional studies are required to examine the potential effects of elevated miR-221 and -222 in other vascular cells (e.g. endothelial cells) as well as potential effects on other signaling pathways in VSMCs. Nonetheless, these data describe a role for ERK-1/2 and miR-221 and -222 in the cardiovascular complications of diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Diabetic mice exhibit increased intimal thickening in response to loss of p27Kip1.
- Loss of p27Kip1 is due to an increased expression of miRNA-221 and -222.
- A change in the molecular regulation of p27Kip1 in response to diabetes is proposed.

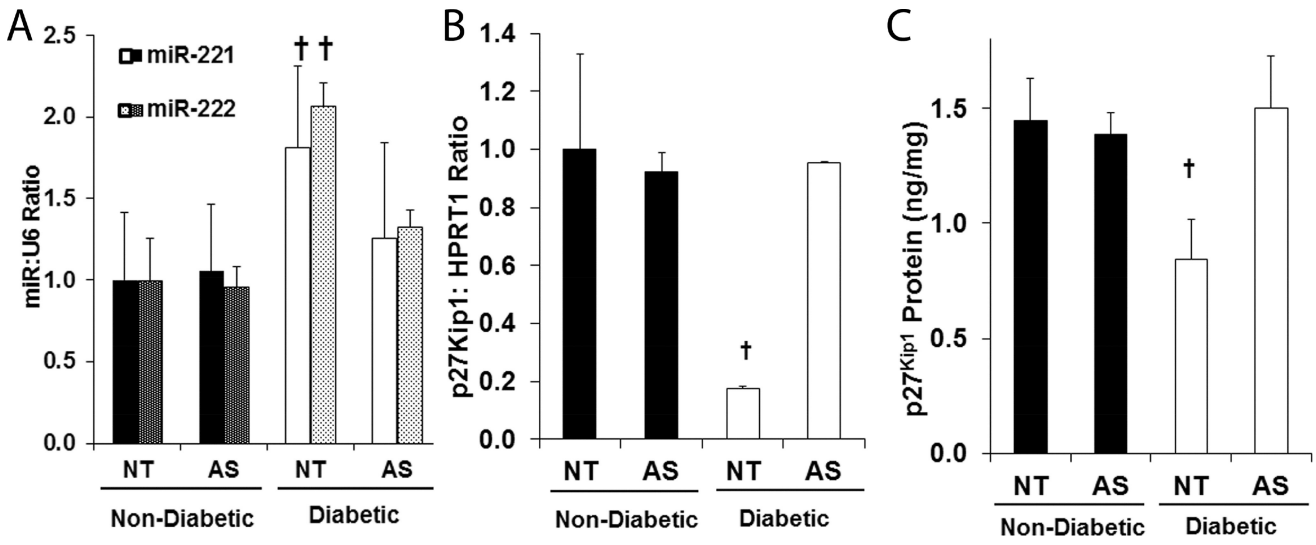

Figure 1. p27^{Kip1} levels are reduced in the arteries of DM mice in response to elevated miR-221 and -222

ND and DM mice were treated with a perivascular Pluronic gel containing non-targeting oligonucleotides (NT) or 2'OMe-miR-222 (AS, n=3 for all groups). Relative levels of (A) miR-221 and -222 levels and (B) p27Kip1 mRNA in the femoral arteries of ND and DM mice. Data is normalized to the ND NT group. † $p < 0.05$. (C) p27^{Kip1} protein levels in the aortae of ND and DM mice. † $p < 0.05$.

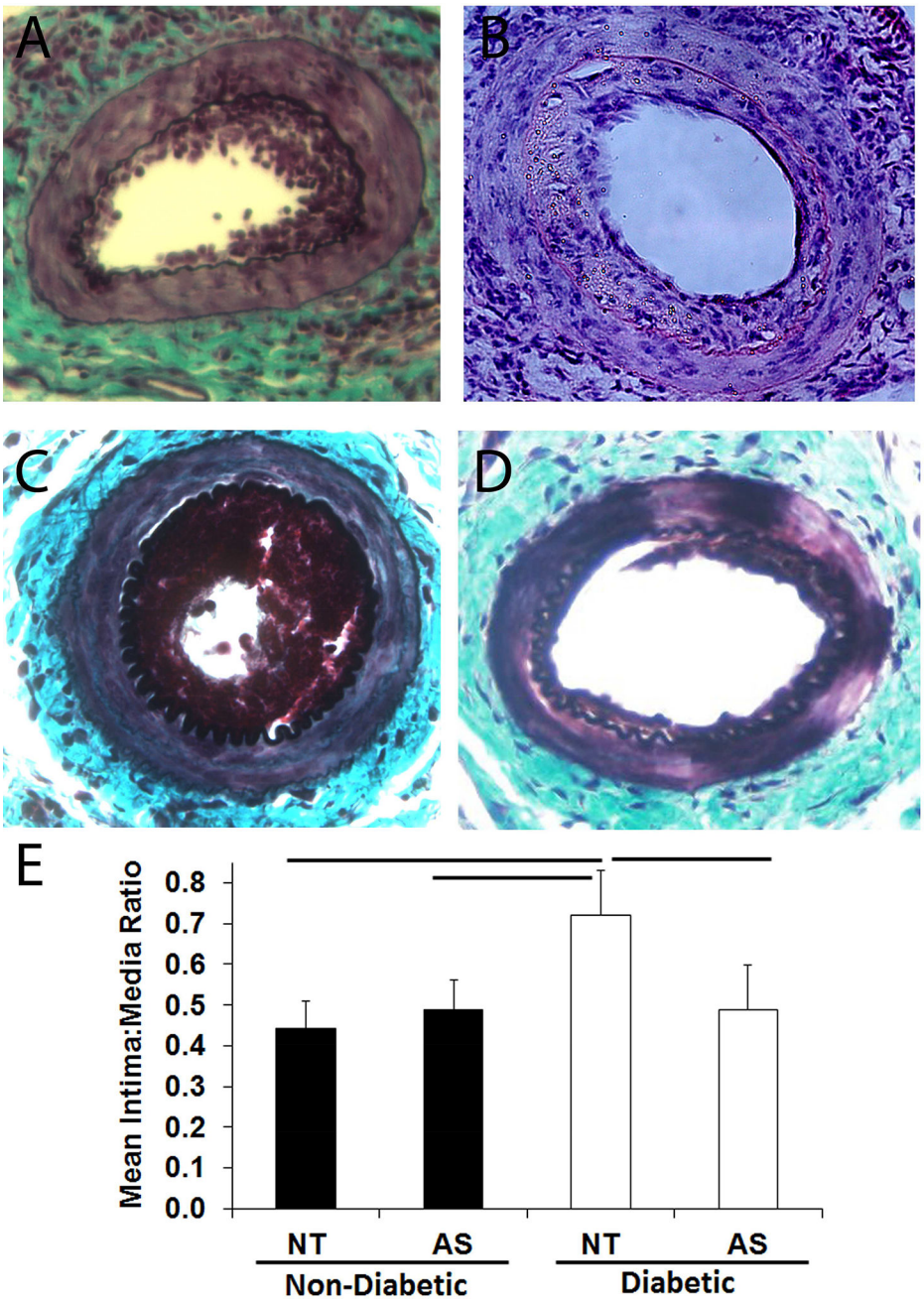

Figure 2. Intimal thickening following wire injury is increased in DM mice in a miR-221 and -222 dependent manner

ND and DM mice underwent femoral artery wire injury coupled with perivascular implantation of a Pluronic gel with either non-targeting oligonucleotide (NT) or 2'OMe-miR-222 (AS). (A–D) Representative micrographs of injured femoral arteries from ND treated with NT (A, n = 8), AS (B, n = 5), and DM mice treated with NT (C, n = 11) and AS (D, n = 6). (E) Intima to media ratio for ND and DM mice. Bar $p < 0.05$.

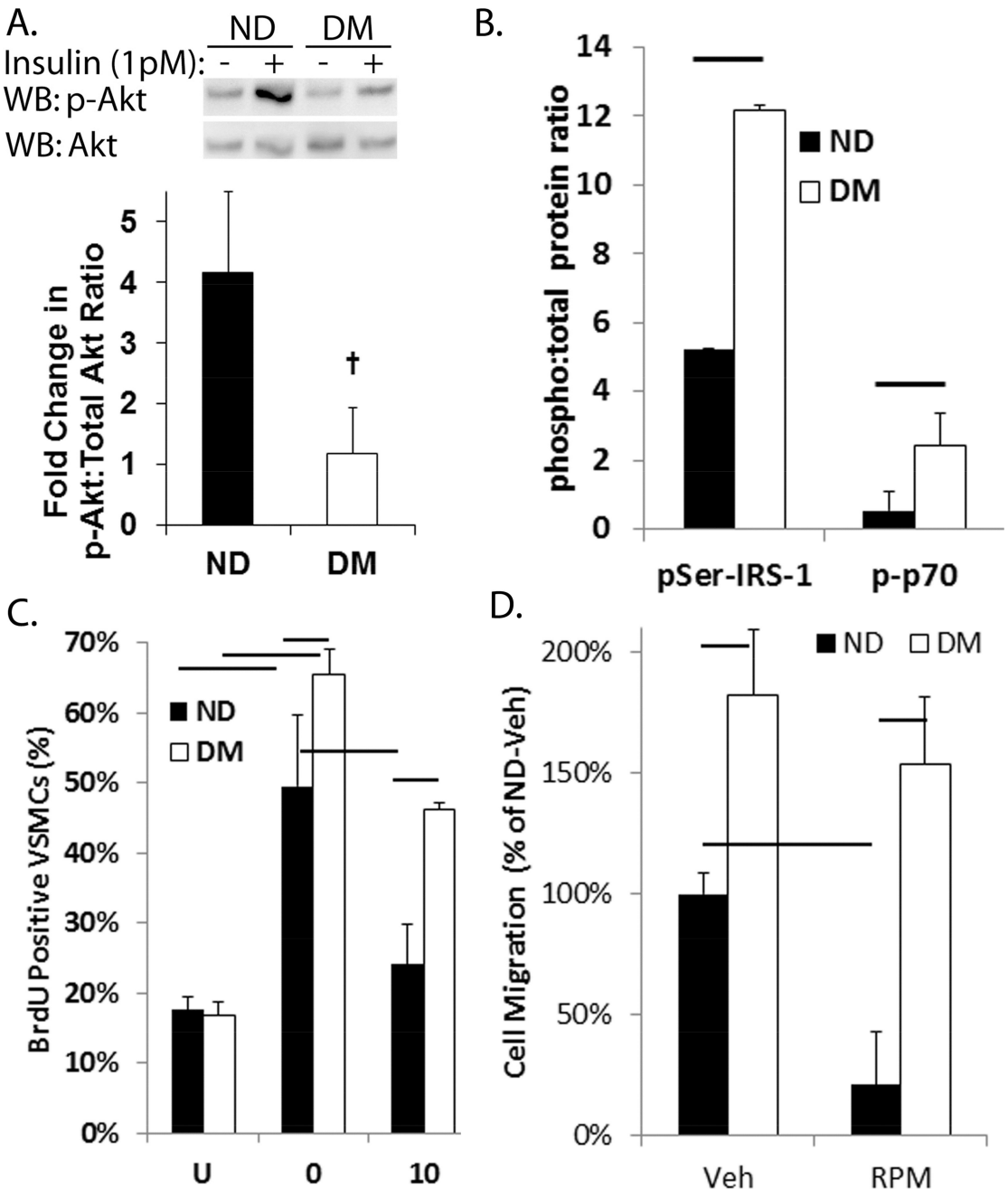

Figure 3. DM-VSMCs exhibit increased proliferation and migration coupled with a relative resistance to rapamycin

(A) Phosphorylation of Akt at threonine 308 residue (p-Akt) is reduced in DM-VSMCs compared to ND-VSMCs following stimulation with 1 pmol/L of insulin. P-Akt is normalized to total Akt. The upper panel shows a representative Western blot and the lower panel shows the densitometric analysis. + $p < 0.05$. (B) Basal phosphorylation of IRS-1 at serine 307 residue and $p70^{S6kinase}$ at tyrosine 421 and serine 424 residues in serum starved DM-VSMCs and ND-VSMCs. (C) Percent of BrdU positive VSMCs following serum starvation overnight (U) and stimulation with 20% serum/DMEM with rapamycin (10 nmol/L) or vehicle (0). Bar $p < 0.05$. (D) Migration of VSMCs toward 10 ng/mL PDGF in a

modified Boyden chamber. VSMCs were incubated in normal media with vehicle (Veh) or 10 nM rapamycin (RPM) overnight prior to seeding in the chamber. All studies were performed in triplicate. Data is expressed as percentage of ND-VSMCs treated with vehicle. Bar $p < 0.05$.

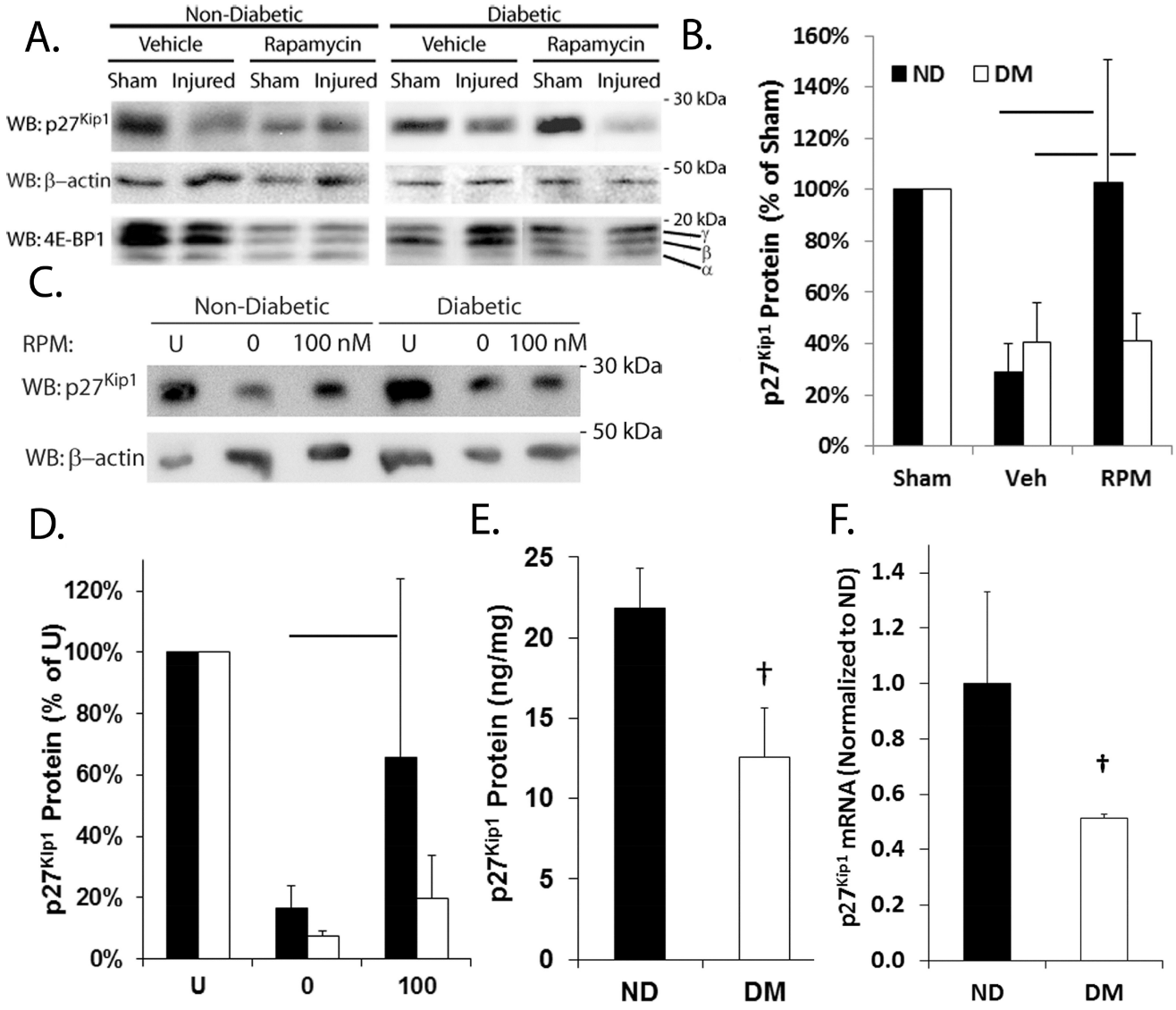

Figure 4. Regulation of p27^{Kip1} by the mTOR pathway is lost under diabetic conditions

(A) Representative Western blots for p27^{Kip1}, β-actin, and 4E-BP1 in ND and DM mice undergoing femoral artery wire injury, coupled with intraperitoneal injection of either vehicle or rapamycin. The contralateral artery underwent a sham procedure. (B) Densitometry of Western blots for p27^{Kip1} for ND (n=4 for vehicle, n=6 for rapamycin) and DM (n=3 for both groups) mice. Data is presented as mean of the percentage of p27^{Kip1} in the injured artery compared to the sham. This results in a reduction of the number of comparisons to 6. (C) Representative Western blots for p27^{Kip1} and β-actin in ND-VSMCs and DM-VSMCs. VSMCs were serum starved overnight (U), stimulated with DMEM/20% FBS supplemented with the indicated dose of rapamycin (RPM). (D) Densitometry of Western blots for p27^{Kip1} for ND-VSMCs and DM-VSMCs treated as indicated in C. All studies were performed in triplicate. Bar $p < 0.05$. (E) p27^{Kip1} protein levels in the aortae of ND and DM mice (n=4 for both groups) as measured by ELISA assay. † $p < 0.05$. (F) p27^{Kip1} mRNA in the aortae of ND and DM mice (n=3 for both groups).

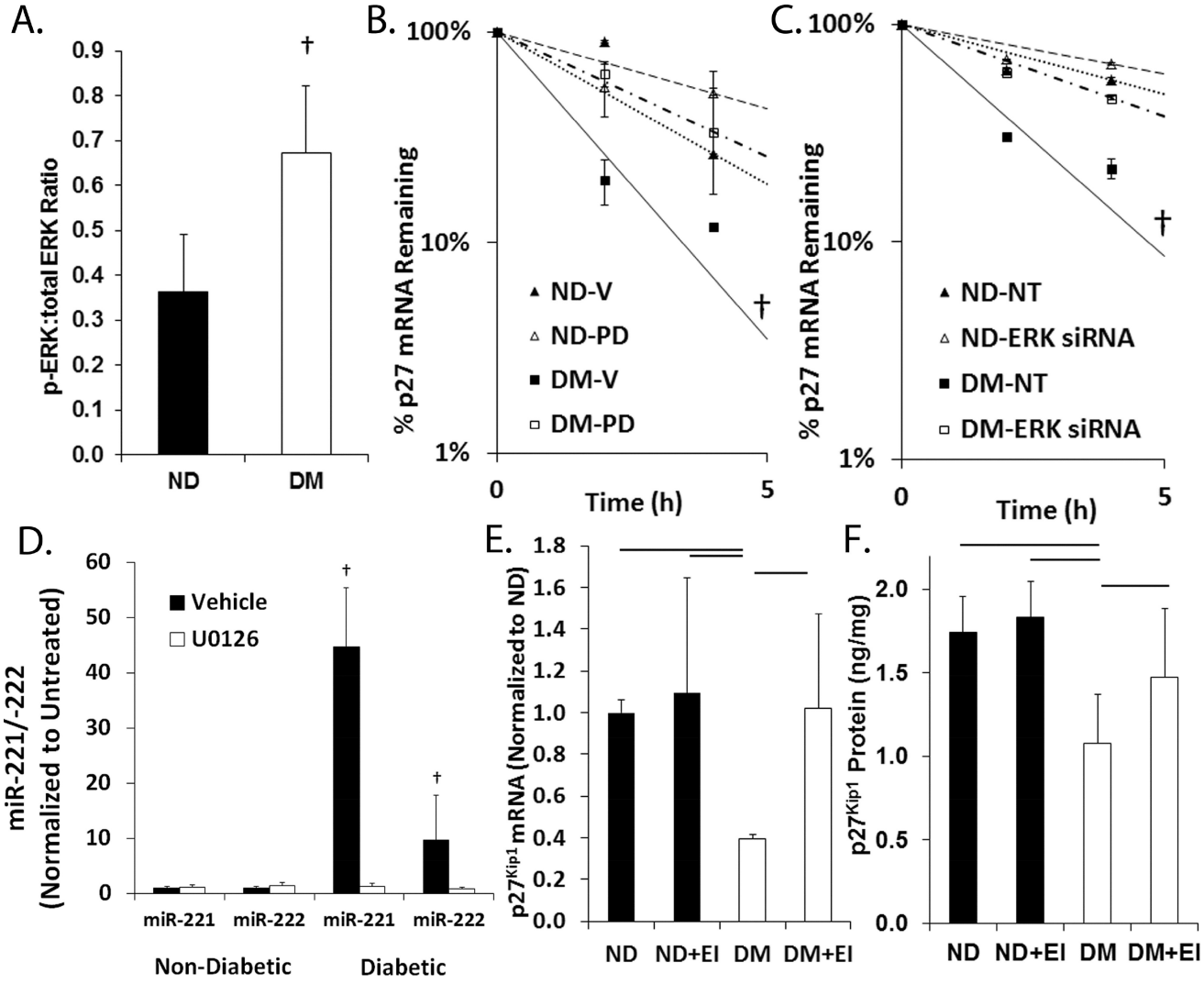

Figure 5. Increased ERK-1/2 activity promotes increased miR-221 and -222 as well as p27^{Kip1} mRNA destabilization

(A) Ratio of phosphorylated to total ERK-1/2 in the aortae of DM (n=3) and ND (n=4) mice as measured by ELISA. Bar $p < 0.05$. (B) Half-life of p27^{Kip1} mRNA in ND-VSMCs and DM-VSMCs treated with vehicle (Veh) and PD98059 (PD). Data are presented as mean of triplicate samples ± standard error of the mean. † $p < 0.05$. (C) Half-life of p27^{Kip1} mRNA in ND-VSMCs and DM-VSMCs transfected with non-targeting control oligonucleotides (NT) or siRNA targeting ERK 1/2 (ERK siRNA). Data are presented as mean of triplicate samples ± standard error of the mean. † $p < 0.05$. (D) Relative levels of miR-221 and -222 in serum stimulated ND-VSMCs and DM-VSMCs treated with vehicle or U0126 (50 μmol/L) overnight. Data are normalized to the vehicle treated samples. Bar $p < 0.05$. (E) Relative p27^{Kip1} mRNA levels in the aortae of ND mice treated with vehicle (ND, n=3) or U0126 (ND+EI, n=5) and DM mice treated with vehicle (DM, n=4) or U0126 (DM+EI, n=4). Data are normalized to ND. Bar $p < 0.05$. (F) Relative p27^{Kip1} protein levels in the aortae of ND

mice treated with vehicle (ND, n=4) or U0126 (ND+EI, n=4) and DM mice treated with vehicle (DM, n=4) or U0126 (DM+EI, n=6). Bar $p < 0.05$.