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Survivin is a mechanosensitive cell cycle regulator in vascular smooth muscle cells

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SUMMARY

Stiffened arteries are a pathology of atherosclerosis, hypertension, and coronary artery disease and a key risk factor for cardiovascular disease events. The increased stiffness of arteries triggers the hypermigration and hyperproliferation of vascular smooth muscle cells (VSMCs), leading to neointimal hyperplasia and accelerated neointima formation, but the mechanism of this trigger is not known. Our analyses of whole-transcriptome microarray data sets from mouse VSMCs cultured on stiff hydrogels simulating arterial pathology and from injured mouse femoral arteries revealed 80 genes that were differentially regulated (74 upregulated and 6 downregulated) relative to expression in control VSMCs cultured on soft hydrogels and in uninjured femoral arteries. A functional enrichment analysis revealed that these stiffness-sensitive genes are linked to cell cycle progression and proliferation. Furthermore, we found that survivin, a member of the inhibitor of apoptosis protein family, mediates stiffness-sensitive cell cycling and proliferation *in vivo* and *in vitro* as determined by gene network and pathway analyses, RT-qPCR, and immunoblotting. The stiffness signal is mechanotransduced via FAK and Rac signaling to regulate survivin expression, establishing a regulatory pathway for how the stiffness of the cellular microenvironment affects VSMC behaviors. Our findings indicate that survivin is necessary for VSMC cycling and proliferation and regulates stiffness-responsive phenotypes.

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INTRODUCTION

Arterial stiffening contributes to the development and progression of a variety of cardiovascular diseases [1-9]. The change in arterial stiffness affects the biophysical input to resident vascular smooth muscle cells (VSMCs), which constitute the biomechanically active and dynamic cell layer in the media of arteries. An increase in arterial stiffness triggers VSMCs to transition from a contractile (or differentiated) state to a synthetic (or dedifferentiated) state, in which they aberrantly migrate, proliferate, and produce extracellular matrix (ECM). This results in pathological neointima formation and further arterial stiffening [8-11]. The stiffness of the ECM surrounding VSMCs thus regulates cardiovascular biology with implications for aging [12] and Hutchinson–Gilford progeria syndrome [13] as well as atherosclerosis [5, 14], hypertension [15, 16], coronary artery diseases [17], and fibrosis [18].

To explore how ECM stiffness affects VSMC function, we and others have used fibronectin- or collagen-coated deformable polyacrylamide hydrogels to create culture matrices mimicking normal (healthy) and pathological (diseased) stiffnesses. Studies with this system showed that ECM stiffness affects cell cycling and proliferation [4, 7, 19], migration [20-22], intracellular stiffness and traction force [7, 19, 23, 24], cell–cell adhesion [19], and ECM synthesis [5, 12]. However, the signaling pathways regulating the response of VSMCs to ECM stiffness have yet to be fully defined.

There is evidence that VSMCs response to vascular injury and stiffening involve survivin [25-27]. Survivin (also known as BIRC5) belongs to the inhibitor of an apoptosis protein (IAP) family and was initially described as an antiapoptotic protein in cancer but later shown to regulate cancer cell migration and proliferation [28, 29]. Survivin is expressed at a low level in healthy adult tissue but is rapidly upregulated in response to vascular injury, atherosclerosis, and hypertension (conditions in which arteries stiffen [4, 5, 7]) in animal models [25, 26, 30]. Moreover, survivin is highly upregulated in proliferating VSMCs in the neointima and media in human atherosclerotic plaques and stenotic vein grafts [26]. Interestingly, overexpression of survivin (via a dominant-negative form of survivin) reduces neointima formation in rabbits [25], suggesting that survivin is a regulator of these effects.

Survivin induction after vascular injury correlates with the expression of cell proliferation genes downstream of focal adhesion kinase (FAK), which activates Rac to promote stiffness-sensitive cell cycle progression of VSMCs [7]. Furthermore, inhibition of FAK [31] or Rac [32] reduces survivin levels in other cell types. Because FAK and Rac are activated at sites of vascular injury and in VSMCs cultured on stiff hydrogels [4, 7, 19], we hypothesized that they contribute to the mechanotransduction of ECM stiffness to induce VSMC responses mediated by survivin. The

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objective of the present study was to identify the regulatory pathway through which the stiffness of the cellular environment affects VSMC behavior. Our findings suggest that ECM stiffness coordinates with survivin and focal adhesion biology in an integrated mechanobiochemical system to control the cell cycle progression and proliferation of VSMCs.

RESULTS

Whole-transcriptome analyses identify the stiffness-sensitive transcriptome of VSMCs

We examined the impact of ECM stiffness on the transcriptome of VSMCs *in vivo* and *in vitro*. For the *in vivo* study, we analyzed previously acquired whole-transcriptome microarray data [7] to identify genes that are differentially expressed after fine-wire injury to the femoral artery of mice (an *in vivo* model of VSMC proliferation and arterial stiffening). For the *in vitro* study, a whole-transcriptome microarray analysis was performed using mRNA samples from mouse VSMCs (mVSMCs) cultured for 24 h on fibronectin-coated soft (2–4 kPa) or stiff (16–30 kPa) polyacrylamide hydrogels, which reflect the elastic moduli of healthy or injured/diseased mouse artery [4, 7, 19]), respectively. Our *in vivo* transcriptome data analysis identified 25,253 genes (11,336 upregulated and 13,917 downregulated genes in injured femoral arteries compared to expression in uninjured arteries) (**Fig. 1A**), and the *in vitro* data analysis identified 21,999 genes (9,627 upregulated and 12,372 downregulated genes in mVSMCs cultured on stiff hydrogels compared to expression in cells on soft hydrogels) (**Fig. 1B**).

These data were then filtered by fold change and *q* value (see Materials and Methods for details), resulting in a list of 667 statistically significant differentially expressed genes (DEGs) for the *in vivo* study (332 upregulated and 335 downregulated; **Fig. 1A** and **Table S1**) and 613 significant DEGs for the *in vitro* study (354 increased and 259 decreased; **Fig. 1B** and **Table S2**). The distributions of these DEGs against the total number of identified genes were plotted as the $-\log_{10}$(*p* value) versus $\log_2$(fold change) values of each detected gene for the *in vivo* (**Fig. 1C**) and *in vitro* (**Fig. 1D**) data sets, with red color denoting significantly upregulated DEGs, green denoting significantly downregulated DEGs, and gray denoting genes with no significant change. Additionally, the DEGs obtained from *in vivo* (**Table S1**) and *in vitro* (**Table S2**) data sets are depicted in heat maps, showing significant clustering (uninjured vs. injured femoral arteries [**Fig. 1E**] and mVSMCs on soft vs. stiff hydrogels [**Fig. 1F**]).

We conducted a gene ontology (GO)-based functional enrichment analysis to identify biological processes associated with the upregulated DEGs. *In vivo* DEGs were highly enriched in cell activation, regulation of biological/cellular processes, response to stress or stimulus, mitotic cell cycle process, migration, and proliferation (**Fig. 1G**), all of which are important in neointima

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formation associated with arterial stiffening. DEGs upregulated *in vitro* were highly enriched primarily in the regulation of cell cycle processes (**Fig. 1H**), which are critical for cell cycle progression and proliferation.

Network analysis identifies survivin as a stiffness-sensitive mediator of cell cycle progression and proliferation

There were 80 DEGS common between the *in vitro* and *in vivo* datasets: 74 genes were commonly upregulated (**Fig. 2A**) and 6 genes were commonly downregulated (**Fig. 2B**). The expression levels of these common DEGs are presented as heat maps with hierarchical clustering, demonstrating groups of genes whose relative expression changes were most similar in the two data sets (**Fig. 2C, D**). We used Cytoscape's String application to couple biological processes with a network analysis of the 74 commonly upregulated DEGs. The functional enrichment of the DEGs in this string network indicated that the genes were involved in cell cycle regulation and nuclear/cell division (**Fig. 2E**). Notably, the most highly connected DEG among them was *Birc5* (baculoviral IAP repeat-containing 5; encodes survivin). *Birc5* was significantly upregulated in both *in vitro* and *in vivo* data sets and linked to all of the biological processes highlighted in this network. We therefore hypothesized that survivin serves as a molecular linchpin that controls the stiffness-mediated cell cycle progression of VSMCs.

Ingenuity Pathway Analysis (IPA; Qiagen) was applied to the *in vitro* DEGs to identify genes that interact with *Birc5* and are involved with cell cycle progression (with a Z-score of 2.124; a Z-score of $\geq$2 is considered significant [7, 33] and associated with cardiovascular disease (**Fig. 2F**)), including *Ccna2*, *Ccne1*, and *Ccne2*. To further visualize the molecular relationships between *Birc5* and cell cycle-associated DEGs, we generated another network with fold change values overlayed from the *in vitro* data set (**Fig. 2G**) using the Path Explorer tool of the IPA program. The survivin-to-cyclin network with the *in vivo* data set (**Fig. S1**) was similar to that for the *in vitro* data.

Survivin in VSMCs is stimulated by pathological ECM stiffness

An analysis of the *in vivo* and *in vitro* whole-transcriptome data sets for IAP family members other than *Birc5* (for survivin) identified *Naip1* (NLR family, apoptosis inhibitory protein 1 [*Birc1*]), *Birc2*, *Birc3, Xiap* (X-linked IAP [*Birc4*]), *Birc6* (baculoviral IAP repeat-containing 6), and *Birc7*. However, only *Birc5* mRNA expression was significantly increased in injured arteries (4-fold compared to that in the uninjured control; **Fig. 3A**) and in mVSMCs grown on stiff hydrogels (2.5-fold compared to that in cells grown on soft hydrogels; **Fig. 3B**). The increases in survivin levels were confirmed

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with real-time quantitative PCR (RT-qPCR) and immunoblotting from mVSMCs (**Fig. 3C, D**) as well as human VSMCs (hVSMCs) (**Fig. 3F, G**) cultured on soft and stiff hydrogels. Furthermore, protein levels of cyclins D1 and A (major targets of ECM stiffness-mediated signaling for cell cycle progression [4, 7, 19]) were sensitive to ECM stiffness in mVSMCs (**Fig. 3D, E**) and hVSMCs (**Fig. 3G, H**), respectively. Together with the results described above from the functional enrichment analysis (**Fig. 2E**), these data suggest that stiffness-sensitive expression of survivin is associated with cell cycle progression.

Survivin is essential for stiffness-mediated cell cycle progression

We next investigated how survivin expression affects cell cycle progression and proliferation. We transfected hVSMCs with control or survivin siRNAs before culturing them on soft or stiff hydrogels. These analyses showed that the targeted siRNAs reduced survivin mRNA (**Fig. 4A**) and protein (**Fig. 4D, E**) expression by 60−70% in hVSMCs cultured on stiff hydrogels compared to that in cells with control siRNAs. Furthermore, knockdown of survivin decreased the stiffness-mediated induction of cyclin D1 (CCDN1; **Fig. 4B, F**) and cyclin A (CCNA; **Fig. 4C, G**). Accordingly, S-phase entry was reduced by survivin siRNAs in cells cultured on stiff hydrogels as determined by the incorporation of EdU (5-ethynyl-2′-deoxyuridine) (**Fig. 4H**). Similarly, blocking the induction of survivin with YM155 (a pharmacological agent that inhibits survivin expression) in cells cultured on stiff hydrogels (**Fig. S2A**) reduced expression of *Ccnd1* (**Fig. S2B**), *Ccna* (**Fig. S2C**), and *Ccnb* (cyclin B) (**Fig. S2D**). S-phase entry was triggered in hVSMCs plated on soft hydrogels with adenovirus-mediated overexpression of survivin (**Fig. 4I**) relative to that in hVSMCs infected with a control virus (for GFP expression). These data demonstrate that survivin in VSMCs mediates the effects of ECM stiffness on cell cycle progression.

FAK-Rac signaling regulates stiffness-mediated survivin expression

Previous studies showed that the activity of FAK and Rac increases with vascular injury and stiffness-induced cell cycle progression and proliferation [4, 7, 19] and that survivin induction after vascular injury correlates with the expression of cell cycle genes downstream of FAK [7]. We therefore investigated whether FAK and Rac activity is responsible for stiffness-mediated survivin expression. Treatment of hVSMCs on stiff hydrogels with PF573228 (PF; FAK-specific inhibitor) or EHT1864 (EHT; Rac-specific inhibitor) markedly reduced the levels of survivin mRNA (**Fig. 5A**) and protein (**Fig. 5B, C**). These effects were confirmed by reduced survivin expression in hVSMCs infected with adenoviruses encoding FAK397 (a nonphosphorylatable form of FAK) or RacN17 (dominant-negative Rac) compared to that in cells expressing the LacZ control (**Fig. 5D**).

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Additionally, siRNA-mediated knockdown of FAK (**Fig. 5E, F**) and adenovirus-mediated overexpression of FAK-related non-kinase (FRNK) (**Fig. 5G, H**) similarly reduced survivin levels in hVSMCs cultured on stiff hydrogels compared to that in controls. Collectively, our results suggest that the mechanotransduction of ECM stiffness activates FAK-Rac signaling, which upregulates survivin and triggers cell cycle progression in VSMCs (**Fig. 5I**).

DISCUSSION AND CONCLUSION

Stiffness-mediated remodeling of the vascular wall involves complex interactions between the local microenvironment and VSMCs. Moreover, accelerated cell cycling and proliferation of VSMCs appear to both cause and result from the arterial stiffening process. To identify the molecular mechanism by which stiffness affects VSMC proliferation, we applied integrative genome-wide analyses to databases from mouse models of vascular injury and cellular models of arterial stiffening and cell proliferation. The evidence suggests that survivin is the molecular linchpin by which arterial or ECM stiffness mediates pathological VSMC behaviors.

Survivin is known as a critical regulator of mitosis and cytokinesis during cancer cell division. Overexpression of survivin in the nuclei of cancer cells promotes the G_1-S cell cycle transition by releasing p21 (known as a cyclin-dependent kinase [Cdk] inhibitor 1] from Cdk4 to activate the cyclin D1/Cdk4 complex, leading to the phosphorylation of the protein retinoblastoma [34, 35]. Furthermore, high ECM stiffness promotes this cyclin D1/Cdk4-dependent phosphorylation of retinoblastoma [4, 7, 19]. We showed that downregulation of survivin interferes with the stiffness-mediated induction of cyclin proteins, including cyclin D1 (expressed in early G_1 phase), cyclin A (expressed in S phase), and cyclin B (expressed in G_2/M phase) in VSMCs grown on stiff hydrogels. Furthermore, ectopic expression of survivin in VSMCs cultured on soft hydrogels was sufficient to promote cell cycling and proliferation. Together with previous studies, our findings suggest a novel mechanism by which the upregulation of survivin in VSMCs in response to ECM/arterial stiffness stimulates mechanosensitive cell cycling and proliferation.

The findings presented here expand on our work showing that stiffness-sensitive cell cycle progression and proliferation are associated with integrin, FAK, and Rac activity [4, 7, 19], suggesting that VSMCs sense ECM stiffness through integrin-dependent signaling. The inhibition of FAK or Rac interfered with stiffness-mediated expression of survivin. This likely involves FAK-Rac targeting of E2F1, which binds the survivin promotor [36] and induces survivin expression [37]. Furthermore, Rac1 binds and activates the transcription factor STAT3 [38], which also regulates the survivin promoter [39]. However, further studies are need to detail how FAK-Rac signaling triggers survivin transcription. Notably, the protein YAP also binds the survivin promoter

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and drives survivin transcription [40] and is involved in the mechanotransduction of ECM stiffness to cell cycling and proliferation [41].

In summary, we demonstrated that ECM/arterial stiffness signals an increase in survivin expression in VSMCs through FAK and Rac activation to induce cell cycle progression. This is a novel mechanism by which alterations to the microenvironment can trigger pathological phenotype switching of VSMCs from a contractile to a synthetic state. These findings also introduce potential targets for therapies for vascular diseases involving aberrant cell proliferation and arterial stiffness.

METHODS

Cell culture

Primary human VSMCs (hVSMCs; catalog number [cat. no.] 354-05a, Cell Applications, Inc.) were maintained in ≤90% Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate, 2% MEM amino acids solution, 50 µg/mL gentamicin solution, 1% penicillin–streptomycin solution, and 10% fetal bovine serum (FBS). Primary mouse VSMCs (mVSMCs) were prepared from explant cultures of thoracic aortae from 2-month-old male C57BL/6 mice and maintained in ≤90% in low-glucose DMEM–F12 medium (1:1) supplemented with 10% FBS, 2 mM glutamine, 25 µM HEPES, 1% penicillin–streptomycin solution, and 50 µg/mL gentamicin solution. Both cell types were maintained in 10% CO_2 at 37°C and used before passage 5. To synchronize VSMCs to the G_0 cell cycle phase, cultures that were near confluence were incubated in serum-free DMEM containing 1 mg/ml heat-inactivated, fatty-acid-free bovine serum albumin (BSA) for 48 h. The starved cells were then treated with 0.05% trypsin–EDTA, centrifuged, resuspended, and plated on soft or stiff hydrogels with fresh medium containing 10% FBS for 24 h.

siRNA transfection: hVSMCs were transfected with 200 nM survivin, FAK, or control siRNAs by using Lipofectamine 2000 reagent in Opti-MEM as previously described [7, 42]. After 4−5 h of siRNA transfection, cells were serum starved in fresh DMEM containing 1 mg/ml BSA for 43−44 h. All siRNA-based experiments were performed 72 h after transfection. The following survivin (*BIRC5*) and FAK (*PTK2*) siRNAs were obtained from Ambion: survivin siRNA #1 (ID no. 121294), 5′-CCACUUCCAGGGUUUAUUCtt-3′; survivin siRNA #2 (ID no. 121295), 5′-GCCAUUCUAAGUCAUUGGGtt-3′; FAK siRNA #1 (ID no. 157448), 5′-CCUAGCAGACUUUAACCAAtt-3′; and FAK siRNA #2 (ID no. 61352), 5′-

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GGCAUGGAGAUGCUACUGAtt-3′. A nonspecific siRNA (cat no. AM4636) was used as an experimental control.

Adenovirus infection: hVMSCs were first incubated in DMEM containing 1 mg/ml BSA for 8−9 h. Cells were then incubated for 20−24 h with adenoviruses encoding wild-type survivin (cat. no. 1611, Vector Biolabs; multiplicity of infection [MOI], 25 and 50) or FRNK (a gift from the Assoian Laboratory; MOI, 600); adenoviruses encoding GFP (cat. no. 1060, Vector Biolabs; MOI, 50) or LacZ (a gift from the Assoian Laboratory; MOI, 600) were used as the respective experimental controls.

Drug treatment: Serum-starved hVSMCs were plated on fibronectin-coated soft or stiff hydrogels with medium containing 10% FBS and were treated with 0.1, 0.5, or 2 μM YM155 (survivin inhibitor; cat. no. 11490, Cayman Chemical), 10 μM PF573228 (FAK inhibitor; cat. no. 14924, Cayman Chemical), 10 μM EHT1864 (Rac inhibitor; cat. no. 17258, Cayman Chemical), or dimethyl sulfoxide (vehicle control).

Preparation of stiffness-tunable hydrogels

The soft (2–4 kPa) and stiff (16–30 kPa) polyacrylamide hydrogels [7, 43] approximate the physiological stiffness of a healthy mouse femoral artery and after vascular injury and atherosclerosis [4, 5], respectively. The protocol for generating stiffness-tunable polyacrylamide hydrogels was previously described [42, 43]. Briefly, glass coverslips were etched homogenously with a 0.1 M NaOH solution for 3 min and then treated with 3-(trimethoxysilyl)propyl methacrylate (cat. no. 440159, Sigma-Aldrich) to introduce amine groups to cross-link with the polyacrylamide hydrogel. Hydrogels of different stiffness were prepared by changing the ratio of 40% acrylamide to 1% bis-acrylamide in a mixed solution with sterilized water, ammonium persulfate (cat. no. A3678, Sigma-Aldrich), TEMED (cat. no. J63734.AC, Thermo Fisher Scientific), and an *N*-hydroxysuccinimide-fibronectin solution prepared by combining 1 part *N*-hydroxysuccinimide solution (cat. no. A8060, Sigma-Aldrich; 1 mg/ml in dimethyl sulfoxide) and 9 parts fibronectin solution (cat. no. 341631, Calbiochem; 100 μl fibronectin at 1 μg/μl dissolved in 1.9 ml Tris base [pH 8.4 pH]). Finally, the fibronectin-coated hydrogels were extensively washed in Dulbecco's phosphate-buffered saline (DPBS) and water to remove unpolymerized polyacrylamide, and unreactive cross-linkers were blocked with 1 mg/ml BSA before VSMCs were seeded.

RNA isolation and RT-qPCR

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hVSMCs and mVSMCs cultured on soft or stiff hydrogels for 24 h were treated with TRIzol reagent to extract total RNA. The RNA was reverse transcribed and analyzed by RT-qPCR as previously described [42]. TaqMan probes (Invitrogen) were used for survivin (Mm00599749_m1 for mouse mRNA and Hs04194392_s1 for human mRNA), GAPDH (Mm99999915_g1 for mouse mRNA and Hs02786624_g1 for human mRNA), cyclin D1 (Hs00765553_m1), cyclin A (Hs00171105_m1), and cyclin B (Hs00259126_m1). The relative change in mRNA expression for each target mouse or human gene was determined by the comparative threshold cycle method using the gene for GAPDH as the reference.

Protein extraction and immunoblotting

As previously described [42], total cell lysates were collected from hVSMCs or mVSMCs cultured on soft or stiff hydrogels by incubating the hydrogels face down for 2 min at room temperature on 5× sample buffer (250 mM Tris [pH 6.8], 10% SDS, 50% glycerol, 0.02% bromophenol blue, and 10 mM 2-mercaptoethanol). Equal amounts of extracted protein were fractionated on reducing 8−12% SDS-polyacrylamide gels, and the fractioned proteins were subsequently transferred electrophoretically onto polyvinylidene fluoride membranes via the Trans-Blot Turbo Transfer System (Bio-Rad). These membranes were blocked in 6% nonfat milk for 1.5 h at room temperature and then probed with antibodies against survivin (cat. no. NB500-201, Novus Biologicals; 1:200), cyclin D1 (cat. no. sc-20044, Santa Cruz Biotechnology; 1:200), cyclin A (a gift from the Assoian Laboratory (Kothapalli, 2003 #54), 1:500), FAK (cat. no. 39-6500, Invitrogen; 1:500), or GAPDH (cat. no. 60004-1-Ig or 10494-1-AP, Proteintech; 1:5,000). Antibody signals were detected using Clarity (cat. no. 1705061, Bio-Rad) or Clarity Max (cat. no. 1705062, Bio-Rad) Western ECL substrate.

EdU incorporation

Serum-starved hVSMCs treated with control or survivin siRNAs or infected with adenovirus encoding wild-type survivin or GFP were plated on fibronectin-coated stiff or soft hydrogels with 10% FBS and then incubated with 20 μM EdU for 24 or 36 h. Cells were fixed in 3.7% formaldehyde and visualized using the Click-iT EdU Alexa Fluor 594 imaging kit (cat. no. C10339, Invitrogen) according to the manufacturer's instructions. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole), and coverslips were mounted on microscope slides. Three to eight fields of view were counted per coverslip to determine the percentage of hVSMCs with DAPI-stained nuclei that were positive for EdU.

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Bioinformatics analysis

(i) Gene expression analysis: Differential gene expression analysis was performed on raw microarray data. Duplicate and blank (no name) gene entries were removed from both data lists, and genes with nonsignificant differential expression values were filtered out before further analysis. For *in vitro* microarray data, differentially expressed genes (DEGs) were defined as genes having a ≥1.5-fold change and a false-discovery rate (FDR)-adjusted *p* value (*q* value) of ≤0.05. For the *in vivo* microarray data, DEGs were defined as genes having a ≥2.0-fold change and a *q* value of ≤0.15. Python's bioinfokit package was used to generate volcano plots and heat maps with hierarchical clustering of DEGs.

(ii) Functional enrichment analysis: Functional enrichment analysis was performed using the g:GOSt tool in gProfiler (https://biit.cs.ut.ee/gprofiler/gost). The statistical domain scope of the analysis was only annotated genes, and the significance threshold was set to the g:SCS algorithm for computing multiple-testing correction for *p* values acquired from Gene Ontology (GO). Significant GO terms were defined by an adjusted *p* value of ≤0.05. The top 25 Biological Process GO terms were presented in histograms on a scale of −log(adjusted *p* value). Cytoscape (https://cytoscape.org/) was used to visualize the functional enrichment results as they pertained to DEGs common to both the *in vitro* and *in vivo* data sets. The list of 74 commonly upregulated DEGs was input into Cytoscape's String application as a protein query, and the *in vitro* expression data were imported into the node table so that the $\log_2$(fold change) values could be used to indicate expression levels, with node color indicating intensity. The only nodes kept were those in the network that displayed a relationship to any other gene in the network at a score of 0.95 and fell under the following Biological Process GO categories: cell cycle (GO:0007049), regulation of cell cycle (GO:0051726), mitotic cell cycle (GO:0000278), mitotic cell cycle processes (GO:1903047), G_2/M transition of mitotic cell cycle (GO:0000086), cell division (GO:0051301), nuclear division (GO:0000280), and mitotic nuclear division (GO:0140014). The resulting network contained 37 genes.

(iii) Network analysis**:** Ingenuity Pathway Analysis (IPA, Qiagen) was used to perform further bioinformatics analysis on the filtered microarray data. A core analysis was run on each of the data sets, which returned information on various mechanistic pathways and enriched functions based on the literature compiled in the Ingenuity Knowledge Base. The "Diseases and Functions" tool was used to identify molecules known to be involved in cell cycle progression within the *in vitro* data set, and the "My Pathway" tool was subsequently used to display known relationships

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between *Birc5* and other genes within the cell cycle progression function. The Z-directional components of the expression analysis were based on the expression log ratio values. Functions with a Z-score of >2 were regarded as having significant activation, whereas those with a Z-score of <−2 were considered as having significant inhibition. The "Molecule Activity Predictor" tool was used to display gene expression levels via node color and intensity and to generate predicted activation states of molecules and interactions based on the results of the Core Analysis.

Statistical analysis

Statistical significance was determined by paired, two-tailed Student's *t* tests. Graphs show means + SEMs from the indicated number of independent experiments.

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AUTHOR CONTRIBUTIONS

J.C.B., A.S., S-J.H, J.K., K.L., and Y.B. designed the research. J.C.B., A.S., J.A.B., A.K., Y.H., and Y.B. performed cell/molecular biology experiments and data analysis. A.S., K.E.P., V.M.T., and Y.B. performed bioinformatic analysis. J.C.B. and Y.B. performed statistical tests. J.C.B., A.S., J.A.B., S-J.H, J.K., K.L., and Y.B. wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1. Whole-transcriptome analyses show the stiffness-sensitive transcriptome of vascular smooth muscle cells (VSMCs). Reductions in data magnitude by applying significance thresholds to the raw *in vivo* data (fold change [FC] ≥ 2.0, *q* value ≤ 0.15) (**A**) and the raw *in vitro* data (FC ≥ 1.5, *q* value ≤ 0.05) (**B**). Volcano plots display the distributions of all detected transcripts, represented as single dots that are not statistically different (gray), significantly upregulated (red), or significantly downregulated [33] from *in vivo* (**C**) and *in vitro* (**D**) data sequencing results. The *y* axis represents each gene's $-\log_{10}$(*p* value), whereas the *x* axis represents their $\log_2$(fold change). Heat maps display the Z-scores of the 667 differentially expressed genes (DEGs) *in vivo* (**E**) and 613 DEGs *in vitro* (**F**). Histograms present the top 25 biological processes enriched for 332 upregulated DEGs in the *in vivo* data set (**G**) and 354 upregulated DEGs in the *in vitro* data set (**H**).

Figure 2. Network analysis identifies survivin (*Birc5*) as a potential stiffness-mediated regulator of cell cycle progression. Venn diagrams representing the number of commonly upregulated (**A**) or downregulated (**B**) differentially expressed genes (DEGs) present within the *in vivo* and *in vitro* data sets. Heat maps representing the $\log_2$(fold change) of the 6 DEGs that were commonly downregulated (**C**) and the 74 DEGs that were commonly upregulated (**D**) in both *in vivo* and *in vitro* data sets. Hierarchical clustering dendrograms are provided to the left of each heat map. (**E**) String network generated using the 74 commonly upregulated DEGs, with node color representing *in vitro* $\log_2$(fold change) values and border colors indicating DEG memberships to GO biological process categories. (**F**) Network diagram providing an overview of the molecular interactions related to Birc5 within the activated cell cycle progression function (Z-score = 2.124) in cardiovascular disease. (**G**) Predictive model for Birc5 regulation of cyclins. Network diagram presenting downstream targets of Birc5 (including cyclins) and potential intermediate molecules, with node color and intensity representing the observed expression levels or predicted activation states based on the *in vitro* data.

Figure 3. ECM stiffness modulates survivin expression in vascular smooth muscle cells (VSMCs). Differential mRNA expression of baculovirus inhibitor of apoptosis repeat-containing (BIRC) gene family after femoral vascular injury (**A**) and from mouse VSMCs (mVSMCs) seeded on fibronectin-coated soft or stiff polyacrylamide hydrogels (**B**). G_0-synchronized mVSMCs (**C–E**) or human (h)VSMCs (**F–H**) were seeded on soft or stiff hydrogels for 24 h. Survivin gene (**C** and **F**) and protein (**D** and **G**) expression was analyzed by RT-qPCR and immunoblotting, respectively.

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Levels of survivin mRNA were normalized to those measured in mVSMCs (**C**) and hVSMCs (**F**) on soft hydrogels; *n = 5* (C) and *n = 8* (F) independent experiments. Average survivin intensity in mVSMCs (**D**) and hVSMCs (**G**) was quantified using ImageJ and normalized to that in VSMCs on soft hydrogels. Representative immunoblot images from four independent biological replicates; GAPDH served as a loading control. Survivin protein levels normalized to that in mVSMCs (**E**) and hVSMCs (**H**) on soft hydrogels. Data are means + SEMs. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 4. Survivin is required for stiffness-mediated cell cycle progression. (**A–G**) Human vascular smooth muscle cells (hVSMCs) were transfected with control siRNA or siRNAs to survivin [1 and #2], synchronized to G_0 via serum starvation, and plated on soft or stiff hydrogels with 10% FBS for 24 h. Total cell lysates were analyzed by RT-qPCR (**A–C**) or immunoblotting (**D–G**) to determine mRNA and protein levels of survivin (**A** and **E**), cyclin D1 (CCND1; **B** and **F**), and cyclin A (CCNA; **C** and **G**). Expression levels were normalized to those in hVSMCs treated with control siRNA on stiff hydrogels. *n = 4* (A), *n = 3* (B), *n = 3* (C), *n = 5* (E), *n = 3* (F), and *n = 5* (G) independent experiments. S-phase entry with survivin knockdown and overexpression was assessed by EdU incorporation, which was normalized to hVSMCs treated with control siRNA on stiff hydrogels (**H**) or infected with GFP adenovirus (Adv) on soft hydrogels (**I**). *n = 5* (H) and *n = 5* (I) independent experiments. Data are means + SEMs. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 5. FAK-Rac signaling regulates stiffness-mediated survivin expression. G_0-synchronized human vascular smooth muscle cells (hVSMCs) treated with FAK inhibitor PF573228 (PF) or Rac inhibitor EHT1864 (EHT) in dimethyl sulfoxide (DMSO) were seeded on fibronectin-coated soft or stiff hydrogels for 24 h. Survivin mRNA (**A**) and protein (**B** and **C**) expression was analyzed by RT-qPCR and immunoblotting, respectively; levels were normalized to those in hVSMCs treated with DMSO on stiff hydrogels; *n* = 3 (A) and *n* = 5 (B and C) independent experiments. (**D**) hVSMCs infected with adenoviruses encoding LacZ, FAK^{397F}, or Rac^{N17} were seeded on soft or stiff hydrogels with 10% FBS for 24 h. Survivin mRNA expression was analyzed by RT-qPCR; levels were normalized to those in hVSMCs infected with LacZ on stiff hydrogels. *n* = 8 independent experiments. (**E** and **F**) G_0-synchronized hVSMCs were transfected with control siRNA or siRNAs to FAK [1 and #2], serum-starved, and plated on stiff hydrogels with 10% FBS for 24 h. FAK and survivin levels in total cell lysates were analyzed by immunoblotting; levels were normalized to those in hVSMCs treated with control siRNA on stiff hydrogels. *n* = 3 independent experiments. (**G** and **H**) hVSMCs infected with adenoviruses encoding LacZ or FRNK were plated on stiff hydrogels for 24 h. Protein expression levels were

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analyzed by immunoblotting. n = 4 independent experiments. (**I**) Model of signal transduction for stiffness-mediated cell cycle progression. Data are means + SEMs. *p < 0.05, **p < 0.01, ***p < 0.001.

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Predictive model for survivin (BIRC5) regulation of cyclins. Network diagram displaying downstream targets of BIRC5 and potential intermediate molecules, with node color and intensity representing the observed expression levels or predicted activation states based on the *in vivo* data.

Figure S2. Pharmacologic reduction of survivin expression decreases cyclin D1, A, and B mRNA expression in human vascular smooth muscle cells (hVSMCs). G_0-synchronized hVSMCs were seeded on soft or stiff hydrogels with or without YM155 (inhibits survivin expression) for 24 h. RT-qPCR was performed to assess the expression of survivin (**A**), cyclin D1 (CCND1; **B**), cyclin A (CCNA; **C**), and cyclin B (CCNB; **D**) mRNA; levels were normalized to those in hVSMCs treated with dimethyl sulfoxide (0 μM YM155) on stiff hydrogels. n = 10–14 (A), n = 3–5 (B), n = 5–9 (C), and n = 3–7 (D) independent experiments. Data are means + SEMs. *p < 0.05, **p < 0.01, ***p < 0.001.

TABLES

Table S1. Differentially expressed gene (DEG) lists of the *in vivo* study

Table S2. Differentially expressed gene (DEG) lists of the *in vitro* study

Survivin is a mechanosensitive cell cycle regulator in vascular smooth muscle cells

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Figure 1

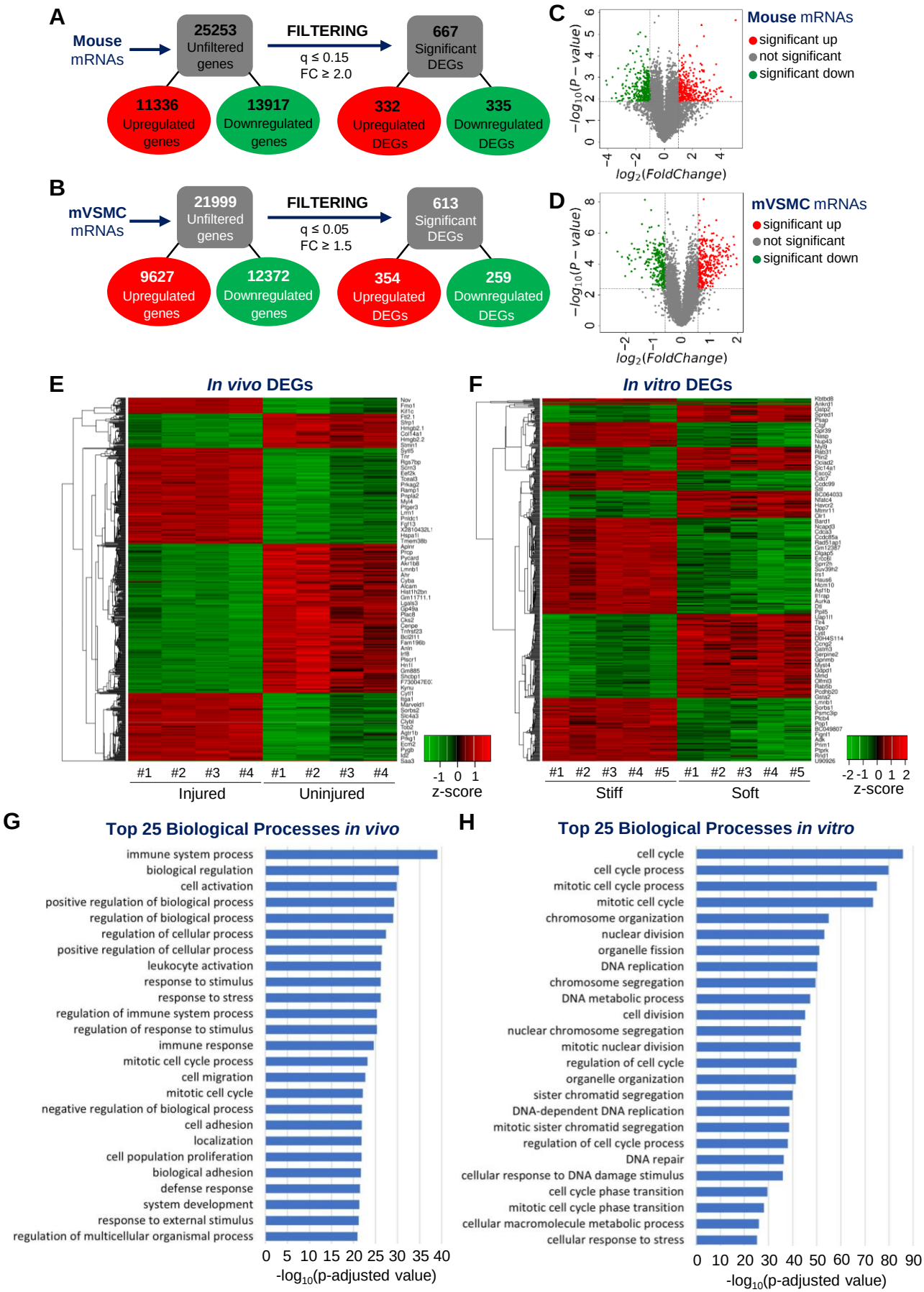

A
Mouse mRNAs
25253 Unfiltered genes
FILTERING
q ≤ 0.15
FC ≥ 2.0
667 Significant DEGs
11336 Upregulated genes
13917 Downregulated genes
332 Upregulated DEGs
335 Downregulated DEGs
B
mVSMC mRNAs
21999 Unfiltered genes
FILTERING
q ≤ 0.05
FC ≥ 1.5
613 Significant DEGs
9627 Upregulated genes
12372 Downregulated genes
354 Upregulated DEGs
259 Downregulated DEGs
C
Mouse mRNAs
significant up
not significant
significant down
−log10(P − value)
log2(FoldChange)
D
mVSMC mRNAs
significant up
not significant
significant down
−log10(P − value)
log2(FoldChange)
E
In vivo DEGs
#1 #2 #3 #4 #1 #2 #3 #4
Injured
Uninjured
-1 0 1
z-score
F
In vitro DEGs
#1 #2 #3 #4 #5 #1 #2 #3 #4 #5
Stiff
Soft
-2 -1 0 1 2
z-score
G
Top 25 Biological Processes in vivo
immune system process
biological regulation
cell activation
positive regulation of biological process
regulation of biological process
regulation of cellular process
positive regulation of cellular process
leukocyte activation
response to stimulus
response to stress
regulation of immune system process
regulation of response to stimulus
immune response
mitotic cell cycle process
cell migration
mitotic cell cycle
negative regulation of biological process
cell adhesion
localization
cell population proliferation
biological adhesion
defense response
system development
response to external stimulus
regulation of multicellular organismal process
0 5 10 15 20 25 30 35 40
-log10(p-adjusted value)
H
Top 25 Biological Processes in vitro
cell cycle
cell cycle process
mitotic cell cycle process
mitotic cell cycle
chromosome organization
nuclear division
organelle fission
DNA replication
chromosome segregation
DNA metabolic process
cell division
nuclear chromosome segregation
mitotic nuclear division
regulation of cell cycle
organelle organization
sister chromatid segregation
DNA-dependent DNA replication
mitotic sister chromatid segregation
regulation of cell cycle process
DNA repair
cellular response to DNA damage stimulus
cell cycle phase transition
mitotic cell cycle phase transition
cellular macromolecule metabolic process
cellular response to stress
0 10 20 30 40 50 60 70 80 90
-log10(p-adjusted value)

Figure 2

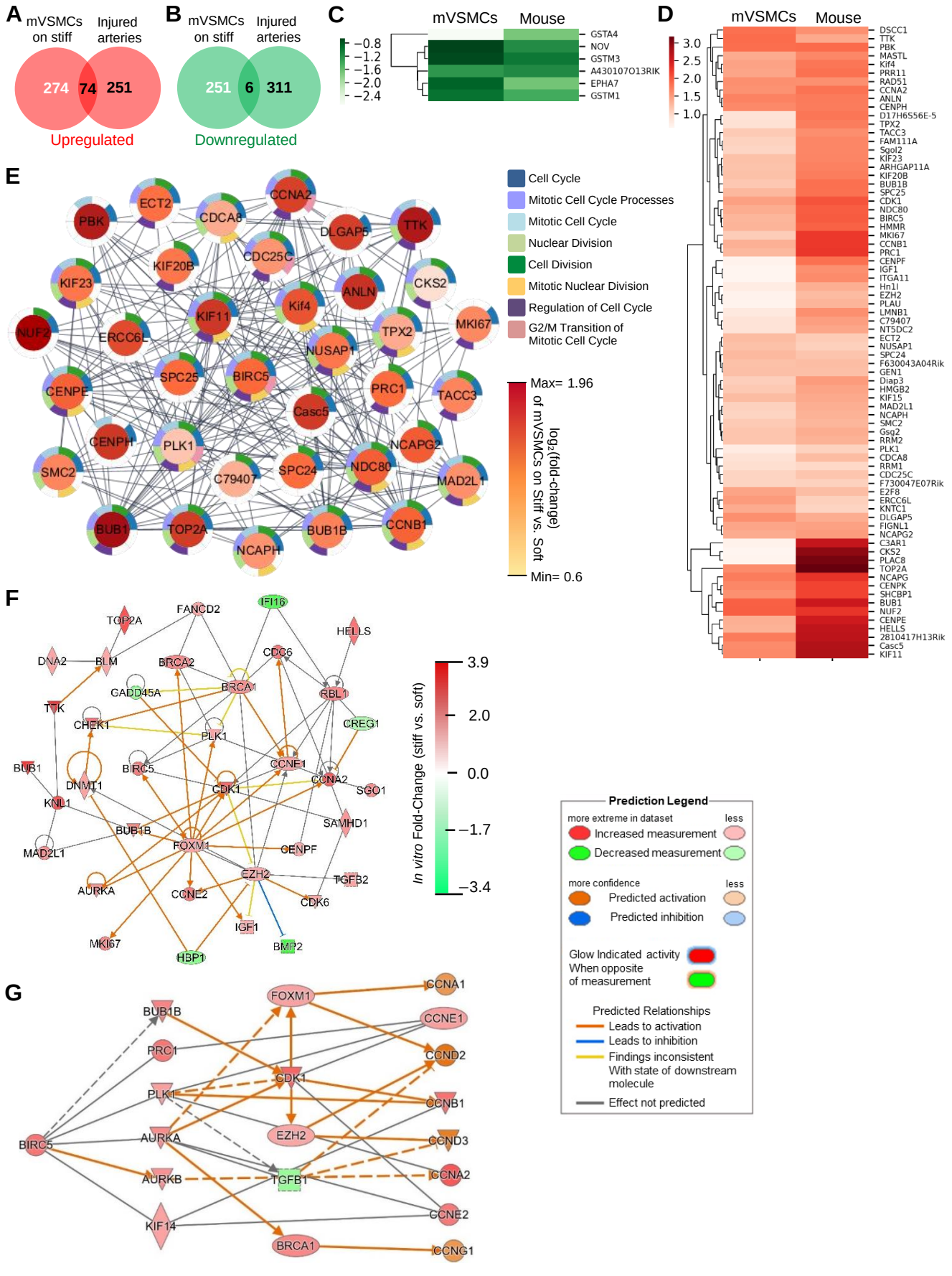

A
mVSMCs on stiff
Injured arteries
274
74
251
Upregulated
B
mVSMCs on stiff
Injured arteries
251
6
311
Downregulated
C
mVSMCs
Mouse
−0.8
−1.2
−1.6
−2.0
−2.4
GSTA4
NOV
GSTM3
A430107O13RIK
EPHA7
GSTM1
D
mVSMCs
Mouse
3.0
2.5
2.0
1.5
1.0
DSCC1
TTK
PBK
MASTL
Kif4
PRR11
RAD51
CCNA2
ANLN
CENPH
D17H6S56E-5
TPX2
TACC3
FAM111A
Sgol2
KIF23
ARHGAP11A
KIF20B
BUB1B
SPC25
CDK1
NDC80
BIRC5
HMMR
MKI67
CCNB1
PRC1
CENPF
IGF1
ITGA11
Hn1l
EZH2
PLAU
LMNB1
C79407
NT5DC2
ECT2
NUSAP1
SPC24
F630043A04Rik
GEN1
Diap3
HMGB2
KIF15
MAD2L1
NCAPH
SMC2
Gsg2
RRM2
PLK1
CDCA8
RRM1
CDC25C
F730047E07Rik
E2F8
ERCC6L
KNTC1
DLGAP5
FIGNL1
NCAPG2
C3AR1
CKS2
PLAC8
TOP2A
NCAPG
CENPK
SHCBP1
BUB1
NUF2
CENPE
HELLS
2810417H13Rik
Casc5
KIF11
E
Cell Cycle
Mitotic Cell Cycle Processes
Mitotic Cell Cycle
Nuclear Division
Cell Division
Mitotic Nuclear Division
Regulation of Cell Cycle
G2/M Transition of Mitotic Cell Cycle
Max= 1.96
log2(fold-change) of mVSMCs on Stiff vs. Soft
Min= 0.6
PBK
ECT2
CDCA8
CCNA2
DLGAP5
TTK
KIF20B
CDC25C
KIF23
ANLN
CKS2
Kif4
KIF11
NUF2
ERCC6L
TPX2
MKI67
NUSAP1
SPC25
BIRC5
CENPE
PRC1
TACC3
Casc5
CENPH
PLK1
NCAPG2
SMC2
C79407
SPC24
NDC80
MAD2L1
BUB1
TOP2A
BUB1B
CCNB1
NCAPH
F
TOP2A
FANCD2
IFI16
HELLS
DNA2
BLM
BRCA2
CDC6
GADD45A
BRCA1
RBL1
TTK
CHEK1
PLK1
CREG1
BUB1
BIRC5
CCNE1
DNMT1
CDK1
CCNA2
SGO1
KNL1
SAMHD1
BUB1B
MAD2L1
FOXM1
CENPF
EZH2
TGFB2
AURKA
CCNE2
CDK6
IGF1
MKI67
BMP2
HBP1
3.9
2.0
0.0
−1.7
−3.4
In vitro Fold-Change (stiff vs. soft)
Prediction Legend
more extreme in dataset
less
Increased measurement
Decreased measurement
more confidence
less
Predicted activation
Predicted inhibition
Glow Indicated activity When opposite of measurement
Predicted Relationships
Leads to activation
Leads to inhibition
Findings inconsistent With state of downstream molecule
Effect not predicted
G
FOXM1
CCNA1
BUB1B
CCNE1
PRC1
CCND2
CDK1
PLK1
CCNB1
BIRC5
AURKA
EZH2
CCND3
AURKB
TGFB1
CCNA2
KIF14
CCNE2
BRCA1
CCNG1

Figure 3

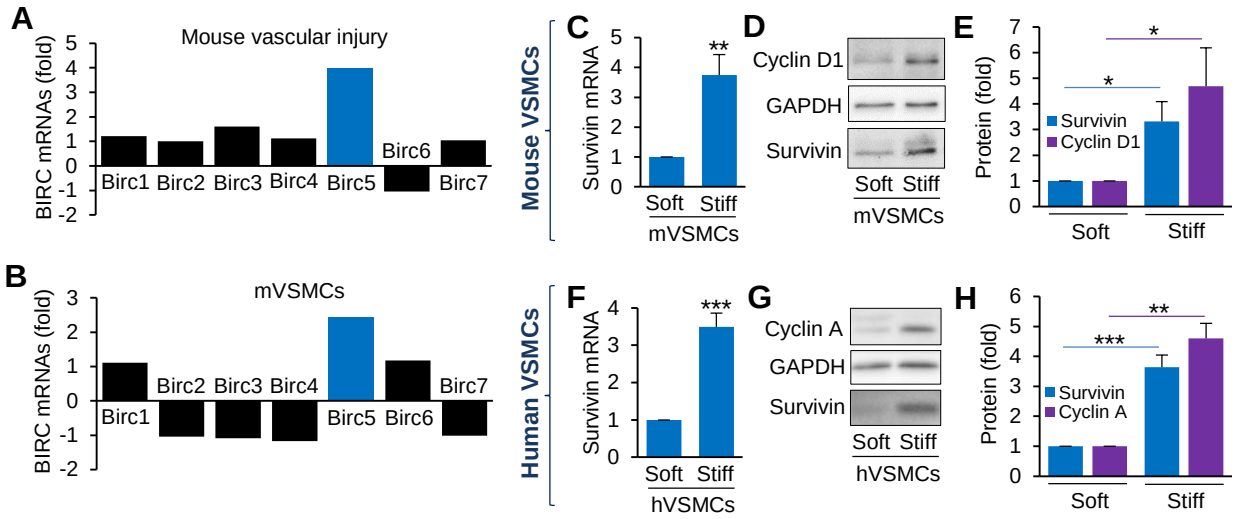
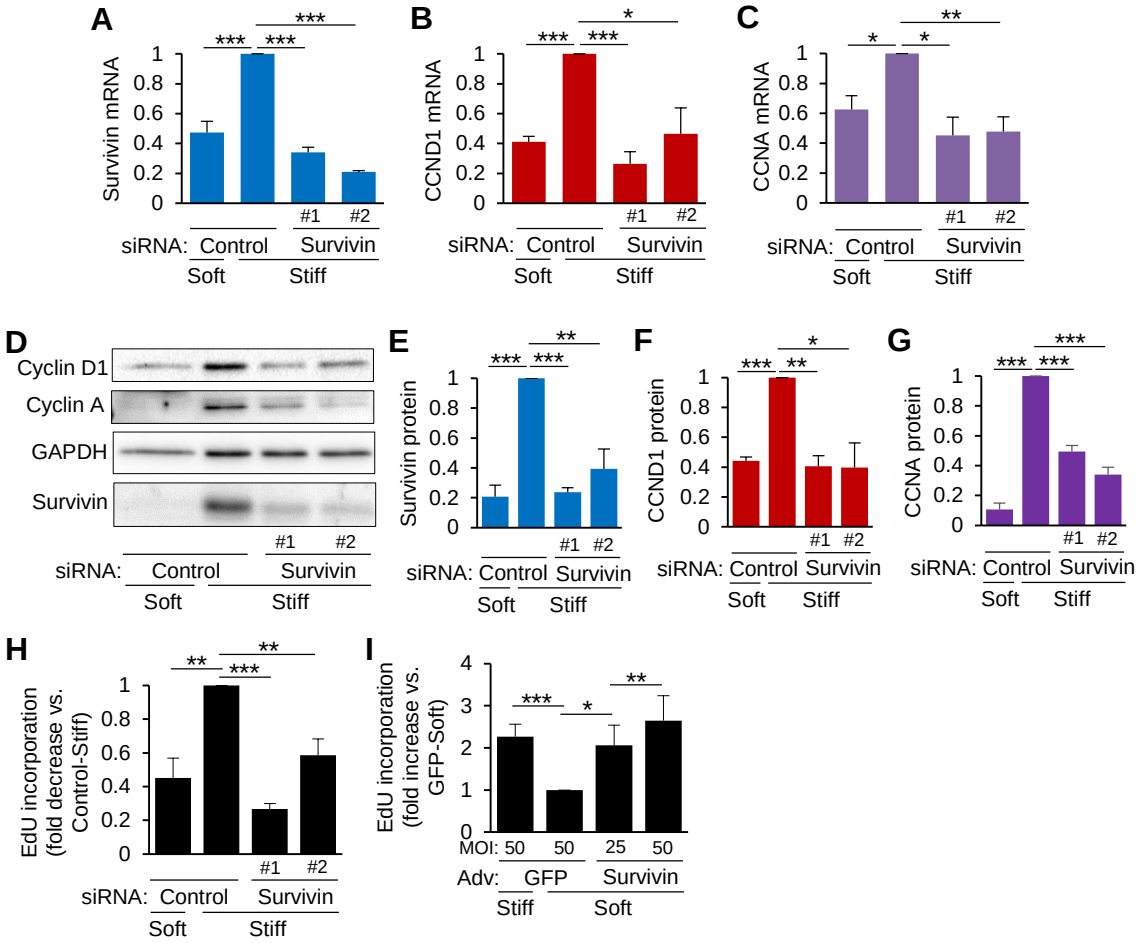
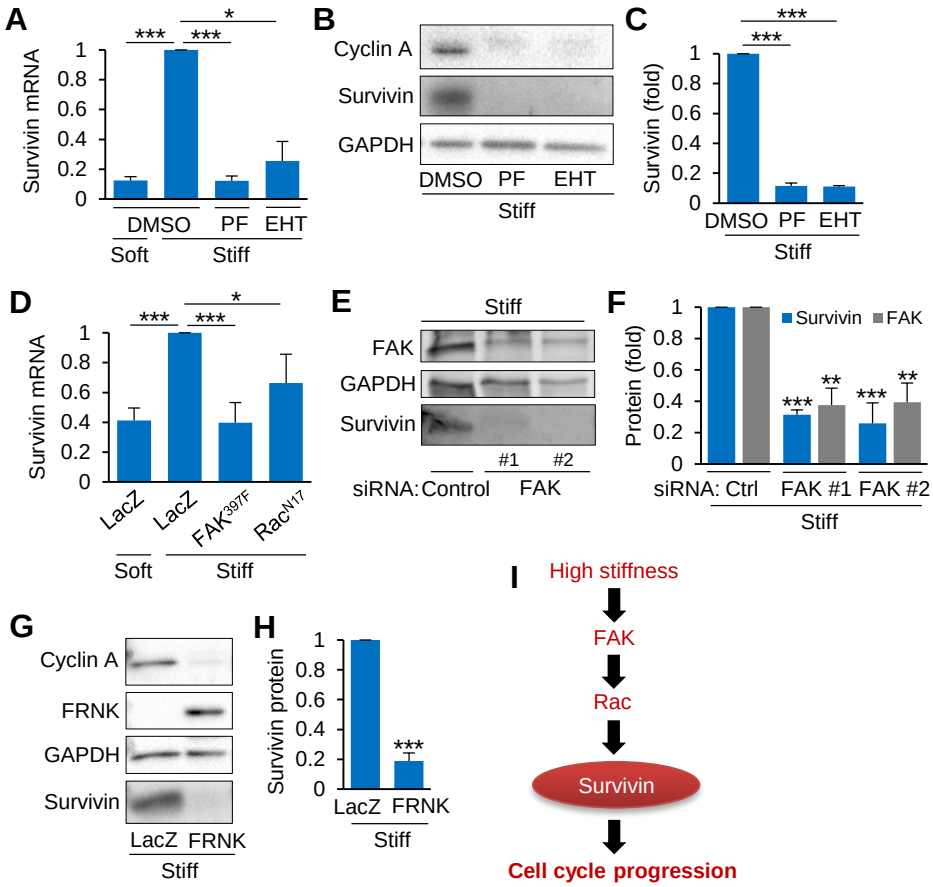
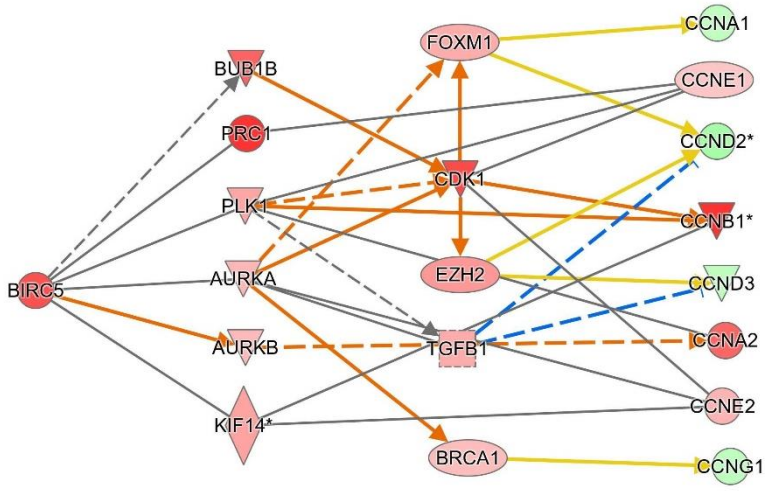
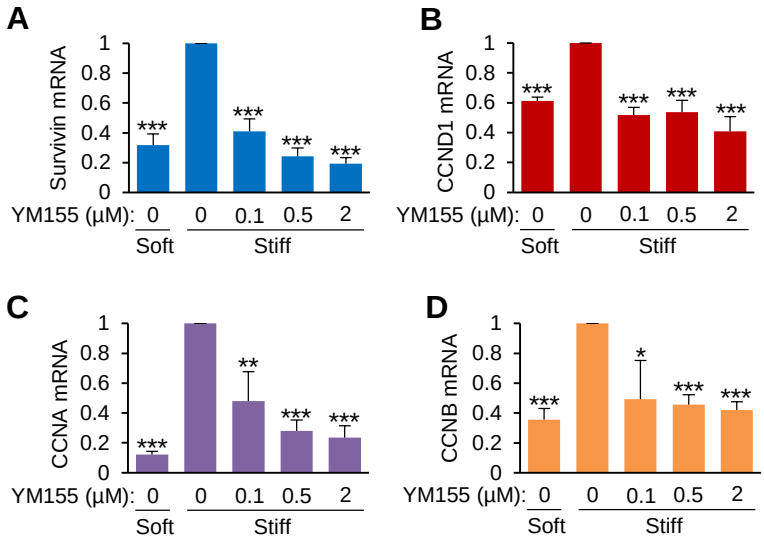

A
Mouse vascular injury
BIRC mRNAs (fold)
Birc1 Birc2 Birc3 Birc4 Birc5 Birc6 Birc7
B
mVSMCs
BIRC mRNAs (fold)
Birc1 Birc2 Birc3 Birc4 Birc5 Birc6 Birc7
Mouse VSMCs
Human VSMCs
C
Survivin mRNA
Soft Stiff
mVSMCs
D
Cyclin D1
GAPDH
Survivin
Soft Stiff
mVSMCs
E
Protein (fold)
Survivin
Cyclin D1
Soft Stiff
F
Survivin mRNA
Soft Stiff
hVSMCs
G
Cyclin A
GAPDH
Survivin
Soft Stiff
hVSMCs
H
Protein (fold)
Survivin
Cyclin A
Soft Stiff

Figure 4


A
Survivin mRNA
siRNA: Control Survivin
#1 #2
Soft Stiff
B
CCND1 mRNA
C
CCNA mRNA
D
Cyclin D1
Cyclin A
GAPDH
Survivin
E
Survivin protein
F
CCND1 protein
G
CCNA protein
H
EdU incorporation (fold decrease vs. Control-Stiff)
I
EdU incorporation (fold increase vs. GFP-Soft)
MOI: 50 50 25 50
Adv: GFP Survivin
Stiff Soft


A
Survivin mRNA
1
0.8
0.6
0.4
0.2
0
***
***
*
DMSO
PF
EHT
Soft
Stiff
B
Cyclin A
Survivin
GAPDH
DMSO
PF
EHT
Stiff
C
Survivin (fold)
***
***
DMSO
PF
EHT
Stiff
D
Survivin mRNA
***
***
*
LacZ
LacZ
FAK397F
RacN17
Soft
Stiff
E
Stiff
FAK
GAPDH
Survivin
#1
#2
siRNA: Control
FAK
F
Protein (fold)
Survivin
FAK
***
**
***
**
siRNA: Ctrl
FAK #1
FAK #2
Stiff
G
Cyclin A
FRNK
GAPDH
Survivin
LacZ
FRNK
Stiff
H
Survivin protein
***
LacZ
FRNK
Stiff
I
High stiffness
FAK
Rac
Survivin
Cell cycle progression

Supplementary Figure 1



Supplementary Figure 2