Metabolic, Endocrine and Genitourinary Pathobiology

Mammalian Target of Rapamycin (mTOR) Induces Proliferation and De-Differentiation Responses to Three Coordinate Pathophysiologic Stimuli (Mechanical Strain, Hypoxia, and Extracellular Matrix Remodeling) in Rat Bladder Smooth Muscle

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Maladaptive bladder muscle overgrowth and de-differentiation in human bladder obstructive conditions is instigated by coordinate responses to three stimuli: mechanical strain, tissue hypoxia, and extracellular matrix remodeling.^{1,2} Pathway analysis of genes induced by obstructive models of injury in bladder smooth muscle cells (BSMCs) identified a mammalian target of rapamycin (mTOR)-specific inhibitor as a potential pharmacological inhibitor. Strain-induced mTOR-specific S6K activation segregated differently from ERK1/2 activation in intact bladder *ex vivo*. Though rapamycin's antiproliferative effects in vascular smooth muscle cells are well known, its effects on BSMCs were previously unknown. Rapamycin significantly inhibited proliferation of BSMCs in response to mechanical strain, hypoxia, and denatured collagen. Rapamycin inhibited S6K at mTOR-sensitive phosphorylation sites in response to strain and hypoxia. Rapamycin also supported smooth muscle actin expression in response to strain or hypoxia-induced de-differentiation. Importantly, strain plus hypoxia synergistically augmented mTOR-dependent S6K activation, *Mmp7* expression and proliferation. Forced expression of wild-type and constitutively active S6K resulted in loss of smooth muscle actin expression. Decreased smooth muscle actin, increased *Mmp7* levels and mTOR pathway activation during *in vivo*

partial bladder obstruction paralleled our *in vitro* studies. These results point to a coordinate role for mTOR in BSMCs responses to the three stimuli and a potential new therapeutic target for myopathic bladder disease. (Am J Pathol 2010, 176:304–319; DOI: 10.2353/ajpath.2010.080834)

Conditions that impede bladder emptying, incite chronic distension, or overstimulate neuromuscular activity in the bladder wall cause high pressure and strain, leading to hypoxia, extracellular matrix (ECM) remodeling, and smooth muscle overgrowth.^{1–8} Other diseases, such as atherosclerosis, have significant consequences triggering “phenotypic switching” of smooth muscle cells (SMCs) from contractile to proliferative, hypertrophic, or synthetic phenotypes. In the bladder, SMC phenotypic alterations resulting from obstruction^{1–4} may lead to dysfunctional micturition, and bladder decompensation.

Distension or wall tension is the stimulus initiating signaling or mechanotransduction in the bladder wall, and can lead to intramural and microvascular compression. Transmural tension and compression of the intramural microvasculature creates tissue hypoxia during bladder obstruction.^{4,8} Both hypoxic and distensive stimuli in

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bladder smooth muscle cells (BSMCs) can lead to matrix metalloproteinase (MMP) activation^{9–11} and matrix remodeling. These remodeling events can expose cryptic epitopes within native matrix elements driving further BSMC growth, which is often self-perpetuating.^{10,11} Despite the widespread and well-recognized clinical sequelae of obstructive uropathies, the signaling mechanisms driving excessive proliferation and phenotypic switching of BSMCs have not been adequately addressed. This knowledge gap has further impeded the development of new pharmacotherapy for obstructive uropathy.

Numerous studies have illustrated the cell-cycle kinases and mitogen activated protein kinases involved in accelerated vascular SMC growth,¹² but in the bladder, the signaling pathways and the critical physiological stimuli driving them are only beginning to be understood.¹³ Bioinformatics analysis of previously identified genes involved in three models of BSMC injury suggested that rapamycin could exert an inhibitory affect on a major associated gene network. Rapamycin, a macro-lide antibiotic, and specific inhibitor of the mammalian target of rapamycin (mTOR, or FRAP1), has been used widely to inhibit development of transplant arteriosclerosis and arterial neointimal thickening of vascular smooth muscle following mechanical and alloimmune injury.¹⁴ Further, this Food and Drug Administration-approved drug¹⁵ has been effective in treating advanced renal cell carcinoma, among other cancer types¹⁶ and strongly prevents organ rejection in renal and other transplants. mTOR plays a pivotal role in cell cycle progression and differentiation in vascular smooth muscle cells via orchestration of kinase activity and protein translation.^{17,18} This signaling pathway directs translation of 5'CAP and 5'TOP mRNAs through phosphorylation of S6 kinases¹⁸ and EIF4E,¹⁸ respectively, augmenting cell size, as well as cell number. The inhibitor of this pathway, rapamycin, was able to modulate BSMC phenotype under the mitogenic conditions of mechanical strain and hypoxia (both together and separately), and denatured matrix, three defining stimuli of the obstructive uropathic microenvironment *in vivo*. Also, mTOR and its inhibitor rapamycin were able to alter expression of smooth muscle actin (SMA), a well-studied early differentiation marker for SMC, in three different physiological models of BSMC injury. Furthermore, we assessed involvement of downstream effectors of mTOR, including S6K1, in differentiation of BSMC.

Materials and Methods

Pathway Analysis

Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Inc.[^] Redwood City, CA) was used to identify highly associated networks of genes and pathways involved in BSMC strain and hypoxia injury. Using genes from previous work^{9–10,19–21} and the known association of muscarinic receptors with obstructive uropathy, focus genes (listed in Table 1) were mapped to gene identifiers in the IPA knowledge base and overlaid on a molecular network

curated by IPA. Gene identifiers were mapped to networks based on their known connectivity and given a score based on the number of focus gene identifiers found in the networks. This score is not a significance score, but simply ranks the networks according to their relevance to the focus gene identifiers. The two most significant networks identified from this analysis were queried for potential chemical or biological inhibitors of these pathways by examining the genes associated with the networks for chemical biological and inhibitors listed in the gene database on IPA. Inhibitors identified were screened for practical applicability based on clinical availability and toxicity. The data were also mapped to canonical pathways and significance of these associations determined by both a ratio of the number of focus gene identifiers mapping to the canonical pathway versus the total number of gene identifiers mapping to the canonical pathway and a one-sided Fisher's exact test was used to uncover pathways of genes with higher odds ratios of containing our focus genes. Some genes identified by the gene networks were in fact groups or complexes of genes, for example "MMP," "MEK," "ERK," or Gαi, as the data curated by IPA in some cases is not specific to one gene but a group of genes.

Primary BSMC Culture

Bladders from neonatal Sprague-Dawley rats (2 to 5 days old) were isolated and SMCs isolated as previously described.⁸ Eagle's minimum essential medium (EMEM; Multicell Technologies, Woonsocket, Rhode Island, CA) containing 10% fetal calf serum (Invitrogen Carlsbad, CA) and antibiotic/antimycotic (Multicell) was used to culture cells at 37°C in 95%O₂/5%CO₂⁹ as described previously. Passages 1 and 2 were used for experiments in this study. To passage and plate cells, BSMC were incubated in 0.25% trypsin, 0.053 mmol/L EDTA (Multicell) briefly, washed in PBS, and re-suspended at 5 × 10⁴ cells/ml. For proliferation assays, 0.5 × 10⁵ cells were seeded into 6 well tissue culture plates or BioFlex plates (Flexcell International, Inc.). Before all experiments, cells were serum-deprived using starvation media (EMEM without serum) for 48 hours to synchronize BSMCs to G₀.

Collagen Gels

Type I bovine collagen (Elastin Products Company, Owensville, Missouri) was gelled by neutralizing the collagen solution in 0.1 mol/L NaOH in 1 × PBS (MultiCell) at 37°C. Collagen was denatured by boiling for 30 minutes, then neutralized in 0.1mol/L NaOH as described.⁹ For denatured collagen (DNC) gels, native collagen (NC) was layered with an equivalent volume of DNC overnight before washing. Both DNC and NC gels were washed in EMEM before plating cells. BSMC were serum-starved for 48 hours before plating at a density of 2 × 10⁴ cells/ml for 3 hours before addition of any pharmacological agent. Cells were incubated for 48 hours at 37°C, 5% CO₂.

Table 1. List of Focus Genes from Figure 1A

Gene	Bladder SMC stimulus	Name and alias	General function in SMC
<i>AKT1</i>	PDGF, strain and pressure induced proliferation	V-akt murine thymoma viral oncogene homolog 1	Proliferation
<i>AKT2</i>	PDGF, strain and pressure induced proliferation	V-akt murine thymoma viral oncogene homolog 2	Differentiation
<i>AKT3</i>	PDGF, strain and pressure induced proliferation	V-akt murine thymoma viral oncogene homolog 3	Proliferation
<i>BMP2</i>	Strain	Bone-morphogenic protein-2	Anti-proliferative, apoptosis
<i>BMPR2</i>	Strain	Bone-morphogenic protein receptor-2	Growth arrest, differentiation
<i>CHRM2</i>	Obstruction	Muscarinic acetylcholine receptor 2	Cholinergic contraction
<i>CHRM3</i>	Obstruction	Muscarinic acetylcholine receptor 3	Cholinergic contraction
<i>CHRM4</i>	Obstruction	Muscarinic acetylcholine receptor 4	Cholinergic contraction
<i>COL3A1</i>	Obstruction, strain	Collagen type III (alpha 1)	Matrix scaffold
<i>F2RL1</i>	Strain	Coagulation factor II (thrombin) receptor-like 1, protease activated receptor 2 (PAR2')	Migration, neuroactive ligand-receptor interaction
<i>HBEGF</i>	Strain	Heparin-binding EGF-like growth factor (HB-EGF)	Proliferation, hypertrophy remodeling
<i>LIF</i>	Strain	Leukemia inhibitory factor	NOS induction
<i>MAPK1</i>	Strain + hypoxia	ERK2, mitogen-activated protein kinase 1	Differentiation, proliferation, apoptosis, migration
<i>MAPK14</i>	Strain	p38, mitogen-activated protein kinase 14	Migration (MMP expression), neointimal growth, apoptosis
<i>MAPK3</i>	Strain + hypoxia	ERK1, mitogen-activated protein kinase 3	Differentiation, proliferation, apoptosis, migration
<i>MAPK8</i>	Strain	JNK1, mitogen-activated protein kinase 8	Proliferation
<i>MMP2</i>	Strain	Matrix metalloproteinase 2	Migration, proliferation
<i>MMP7</i>	Strain + hypoxia	Matrix metalloproteinase 7	Proliferation
<i>MMP9</i>	Strain	Matrix metalloproteinase 9	Migration, proliferation
<i>PTGS2</i>	Strain	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Proliferation
<i>STAT3</i>	Strain + obstruction	Signal transducers and activator of transcription 3	Proliferation, angiogenesis, apoptosis (IFN γ -induced)

Mechanical Strain in Vitro

Cells were mechanically strained on a vacuum modulated device (Flexcell 4000, Flexcell International Corporation), 5×10^4 cells/ml were plated onto Bioflex Collagen I strain plates. At 50% confluency, cells were serum-starved for 48 hours to arrest cells at the G₀. All strain experiments were conducted using a static pattern with an initial ramping of 2% and 4% elongation for 1 hour each, then 5% elongation for a total of 16 or 18 hours strain.¹⁰ This static patterning is more reminiscent of slow bladder filling and chronic distension, rather than rapidly cyclic or oscillating straining patterns more appropriate for vascular SMC.¹⁰

Hypoxia

To generate a controlled, low oxygen environment, a humidified hypoxic chamber (Biospherix Redfield, New York) was used to condition BSMC. Variables were set at 3%O₂/5%CO₂ as in our previous study as well as 1%O₂/5%CO₂ with the balance N_{2(gas)}.¹¹ Normoxic controls were identical to hypoxic conditions, with the exception of oxygen levels, which were at atmospheric levels of 21%O₂.

Drug Treatments

BSMCs were pretreated in serum-free EMEM containing 25 mol/L PD98059 (Calbiochem, San Diego, CA) or 5 to

15 ng/ml rapamycin (Calbiochem, San Diego, CA) for 60 minutes before mechanical strain or hypoxia induction. Cells on collagen gels were treated after attachment (3 hours after plating cells) to denatured or native collagen gels to prevent interference with cell attachment.

Thymidine Incorporation and Cell Counting

In all BSMC experiments, serum-starved cells were incubated in ³H-thymidine (GE Healthcare, Piscataway, NJ) at 2 μ Ci/ml before conditioning. At the conclusion of each experiment, radiolabeled counts were fixed in ice-cold methanol, precipitated with ice-cold 5% trichloroacetic acid, solubilized in 0.4 mol/L NaOH + 0.5% SDS and counted as previously described.^{9,10}

Western Blotting

As previously described,^{9,10} Western blotting was performed against whole cell lysates or tissue lysates isolated by crushing tissue under N_{2(liq)}. Antibodies for blotting comprised phospho-specific antibodies for threonine³⁸⁹-ribosomal S6K, serine^{235/236}-S6, threonine^{197/202}-MNK1, serine⁶⁵-4EBP, -EIF4 α , tyrosine⁷⁰⁵-STAT3 (all at 1:1000; Cell Signaling Danvers, MA), and SMA (Abcam Cambridge, MA, 1:500). Bands were normalized to total actin (Sigma St. Louis, MO), total p70 S6K or pan-ERK1/2 (1:500; Cell Signaling). Densitometric analysis was performed with Image J as described.^{9,10}

Table 2. Real-Time PCR Primers

Gene	Forward	Reverse
<i>Sma</i>	5'-GATCACCATCGGGAATGAACGC-3'	5'-CTTAGAAGCATTTGCGGTGGAC-3'
<i>Mmp7</i>	5'-TGGGTCTGGGTCACTCTTCT-3'	5'-CACAGCTTGTTCTCTTTCC-3'
<i>Rpl32</i>	5'-CATCTGTTTGCGGCATCA-3'	5'-CACCTGTGTGTCGATGCCTC-3'
<i>Gapdh</i>	5'-GATCGTGAAGGGCTAATGA-3'	5'-GAGCTCTGGGATGACTTTGC-3'

Immunocytochemistry for SMA

As described in Herz et al (2003),¹¹ cells were fixed in ice-cold methanol or 4% paraformaldehyde, and permeabilized with 0.2% Triton-X 100. BSMCs were blocked with 5% goat serum and stained with anti-SMA-Cy3 (1:200; Sigma). Nuclei were counterstained with Hoechst and cells mounted in Dako fluorescent mounting medium. Cells transfected with rat HA-S6K1 plasmids (From Addgene Cambridge, MA)¹⁸ were double-stained for the hemagglutinin (HA) tag using a mouse monoclonal anti-HA antibody (Covance, Princeton, NJ), and a rabbit polyclonal anti-SMA antibody (Abcam) and secondary goat anti-rabbit-Cy3 and goat anti-mouse-Cy2, respectively (both 1:200; Jackson Immunolabs). Nuclei were counterstained with Hoechst.

Immunofluorescence of Distended Bladder

Bladders from Sprague-Dawley 100 to 120 g female rats were mechanically strained by distension during *ex vivo* whole organ culture for the indicated times (0 to 120 minutes). To perform *ex vivo* distension, bladders were first catheterized *in vivo* under anesthesia. Ureters were ligated, and the urethra sutured around the catheter tightly five times. Bladders were distended to 40 cm of hydrostatic pressure by manometry, as described in Capolicchio et al.^{22,19} We found that 40 cm hydrostatic pressure induces strain injury in the bladder, sufficient to alter ECM gene expression,²² ERK and STAT3 signaling^{10,19} and induce hematuria.²³ After harvesting, bladders were placed briefly in ice-cold 0.25 mol/L sucrose then embedded in optimal cutting temperature compound under N₂(liq). Bladders were cryosectioned, fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton-X 100, and blocked in 5% goat serum. Primary antibodies (mouse anti-phospho-threonine³⁸⁹-S6K, rabbit anti-phospho-S6 and mouse anti-phospho-ERK1/2 from Cell Signaling) were applied at 2 μ g/ml at 4°C overnight, and secondary antibodies and Hoechst applied as in immunocytochemistry. Using ImageJ, fluorescence intensities of each channel from the detrusor muscle were analyzed by subtracting the mean background intensity from the mean intensity then normalizing to nuclei. Statistical differences were analyzed by two-tailed *t*-test using >3 measurements of fields of view (*r* = 3) for *n* = 3 samples.

Transfections with myc-mTOR and HA-S6K

Rat wild-type (pRK7-HA-S6K1-WT) and constitutively active S6K clones (pRK7-HA-S6K1-E389, -E389D3E)⁷ were obtained from Addgene. Primary BSMC were transfected

using LTX and Plus reagent as recommended by the manufacturer (Invitrogen, Carlsbad, CA). Briefly, LTX and DNA in OptiMEM were incubated for 15 minutes and added to BSMC plated on six-well Bioflex collagen I plates at 50% confluency for 4 hours. Media was replaced with 10% fetal calf serum/EMEM for 48 hours before fixation and immunostaining using anti-HA and anti-SMA antibodies, as above.

Real-Time PCR for SMC Differentiation Markers and Mmps

RNA from SMC treated with mechanical strain or hypoxia, was extracted using Trizol (Invitrogen). Reverse transcription was performed using Superscript III and oligo(dT) as described previously.⁹ Real-time PCR for *SMA*, *Mmp7*, *Rpl32* and *Gapdh* was performed on the Peltier Thermal Cycler-200 (MJ Research) using primers (Table 2) and the 2 $\times$ Dynamo SYBR-green Master Mix (Finnzymes, New England Biolabs Ipswich, MA). Relative levels of transcripts were determined by comparing housekeeping gene levels (*Rpl32*, *Gapdh*) to targets using the deltaC(t) methodology.¹⁴ *Mmp*-2, -9, and -14^{9,10} levels for *in vivo* studies were determined by semiquantitative reverse transcription (RT)-PCR on the Perkin Elmer Thermocycler 2000 using Taq polymerase (Roche, Indianapolis, IN). Normalization to *Gapdh* was performed as described previously.²²

Chronic *in Vivo* Partial Bladder Outlet Obstruction

As described by Elkini et al (2009),²⁰ urethras from Sprague-Dawley female rats were partially obstructed by ligation with a 2-0 silk suture. Rats were palpated abdominally every 6 to 8 hours to ensure bladder emptying, as required by the approved protocol of the animal care committee of the University Health Network. Bladders were harvested, and flash frozen in N₂(liq).

Statistical Analysis

Using one or two-factor analysis of variance program (SuperANOVA), results were assessed for significance from controls and expressed as a mean of *n* = 3 or 6, as indicated in legends, for hypoxia, matrix and strain experiments. Significance was assumed for a value of *P* < 0.05 using analysis of variance or a posthoc Student-Neuman-Keul's *t*-test (one or two-tailed, as indicated).

Results

FRAP1/mTOR Is a Significant Interacting Partner in the Response to Bladder SMC Injury

We wished to uncover pathways induced by BSMC injury models by determining whether or not common pathways

could be found among proteins activated or induced during bladder strain injury, *in vitro* and *ex vivo*, as well as BSMC hypoxia. We used IPA to examine a set of genes for their interactions with known networks of genes or gene products to identify the most significant pathways and networks of genes and their products (Figure 1A and Table 1). IPA is a bioinformatics tool for the identification of interacting proteins and pathways through comparison of focus genes (or entire datasets) with a curated knowledge base. Some of the focus genes (ERK,^{10,11} STAT3,¹⁹ MMP7,⁹ MMP2,¹⁰ MMP9^{10,11}) were derived from the literature on strain and hypoxia-injured BSMC, co-stimuli present during bladder obstruction. We also included: (a) microarray data of stretched human BSMCs, confirmed in distended bladders *ex vivo* (13 genes up-regulated >twofold)²¹; and (b) gene products known to be involved in obstructive bladder disease (AKT,²⁴ CHRM-2, -3, -4). IPA uses an unbiased method of associating genes based on their known interactions in the database, according to a significance score based on the likelihood on these genes randomly associating with each other by a Fisher's exact test. Networks (Table 3, Figures 1B, C) can also be queried for therapeutic targets using the Build options in their pathway software. One of the most significantly associated networks ($P < 0.00005$) demonstrated potential interactions of 8 focus genes with each other and 10 other genes (Figure 1C). The network also exposed S6K as significant interacting partner, which is a potential target of rapamycin through its association with mTOR. The focus genes MAPK3/1, or ERK1/2, figured prominently in networks 1 and 2 (Figure 1, B and C), interacting with 14 other nodes, including Ras, Raf, and Mek, upstream of ERK1/2. Other genes interacted strongly, including, p38, JNK, insulin, TGF β , MMPs and Ras homolog (e.g., RHOK), which have been studied in the BSMC literature. The utility of this method is that interactivities between genes that have not been highly studied will be exposed; these interactivities can

Figure 1. Integrated Pathway Analysis of focus genes from studies of strain and hypoxia stimulated bladder SMC. Genes identified through our own work or by microarray analysis were entered into the Ingenuity Pathway Analysis program (Ingenuity Systems, Inc.). The pathway (A) was generated by analyzing the focus genes or proteins (nodes) identified through the literature on strain (red) and/or hypoxia (blue) mediated injury in BSMC and *ex vivo* bladders using integrated pathway analysis on IPA. The two most significant networks associated with these genes as proposed by IPA are shown (B and C) with the focus genes from (A) highlighted as before. Table 3 shows these pathways, list of genes, network z-scores and functions. Due to the nature of the database, some nodes are duplicated due to changes in the nomenclature of the gene, eg, MAP2K1/2 and Mek, and ERK and MAPK1/3. Nodes or genes highlighted in green were identified as through the IPA curated database as genes relevant to these networks. The networks (B and C) were queried for druggable targets using information on each target in the IPA database. Targets with clinically approved pharmacotherapeutic agents affecting them were circled in green (new to bladder) or red (previously examined in bladder). In (C), IPA uncovered a target previously unstudied in bladder smooth muscle: S6K, a target of rapamycin through its association with mTOR. Other targets include those studied in the context of the bladder, such as the cyclooxygenase-2 pathway (PTGS2 and CYCS), MMPs,¹⁴ the EGF receptor pathway (HBEGF), NMDA receptor, Insulin, and NFAT/Calcineurin,³⁵ which are circled in red. Further targets may become apparent as the database of IPA increases. The edges (relationships) between each of the nodes (genes) can be queried in an interactive html format of these images (available in online Supplemental Figure S1 at <http://ajp.amjpathol.org>). IgE, TCR, FSH, CD3, and other nodes possibly unexpressed in BSMC were omitted from the figures of the networks (full network list available in Table 3).

Table 3. IPA Networks Identified via Analysis of Focus Genes. Network 1 and 2 Are Illustrated in Figures 1B and C, Respectively. Network 3 Not Shown

Network	Gene identifiers	Score	Focus genes	Top functions
1	<i>Akt, AKT1, AKT2, AKT3, alcohol group acceptor phosphotransferase, ALP, Ap1, Beta ark, BMP2, BMPR2, CHRM2, CHRM3, CHRM4, Cytochrome c, ERK1/2, FSH, G alpha, G protein $\beta + \gamma$, hCG, Hsp70, IFNβ, Insulin, Interferonα, LDL, Mapk, MAPK1, MAPK8, MAPK14, NFkB, NMDA Receptor, PDGF BB, Pkc(s), PLC, PP2A, Ras homolog</i>	27	11	Digestive system development and function, hepatic system development and function, organ morphology
2	<i>Calcineurin protein(s), CD3, ERK, F2RL1, Fgf, Fibrin, Gai, HBEGF, Hsp27, Irfnγ, IgE, IKK, IL1, Interferonβ, Jnk, LIF, MAP2K1/2, MAPK3, Mek, Mmp, MMP7, MMP9, Nfat, P38 MAPK, p70 S6K, Pdgf, PI3K, PTGS2, Raf, Ras, STAT3, TCR, Tgfβ, Tnf receptor, Vegf</i>	18	8	Embryonic development, reproductive system development and function, organ morphology
3	<i>C21ORF33, CCL1, CLDN4, COL3A1, DAB2IP, DDR2, ELP2, ELP3, FSTL1, GSTA4, Histone h3, HRSP12, HSP, IFIT1L, IL21, IL18BP, MMP2, MMP16, MMP17, NAIP, NCR1, NOS2, OGN, PKMYT1, PSCDBP, Rac, RNA po2-transcription factor, TNA polylmerase II, SBF1, TFIIIF, TGTP, TNF, Vacuolar H+ATPase, XCL1</i>	3	2	Inflammatory disease, skeletal and muscular disorders, connective tissue disorders

Data generated using Ingenuity Systems, Inc. (©2000–2006; data used with permission).

be queried in an interactive format in the Supplemental Figure S1 (online at <http://ajp.amjpathol.org>). Interestingly, G α i was similarly implicated by our network, but was ruled out as a player in the down-regulation of calcium sensitization in a rabbit model of partial obstruction,²⁵ though its role in other pathological processes cannot be ruled out. Other G proteins α , β , and γ were also highlighted. Inflammatory mediators, such as interferons, IL1 and TNF receptor were also significant interacting partners. Though such proteins have been implicated and targeted in strain-mediated cardiovascular disease, their roles in bladder muscle are less defined and may be of interest in future studies. NFAT/calcineurin was also exposed in these networks; it appeared to be a significant interacting node with potential as a therapeutic target, one of which has been explored by other groups.^{26,27}

Furthermore, querying for potential therapeutic approaches to the interacting partners in this network revealed the specific inhibitor of the S6K1/mTOR(FRAP1) pathway, rapamycin. S6K1 is regulated in part through phosphorylation of threonine-³⁸⁹ by mTOR. To confirm that the S6K1/mTOR pathway responds to mechanical strain of the bladder, we performed bladder strain injury in our *ex vivo* bladder organ culture model using pressure-specific volume distension by catheterization per urethra, at 40 cm of H₂O pressure and examined mTOR-specific phosphorylation of S6K, downstream phosphorylation of S6 itself, and phospho-ERK for comparison. S6K activation was seen most dramatically at 30 minutes in the detrusor (Figure 2, 0 vs. 30 minutes, S6K muscle staining, $P < 0.05$, by two-tailed *t*-test), decreasing thereafter. S6 phosphorylation, downstream of S6K was also increased above control levels during distension, with levels significantly increasing 11-fold (± 3.7) above control levels (1 ± 0.4) at and beyond 60 minutes ($P < 0.05$ by *t*-test) persisting longer than S6K activation. ERK

staining increased, consistent with previous studies¹⁰ by 40-fold at 60 minutes ($P < 0.04$ by two-tailed *t*-test). ERK activation was interestingly localized in smooth muscle of blood vessels and detrusor after only 5 minutes of distension. At 120 minutes of distension, a distinct suburothelial compartment stained very strongly for phosphorylated ERK. Phospho-S6 localized to the muscle, interstitial spaces and urothelium. Some colocalization of phospho-S6 and -ERK was apparent in distended samples in the detrusor muscle.

Rapamycin, a Specific Inhibitor of mTOR, Inhibited Proliferation of BSMCs in Response to Mechanical Strain, Hypoxia, and Damaged ECM

To determine the safe dose range of rapamycin for BSMC, a dose-response curve was generated *in vitro*. Rapamycin at 20 ng/ml significantly decreased BSMC proliferation compared with controls suggesting non-toxic concentrations were lower than 20 ng/ml (data not shown). Previously we found that 3% O₂ increases BSMC proliferation, a finding replicated in this study (Figure 3A). Over 18 hours at 3% O₂ in the humidified hypoxia chamber, rapamycin significantly reduced proliferation in response to hypoxia *in vitro* ($P < 0.05$, Figure 3A). Both normoxia alone (21% O₂) and normoxia plus rapamycin controls had similar baseline proliferation levels. Our previous studies demonstrated that BSMCs proliferate when subjected to sustained static strain.^{10,19} During mechanical strain of BSMC, we noted a 10-fold increase in thymidine incorporation (Figure 3B). Rapamycin significantly reduced strain-induced proliferation to near control levels (Figure 3B, $P < 0.0001 \times 2$ -factor analysis of variance). Similarly, DNC matrices

Figure 2. Distension of intact *ex vivo* bladder activates effectors of the mTOR pathway. **A:** S6 kinase and ERK1/2 phosphorylation was detected in distended *ex vivo* bladders by immunofluorescence on cryosections using monoclonal anti-phospho-S6 kinase and -phospho-ERK1/2 antibodies (Cell Signaling) and secondary anti-mouse-Cy3 (red). Phospho-S6 activation was also examined with polyclonal anti-phospho-S6 and anti-rabbit-Cy2 (green) with Hoechst (blue) nuclear counterstaining. Localization of mTOR pathway and ERK1/2 activation was examined over a time course from 0 to 120 minutes, revealing a rise in S6K at 30 minutes and gradual increases in both ERK and S6 activation over the longer term. Original magnification, $\times 200$. Scale bar = 80 μ m. Representative photos of $n = 3$ bladders. **Yellow arrow** = phospho-ERK positive vessels in the mucosa. **White arrow** = phospho-ERK positive suburothelial compartment. Immunofluorescent intensities of the detrusor muscle from $N = 3$ bladders were analyzed on ImageJ, in individual channels. **B:** ERK increased in phosphorylation early (5 minutes, $P < 0.05$ by two-tailed *t*-test). Both activated S6 (long dashed line) and ERK (straight line) appeared to increase past 60 and 120 minutes of distension ($**P < 0.05$, by *t*-test; $***P < 0.05$, by two-tailed *t*-test, respectively). Phosphorylation of S6K (short dashed line) was increased at 30 minutes of distension, $****P < 0.05$ by two-tailed *t*-test.

have been shown by others¹⁷ and our own work^{10,11} to increase proliferation of SMC, a finding reiterated here ($P < 0.04 \times 2$ -factor analysis of variance; Figure 3C). The mitogenic response to damaged matrix was also inhibited by rapamycin ($P < 0.04$). The three physiological stimuli, mechanical strain, hypoxia, and denatured matrix each induced BSMC proliferation, which in all three cases was significantly inhibited by rapamycin.

Hypoxia Plus Mechanical Strain Have Synergistic Effects on Proliferation of BSMCs

Slightly milder degrees of hypoxia and mechanical strain in combination induced a higher proliferative response than either individually. The individual stimuli of hypoxia at 4% O_2 (vs. 3% in Figure 3A) and mechanical strain at 5% for only 1 hour (vs. for 16 hours) still significantly

augmented proliferation (Figure 3D), though the increases were less robust than those seen in Figure 3, A and B. Furthermore, the combination of the two stimuli caused augmented proliferation beyond their individual responses (hypoxia versus hypoxia plus strain, $P < 0.01$, strain versus hypoxia plus strain, $P < 0.05$, by two-tailed *t*-test). In all cases, rapamycin abrogated the proliferative responses to the various stimuli and the combined stimuli ($P < 0.01$).

Downstream of mTOR, S6K Is Activated by Mechanical Strain and Hypoxia, and the Combination of the Two

Initially we sought to explore the downstream signaling intermediates of mTOR induced by physiological stimuli

Figure 3. Rapamycin inhibits proliferation in response to mitogenic stimuli in BSMC. **A:** Hypoxia (3% O₂) for 18 hours induced proliferation of BSMC as compared with normoxia (21%O₂) by ³H-thymidine incorporation. Hypoxia was induced using the Pro-ox controller in a humidified hypoxia chamber (Biospherix). Rapamycin (5 ng/ml) did not inhibit control levels of BSMC proliferation, but hypoxia-induced BSMC proliferation was significantly inhibited by rapamycin (**P* < 0.05, by 2-factor analysis of variance, ***P* < 0.04). Each group represents means (±SD) from *n* = 6. **B:** Proliferation of BSMC in response to strain for 16 hours is inhibited by rapamycin. Quiescent BSMC at 60 to 70% confluency were incubated ± rapamycin and strained (or not). Elongation was performed with a static pattern, slowly ramping strain up from 2% for 1 hour, 4% 1 hour to 5% for 14 hours¹⁰ increasing slowly to a 5% elongation. Under strain conditions, rapamycin showed a significant inhibitory effect on BSMC proliferation (* vs. others, *P* < 0.0001, by 2-factor analysis of variance). There was no significant difference between samples without strain ± rapamycin (***P* = 0.4303). Each group represents a mean (±SD) of *n* = 6. **C:** Denatured matrix induced proliferation is inhibited by rapamycin in BSMCs. Cell counting revealed augmented proliferation on denatured collagen type I matrices, as reported previously.¹¹ This increase in proliferation was inhibited by rapamycin (* vs. others, *P* < 0.04, by one-way analysis of variance, *n* = 3 gels with 10 fields counted each). **D:** Hypoxia and mechanical strain synergize to increase proliferation of SMCs. BSMC serum-starved and pretreated with rapamycin, were stimulated with nothing, 4% O₂, 5% static mechanical strain for 1 hour, or both 5% strain and 4%O₂ hypoxia, *n* = 3. The strain and hypoxia parameters each induced lower increases of proliferation individually than seen with higher degrees of these stimuli (**P* < 0.01). However, *in combination*, the milder hypoxia and milder strain induced a significant increase in proliferation compared with the individual stimuli (**P* < 0.01, ***P* < 0.05, ****P* < 0.001). All stimulated groups (strain, hypoxia and hypoxia plus strain) were inhibited by rapamycin treatment (*P* < 0.01, in all cases). Analysis by two-tailed *t*-test.

in vitro. Immediately downstream of mTOR, S6K is phosphorylated at a critical mTOR-specific site for activation, threonine³⁸⁹. We observed increased S6K-Thr³⁸⁹ phosphorylation after 30 minutes of mechanical strain plus hypoxia, or hypoxia alone (Figure 4). 10 minutes of mechanical strain increased S6K activation, which was significantly inhibited by rapamycin (*P* < 0.05; see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Interestingly, the combination of strain and hypoxia, as encountered *in vivo*, had a synergistic effect on S6K activation after 30 minutes (Figure 4). This is the first time that SMC signaling due to strain in conjunction with hypoxia has been studied using defined parameters *in vitro*. Furthermore, rapamycin blocked S6K Thr³⁸⁹ phosphorylation induced by both strain and hypoxia alone or in combination with hypoxia.

Downstream of S6K, S6 integrates the cumulative effect of S6K phosphorylation. S6 showed a similar increase in phosphorylation at 20 minutes of strain (Figure 5A). As an independent control for rapamycin inhibition, another effector of translation control, activated separately from the mTOR pathway, MNK1, was also examined in response to strain. As expected, strain-induced

MNK1 activation was not significantly inhibited by rapamycin (Figure 5A) though MEK inhibition abrogated the ERK-dependent phosphorylation of MNK1 (*P* < 0.05). Similarly strain-induced ERK activation itself was not significantly affected by rapamycin treatment (Figure 5B). Signaling through mTOR also inhibits 4EBP, which in turn impedes EIF4E, an inducer of translation of 5'CAP mRNAs. While strain raised EIF4E phosphorylation above basal levels (*P* < 0.04), EIF4E was only marginally affected by rapamycin treatment during strain (Figure 5C; Supplemental Figure S3 available online at <http://ajp.amjpathol.org>). Strain induced EIF4E activation was inhibited by EGFR inhibitor PD153035, but not PD98059 nor rapamycin, possibly reflecting predominantly non-mTOR regulation of EIF4E downstream of the EGFR receptor. Strain can also induce phosphorylation of threonine-⁴²¹ on S6K at an ERK-dependent autophosphorylation site. As such, rapamycin had no effect on this site, while MEK inhibitor, PD98059, reduced its phosphorylation (data not shown). Previously we had examined the ability of STAT3 to modulate proliferation and differentiation of BSMCs.¹⁹ We examined the role of rapamycin on strain-induced activation of STAT3 at the Tyr⁷⁰⁵ site. Interestingly, while

Figure 4. Mechanical strain and hypoxia activates S6K downstream of mTOR. BSMCs plated on collagen type I Bioflex plates were serum starved for 48 hours before stimulating with static strain and/or hypoxia. S6K phosphorylation synergistically increased in response to 5% strain in combination with hypoxia. Hypoxia was performed using mixed gas to replace the oxygen in the atmosphere, lowering O₂ to 3%. Combinations of strain with hypoxia were performed using a unique chamber (Biospherix) designed for the use of the Flexcell baseplates in combination with hypoxia. Protein harvested after indicated time points was analyzed by Western blotting for phospho-S6K, total S6K, total actin. Representative autoradiographs from $n = 3$ blots shown. Densitometry on $n = 3$ blots was statistically analyzed by two-factor analysis of variance * $P < 0.02$, ** $P = 0.0007$; *** $P < 0.002$, $n = 3$.

strain increased phospho-Tyr,⁷⁰⁵ rapamycin seemed to promote basal phosphorylation ($P = 0.031$, by two tailed t -test) and had little effect on strain-induced STAT3 activation (Figure 5C).

Rapamycin Restores Differentiation of BSMCs Exposed to Mitogenic Stimuli

In addition to obstruction-induced BSMC proliferation, we were also interested in the relationship between proliferation and de-differentiation, and if rapamycin treatment can restore differentiation in BSMC. SMA is an early marker of differentiation of both vascular and visceral SMC, widely used to ascertain phenotypic differentiation.^{11,19} Cell strain resulted in decreased SMA expression (Figure 6A) supportive of BSMC de-differentiation. Phenotypically, BSMC also assumed a less differentiated morphology and stained less intensely for SMA after strain or denatured matrix exposure (Figure 6B+C) compared with unstrained or native collagen gel controls, respectively. Interestingly, rapamycin prevented strain-induced decreases in differentiation marker expression, maintaining levels of SMA expression, as well as differentiated cell morphology. Rapamycin treatment of BSMC exposed to hypoxia also showed a trend toward increasing SMA levels compared with untreated hypoxia-stimulated BSMC (Figure 6D, $P < 0.17$, by t -test, $n = 3$).

To confirm the regulation of SMA expression by the mTOR pathway, we overexpressed wild-type and constitutively active HA-S6K in BSMC and analyzed SMA staining relative to expression of the constructs. Cells expressing high levels of the transfected active S6K constructs expressed less SMA compared with non-transfected adjacent cells (Figure 7, $P < 0.0002$, by t -test).

Rapamycin Reduces Mmp7 Expression in Response to Mitogenic Stimuli

Previous studies identified MMP activity as an important mediator of BSMC proliferation.^{9,10} Damaged collagen induced further breakdown of collagen gels than native collagen (see online Supplemental Figure S4 at <http://ajp.amjpathol.org>). When cells plated on DNC were treated with rapamycin, the breakdown of this matrix was inhibited. Previously we found that *Mmp7*, which activates BSMC mitogens, is uniquely expressed in response to hypoxia, whereas transcription of other *Mmps* (*Mmp2*, 3, 9, 14) was not increased.¹¹ In contrast to *SMA*, *Mmp7* expression increased under strain. Interestingly, the combination of strain and 1%O₂ hypoxia had a profound synergistic effect on *Mmp7* expression (Figure 8), as compared with strain alone in BSMC *in vitro* ($P < 0.05$). Strain of SMC in conjunction with hypoxia has not previously been studied using controlled *in vitro* parameters.

Given the role of MMPs in bladder hyperplasia, we were interested in the effect of rapamycin on *Mmp7* expression. Rapamycin treatment inhibited the effects of strain on *Mmp7* expression and was able to reduce the extremely high *Mmp7* levels seen with the combined strain plus hypoxia stimuli.

In Vivo Obstruction Results in Reduced SMA, Increased Mmp7 Expression and Increased mTOR Signaling

In vivo partial bladder outlet obstructions (PBO) were surgically performed by suturing the outlet.²⁰ This condition resulted in greatly increased bladder mass by 3 and 6 weeks.²⁰ Interestingly, *Mmp7* mRNA levels by real-time PCR were significantly increased at 6 weeks of PBO ($P < 0.05$; Figure 9A). RT-PCR by semiquantitative methods for other MMPs (*MMP-2*, -9, -14) showed no increase in expression levels at any timepoints (data not shown). In contrast, SMA expression was decreased at 6 weeks of PBO ($P < 0.05$; Figure 9, B and C).

To further confirm that *in vivo* PBO bladders demonstrate mTOR pathway activation, we studied the activation of two arms of this pathway, S6K and EIF4E, by Western blotting with anti-phospho-S6K and -4EBP antibodies, respectively. The increased phospho-S6K and decreased phospho-4EBP clearly demonstrate (Figure 9D) that the mTOR pathway is activated during PBO.

Figure 5. Mechanical strain activates many downstream effectors of mTOR. **A–C:** Downstream effectors were examined in response to strain alone. Cells were statically strained on the Flexcell 4000 system with 5% elongation for **A, C:** 20 minutes; **B:** 10 minutes. Whole cell lysates harvested after indicated time points were analyzed by Western blotting for phospho-S6, - MNK1, -ERK1/2, -STAT3, -EIF4E, total actin, and pan-ERK1/2. Representative autoradiographs from $n = 3$ blots shown. Densitometry on $n = 3$ blots was statistically analyzed by: **(A)**, phospho-S6: t -test, $*P < 0.10$; phospho-MNK1: t -test, $*P < 0.05$, $**P < 0.01$, $n = 3$; **(B)**, phospho-ERK: t -test, $*P < 0.02$, $n = 3$; **(C)**, phospho-STAT3: t -test, $*P < 0.04$, $**P < 0.03$, $n = 3$; phospho-EIF4E: t -test, $*P < 0.04$, $n = 3$.

Discussion

In the 40 years since their inception, no new pharmacotherapy beyond muscarinic anticholinergic drugs and their derivatives has been approved to reverse or stabilize bladder muscle wall overgrowth and dedifferentiation in response to obstruction. For the first time in BSMCs, we demonstrate that rapamycin, an orally active inhibitor of S6K1/mTOR can stabilize differentiation of BSMCs, under the influence of three canonical stimuli that drive myopathic damage and BSMC dedifferentiation during bladder obstruction. In urinary pathologies, such as neurogenic bladder and posterior urethral valves, bladder hyperdistension and hypercontraction lead to excessive BSMC proliferation and decreased BSMC differentiation with progressive loss of muscle function. Bladder wall distension compresses surrounding intramural blood vessels inciting hypoxia. Both strain and hypoxia induce expression and activation of MMPs (as seen in Figure 8 and elsewhere,^{10,11,30}) ultimately restructuring the ECM. Furthermore, tissue hypoxia and mechanical strain incite alterations in MMPs, which contribute to matrix remodeling and muscle tissue damage. Through a process

termed dynamic reciprocity,^{31,32} damaged matrix itself has been found to elicit proliferative responses in both bladder¹¹ and vascular SMC,²⁹ providing a mechanism whereby MMPs induced by mechanical or hypoxic injury may lead to longer term alteration in differentiation of BSMC. Three different types of stimulation—mechanical strain,¹⁰ hypoxia,⁹ and exposure to abnormal ECM^{11,29} induce remarkably similar increases in vascular and as seen here, BSMC growth, *Mmp7* expression, and loss of differentiation markers. The contractile or differentiated SMC phenotype is associated with increased expression of α -smooth muscle actin (SMA).^{5,33,34} The conserved response to three different microenvironmental conditions prevalent during obstructive uropathy, further suggests they are pathophysiologically related.

Rapamycin was able to dramatically suppress proliferation and maintain differentiation of BSMCs exposed to these three coordinate but distinct stimuli found in obstructive bladder disease: cell strain, cell hypoxia, and denatured matrix. This implicates mTOR as a critical regulator of BSMCs in obstructive uropathy, similar to studies in vascular SMCs showing that mTOR is a key

Figure 6. Rapamycin reverses strain-, hypoxia- and denatured matrix-induced loss of differentiation phenotype. **A:** Loss of *SMA* mRNA during strain is significantly restored by rapamycin ($*P < 0.05$, by *t*-test). *SMA* mRNA was assessed by real-time PCR via the deltaC(t) method: the change in expression is compared with housekeeping genes (*Rpl32* and *Gapdh*), then to control unstrained levels. **B:** BSMCs were plated on collagen type I Flexcell plates and statically strained for 18 hours. Cells were fixed in 4% paraformaldehyde, and stained for SMA using anti-SMA-Cy3 (Sigma) and Hoechst after permeabilization with 0.2% TritonX-100. Loss of SMA expression during strain is restored by rapamycin treatment. **C:** SMA staining and morphology is altered in denatured matrix-stimulated and strained BSMCs. Serum-starved BSMCs were cultured on denatured collagen versus native collagen gels for 24 hours, with and without rapamycin treatment BSMCs on DNC plates had significantly lower SMA expression ($*P < 0.005$, by *t*-test, using a total of $n = 4$), which was partially recovered by rapamycin treatment ($**P = 0.03$, by *t*-test, using a total of $n = 3$). **D:** *Sma* expression is increased in BSMC treated with rapamycin $\pm$ hypoxia (1% O_2). *Sma* expression was assessed by real-time PCR using the deltaC(t) method, where the change in expression is compared with housekeeping genes (*Rpl32* and *Gapdh*), and compared with control normoxic levels.

regulator of proliferation and de-differentiation.^{17,35,36} Vascular smooth muscle cells proliferation following mechanical strain during arterial distension by balloon angioplasty was inhibited with rapamycin, similar to our mechanical strain responses in BSMCs.³⁷ Two main families of mTOR- dependant signaling kinases are responsible for mRNA translation³⁸: the S6 kinases (S6Ks) and the eukaryotic initiation factor 4E (EIF4E)-binding proteins, which mediate 5'-terminal oligopolyrimidine (5'-TOP) mRNA translation and 5'-cap-dependent mRNA translation, respectively.^{39,40} Overall, the mTOR pathway, relatively unexplored in visceral SMC growth and differentiation, may act as a key regulator of stimulated cell cycle progression.

Rapamycin prevents acute phosphorylation of S6K at Threonine³⁸⁹, the linker between catalytic and auto-inhibitory domains⁴¹ and blocks proliferation. Strain and hypoxia activate mTOR pathway (eg, S6K) (Figure 4),

resulting in BSMC growth (Figure 3). As strain is a physiological stimulus and not a discrete molecular stimulus, other BSMC pathways are activated during strain, such as MNK1, ERK1/2, as well as STAT3. Although neither ERK1/2 nor STAT3 are strongly inhibited by rapamycin, our previous studies have shown that ERK and STAT3 both play roles in BSMC proliferation.^{9–11,19} Although ERK1/2 mitogen activated protein kinase, and mTOR pathway effectors herein are both activated during whole bladder strain injury and play a role in BSMC growth^{9–11} (Figure 3), they do not consistently colocalize in distended intact bladder. Different BSMC populations heterogeneous in SMA and iNOS expression,⁵ ERK and mTOR signaling, may be regulated by distinct pathways.

For the first time, this study shows a dependency of SMA expression in BSMC on down-regulation of mTOR. Rapamycin clearly increases SMA expression *in vitro* in stimulated BSMC which suggests that decreased SMA

HA-S6K transfection

Figure 7. S6K1 overexpression in BSMCs is associated with decreased SMA expression. BSMCs were plated at 50% confluency in six-well plates, and transfected with rat hemagglutinin(HA)-tagged S6K1 constructs^{18,35} (Addgene) using LTX with plus reagent in OptiMem. After 4 hours, media was replaced with growth media and cells incubated for 2 days. HA-S6K1 was detected using mouse anti-HA antibody (Covance) and anti-mouse-Cy2. SMA was detected using rabbit anti-SMA (Abcam) and anti-rabbit-Cy3. HA expression (a tag for transgene expression) particularly in cells transfected with the constitutively active mutants (D3E E389) was associated with a down-regulation of SMA staining. The cells with the highest HA- tag immunoreactivity (S6K expression) had significantly lower SMA expression compared with low or nontransfected cells (* $P < 0.002$, ** $P < 0.007$, by two-tailed t -test; data are presented as means $\pm$ SEM).

expression during partial bladder obstruction may be due to mTOR activation. Previous studies have shown that dedifferentiation due to mechanical strain in SMC is associated with reduced SMA protein.^{42,43} The decrease in SMA protein seen here (Figure 9) is concordant with SMA mRNA expression. Rapamycin may be able to not only inhibit excessive BSMC growth, but also in turn stabilize the differentiation state of the SMC. Our *in vitro* transfections also show an inverse relationship between the activity of the S6K and SMA immunostaining, which parallels the de-differentiation observed in S6K1-overexpressing cells. Overexpression of S6K1 may lead to alterations in SMC gene transcription, but could also alter the translation of

SMC genes. S6K1 overexpression in other cells results in increased activity of 4EBP, a downstream component of the mTOR normally suppressed during mTOR pathway activation.^{41,43}

This may augment 4EBP-mediated inhibition of EIF4E, which lies immediately downstream of 4EBP, thus muting translation of 5'Cap mRNA of SMA. Similar studies with HA-S6K1 clones in vascular SMC also noted a decrease in SMA with increased levels of S6K1 activity.^{35,44} Similarly, while mTOR appears to control SMA expression translation and transcription in vascular SMC, this is the first report of a role for mTOR in visceral SMC. Interestingly, our *in vivo* data shows increased S6K activity alongside decreased SMA expression and 4EBP activity. Ongoing studies in our lab are examining whether this regulation of SMA extends to other markers of SMC differentiation.

Previously, we and others found a role for STAT3 in proliferation in a static model of strain and hypertrophy during obstruction *in vivo*,^{26,27,45} but not de-differentiation in BSMCs *in vitro*.¹⁹ Here we see that mTOR/TORC1 has a negative regulatory effect on basal STAT3 phosphorylation of Tyr-⁷⁰⁵ (Figure 5C) in BSMCs either non-strained or on native collagen. This increased STAT3 phospho-Tyr-⁷⁰⁵ in rapamycin-treated unstimulated cells was concordant with de-differentiation in unstimulated BSMCs (Figure 6). Conversely, STAT3 inhibition (by JAK2 inhibitor AG490) prevented proliferation *in vitro*.¹⁹ STAT3 phospho-Tyr⁷⁰⁵ the site for dimerization and activity of STAT3 might be increased in response to rapamycin due to feedback onto stimulatory pathways such as PI3K.⁴⁶

Alternatively, other phospho-sites on STAT3 may be regulated differently in response to rapamycin. Inhibition of mTOR, which phosphorylates STAT3 at Ser⁷²⁷ (a negative regulator of Tyr⁷⁰⁵STAT3 phosphorylation), may in-

Figure 8. Rapamycin reduces *Mmp7* expression induced by mitogenic stimuli. Real-time PCR was performed on cDNA from BSMC, plated on Bioflex plates that were serum-starved and stimulated by 5% equibiaxial strain $\pm$ 1% O₂ hypoxia, for 18 hours. Strain plus hypoxia induced a significant upregulation of *Mmp7* as compared with unstimulated cells (* $P < 0.0008$), rapamycin treated or hypoxia stimulated cells (** $P < 0.003$) and strained cells (** $P = 0.05$). Hypoxia (1% O₂) alone did not increase *Mmp7* mRNA levels, though in previous work 3% O₂ increased *Mmp7* transcription.⁹ A trend toward increased *Mmp7* was seen in the strain alone group (** $P = 0.06$), which was decreased significantly in the presence of rapamycin (* $P < 0.04$). Data are presented as means $\pm$ SEM ($n = 3$) and P values calculated using 3-factor analysis of variance.

Figure 9. *In vivo* partial bladder outlet obstruction (PBO) is associated with decreased SMA and increased *Mmp7* expression. Outlet obstructions were performed by ligation of the urethra and tube, and removal of the tube, to cause a permanent partial obstruction of the urethra ($n = 4$). Sham obstructed bladders ($n = 3$) were not ligated, but all other manipulations were performed. Harvested tissue was crushed under liquid nitrogen for RNA isolation, cDNA synthesized using Superscript III (Invitrogen) and real-time PCR performed using SyBr green. The delta C(t) method of quantitation of real-time PCR results of *Mmp7*, *Sma*, and *Gapdh* (housekeeping gene) revealed that: **(A)** *Mmp7* mRNA was up-regulated during PBO ($*P < 0.002$, by *t*-test), and **(B)** *Sma* mRNA was down-regulated during PBO ($*P = 0.05$, by *t*-test). The pattern of up-regulated *Mmp7* and down-regulated *Sma* recapitulates the *in vitro* data observed with strain and hypoxia. **C:** SMA protein expression is down-regulated during PBO, by Western blotting (Sigma monoclonal against SMA), $*P < 0.05$ by *t*-test. **D:** mTOR pathway is activated *in vivo* during PBO. Activation of S6K and 4EBP was assessed by Western blotting using phospho-specific antibodies to probe tissue lysates harvested by crushing under N₂ (liq). Increased phosphorylation of S6K ($P = 0.01$) and decreased phosphorylation of 4EBP ($P = 0.05$) were indicative of activation of the two main arms of translation control during PBO (by two-tailed *t*-test, $n = 3$).

crease Tyr⁷⁰⁵ phosphorylation. The possibility also exists that rapamycin differentially affects BSMC subpopulations under different conditions, though the differentiating effect of rapamycin is consistently seen in cells stimulated by hypoxia, DNC or strain. To avoid adverse effects on STAT3 by rapamycin in quiescent cells, targeting rapamycin to de-differentiated cells *in vivo* may be needed to avoid deleterious effects of STAT3 activation. Further, as rapamycin inhibits TORC1, it remains to be seen if STAT3 is similarly activated during inhibition of both TORC1 and TORC2 complexes. SMA expression could also be regulated by EIF4E, which is independently regulated in our models of strain injury, possibly by EGFR (Figure 4), ERK1/2, or p38, molecules activated during BSMC injury and proliferation.^{9,10,47,48}

EGFR appears to be upstream of many strain-induced pathways: mTOR, S6, ERK, and STAT3 (Figure 10). Iressa, an EGFR inhibitor, can prevent BSMC proliferation in distended *ex vivo* bladders cultures⁴⁸ and induce apoptosis in vascular SMC.⁴⁹ Such upstream inhibitors may be of clinical utility to prevent STAT3, MAPK and mTOR activation, though they may be less specific in their downstream pathway targets. Furthermore, elucidating the mechanisms mediating the inhibition of proliferation/

de-differentiation by disparate pathways of JAK2/STAT3, ERK and mTOR/S6K is best addressed with molecular approaches. Our approach using transfections of wild-type and constitutively active S6K has shown that this pathway is sufficient to induce a loss of SMA in visceral SMC.

Our *in vivo* transcription data suggests that reciprocal transcriptional controls occur in both *in vitro* and *in vivo*, as *Mmp7* and SMA are similarly down- and up-regulated, respectively. This study also raises new questions. How does rapamycin reduce cell cycle progression? One prominent theory suggests that mTOR functions in parallel and downstream of the PI3K/Akt pathway which is involved in cell cycle progression, cell migration, growth, and survival.⁵⁰ Both mTOR^{18,38,39} and PI3K-dependant signals coordinately control S6Ks, STAT3, and EIF4E proteins. Recall that rapamycin can lead to a paradoxical increase in basal STAT3⁷⁰⁵ phosphorylation and no effect on EIF4E, despite its inhibition of S6. As multiple pathways converge on the regulation of BSMC growth and differentiation, this study begins to address their interactivity.

Another factor of interest is MMP7, whose importance in BSMC injury is highlighted by the fact that expression

Figure 10. Model of pathway induction after pathophysiologic stimulation of BSMC by three coordinate stimuli: strain, hypoxia, and damaged matrix. Bladder obstruction leads to strain injury of the bladder smooth muscle. Strain injury is associated with microvascular compression and consequent hypoxia^{8,55–57} as well as matrix metalloprotease activation and consequent alteration of the ECM. These three coordinate stimuli, hypoxia, strain (directly) and damaged matrix can lead to increased signaling through mTOR, inhibited by rapamycin, as well as parallel pathways ERK, JAK2/STAT3 and p38.⁴⁷ EIF4E is strain-activated, but not in an mTOR dependent manner, suggesting that in BSMC strain activates EIF4E through other pathways (EGFR-dependent, p38-dependent), which prevent inhibition by rapamycin under strain 4EBP upstream of EIF E and downstream of mTOR is inhibited by *in vivo* obstruction in contrast to strain *in vitro*. (see Discussion). STAT3 phosphorylation is also induced by strain, but is also basally induced by rapamycin. Phosphorylation of S6K on the other hand is augmented by strain, hypoxia, and, as with S6, is inhibited by rapamycin. S6K appears to initiate the de-differentiating response. However, STAT3, ERK and S6K inhibition all prevent proliferation in response to obstruction-related stimuli in BSMC, suggesting a common intermediary in all three pathways.

of this MMP increases in response to both hypoxia $\pm$ strain (*in vitro*) as well as uniquely in obstruction (*in vivo*) (Figures 8, 9). Its expression in many cell types including BSMC is associated with proliferation.^{9,51–53} Furthermore, diabetic bladder (a condition of decreased neural and vascular activity, increased inflammation) is associated with bladder SMC proliferation, and a 600-fold increase in *Mmp7* by microarray analysis of bladder smooth muscle.⁵⁴ BSMC mitogens, such as HBEGF, rely on MMP7 for activation, through modifying receptors (e.g. CD44), binding factors and ECM. MMP7 may play a critical role in matrix remodeling processes as well (Supplemental Figure S4, <http://ajp.amjpathol.org>), especially as its expression is regulated by rapamycin, and HBEGF has a known role in BSMC response to strain.⁴⁷

As mentioned throughout this study, de-differentiation may result from hyperdistension and tissue hypoxia. As bladder wall hypoxia occurs alongside distension, and not independently during obstruction, it may be considered a modulator of distension. As such, it is logical to devise an experimental model incorporating both strain and hypoxia simultaneously. Here we have seen that strain in conjunction with hypoxia leads to significantly increased *Mmp7* mRNA, S6K phosphorylation levels and proliferation over levels due to strain alone, and co-segregates with decreased SMA levels (Figures 5, 7, 8, and 9). Indeed, this dual stimulatory approach has not been to our knowledge applied in the study of SMC molecular responses. While stretch and hypoxia have oft been considered coordinate physiological stimuli in theory,⁵⁸ they have not often been examined experimentally in conjunction with one another. This confirms that these *in vitro* models are a functional representation of obstructive uropathy *in vivo* and provides a rationale for studying the effects of rapamycin on these markers *in vitro*. Such an approach may be of particular benefit for modeling other *in vivo* systems and testing new therapies.

This study also illustrates the utility of pathway analysis to identify novel pharmacological targets, as S6K1/mTOR is clearly involved in proliferation and de-differentiation in BSMC. We have expanded our knowledge of cellular responses during bladder injury by unveiling a new model of the role of mTOR in bladder strain injury (Figure 10) and new therapeutic avenue. *In vivo*, oral or intravesical rapamycin could be used to prevent proliferative and de-differentiation of BSMCs during particular phases of clinical conditions characterized by bladder outlet obstruction. Whether rapamycin therapy is beneficial during the decompensatory or compensatory phase, remains to be determined with further *in vivo* studies. In future, we will be testing various dosing and treatment regimens to optimize the effects of rapamycin *in vivo*. In vascular diseases, rapamycin coated stents have been used to inhibit neointimal SMC growth.^{15,37} Novel delivery methods of rapamycin to the most proliferative regions of the bladder may be required, as simple oral rapamycin may be inhibitory to cells required to protect the bladder from infection. One possible approach is a drug-eluting pouch proximal to bladder smooth muscle. Alternatively, RGD- or $\alpha v\beta 3$ Ab-coated rapamycin nanoparticles used for treatment of stenosis *in vivo*⁵⁹ might target aberrantly proliferating smooth muscle cells without affecting normal bladder cells. As RGD-peptides are able to block strain induced proliferation,⁶⁰ and $\alpha v\beta 3$ is associated with proliferative SMC phenotypes,⁶¹ targeted nanoparticles eluting rapamycin provide an alternative method to treat obstructive disease. With the present paper, a greater understanding of smooth muscle pathobiology provides the basis for identifying the appropriate use of mTOR and other inhibitors in myopathic diseases such as obstructive uropathy.

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