Cytoplasmic provenance of STAT3 and PY-STAT3 in the endolysosomal compartments in pulmonary arterial endothelial and smooth muscle cells: implications in pulmonary arterial hypertension

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PULMONARY ARTERIAL HYPERTENSION (PAH) is characterized by enlarged, vacuolated (“megalocytotic”) pulmonary arterial endothelial (PAEC) and smooth muscle cells (PASMC). We have recently proposed that dysfunction of vesicle tethers, soluble N-ethylmaleimide-sensitive factor attachment proteins (SNAPs), and SNAP receptors (SNAREs), leading to disruptions of intracellular trafficking from the Golgi to plasma membrane (centrifugal) and the plasma membrane to cell interior (centripetal) directions might represent a key subcellular mechanism leading to the changes observed in endothelial and vascular smooth muscle cells in PAH. We (24, 26–28, 41–43, 47) have previously provided evidence for disruption of centrifugal Golgi to plasma membrane trafficking, and thus a Golgi blockade, in models of PAH. In this article, we explore aspects of centripetal cytokine and growth-factor signaling in endothelial and vascular smooth muscle cells in PAH.

IL-6 and its activation of STAT3 signaling is increasingly viewed as contributing to the pathobiology of PAH. IL-6 mRNA has been reported to be elevated in lungs of monocrotaline (MCT)-treated rats with PAH and inhibition of IL-6 production with dexamethasone-attenuated MCT-induced PAH (4). Serum IL-6 levels were increased in idiopathic PAH in human (18). Transgenic mice overexpressing IL-6 developed mild PAH, which could be exacerbated by hypoxia (13). Additionally, IL-6 levels were dramatically increased by more than 15-fold in transgenic mice expressing a mutant isoform of BMPR2 that spontaneously developed PAH (15). A negative feedback loop in which IL-6 inhibits bone morphogenetic protein receptor 2 (BMPR2) signaling has been proposed in this model (15).

In 2004, working with the rat/MCT model of PAH, we reported the reciprocal relationship between the loss of caveolin-1 (cav-1) in plasma membrane rafts/caveolae in PAECs and hyperactivation of the pro-proliferative, IL-6-activated transcription factor STAT3 [increase in tyrosine-phosphorylated STAT3 (PY-STAT3); Ref. 24]. The loss of cav-1 and hyperactivation of PY-STAT3 was observed within 2–4 days after administration of MCT to rats (i.e., before the onset of PAH at 10–14 days; Ref. 24). Close examination of individual cells in vivo showed that the specific endothelial cells that had a loss of cav-1 expression were the same cells with hyperactivation of PY-STAT3 and increased DNA synthesis (24). This reciprocal relationship between cav-1 downregulation and STAT3 activation in PAH in the rat/MCT model was later confirmed by Jasmin and colleagues (19). That the increase in PY-STAT3 levels was seen before the onset of PAH in MCT-treated rats (19, 24) and that the introduction of a cav-1 scaffolding domain peptide blocked PY-STAT3 hyperactivation and ameliorated MCT-induced PAH (19) indicated that STAT3 played a role in
the initiation of PAH, at least in this animal model. Moreover, Park and colleagues (33) reported the hyperactivation of PY-STAT3 in lungs of cav-1−/− mice, which spontaneously developed PAH. Additionally, the increase in PY-STAT3 levels has now been confirmed in pulmonary arterial lesions of idiopathic PAH in humans (23), and a similar increase in STAT3 was also reported in lungs of mice with hypoxia-induced PAH (53). Thus the mechanisms of IL-6/STAT3 signal transduction from the plasma membrane to the cell interior in pulmonary arterial endothelial and vascular smooth muscle cells are of relevance to the pathobiology of PAH. However, considerations of centripetal signaling from the plasma membrane to the cell interior in the pulmonary hypertension literature have largely represented transcytoplasmic transit of activated signaling molecules as taking place exclusively as a soluble cytoplasmic process (reviewed in Refs. 42, 43).

To focus on IL-6/STAT3 signaling, STATs were viewed as monomeric cytosolic proteins that, upon cytokine stimulation, were phosphorylated on specific tyrosine residues and then dimerized, diffused through the cytosol to the region of nuclear pores, and then imported to the nucleus, which led to activation of gene transcription (reviewed in Refs. 11, 25, 40, 44, 51). Over the past few years, several key tenets of this classic JAK-STAT signaling paradigm have undergone revision. We and others have shown that “inactive” nonphosphorylated STATs were not free monomers in the cytoplasm but largely preexist in dimers and dimer-plus high molecular weight “statosome” complexes together with other proteins including clathrin heavy chain (CHC), glucose regulated protein 58 (GRP58), and heat shock protein 90 (HSP90) (14, 20, 29, 31). Moreover, we and others have established that the transcytoplasmic trafficking of PY-STAT3 was not a free cytosolic process but was initiated at plasma membrane raft microdomains (with or without cav-1) and proceeded along the cytoplasmic endocytic pathway at least in human hepatocytes (HeP3B cells) and in murine NIH/3T3 and BALB/c 3T3 fibroblast cells (“signaling endosome hypothesis”; Refs. 5, 39, 46). In these cell types, components of the endocytic pathway (such as dynamin, epsin, Eps15, and amphiphysin A1) were essential for optimal transcriptional activity of PY-STAT3 (5, 46). Additionally, other groups have established that the STAT3 constitutively shuttled between the nucleus and the cytoplasm (35) and that phosphorylation of STAT3 was not a prerequisite for transcriptional activity (57). However, nonphosphorylated STAT3 transcriptionally regulated different subsets of genes than PY-STAT3 (57).

With these insights, we asked whether the transcytoplasmic transit of STAT3 and PY-STAT3 in PAECs and PASMCs might also be membrane-associated and take place along the endocytic pathway, whether cav-1 might shunt this intracellular trafficking towards the lysosomal compartment as it does for TGF-β/Smad signaling (12), and whether this centripetal trafficking might be affected in PAH.

**MATERIALS AND METHODS**

**Cell culture.** Growth of primary bovine PAECs, human PASMCs (HPASMC), and the human Hep3B cell line in T-75 flasks, six-well plates, or eight-well chamber slides was done essentially as described by us previously (24, 26–28, 41, 46). PAECs were used between passages 4–20. HPASMC cultures (from Cascade Biologics, Portland, OR) were initially grown in Medium 231 supplemented with smooth muscle growth supplement containing basic fibroblast growth factor, epidermal growth factor, and insulin (as supplied by Cascade Biologics) to stimulate proliferation. Before experimental use, HPASMC cultures were transferred to Medium 231 supplemented with smooth muscle differentiation supplement containing 30 μg/ml heparin (Cascade Biologics) for >7 days as per manufacturer’s protocols. Monocrotaline pyrrole (MCTP) was prepared from MCT (Trans World Chemicals, Rockville, MD) as described by us previously (41) and used at a final concentration of 200 μM for 4 days. By mass spectrometry, ~30–50% of the input MCT was converted to active pyrrolic derivative (data not shown). Recombinant human IL-6 (R&D Systems, Minneapolis, MN) was used at 10 ng/ml for 30 min unless otherwise specified (24, 25, 29, 39). Phenylarsine oxide (PAO) was from Sigma-Aldrich (St. Louis, MO) and was used at 5 μM for 30 min (46).

**Immunofluorescence analyses of cells in culture.** Immunofluorescence analyses of PAECs and PASMCs were performed essentially as described by us earlier (24, 26–28, 41, 46). Cells were fixed and permeabilized using either the 4% cold paraformaldehyde-0.1% Triton X-100 protocol or 3.7% warm formalin (at 37 C)-0.1% Triton X-100 protocol as previously described by us (24, 26–28, 41, 46). Secondary antibodies used were Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes, Eugene, OR). Nuclei were demarcated using 4,6-diamidino-2-phenylindole (DAPI). Images were captured using either a Leitz epifluorescence microscope system equipped with a black-and-white charge-coupled device (CCD) camera and then rendered in pseudocolor, or a MRC 1024 ES (Bio-Rad) confocal laser scanning microscopy system with a black-and-white CCD camera and then rendered in pseudocolor, or a Zeiss epifluorescence microscope system equipped with an RGB camera (for immunomorphometry of lung sections). All images within each specific experiment were collected under identical imaging, laser, and microscopy settings. Images were deconvolved using the Iterative Deconvolve 3D plugin of the NIH Image software.

**Plasmids.** The following constitutive expression constructs were used in our studies: 1) N1-EGFP and STAT3-EGFP from Dr. Taddaki Miyazaka, Hokkaido University, Sapporo, Japan (54); 2) dominant-negative (DN) dynamin II K44A (Dyn II K44A) from Dr. Lois Green, National Institutes of Health (NIH), Bethesda, MD (55); 3) murine cav-1 wild-type (WT) from Dr. Ferruccio Galbiati, University of Pittsburgh, Pittsburgh, PA (52); 4) myc-tagged canine cav-1 WT and cav-1-S80A-myc and cav-1-S80E-myc, which are single point mutations in the scaffolding domain of cav-1 from Dr. Jeffery Pessin, SUNY Stony Brook, Stony Brook, NY (48); 5) a myc-tagged double point mutation in the scaffolding domain of cav-1 (canine species) cav-1-F92A/V94A and the corresponding myc-tagged cav-1 WT from Dr. Michael Quon, NIH (30); 6) human GRP58-WT from Drs. Mohammed Bourdi and Lace Pohl, NIH (8); and 7) pCMV5M4-luciferase, a well-characterized STAT3 responsive luciferase construct, which contains four copies of STAT3 DNA-binding element from the human angiotensin promoter from Dr. Ashok Kumar, New York Medical College, Valhalla, NY (26).

**Live cell imaging and fluorescence protease protection assays.** For live cell imaging experiments with STAT3-GFP, PAEC or Hep3B cells were cultured in six-well plates and transfected 24 h after plating either with STAT3-GFP or N1-GFP alone or in combination with Dyn II K44A or cav-1 WT using procedures described earlier (56). In cotransfection experiments, the ratio of the GFP-tagged plasmid to the non-GFP-tagged plasmid was 1:4 by mass (56). In each experiment, pCDNA3.1 was used as the “empty” control vector. Transfections were performed using PolyFect (Qiagen, Valencia, CA) as per manufacturer’s protocol. Cultures were viewed 24–36 h after transfection using confocal laser microscopy. For dual-channel live cell imaging to visualize lysosomes, LysoTracker (Molecular Probes) was added to the medium at a final concentration of 100 nM as described by us earlier (28). Fluorescence protease protection (FP) assays were
performed as described by us recently (22, 56). Briefly, cultures were transferred to warm HBSS to a final volume of 1 ml. After identification of a region of interest, at discrete time intervals, 1 ml each of a 2× strength solution of digitonin in warm HBSS followed by a 3× strength solution of trypsin in warm HBSS and finally a 4× strength solution of Triton X-100 in warm HBSS were added. The final effective concentrations of digitonin, trypsin, and Triton X-100 were 50 μg/ml, 100 μg/ml, and 0.5% vol/vol, respectively (56). Images were captured using the MRC 1024 ES confocal microscope. There was no spillover between the red and green channels.

IL-6/STAT3-responsive reporter/luciferase assays. Luciferase reporter assays were carried out essentially as described by us earlier (26, 46). PAEC or Hep3B cells in six-well plates were transfected with the p950M4-luciferase reporter together with the constitutive β-galactosidase expression plasmid pCH110 and either murine cav-1-WT, myc-tagged canine cav-1-WT, cav-1-S80A-myc, cav-1-S80E-myc, cav-1-F92AV94A, or GRP58-WT using PolyFect. All assays were carried out in triplicate for each variable. Cultures were incubated at 37°C for another 24 h and then treated with IL-6 (10 ng/ml) for 6 h followed by harvesting the cells in 300-μl passive lysis buffer (Promega, Madison, WI). Fifty-microliter aliquots of the clarified extracts were used to assay luciferase and β-galactosidase activity using respective assay kits/reagents from Promega and Roche (Indianapolis, IN), respectively, and the manufacturer’s protocols. The luciferase activity was then normalized with reference to the β-galactosidase activity in the extract (to control for transfection efficiency) and expressed in terms of that for the IL-6-free, cav-1- and GRP58 cotransfection-free culture.

Immunofluorescence studies of MCT-treated rat lungs. Immunofluorescence analyses of rat lung sections were carried out essentially as described earlier (24, 41). All protocols were approved by the Institutional Animal Care and Use Committee. Four- to six-week-old juvenile male Sprague-Dawley rats were injected with MCT (60 mg/kg) subcutaneously. Control animals received equivalent amount of PBS. Lungs were harvested for 8–10 min in PBS. Fixation in formaldehyde and embedded in paraffin, and 4-μm sections were processed for immunofluorescence. Antibody reaction was performed by both indirect immunoperoxidase and in PBS.

Immunofluorescence studies of human PAH. Sections of formalin-fixed, paraffin-embedded archived lung tissue from patients with idiopathic PAH and from controls without PAH (n = 2 each) were obtained from the Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD. Antibody reaction and immunofluorescence analyses were carried out as for the MCT rat lung sections above.

Image analyses. All experimental and control images of the immunofluorescence data were collected at identical imaging settings. Image analyses were performed using NIH ImageJ (available as a free download from http://rsb.info.nih.gov/ij/). Per cell pixel intensities were measured by visually defining cell outlines and measuring integrated and mean pixel intensities for that area (28). For each image, the “background” was adjusted to 0 pixels to reduce variance in intensities between experimental and control sets. Colocalization analyses were performed using the NIH ImageJ Just Another Co-localization Plugin (JACoP) (6). JACoP directly provides the Pearson R correlation, Manders’ M1 and M2 coefficients, and Costes’ randomization coefficients for a pair of images in the red and green channels. Briefly, Pearson correlation coefficient (R) measures the pattern similarities between two images. Values of Pearson R range from +1, indicating complete colocalization, to −1, indicating negative colocalization. A Pearson R coefficient value of 0 indicates no correlation between the two images. Manders’ M1 is defined as the ratio of the summed intensities of pixels from the green channel for which the intensity in the red channel is above 0 to the total intensity in the green channel; Manders’ M2 is the reverse. These coefficients are good indicators of the level of colocalization between red and green channels (and vice versa) and are independent of the intensity of fluorescence. Costes’ randomization is a useful tool to investigate whether the observed colocalization is purely due to chance or is statistically significant. This coefficient is based on a Monte Carlo protocol that creates random images by shuffling pixels in the green channel and then calculating a new Pearson R for each randomized image in the green channel (the image in the red channel is kept unchanged). The Pearson R of the original image in the green and red channel is then compared with each new Pearson R obtained from the randomized green images with the red channel. The final result is obtained as the likelihood that the Pearson R calculated by randomizing the green images is greater than the original Pearson R observed (7).

Antibody reagents. Rabbit polyclonal antibodies (pAbs) to STAT3, PY-STAT3, cav-1 and corresponding competing peptides were from Santa Cruz Biotechnology (Santa Cruz, CA). Murine MAbs to CHC, early endosome antigen 1 (EEA1), Golgi matrix 130 (GM130), lysosome-associated membrane protein 1 (LAMP1), Rab5, and Rab11 were from BD Biosciences (Eugene, OR).

Statistical analyses. Statistical analyses were performed using the two-tailed Student’s t-test and Microsoft Excel software.

RESULTS

Increased targeting of STAT3 and PY-STAT3 to cytoplasmic vesicles in MCTP-induced megalocytosis in PAECs. We (26) have previously shown that in MCTP-induced megalocytosis of PAEC in culture, there was a reciprocal relationship between loss of cellular cav-1 and hyperactivation of IL-6/STAT3 signaling as assayed in terms of an increase in PY-STAT3 in Western blotting assays. However, this increase in total and cytoplasmic PY-STAT3 did not convert into an enhancement of IL-6-activated STAT3 transcriptional luciferase-reporter activity compared with IL-6-treated PAEC not exposed to MCTP (26). Thus we asked whether PY-STAT3 was aberrantly sequestered in the cytoplasm of MCTP-treated PAEC.

Immunofluorescence assays for STAT3 localization in untreated PAECs in culture showed that STAT3 was constitutively localized in punctuate plasma membrane caveolae and in the nucleus (Fig. 1A, top, left). Exposure to IL-6 for 30 min led to a relative reduction in STAT3 in plasma membrane caveolae with an increase in the nuclear pool (Fig. 1A, top, right). Treatment of PAECs with MCTP for 4 days, which induced an increase in cell size (megacell), led to a dramatic redistribution of STAT3 from plasma membrane caveolar puncta to large cytoplasmic vesicles (Fig. 1A, bottom, left). This cytoplasmic vesicular STAT3 persisted in megalocytotic PAECs exposed to IL-6 (Fig. 1A, bottom, right). Thus, in MCTP-induced megalocytosis in PAECs, there was a loss of caveolar STAT3 and sequestration of this transcription factor in cytoplasmic structures.

To investigate whether this sequestration was in components of the endocytic pathway, we performed double-label immunofluorescence analyses of PAECs treated with MCTP for 4 days together with immunostaining for the endosomal markers Rab5, Rab11, CHC, and the lysosomal marker LAMP1. The data in Fig. 1B show that the STAT3 vesicles in MCTP-treated PAEC represented a heterogeneous population of endocytic pathway components that partially colocalized with the early endosome marker Rab5, the recycling endosome marker Rab11, the endocytic coat protein CHC, and the lysosome marker LAMP1. Costes’ randomization performed using NIH ImageJ confirmed the statistical significance of each of these colocalizations (see legend to Fig. 1). Moreover, peptide competition controls (Fig. 1C) con-
firmed the specificity of the anti-STAT3 antibody used in these analyses.

Figure 2, A and B, extends the above observations to activated PY-STAT3. Figure 2A, top, left, shows that, in control PAECs, there was constitutive presence of activated PY-STAT3 in plasma membrane caveolar puncta, in discrete cytoplasmic vesicles, as well as in the nucleus. Exposure to IL-6 led to an increase in nuclear PY-STAT3 (Fig. 2A, top,
right). However, a pool of PY-STAT3 persisted in discrete puncta both at plasma membrane caveolae as well as in the cytoplasm. Exposure of PAECs to MCTP led 4 days later to a marked accumulation of PY-STAT3 in cytoplasmic vesicles and persistence in the nucleus. Additional exposure to IL-6 did not change this distribution in MCTP-treated PAEC (peptide competition controls that verify the specificity of the anti-PY-STAT3 pAb used are in Fig. 9, E and F; data confirming the detection of the correct PY-STAT3 protein by this antibody in Western blots has been shown in Refs. 24, 26, 46). This cytoplasmic sequestration of PY-STAT3 was not in the enlarged Golgi elements previously reported by us in such cells (26, 27, 28, 41) in that Fig. 2B shows that the bulk of this cytoplasmic PY-STAT3 did not colocalize with the Golgi tether GM130. The subcellular redistribution of STAT3 and PY-STAT3 after MCTP was accompanied by changes in the localization of the STAT3-associated protein and chaperone GRP58 (14) from the cytoplasm to the nucleus (Fig. 2C).

Live cell imaging of the cytoplasmic trafficking of STAT3 in PAECs. To obtain further evidence in support of membrane-associated trafficking of STAT3 in the endothelial cell cytoplasm, we used a live cell imaging approach using a GFP-tagged STAT3 expression construct together with treating the cells with LysoTracker (which demarcates acidic late endosomes/lysosomes) and cotransfection with an expression vector for the DN K44A mutant of the GTPase dynamin II (Dyn II K44A). The DN mutant Dyn II K44A disrupts endocytic membrane trafficking (10). Figure 3A shows that after 30 min of IL-6, STAT3-GFP, but not the N1-GFP tag by itself, was targeted to the both the nucleus and to discrete, large cytoplasmic vesicles, which were predominantly free of LysoTracker. As with the data in Fig. 1, Fig. 3B, top shows that this targeting of STAT3-GFP to the nucleus and to large cytoplasmic vesicles occurred constitutively (Fig. 3B, top), although IL-6 increased the relative amount of STAT3-GFP in the nucleus. The cytoplasmic structures to which STAT3-GFP was targeted were part of the endocytic compartment in that overexpression of Dyn II K44A led to marked accumulation of STAT3-GFP in large cytoplasmic vesicles with concomitant depletion of STAT3-GFP from the nucleus in both control and MCTP-treated PAEC (Fig. 3B, middle and bottom). Further exposure to IL-6 failed to drive STAT3-GFP to the nucleus in the presence of K44A. (In separate control experiments, we have confirmed the coexpression of the Dyn II K44A mutant specifically in cells expressing STAT3-GFP; see Ref. 56). These live cell Dyn II K44A cotransfection data provide unambiguous evidence for constitutive membrane-associated trafficking of STAT3 along the endocytic pathway in the endothelial cell cytoplasm (also see Ref. 56).

Targeting of a pool of cytoplasmic STAT3-GFP to lysosomes. Whereas the majority of cytoplasmic STAT3-GFP vesicles (with and without Dyn II K44A cotransfection) were free of LysoTracker, we observed that a small subset of STAT3-GFP vesicles did colocalize with LysoTracker (Fig. 3C). This observation, combined with the data in Fig. 1B showing that endogenous STAT3 colocalized with the late endosomal/lysosomal marker LAMP1 in PAECs, suggested that a pool of STAT3 may eventually reach the lysosome, reminiscent of TGF-β receptor (TGF-βR)/Smad signaling (12). In these LysoTracker and STAT3-GFP double-positive vesicles, we asked whether the STAT3 was inside the lysosomal lumen or was on its external surface. Figure 3C summarizes data from a FPP assay using sequential exposure of PAECs transfected with STAT3-GFP and treated with LysoTracker to digitonin (50 μg/ml), trypsin (100 μg/ml), and Triton X-100 (0.1% vol/vol). [This assay is based on the principle that digitonin permeabilizes mainly the plasma membrane releasing cytosolic proteins. The addition of trypsin after digitonin provides trypsin access to proteins that are on the cytosolic face of vesicles. Thus, if a protein is on the external face of a vesicle, it will be digested by the addition of trypsin after digitonin, and its signal will be lost. Proteins that are within a limiting membrane, such as inside endosomes or lysosomes, require the addition of Triton X-100 before they can be digested by trypsin (22). The retention of LysoTracker until the last Triton X-100 step serves as a positive internal control]. The data in Fig. 3C show I) the STAT3-GFP that did not colocalize with LysoTracker was released by digitonin, but 2) the STAT3-GFP that colocalized with LysoTracker was not released by digitonin nor digested by the subsequent addition of trypsin alone. It was only with the addition of Triton X-100 that the STAT3-GFP signal in the LysoTracker-positive compartment was solubilized. Thus a subset of STAT3-GFP in endothelial cells colocalized with lysosomes and was present inside the lysosomal lumen.

Cav-1 overexpression increases targeting of STAT3 to lysosomes and inhibits STAT3 transcriptional activity. WT cav-1 enhances the lysosomal targeting of the TGF-β/TGF-βRs endocytic complexes resulting in inhibition of transcriptional signaling (12). Thus we investigated whether cav-1 might have a similar effect on STAT3 endocytic trafficking and transcriptional activity. We first investigated an RNA interference (RNAi) approach to downregulate cav-1 expression. In preliminary experiments, although cav-1 RNAi enhanced IL-6/
STAT3 transcriptional signaling, a significant reduction in cav-1 levels per se was difficult to verify by Western blotting, and a similar enhancement of IL-6/transcriptional signaling was observed using an irrelevant RNAi control oligonucleotide. Thus we turned to an alternative approach, overexpression of cav-1 and its mutants in the human Hep3B cell line that expresses only low levels of endogenous cav-1. Overexpression of WT cav-1 in these cells by transient transfection methods led to the targeting of STAT3-GFP to LysoTracker-positive lysosomes (Fig. 4A). Quantitative colocalization anal-

Fig. 2. Aberrant subcellular localization of tyrosine-phosphorylated STAT3 (PY-STAT3) and glucose regulated protein 58 (GRP58) in MCTP-induced megalocytosis in PAECs. PAECs in 6-well plates were treated with MCTP or DMF for 4 days and then fixed and processed for immunofluorescence using rabbit pAbs to PY-STAT3 (A and B) and GRP58 (C) and an MAb to Golgi matrix 130 (GM130) as indicated. Nuclei were visualized using DAPI in A. Single arrowheads in A and B indicate caveolar PY-STAT3, and double arrowheads indicate cytoplasmic, vesicular PY-STAT3. Scale bars = 25 μm.
yses performed using NIH ImageJ software (using multiple 983-× 983-μm images) showed that cotransfection of the STAT3-GFP construct together with one expressing WT cav-1 led to a statistically significant increase in the colocalization of STAT3-GFP with LysoTracker in both the Pearson R and Manders’ M2 statistics (Fig. 4B). That the colocalization of LysoTracker (red) with STAT3-GFP (green) did not significantly change in the Manders’ M1 statistic acts as an
Fig. 4. Epigenetic regulation of STAT3 signaling: enhanced targeting of STAT3 to lysosomes by cav-1 and inhibition of transcriptional activity by cav-1 and GRP58. A: human Hep3B cells with low endogenous cav-1 expression were plated in 6-well plates and 1 day later transfected with STAT3-GFP and murine cav-1 wild-type (WT) expression vector (total DNA per well = 1 µg; 1:4 ratio of STAT3-GFP to cav-1). Thirty-six hours later, cultures were exposed to IL-6 (30 min) and LysoTracker and imaged. Scale bar = 25 µm. B: Hep3B cells in 6-well plates were transfected either with STAT3-GFP and pcDNA3.1 or STAT3-GFP and murine cav-1-WT expression vectors (1:4 ratio of STAT3-GFP to non-GFP plasmids; total DNA per well = 1 µg). Thirty-six hours later, the cultures were exposed to LysoTracker and extensively imaged (at least >5,983 µm × 983 µm fields per variable). Formal colocalization analyses (of the green STAT3-GFP with the red LysoTracker pixels) were then carried out on all the imaged frames using the NIH ImageJ Just Another Co-localization Plugin (JACoP) and expressed in terms of the Pearson R and Manders’ M1 and M2 coefficients as described in MATERIALS AND METHODS (means ± SE). *P < 0.05 in comparing cav-1-transfected culture with that transfected with the pcDNA3.1 control; n.s., not significant (P ≥ 0.05). C: Hep3B cells in 6-well plates were transfected (in triplicate per variable) with p950M4-luciferase, a constitutive β-galactosidase expression construct, and either of pcDNA3.1, murine cav-1-WT, canine cav-1-WT-myc, or cav-1 scaffolding domain mutants cav-1 S80A-myc or cav-1 S80E-myc expression constructs. Basal and IL-6-induced luciferase and β-galactosidase activities were assayed as described in MATERIALS AND METHODS. Luciferase activity, normalized to respective β-galactosidase activity, is expressed in terms of that in the IL-6-free, pcDNA3.1-transfected cultures (means ± SE). *P = 0.05 in comparisons of basal (IL-6-free) luciferase activity in cav-1 cotransfected cultures to that of the control pcDNA3.1 transfected-free culture; **P ≤ 0.05 in comparisons of IL-6-induced luciferase activity in cav-1 cotransfected cultures to that of the control pcDNA3.1 treated-free culture. A.U., arbitrary units. D: PAECs in 6-well plates were transfected with p950M4-luciferase, a constitutive β-galactosidase expression construct and either of pcDNA3.1, murine cav-1-WT, or the cav-1 scaffolding domain mutant cav-1 F92A/V94A. Basal and IL-6-induced luciferase and β-galactosidase activity was assayed as described in C. *P ≤ 0.05 in comparisons of basal (IL-6-free) and **P ≤ 0.05 in comparisons of IL-6-induced luciferase activity. E: Hep3B cells in 6-well plates were transfected with p950M4-luciferase, a constitutive β-galactosidase expression construct and either pcDNA3.1 or an expression construct for human GRP58-WT. Basal and IL-6-induced luciferase and β-galactosidase activity was assayed as described in MATERIALS AND METHODS and expressed as in C. **P ≤ 0.05 in comparisons of IL-6-induced luciferase activity.

Did this increased targeting of STAT3 to lysosomes by WT cav-1 affect IL-6-induced STAT3 transcriptional activity? Figure 4C shows that overexpression of WT cav-1 led to a significant inhibition of both constitutive (single asterisk) and IL-6-induced (double asterisk) STAT3-luciferase reporter activity. Cav-1-induced inhibition of STAT3-luciferase reporter activity was observed using two different WT cav-1 expression vectors and was not dependent on the integrity of the scaffolding domain in cav-1 in that mutations in this domain (either S80A or S80E) were just as effective in inhibiting IL-6-induced STAT3 transcriptional activity (Fig. 4C). Thus this downregulation is not at the level of rafts/caveolae but reflects an event at a site internal to the cell. Figure 4D confirms that internal control in this experiment that because 100% of cells pick up LysoTracker in the red channel (generating a large denominator approaching 100%) but only a minority of these are cotransfected by the STAT3-GFP construct (thus generating a small numerator to begin with), we do not expect significant changes in colocalization of the green STAT3-GFP pixels (small numerator and thus only small additional changes due to cav-1 overexpression) to be apparent against the background of a large denominator of red LysoTracker pixels. As a further internal control for this experiment, overexpression of cav-1 did not change the level of colocalization of N1-GFP with LysoTracker even when using the Manders’ M2 statistic.
overexpression of WT cav-1 or the scaffolding domain mutant F92A/V94A (a different mutation from the S80A or S80E) inhibited IL-6/STAT3 transcriptional activity in PAECs. Figure 4E shows that the increased mislocalization of the chaperone GRP58 in the nucleus in MCTP-treated PAEC (as in Fig. 2C) also likely contributes to reduced STAT3 transcriptional activity in that overexpression of GRP58 inhibits IL-6/STAT3 transcriptional activity. Taken together, the data in Figs. 1–4 point to significant layers of epigenetic control of STAT3 transcriptional activity in PAECs due to modulation of traf-
Fig. 6. Colocalization of STAT3 with the early endosome and lysosome compartments in HPASMCs. HPASMCs grown as in Fig. 5 were fixed, and immunofluorescence analyses carried out using anti-STAT3 pAb and MAbs against early endosome antigen 1 (EEA1) and LAMP1. Arrowheads show colocalization of STAT3 vesicles with EEA1 and LAMP1. Costes’ randomization analyses performed using NIH ImageJ showed that for each of the double-label immunofluorescence analyses, the probability that the colocalization was merely due to chance was <0.001 (\( R_{\text{org}} > R_{\text{rand}} \) in 100% of recreated images). Scale bar = 25 μm.
ficking of activated PY-STAT3 along the endolysosomal pathways.

Association of STAT3 and PY-STAT3 with caveolar and endolysosomal subcellular compartments in HPASMC. The pulmonary arterial vascular smooth muscle cell is an important contributor to the pathobiology of PAH in humans (reviewed in Refs. 42, 43). Data from double- and triple-label confocal immunofluorescence analyses of HPASMCs in culture summarized in Figs. 5–8 provide evidence for the association of STAT3 with the endolysosomal pathway in this cell type.

Fig. 7. Colocalization patterns of STAT3 and cav-1 with lysosomes in HPASMCs. HPASMCs grown in 6-well plates as in Fig. 5 were fixed and treated with MCTP for 4 days, and immunofluorescence analyses were carried out using anti-STAT3 pAb and LAMP1 MAb (A) and additionally with cav-1-FITC (B). Single arrowheads in A show colocalization between STAT3 and LAMP1. Single arrowheads in B show colocalization among all 3, STAT3, cav-1, and LAMP1, and double arrowheads in B show colocalization between STAT3 and cav-1 only in LAMP1-free vesicles. Costes’ randomization analyses showed that, for each of the double-label immunofluorescence analyses and individually for a set of 2 labels in the triple-label analyses, the probability that the colocalization was merely due to chance was <0.001 (\(R_{\text{stat}} > R_{\text{rand}}\) in 100% of recreated images). Scale bars = 25 μm.
Fig. 8. Targeting of PY-STAT3 to cytoplasmic destinations in vascular smooth muscle cells. HPASMCs cultured in 6-well plates were treated with IL-6 for 30 min or left untreated and then fixed and stained with anti-STAT3 pAb (A), anti-PY-STAT3 pAb (B), or a combination of anti-PY-STAT3 pAb and anti-LAMP1 MAb. In A and B, the boxed areas are shown in high magnification (High mag). Scale bars in A and B: low magnification (top) = 25 μm; high magnification (bottom) = 5 μm. Scale bar in C = 10 μm. Arrowheads in B show targeting of PY-STAT3 to cytoplasmic vesicles, and those in C to focal adhesions.
Cytoplasmic vesicles bearing STAT3 in HPASMCs partially colocalized with both CHC and cav-1 as well as with α-adaptin (Fig. 5), with the early endosome marker EEA1 (Fig. 6), and with the lysosome marker LAMP1 (Figs. 6 and 7A). The triple-label analyses in Fig. 7B show that, in HPASMCs, cav-1 colocalized with STAT3 in a subset of LAMP1-positive lysosomes (Fig. 7B, single arrowheads) as well as in vesicles free of LAMP1 (Fig. 7B, bottom, double arrowhead). Thus, overall, the colocalization of STAT3 with endolysosomal/cav-1 markers was partial in each instance and pointed to further complexity among STAT3-positive endosomes and their vesicular trafficking in vascular smooth muscle cells.

Figure 8 summarizes experiments aimed at investigating IL-6 effects on STAT3 and PY-STAT3 trafficking in HPASMCs. First, Fig. 8A shows that STAT3 was constitutively (i.e., in the absence of exogenously added IL-6) present in the nucleus of HPASMCs in culture. The high magnification panels in Fig. 8A show that, in addition to the nucleus, STAT3 was also targeted to vesicular structures in the cytoplasm of smooth muscle cells even in the absence of exogenously added IL-6. Figure 8B shows that STAT3 was also constitutively present both in the nucleus and in cytoplasmic vesicles. Remarkably, in these cells in culture, the exposure to IL-6 did not significantly increase the nuclear pool of PY-STAT3 but increased the cytoplasmic targeting of PY-STAT3 to elongated LAMP1-negative focal adhesions (Fig. 8C). Parenthetically, the targeting of IL-6-activated PY-STAT3 to focal adhesions has been previously reported in cancer cell lines (49). These data again highlight the complexity of the cytoplasmic trafficking of activated PY-STAT3 in PASMCs and the different cytoplasmic destinations involved.

Almost exclusive cytoplasmic provenance of PY-STAT3 in PAECs in MCT-treated rats. The data in Figs. 1–8, taken together, revealed an unexpected level of complexity in the centripetal trafficking of STAT3 and PY-STAT3 in endothelial and vascular smooth muscle cells along the endocytic pathway. Moreover, the customary expectation that all activated PY-STAT3 automatically trafficked to the cell nucleus was clearly an oversimplification. With these insights, we investigated the subcellular localization of PY-STAT3 in endothelial cells in vivo in the rat/MCT model of PAH. The data in Fig. 9, A–C, show that there was a ~5-fold increase in total PY-STAT3 on a per cell basis in PAECs in rats that had developed MCT-induced PAH (by 4 wk) compared with that in controls. This increase in PY-STAT3 was restricted to arterial luminal PAECs and did not extend to vascular smooth muscle (Fig. 9, B and C; Table 1). Unexpectedly, high magnification imaging of the luminal PAECs that had increased PY-STAT3 content showed that >75% of cells (n = 363) displayed an exclusively cytoplasmic sequestration of PY-STAT3 and that this PY-STAT3 was present in discrete, cytoplasmic vesicles (Fig. 9, D and G). The specificity of the PY-STAT3 immunostaining was confirmed in peptide competition assays using relevant and irrelevant peptides (Fig. 9, E and F). Thus, in the MCT-treated rat, the markedly increased content of PY-STAT3 in the luminal PAEC was predominantly sequestered in cytoplasmic vesicles.

Marked cytoplasmic provenance of PY-STAT3 in pulmonary arterial lesions in idiopathic PAH in humans. The cytoplasmic predominance of PY-STAT3 in PAECs in the MCT rat model led us to investigate the subcellular localization of PY-STAT3 in pulmonary arterial lesions of idiopathic PAH in humans. By quantitative immunomorphometry, there was a ~3-fold increase in PY-STAT3 content on a per cell basis in the smooth muscle actin (SMA)-positive cells in the proliferative intima and media of affected pulmonary arteries (Fig. 10, A and B, and data not shown). In PAH in humans, the major cell type with marked increases in PY-STAT3 was the SMA-positive cell, however, PY-STAT3-positive PAECs were clearly evident (Fig. 10, A–D; Table 1). As in the rat/MCT model, the majority (>75%) of the SMA-positive cells showed marked cytoplasmic sequestration of PY-STAT3 (Fig. 10, A and C; Table 1). Only minority subsets of SMA-positive cells and of PAECs had predominantly nuclear PY-STAT3 in the proliferative arterial lesions (Fig. 10D; Table 1). Examination of plexiform lesions in human PAH showed two distinct subpopulations of cells: the majority population (>59%) with markedly increased cytoplasmic PY-STAT3 and a second, smaller population with predominantly nuclear PY-STAT3 (Fig. 10, E and F; Table 1). Thus marked cytoplasmic sequestration of activated PY-STAT3 in affected cells was a common feature in both the rat MCT model and in cells in the proliferative arterial and plexiform lesions in PAH in human.

Relationship between cytoplasmic sequestration of PY-STAT3 and dysfunctional intracellular trafficking in PAH. The fact that the PY-STAT3 was sequestered in the cytoplasm of a large number of affected PAECs and PASMcs in the MCT rat model and in human PAH led us to investigate the relationship between dysfunctional intracellular trafficking and trapping of PY-STAT3 in the cytoplasm. Specifically, we asked whether cells that had increased cytoplasmic PY-STAT3 were the same cells that had dysfunctional intracellular trafficking as assayed by aberrant subcellular accumulation of vesicle tethers, SNAREs, and SNAPs. To address this question, we performed triple-labeled immunofluorescence analyses of sections from lungs of MCT-treated rats using a pAb to PY-STAT3 in combination with an MAb to either α-SNAP (a protein required for all SNARE disassembly reactions), Vti1a (a v-SNARE), or GS28 (a Golgi v-SNARE) (see Ref. 41 and citations therein). Figure 11A shows that, at the single cell level, the specific PAECs that had elevated cytoplasmic PY-STAT3 were the ones with marked increases in the accumulation of α-SNAP, Vti1a, and GS28, albeit in different vesicular structures. Thus, in the rat MCT model, the PY-STAT3-positive PAECs have dysfunctional intracellular trafficking. Similar data were obtained in colocalization analyses of PY-STAT3 with α-SNAP in the SMA-positive nonluminal cells in human PAH (Fig. 11B).

DISCUSSION

The entire cytoplasmic pool of activated PY-STAT3 has been customarily viewed as destined for import into the nucleus to mediate transcriptional regulatory functions. The most dramatic aspects of the present data are the observations that in the majority of PAECs and PASMcs in vivo the bulk of PY-STAT3 remains sequestered in cytoplasmic vesicles (Figs. 9G and 10F; Table 1). In both the rat MCT and human PAH situations, only a minority (~10%) of PAECs and PASMcs showed a nuclear localization of PY-STAT3. The in vivo observations reported here led us to reexamine our thinking about the biology of PY-STAT3. Clearly, activated STAT3 is not exclusively a nuclear transcription factor but can also be
targeted and retained in cytoplasmic destinations. Indeed, we have recently reported live cell imaging data showing the targeting of IL-6-activated STAT3-GFP to cytoplasmic sequestering endosomes colocalizing with the signaling adaptor protein myeloid differentiation factor 88 (MyD88) even in the absence of a centripetal trafficking defect (Fig. 12; Ref. 56). From our point of view, the discovery of the almost exclusive cytoplasmic provenance of PY-STAT3 in PAECs and

A

B

C

D

E

F

G

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Table 1. Cytoplasmic provenance of PY-STAT3 in PASMCs and PAECs in human and rat lung with pulmonary hypertension

<table>
<thead>
<tr>
<th>Model</th>
<th>Cell Type</th>
<th>Subcellular Localization of PY-STAT3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human PAH</td>
<td>PASMCs in pulmonary arteries</td>
<td>Cytoplasmic  76.3</td>
</tr>
<tr>
<td></td>
<td>Nuclear  9.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic + Nuclear  13.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells, n  1,192</td>
<td></td>
</tr>
<tr>
<td>Human PAH</td>
<td>Luminal PAECs in pulmonary arteries</td>
<td>Cytoplasmic  55.3</td>
</tr>
<tr>
<td></td>
<td>Nuclear  24.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic + Nuclear  19.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells, n  141</td>
<td></td>
</tr>
<tr>
<td>Human PAH</td>
<td>Plexiform lesion (factor VIII- and SMA-positive cells)</td>
<td>Cytoplasmic  59.7</td>
</tr>
<tr>
<td></td>
<td>Nuclear  32.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic + Nuclear  7.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells, n  97</td>
<td></td>
</tr>
<tr>
<td>Rat-MCT PAH</td>
<td>Luminal PAECs in pulmonary arteries</td>
<td>Cytoplasmic  78.2</td>
</tr>
<tr>
<td></td>
<td>Nuclear  1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic + Nuclear  0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells, n  363</td>
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</tr>
</tbody>
</table>

*Expressed as % of total cells characterized per specific cell type/lesion. Immunomorphometric classification of pulmonary arterial lesions based on Refs. 1 and 36. PY-STAT3, tyrosine-phosphorylated STAT3; PASMCs, pulmonary arterial smooth muscle cells; PAECs, pulmonary arterial endothelial cells; PAH, pulmonary arterial hypertension; Rat-MCT, monocrotaline-treated rats.

PASMCs in rat and human lungs represents a major departure from existing thinking in the STAT3 signaling field. [As a contrast, the reader is referred to the depiction of JAK-STAT signaling as an exclusively cytosolic process destined solely for nuclear transcription in the “Snapshot” figure presented by Mertens and Darnell in 2007 (25). The present in vivo data in the context of PAH showing the paucity of PY-STAT3 in the nucleus are in contrast to those derived from studies of cells in culture that show abundant PY-STAT3 in the nucleus (Figs. 1–8) and the marked localization of PY-STAT3 in the nucleus of cancer cells in many tumors in vivo (3). That cytoplasmic retention of PY-STAT3 was observed in the very same cells that showed increased accumulation of α-SNAP and the SNAREs Vti1a and GS28 (Fig. 11, A and B) suggests modulation of centripetal vesicular trafficking in the context of PAH.

The present data highlight the need to include considerations of the epigenetic regulation of centripetal cytokine and growth-factor signaling pathways at the level of transcytoplasmic transit of signaling molecules from the plasma membrane to the cell interior in the pathobiology of PAH (Fig. 12). Membrane-associated trafficking pathways are involved in productive transcriptional signaling in several pathways critically implicated in the pathobiology of PAH: IL-6/STAT3, BMP/Smad, TGF-β/Smad, angioptoinetin-1 (Ang1)/Tie2, and VEGF/VEGFR-1 and -2 signaling (Refs. 1, 24, 36, 37; reviewed in Refs. 42, 43; Fig. 12). We (46) and others (5) have previously shown that optimal IL-6/STAT3 signaling is dependent on a functional endosomal and caveolar system in diverse cell types. Moreover, it has been established that the TGF-β/Smad signaling pathway critically depends on the early endocytic pathway. TGF-βRs are internalized via both clathrin- and caveolin-mediated endocytic pathways (12, 32, 50). Internalization of TGF-βR via clathrin-mediated endocytosis into the endosomal compartment (21, 38). Moreover, membrane-associated centripetal trafficking also regulates the Ang1/Tie2 signaling pathway. Approximately 70% of phosphorylated Tie2 is internalized from the plasma membrane and targeted for degradation in lysosomes within 30 min of Ang1 stimulation in endothelial cells (6). The proper elucidation of the role of these signaling pathways in PAH requires our appreciation of the issue of regulated membrane-associated centripetal trafficking from the plasma membrane to the cell interior (Fig. 12).
Data derived from PAECs and PASMCs in culture revealed that STAT3 and PY-STAT3 were constitutively associated with heterogeneous components of the endolysosomal system including EEA1- and Rab5-positive early endosomes, cav-1-positive caveosomes, Rab11-positive recycling endosomes, and LAMP1-positive lysosomes. The targeting of STAT3 to lysosomes was novel in that STAT3 has not been known to traffic to this organelle. Due to the potential similarities between the TGF-βR/Smad signaling paradigm where cav-1 leads to lysosomal targeting and attenuates transcriptional
signaling and IL-6/STAT3 signaling, we investigated the role of cav-1 on the lysosomal targeting and transcriptional activity of STAT3. That cav-1 increased the lysosomal targeting of STAT3 while simultaneously reducing IL-6/STAT3-responsive luciferase reporter activity provides a mechanistic basis for understanding the reciprocal relationship between activation of STAT3-signaling and cav-1 downregulation in PAH.

Achcar et al. (1) have demonstrated that both factor VIII- and SMA-positive cells in plexiform lesions in idiopathic PAH in humans have marked decreases in the expression levels of cav-1 and cav-2. However, PAECs and PASMCs in remodeled pulmonary arteries with medial hypertrophy in the same set of patients did not show a change in cav-1 (or cav-2) expression compared with controls. Interestingly, the loss of cav-1 observed in plexiform lesions of idiopathic PAH was specific for these cells in that these authors did not find a decrease in cav-1 levels by Western blot assays of whole lung extracts. A similar decrease in cav-1 and cav-2 was also observed in a model of severe PAH in the rat caused by administration of the VEGF receptor antagonist SU-5416 followed by exposure of the animals to 3 wk of chronic hypoxia (1, 37). Although there is evidence from several groups showing the pivotal role played by the downregulation of cav-1 in pulmonary arterial lesions of idiopathic PAH in humans and in diverse experimental models (MCT, hypoxia, and SU-5416 + hypoxia), a recent report by Patel et al. (34) appears to have reached a different conclusion. The latter investigators reported that although there was a decrease in total cav-1 levels in whole lung extracts assayed by Western blotting in idiopathic PAH patients compared with controls, there was a paradoxical increase in cav-1 expression and caveolae numbers in PASMCs in vivo in this disease and in cultured PASMCs derived from such patients. However, the immunomorphometric data showing increased cav-1 expression in PASMCs in idiopathic PAH are unclear (34). Furthermore, these authors (34) misquote Achcar et al. (1) when they state that Achcar et al. observed a decrease in cav-1 expression in whole lung extract Western blot assays in idiopathic PAH (whereas Achcar et al. explicitly mention the specific loss of cav-1 in plexiform lesions only). The present data highlighting the epigenetic regulation of STAT3 transcriptional activity underline the importance of a careful cell type-specific evaluation of the relevant parameters and components in discussions of the pathobiology of PAH.

A pertinent question to ask at this point is if PAH is a disease associated with downregulation of cav-1, what is the relevance of the cav-1-induced targeting of STAT3 to lysosomes in the pathobiology of PAH? The key to answering this question is to focus not on cav-1 content at the tissue or whole cell level but in specific subcompartments within the cytoplasm. We suggest that the role of cav-1 at the level of the plasma membrane raft with loss of cav-1 leading to hyperactivation of STAT3 signaling is different from the role of cav-1 in targeting signaling molecules to the lysosomal compartment. To clarify further, first, we have shown that STAT3 interacts with cav-1 in diverse subcellular compartments including plasma membrane lipid rafts, other intracellular membrane-associated compartments like endosomes, as well as in the membrane-free cytoplasmic compartment (see Fig. 5 and Ref. 45 for details). Second, whereas cav-1 is a negative regulator of STAT3 tyrosine phosphorylation at the plasma membrane lipid raft level (see Ref. 19 and citations therein for details of how cav-1 negatively regulates signaling at the plasma membrane), the effect of the interaction of STAT3 with cav-1 at other subcellular compartments (like endosomes) is likely different and not known. Third, from work in this laboratory in the MCT model of PAH, the downregulation of cav-1 seen at the whole cell level is largely representative of a loss of cav-1 from the plasma membrane rafts/caveolae (24). Thus this loss of cav-1 relates to hyperactivation of PY-STAT3 signaling at the level of the raft/caveolae. Mechanistically, this loss of cav-1 from caveolae/rafts results from the trapping of cav-1 in the Golgi organelle (reviewed in Refs. 42, 43) from where this trapped cav-1 can aberrantly traffic to other cytoplasmic compartments. Finally, that scaffolding domain mutants of cav-1 were equally effective in inhibiting transcriptional IL-6/STAT3 signaling compared with WT cav-1 (Fig. 4) suggests that overexpression of cav-1 can inhibit PY-STAT3 function independent of events at the level of the plasma membrane caveolae/rafts. Thus the role of cav-1 in PAH needs to be understood at the level of discrete intracytoplasmic subcompartments and not merely in cell surface caveolae.

Recently, Masri and colleagues (23) reported increased PY-STAT3 in cellular elements in vascular lesions in idiopathic PAH in humans. However, the immunoperoxidase assays used to detect PY-STAT3 histologically did not have sufficient resolution to reveal the subcellular localization of PY-STAT3. These investigators also reported on the activation of PY-STAT3 in cultures of endothelial cells derived from patients with PAH. However, these investigators carried out Western blot assays for PY-STAT3 and DNA-binding assays using whole cell extracts, and thus the data obtained are not informative with respect to the subcellular localization of the PY-STAT3. Indeed, we have extensively shown that PY-STAT3 in the cytoplasm as assayed by Western blotting is competent in DNA-binding gel-shift assays (29). The present data showing that PY-STAT3 was sequestered in the cytoplasm in the majority of PAECs and PASMCs in pulmonary arterial lesions in human and experimental PAH underlines the...
significance of evaluating the subcellular localization of activated transcription factors.

The observation that, at the single cell level in vivo, the increase in cytoplasmic PY-STAT3 occurred in the same cells that also showed increased accumulation α-SNAP, Vti1a, and GS28 suggests that this cytoplasmic sequestration of PY-STAT3 may reflect a consequence of the dysfunction of vesicle tethers, SNAREs, and SNAPs in this disease. However, we do not exclude the possibility that cytoplasmically sequestered PY-STAT3 may mediate novel biological functions in the cytoplasm as such relevant to the pathobiology of PAH. One possibility is that the cytoplasmic sequestration of PY-STAT3 is a mechanism for the cell to temporally regulate the amount of STAT3 entering into the nucleus. Recently, we (56) reported that, in Hep3B cells, IL-6 induced the targeting of STAT3 and PY-STAT3 to long-lived, sequestering endosomes colocalizing with the signaling adaptor MyD88. MyD88 has primarily been thought of as an adaptor protein required for Toll-like receptor and IL-1 signaling (see our Ref. 56 and citations therein). Additional functions of MyD88 include enhanced mRNA stabilization via activation of the p38 MAPK pathway (see Ref. 56 and citations therein). We entertain the possibility that the cytoplasmic PY-STAT3 signals to MyD88-positive endosomes to regulate mRNA stability. IL-6 and other acute phase cytokines, in addition to increasing the transcription of certain target plasma protein genes (like α2-macroglobulin, complement C3, etc.), also stabilize their mRNAs in the cytoplasm (see our Ref. 56 for details). Thus the same transcription factor (activated PY-STAT3) might enhance transcription of a gene in the nucleus and also mediate enhanced stabilization of the product mRNA in the cytoplasm. In the context of PAH (9), it has recently been shown that NO increases the decay of matrix metalloproteinase-9 (MMP-9) mRNA by modulating the p38 kinase/HuR pathway (2). Thus MMP-9 mRNA represents a potential target for investigations of the biological functions of cytoplasmic PY-STAT3 in endothelial cells in the specific context of PAH.

To summarize, we show that the transcription factor STAT3 and its tyrosine-activated form, PY-STAT3, traffic through the endothelial and vascular smooth muscle cell cytoplasm in association with components of the endolysosomal pathway and that MCTP affects this centripetal trafficking in PAECs. More dramatically, we demonstrate the marked sequestration of PY-STAT3 in cytoplasmic vesicles in lung vascular cells

Fig. 11. Relationship between dysfunctional intracellular trafficking and elevated cytoplasmic PY-STAT3 in rat/MCT and human PAH. Triple-label immunofluorescence analyses were performed using lung sections of MCT-treated rats (A) or a patient with idiopathic PAH (B; same as in Fig. 10). Sections were probed with the pAb against PY-STAT3 and MAbs against α-soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP), Vti1a (a v-SNARE), and GS28 (a Golgi v-SNARE). Arrowheads point to cells with elevated PY-STAT3 and the indicated SNAP receptor (SNARE) or SNAP. Scale bars = 5 μm.

Fig. 12. Epigenetic regulation of centripetal transcytoplasmic transit of signaling by cytokines and growth-factors relevant in pulmonary hypertension, SARA, Smad anchor for receptor activation; ANG1, angiotensin-1; TGF-βR, TGF-β receptor; MVB, multivesicular bodies; HRS, hepatocyte stimulating factor receptor substrate; NF2, neurofibromatosis gene product 2.
in vivo in the rat MCT model and in human PAH. These observations are consistent with disruptions of centripetal intracellular trafficking in this disease.

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