p38 MAPK Is an Early Determinant of Promiscuous Smad2/3 Signaling in the Aortas of Fibrillin-1 (Fbn1)-null Mice*

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Excessive transforming growth factor-β (TGF-β) signaling characterizes the progression of aortic aneurysm in mouse models of Marfan syndrome, a systemic disorder of the connective tissue that is caused by mutations in the gene encoding the extracellular matrix protein fibrillin-1. Fibrillin-1 mutations are believed to promote abnormal Smad2/3 signaling by impairing the sequestration of latent TGF-β complexes into the extracellular matrix. Here we report that promiscuous Smad2/3 signaling is the cell-autonomous phenotype of primary cultures of vascular smooth muscle cells (VSMC) explanted from the thoracic aortas of Fbn1 mutant mice with either neonatal onset or progressively severe aortic aneurysm. This cellular phenotype was characterized in VSMC isolated from Fbn1-null (mgN/mgN) mice, which recapitulate the most severe form of Marfan syndrome. We found that loss of fibrillin-1 deposition promotes the production of intracellular reactive oxygen species and abnormal accumulation of phosphorylated TGF-β-activated kinase 1 and p38 MAPK, in addition to increasing the levels of endogenous phospho-Smad2. We showed that improper Smad2/3 signaling in Fbn1-null VSMC is in part stimulated by phospho-p38 MAPK, which is in turn activated in response to signals other than those mediated by the kinase activity of the ALK5 receptor. Consistent with these cell culture data, in vivo analyses documented that phospho-p38 MAPK accumulates earlier than phospho-Smad2 in the aortic wall of mgN/mgN mice and that systemic inhibition of phospho-p38 MAPK activity lowers the levels of phospho-Smad2 in this tissue. Collectively, these findings indicate that improper activation of p38 MAPK is a precursor of constitutive Smad2/3 signaling in the aortic wall of a mouse model of neonatal lethal Marfan syndrome.

Marfan syndrome (MFS) is a systemic disorder of the connective tissue with predominant manifestations in the cardiovascular, musculoskeletal, and ocular systems (1). MFS is caused by heterozygous mutations that affect the structure or decrease the expression of fibrillin-1, the major constituent of extracellular microfibrils, with an average diameter of 10 nm (1–4). Fibrillin-rich microfibrils are widely distributed architectural components of the extracellular matrix (ECM) that impart specific physical properties to connective tissues, either as obligatory constituents of elastic fibers or as elastin-free assemblies (2–4). Contrary to the earlier suggestion that MFS pathogenesis is solely accounted for by the loss of connective tissue integrity, mouse models of MFS have revealed that mutations in the fibrillin-1 gene (Fbn1) also promote promiscuous activation of latent TGF-β with adverse consequences to cellular activities (1). Relevant to aortic aneurysm, abnormal Smad2/3 signaling has been associated with disease progression in mice lacking or underexpressing fibrillin-1, which die at postnatal days 10–14 (P10–P14) (mgR/mgR mice) and at 2–6 months of age (mgR/mgR mice), respectively, and in mice heterozygous for a missense mutation in fibrillin-1, which display vascular abnormalities that do not reach a clinical end point (C1039G/+ mice) (5–7). Importantly, TGF-β antagonism has recently been shown to mitigate aneurysm progression in C1039G/+ mice and in severely affected MFS children (8, 9).

Fibrillin-1 is a large, cysteine-rich glycoprotein that can interact with cell surface receptors, such as integrins ααββ/ααββ and αβ/αβ, and other extracellular molecules, including latent TGF-β-binding proteins 1 and 4 (10–13). Interaction of fibrillin-1 with integrins is believed to modulate cell behavior and/or guide microfibril biogenesis, whereas association with latent TGF-β-binding proteins has been shown to promote the storage of latent TGF-β complexes in the ECM (1–4). Thus, it has been argued that fibrillin-1 mutations in MFS render latent TGF-β more prone to activation by destabilizing its interaction with the extracellular microfibrils (14). Activators of latent TGF-β complexes include reactive oxygen species (ROS), matrix metalloproteases, thrombospondin-1, and integrins (15–20). Once activated, TGF-β signals through serine/threo-

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3 The abbreviations used are: MFS, Marfan syndrome; ECM, extracellular matrix; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell(s); TGF-β, transforming growth factor β; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; Pn, postnatal day n. 
nine kinase receptors (TGFBR1 and ALK5) and intracellular Smad2/3, Smad4 protein complexes that translocate into the nucleus and bind specific DNA elements in cooperation with other transcription factors (21). TGF-β can also signal through MAPK-dependent pathways, and molecules that transduce MAPK signals can directly or indirectly influence Smad2/3 activity (22).

The present study was designed to identify other molecular determinants of aneurysm progression in MFS by examining primary vascular smooth muscle cell (VSMC) cultures explanted from the thoracic aortas of MFS-like mice. Our results demonstrate that Smad2/3 signaling is constitutively active in VSMC cultures from mgN/mgN, mgR/mgR, and C1039/+ mice. We also present in vitro and in vivo evidence indicating that p38 MAPK is an early contributor to promiscuous Smad2/3 signaling during aneurysm progression in Fbn1-null mice. These findings extend our understanding of the molecular events associated with aneurysm progression in MFS.

**EXPERIMENTAL PROCEDURES**

*Primary VSMC Cultures*—Primary VSMC strains were established after isolating the cells from the thoracic aortas of 10-day-old mgN/mgN mice, 5-month-old mgR/mgR mice, 9-month-old C1039G/+ mice, and the respective wild-type littermates (23, 24). Aneurysm progression in mgR/mgR and C1039G/+ mice was monitored by echocardiography prior to the isolation of VSMC (8). Primary VSMC were expanded on 100-mm Primaria Plates (BD Biosciences) in 1× Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT) and supplemented with streptomycin, penicillin, and fungizone; cell aliquots were frozen and stored in liquid nitrogen for subsequent use. VSMC purity (evaluated by immunostaining with anti-SMA anti-serum and stored in liquid nitrogen for subsequent use. VSMC cultures were treated with 50 μM dichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, OR) and 5.5 mM glucose. This was followed by an additional 10-min incubation in Dulbecco’s modified Eagle’s medium containing 10% FBS, fixation in 4% paraformaldehyde, and viewing with a Zeiss LSM 410 confocal microscope (Carl Zeiss SMT Inc., Peabody, MA) at λ = 488 nm. The intensity of the fluorescent signal was measured in several randomly chosen fields (each containing a minimum of 30 cells) with the aid of the MetaMorph version 6.3 software (Molecular Devices, Sunnyvale, CA), and it was expressed as the average combined value of all fields. Statistical analyses were performed using Student’s *t* test, assuming a *p* value of ≤0.05 as significant.

*Detection of ROS*—Levels of intracellular ROS were assessed using a modification of the published protocols (28, 29). Briefly, subconfluent VSMC cultures were washed with phosphate-buffered saline at 37 °C and incubated for 10 min in the dark in a phosphate-buffered saline-based buffer containing 10 μM 2,7-dichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, OR) and 5.5 mM glucose. This was followed by an additional 10-min incubation in Dulbecco’s modified Eagle’s medium containing 10% FBS, fixation in 4% paraformaldehyde, and viewing with a Zeiss LSM 410 confocal microscope (Carl Zeiss SMT Inc., Peabody, MA) at λ = 488 nm. The intensity of the fluorescent signal was measured in several randomly chosen fields (each containing a minimum of 30 cells) with the aid of the MetaMorph version 6.3 software (Molecular Devices, Sunnyvale, CA), and it was expressed as the average combined value of all fields. Statistical analyses were performed using Student’s *t* test, assuming a *p* value of ≤0.05 as significant.

*Immunoblots, Immunohistochemistry, and in Vitro Kinase Assay*—VSMC were cultured for 4 days in 10% FBS and for 2 additional days in 2% FBS. In some experiments, the culture medium included MAPK or ALK5 inhibitors at the aforementioned concentrations or 1 μM diphenylene iodonium (Calbiochem-EMD Biosciences). Cell layers were scraped into ice-cold Tris-buffered saline solution (pH 7.4) and flash frozen in liquid nitrogen. Cell and tissue extracts were prepared and assayed for total protein content using the BCA kit (Pierce) (7). Protein extracts (10–25 μg/lane) were fractionated by 10% (w/v) SDS-PAGE and electroblotted onto an Immobilon-P membrane (Millipore, Billerica, MA). Membranes were incubated first with antibodies against phosphorylated TGF-β-activated kinase 1 (phospho-TAK1 (TGF-β-activated kinase 1)), phospho-Smad2, phospho-p38 MAPK, or phospho-ATF2 (1:1000 dilution; Cell Signaling Technology, Danvers, MA) and subsequently with biotin-labeled anti-rabbit IgG antibody (1:25,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) and horseradish peroxidase-conjugated Streptavidin (Millipore). Immunoreactive products were visualized by chemiluminescence using the ECL Plus kit (Amershams Biosciences), and their relative intensity was evaluated with the aid of Adobe Photoshop software (Adobe Systems Inc., San Jose, CA). To assay p38 MAPK activity *in vitro*, cell lysates from wild-type and mutant VSMC cultures were immunoprecipitated overnight at 4 °C with an immobilized anti-phospho-p38 MAPK monoclonal antibody (Thr-180/Tyr-182; Cell Signaling Technology). Immunoprecipitates were subsequently incubated for 30 min at 30 °C with 1 μg of recombinant ATF2 and 200 μM ATP as per the manufacturer’s protocol. Products of the reaction were resolved by SDS-PAGE and immunoblotted with an antibody against phospho-ATF2 (Thr-71). Positive bands were visualized and quantified as described above. Statistical analyses in all
FIGURE 1. Smad2/3 signaling is abnormally high in Fbn1 mutant VSMC cultures. A, representative phospho-Smad2 immunoblots of protein extracts from VSMC cultures of the indicated genotypes (n = 3) with the bar graphs below summarizing the results of control (black) and experimental samples (white) normalized against β-tubulin levels. B, transcriptional activity of the p3TP-Lux reporter plasmid transiently transfected into VSPCM of the indicated genotype (n = 3) without or together the DN-Smad3 expression plasmid. Black and white bar graphs summarize the results of three independent tests performed in duplicate for each of the experimental and control samples, respectively. In both panels, wild-type values are arbitrarily expressed as 1 unit, and asterisks indicate values statistically different from controls (Student’s t test, p ≤ 0.05).

FIGURE 2. Multiple signaling molecules are activated in Fbn1-null VSMC. A, transcriptional activity of p3TP-Lux transfected into wild-type (black) or mgN/mgN (white) VSMC (n = 3) cultured with or without the indicated chemical inhibitors. The bar graphs summarize the results of three independent tests performed in duplicate for each of the experimental and control samples. B, immunodetection of phospho-Smad2 accumulation (top) and confocal microscopy of ROS production (bottom) in wild-type VSMC (WT) and 1106C cultures (Mt). In the latter sample, the intensity of the fluorescence signals in control and experimental samples were estimated to be 2.068 ± 0.322 and 2.95 ± 0.32 (respectively (p = 0.0098). C, representative immunoblot of phospho-TAK1 (arrowhead) in wild-type and 1106C cells. D, representative immunoblot of phospho-p38 MAPK (phospho-p38) in wild-type and 1106C cells. E, p38 MAPK-directed phosphorylation of ATF2 (pATF2) in vitro using protein extracts from wild-type and 1106 cells. The bar graphs below the immunoblots of C and D summarize the results of experimental (white) and control samples (black) normalized against β-actin levels and recombinant ATF2 input, respectively. The faster migrating band highlighted in the TAK1 immunoblot corresponds to the TAK1c/d isoforms (39). Experiments involving 1106C cells were each performed at least three different times. In relevant panels, wild-type values are arbitrarily expressed as 1 unit, and asterisks indicate values statistically different from controls (Student’s t test, p ≤ 0.05).

RESULTS

Smad2/3 Signaling Is Constitutively Active in Fbn1 Mutant VSMC Cultures—Previous studies have associated the emergence of TGF-β-driven unproductive remodeling of the aortic wall with the progression of vascular disease in mice that harbor different Fbn1 mutations and display discrete phenotypic outcomes (5–7). Here we evaluated TGF-β signaling (as evidenced by the accumulation of endogenous phospho-Smad2 and the transcription of TGF-β-responsive plasmids) in primary VSMC explanted from the thoracic aortas of these same of these experiments were performed using the Student’s t test, assuming a p value of ≤ 0.05 as significant. Tissue samples for immunohistochemistry were isolated from the thoracic aortas of wild-type and mgN/mgN mice, processed as previously described (7), and viewed with a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Yokohama, Japan).

Experimental Animals—FR167653 (kindly provided by Astellas Pharma Inc., Tokyo, JP) was dissolved in saline solution containing 1% carboxymethylcellulose and administered to mgN/mgN mice and wild-type littermates (n = 6/genotype) by daily subcutaneous injections (from P1 to P10) at the dose of 32 mg/kg (30). An equivalent number of control mice was only treated with vehicle. Mice were sacrificed at P10 by carbon dioxide inhalation, and the thoracic aorta was transsected, placed immediately in ice-cold Tris-buffered saline solution (pH 7.4), cleaned-up, and then flash frozen and stored in liquid nitrogen to be subsequently processed for protein analyses as described above. In parallel experiments, thoracic aortas from untreated wild-type and mgN/mgN mice sacrificed at P4 and P10 were similarly processed for protein analyses. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine.
Smad3, a dominant-negative Smad3 protein that lacks the MH2 domain of Smad3, was designed to provide an in vitro system to investigate abnormal signaling events associated with the deposition of a microfibril-deficient matrix.

Several Signaling Molecules Are Abnormally Activated in Fbn1-null VSMC—Cross-talk between TGF-β and MAPK-dependent pathways modulates cellular responses to specific stimuli (22). In order to verify whether MAPK-transduced signals contribute to elevated Smad2/3 activity in Fbn1-null VSMC, we compared luciferase expression of TGF-β-responsive plasmid (CAGA)12MLP-Luc and phospho-p38 MAPK (p38 MAPK) levels in mutant VSMC cultures. We found that 1106C cultures display increased nuclear localization of phospho-Smad2 as well as abnormally high levels of ROS, phospho-TAK1, and phospho-p38 MAPK (Fig. 2, A–D). Heightened p38 MAPK activity in Fbn1-null VSMC was independently confirmed by an in vitro assay that measured phosphorylation of the downstream effector ATF2 (Fig. 2E) (35). These observations therefore demonstrated that multiple signaling molecules are abnormally activated in primary VSMC cultures lacking fibrillin-1 microfibrils.

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p38 MAPK Stimulation Decreases Smad2/3 Signaling in Fbn1-null Aortas—Next, chemical inhibitors were employed to assess the potential contribution of TGF-β-elicited signals to p38 MAPK stimulation and, implicitly, to phospho-p38 MAPK-driven Smad2/3 activation in 1106C cultures. In contrast to the ~35% decrease of phospho-p38 MAPK levels in mutant VSMC treated with the NADPH-oxidase inhibitor diphenylene iodonium (Fig. 3A), we found comparable amounts of phospho-p38 MAPK in 1106C cells cultured with or without the ALK5 kinase inhibitor SB431542 (Fig. 3B). Together, these results excluded a significant role of Smad-mediated signals in p38 MAPK activation. Surprisingly, the analyses also revealed that the p38 MAPK inhibitor SB203580 lowers the levels of endogenous phospho-Smad2 by ~30% (Fig. 3C), thus significantly less than the activity of the TGF-β-responsive plasmids (Fig. 2A). We believe that the discrepancy is likely to reflect experimental differences in the two assays and indirectly support this contention by noting that a different p38 MAPK inhibitor (FR167653) lowers phospho-Smad2 levels to the same extent as the SB203580 inhibitor both in vitro (data not shown) and in vivo (see below). Along the same lines, a ~30% reduction in phospho-Smad2 levels was also observed in Fbn1-null cells in which p38 MAPK production was nearly abrogated by RNA interference (Fig. 3D).

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p38 MAPK Inhibition Decreases Smad2/3 Signaling in the Aortas of mgN/mgN Mice—The last set of experiments was designed to provide an in vivo validation of p38 MAPK role in promiscuous Smad2/3 signaling. Accordingly, phospho-p38
MAPK levels were first shown to be abnormally elevated in the thoracic aortas of mgN/mgN mice (Fig. 4A). Next, p38 MAPK activity (as evidenced by the accumulation of the downstream effector phospho-ATF2 (35) and phospho-Smad2 levels were compared in the thoracic aortas of mgN/mgN mice and wild-type littermates, which had been systemically treated with the phospho-p38 MAPK inhibitor FR167653 from birth until P10 (30). The results of these in vitro inhibitions (summarized as relative protein ratios of experimental over control samples) demonstrated that treatment with FR167653 reduces the abnormal accumulation of phospho-Smad2 in the Fbn1-null aortas by ~30% (Fig. 4B). Last, Western blot experiments revealed a statistically significant increase of phospho-p38 MAPK in the thoracic aortas of untreated mgN/mgN mice earlier (P4) than phospho-Smad2 (P10) (Fig. 4C). Together, these in vivo data indicated that improper Smad2 stimulation in the aortic wall of mgN/mgN mice is temporally and causally related to p38 MAPK activation.

**DISCUSSION**

*In vitro* and *in vivo* experiments were performed to delineate the signaling profile of aneurysm progression in Fbn1 mutant mice. These analyses have yielded two major new findings. First, they have documented that constitutive Smad2/3 signaling is maintained *in vitro* by VSMC explanted from the aortas of mice with different Fbn1 mutations, phenotypic manifestations, and clinical outcomes. Second, they have demonstrated that abnormal p38 MAPK activation is an early contributor to Smad2/3 stimulation in the aortic wall of Fbn1-null mice. Together, these observations extend our understanding of the molecular events associated with aortic disease in MFS.

Previous studies of mouse models of MFS have demonstrated that connective tissue integrity and homeostasis depend on the threshold level of functionally competent microfibrils, which when it is breached by primary or secondary fibrillin-1 deficiencies triggers a destructive cascade involving unproductive tissue repair driven by dysregulated TGF-β activity (5–8, 14). Here we showed that the TGF-β-activated phenotype of aortas with structural, hypomorphic, or null mutations of Fbn1 is replicated *in vitro* by the corresponding VSMC. Indeed, DNA microarray analyses have identified TGF-β as the only dysregulated pathway in common among these three mutant aortas.4 These findings thus support the notion that constitutive activation of latent TGF-β is the common outcome of mutations that affect either the structure or the expression of fibrillin-1. Relevant to latent TGF-β activation, preliminary evidence suggests that one or more matrix metalloproteases are upregulated in Fbn1-null VSMC. *In vitro* retention of the TGF-β-activated phenotype may in principle reflect a chronic response by the cultured cells to a structurally abnormal ECM, earlier developmental events that have altered cell fate, or a combination of both mechanisms. Ongoing studies are addressing these possibilities in VSMC from different Fbn1 mutant mice as well as in cells derived from other mutant tissues.

The mgN/mN mice represent an informative model in which to investigate early events of dysregulated TGF-β activity in the diseased aorta (7). Although mgN/mgN aortas express molecular markers of TGF-β-driven tissue repair (7), loss of fibrillin-1 deposition leads to the extremely rapid physical collapse of the aortic wall without participation of

4 L. Carta, unpublished results.
the destructive cascade that characterizes the progressively severe phenotype of mgR/mgR and C1039G/+ mice (5, 6). As such, early demise of mgN/mgN mice eliminates the problem of phenotypic heterogeneity noted in the other two MFS-like mice as they age. The downside of the Fbn1-null model is that potentially beneficial interventions can only be evaluated as changes in early molecular markers of disease progression rather than in improved aortic tissue architecture. With this limitation in mind, the study of cultured VSMC from mgN/mgN mice has revealed that loss of fibrillin-1 deposition elicits improper activation of several signaling molecules. We have examined the functional relationship between p38 MAPK and Smad2/3 because of the central role that the former molecule plays in multiple stress response pathways and in TGF-β signaling as well (31–33). Experiments using chemical inhibitors have documented the contribution of phospho-p38 MAPK to the transcription of TGF-β-responsive plasmin and to the accumulation of endogenous phospho-Smad2 in Fbn1-null VSMC. They have also demonstrated the ability of ROS but not ALK5 kinase to promote phospho-p38 MAPK accumulation. The latter finding is consistent with recent reports demonstrating that TGF-β activates c-Jun N-terminal kinase and p38 MAPK in an ALK5 kinase-independent manner (32, 33). It is also formally possible that activation of p38 MAPK in Fbn1-null VSMC cultures may be part of the stress response signals induced by impaired cell-cell interactions. Irrespective of the underlying mechanism, our in vivo data indicate that phospho-p38 MAPK accumulates in the thoracic aortas of mgN/mgN mice before they progress to end-stage vascular disease, when heightened levels of phospho-Smad2 are first noticeable (7). Thus, we propose that loss of fibrillin-1 in mgN/mgN aortas promotes TGF-β- and/or ECM-mediated stimulation of p38 MAPK, and this in turn augments Smad2/3 signaling and perhaps induces TGF-β activators. Early activation of p38 MAPK and conceivably other MAPKs may also cooperate with Smad signaling in executing specific cellular programs that further contribute to aneurysm progression.

The discovery that TGF-β plays a central role in MFS pathogenesis has provided the rationale to explore therapeutic strategies against free TGF-β latent complex activators and/or Smad2/3 modulators (1). Although its precise mechanism of action is unknown, losartan has already proven to be an effective means to counteract TGF-β-driven manifestations in C1039G/+ mice and MFS children (8, 9). Consistent with earlier observations (5, 36), the matrix metalloproteinase inhibitor doxycycline has also been shown to improve aortic wall architecture in C1039G/+ mice and delay aneurysm rupture in mgR/mgR mice (37, 38). The demonstration that p38 MAPK is implicated in constitutive activation of Smad2/3 signaling in the aortas of mgN/mgN mice points to another potential opportunity for therapy in MFS. Validation of this possibility, however, requires additional studies to establish whether p38 MAPK is also involved in the early stage of vascular disease in C1039G/+ and mgR/mgR mice or whether MAPKs are differentially activated in various mouse models of MFS.

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REFERENCES

1. Ramirez, F., and Dietz, H. C. (2007) Curr. Opin. Genet. Dev. 17, 252–258
2. Kidlty, C. M., Sheratt, M. J., and Shuttleworth, C. A. (2002) J. Cell Sci. 115, 2817–2828
3. Ramirez, F., Sakai, L. Y., Dietz, H. C., and Rifkin, D. B. (2004) Physiol. Genomics 19, 151–154
4. Hubmacher, D., Tiedemann, K., and Reinhardt, D. P. (2006) Curr. Top. Dev. Biol. 75, 93–123
5. Pereira, L., Lee, S. Y., Gayraud, B., Andrìkopoulos, K., Shapiro, S. D., Bunton, T., Biery, N. J., Dietz, H. C., Sakai, L. Y., and Ramirez, F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3819–3823
6. Judge, D., Biery, N. J., Keene, D. R., Greub, J., Myers, L., Hsu, D. L., Sakai, L. Y., and Dietz, H. C. (2004) J. Clin. Invest. 114, 172–181
7. Carta, L., Pereira, L., Arteaga-Solis, E., Lee-Arteaga, S. Y., Lenart, B., Starcher, B., Merked, C. A., Sukoyan, M., Kerks, A., Hazeki, N., Keene, D. R., Sakai, L. Y., and Ramirez, F. (2006) J. Biol. Chem. 281, 8016–8023
8. Habashi, J. P., Judge, D. P., Holm, T. M., Cohn, R. D., Loey, B. L., Cooper, T. K., Myers, L., Klein, E. C., Liu, G., Calvi, C., Podowski, M., Neptune, E. R., Halushka, M. K., Bedja, D., Gabrielsson, K., Rifkin, D. B., Carta, L., Ramirez, F., Hsu, D. L., and Dietz, H. C. (2006) Science 312, 117–121
9. Brooke, B. S., Habashi, J. P., Judge, D. P., Patel, N., Loey, B., and Dietz, H. C. (2008) N. Engl. J. Med. 358, 2787–2795
10. Sakamoto, H., Broekelmann, T., Cheres, D. A., Ramirez, F., Rosenbloom, I., and Mechem, R. P. (1996) J. Biol. Chem. 271, 4916–4922
11. Bax, D. V., Bernard, S. E., Lomas, A., Morgan, A., Humphries, J., Shuttleworth, C. A., Humphries, M. J., and Kidlty, C. M. (2003) J. Biol. Chem. 282, 36405–36416
12. Jovanovic, J., Takagi, J., Choulier, L., Abrescia, N. G., Sturt, D. L., van der Merwe, P. A., Mardon, H. J., and Handford, P. A. (2007) J. Biol. Chem. 282, 6743–6751
13. Isogai, Z., Ono, R. N., Ushiro, S., Keene, D. R., Chen, Y., Mazzieri, R., Charbonneau, N. L., Reinhardt, D. P., Rifkin, D. B., and Sakai, L. Y. (2003) J. Biol. Chem. 278, 2750–2757
14. Neptune, E. R., Frischmeyer, P. A., Arking, D. E., Myers, L., Bunton, T. E., Gayraud, B., Ramirez, F., Sakai, L. Y., and Dietz, H. C. (2003) Nat. Genet. 33, 407–411
15. Rifkin, D. B. (2005) J. Biol. Chem. 280, 7409–7412
16. ten Dijke, P., and Arthur, H. M. (2007) Nat. Rev. Mol. Cell. Biol. 8, 857–869
17. Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P., and Bouck, N. (1998) Cell 93, 1159–1170
18. Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., Pettet, J. F., Kaminski, N., Garat, C., Matthey, M. A., Rifkin, D. B., and Sheppard, D. (1999) Cell 96, 319–328
19. Wipff, P. J., Rifkin, D. B., Meister, J. J., and Hinz, B. J. (2007) Cell Biol. 179, 1311–1323
20. Jenkins, G. (2008) Int. J. Biochem. Cell Biol. 40, 1068–1078
21. Shi, Y., and Massague, J. (2004) Cell 113, 685–700
22. Moustakas, A., and Heldin, C. H. (2005) J. Cell Sci. 118, 3573–3584
23. Travo, P., Barrett, G., and Burnstock, G. (1980) Blood Vessels 17, 110–116
24. Sprague, E. A., Kelley, J. L., and Schwartz, C. J. (1983) Exp. Mol. Pathol. 37, 48–66
25. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massague, J. (1992) Cell 71, 1003–1014
26. Dennler, S., Itoh, S., Siviven, D., ten Dijke, P., Huet, S., and Gauthier, I. M. (1998) EMBO J. 17, 3091–3100
27. Zhang, W., Ou, J., Inagaki, Y., Greenwel, P., and Rifkin, F. (2000) J. Biol. Chem. 275, 39237–39245
28. Bass, D. A., Parce, J. W., Dechateau, L. R., Szedja, P., Seeds, M. C., and Thomas, M. (1983) J. Immunol. 130, 1910–1917
29. Ohba, M., Shibamuna, M., Kuroki, T., and Nose, K. J. (1994) Cell Biol. 126, 5635
30. Ohashi, N., Matsumori, A., Furukawa, Y., Ono, K., Okada, M., Iwasaki, A., Miyamoto, T., Nakano, A., and Sasayama, S. (2000) Artherioscler. Thromb. Vasc. Biol. 20, 166–172
31. Winter-Vann, A. M., and Johnson, G. L. (2007) J. Cell Biochem. 102, 848–858
32. Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow, V., Schuster, N., Zhang, S., Heldin, C. H., and Landström, M. (2008) Nat. Cell Biol. 10, 1199–1207
33. Yamashita, M., Faytol, K., Jin, C., Wang, X., Liu, Z., and Zhang, Y. E. (2008) Mol. Cell 31, 918–924
34. Koli, K., Myllarniemi, M., Keski-Oja, J., and Kinnula, V. L. (2008) Antioxid. Redox Signal. 10, 333–342
35. Morton, S., Davis, R. J., and Cohen, P. (2004) FEBS Lett. 13, 177–183
36. Bunton, T. E., Biery, N. J., Myers, L., Gayraud, B., Ramirez, F., and Dietz, H. C. (2001) Circ. Res. 88, 37–42
37. Xiong, W., Knispel, R. A., Dietz, H. C., Ramirez, F., and Baxter, B. T. (2008) J. Vasc. Surg. 47, 166–172
38. Chung, A. W., Yang, H. H. C., Radomski, M. W., and van Breemen, C. (2008) Circ. Res. 102, e73–e85
39. Watkins, S. J., Jonker, L., and Arthur, H. M. (2006) Cardiovasc. Res. 69, 432–439