Shear Stress Increases Expression of the Arterial Endothelial Marker EphrinB2 in Murine ES Cells via the VEGF-Notch Signaling Pathways

Tomomi Masumura, Kimiko Yamamoto, Nobutaka Shimizu, Syotaro Obi, Joji Ando

Objective—Arterial-venous specification in the embryo has been assumed to depend on the influence of fluid mechanical forces, but its cellular and molecular mechanisms are still poorly understood. Our previous in vitro study revealed that fluid shear stress induces endothelial cell (EC) differentiation by murine embryonic stem (ES) cells. In the present study we investigated whether shear stress regulates the arterial-venous specification of ES-cell-derived ECs.

Methods and Results—When murine ES cell–derived VEGFR2$^+$ ES cells were exposed to shear stress, expression of the arterial EC marker protein ephrinB2 increased dose-dependently. The ephrinB2 mRNA levels also increased in response to shear stress, whereas the mRNA levels of the venous EC marker EphB4 decreased. Notch cleavage and translocation of the Notch intracellular domain (NICD) into the nucleus occurred as early as 30 minutes after the start of shear stress and increased with time. Gamma-Secretase inhibitors (DAPT and L685 458), and the recombinant extracellular domain of the Notch ligand DLL4 abolished the shear stress–induced NICD translocation, and that, in turn, blocked the shear stress–induced upregulation of ephrinB2 expression. In addition, the VEGF receptor kinase inhibitor SU1498 was found to suppress both the shear-stress-induced Notch cleavage and up-regulation of ephrinB2 expression.

Conclusion—Exposure to shear stress induces an increase in expression of ephrinB2 in murine ES cells via VEGF-Notch signaling pathways. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: embryonic stem cells ■ arterio-venous differentiation ■ endothelial cells ■ shear stress ■ vascular biology

Whether a blood vessel is called an artery or a vein depends on the direction of blood flow in the vessel in relation to the heart, and there are structural and functional differences between them. Recent studies have shown that arteries and veins can also be distinguished on the basis of the presence or absence of several molecules that are specifically expressed by the endothelial cells (ECs) of arteries or veins alone. For example, ephrinB2, an Eph family transmembrane ligand, marks arterial ECs, whereas EphB4, a receptor for ephrinB2, marks venous ECs. The development of arteries and veins in the embryo has long been assumed to depend largely on the influence of physiological conditions in the environment, such as oxygenation, blood pressure, and shear stress. However, recent evidence suggests that the identity of ECs that line arteries and veins is established by genetic mechanisms before the onset of blood circulation. There is also evidence that despite expressing arterial or venous markers ECs remain plastic in regard to arterial-venous differentiation until late in embryonic development. The cellular and molecular mechanisms governing the complex processes of arterial–venous differentiation are still poorly understood.

Notch signaling is well known to play a fundamental role in determining the fate of a variety of cell types. Four Notch receptors (Notch1–4) and 5 Notch ligands (Jagged1–2, and Delta-like1, –3 and –4) have been identified in mice and humans. When Notch is activated by ligands expressed on adjacent cells, the intracellular domain of the Notch receptor (NICD) is cleaved by γ-secretase and translocates into the nucleus, interacting with the DNA-binding factor CSL (also known as RBP-Jk and CBF1), resulting in trans-activation of various gene promoters, such as those of the HES and HEY families. A variety of evidence suggests that Notch has an important role during embryonic vascular development and functions in arterial-venous differentiation. During development of the zebrafish dorsal aorta, loss of Notch or the downstream Notch-target gene gridlock, a homologue of mammalian HES, leads to failure to express the arterial cell marker ephrinB2 and an increase in expression of the venous marker EphB4. Conversely, constitutive
activation of Notch suppresses expression of the venous cell marker Flt4. Thus, it seems that Notch signaling promotes development of arteries and inhibits development of veins in the embryo. Sonic hedgehog (SHH) and vascular endothelial growth factor (VEGF) are known to act upstream of the Notch pathway, and a SHH-VEGF-Notch-ephrinB2 cascade is assumed to regulate arterial-venous specification.

It is generally known that many types of cells respond to mechanical forces in their environment. For example, vascular ECs respond to shear stress generated by flowing blood by undergoing changes in cell function and gene expression that play an important role in maintaining vascular homeostasis. It has recently become apparent that mechanical forces affect organogenesis in the developing embryo. For example, blocking blood flow, and thereby reducing shear stress, has been found to result in heart defects in zebrafish, and nodal flow influencing blood flow, and thereby reducing shear stress, has been shown to regulate arterial-venous specification.

It has recently become apparent that mechanical forces affect organogenesis in the developing embryo. For example, blocking blood flow, and thereby reducing shear stress, has been found to result in heart defects in zebrafish, and nodal flow influencing blood flow, and thereby reducing shear stress, has been shown to regulate arterial-venous specification.

Shear Stress Experiments
Cells were exposed to laminar shear stress with a parallel plate-type device, as described previously. The intensity of the wall shear stress (τ, dynes/cm²) on the cell layer was calculated by the formula $\tau = 6\pi \eta Q/a b$, where $\eta$ is the viscosity of the perfusate (poise), $Q$ is the flow volume (ml/s), and $a$ and $b$ are the cross-sectional dimensions of the flow path. The shear stress used in this study ranged from 1.5 dynes/cm² to 20.0 dynes/cm²; all experiments were performed at 37°C in a CO₂ incubator.

Real-Time PCR Analysis
Total RNA samples were prepared from cells with ISOGEN (Nippon Gene, Tokyo, Japan), and first-strand cDNAs were generated by using Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Roshe) and RNA primed with oligo dT-primer. After reverse transcription of the RNA into cDNA, real-time PCR with a Smart Cycler (Cepheid) was used to monitor gene expression according to the standard procedure. PCR was performed with a TaKaRa EX Taq R-PCR kit (TaKara), as described previously, and the primer pairs shown in Table.

Immunohistochemistry
Culture cells were immunostained as described previously. The cleaved intracellular domain of Notch (NICD) was stained by using cleaved Notch1 antibody (Cell Signaling Technology) as the primary antibody and the TSA Biotin System (PerkinElmer). NICD was visualized by using Alexa Fluor 488-conjugated streptavidin (Invitrogen). Stained cells were photographed through a confocal laser microscope (SP2, Leica).

Western Blot Analysis
Western blot analysis was performed as described previously. Briefly, cells were dissolved in RIPA buffer and centrifuged at 26,000g for 30 minutes. The cell lysate was separated by SDS-polyacrylamide gel electrophoresis and then transferred onto an Immobilon membrane (Millipore). The membrane was incubated with antibodies against ephrinB2 (Santa Cruz), CD31 (Pharmingen), Notch1 (BD Bioscience), Notch4 (Millipore), cleaved Notch1 (Cell Signaling Technology), DLL4 (R&D systems), Jagged-1 (BD Bio-

Table. Oligonucleotide Primers Used for Gene Expression Analysis by Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primers, 5’-3’</th>
<th>Reverse Primers, 5’-3’</th>
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<tbody>
<tr>
<td>Ephrin B2</td>
<td>GACCTCCGGAAGCTTGGACCATC</td>
<td>AACAGCCAATCCATACAGGCAGGTA</td>
</tr>
<tr>
<td>Eph B4</td>
<td>CGTTGATGACCTATACCTGTTGAGG</td>
<td>GAGTACCTCACTTCCCTCATGTTCT</td>
</tr>
<tr>
<td>Notch 1</td>
<td>GACACACGGCCAGACCTGTA</td>
<td>ACTTCGACATGCTGAGCAGC</td>
</tr>
<tr>
<td>Notch 4</td>
<td>ACGGACTACACCTTGGTGCT</td>
<td>GGGAATTTTATCCCCCTCCA</td>
</tr>
<tr>
<td>DLL4</td>
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<td>TCCCTACAGGATGCGATGTA</td>
</tr>
<tr>
<td>Jagged 1</td>
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<td>GCTGTGGTTGCTGAGGGAAG</td>
</tr>
<tr>
<td>Jagged 2</td>
<td>GATGCGACGGGCTGAGT</td>
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</tr>
<tr>
<td>β-actin</td>
<td>GTGCACACAGGCGATGATG</td>
<td>GCAATGCGTGAGCTGATG</td>
</tr>
</tbody>
</table>

Materials and Methods

Cell Culture
MGZ5 ES cells (a gift from H. Niwa of Riken, CDB, Kobe, Japan) were initially maintained undifferentiated without a feeder layer on gelatin-coated tissue culture dishes in DMEM (IBL) containing 15% FBS (JRH), 10 U/mL LIF (ESGRO; Chemicon), 1× nonessential amino acid (ICN), and 5 × 10⁻⁵ mol/L β-mercaptoethanol (Sigma), and subsequently maintained, differentiated, cultured, and sorted as described previously. To initiate ES cell differentiation, trypsinized cells were plated on type-IV collagen-coated Petri dishes and cultured without LIF in α MEM (Gibco) containing 10% FBS, 50 U/50 μg/mL penicillin-streptomycin (ICN), and 5 × 10⁻⁵ mol/L β-mercaptoethanol. On day 4, VEGFR2 cells were known as common vascular progenitors were isolated by using an anti-mouse VEGFR2 antibody (Clone Avas 12a1; Pharmingen) and standard immunomagnetic techniques (MACS; Miltenyi Biotech), and then plated in differentiation medium on type-IV collagen-coated coverslips. After culture for 3 days, flow-loading experiments were performed.

Western Blot Analysis
Western blot analysis was performed as described previously. Briefly, cells were dissolved in RIPA buffer and centrifuged at 26,000g for 30 minutes. The cell lysate was separated by SDS-polyacrylamide gel electrophoresis and then transferred onto an Immobilon membrane (Millipore). The membrane was incubated with antibodies against ephrinB2 (Santa Cruz), CD31 (Pharmingen), Notch1 (BD Bioscience), Notch4 (Millipore), cleaved Notch1 (Cell Signaling Technology), DLL4 (R&D systems), Jagged-1 (BD Bio-

Materials and Methods

Cell Culture
MGZ5 ES cells (a gift from H. Niwa of Riken, CDB, Kobe, Japan) were initially maintained undifferentiated without a feeder layer on gelatin-coated tissue culture dishes in DMEM (IBL) containing 15%
increase in number of ephrinB2 expression seen in all cells is the result of both an
independent and CD31 cells. EphrinB2 expression increased dose-
dependently in response to shear stress. B, Changes in ephrinB2 expression in all cells and in EC marker CD31 cells. CD31 cells were obtained by using anti-CD31 antibody (Pharmingen) and immunomagnetic techniques. Shear stress (10 dynes/cm²) significantly increased ephrinB2 expression in all cells and in CD31 cells, indicating that the increase in ephrinB2 expression seen in all cells is the result of both an increase in number of ephrinB2/CD31 cells and an increase in ephrinB2 expression in individual CD31 cells. Data from 5 separate samples are expressed as means ± SD. *P < 0.01 vs the static control.

Figure 1. Effect of shear stress on expression of the arterial EC marker ephrinB2. Expression of ephrinB2 protein in cells cultured under static conditions (Static) and in cells exposed to shear stress for 48 hours (Shear) was assessed by Western blotting. Beta-actin was used as a protein loading control. A, Changes in ephrinB2 expression in the ES cell–derived VEGFR2+ cells. EphrinB2 expression increased dose-dependently in response to shear stress. B, Changes in ephrinB2 expression in all cells and in EC marker CD31 cells. CD31 cells were obtained by using anti-CD31 antibody (Pharmingen) and immunomagnetic techniques. Shear stress (10 dynes/cm²) significantly increased ephrinB2 expression in all cells and in CD31 cells, indicating that the increase in ephrinB2 expression seen in all cells is the result of both an increase in number of ephrinB2/CD31 cells and an increase in ephrinB2 expression in individual CD31 cells. Data from 5 separate samples are expressed as means ± SD. *P < 0.01 vs the static control.

Statistical Analysis

All results are expressed as the mean ± SD. Statistical significance was evaluated by an ANOVA and a Bonferroni adjustment applied to the results of a t test performed with SPSS software (SPSS Inc). A probability value of < 0.01 was regarded as being statistically significant.

Results

Shear Stress Increased EphrinB2 Expression but Decreased EphB4 Expression

ES cell–derived VEGFR2+ cells were exposed to laminar shear stress (5, 10, 15, and 20 dynes/cm²) for 24 hours and examined for changes in expression of the arterial EC marker ephrinB2. Expression of ephrinB2 protein in the cells exposed to shear stress increased dose-dependently (Figure 1A). Shear stress increased expression of the EC marker CD31, as previously reported, and it also significantly increased expression of ephrinB2 protein in CD31+ cells (Figure 1B).

This would seem to indicate that the increase of ephrinB2 protein was attributable to both an increase in number of ephrinB2+/CD31+ cells and an increase in ephrinB2 expression in individual CD31+ cells.

VEGFR2+ cells were exposed to laminar shear stress (10 dynes/cm²) for 24, 48, and 72 hours and examined for changes in the mRNA levels of ephrinB2 and a venous EC marker EphB4. Under static culture conditions, the mRNA levels of ephrinB2 and EphB4 increased with time (Figure 2A and 2B). When exposed to shear stress, however, the ephrinB2 mRNA level increased much more than in the static controls, but the EphB4 mRNA level decreased. The ephrinB2 mRNA level, which had increased in response to shear stress, returned to the basal level within 24 hours under static conditions (Static after shear). D, Effect of γ-secretase inhibitors. VEGFR2+ cells were exposed to a shear stress of 10 dynes/cm² for 24 hours in the presence or absence of DAPT (2.5 μmol/L, Sigma) or L685 458 (0.1 μmol/L, Calbiochem). γ-secretase inhibitors that block Notch cleavage by presenilin-1. The inhibitors abolished the shear stress–induced upregulation of ephrinB2 expression, but had no effect on the downregulation of EphB4. Data are means ± SD of 6 samples. *P < 0.01 vs the static control.

Figure 2. Involvement of Notch in the effect of shear stress on expression of ephrinB2 and EphB4. A, ephrinB2 mRNA levels. B, EphB4 mRNA levels. Shear stress (10 dynes/cm²) increased the ephrinB2 mRNA level time-dependently but decreased the EphB4 mRNA level. C, Reversible effect of shear stress on ephrinB2 expression. The ephrinB2 mRNA level, which had increased in response to 24-hour exposure to shear stress (10 dynes/cm², Shear), returned to the basal level within 24 hours under static conditions (Static after shear). D, Effect of γ-secretase inhibitors. VEGFR2+ cells were exposed to a shear stress of 10 dynes/cm² for 24 hours in the presence or absence of DAPT (2.5 μmol/L, Sigma) or L685 458 (0.1 μmol/L, Calbiochem). γ-secretase inhibitors that block Notch cleavage by presenilin-1. The inhibitors abolished the shear stress–induced upregulation of ephrinB2 expression, but had no effect on the downregulation of EphB4. Data are means ± SD of 6 samples. *P < 0.01 vs the static control.

Notch Signaling Is Involved in the Increase in EphrinB2 Expression by Shear Stress

VEGFR2+ cells were exposed to shear stress (10 dynes/cm²) for 24 hours in the presence or absence of the γ-secretase inhibitors DAPT and L685 458, which block proteolytic cleavage of Notch, and they were examined for changes in the mRNA levels of ephrinB2 and EphB4. Both DAPT and
L685,458 completely abolished the shear stress-induced increase in the EphrinB2 mRNA level, but they had no effect on the decrease in the EphB4 mRNA level (Figure 2D). These findings suggested that Notch signaling is involved in the effect of shear stress on ephrinB2 expression, but not in its effect on EphB4 expression.

Shear Stress Increases Expression of Notch Receptors and Ligands
Changes in the expression of Notch receptors and Notch ligands were examined in VEGFR2^+ cells exposed to shear stress. The protein levels of Notch 1, Notch 4, DLL4, Jagged 1, and Jagged 2 increased in response to shear stress, and the increases were both dose- and time-dependent (Figure 3A). The mRNA levels of these Notch receptors and ligands also increased in response to shear stress, although the temporal profiles of the gene responses differed (Figure 3B). It should be noted that Notch 2 and 3, and DLL1 and 3 were not included in this study because their gene expression was not significantly increased expression of all of these Notch receptors and ligands at both the protein level and mRNA level. The experiments were repeated 3 times, and similar results of the Western blot analysis were obtained each time. Data from real-time PCR are means±SD of 6 samples. *P<0.01 vs the static control.

Notch Cleavage Occurs in VEGFR2^+ ES Cells in Response to Shear Stress
To investigate whether shear stress activates Notch signaling, VEGFR2^+ cells were exposed to shear stress (10 dynes/cm^2) for 30 minutes, 1 hour, and 4 hours, and the cells were examined for translocation of NICD into the nucleus. Immunofluorescence staining showed marked translocation of NICD into the nucleus of the cells exposed to shear stress for 1 hour or to VEGF (Figure 4A), and these findings were confirmed by Western blot analysis (Figure 4B). The amount of NICD in the nucleus began to increase as early as 30 minutes after the application of shear stress, and it increased with time. These findings indicated that shear stress induces Notch cleavage in VEGFR2^+ cells.

The shear stress–induced Notch cleavage was completely blocked by treating the cells with the γ-secretase inhibitors L685,458 (Figure 4A and 4B) and DAPT (Figure 4B) and with a recombinant extracellular domain of murine DLL4 (mDLL4) whose signal peptide had been removed proteolytically (Figure 4A and 4B), indicating involvement of γ-secretase and DLL4 in the shear-stress-induced Notch activation.

VEGF Signaling Is Linked to Notch Activation and the Subsequent Increase in EphrinB2 Expression
Because our previous study showed that shear stress induces VEGFR2 phosphorylation in a ligand-independent manner, which, in turn, leads to VEGFR2^+ cell differentiation into vascular ECs,^{25} we investigated whether VEGF signaling is associated with the shear stress–induced Notch activation and upregulation of ephrinB2 expression. The VEGF receptor kinase inhibitor SU1498 abolished the effect of shear stress on both Notch cleavage (Figure 4) and ephrinB2 expression (Figure 5B). Shear stress–induced Notch activation and upregulation of ephrinB2 were also blocked by inhibitors of protein kinase C (PKC), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase kinase (MAPKK), phosphatidylinositol 3-kinase (PI3K), and Akt kinase, but not by c-jun N-terminal kinase (JNK) or p38MAPK. These findings indicate that VEGF phosphorylation and activation of 2 signaling pathways (ie, the PKC-MAPKK-ERK pathway and PI3K-Akt pathway), both of which are known to be
activated downstream of VEGFR phosphorylation, are involved in the shear stress–induced Notch activation, leading to upregulation of ephrinB2 expression.

Discussion

The present study demonstrated that exposure to shear stress induced an increase in expression of the arterial EC marker ephrinB2 in ES cell–derived VEGFR2+ cells. The effect of shear stress was time- and dose-dependent, and reversible. VEGFR2+ cells are detected within the lateral plate mesoderm of the developing embryo as early as stage E8.0.28,29 Because the cells used in the shear stress experiments were VEGFR2+ cells that had been isolated from ES cells and cultured for 3 days, we assumed that the VEGFR2+ cells are around stage E11.0 and that they would have been exposed to shear stress in an early vascular network through which flow begins after the heartbeat starts at stage E9.0.30 The opposite effect of shear stress on ephrinB2 expression has been reported in mature ECs, such as human umbilical vein ECs (HUVECs) and human coronary artery ECs.31 Laminar shear stress of 15, 30, and 50 dynes/cm², but not of 1, 5, and 10 dynes/cm², significantly decreased the ephrinB2 mRNA levels in these ECs, and although the exact reason for the discrepancy is unclear, it seems to be attributable to the difference between immature ES cells and mature ECs.

Different gene responses to shear stress by human ES cells and mature ECs have been demonstrated, eg, the mRNA levels of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) have been shown to decrease in response to shear stress in human ES cells, but not in mature ECs, such as HUVECs or human microvascular ECs.32 The results of our in vitro study suggest that shear stress plays a role in the artery and vein EC specification of ES cells. In vivo, the differentiation of arteries and veins in the chick embryo have been found to be governed by hemodynamic forces, and expression of the arterial marker ephrinB2 has been demonstrated to be controlled by the blood flow.33 Thus, the results of the present study provide evidence in support of the concept that factors in the local environment such as fluid mechanical forces, as well as the genetic program, play a critical role in artery-vein specification during blood vessel development in the embryo.
mediated signaling pathways. It is an established fact that Notch cleavage occurs downstream of VEGF signaling, but the mechanisms by which the cleavage occurs remain unclear. Although as yet no evidence has been made available, direct involvement of shear stress in Notch cleavage cannot be ruled out. Shear stress may increase the probability of association between Notch receptors and Notch ligands by acutely increasing their cell surface expression or changing their distribution, or shear stress may cause a conformational change in Notch receptor molecules that increases their affinity for proteases involved in Notch cleavage, including the ADAM family of proteases and γ-secretase. shear stress may also promote Notch cleavage by affecting the amount, distribution, and activity of such proteases. Expression of Notch receptors (Notch 1 and Notch 4) and Notch ligands (DLL4, Jagged 1, and Jagged 2) significantly increased at both the mRNA and protein levels in response to shear stress, although the temporal profiles of the responses differed.

Because there was no significant change in Notch protein expression for an hour after the start of shear stress, it is unlikely that the Notch cleavage that occurred as early as 30 minutes after the start of shear stress is attributable to the increase in the expression of Notch receptors and Notch ligands. Recombinant murine DLL4 and γ-secretase inhibitors (DAPT and L685 458) abolished the shear stress–induced Notch cleavage and also blocked the shear stress–induced increase in ephrinB2 expression, indicating that Notch signaling mediates VEGFR2⁺ cell differentiation into arterial ECs in response to shear stress. Although this study did not identify the signaling pathway downstream of the NICD translocation that leads to the increase in ephrinB2 expression, involvement of Notch signaling in the regulation of ephrinB2 expression and the determination of arterial identity has been well documented.34,35

In our previous study we found that shear stress caused phosphorylation of VEGFR2 (Flk-1) in a ligand-independent manner and that suppression of the shear stress–induced phosphorylation of VEGFR2 by the VEGFR kinase inhibitor SU1498 abolished the induction of VEGFR2⁺ cell differentiation into vascular ECs by shear stress.26 In the present study SU1498 was found to block the shear stress–induced Notch cleavage in ES cell–derived VEGFR2⁺ cells. These findings taken together suggest that VEGFR2 phosphorylation and the subsequent signaling pathways lead to the activation of Notch signaling. Recent studies have shown that exposing cultured mammalian ECs to VEGF results in immediate activation of 2 distinct signaling pathways.36 One pathway involves activation of PLCγ and results in activation of PKC, which then triggers ERK/MAPK. The other pathway involves activation of PI3K and is followed by Akt activation. The 2 signaling pathways are thought to govern artery-vein specification through Notch activation in a cross-talk manner.37 The results obtained by using protein kinase inhibitors suggested that both the PKC–MAPKK–ERK pathway and the PI3K–Akt pathway are involved in the shear stress–induced Notch cleavage and increase in ephrinB2 expression. Based on all of these findings, shear stress appears to have induced differentiation of ES cell–derived VEGFR2⁺ cells into arterial ECs via the VEGF-Notch signaling pathway. It should be noted, however, that the data obtained by using the inhibitors must be interpreted carefully with their specificity in mind. More recently, it has been shown that some transcription factors function downstream of VEGF signaling and affect Notch activation. Foxc factors, members of the forkhead/Fox transcription factor family, have been shown to activate expression of DLL4 and to play a critical role in linking the VEGF signal to the Notch pathway.38 Moreover, an orphan nuclear receptor, COUP-TFII (chicken ovalbumin upstream promoter-transcription factor II), has been found to be expressed specifically in venous ECs, and its function is assumed to be to maintain venous identity by repressing Notch signaling.39 Clarification of these VEGF-Notch signaling pathways would lead to a better understanding of the molecular mechanism by which shear stress regulates arterial-venous differentiation in the embryo.
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Disclosures

None.

References

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