The Q510E mutation in Shp2 perturbs heart valve development by increasing cell migration

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Edwards MA, Crombie K, Schramm C, Krenz M. The Q510E mutation in Shp2 perturbs heart valve development by increasing cell migration. J Appl Physiol 118: 124–131, 2015. First published October 30, 2014; doi:10.1152/japplphysiol.00008.2014.—Tightly regulated cellular signaling is critical for correct heart valve development, but how and why signaling is dysregulated in congenital heart disease is not well known. We focused on protein tyrosine phosphatase Shp2, because mutations in this signaling modulator frequently cause valve malformations associated with Noonan syndrome or Noonan syndrome with multiple lentigines (NSML). To model NSML-associated valve disease, we targeted overexpression of Q510E-Shp2 to mouse endocardial cushion cells (ECs) using a Tie2-Cre-based approach. At midgestation, Q510E-Shp2 expression increased the size of atrioventricular valves by 80%. To dissect the underlying cellular mechanisms, we explanted ECs from chick embryonic hearts and induced Q510E-Shp2 expression using adenoviral vectors. Valve cell outgrowth from cultured EC explants into surrounding matrix was significantly increased by Q510E-Shp2 expression. Because focal adhesion kinase (FAK) is a critical regulator of cell migration, we tested whether FAK inhibition counteracts the Q510E-Shp2-induced effects in explanted ECs. The FAK/src inhibitor PP2 normalized valve cell outgrowth from Q510E-Shp2-expressing ECs. Next, chick ECs were further dissociated to assess cell proliferation and migration. Valve cell proliferation was not increased by Q510E-Shp2 as determined by label incorporation. In contrast, valve cell migration as reflected in a wound-healing assay was increased by Q510E-Shp2 expression, indicating that increased migration is the predominant effect of Q510E-Shp2 expression in ECs. In conclusion, PP2-sensitive signaling mediates the pathogenic effects of Q510E-Shp2 on cell migration. This leads to enlarged valve primordia (3, 24). Furthermore, ERK hyperactivation is both necessary and sufficient to induce valve malformation in mouse and in vitro models of Noonan syndrome (24, 25).

Paradoxically, loss-of-function mutations in Shp2 also cause valve defects. In particular, dominant-negative Shp2 mutations were identified in patients with Noonan syndrome with multiple lentigines (NSML, also termed LEOPARD syndrome) (10, 26). Notably, patients with NSML often suffer from hypertrophic cardiomyopathy and valve abnormalities. Therefore, NSML can be difficult to distinguish from Noonan syndrome, particularly in infants (27). Despite this clinical overlap, the NSML pathomechanism must be distinct from that in Noonan syndrome, because NSML mutations in Shp2 disrupt rather than enhance the catalytic activity of the protein.

To date, nothing is known about the valve disease mechanism(s) in NSML. Recently, we studied the effects of a particularly aggressive NSML mutation in Shp2, Q510E-Shp2, on signaling in cardiomyocytes. We found that hyperactivation of signaling through focal adhesion kinase (FAK) and Akt is responsible for the cardiomyocyte-specific phenotype (40, 41). Because conditional FAK deletion causes septal defects, outflow tract malalignment, and thickened semilunar valve leaflets (15), we hypothesized that dysregulated FAK signaling is responsible for NSML-associated valve malformation.

The goal of the present study was to characterize cellular mechanisms and the role of FAK in appropriate models of Q510E-Shp2-induced valve disease. Valve development is highly conserved across species (18, 28). We previously used mouse and chick embryos side by side to investigate Noonan syndrome-related valve defects and obtained consistent results in the two models (24, 25).

For the current study, we generated transgenic mice to test the effects of Q510E-Shp2 expression on valve development. To complement these studies and better dissect cellular mechanisms, we used cultured endocardial cushion (EC) explants and valve interstitial cells (VICs) from chick embryos. This allowed us to take an in vitro pharmacological approach to examine the role of FAK/src as a mediator of the pathogenic...
demonstrate robust Q510E-Shp2 expression in the myocardium of irreversible switches on transcription of Q510E-Shp2.

**MATERIALS AND METHODS**

Transgenic mice. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Missouri-Columbia and in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training.

Our goal was to generate a tissue-specific mouse model of NSML-associated valve disease in which the mutated Shp2 protein is expressed within the developing valves. Previously, we successfully induced transgene expression in the valve primordia using a conditional approach (24). Therefore, we now chose a similar strategy and generated CAG-CAT-Q510E-Shp2 (CC-Q510E) transgenic mice as the responder element. The CAG-CAT promoter cassette, which contains a cytomegalovirus (CMV) enhancer and chicken β-actin gene (CAG) promoter, linked to the chloramphenicol acetyltransferase (CAT) gene, flanked by loxP sites (2). In the absence of Cre recombinase, transcription driven by this promoter is terminated after the CAT cDNA. As depicted in the schematic in Fig. 1A, Cre recombinase excises the CAT cassette, including the poly(A) stop sequences, and thereby irreversibly activates transcription of any gene inserted after the second loxP site (here, Q510E-Shp) in the respective cell lineage.

The Q510E-Shp2 mutation was introduced into wild-type (WT) Shp2 cDNA by using polymerase chain reaction (PCR)-based mutagenesis. Subsequently, a C-terminal flag tag was added and the full-length Q510E-Shp2 construct was inserted into the CAG-CAT cassette by positional cloning and used to generate transgenic mice (FVB/N background). To activate transgene expression, transgenic animals were mated with Tie2-Cre mice obtained from Jackson Laboratories or with β-MHC-Cre mice (FVB/N background) kindly shared by Dr. Katherine Yutzey (37). Tie2-Cre mice were crossed into the FVB/N background for at least 12 generations before starting the crosses with the CAG-CAT-Q510E-Shp2 mice.

**Western blotting, immunohistochemistry, and wheat germ agglutinin staining.** Proteins extracted from flash-frozen mouse ventricular tissue were separated by gel electrophoresis and probed with anti-Shp2 (C-1; Santa Cruz Biotechnology, Santa Cruz, CA) to determine transgene expression. As a loading control, anti-GAPDH (Cell Signaling Technologies) was used.

For immunohistochemistry, paraformaldehyde-frozen embryo sections were stained with anti-Shp2 (C-1; Santa Cruz Biotechnology) or anti-phospho-Histone H3 (Ser10; Abcam) followed by Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). As a nuclear counterstain, 0.1 mg/ml bisbenzamide was used.

To delineate cell borders, sections were stained with fluorescein-labeled wheat germ agglutinin (WGA; Invitrogen, Carlsbad, CA).

**Fig. 1. Cre-mediated tissue-specific expression of Q510E-Shp2.** A: schematic of the CreloxP-based approach. Excision of the CAT gene by Cre recombinase irreversibly switches on transcription of Q510E-Shp2. B: Western blot to demonstrate robust Q510E-Shp2 expression in the myocardium of β-MHC-Cre;CC-Q510E-Shp2 double transgenic (DTG) mice. Notably, the Q510E-Shp2 protein runs slightly higher on the protein gel due to the presence of a C-terminal flag tag. D and C: immuno-staining for total Shp2 in the septal leaflets of the atriointerventricular valves. Arrows point at septal leaflets, a atrium, s interventricular septum. Scale bars = 50 μm.
Proliferation and wound-healing assays in dissociated chick EC cells. To assess proliferation rates in embryonic valve cells, chick AV ECs (Stage 25 to 26; Hamburger-Hamilton) were isolated and the adjacent myocardial layer removed. ECs from 10 to 12 embryos were pooled and dissociated with trypsin/EDTA for 2 to 3 min, passed three times through a 25-gauge needle, and plated onto chamber glass slides treated with 0.01% rat tail collagen I (Sigma). Cells were cultured in M-199 with 10% FBS, 1% chick embryo extract, and 1% penicillin/streptomycin. For adenovirus infection, adenovirus was added on plating and remained for 24 h. Forty-eight hours after infection, cells were incubated with 5-bromo-2′-deoxyuridine (BrDU) label for 2 h and then fixed in 70% ethanol. BrdU-positive nuclei were identified using immunohistochemistry (Zymed Laboratories). The majority of cells growing on the collagen coating displayed the characteristic spindle-like shape of mesenchymal cells. Occasionally, we could observe small patches of endothelial cells growing in a cobblestone pattern; these areas were excluded from the analysis.

For wound-healing assays, dissociated EC cells were obtained and infected with the respective adenoviruses as for the proliferation assays. When reaching near confluency, a wound was created in each well using a P200 pipette tip, and the area photographed hourly using an inverted phase-contrast microscope (Olympus IX51). The wound area for each time point was quantified using ImageJ software.

ELISA signaling protein activity assays in dissociated chick EC cells. To assess the degree of p70S6K and ERK1/2 pathway activation, dissociated chick VICS were prepared and cultured as described above for the proliferation and wound-healing assays. Forty-eight hours after adenovirus infection, cells were harvested and lysed. P70S6K and ERK1/2 activity was measured in these lysates using a commercial kit (InstantOne ELISA, eBioscience) according to the manufacturer’s instructions. In short, these ELISAs are based on phospho-specific antibodies against p70S6K (Thr389) and ERK1/2 (Thr202/Tyr204, Thr185/Tyr187) and detection with 3,3′,5,5′-tetramethylbenzidine (TMB) colorimetric substrate for horseradish peroxidase.

Statistics. All values are given as average ± SE. Unpaired Student’s t-tests were used for comparisons of two groups (Figs. 1, 2, 3, 6). Two-way ANOVA with repeated measures was used for comparisons of data from two groups measured at multiple time points (Figs. 4, 7). Four groups were compared using two-way ANOVA (Fig. 5). The Holm–Sidak approach was used for post hoc analyses; P < 0.05 was considered significant (GraphPad).

RESULTS

First, we used β-MHC-Cre mice to trigger Q510E-Shp2 expression and to confirm recombination and correct expression of the Q510E-Shp2 protein. The β-myosin heavy chain (β-MHC) promoter drives Cre expression in skeletal muscle and in the myocardium before birth. Left ventricular tissue samples from newborn β-MHC-Cre;CC-Q510E double transgenic (DTG) mice were collected and Shp2 protein expression was quantified using Western blots (Fig. 1B). Compared with single-transgenic control hearts, Q510E-Shp2 was expressed 2.6-fold over endogenous Shp2 levels in the DTG hearts.

Next, we induced prenatal expression of Q510E-Shp2 in the endothelial-derived cell lineage by mating CC-Q510E mice with transgenic mice expressing Cre under the control of the Tie2 promoter (22, 39). All Tie2Cre;CC-Q510E DTG embryos died by E14.5; therefore, we collected embryos at E13.5 to assess transgene expression and cardiac phenotype. At E13.5, live DTG embryos were found at the expected Mendelian ratios (data not shown), indicating that Q510E-Shp2 does not induce embryonic lethality at earlier time points. Using immunohistochemistry, we confirmed robust Q510E-Shp2 expression in the developing atrioventricular EC (Fig. 1, C and D) compared with single-transgenic control littermates.

Next, we determined EC size and morphology in Tie2Cre; CC-Q510E DTG embryos using H&E-stained histological sections. Fig. 2A shows representative longitudinal sections through the middle of E13.5 embryonic hearts. In particular, the septal leaflets of the AV valves were thicker. For quantification of EC volumes, areas of the valve primordia were traced (ImageJ) in serial sections through the entire heart. As summarized in Fig. 2B, EC volumes were significantly increased by 81% in DTG embryos. Notably, we previously used the same conditional approach to overexpress WT-Shp2 and observed no effect on valve development, thus excluding potential secondary effects due to the transgenic approach (24).

To test whether increased valve cell proliferation contributed to the increased EC volumes, we also stained E13.5 mouse embryo sections for phospho-Histone H3 (Fig. 2C). The frequency of phospho-Histone H3-positive nuclei within the atrioventricular EC from DTG embryos was not different compared with controls (Fig. 2D).

Comparing EC tissue morphology in H&E-stained sections at higher magnification, control and DTG embryonic valves did not appear to be substantially different (Fig. 3, A and B). First, we quantified the total number of nuclei per EC (i.e., without distinction between endothelial/subendothelial vs. mesenchymal areas). Averaged across the entire EC, control and DTG valve primordia did not exhibit any differences in nuclear density (Fig. 3C). We also analyzed the EC mesenchyme separately. As shown in Fig. 3D, again, no differences in nuclear density between control and DTG valves were detectable in this subset of cells. Next, E13.5 sections of the valve primordia were stained with WGA to delineate the
plasmalemma of the valve cells (Fig. 3, E and F). The cross-sectional areas of mesenchymal VICs were measured, and random regions of interest within the EC mesenchyme were chosen to determine the ratio of intracellular vs. extracellular space. Q510E-Shp2 expression did not affect either parameter (Fig. 3, G and H). Taken together, these data indicate that the increase in EC volume in DTG embryos is neither due to increased VIC size nor to increased deposition of extracellular matrix. Because we already excluded increased mesenchymal VIC proliferation (Fig. 2, C and D), we concluded that the underlying cellular mechanism may be increased migration of cells into the EC mesenchyme.

Because histological sections provide only a static image of the different embryological stages, we switched to tissue culture systems to further dissect the underlying mechanisms. Previously, we successfully used a valve tissue explant system to model certain steps of valve development in vitro and therefore now employed a similar system (25). Fertilized white leghorn eggs were incubated for 4.5 days and AV ECs were dissected away from chick embryos, removed the myocardial layers, and used a trypsin digest to dissociate the tissue explants. This is consistent with our hypothesis that FAK acts as a downstream mediator of the effects of Q510E-Shp2.

In Matrigel cultures, effects on cell proliferation vs. cell migration cannot be easily distinguished. We therefore dissected valve primordia away from chick embryos, removed the myocardial layers, and used a trypsin digest to dissociate the tissue explants. Right: quantification of outgrowth diameters normalized to original explant diameter. Q510E-Shp2 expression in the tissue explants resulted in significantly increased cellular outgrowth into the surrounding matrix when comparing diameters at each time point. *P < 0.05 vs. β-gal at the same day; n = 16–18 EC explants from three independent isolates.

Next, we tested whether intervention at the level of FAK could counteract the Q510E-Shp2-induced effects in culture. We recently studied the effects of Q510E-Shp2 expression on signaling in mouse ventricles and in isolated cardiomyocytes (40, 41). We found that Q510E-Shp2 fails to dephosphorylate FAK, which leads to hyperactivation of Akt and mammalian target of rapamycin (mTOR) signaling farther downstream. Because FAK has been shown to play an essential role in heart morphogenesis and valvuloseptal development (15), we hypothesized that FAK hyperactivation is also critical for the alterations in our explant cultures. Therefore, we tested the effects of the src/FAK inhibitor PP2 in our model system. We titrated the PP2 dose to the lowest effective dose that would not affect normal outgrowth. As shown in Fig. 5, 250 nM PP2 did not alter the diameter of outgrowth from the tissue explant in the β-gal-infected control group. However, PP2 normalized outgrowth diameter from the Q510E-Shp2-expressing tissue explants. This is consistent with our hypothesis that FAK acts as a downstream mediator of the effects of Q510E-Shp2.

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Fig. 4. Q510E-Shp2 expression increases cellular outgrowth from Matrigel-embedded EC explants. Left and middle: representative images of day 4 EC explant cultures infected with β-gal control or Q510E-Shp2 adenovirus. Right: quantification of outgrowth diameters normalized to original explant diameter. Q510E-Shp2 expression in the tissue explants resulted in significantly increased cellular outgrowth into the surrounding matrix when comparing diameters at each time point. *P < 0.05 vs. β-gal at the same day; n = 16–18 EC explants from three independent isolates.

Fig. 5. The FAK/src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) normalizes tissue explant outgrowth in Q510E-Shp2-expressing cultures. Left: representative images of tissue explants expressing either β-gal or Q510E-Shp2 cultured in the presence of vehicle or PP2. Right: quantification of outgrowth diameters normalized to original tissue explant diameter. PP2 (250 nM) but not vehicle alone, effectively counteracted the effects of Q510E-Shp2 on the diameter of tissue explant outgrowth on day 3. *P < 0.05 for indicated comparisons; n = 12–20 EC explants from four independent isolates.

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cells, expression of Q510E-Shp2, but not WT-Shp2, increased an ELISA-based approach. In cultured dissociated chick EC cells grew to near confluency and then were scratched. The wound area remaining after 6h was quantified. In Q510E-Shp2-expressing cultures, the wound area was smaller than in the β-gal-expressing controls. In contrast, WT-Shp2 expression had no effect on wound healing in this assay. This supports that cell migration is increased by Q510E-Shp2 expression.

We also assessed activation of p70S6K and ERK1/2 using an ELISA-based approach. In cultured dissociated chick EC cells, expression of Q510E-Shp2, but not WT-Shp2, increased the level of phosphorylation of both p70S6K and ERK1/2.

DISCUSSION

In summary, this study demonstrates that Q510E-Shp2 expression in the endothelial-derived cell lineage results in enlarged ECs in mouse embryos. This effect is not due to increased valve cell sizes or increased deposition of extracellular matrix, but is caused by increased overall cell numbers per EC. In a more reductionist system, cultured EC explants expressing Q510E-Shp2 demonstrate increased cellular outgrowth. This effect is reversed by the FAK/src inhibitor, PP2, suggesting that FAK/src activation is a mediator downstream of Q510E-Shp2. Furthermore, Q510E-Shp2 expression in dissociated VICs results in increased cell migration, not proliferation. Consistent with these findings, VIC proliferation is also not increased in mouse EC. Presumably, increased valve cell migration triggered by Q510E-Shp2 expression promotes recruitment of more cells to the valve mesenchyme in vivo, thus explaining the EC hyperplasia observed in our transgenic mouse model.

Our study provides novel insight into the underlying cellular mechanisms of NSML-associated valve disease. This is the first report showing that cell migration is altered by Q510E-Shp2 expression and that the pathogenic effects of Q510E-Shp2 in developing valves are likely to be mediated by FAK. The current findings are further supported by a number of recent studies, including our own, indicating that NSML-associated mutations in Shp2 cause hyperactivation of FAK/Akt/mTOR signaling (11, 21, 31, 40, 41). However, these previous studies focused mainly on cardiomyocytes or cultured striated muscle cells and prohypertrophic mechanisms, whereas valve-specific disease mechanisms in NSML were not studied. Our current study therefore addresses an important gap and provides evidence that although cellular effects are very different in the developing valves vs. cardiomyocytes, the underlying signaling mechanisms are similar.

Interestingly, the valve disease mechanism identified in this study is very different from the one we discovered previously in a Noonan syndrome model using the gain-of-function mutation Q79R-Shp2 in Matrigel-embedded chick EC explants (25). In that study, hyperproliferation of VICs was the central pathogenic mechanism, whereas VIC migration did not appear to be affected. In our current NSML model, the pathomechanism appears to be the direct opposite, at least as far as it can be recapitulated in culture. Regarding VIC proliferation, we even observed a trend toward reduced proliferation both in vivo and in vitro. This is consistent with our earlier study in which we observed slightly reduced proliferation in embryonic mouse cardiomyocytes expressing Q510E-Shp2 (41). In the current mouse model, even a small reduction in proliferation could over time add up to a detectable decrease in EC size. We therefore suspect that the effects of Q510E-Shp2 on VIC migration in vivo are substantial and possibly outweigh the small reduction in proliferation. Notably, the two mouse models of NSML and Noonan syndrome with Q510E- and Q79R-
Shp2 expression, respectively, in the developing valves are histologically very similar. In particular, ECs at E13.5 are enlarged to nearly twice the normal size in both models. In the clinic, the valve phenotypes in Noonan syndrome and NSML are also very similar, if not indistinguishable. How the biochemically opposite Noonan syndrome and NSML mutations in Shp2 can cause similar disease characteristics has been a longstanding paradox in the field (12). Our data now indicate that although the cellular mechanisms are different, both increased proliferation and increased migration lead to the same result (i.e., EC enlargement). This is a new step toward resolution of the current paradox.

Trying to explain this paradox on the molecular signaling level, recent studies support the notion that Noonan syndrome and NSML mutations lead to differential activation of two distinct pathways that both promote growth of the valve primordia. It has been consistently shown in various models that Noonan syndrome mutations in Shp2 lead to hyperactivation of ERK signaling (4, 9, 14, 24, 25, 34, 35). In contrast, it is thought that NSML mutations primarily induce disease through FAK/AKT/mTOR signaling (11, 21, 31, 40, 41). However, the extent to which dysregulation of ERK signaling also plays a pathogenic role in NSML remains highly controversial. In some models, NSML mutations downregulate the level of ERK1/2 activity after growth factor stimulation (23, 31, 41). However, in other models, expression of Shp2 proteins carrying various NSML mutations lead to the opposite effect [i.e., increased activation of ERK1/2 (33, 36, 48)]. In a Drosophila model, ERK inhibition rescued the wing and eye phenotypes induced by mutant Shp2 (36), indicating that ERK hyperactivation is not just an epiphenomenon, but it plays a causative role for the disease phenotype in this particular model. Interestingly, induced pluripotent cells generated from NSML patient fibroblasts also showed dysregulation of ERK1/2 signaling (7). These cells exhibited higher baseline activation of ERK1/2, which could not be further increased by growth factor stimulation.

Our current data are consistent with the latter studies demonstrating upregulation of ERK signaling by NSML mutants and also match our earlier findings in transgenic mouse hearts showing upregulation of ERK1/2 phosphorylation under unstimulated conditions. However, when we used cultured neonatal cardiomyocytes, we previously found a trend toward lower levels of ERK1/2 phosphorylation under serum-free conditions (40) and downregulation of ERK1/2 activity after serum stimulation (41). Our dissociated VIC cultures cannot be serum-starved because this leads to rapid detachment of the cells from the dish; therefore, they were cultured in the presence of serum. We suspect that the effect of Q510E-Shp2 on ERK activation depends on the cell type and also on the culture conditions; in particular, the continued presence or absence of growth factors.

Importantly, a role for ERK1/2 in the NSML pathomechanism cannot be excluded at this point. One argument against a causal role of ERK in our current model is that the FAK/src inhibitor PP2 prevented the effects of Q510E-Shp2 on tissue explant outgrowth in our current model. PP2 is a selective tyrosine kinase inhibitor with an IC50 of 4 and 5 nM for Lck and Fyn, respectively (16). A recent study has shown that this inhibitor may not be as selective as originally thought (6). However, it has been demonstrated that PP2 does not inhibit ERK1/2 activation at concentrations up to 100 μM (47). We therefore feel that ERK1/2 activation is unlikely to be critical for NSML-associated valve disease, but are planning highly specific interventions using adenoviral inhibitors of ERK signaling to definitively answer this question in the future.

FAK plays a central role in the developing heart. In particular, inhibition of FAK in early stages of heart formation leads to severe defects in ventricular septum formation and outflow tract alignment, showing that FAK is essential for cardiac morphogenesis (15). Furthermore, FAK is required for neural crest cell morphogenesis during mouse cardiovascular development (45). Notably, Shp2 interacts with and modulates the activity of FAK in various cell types (1, 20, 30, 46). Recently, it has been shown that Shp2 promotes growth factor-induced cell migration by dephosphorylating FAK in breast cancer cells (17). How Shp2 interacts with FAK in valve cells has not yet been explored, but our current data suggest that the two proteins are also closely linked in this tissue and are critical for normal valve development.

However, a limitation of our study is that the role of FAK in NSML-associated valve disease still needs to be better defined. We hypothesize that Q510E-Shp2 expression upregulates FAK activity but could not test this due to lack of a specific antibody that can detect phospho-FAK in chick tissue. We confirmed that Q510E-Shp2 expression increases activity of mTOR/p70S6K in chick VICs, but whether FAK is the critical upstream mediator remains to be shown. Furthermore, the effects of Q510E-Shp2 expression during earlier stages of valve development have not yet been characterized either. In the beginning stages of valve development, endothelial cells at the future valve insertion site undergo endothelial-to-mesenchymal transformation (EndoMT) and migrate into the EC mesenchyme. Our data were obtained using EC explants after EndoMT was completed, and therefore are mainly relevant for EC growth, but they would be compatible with increased and/or prolonged EndoMT as well. Therefore, we will also direct our future studies at earlier developmental stages to address the question of whether effects on EndoMT contribute to the NSML pathomechanism.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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