Genomic analysis distinguishes phases of early development of the mouse atrio-ventricular canal

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Vrljicak P, Chang AC, Morozova O, Wederell ED, Niessen K, Marra MA, Karsan A, Hoodless PA. Genomic analysis distinguishes phases of early development of the mouse atrio-ventricular canal. Physiol Genomics 40: 150–157, 2010.—Valve formation during embryonic heart development involves a complex interplay of regional specification, cell transformations, and remodeling events. While many studies have addressed the role of specific genes during this process, a global understanding of the genetic basis for the regional specification and development of the heart valves is incomplete. We have undertaken genome-wide transcriptional profiling of the developing heart valves in the mouse. Four Serial Analysis of Gene Expression libraries were generated and analyzed from the mouse atrio-ventricular canal (AVC) at embryonic days 9.5–12.5, covering the stages from initiation of endothelial to mesenchymal transition (EMT) through to the beginning of endocardial cushion remodeling. We identified 14 distinct temporal patterns of gene expression during AVC development. These were associated with specific functions and signaling pathway members. We defined the temporal distribution of mesenchyme genes during the EMT process and of specific Notch and transforming growth factor-β targets. This work provides the first comprehensive temporal dataset during the formation of heart valves. These results identify molecular signatures that distinguish different phases of early heart valve formation allowing gene expression and function to be further investigated.

serial analysis of gene expression; epithelial to mesenchymal transition; Notch; transforming growth factor-β

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CONGENITAL MALFORMATIONS of the cardiovascular system are observed in at least 1% of newborn babies, with abnormal development of the valves and septal structures accounting for a majority of these defects (17). Although many congenital valve defects occur as part of well-defined clinical syndromes, the genetic causes for a large proportion remain undetermined. Given the high morbidity and mortality associated with these defects, an increased molecular understanding of the processes involved in valve formation is crucial to the development of new therapies.

In the embryo, the mitral and tricuspid valves and part of the atrio-ventricular septum develop from cardiac cushions that form in the atrio-ventricular canal (AVC). Beginning at embryonic day (E) 9.0–9.5, signals from the myocardium induce an epithelial to mesenchymal transition (EMT) of endocardial cells in the AVC, which delaminate and invade the cardiac jelly to form endocardial cushions. The Notch and transforming growth factor (TGF)-β pathways have emerged as critical regulators of this process. TGF-β2 from the myocardium induces EMT (6, 9, 31), while Notch signaling establishes a boundary for EMT responsiveness (19, 30), making endocardial cells lining the AVC competent to respond (13, 36, 38).

Although knowledge about the signaling pathways driving the onset of EMT is accumulating, little is known about the downstream targets of these pathways and the mechanisms governing the events following EMT. After initial invasion, the endocardial cushions undergo remodeling through proliferation, differentiation and apoptosis of the newly formed mesenchyme cells (28). These highly coordinated events are accompanied by changes in gene expression.

Serial Analysis of Gene Expression (SAGE) examines gene expression profiles without prior knowledge of the genes involved. SAGE permits the simultaneous evaluation of thousands of expressed transcripts (37), generating absolute values that are easily compared and achieving extensive transcriptome coverage (33). Previous studies have used SAGE to characterize the transcriptome of heart-derived tissue and cell lines (2, 3, 27, 33).

We describe the first comprehensive SAGE analysis of mouse embryonic AVC development from E9.5 to E12.5. We identified temporal expression patterns in the developing AVC to determine subsets of transcripts likely involved in key developmental stages. We show that these temporal expression patterns are associated with specific functional processes occurring in the AVC. We also define the temporal distribution of mesenchyme genes during EMT and of specific Notch and TGF-β targets. This resource will be useful in the identification of novel AVC genes and the study of their function and regulation during early valve development.

MATERIALS AND METHODS

SAGE. C57BL/6J mouse embryos were dissected following procedures approved by the Animal Care Committee at the University of British Columbia. Mice were assigned to the appropriate Theiler stage at the time of tissue collection to ensure uniformity in the classification of developmental stages. To isolate the heart, embryos of the appropriate stage were collected from timed-pregnant females and placed in ice-cold PBS. AVCs containing endothelial, mesenchyme, and myocardial cell populations were manually dissected via 30G needles. Samples from multiple litters were pooled to obtain sufficient RNA for SAGE library construction and avoid possible bias created by spontaneous mutations in the colony. Blood was removed by puncturing the heart chambers and washing the tissue with PBS. Further details are available from the Mouse Atlas of Gene Expression website (http://www.mouseatlas.org/). Dissected mouse tissue samples were collected in either RNAlater (Ambion) or TRIzol reagent.
(Invitrogen). After RNA isolation, RNA quality was assessed using an Agilent Bioanalyzer and the RNA was stored at −80°C until SAGE library construction.

SAGE libraries were constructed using standard protocols (32), and the data are available at http://www.mouseatlas.org/, Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/), and Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). SAGE data were analyzed and mapped to genes using DiscoverySpace v4.0 (29). Unambiguous sense mappings to the RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq/) were used. Genes represented by multiple tag types were manually curated to determine the most likely representative.

Cluster analysis. Poisson-based k-means clustering (PoissonC) for SAGE data (8) was conducted >100 iterations and the data combined to obtain consensus clusters. To estimate the optimal number of clusters (k), the within-cluster dispersion was computed as described (7) for values of k from 1 to 50 over 50 iterations. The largest drop in within-cluster dispersion occurred for k values of 5 to 15. K = 14 was chosen after visual inspection of the resulting patterns.

Gene Ontology (GO) term enrichment analysis was performed using Expression Analysis Systematic Explorer (EASE) as described (7, 18). Raw EASE scores of <0.05 were considered significant.

RESULTS

To examine global gene expression changes during the initial phase of heart valve formation, we generated and analyzed four SAGE libraries from the mouse AVC at E9.5–E12.5. These libraries represent endothelium, mesenchyme, and myocardial cell expression in the AVC and cover the stages from initiation of EMT through to the beginning of cushion remodeling. For SAGE libraries a sequencing depth of 120,000 tags is comparable to fluorescent-based microray arrays (32), and new transcript discovery reaches a plateau at 300,000 tags (1). We therefore sequenced a minimum of 300,000 tags per library for a total of 1,274,310 tags representing 211,971 different tag types (Table 1).

In these libraries, 71% of the tags mapped to known transcripts using the RefSeq database. A further 8% of the tags mapped to the genome, likely representing unannotated transcripts. Of the remaining tags, many were only present once and may have been generated by sequencing, PCR, or other errors (34). However, 4,869 unmapped tag types were found at a significant level (>5 tags) suggesting these tags may represent valid, novel transcripts. Importantly, these AVC SAGE libraries exhibit a large dynamic range, covering over three orders of magnitude in expression levels from genes with >1,000 tags for myosins (e.g., Myl2, Myl4, and Myl7), to genes with only a few tags (e.g., transcription factors).

To test whether our SAGE libraries offer an accurate portrayal of transcripts present during the EMT process, we searched for tags representing genes previously shown to be involved in AVC development. We observed tags representing all of these genes, including the highly expressed extracellular matrix (ECM) molecule Periostin (22), and the lowly expressed transcription factor Tbx20 (20) (Supplemental Table S4). Furthermore, tags for genes known to be more highly expressed in the AVC were overrepresented in the AVC SAGE libraries compared with other heart libraries in the Mouse Atlas (32) (Supplemental Tables S3 and S5). These results suggest that our SAGE library dataset is sufficiently comprehensive at 300,000 tags per library to recapitulate patterns of gene expression observed in vivo over a large dynamic range. Significantly, the vast majority of tags in our libraries represent genes not previously characterized in the context of AVC development, suggesting that our database is a rich source of novel AVC genes.

Clustering of AVC libraries reveals temporal expression patterns. Genes that share temporal expression patterns may participate in similar biological processes. To determine if coordinated patterns of gene expression could be identified in the developing AVC, we performed cluster analysis. To exclude tags with constant expression levels, we conducted pair-wise comparisons of tag type expression patterns using Audic-Claverie statistics (4), which account for different library sizes and was designed for the quantitative, absolute comparison of SAGE gene expression profiles. We identified 8,839 tag types representing 3,424 genes with at least one significant difference (P < 0.05) and grouped them into clusters using a Poisson model-based k-means algorithm designed specifically for SAGE data (8). We used this algorithm over

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Table 1. Tissues and stages sampled

<table>
<thead>
<tr>
<th>Library ID</th>
<th>Description</th>
<th>Total RNA</th>
<th>Total Tags*</th>
<th>Tag Types†</th>
<th>Tag Count‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2–5</td>
<td>6–49</td>
</tr>
<tr>
<td>SM206 and SM246</td>
<td>E9.5 AVC (Theiler stage 15)</td>
<td>550, ng</td>
<td>314,093</td>
<td>65,103</td>
<td>47,395</td>
</tr>
<tr>
<td>SM234</td>
<td>E10.5 AVC (Theiler stage 17)</td>
<td>2.5, μg</td>
<td>301,949</td>
<td>75,375</td>
<td>54,504</td>
</tr>
<tr>
<td>SM008 and SM241</td>
<td>E11.5 AVC (Theiler stage 19)</td>
<td>6.3, μg</td>
<td>345,463</td>
<td>82,704</td>
<td>61,311</td>
</tr>
<tr>
<td>SM238</td>
<td>E12.5 AVC (Theiler stage 20)</td>
<td>2.5, μg</td>
<td>312,805</td>
<td>71,385</td>
<td>51,861</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>1,274,310</td>
<td>211,971</td>
<td>158,211</td>
</tr>
</tbody>
</table>

Atrio-ventricular canal (AVC) tissue was isolated from 4 time points for serial analysis of gene expression (SAGE) library construction. *Total number of tags sequenced; †unique tag sequences; ‡proportion of tags at different expression levels. E, embryonic day.

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100 iterations to resolve the tag types into 14 distinct expression patterns (Fig. 1 and Supplemental Table S6). These patterns were ordered by visual inspection and hierarchical clustering on the median expression at each time point.

To examine the genes represented in each cluster, tag types were mapped to the RefSeq database (Table 2 and Supplemental Table S7). We used EASE (18) to determine if specific GO terms were overrepresented (Table 3 and Supplemental Table S8). Clusters that showed peak expression at E9.5–E10.5 and decrease over time (particularly clusters B–D) contained many markers for endothelial cells (e.g., Vcam1, Edg1, Edf1, and Ednra), while clusters that increase over time (clusters K–N) included factors involved in ECM structure (e.g., Mmp2, Periostin, Biglycan, and collagens). Clusters with peak expression at E10.5 (cluster E), E11.5 (cluster J), and both time points (cluster G) included many transcription factors and signaling components, particularly of the Notch, Wnt, and TGF-β pathways such as Jag1, Bmp2, and Gsk3β. Interestingly, clusters that peak at E11.5 (clusters I and J) were significantly enriched for genes involved in cell proliferation (P < 0.05; e.g., Cdc2a, Cdc25c, and Dusp1). The cluster with the most pronounced peak at E12.5 (cluster N) was significantly enriched for cell adhesion and apoptosis genes (P < 0.05). Both proapoptotic and antiapoptotic genes (e.g., TNF receptor 12a and Bcl2l1, ...
respective) were represented suggesting a balance between proapoptotic and antiapoptotic signals. Thus, our data show dynamic patterns of gene expression in the developing AVC.

To further confirm our SAGE results, we performed quantitative RT-PCR validation on genes with different temporal expression patterns (Fig. 2A). Our SAGE and RT-PCR results were highly correlated even for lowly expressed transcription factors (i.e., Lef1, Tbx20, Sox9, Twist1). Interestingly, in situ hybridization analysis revealed differences in the spatial expression within the AVC region (Fig. 2B). Sox9, Twist1, and Periostin were expressed exclusively in mesenchyme cells throughout the E9.5–E12.5 timeframe, while Lef1 and Tbx20 were also expressed in the myocardium.

Epithelial/endothelial to mesenchymal transition in the AVC. To examine the transition of gene expression from epithelial to mesenchymal morphology in the AVC on a global scale and to address the gene expression differences within the various cell types of the AVC, we used epithelial-mesenchymal library pairs from our SAGE data collection (32) to examine the temporal expression pattern of epithelial- and mesenchymal-enriched genes. Six pairs of libraries, representing the dissociated epithelial and mesenchymal components of kidney, lung, male and female urogenital sinus, as well as large and small intestine, were used to calculate a mesenchymal-epithelial score ranging from +6 (mesenchyme enrichment) to −6 (epithelial enrichment). A mesenchyme-epithelial score was assigned to each tag type, and the median score per cluster was calculated (Fig. 3). Clusters peaking at E9.5 (i.e., clusters A–C) were more likely to contain tag types with higher expression in epithelial cells compared with mesenchyme. Clusters with peaks at E10.5 or E11.5 (e.g., clusters G–J) included tag types more commonly expressed in mesenchymal tissues, supporting the production of mesenchyme in the AVC at these time points. GO analysis of the mesenchyme-enriched genes with mesenchyme-epithelial score > +1.5 in the clusters demonstrated that genes in these clusters were more likely to be involved in signal transduction and transcriptional regulation (data not shown). The cluster with highest mesenchymal score (cluster K) contained tag types that consistently increased in expression from E9.5 to E11.5 with expression maintained at E12.5. This cluster is significantly enriched for many of the ECM components necessary to support mesenchymal cells (P = 0.009). Finally, clusters peaking at E12.5 (cluster N) included tag types with higher expression in epithelium, suggesting that at E12.5 the gene expression program is dramatically changing. The increased proportion of epithelial genes with expression peaking at E12.5 indicates that mesenchyme production is downregulated by E12.5. Interestingly, GO analysis of the genes with positive mesenchyme scores suggests that clusters that peak at E12.5 are significantly enriched in genes involved in bone remodeling (P = 0.034), supporting work demonstrating that heart valve maturation shares regulatory mechanisms active in developing cartilage, tendon, and bone (11, 23). Taken together, this global analysis of epithelial and mesenchymal markers defines the temporal events of EMT in the AVC and supports the functional relationships between the genes grouped in the gene expression clusters. Moreover, since our libraries were from the whole AVC, which included both the endothelial and mesenchymal cells, this analysis allows prediction of the cellular expression of genes in the clusters.

Table 3. Coexpressed genes share specific functions

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Biological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>biosynthesis P = 0.002, amino acid biosynthesis P = 0.018</td>
</tr>
<tr>
<td>B</td>
<td>protein biosynthesis P = 0.034</td>
</tr>
<tr>
<td>C</td>
<td>protein amino-acid phosphorylation P = 0.034, transcription/DNA-dependent P = 0.042, energy reserve metabolism P = 0.043</td>
</tr>
<tr>
<td>D</td>
<td>morphogenesis P &lt; 0.005, skeletal dev. P = 0.003, cell communication P = 0.009, signal transduction P = 0.011</td>
</tr>
<tr>
<td>E</td>
<td>proton transport P = 0.043; nucleobase, nucleoside, nucleotide, and nucleic acid metabolism P = 0.048</td>
</tr>
<tr>
<td>F</td>
<td>DNA metabolism P = 0.038</td>
</tr>
<tr>
<td>G</td>
<td>protein biosynthesis P = 0.010, macromolecule biosynthesis P = 0.016</td>
</tr>
<tr>
<td>H</td>
<td>mitotic cell cycle P = 0.003, main pathways of carbohydrate metabolism P = 0.004, electron transport P = 0.021, pyridine nucleotide metabolism P = 0.023, protein transport P = 0.039</td>
</tr>
<tr>
<td>I</td>
<td>mitosis P = 0.020, intracellular signaling cascade P = 0.030, intracellular transport P = 0.049</td>
</tr>
<tr>
<td>J</td>
<td>fatty acid metabolism P = 0.004, protein complex assembly P = 0.008, cell adhesion P = 0.05</td>
</tr>
<tr>
<td>K</td>
<td>cell adhesion P = 0.002, cell-matrix adhesion P = 0.015, communication P = 0.024, regulation of apoptosis P = 0.033</td>
</tr>
</tbody>
</table>

Enriched Gene Ontology (GO) biological process categories were determined for each cluster using Expression Analysis Systematic Explorer (EASE) (18). P values represent raw EASE scores for the respective categories. Representative biological process GO categories are indicated. Clusters A, L, and M do not contain any statistically significant biological process GO categories. Full list of significant GO categories (including molecular function and cellular component) is provided in Supplemental Table S8.
Of the 336 genes showing at least 1.5-fold change in expression in response to TGF-β1/H9252 treatment, 129 (35%) were detected in our AVC SAGE libraries, and 80% of these (104, 30% of total) showed a dynamic temporal expression pattern in the AVC and were present in one of our clusters. Interestingly, TGF-β-responsive genes are overrepresented in specific clusters (Fig. 4A and Supplemental Table S9) forming a pattern that suggests multiple phases of TGF-β activity in the AVC. The first phase corresponds to tag types whose expression is highest at E10.5 (clusters D, E, and F). It includes transcription factors involved in cell differentiation such as Atf3 and Nab1. A second phase of TGF-β-responsive genes corresponds to tag types whose expression peaks at E11.5 (cluster J) and includes regulators of apoptosis (e.g., Sod2 and Dedd). Finally a third phase corresponds specifically to tag types whose expression dramatically increases at E12.5 (cluster N). It includes ECM components Biglycan and Von Willebrand factor homolog (Vwf).

The Notch pathway has also been implicated in EMT during AVC development. To identify genes regulated by Notch and to determine if targets of the Notch pathway show similar patterns to those of TGF-β, we analyzed microarray data generated from HUVECs activated by overexpression of the...
Notch ligand Dll4 (15). From the 695 genes showing at least a 1.5-fold change in the microarray experiments, 535 (77%) were detected in the AVC SAGE libraries, and 242 (35% of the total) showed differential expression over time and were present in one of our clusters. As with TGF-β, clusters with peak expression at E10.5, E11.5, and E12.5 showed a higher proportion of targets determined by microarray analysis, indicating high signaling activity at these time points (Fig. 4B). Four phases of Notch activity were observed in our clusters (Fig. 4B and Supplemental Table S9). The first phase of Notch responsive genes, which includes signaling pathway members TGF-β2, Jag1, Smad7, and Igbp3, corresponds to genes with highest expression at E10.5 (clusters D and E). The second phase corresponds to tag types whose expression peaks at E10.5 and E11.5 (cluster G). Genes in this peak include cell-adhesion molecules such as Itga5, the regulator of apoptosis Cflar, and the microRNA processing enzyme Dicer. A third phase corresponds to tag types whose expression peaks at E11.5 (cluster J), including the endopeptidase inhibitor Reck, a modulator of the Notch pathway. Finally a fourth phase corresponds to tag types whose expression dramatically increases at E12.5 (cluster N). It includes ECM components Elastin and Periostin. As with TGF-β, the distribution of Notch signaling targets suggests that there are multiple phases of signaling activity occurring in the AVC, each associated with distinct functions. Interestingly, there was considerable overlap between the phases of Notch and TGF-β signaling activity since both ligands activated a high number of genes in cluster E (peaking at E10.5), supporting a role for both ligands in the early EMT phase of AVC development. However, distinct differences were observed between Notch and TGF-β activity since cluster G (peaking at E10.5 and E11.5) contained a high number of Notch-responsive genes but only a few TGF-β-responsive genes. Cluster J, which peaks at E11.5 and is associated with GO categories related to proliferation, contained the highest proportion of TGF-β-responsive genes, suggesting that TGF-β may play an important role during this phase of AVC development. Importantly, 28 genes (8%) were found to be regulated by both TGF-β1 and Dll4, including members of the Notch and TGF-β pathways (Jag1 and Tgfr2). These observations suggest cross talk between these pathways and suggest that these two ligands also have distinct functions in AVC development.
patterns are more prevalent during AVC development. Specifically, a large proportion (34%) of genes showed peaks of expression at E10.5, E11.5, or both (clusters E, G, and J). These clusters contained many TGF-β, Notch, and BMP pathway members, supporting the importance of these pathways at these stages.

Our AVC SAGE libraries include mixed populations of cells; therefore, a change in the proportion of transcripts in our libraries could reflect both a change of expression within cells as well as a change in the proportion of the different cell populations in the AVC. To investigate the distribution of mesenchyme-enriched genes, we compared six epithelial-mesenchymal library pairs (32) to calculate the mesenchyme enrichment of tag types. This permitted the temporal evaluation of mesenchyme-enriched genes on a global scale. Previous studies have focused on E9.5–10.5 as the critical time in EMT in the AVC (9). We show that some mesenchyme-enriched genes begin to be expressed at E10.5 and later (clusters G–J), suggesting that mesenchyme differentiation continues throughout the entire timeframe.

Following EMT, the mesenchyme of the AVC expands through proliferation (16, 21). Though proliferation decreases as valves mature, no studies have shown the temporal changes in proliferation between E9.5 and E12.5. In clusters I and J, which peak at E11.5, the expression of genes associated with mitosis and cell division is remarkably overrepresented compared with the other stages, suggesting that a burst of proliferative activity occurs at E11.5. As EMT concludes, the AVC is remodeled through differentiation of mesenchymal cells. Mesenchymal cells near the endocardial layer remain undifferentiated and are highly proliferative (14, 35), while cells near the myocardial layer are less proliferative and express markers of differentiation (25). We observed that genes correlated with an undifferentiated state, such as MsexI and Twist1, show a peak of expression at E10.5–11.5 (cluster D and G, respectively). Meox1, a marker of differentiating mesenchyme, increased gradually after E10.5 (cluster K) (10). Moreover, genes involved in bone remodeling were enriched in the mesenchymal fraction at E12.5, indicating that the program leading to remodeling of the ECM has initiated by this time point.

Finally, apoptosis removes excess cells and remodels the valves into leaflets. In the developing mouse heart, apoptotic cells were absent from E9.5–11.5 AVCs but began to appear in the fusion seam of the AV cushion at E12.5–13.5 (40). Correspondingly, we found that the cluster with the most dramatic increase in expression at E12.5 (cluster N) is enriched for genes that regulate apoptosis. Together, our data provide molecular signatures distinguishing key morphogenetic events during early AVC development.

TGF-β and Notch pathways control EMT during AVC development. Less is known about their role after EMT. Thus, we characterized the temporal expression of TGF-β and Notch pathway members. Interestingly, different ligands of the TGF-β and Notch pathways showed distinct expression patterns in our dataset, suggesting that there are multiple phases of TGF-β and Notch pathway activity over the course of AVC development. For example, expression of TGF-β2 was found to be high at E9.5–10.5 and then decrease over time (cluster D), while TGF-β3 expression peaked at E12.5 (cluster N). Previous work has found TGF-β2 to be essential for EMT in mouse knockout embryos and in vitro explant assays (5, 9, 31). Significantly, the high expression of TGF-β3 at E12.5 suggests that it plays a role in the remodeling process. Similarly, expression of the Notch ligand Jagl peaked at E10.5 (cluster E), while the Notch ligand Dll4 was highest at E12.5 (cluster N), suggesting that they control different processes during AVC development.

Using endothelial cell microarray data, we overlaid our temporal expression patterns with targets of TGF-β and Notch pathways. Out of 1,031 genes differentially expressed in the microarray experiments, 664 were found in our AVC SAGE libraries and 346 had a dynamic temporal expression pattern. Notably, there was a significant difference between the types of TGF-β and Notch target genes peaking at different time points. For example, TGF-β-responsive genes peaking at E9.5–10.5 (cluster D) included many genes involved in transcription factor activity or chromatin remodeling, while those peaking at E12.5 included many ECM proteins and cell-adhesion molecules. Significantly, several members of the TGF-β pathway were found downstream of Notch signaling and vice versa, indicating that there is considerable cross talk between the pathways.

In conclusion, we describe the creation of a database for AVC development, which we have used to analyze the temporal expression of genes. This resource is valuable for the elucidation of the molecular mechanisms underlying heart development.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


