Mesenchymal cell replacement corrects thymic hypoplasia in murine models of 22q11.2 deletion syndrome

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Introduction

Chromosome 22q11.2 deletion syndrome (22q11.2DS) is the most common human chromosomal microdeletion disorder reported, affecting approximately 1 of 4,000 individuals (1–4). Patients with this syndrome have developmentally linked congenital malformations, thymic hypoplasia or aplasia resulting in low T cell levels, cardiac defects, and hypoparathyroidism leading to low calcium and/or dysmorphic facial features (1, 3–5). Overall, many patients will have learning disabilities, autism, attention deficit disorders, and/or schizophrenia (1, 3, 6–9). The diverse and variably penetrant clinical presentations result from either a 3 Mb or nested 1.5 Mb deletion on chromosome 22q (4, 5). Both deletions lead to a haploinsufficiency of the T-box 1 (TBX1) transcription factor, a master regulator of pharyngeal patterning during embryogenesis (3, 10–13). While TBX1 plays a key role in the congenital malformations, various other genetic and epigenetic regulators can influence the penetrance and severity of the clinical phenotypes of 22q11.2DS (4).

Sixty to seventy percent of patients with 22q11.2DS have varying degrees of T cell lymphopenia due to thymic hypoplasia (often termed DiGeorge syndrome) (1–4, 14). T cell development remains normal for most, with lower T cell numbers the prevailing clinical presentation, resulting in mild SCID (15). Less than 1% of patients with 22q11.2DS have thymic aplasia, resulting in severe immunodeficiency due to the absence of T cells (16, 17). Some T cell development can be restored for these individuals with an allogenic thymic tissue implant (18, 19). Depleted of most hematopoietic cells, the engrafted thymus is composed of stromal cells that recruit host-derived stem cells, which develop into thymocytes (18–21). This clinical approach confirms that defects in host stromal tissues (mesenchymal cells, thymic epithelial cells [TECs], and/or endothelial cells) are the basis of thymic hypoplasia in 22q11.2DS (4, 20, 22). Among these stromal cell populations, mesenchymal cells are derived from the neural crest and form the thymus capsule and vasculature (23–27). These cells interact with endothelial cells and TECs to support the formation of the thymus (25–34). TECs release chemokines to recruit thymic seeding progenitors from the bone marrow, provide growth factors for thymocyte proliferation, and express self-peptide/self-MHC complexes that dictate

22q11.2 deletion syndrome (22q11.2DS) is the most common human chromosomal microdeletion, causing developmentally linked congenital malformations, thymic hypoplasia, hypoparathyroidism, and/or cardiac defects. Thymic hypoplasia leads to T cell lymphopenia, which most often results in mild SCID. Despite decades of research, the molecular underpinnings leading to thymic hypoplasia in 22q11.2DS remain unknown. Comparison of embryonic thymuses from mouse models of 22q11.2DS (Tbx1 neo2/neo2) revealed proportions of mesenchymal, epithelial, and hematopoietic cell types similar to those of control thymuses. Yet, the small thymuses were growth restricted in fetal organ cultures. Replacement of Tbx1 neo2/neo2 thymic mesenchymal cells with normal ones restored tissue growth.

Comparative single-cell RNA-Seq of embryonic thymuses uncovered 17 distinct cell subsets, with transcriptome differences predominant in the 5 mesenchymal subsets from the Tbx1 neo2/neo2 cell line. The transcripts affected included those for extracellular matrix proteins, consistent with the increased collagen deposition we observed in the small thymuses. Attenuating collagen cross-links with minoxidil restored thymic tissue expansion for hypoplastic lobes. In colony-forming assays, the Tbx1 neo2/neo2–derived mesenchymal cells had reduced expansion potential, in contrast to the normal growth of thymic epithelial cells. These findings suggest that mesenchymal cells were causal to the small embryonic thymuses in the 22q11.2DS mouse models, which was correctable by substitution with normal mesenchyme.
the selection and maturation of T cells capable of recognizing but not responding to self-peptides (32, 33, 35). TEC functionality is determined by the master transcriptional regulator Forkhead box N1 (FOXN1) (36–39). Autosomal recessive (AR) FOXN1 mutations result in a nude and SCID phenotype, the latter a consequence of thymic hypoplasia and aplasia (40–45). Like 22q11.2DS, the treatment option for patients with such AR FOXN1 mutations is an allogenic thymus implant, revealing the importance of stromal cell populations (18, 46, 47). Our understanding of the various cell types required for thymus formation and function has significantly advanced with the use of single-cell RNA-Seq (scRNA-Seq) (48).

Profiling of embryonic and adult thymuses reveals many distinct mesenchymal, TEC, endothelial, and hematopoietic cell subsets in thymuses at both early developmental stages and during the aging and involution of this tissue (49–51).

Despite decades of research, the molecular defects leading to the formation of a size-restricted thymus in 22q11.2DS remain poorly defined (3, 15, 22, 52–54). Multiple mouse models of 22q11.2DS have been developed, with thymic hypoplasia most often evident in mice on a C57BL/6 background. The small thymus severity and penetrance are Tbx1 gene dosage dependent in mouse models, with a more penetrant hypoplasia occurring when Tbx1 levels are less than 50% normal (10, 55–57) (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI160101DS1). To determine which cell populations are causal to thymic hypoplasia, we used 2 mouse models of 22q11.2DS (Df1/+ and Tbx1<sup>neo2/neo2</sup>) and compared the cell types necessary for the formation and function of the thymus (32). This was complemented with an analysis of human thymuses from healthy individuals and patients with 22q11.2DS. With reaggregate thymus organ culture procedures, we report that neural crest–derived mesenchymal cells were primarily responsible for the formation of size-restricted embryonic thymuses developing in the Tbx1<sup>neo2/neo2</sup> mouse model of 22q11.2DS. scRNA-Seq revealed 17 distinct cell subsets in the developing fetal thymus, with the 5 mesenchymal cell subsets and 1 endothelial cell population from the hypoplastic thymuses having the most divergent transcriptomes. The differentially expressed transcripts included extracellular matrix (ECM) proteins such as collagens. We report that the drug minoxidil restored thymus size and cellularity for the hypoplastic tissues, in part by suppressing transcripts required for collagen synthesis and cross-linking.

Results

Hypoplastic embryonic thymuses from 22q11.2DS mouse models maintain normal thymopoiesis. 22q11.2DS causes congenital malformations affecting the thymus, heart, and parathyroids (2–4). These are phenocopied in several mouse models of 22q11.2DS, including Tbx1-modified lines (10, 55–57). We noted a mildly penetrant thymic hypoplasia in thymuses at both early developmental stages and during the aging and involution of this tissue (49–51).

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**RESEARCH ARTICLE**

**A** E18.18.5 fetal thymic lobes

- Tbx1<sup>++</sup>
- Tbx1<sup>t<sub>neo</sub>2</sup>
- Tbx1<sup>t<sub>neo</sub>2 e.g.1</sup>
- Tbx1<sup>t<sub>neo</sub>2 e.g.2</sup>

Top row: H&E
Middle row: red = cytokeratin 8 (cTECs); green = cytokeratin 14 (mTECs); blue = DAPI
Bottom row: cytokeratin 14 (immature TECs, mTECs)

**B**

- Tbx1<sup>++</sup>
- Tbx1<sup>t<sub>neo</sub>2</sup>
- Tbx1<sup>t<sub>neo</sub>2 e.g.3</sup>
- Tbx1<sup>t<sub>neo</sub>2 e.g.4</sup>

**C**

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*P = 0.0002*
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E18–18.5 embryonic thymuses were obtained from Tbx1+/+ intercrossed mouse lines. (A) Live cell images from the cardiothoracic regions of Tbx1+/+, Tbx1+/neo2, and Tbx1 neo2/neo2 embryos. Thymic lobes are indicated with black arrows. An interrupted aortic arch (white arrow) often copresented with thymic hypoplasia in the Tbx1 neo2/neo2 embryos (Tbx1 neo2/neo2, e.g. 1). Scale bars: 50 μm. (B) Thymic tissue sections were processed for H&E staining and IHC. Top row: In the H&E-stained images, the cortical and medullary regions are dark and light purple, respectively. A medullary region is indicated by the boxed area. Scale bars: μm. Original magnification, ×10. Middle and bottom rows: With IHC, staining with antibodies selective for cortical (cytokeratin 8, red; middle row) and medullary (cytokeratin 14, green; bottom row) TECs is shown; DAPI staining revealed nuclei (blue; middle row). Coexpression of both cytokeratins (green and red) represents immature TECs. Original magnification, ×20 (middle and bottom rows). (C) T cell development was assessed by staining single-cell suspensions with antibodies selective for the CD4 and CD8 coreceptor proteins. The 4 thymocyte subsets are distinguished by electronic gating for the CD4 CD8 (DN), CD4 CD8 (DP), and the CD4 CD8 (CD4 and CD8; SP) subsets. (D) DN cells are further categorized by CD4 and CD25 cell-surface expression. This identifies the DN1 (CD4+CD25+), DN2 (CD4+CD25+), DN3 (CD4+CD25-), and DN4 (CD4+CD25-) subpopulations in the Tbx1+/+, Tbx1 neo2, and Tbx1 neo2/neo2 thymuses. (E) The total cell number and percentages of DP thymocytes. DN4 subpopulation of DN thymocytes, and cTECs were compared among the Tbx1+/+, Tbx1 +/neo2, and Tbx1 neo2/neo2 thymuses. (F) The number of all the thymocyte subpopulations and of the mesenchymal cells, and cTECs was slightly reduced in the Tbx1neo2/neo2 FTOC (Supplemental Figure 3). These findings point to a differentiation and/or functional defect among the mesenchymal cells, endothelial cells, and/or TECs. As mesenchymal cells produce growth factors to support FTOC expansion, reduced levels of these in Tbx1 neo2/neo2 lobes could account for the reduced lobe expansion (26, 27, 29).

To address this possibility, we placed paired hypoplastic lobes in FTOC surrounded by 4 pairs of control lobes. Despite the tissue expansion of the surrounding control lobes, we found that the centrally positioned Tbx1 neo2/neo2 lobes did not expand (Supplemental Figure 4, A–D). Taken together, our results reveal that at early stages of thymus formation, the thymus from the 22q11.2DS mouse model was growth restricted in FTOC.

Normal mesenchymal cells restore cellularity to hypoplastic thymuses. The FTOC findings suggest that mesenchymal cells, TECs, and/or endothelial cells could be functionally compromised in 22q11.2DS (Figure 3). To define whether 1 or more of these cell types were causal to the thymic hypoplasia, we performed regreaggregated thymus organ culture (RTOC) assays (Figure 4A) (59, 60). The RTOC assay was modified by initially sorting 3 different subgroups of cells by flow cytometry: mesenchymal cells (I), TECs (II), and a pool of all the remaining cell populations (III; endothelial cells and ETPs along with other hematopoietic cells). This modification enabled us to substitute different cell types from the Tbx1+/+, Tbx1 +/neo2, and Tbx1 neo2/neo2 thymuses prior to reaggregation with an equivalent starting number of reaggregated cells (minimum of ~30,000 cells) (Figure 4A and Supplemental Figure 5).

Recombinating the 3 subgroups of sorted cells from control thymuses (Tbx1+/+, Tbx1 +/neo2, Tbx1 neo2/neo2) resulted in thymic tissue growth (Figure 4, B–D). Thymocyte numbers increased, with DP and SP thymocyte subsets evident after 10 days of RTOC (Figure 4, B–D). Reaggregating identical numbers and proportions of the cell subgroups from the Tbx1 neo2/neo2 hypoplastic thymocytes failed to sustain normal tissue growth (Figure 4, B–D). However, substitution of the Tbx1 neo2/neo2 mesenchymal cells (Sub Tbx1 neo2/neo2 Mes) with an equivalent number of control mesenchymal cells (Ctl Mes) restored thymic tissue expansion and thymopoiesis (Figure 4, B–D). We observed a 10-fold increase in cellularity, matching the cell numbers achieved with controls (Figure 4, D and E). Cell viability and DP cell percentages also equaled control RTOC levels (Figure 4, F and G). Substitution of TECs from the Tbx1 neo2/neo2 thymuses (Sub Tbx1 neo2/neo2 TECs) with normal TECs (Ctl TECs) did not sustain tissue regeneration, as the cell number, cell viability, and DP cell percentages were significantly reduced compared with control and mesenchymal cell substituted RTOCs (Figure 4, D–G). Despite representing only 1%–4% of the cells in the embryonic thymus, we also examined the contributions of endothelial cells. Substituting the Tbx1 neo2/neo2 endothelial cells with normal ones (Sub Tbx1 neo2/neo2 nEndo) had an effect similar to that seen with TEC-replaced cultures, with only limited tissue expansion evident (Figure 4, B–G). These experiments established that replacement of mesenchymal cells, but not TECs or endothelial cells, in the Tbx1 neo2/neo2 thymuses restored tissue growth to normal levels. To determine whether the Tbx1 neo2/neo2 mesenchymal cells had a negative effect on thymopoiesis, RTOCs were grown with these cells used as substitutes for normal mesenchymal cells (Sub nMes). While tissue expansion and T cell development were evident, these were not as effective as the control tissue expansion with RTOC assays (Supplemental Figure 6).

Cellular composition of E13 embryonic thymuses. There are multiple cell subsets in a developing fetal thymus, and how these are affected by 22q11.2DS remains undefined. To address this, we performed scRNA-Seq to identify and compare all the cell subsets present in embryonic Tbx1+/+ and Tbx1 neo2/neo2 thymuses. This technique enables a precise delineation of the different cell types.
**RESEARCH ARTICLE**

A. E13-13.5 embryos

- **Tbx1**^+/+^  
- **Tbx1**^\text{neo2}\text{neo2}^  

B. E13-13.5 embryos

- **Tbx1**^+/+^  
- **Tbx1**^\text{neo2}\text{neo2}^  

C. 

- **Tbx1**^+/+^  
- **Tbx1**^\text{neo2}\text{neo2}^  
- **Tbx1**^\text{neo2}\text{neo2}^ \text{e.g.1}  
- **Tbx1**^\text{neo2}\text{neo2}^ \text{e.g.2}  

Pdgfrα = red; Pdgfrβ = yellow; cytokeratin = green

D. Mesenchymal & epithelial cells

- **Tbx1**^+/+^  
- **Tbx1**^\text{neo2}\text{neo2}^  

E. Thymic progenitors

- **Tbx1**^+/+^  
- **Tbx1**^\text{neo2}\text{neo2}^  

F. Total cell number  

- **Mesenchymal cell number**  

- **TEC cell number**  

- **Ratio mes/TECs**  

G. Mesenchymal cell percentage  

- **TEC percentage**  

- **ETP cell percentage**
in a developing tissue along with key transcriptome information (51, 61, 62). Hypoplastic thymic lobes from embryos harboring mutations in the Foxn1 transcription factor were also used in our analysis, since this gene is essential for TEC development (22, 63). AR mutations Foxn1 (Foxn11089/1089, c.1089_1103del15) result in thymic hypoplasia in mice that is similar to that in the Tbx1neo2/neo2 lines (Supplemental Figure 7).

Cells from the various E13–E13.5 lobes were encapsulated in nonadherent droplets with primer-containing beads for barcoding, followed by RNA isolation, cDNA synthesis, and sequencing. We used between 5,700 and 12,440 cells per thymus, providing an average read count of 69,000/cell (Supplemental Table 2). Unsupervised hierarchical clustering revealed 17 distinct clusters in control E13.0–E13.5 thymic lobes (Figure 5A). Cellular identities were defined with singular and combinatorial gene signatures for mesenchymal cells (Pdgfra and Col1A2), epithelial cells (EpCam and various keratin genes), endothelial cell populations (Cd55 and/or Pecam), hematopoietic lineage cells (Ptprc, Lck, Cd3d, and/or Cd3g) and RBCs (Hbb genes) (Table 1). We selected additional lineage and cell type markers on the basis of their identification in previous RNA-Seq and scRNA-Seq experiments using normal embryonic thymic lobes (51, 61, 62). Of the 17 distinct cell clusters, we identified 5 mesenchymal cell subgroups (M-1 to M-5), 6 TEC subgroups (E-1 to E-6), 4 hematopoietic cell clusters (H-1 to H-4), 1 endothelial cell population (En-1), and 1 group corresponding to RBCs (U-1) (Figure 5, A and B, and Table 1). A single cluster contained mitochondrial genes (U-6). The 20 most significantly differentially expressed genes (DEGs) evident in these clusters are listed in Table 1 and Supplemental Table 3. A complete list of all DEGs among the 17 clusters is provided in Supplemental Data File 1.

The number and composition of the cell clusters in the normal and hypoplastic thymic lobes were compared first. At E13–E13.5, a normal thymus was primarily composed of nonhematopoietic cell types (Table 2). Relative to the Tbx1+/+ controls, the Tbx1neo2/neo2 thymuses lacked most of the E-5 cell population, had reduced cells numbers in E-1, E-3, and E-4 cell clusters, and more cells in E-6 and M-5 (Figure 5A and Table 2). Compared with Foxn11089/1089, the Tbx1neo2/neo2 lobes were more affected in clusters M-5 (7-fold increase), E-5 (16-fold decrease), and E-6 (26-fold increase) (Table 2). The M-5 cell cluster expressed Pdgfrα, which is a marker for mesenchymal cells that develop into pericytes and vascular smooth muscle (64). In E-6, the presence of prolactin (Prl), bone morphogenetic protein 5 (Bmp5), and the long noncoding RNA Rmst (possible neural crest cell marker) suggested a mixed population in the Tbx1neo2/neo2 embryonic thymuses that included parathyroid cells. Both the Tbx1neo2/neo2 and the Foxn11089/1089 hypoplastic lobes had reduced numbers of hematopoietic cells (H-1, H-3, and H-4 clusters), consistent with the diminished effectiveness of thymopoiesis. Unique to the Foxn11089/1089 thymic tissues were cellular increases in E-5 (TEP subset) and H-3 (ETPs, early thymic progenitor cells). Differences in the levels and types of transcripts were visualized with dot plots, with the percentages of cells expressing a particular transcript and the relative levels of these transcripts in 16 of the 17 cell subsets (RBC cluster excluded) shown in Figure 5B and Supplemental Figures 8, 10, and 11. We used heatmaps to reveal the transcripts associated with mesenchymal, epithelial, and endothelial cell functions (Figure 5C). As demarcated with the dashed red box, mesenchymal cell subsets of the Tbx1neo2/neo2 genotype had elevated expression of Pdgfrα, Fgfr1, multiple collagens (Col1a2, Col3a1, Col4a1, Col5a1), and a cluster of genes coupled to ECM proteins and growth factor receptors. Among these were actinin 1 (Actn1), ADAM metallopeptidase with thrombospondin type 1 (Adams2), calpain 6 (Capn6), trooepoetin 1 (Eln1), elastin microfibril interactor 1 (Emlin1), fibulin 5 (Fbn5), Forkhead box transcription factor p1 (Foxp1), frizzled family receptor 1 (Fzd1), IGF-binding protein 10 (Igfbp10), also known as cysteine-rich angiogenic inducer 61 (Cyr61), matrix gla protein (Mgp), Pr domain-containing protein 6 (Prdm6), a histone-lysine methyltransferase), procollagen C-endopeptidase enhancer (Plocic), and β catenin (Ctnnb1) (Figure 5C and Supplemental Figure 8). The 6 epithelial cell subsets (E-1 to E-6) had remarkably similar transcript levels when comparing the control and Tbx1neo2/neo2 thymuses (Figure 5C). Similar levels of TEC-specific transcripts were also revealed in E16.5 thymic lobes isolated from the Dfl1/mouse model of 22q11.2DS when we compared hypoplastic and normal paired lobes and controls (Supplemental Figure 1B and Supplemental Figure 9). These observations contrast with the dramatic TEC transcript differences among the E-1 to E-6 subsets that were uniquely impacted in the Foxn11089/1089 thymus (Figure 5C). Therein, many of the key genes needed to support thymocyte trafficking and development were severely underexpressed (Supplemental Figure 10). Comparing the transcript levels in the single endothelial cell cluster also revealed some DEGs in Tbx1neo2/neo2-derived tissues that overlapped with those in the mesenchymal subsets (Figure 5C and Supplemental Figure 11). Pathway analyses revealed that Wnt/β-catenin, tight junction, hepatic fibrosis, hotair, IL-8, integrin, and ILK signaling pathways were all
Figure 3. Hypoplastic fetal thymic lobes from 22q11.2DS mouse models have diminished thymopoiesis potential in culture. (A) Paired normal-sized (Tbx1+/+ or Tbx1+/neo2) and hypoplastic (Tbx1neo2/neo2) thymic lobes (E13–E13.5) were cultured for 4 days and 8 days. Live cell imaging revealed changes in thymus size, which were limited in the Tbx1neo2/neo2 22q11.2DS mouse model. Scale bars: 1 mm. (B) T cell development was assessed by comparing the percentage of DN, DP, and SP thymocytes using electronic gating following antibody staining for surface CD4, CD8, and the TCR-β subunit expression. (C) The percentages of mesenchymal cells (Pdgfra+) and TECs (EpCAM+) were determined after 4- and 8-day cultures via flow cytometric analyses. (D) After 4 and 8 days of FTOC, thymic lobes were processed, and total cell numbers along with the percentages of mesenchymal cells (Mes), TECs, and DP thymocytes were determined. Tbx1+/+ (n = 8), Tbx1+/neo2 (n = 14), and Tbx1neo2/neo2 (n = 12) embryonic thymuses were used. (E) Eight days after FTOC, the total cell numbers and percentages of live cells, DP thymocytes, and TECs were determined. Note that by day 8, relatively few Pdgfra+ cells remained due to the differentiation of these cells. Tbx1+/+ (n = 4), Tbx1+/neo2 (n = 6), and Tbx1neo2/neo2 (n = 10) embryonic thymuses were used. Statistically significant differences were established by 1-way ANOVA (Brown-Forsythe and Welch tests).
affected in the Tbx1neo2/neo2 mesenchymal subsets relative to control and Foxn1 thymuses (Figure 5D and Supplemental Table 4). The aggregates appear as a small dot in the yellow circled area. Endothelial cell replacements required sorting of CD31+ cells from the remaining cell subsets prior to reaggregating culture. (B) Live cell imaging was used to visualize RTOCs after 10 days of culturing. The control corresponds to the 3 subgroups of cells from Tbx1+/+ or Tbx1neo2/neo2 embryos. In the second column, 22q11.2DS hypoplastic thymocytes were from Tbx1neo2/neo2 embryos. In the third column, normal mesenchymal cells were used as substitutes for those in the 22q11.2DS tissues (Sub Tbx1+/+;+/neo2 Mes). In columns 4 and 5, normal TECs or endothelial cells were used as substitutes for Tbx1neo2/neo2 TECs (Sub Tbx1neo2/neo2 TECs) or endothelial cells (Sub Tbx1neo2/neo2 Endo), respectively. Scale bars: 1 mm. (C) Cell viability (top row) and thymopoiesis (DN to DP and then SP progression, bottom row) are shown for the cells after 10 days of RTOC. (D) Cumulative cell numbers are shown for a representative RTOC experiment. (E–G) The fold increase in cell numbers following 10 days of RTOC along with cell viability and the percentage of DP cells developing over this period. n = 37, 28, 13, 8, and 5 experiments per group, respectively, for E–G. Statistical analyses done with 1-way ANOVA (Brown-Forsythe and Welch tests).

Discussion

Stromal and epithelial cell defects leading to thymic hypoplasia are evident in several human conditions, including 22q11.2DS, CHARGE syndrome (featuring coloboma, heart defects, atresia choanae, percentage of Pdgfrα/Pdgfra+ cells was evident in the Tbx1neo2/neo2 thymuses relative to controls (Supplemental Figure 12).

To determine whether increased ECM deposition and/or collagen cross-linking contributed to the thymic hypoplasia, we incubated RTOCs in the presence of several inhibitors of collagen and ECM deposition. Among these were verteporfin, minoxidil, and β-aminopropionitrile (BAPN) (65–69). Verteporfin was toxic to the cultures, whereas BAPN had no effect. Control RTOCs grown with minoxidil had cellularity, cell viability, and thymocyte subset percentages similar to those without the drug (Figure 7, A–C). RTOCs with an equivalent number of cells from the Tbx1neo2/neo2 embryonic thymuses consistently failed to expand (Figure 7, A–C, and Figure 4). Tissue expansion in the Tbx1neo2/neo2 RTOCs was restored in the presence of minoxidil, as revealed by increased cellularity and improved cell viability matching those of normal controls (Figure 7, A–C). Only the percentage of DP cells, which increased in the presence of minoxidil, did not reach the same levels as those in the control RTOCs cultured with minoxidil (Figure 7C). Since minoxidil reduces the expression of enzymes linked to collagen deposition and cross-linking, including Pld1 and Colla1 family members, we examined the transcript levels of these genes following RTOC. Normal RTOCs in the presence of minoxidil had statistically significant reduced expression of Pld1, Pld2, Colla1, and Colla2 on both days 3 and 4 of culturing (Figure 7D). These experiments confirmed that minoxidil affected collagen deposition and cross-linking, effectively improving tissue expansion for embryonic hypoplastic thymus.
growth retardation, genital abnormalities, and ear abnormalities), nude/SCID (AR FOXN1 mutations), and diabetic embryopathies (22). While FOXN1 mutations directly impact TECs, the cell populations affected in the other syndromes remain less well defined. We report here that thymic hypoplasia and aplasia in 22q11.2DS are linked to mesenchymal cell defects. This was confirmed by RTOC, scRNA-Seq data, and blocking of ECM deposition in developing thymuses using the Tbx1\textsuperscript{neo2/neo2} mouse model of 22q11.2DS. Importantly, normal fetal thymic mesenchymal cells restored thymic tissue growth and thymopoiesis when used as substitutes for cells from Tbx1\textsuperscript{neo2/neo2} thymuses (Figure 4).

During thymus specification and expansion, mesenchymal cells produce ECM proteins such as collagen, cell adhesion molecules, and growth factors to support both endothelial and TEC differentiation and expansion (reviewed in ref. 32). Prior studies have shown that wild-type embryonic thymuses, when stripped of the mesenchymal capsule, only expand upon readdition of this stromal tissue (29, 30). Capsule-depleted embryonic thymuses even fail to expand when transplanted under the kidney capsule, wherein adult mesenchyme surrounds the tissue (26). Despite reduced cell numbers, T cell development is normal in the capsule-stripped thymuses. In the Tbx1\textsuperscript{neo2/neo2} hypoplastic embryonic thymuses analyzed in the present study, higher levels of ECM proteins were apparent, and blocking collagen cross-linking with minoxidil restored tissue expansion to normal levels. Our findings are supported by recent studies that inhibited lysyl hydroxylases (LHs, encoded by Plod) (65, 66, 68). Minoxidil improved hypoplastic thymus expansion in both FTOC and RTOC assays, which correlated with the inhibition of Plod1, Plod2, Col1A1, and Colla2 genes. Mesenchymal cell subsets are the predominant sources of collagens and other ECM protein in the embryonic thymus, again pointing to a key role for these cells in the phenotypes of 22q11.2DS. Minoxidil could have additional effects on thymus growth, as it reportedly increases growth factor production (75). Although our experiments did not reveal this possibility, there may be other effects imparted by minoxidil (Supplemental Figure 5). At present, our data continue to support the idea that increased collagen cross-linking and subsequent ECM deposition limit thymus expansion in mouse models of 22q11.2DS, potentially impacting mesenchymal-endothelial cell functions.

Our scRNA-Seq results provide additional evidence that mesenchymal cells and, consequently, endothelial cells were affected by 22q11.2DS in the mouse model. While both normal and hypoplastic embryonic thymuses had the same 5 mesenchymal cell subsets (Table 1: M-1 to M-5), their transcriptomes were distinct. The expression of several ECM transcripts was increased in the hypoplastic lobes. Many of these transcripts were coupled to tissue remodeling pathways including elevations in the Wnt signaling pathway (Figure 5D and Supplemental Table 4). Increased Wnt signaling disrupts thymus organogenesis (76). With regard to specific mesenchymal subsets, we found that M1-M3 were the least affected, whereas M-5 was overrepresented in the hypoplastic lobes (Tbx1\textsuperscript{neo2/neo2} genotype) (Table 2). M-5 marks pericytes and vascular smooth muscle cells, which are cells that surround the endothelial vasculature and regulate T cell entry and egress from the thymus (31). The M-5 population, in the Tbx1\textsuperscript{neo2/neo2} setting, produced more ECM and collagens (Figure 5C). With the 1 endothelial cell cluster in the Tbx1\textsuperscript{neo2/neo2} line, the transcriptome changes had some overlap with the mesenchymal pattern. While the endothelial cells represented only 1%-4% of all the cells in an E13 thymus, their disrupted transcriptome was likely a direct consequence of their interactions with mesenchymal cells. Current experiments are underway to define how these 2 cell types coordinate thymic tissue expansion in the embryo.

Our comparison of control, Tbx1\textsuperscript{neo2/neo2}, and Foxn1\textsuperscript{1089/1089} thymuses provided strong evidence that TEC functions were normal in the 22q11.2DS mouse model. T cell development was similar, with only a delay in the developmental progression of thymocytes to the DP stage noted in the FTOC assays. scRNA-Seq revealed similar transcriptome patterns in the TEC subsets designated as E-1 to E-4 and E-6. Key transcripts needed for T cell development were present, including Foxn1, Cd25, Pmish11, Prss16, Cd44, and Aire (Figure 5, B and C). One difference was an underrepresentation of the E-5 population. In control thymuses, E-5 retained some parathyroid-related genes (Pth, Pax8, Chga), supporting prior evidence that the developing thymus contains some parathyroid precursor cells (77). We also performed quantitative reverse transcription PCR (qRT-PCR) with E16.5 thymic lobes using the D1/+ mouse model of 22q11.2DS, comparing a hypoplastic lobe separated from its paired normal-sized lobe (Supplemental Figure 1B and Supplemental Figure 9A). The key
transcripts required for TEC functions, including Foxn1, AIRE, and a Foxn1 target, E2F1, were present at normal levels in the hypoplastic lobes (Supplemental Figure 9A). In addition, transcriptome comparisons revealed that 14 of the 22 mRNAs affected by a hemizygous deletion of the chromosome 16 genes (22q11.2 equivalent) were expressed in the developing thymus (chromosome 16 in the mouse) (Supplemental Figure 9B). A prior study compared transcripts in the third pharyngeal pouch in normal and Df1/+ embryos. In a second 22q11.2DS mouse model that was generated in-house, the LOF of approximately 1 Mb on human chromosome 22q11.2 was caused by the insertion of Tbx1 into the downstream of the gene spanning approximately 22 orthologs of the genes spanning approximately 1 Mb on human chromosome 22q11.2 (Supplemental Figure 1A). In a second 22q11.2DS mouse model that was generated in-house, the Tbx1 null mouse was used, which was already bred on a C57Bl/J background (Supplemental Figure 1A) (55, 82). There was selective targeting of Tbx1 in this mouse line, which occurred via the insertion of neomycin into intron 5. Timed pregnancies were established by setting up breeding pairs in the late evening and screening for vaginal plugs the following morning. This was designated as day 0–0.5, primarily because the duration of the cell isolation often took an entire morning.

In summary, the neural crest–derived mesenchymal cells in embryonic thymus from mouse models of 22q11.2DS had transcriptome alterations with increased production of ECM proteins such as collagen. These changes, along with cell-cell interaction alterations, affected both mesenchymal and endothelial cells. We believe our findings are important in the context of efforts to regenerate thymuses for patients with aplasia, individuals who have thymectomies due to cardiac surgeries or the autoimmune disease myasthenia gravis, as well as for individuals undergoing rigorous chemotherapy treatments (32). Addition of appropriate mesenchymal cell populations that aid in endothelial vascularization along with TEC expansion and differentiation may provide a novel strategy for thymus organoid technologies, which are much needed in numerous clinical settings (32).

Methods

Human studies. Patients in the cardiothoracic group at Children’s Health, Dallas, Texas, for corrective surgeries to treat IAA-type B, truncus arteriosus, and/or tetralogy of Fallot. Affected individuals were screened for 22q11.2DS (often clinically listed as DiGeorge syndrome). The thymus was obtained from the patients if a partial thymectomy was performed. The thymus size was variable from patient to patient. Samples were taken and processed for histological analyses. Thymic tissue sections were prepared and stained with H&E at UT Southwestern Medical Center’s Molecular Pathology Core.

Mouse models. Mice were housed in a specific pathogen–free (SPF) facility at UT Southwestern Medical Center. One of the murine models of 22q11.2DS used in the study, termed the Df1/+ line (Del(16E-3)F10), was backcrossed over 12 generations with mice on a C57BL/6 background (10, 81). These Df1/+ mice were haploinsufficient in approximately 22 orthologs of the genes spanning approximately 1 Mb on human chromosome 22q11.2 (Supplemental Figure 1A). In a second 22q11.2DS mouse model that was generated in-house, the Tbx1null mouse line was used, which was already bred on a C57BL/J background (Supplemental Figure 1A) (55, 82). There was selective targeting of Tbx1 in these mouse lines, which occurred via the insertion of neomycin into intron 5. Timed pregnancies were established by setting up breeding pairs in the late evening and screening for vaginal plugs the following morning. This was designated as day 0–0.5, primarily because the duration of the cell isolation often took an entire morning. RNA isolation and transcriptome analysis. Total RNA was isolated from fetal thymic lobes, the pharyngeal region, or sorted cells. RNA was prepared and purified using the miRNana kit (Ambion, Thermo Fisher Scientific), and for small numbers of cells, the MicroRNA Isolation kit (Zymo Research) was used. Contaminating DNA was removed by DNase treatment (Applied Biosystems, Thermo Fisher Scientific) and in-column DNase digestion (Zymo Research). RNA was reverse transcribed (Applied Biosystems, Thermo Fisher Scientific) to cDNA. qRT-PCR was performed with SYBR qPCR Master Mix (Thermo Fisher Scientific) to check the expression of Ploids and ECM genes, which

### Table 1. Clustering of cell types from E13.0 normal and hypoplastic thymuses

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Subset ID</th>
<th>Cluster number</th>
<th>Gene identifiers</th>
<th>Additional transcripts coupled to cell differentiation; lineage specification, and/or function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesenchymal cells</td>
<td>M-1,0</td>
<td>Pdgfra, Cola2a, Gdf8, Aldh1a2, Snf2b, Cola2a</td>
<td>Foxn1, Mpl1, Nrl2, Wnt5a, Fgfr10, fibroblast type 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M-2,7</td>
<td>Pdgfra, Cola2a, Dlk1, Dcn1, Gdf8, Cola2a</td>
<td>Foxn1, Mpl1, Nrl2, fibroblast type 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M-3,8</td>
<td>Pdgfra, Cola2a, Mesp, Lum</td>
<td>Foxn1, Mpl1, Nrl2, fibroblast type 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M-4,10</td>
<td>Cola2a Vim, Myo, Itm2a, Sparc</td>
<td>Foxn1, Mpl1, Nrl2, fibroblast type 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M-5,17</td>
<td>Cola2a, Maxm, Csp4a, Actn1, Actg2, Sf7p2</td>
<td>Mefa2a/1/2/2d, Pdgfrb/t, Rgs5, Actu5, pericentrical organogenesis/vascular smooth muscle</td>
<td></td>
</tr>
<tr>
<td>TECs (Foxn1)</td>
<td>E-1,2</td>
<td>EpCam, Krt8</td>
<td>Foxn1, H-2A, Pmbn1, cortical TECs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E-2,3</td>
<td>Krt8, Krt18</td>
<td>Foxn1, H-2A, Pmbn1, cortical TECs</td>
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</tr>
<tr>
<td></td>
<td>E-3,4</td>
<td>EpCam, Krt8</td>
<td>Foxn1, H-2A, Pmbn1, cortical TECs</td>
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</tr>
<tr>
<td></td>
<td>E-4,9</td>
<td>EpCam, Ktf5, Krt8, Krt17, Krt19</td>
<td>Foxn1, H-2A, Pmbn1, cortical TECs</td>
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</tr>
<tr>
<td></td>
<td>E-5,12</td>
<td>EpCam Ptaha, Ogo, Col2a, Sup1</td>
<td>Foxn1, H-2A, Pmbn1, cortical TECs</td>
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<tr>
<td></td>
<td>E-6,16</td>
<td>EpCam, Nkx2.1, Hhex</td>
<td>Foxn1, H-2A, Pmbn1, cortical TECs</td>
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</tr>
<tr>
<td>Endothelial cells</td>
<td>E-1,13</td>
<td>Pecam1, Cdh8, Cdx1, Foxg7</td>
<td>Scx18, endothelial cells</td>
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</tr>
<tr>
<td>Hematopoietic cells</td>
<td>H-1,5</td>
<td>Ptprca, Cdz1, Cdz2, Lck Mtb1</td>
<td>Runx1/3, Tgf7, Gata2, Lat, DN thymocytes</td>
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</tr>
<tr>
<td></td>
<td>H-2,11</td>
<td>Ptcrc</td>
<td>Cpf4, Ptf4, microphages/macrophages</td>
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<td></td>
<td>H-3,14</td>
<td>Ptprca, Cdz2, Cdz4, Lck</td>
<td>Itga9, Cds5, Cds2, Itf7, Itf2, Itf3, early seeding cells, thymic progenitors</td>
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</tr>
<tr>
<td></td>
<td>H-4,15</td>
<td>Ptprca</td>
<td>CD80, CD86, DCs</td>
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</tr>
<tr>
<td>RBCs</td>
<td>U-1,1</td>
<td>Hba1, Hbb1</td>
<td>RBCs</td>
<td></td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>U-6,6</td>
<td>Mt gene</td>
<td>Mitochondria</td>
<td></td>
</tr>
</tbody>
</table>

*Uniquely elevated in Tbx1null hypoplastic thymus. *Uniquely reduced (E-3, E-5) or elevated (E-6) in Tbx1null versus Foxn1null hypoplastic thymus.
were normalized to Gapdh. scRNA-Seq and data analysis are described in the Supplemental Methods.

**FTOC and RTOC.** FTOC assays and antibodies used for staining are detailed in the Supplemental Methods. For cell viability and proliferation assays, FTOC was performed for 4 and 8 days. After 4 days in FTOC, a single-cell suspension of lobes was made and divided into 2, and 1 set was stained with anti–EpCAM-FITC, anti–PDGFRα-PE, anti–CD45-PE, and anti–PDGFRα stained with anti–EpCAM-FITC, anti–PDGFRα was added 10 minutes prior to analysis. The second set of cells was cultured in a CO₂ incubator for 10 days at 37°C with an input of 7.5% CO₂. Minoxidil-supplemented media contained M minoxidil in certain cultures. RTOCs were performed for 10 days in FTOC, a single-cell suspension of lobes was made and divided into 2, and 1 set was stained with anti–EpCAM-FITC, anti–PDGFRα-PE, and anti–CD45-APC-Cy7 antibodies, followed by intracellular staining with anti-Ki67-APC antibody following the manufacturer’s recommendations (Invitrogen Fix and Perm kit, Thermo Fisher Scientific). Analyses were done on a BD FACSaria Zika.

For reaggregate assays, normal and hypoplastic fetal thymic lobes, at a gestational age between E13.0 and E13.5, were isolated and collected in thymus organ culture (TOC) media. The media consisted of RPMI, 20% FCS supplemented with HEPES, L-glutamine, sodium pyruvate, penicillin, streptomycin, 5 × 10⁻⁵ M 2-mercaptoethanol, and nonessential amino acids. A minimum of 6–8 hypoplastic lobes were needed for a single RTOC assay (30,000 cells total). Lobes were washed with PBS and digested in 0.25% trypsin and 0.02% EDTA at 37°C for 6–10 minutes, followed by pipetting until single-cell suspensions were obtained. Digestion was stopped by addition of TOC media. Cells were washed, resuspended in volumes of less than 250 μL/6–20 lobes, and an aliquot counted with a hematocytometer. Cells were stained with antibodies specific for mesenchymal cells (PDGFRα-PE) and TECs (EpCAM-FITC) under sterile conditions. After washing, the cells were sorted into 3 populations: mesenchymal, epithelial, and the remaining cells (EpCAM PDGFRα; precursor thymocytes, DCs, endothelial cells, macrophages). Sorting was done with an FACSaria Zika machine. RTOC was performed by reaggregating the 3 cell populations — EpCAM⁺ (~30%), PDGFRα⁺ (~30%), and EpCAM PDGFRα⁻ (~40%) — in a 1.5 mL tube in varying combinations (Figure 4A). Cells were centrifuged consecutively for 5 minutes and 10 minutes at 100g and 400g, respectively. After the second spin, the supernatant was removed, leaving behind 2–4 μL aggregated cells, which were placed on ice for 10 minutes. The cell pellet was gently dispersed, and the mixture was drawn into a pulled glass pipette and delivered as a single drop onto a Millipore nitrocellulose filter (MilliporeSigma). The filter was placed on top of a sterilized foam sponge (2 mm thick) in a single well of a 6-well tissue culture plate (60 mm diameter). The foam sponge had been soaked in 3 mL TOC medium, with air pockets removed by gentle compression with the flat end of a 1 mL syringe plunger. Reaggregated thymic lobes were cultured in a CO₂ incubator for 10 days at 37°C with an input of 7.5% CO₂. After 10 days of organ culture, reaggregated thymic lobes were harvested in PBS (Ca²⁺- and Mg²⁺-free) supplemented with 2% FCS, and single cells were prepared by gentle squishing and pipetting of the lobes. Cells were counted using a hematocytometer. An aliquot was used for flow cytometric analysis after staining the cells with antibodies against CD8-FITC, CD4-PE, and TCRβ-PerCP-Cy5.5. Samples were analyzed on a FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (Tree Star). For RTOC assays in the presence of minoxidil, single-cell suspensions from the thymic lobes were prepared. The cells were reaggregated in batches of 30,000 cells/group for RTOC. As indicated, the media were supplemented with 3 μM minoxidil in certain cultures. RTOCs were performed for 10 days at 37°C with an input of 7.5% CO₂. Minoxidil-supplemented media were renewed every 4 days. After 10 days, the lobes were processed into a single-cell mixture. Cells were stained with antibodies specific for various cell-surface proteins.

**IHC analyses.** E10.5–E13.5 embryos and E18.5 fetal thymuses and fragments from human thymuses were fixed for 24 hours in
deparaffinized in xylene and rehydrated using a descending ethanol gradient (100%, 95%, 90%, 80%, 70%, and 50% ethanol). Antigen retrieval was performed for 15 minutes at 95°C in Antigen Retrieval R Buffer A, pH 6 (Electron Microscopy). Slides were blocked in CAS Block (Invitrogen, Thermo Fisher Scientific) for 2 hours at room temperature. Anti–cytokeratin 14, anti-cytokeratin, anti-Pdgfrα, anti-Pdgfrβ, anti–E cadherin, anti-laminin, anti-CD31, anti-endomucin, anti–collagen I, anti-Mcam, anti-Cspg4, and anti–collagen I antibodies were used to stain slides overnight at 4°C. The antibodies are listed in Supplemental Table 5. Secondary antibodies were used according to the manufacturer’s instructions (Invitrogen, Thermo Fisher Scientific). The slides were stained with DAPI (Molecular Probes) prior to being mounted with Prolong Gold Anti-fade Reagent (Invitrogen, Thermo Fisher Scientific). Images were taken on a Keyence Fluorescence microscope, and images were analyzed using ImageJ software (NIH). H&E staining was performed as described previously (83). Images were also taken on a Leica TCS SP5 confocal microscope and analyzed using ImageJ.

Sections stained with H&E were imaged on an Axiovert 200M inverted fluorescence microscope (Zeiss).

Mesenchymal and epithelial cell differentiation assays. E13–E13.5 control and Tbx1neo2/neo2 thymic lobes were prepared as per the RTOC
experiments. Mesenchymal cells (Pdgfra⁺) and TECs (EpCam⁺) were isolated by flow sorting as in the RTOC assays. Between 6,000 and 8,000 cells/experiment were seeded onto 6-well tissue culture plates containing RPMI media supplemented with MesenCult Expansion or EpiCult media for murine cells (both from STEMCELL Technologies). The cells from either normal or Tbx1 neo2/neo2 thymuses were reaggregated with equivalent starting clusters of approximately 30,000 cells/group. Cultures were maintained in media alone or supplemented with 3 μM minoxidil. (A) Live cell imaging revealed cell expansion after 10 days of culturing. Scale bars: 1 mm. (B) Thymopoiesis was compared using antibodies specific for CD4 and CD8. (C) Cell numbers, cell viability, and the percentage of DP cells are shown. Note that the number of cells in Tbx1 neo2/neo2 thymuses was severely limited, as established in Figure 4, B, D, and E. n = 10, 10, 3, and 3 for the indicated groups, from left to right, in each panel. Statistical significance was determined by 1-way ANOVA. (D) Control FTOCs were grown in the absence or presence of minoxidil. On day 3 and day 4 after culturing, the cells were processed for qRT-PCR using probes detecting 2 Plod and 2 Col1a genes, along with GAPDH for normalization. Day 3, n = 5; day 4, n = 4. (E) Mesenchymal cells and TECs from E13–E13.5 embryonic thymuses from Tbx1+/+ or Tbx1 neo2/neo2 embryos were flow sorted. Mesenchymal sorted cells were grown in MesenCult differentiation media. After 15 days of culturing, the cells were fixed, and live cell images were obtained. The well was from a 6-well tissue culture plate. Bottom image: A representative cluster of cells was imaged following crystal violet staining. Scale bars: 1 mm. (F) Total number of pixels in the images in E in conjunction with 5 additional independent experiments were calculated. These values were divided by the total number of mesenchymal cells seeded in each experiment and plotted as pixel area divided by the total cell number. This was compared with TECs grown in EpiCult. These cells were enumerated by cell counting, as shown. Statistical significance was determined by Student’s t test.

The plates were photographed using a ChemiDoc Imaging system (BioRad). Adherent colonies containing more than 20 cells were counted as a colony. For epithelial cells, the colonies were dispersed with 0.25% trypsin, washed, and enumerated with a hemocytometer.
Data and materials availability. The Df1/+ and Tbx1<sup>1neo2</sup> mouse lines described in this study were obtained from outside investigators, as indicated in this article. The scRNA-Seq data were deposited in the NCBI’s Gene Expression Omnibus (GEO) database (GEO GSE170686).

Statistics. Statistically significant differences among the different test groups were determined by 1-way ANOVA. A P value of less than 0.05 was considered significant. Data represent the mean ± SEM. As indicated in the figure legends, for 1-way ANOVA, Brown-Forsythe and Welch tests were sometimes applied, as indicated. A 1-tailed Student’s t test was applied for certain experiments in which only 2 distinct samples were compared, as indicated in the figure legends.

Study approval. Informed consent was obtained for human studies under a protocol approved by the IRB of UT Southwestern Medical Center (STU-072010-003, STU-2019-1087). The animal work described in this study was approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee (APN numbers 2015-101163 and 2015-101247).

Author contributions
PB, QD, MTDL-M, and NSCVO conceptualized the study. PB, QD, AK, CX, ID, AM, CAW, OBC, AB, and NSCVO designed the study methodology. PB, QD, AK, CX, ID, AM, CAW, OBC, TJP, MLM, MDLM, AB, NSCVO performed studies. PB, QD, AK, MDLM, AB, and NSCVO designed experiments and interpreted results. CAW, MDLM, and NSCVO acquired funding. CAW, TJP, MLM, MTDL-M, and NSCVO conducted clinical discussions. NSCVO supervised the study. QD and NSCVO wrote the original draft of the manuscript. PB, QD, CAY, TJP, MLM, MTDL-M, AB, and NSCVO reviewed and edited the manuscript.

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