Title: Mesenchymal cell replacement corrects thymus hypoplasia in murine models of 22q11.2 deletion syndrome

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Conflict of Interest

The authors have declared that no conflict of interest exists.

**One Sentence Summary:** Thymus hypoplasia in 22q11.2 deletion syndrome results from mesenchymal alterations
Abstract

22q11.2 deletion syndrome (22q11.2DS) is the most common human chromosomal microdeletion, causing developmentally linked congenital malformations; thymus hypoplasia, hypoparathyroidism and/or cardiac defects. Thymus hypoplasia leads to T cell lymphopenia, which most often results in mild SCID. Despite decades of research, the molecular underpinnings leading to thymus hypoplasia in 22q11.2DS remain unknown. Comparing embryonic thymuses from mouse models of 22q11.2DS (Tbx1\textsuperscript{neo2/neo2}) revealed similar proportions of mesenchymal-, epithelial- and hematopoietic- cell types as controls. Yet, the small thymuses were growth restricted in fetal organ cultures. Replacement of Tbx1\textsuperscript{neo2/neo2} thymus mesenchymal cells with normal ones restored tissue growth. Comparative single cell RNA sequencing of embryonic thymuses uncovered 17 distinct cell subsets, with transcriptome differences predominant in the 5 mesenchymal subsets from the Tbx1\textsuperscript{neo2/neo2} line. Transcripts impacted include extracellular matrix (ECM) proteins, consistent with increased collagen deposition seen in the small thymuses. Attenuating collagen cross-links with minoxidil restored thymus tissue expansion for hypoplastic lobes. In colony forming assays, the Tbx1\textsuperscript{neo2/neo2}-derived mesenchymal cells had reduced expansion potential, contrasting the normal growth of thymus epithelial cells. These findings suggest that mesenchymal cells are causal to the small embryonic thymuses in 22q11.2DS mouse models, correctable by substituting with normal mesenchyme.
Introduction

Chromosome 22q11.2 deletion syndrome (22q11.2DS) is the most common human microdeletion disorder reported, affecting approximately 1/4000 individuals (1-4). Patients with this syndrome have developmentally linked congenital malformations, thymus hypoplasia/aplasia resulting in low T cell levels, cardiac defects, hypoparathyroidism leading to low calcium and/or dysmorphic facial features (1, 3-5). Over time, 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 transcription factor (TBX1), 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 22q11.2DS patients have varying degrees of T cell lymphopenia due to their thymus 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 a mild SCID (15). Less than 1% of 22q11.2DS patients have thymus aplasia, resulting in a 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 thymus hypoplasia in 22q11.2DS (4, 20, 22).
Among these stromal 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 thymus seeding progenitors from the bone marrow, provide growth factors for thymocyte proliferation and express self-peptide/self-MHC complexes that dictate 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 thymus hypoplasia/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 sequencing (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 a thymus hypoplasia most often evident in those on a C57Bl/6 background. The small thymus severity and penetrance is Tbx1 gene dosage dependent in mouse models, with a more penetrant hypoplasia occurring when Tbx1 levels are < 50% normal (Supplemental Table S1) (10, 55-57). To determine which cell populations are causal to thymus hypoplasia, we used two mouse models of 22q11.2DS (Df1/+ and Tbx1\textsuperscript{neo2/neo2}) 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 normal and 22q11.2DS patients. With reaggregate thymus organ culture procedures, we report that that neural crest-derived mesenchymal cells are primarily responsible for the formation of size-restricted embryonic thymuses developing in the Tbx1\(^{neo2/neo2}\) mouse model of 22q11.2DS. Single cell RNA sequencing reveals 17 distinct cell subsets in the developing fetal thymus, with the 5 mesenchymal cell subsets and 1 endothelial population from the hypoplastic thymuses having the most divergent transcriptomes. The differentially expressed transcripts include extracellular matrix proteins such as collagens. We report that the drug minoxidil restores 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 impacting the thymus, heart and parathyroids (2-4). These are phenocopied in several mouse models of 22q11.2DS, including Tbx1 modified lines (10, 55-58). We noted a mildly penetrant thymus hypoplasia in the Df1/+ line, wherein a 1 Mb deletion orthologous to chromosome 22q11.2 is deleted, leading to a Tbx1 haploinsufficiency (Supplemental Figure 1A-B). The penetrance and severity of thymus hypoplasia is much higher in embryos from the Tbx1\(^{neo2/neo2}\) mouse model of 22q11.2DS due to ~35% normal levels of Tbx1 (Supplemental Table 1) (55). Thymuses were compared between Tbx1\(^{+/+}\), Tbx1\(^{+/neo2}\) and Tbx1\(^{neo2/neo2}\) e18-18.5 embryos, derived from Tbx1\(^{+/neo2}\) intercrosses (Figure 1A-B). Paired thymus lobes from the Tbx1\(^{neo2/neo2}\) embryos were consistently smaller than Tbx1\(^{+/+}\) or Tbx1\(^{+/neo2}\) controls (Figure 1A, black arrows). The magnitude of the size reduction was variable, much like
that reported for individuals with 22q11.2DS (Figure 1A, e.g., 1 vs. 2, Supplemental Table 1). In the mouse model, thymus hypoplasia co-presented with an interrupted aortic arch type B, also common in 22q11.2DS patients (Figure 1A, white arrow, movie S1) (55).

The thymuses were sectioned and compared with H&E staining and immunohistochemistry (IHC). Structurally, the small thymus lobes from the Tbx1 neo2/neo2 embryos resembled controls (Tbx1+/+ and Tbx1+/neo2) with cortical (dark area) and medullary (lighter area) regions present (Figure 1B, see boxed area). The magnitude of thymus size restriction was variable, with two representative phenotypes shown (Figure 1B, e.g., 3 and 4). Cortical TECs (cytokeratin 8), immature TECs (co-expression of cytokeratin 8 and 14) and small numbers of mTECs (cytokeratin 14) were evident, revealing a normal TEC composition in the Tbx1 neo2/neo2 embryos (Figure 1B). Note that at embryonic stages of thymopoiesis, mTECs are limited in number as they require SP thymocytes to develop. With regards to thymocyte development, percentages of double negative (DN; CD4 CD8-), double positive stage (DP; CD4+CD8+) and single positive thymocytes (SP; CD4+CD8- and CD4-CD8+) were similar in all the embryos (Figure 1C). Subdividing the DN thymocytes into defined developmental stages termed DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3 (CD44-CD25+) and DN4 (CD44-CD25-) revealed similar percentages of each subset in the hypoplastic lobes (Figure 1D). The major distinction with the Tbx1 neo2/neo2 thymuses was a statistically significant reduction in cellularity in the setting of normal percentages of DP, DN and TECs (Figure 1E). These data concur with normal percentages of DN, DP, and SP reported for most humans with 22q11.2DS (Supplemental Figure 2, Panel C) (15). Only in the rare cases of severe thymus hypoplasia/aplasia are major defects in thymopoiesis noted (Supplemental Figure 2, e.g., Pt.2).
We analyzed thymuses at earlier development times. Paired thymic lobes from e13-13.5 Tbx1\(^{neo2/ne2}\) embryos were smaller and more rounded than controls (Tbx1\(^{+/+;+/neo2}\)) (Figure 2A, dotted lines). A co-presenting interrupted aortic arch type B (IAA-B) was also evident (Figure 2A, white arrow). Despite being smaller, hypoplastic thymus lobes were structurally similar (Figure 2B, H&E staining e.g., 1 and 2). The hypoplastic lobes were not always paired symmetrically, as seen with a missing lobe in one section (Figure 2B). The capsular region (Pdgfra), vasculature (Pdgfrb) and TECs (cytokeratin) were evident in the Tbx1\(^{neo2/ne2}\) thymuses (Figure 2C). Between e13-e13.5, mesenchymal cells and TECs are the predominant stromal cell types in the thymus, with both present at similar percentages in hypoplastic lobes as controls (Figure 2D, F-G). In addition, the percentage of early thymus progenitor cells (ETPs) (CD45\(^+\)CD117\(^+\)CD25\(^-\)) was equivalent (Figure 2D, G). Overall, Tbx1\(^{neo2/ne2}\) embryonic thymuses had statistically significant lower cell numbers, including mesenchymal cells and TECs, with an average 3.4-fold lower cell number (4784 cells/lobe) than controls (16526 cells/lobe) (Figure 2F). This impacted mesenchymal cells and TECs equally, as their proportions were equivalent to controls (Figure 2F-G).

**Thymic lobes from 22q11.2DS embryos do not support thymus tissue expansion.** To examine the growth potential of the hypoplastic lobes, fetal thymus organ culture (FTOC) assays were performed (29, 59). Paired thymus lobes from e13-13.5 lobes Tbx1\(^{+/+}\) embryos increased in size, evident at 4- and 8-days of FTOC (Figure 3A-B). This was due to the rapid expansion of thymocytes, with 50-60% of the cells having reached the DP stage at these time points (Figure 3B). As the thymocytes expanded and differentiated, the percentages of mesenchymal (Pdgfra) and TECs (EpCAM\(^+\)) declined (Figure 3C). Tbx1\(^{neo2/ne2}\) thymus lobes were much smaller than controls by days 4 and 8 of FTOC, with the tissues often dispersed on the membrane (Figure 3A-
C, e.g., 1 & 2, Supplemental Figure 4). These thymuses had significantly lower numbers of cells and a reduced percentage of DP thymocytes, both at days 4 and 8 of FTOC (Figure 3D-E, Supplemental Figure 3). This correlated with the higher mesenchymal and TEC percentages (Figure 3D-E). Cell death and proliferation were also compared among the different genotypes. Overall, the levels of cell death, measured by co-expression of 7-AAD+ and Annexin V+, were very low (5%) in all the tissues. A small decrease in the percentage of mesenchymal cell death by day 4 was noted in the Tbx1 neo2/neo2 FTOC (Supplemental Figure 3). Yet, thymocyte proliferation was not statistically different among the groups compared, while the percentage of proliferating TECs (Ki-67+) was slightly reduced in the Tbx1 neo2/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, paired hypoplastic lobes were placed in FTOC surrounded by 4 pairs of control lobes. Despite the tissue expansion of the surrounding control lobes, the centrally positioned Tbx1 neo2/neo2 lobes did not expand (Supplemental Figure 4A-D). Taken together, our results reveal that at early stages of thymus formation, the thymus from the 22q11.2DS mouse model is 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 one or more of these cell types were causal to the thymus hypoplasia, reaggregate thymus organ cultures (RTOC) assays were performed (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, ETPs along with other hematopoietic cells). This modification enabled us to substitute different cell types from the Tbx1^{+/+;+/-neo2} and Tbx1^{neo2/neo2} thymuses prior to reaggregation with an equivalent starting number of reaggregated cells (minimum ~30,000 cells) (Figure 4A, Supplemental Figure 5).

Re-combining the 3 subgroups of sorted cells from control thymuses (Tbx1^{+/+;+/-neo2}) resulted in thymus tissue growth (Figure 4B-D). Thymocyte numbers increased, with DP and SP thymocyte subsets evident after 10 days of RTOC (Figure 4B-D). Reaggregating identical numbers and proportions of the 3 cell subgroups from the Tbx1^{neo2/neo2} hypoplastic thymuses failed to sustain normal tissue growth (Figure 4B-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 thymus tissue expansion and thymopoiesis (Figure 4B-D). A 10-fold increase in cellularity was observed, matching the cell numbers achieved with controls (Figure 4D-E). Cell viability and DP cell percentages also equaled control RTOC (Figure 4F-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 to control and mesenchymal substituted RTOCs (Figure 4D-G). Despite representing only 1-4% of the cells in the embryonic thymus, we also examined the contributions of endothelial cells. Replacing the Tbx1^{neo2/neo2} endothelial cells with normal ones (Sub Tbx1^{neo2/neo2} nEndo) had a similar effect as TEC substituted cultures, with only limited tissue expansion evident (Figure 4 B-G). These experiments establish that replacement of mesenchymal cells but not TECs or endothelial cells in the Tbx1^{neo2/neo2} thymuses restores tissue growth to normal levels. To determine if the Tbx1^{neo2/neo2} mesenchymal cells had a negative impact on thymopoiesis, RTOCs were performed with these cells used as substitutes for
normal mesenchyme (Sub normal Mes). While tissue expansion and T cell development was evident, these were not quite 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 impacted by 22q11.2DS remains undefined. To address this, we used single cell RNA sequencing (scRNA Seq) to identify and compare all the cell subsets present in embryonic Tbx1+/+ and Tbx1<sup>neo2/neo2</sup> thymuses. This technique enables a precise delineation of the different cell types in a developing tissue along with key transcriptome information (51, 61, 62). Hypoplastic thymic lobes from embryos harboring mutations in the *Forkhead Box N1 (Foxn1)* transcription factor were also used in our analysis since this gene is essential for TEC development (22, 63). Autosomal recessive mutations *Foxn1* (*Foxn1<sup>1089/1089</sup>; c.1089_1103del15;*) results in a similar thymus hypoplasia in mice as the Tbx1<sup>neo2/neo2</sup> lines (Supplemental Figure 7).

Cells from the various e13-13.5 lobes were encapsulated in nanoliter droplets with primer-containing beads for barcoding, followed by RNA isolation, cDNA synthesis, and sequencing. Between 5700 and 12,440 cells per thymus were used, providing an average read count of 69,000/cell (Supplemental Table 2). Unsupervised hierarchical clustering revealed 17 distinct clusters in control e13.0-13.5 thymus lobes (Figure 5A). Cellular identities were defined with singular and combinatorial gene signatures for mesenchymal cells (*Pdgfr-alpha* and *Col1A2*), epithelial cells (*EpCam* and various *keratin* genes), endothelial populations (*Cdh5* and/or *Pecam*), hematopoietic lineage cells (*Ptprc, Lck, Cd3d* and/or *Cd3g*) and red blood cells (*Hbb* genes) (Table 1). Additional lineage and cell type markers were selected based on their identification in previous RNA-Seq and scRNA-Seq experiments using normal embryonic
thymic lobes (51, 61, 62). Of the 17 distinct cell subsets, 5 mesenchymal subgroups (M-1 to M-5), 6 TEC subgroups (E-1 to E-6), 4 hematopoietic clusters (H-1 to H-4), 1 endothelial population (En-1) and 1 group corresponding to red blood cells (U-1) were identified (Figure 5A-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 (Table 1 and Supplemental Table 3). A complete list of all DEGs among the 17 clusters is provided in a Supplemental Excel Spreadsheet 1.

The number and composition of the cell clusters in the normal and hypoplastic thymus lobes was compared first. At e13-13.5, a normal thymus was majorly composed of non-hematopoietic cell types (Table 2). Relative to Tbx1+/+ controls, Tbx1neo2/neo2 thymuses lacked most of the E-5 population, had reduced cell numbers in E-1, E-3, E-4 and more cells in E-6 and M-5 (Figure 5A and Table 2). Compared to Foxn11089/1089, the Tbx1neo2/neo2 lobes were more impacted in clusters M-5 (7-fold increase), E-5 (16-fold decrease) and E-6 (26-fold increase) (Table 2). M-5 expressed Pdgfrb, which is a marker for mesenchymal cells that develop into pericytes and vascular smooth muscle (64). In E-6, the presence of Prl (prolactin), Bmp5 (Bone morphogenic protein 5) and the long noncoding RNA Rmst (possible neural crest cell marker) suggest a mixed population in the Tbx1neo2/neo2 embryonic thymuses that includes 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 (TEC subset) and H-3 (ETPs, early thymus 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 (Figure 5B, Supplemental Figures 8, 10-11). Heat maps were used to reveal the transcripts associated with mesenchymal, epithelial, and endothelial functions (Figure 5C). As demarcated with the dashed red box, mesenchymal cell subsets from Tbx1\textsuperscript{neo2/neo2} genotype had elevations in \textit{Pdgfrb}, \textit{FgfR1}, multiple collagens (\textit{Colla2}, \textit{Col3a1}, \textit{Col4a1}, \textit{Col5a1}) and a cluster of genes coupled to extracellular matrix proteins and growth factor receptors. Among these were \textit{Actn1} (actinin alpha 1), \textit{Adams2} (ADAM metallopeptidase with thrombospondin type 1), \textit{Capn6} (calpain 6), \textit{Eln1} (tropoelastin 1), \textit{Emilin1} (Elastin Microfibril Interfacer 1), \textit{Fbln5} (fibulin 5), \textit{Foxp1} (Forkhead box transcription factor p1), \textit{Fzd1} (Frizzled Family Receptor 1), \textit{Igfbp10} (IGF binding protein 10) also known as \textit{Cyr61} (Cysteine-rich angiogenic inducer 61), \textit{Mgp} (Matrix gla protein), \textit{Prdm6} (Pr domain containing protein 6, a histone-lysine methyltransferase), \textit{Pcolce} (Procollagen C-Endopeptidase enhancer), and \textit{Ctnnb1} (Beta catenin) (Figure 5C and Supplemental Figure 8). The 6 epithelial cell subsets (E1 to E6) had remarkably similar transcript levels comparing the control and Tbx1\textsuperscript{neo2/neo2} thymuses (Figure 5C). Similar levels of TEC specific transcripts were also revealed in e16.5 thymus lobes isolated from the Df1/+ mouse model of 22q11.2DS, comparing hypoplastic and normal paired lobes and controls (Supplemental Figure 1B, Supplemental Figure 9). This contrasts the dramatic TEC transcript differences among the E-1 to E-6 subsets uniquely impacted in the Foxn1\textsuperscript{1089/1089} thymus (Figure 5C). Therein, many of the key genes needed to support thymocyte trafficking and development were severely under-expressed (Supplemental Figure 10). Comparing the transcript levels in the single endothelial cell cluster also revealed some DEGs in Tbx1\textsuperscript{neo2/neo2} derived tissues that overlapped with those in the mesenchymal subsets (Figure 5C, Supplemental Figure 11).

Pathway analyses revealed Wnt/beta-catenin, tight junction, hepatic fibrosis, hotair, IL-8, integrin and ILK signaling pathways were all impacted in the Tbx1\textsuperscript{neo2/neo2} mesenchymal subsets
relative to control and Foxn1 thymuses (Figure 5D, Supplemental Table 4). Some of these same pathways were also impacted in the single endothelial cluster (Figure 5E). Taken together, the transcriptome data provide further evidence of a mesenchymal abnormality in the Tbx1\textsuperscript{neo2/neo2} thymuses that impacts endothelial tissues.

*Developmental alterations of the embryonic thymus in Tbx1\textsuperscript{neo2/neo2} lines can be over-ridden by blocking collagen cross-linking with minoxidil.* The scRNA comparisons revealed that mesenchymal cells had elevated transcript levels for extracellular matrix (ECM) proteins such as collagen. IHC was used to assess several such ECM proteins in e13.5 tissue sections (Figure 6A). Despite the reduced thymus size in the Tbx1\textsuperscript{neo2/neo2} lines, the vasculature was like controls (Figure 6A, e.g., 1 and 2). However, the hypoplastic lobes had higher levels of collagen (Figure 6A, e.g., 1 and 2). Additional ECM proteins including Cspg4 and Mcam were higher in both the Tbx1\textsuperscript{neo2/neo2} and Tbx1\textsuperscript{+/neo2} thymuses, with altered levels even more pronounced in/around the carotid artery (Figure 6B, yellow arrow). This suggests that some ECM changes are already occurring in the setting of a Tbx1 haploinsufficiency in the mouse model of 22q11.2DS. Such ECM changes were restricted to the tissues impacted by 22q11.2DS (thymus and heart), as the distribution of these proteins was normal in the vagal trunk (Figure 6B, light gray arrow). The endothelial layer, detected by CD31, was similar when comparing the various thymuses, suggesting normal vascularization had occurred in the hypoplastic thymuses (Fig. 6B). The scRNA sequencing data revealed an increase in the M-5 subtype, a population that represents pericytes, defined by Pdgfrb expression (Figure 5C) (64). This was confirmed by flow cytometric assays. Thus, a statistically significant increase in the percentage of Pdgfrb\textsuperscript{+}Pdgfra\textsuperscript{-/lo} cells was evident in the Tbx1\textsuperscript{neo2/neo2} thymuses relative to controls (Supplemental Figure 12).
To determine if increased ECM deposition and/or collagen cross-linking contributed to the thymus hypoplastic, we incubated RTOC in the presence of several inhibitors of collagen and ECM deposition. Among these were verteporfin, minoxidil and beta amino propionitrile (BAPN) (65-69). Verteporfin was toxic to the cultures while BAPN had no effect. Control RTOCs cultured with minoxidil had similar cellularity, cell viability and thymocyte subset percentages as those without the drug (Figure 7A-C). RTOC cultures with an equivalent number of cells from the Tbx1neo2/neo2 embryonic thymuses consistently failed to expand (Figure 7A-C and Figure 4). Tissue expansion in the Tbx1neo2/neo2 RTOCs was restored in the presence of minoxidil, as revealed with an increased cellularity and improved cell viability matching that of normal controls (Figure 7A-C). Only the percentage of DP cells, which increased in the presence of minoxidil, did not reach the same levels as with the control RTOC cultured with minoxidil (Figure 7C). Since minoxidil reduces the expression of enzymes linked to collagen deposition and cross-linking, including Plods and Col1a family members, we examined the transcript levels of these genes following FTOC. Normal FTOCs in the presence of minoxidil had statistically significant reduced expression of Plod1, Plod2, Col1a1 and Col1a2 at both days 3 and 4 of culture (Figure 7D). These experiments confirm that minoxidil impacts collagen deposition and cross-linking, effectively improving tissue expansion for embryonic hypoplastic thymuses from the Tbx1neo2/neo2 mouse model of 22q11.2DS (Figure 7D).

To expand on our observations that collagen/ECM deposition occurs in the setting of 22q11.2DS, IHC comparisons were done with human thymus tissue sections from controls and patients. With post-natal tissues, increased levels of collagen were selectively observed in the 22q11.2DS hypoplastic human thymuses (Supplemental Figure 13). These results suggest ECM
proteins such as collagen remain elevated in human thymuses from 22q11.2DS after the initial formation of the thymus.

The IHC, RTOC and scRNA sequencing results are consistent of a functional impairment among the Tbx1\(^{neo2/neo2}\) mesenchymal cells as opposed to TECs. To further address functional issues with this cell type, a mesenchymal colony forming cell (CFU) assay was undertaken. Mesenchymal cells (Pdgfra\(^+\)) were flow sorted from e13-13.5 thymuses using control (Tbx1\(^{+/+/neo2}\)) and Tbx1\(^{neo2/neo2}\) embryos. Equivalent cell numbers were cultured in Mesencult\(^{TM}\) media to induce colony formation. After 14-days in culture, the number of clusters of cells (CFUs/plate) was similar in control and Tbx1\(^{neo2/neo2}\) sorted mesenchymal cells, as seen with live cell imaging and crystal violet staining of one such cluster (Figure 7D-E). However, the Tbx1\(^{neo2/neo2}\) clusters had a statistically significant reduction in mesenchymal cell expansion (Figure 7D-F). This contrasted the normal differentiation/expansion of TECs from control and Tbx1\(^{neo2/neo2}\) thymuses, after these cells had been sorted and cultured in Epicult\(^{TM}\) differentiation media (Figure 7F).

Discussion

Stromal-epithelial cell defects leading to thymus hypoplasia are evident in several human conditions, including 22q11.2DS, CHARGE syndrome (Coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities), Nude/SCID (autosomal recessive \(FOXN1\) 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 herein that thymus hypoplasia/aplasia in 22q11.2DS is linked to mesenchymal cell problems. This was confirmed by RTOC, scRNA sequencing data, and
blocking ECM deposition in developing thymuses using the Tbx1

ne2/ne2 mouse model of 22q11.2DS. Importantly, normal fetal thymus mesenchymal cells restore thymus tissue growth and thymopoiesis when used as substitutes for those from the Tbx1

ne2/ne2 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 (32)). Prior studies have shown that wild-type embryonic thymuses, when stripped of the mesenchymal capsule, only expand upon re-addition 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 developmental is normal in capsule stripped thymuses. In the Tbx1

ne2/ne2 hypoplastic embryonic thymuses analyzed in the present study, higher levels of ECM proteins are apparent, and blocking collagen cross-linking with minoxidil restores tissue expansion to normal levels. Our findings are supported by recent experiments with human tissues. Blood vessel organoids, developed from induced pluripotent stem cells derived from 22q11.2DS patients, are smaller than controls, with upregulation of ECM and collagen evident (70). This results in diminished vascular developmental processes. During embryogenesis, thymus mesenchyme differentiates into pericytes, which envelope and support the emerging endothelial vasculature (31, 71). Eliminating mesenchyme in the developing chick embryo, done by ablating the neural folds, leads to both thymus hypoplasia and cardiac outgrowth vessel defects (72). This suggests that, like the thymus hypoplasia, the congenital heart malformations in 22q11.2DS may be linked to increased collagen cross-linking and ECM deposition (13, 73, 74). This was experimentally validated with the use of minoxidil, a drug that inhibits lysyl hydroxylases (LHs, encoded by Plods) (65, 66, 68). Minoxidil improves hypoplastic thymus
expansion in both FTOC and RTOC assays, which correlates with the inhibition of Plod1, Plod2, Col1a1 and Col1a2 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 the thymus growth, as it reportedly increases growth factor production (75). While our experiments did not reveal this possibility, there may be other effects imparted by minoxidil (Supplemental Fig. 5). At present, our data continue to support the idea that increased collagen cross-linking and subsequent EMC deposition is limiting thymus expansion in mouse models of 22q11.2DS, potentially impacting mesenchymal-endothelial cell functions.

The scRNA sequencing results provide additional evidence that mesenchymal cells and consequently, endothelial cells are affected by 22q11.2DS in the mouse model. While both normal and hypoplastic embryonic thymuses have the same 5 mesenchymal cell subsets (Table 1: M-1 to M-5), their transcriptomes are distinct. Several ECM transcripts are increased in the hypoplastic lobes. Many of these transcripts are coupled to tissue remodeling pathways including elevations in the Wnt signaling pathway (Figure 5D, Supplemental Table 4). Increased Wnt signaling disrupts thymus organogenesis (76). With regards to specific mesenchymal subsets, M1-M3 are the least impacted, while M-5 is over-represented in the hypoplastic lobes (Tbx1 neo2/ne2 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 neo2/ne2 setting, produces more ECM and collagens (Figure 5C). With the one endothelial cluster in the Tbx1 neo2/ne2 line, the transcriptome changes have some overlap with the mesenchymal pattern. While the endothelial cells represent only 1-4% of all the cells in an e13 thymus, their disrupted transcriptome is likely a direct consequence
of their interactions with mesenchymal cells. Current experiments are underway to define how these two cell types coordinate thymus tissue expansion in the embryo.

Comparing control, Tbx1\textsuperscript{neo2/neo2} and Foxn1\textsuperscript{1089/1089} thymuses provide strong evidence that TEC functions are normal in the 22q11.2DS mouse model. T cell development is similar, with only a delay in the developmental progression of thymocytes to the DP stage noted in FTOC. ScRNA sequencing reveals similar transcriptome patterns in the TEC subsets designated as E-1 to E-4 and E-6. Key transcripts needed for T cell development are present, including Foxn1, Ccl25, Psmb11, Prss16, Cd44 and AIRE (Figure 5B-C). One difference is an under-representation of the E-5 population. In control thymuses, E-5 retains some parathyroid related genes (Pth, Pax8, Chga), supporting prior evident that the developing thymus contains some parathyroid precursor cells (77). We also performed qRT-PCR with e16.5 thymus lobes using the Df1/+ mouse model of 22q11.2DS, comparing a hypoplastic lobe separated from its paired normal-sized lobe (Supplemental Figure 1B, 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 did reveal that 14 of the 22 mRNAs impacted by a hemizygous deletion of the chromosome 16 genes (22q11.2 equivalent) are expressed in the developing thymus (chromosome 16 in the mouse) (Supplemental Figure 9B). A prior study compared transcripts in the 3\textsuperscript{rd} PP in normal and Df1/+ embryos. Pax1, Hoxa3, Eya1, and Foxn1 are expressed at similar levels (78). Pax9 and Gcm2 were reduced (79). Taken together, the findings suggest that 22q11.2DS has a minimal impact on TEC functions despite their lower overall number in the hypoplastic lobes. While Tbx1 haploinsufficiency is responsible for the heart defects in 22q11.2,
it is not expressed in the embryonic thymus, and if forcibly expressed in this tissue, abrogates T cell development (80).

In summary, the neural crest-derived mesenchymal cells in embryonic thymuses from mouse models of 22q11.2DS have transcriptome alternations with increased production of ECM proteins such as collagen. These changes along with cell-cell interaction alterations impact both mesenchymal and endothelial cells. Our findings are important in the context of efforts to regenerate thymuses for patients with aplasia’s along with individuals who have thymectomies due to cardiac surgeries and the autoimmune disease myasthenia gravis, along with those 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, much needed in numerous clinical settings (32).

Methods

Human studies. Patients presented to the cardiothoracic group at Children’s Health, Dallas, for corrective surgeries due to 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 hematoxylin and eosin by the Molecular Pathology Core at UT Southwestern Medical Center.

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(16Es2el-Ufd1l)217Bld), was backcrossed over 12 generations onto the C57BL/6 mice (10, 81). These Df1/+ mice are haploinsufficient in ~22 orthologs of the genes spanning ~1Mb on human chromosome 22q11.2 (Supplemental Figure 1A). A second 22q11.2DS mouse model used the Tbx1+/neo2 line, provided by Dr. Antonio Baldini, which was already bred with a C57Bl/j background (Supplemental Figure 1A) (55, 82). There is a selective targeting of Tbx1 in this mouse line, which occurs via the insertion of neomycin in 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 purified using the miRVana kit (Ambion, Thermo-Fisher) and for small numbers of cells, the microRNA isolation kit (Zymo Research). Contaminating DNA was removed by DNase treatment (ABI-ThermoFisher) or in-column DNase digestion (Zymo Research). RNA was reverse transcribed (ABI-ThermoFisher) to cDNA. Quantitative RT PCR was performed (SYBr qPCR Master Mix, ThermoScientific) to check the expression of Plods and ECM genes, which were normalized to Gapdh. Single cell RNA sequencing and data analysis are described in the supplemental materials and methods.

**Fetal thymus organ culture and reaggregate thymus organ culture.** Fetal thymic organ culture assays and antibodies used for staining are detailed in supplementary materials and 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 in two, one set was stained with EpCAM-FITC, PDGFRa-PE, CD45-APC Cy7 and Anti-Annexin V antibodies. Annexin V staining was performed at the last step using an Annexin staining buffer (0.01 M
HEPES, 140 mM NaCl, and 2.5 mM CaCl\(_2\)). 7-AAD was added 10 minutes prior to analysis. The second set of cells was stained with EpCAM-FITC, PDGFRa-PE and CD45-APC-Cy7, followed by intracellular staining with anti-Ki67-APC following the manufacturer’s recommendations (Invitrogen Fix and Perm kit, Thermo-Fisher). Analyses done on an Aria Zelda.

For reaggregate assays, normal and hypoplastic fetal thymic lobes, between e13.0-13.5 gestational age, were isolated and collected in thymus organ culture media (TOC). This media consisted of RPMI, 20% fetal calf serum supplemented with HEPES, l-glutamine, sodium pyruvate, penicillin, streptomycin, 5 x 10\(^{-5}\) M 2-mercaptoethanol and non-essential 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, 0.02% EDTA at 37°C for 6-10 min, followed by pipetting until single cell suspensions were obtained. Digestion was stopped by addition of TOC media. Cells were washed, resuspended in volumes <250 µl/6-20 lobes and an aliquot counted with a hematocytometer. Cells were stained with antibodies specific for mesenchymal cells (Pdgfra-PE) and TECs (EpCAM-FITC) under sterile conditions. After washing, the cells were sorted into three populations; mesenchymal, epithelial and the remaining cells (EpCAM\(^+\) Pdgfra\(^-\); precursor thymocytes, dendritic cells, endothelial cells, macrophages). Sorting was done with an Aria Zelda FACS machine. RTOC was performed by reaggregating the 3 cell populations, EpCAM\(^+\) cell (~30%), Pdgfra\(^+\) (~30%) and EpCAM\(^-\) Pdgfra\(^-\) (~40%) in a 1.5 ml tube in varying combinations (Figure 4A). Cells were centrifuged consecutively for 5’ and 10’ at 1000 rpm and 2000 rpm, respectively. After the 2\(^{nd}\) spin, the supernatant was removed, leaving behind 2-4 µl of aggregated cells, which was 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. The filter was placed on top of a sterilize 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 of 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 prepared by gently squishing/pipetting of the lobes. Cells were counted using a hematocytometer. An aliquot was used for flow cytometry analysis after staining the cells with antibodies CD8-FITC, CD4-PE and TCRβ-PerCP-Cy5.5. Samples were analyzed on a FACS Caliber FACS Caliber (BD Bioscience), and data was analyzed via FlowJo (Tree Star Inc.). For RTOC assays in the presence of minoxidil, single cell suspensions from the thymus lobes were prepared. The cells were reaggregated in batches of 30,000 cells/group for RTOC. As indicated, the media was 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 was 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.

**Immunohistochemistry.** E10.5-e13.5 embryos and e18.5 fetal thymuses and fragments from human thymuses were fixed for 24 hours in 4% paraformaldehyde (in PBS) at 4°C. These were then dehydrated in a stepwise ethanol gradient of 25, 50, 75, and 100% ethanol, prepared in PBS. After a subsequent wash in xylene, the tissues were embedded in paraffin and sectioned (4 μm thick). Slides were de-paraffinized in xylene and rehydrated using a descending ethanol gradient (100, 95, 90, 80, 70, and 50% ethanol). Antigen retrieval was performed for 15 min at
95°C in Antigen retrieval R Buffer A pH 6 (Electron Microscopy). Slides were blocked in CAS Block (Invitrogen) for 2hr at RT. Anti-cytokeratin 14, anti-cytokeratin, anti-Pdgfra, anti-Pdgfrb, anti-E cadherin, anti-Laminin, anti CD31, anti Endomucin, anti-collagen I, anti Mcam, anti-Cspg4, and anti-SMA antibodies were used to stain slides O/N at 4℃. These are listed in Supplemental Table S5. Secondary antibodies were used according to 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).
Images were taken on a Keyence Fluorescence microscope and images were analyzed using Image J software. H&E staining was performed as described (83). Images were also taken on a Leica TCS SP5 confocal microscope and images were analyzed using Image J software. Sections stained with H&E were imaged on an Aziovert 200M inverted fluorescent microscope.

Mesenchymal and epithelial cell differentiation assays. E13-13.5 control and Tbx1 neo2/neo2 thymus lobes were prepared as per RTOC experiments. Mesenchymal cells (Pdgfra+) and TECs (EpCam+) were isolated by flow sorting as in RTOC. Between 6000-8000 cells/experiment were seeded into 6-well tissue culture plates containing RPMI-media supplemented with MesenCult™ Expansion or Epicult Expansion media for murine cells (MesenCult or EpiCult medica, Stem Cell Technologies, Vancouver, Canada). The cells were cultured at 37°C in 7.5% CO₂. The culture medium was changed every 4 days. After 14-days, the cell colonies in the mesenchymal cultures were rinsed in PBS and then fixed in 100% methanol. Clusters were visualized by staining in a 1% Crystal Violet solution (Sigma Chemical Co.). The plates were photographed using a Chemidoc™ Imaging system. 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.
Statistics. Statistically significant differences among the different test groups were determined by one-way ANOVA. A P value less than 0.05 was considered significant. Data represent the mean +/- SEM. As indicated in the figure legends, for one-Way ANOVA, Brown-Forsythe and Welch tests were sometime applied, as indicated in the legends. A 1-tailed Student’s t test was applied for certain experiments wherein only two distinct samples were compared, as presented in the figure legends.

Study Approval. Informed consent was obtained for human studies under a protocol approved by the Institutional Review Board at UT Southwestern Medical Center (STU-072010-003, STU-2019-1087). Animal work described in this manuscript has been 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
Conceptualization: QD, MDLM, NVO
Methodology: PB, QD, AK, CX, ID, AM, CAW, OBC, AB, NVO
Investigation: PB, QD, AK, CX, ID, AM, CAW, OBD, TP, MLM, MDLM, AB, NVO
Experimental Design and Interpretation: PB, QD, AK, MDLM, AB, NVO
Funding acquisition: CAW, MDLM, NVO
Clinical discussions and information: CAW, TP, MLM, MDLM, NVO
Supervision: NVO
Writing – original draft: QD, NVO
Writing – review & editing: PB, QD, CAW, TP, MLM, MDLM, AB, NVO
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Data and materials availability

The Df1/+ and Tbx1⁺/neo2 mouse lines described in this study were obtained from outside investigators, and this is indicated in our manuscript. The Foxn1¹⁰⁸⁹/¹⁰⁸⁹ mouse line was developed in the van Oers lab and is available upon request and if appropriate IACUC is received. The single cell RNA sequencing data is deposited at the GEO database (GSE170686).
Figures

Figure 1. Hypoplastic embryonic thymuses isolated from 22q11.2DS mouse models have normal proportions of thymocytes and TEC subsets. (A-E) E18-18.5 embryonic thymuses were obtained from Tbx1<sup>+/neo2</sup> intercrossed lines. (A) Live cell images from the cardiothoracic regions of Tbx1<sup>+/+</sup>, Tbx1<sup>+/neo2</sup>, and Tbx1<sup>neo2/neo2</sup> embryos are shown. Thymus lobes are indicated with black arrows. An interrupted aortic arch (white arrow) often co-presented with thymus hypoplasia in the Tbx1<sup>neo2/neo2</sup> embryos (e.g., 1). Bars within the images are 1 mm. (B) Thymus tissue sections were processed for H&E staining and immunohistochemistry (IHC). In the H&E images, cortical and medullary regions are dark and light purple, respectively. A medullary is indicated by the boxed area. With IHC, staining with antibodies selective for cortical (cytokeratin 8 = red) and medullary (cytokeratin 14, green) TECs is shown, while DAPI staining reveals nuclei (shown in blue). Co-expression of both cytokeratins (green and red) represent immature TECs. (C) T cell development was assessed by staining single cell suspensions with antibodies selective for the CD4 and CD8 co-receptor proteins. The 4 thymocyte subsets are distinguished by electronic gating for the CD4<sup>-</sup>CD8<sup>-</sup> (DN), CD4<sup>+</sup>CD8<sup>-</sup> (DP), and the CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>-</sup>CD8<sup>+</sup> (SP) subsets. (D) DN cells are further categorized by CD44 and CD25 cell surface expression. This identifies the DN1 (CD44<sup>+</sup>CD25<sup>-</sup>), DN2 (CD44<sup>+</sup>CD25<sup>+</sup>), DN3 (CD44<sup>-</sup>CD25<sup>+</sup>) and DN4 (CD44<sup>-</sup>CD25<sup>-</sup>) subpopulations in the Tbx1<sup>+/+</sup>, Tbx1<sup>+/neo2</sup> and Tbx1<sup>neo2/neo2</sup> thymuses. (E) The total cell number and the percentages of DP thymocytes, DN4 subpopulation of DN thymocytes and cTECs was compared among the Tbx1<sup>+/+</sup> (n = 7-10), Tbx1<sup>+/neo2</sup> (n = 10-15) and Tbx1<sup>neo2/neo2</sup> (n = 4-7) genotypes. Statistically significant differences among the 3 groups were determined by one-way ANOVA (Brown-Forsythe and Welch tests).
A. E18-18.5 fetal thymic lobes

B. Tbx1^+/+ Tbx1^+/neo2 Tbx1^neo2/neo2 e.g.1 Tbx1^neo2/neo2 e.g.2

Top row 10X; H&E
Middle row 20X; red = cytokeratin 8 (cTECs); green = cytokeratin 14 (mTECs); blue = DAPI
Bottom row 20X; cytokeratin 14 (immature TECs, mTECs)

C. Thymocyte subsets

D. DN1-DN4 subsets

E. Table of cell number, DP percentage, DN4 percentage, and cTEC percentage

- **Cell number**
  - Tbx1^+/+
  - Tbx1^+/neo2
  - Tbx1^neo2/neo2

- **Percent DP thymocytes**
  - Tbx1^+/+
  - Tbx1^+/neo2
  - Tbx1^neo2/neo2

- **Percent DN4 thymocytes**
  - Tbx1^+/+
  - Tbx1^+/neo2
  - Tbx1^neo2/neo2

- **Percent cTECs (Ly5^+)**
  - Tbx1^+/+
  - Tbx1^+/neo2
  - Tbx1^neo2/neo2

- **p-values:**
  - p = 0.0002
  - ns (not significant)
Figure 2. Hypoplastic embryonic thymuses have similar proportions of mesenchymal cells and TECs as normal control tissues. (A-G) E13.13.5 embryonic thymuses from Tbx1+/neo2 intercrossed time pregnant mice were genotyped and analyzed by live cell imaging, IHC and flow cytometry. (A) Live cell images reveal the size and location of the developing thymus in the Tbx1+/+, Tbx1+/neo2 and Tbx1neo2/neo2 embryos (demarcated with dotted lines). In the Tbx1neo2/neo2 genotype, an interrupted aortic arch type B is routinely visualized (white arrow). Bars are 0.5 mm. (B) Transverse sections comprising the thymus region were processed for H&E. Black arrows point to the thymus, with Trachea (Tr) and Esophagus (Eso) locations shown. Bars are 50 μm in length. In the Tbx1neo2/neo2 genotyped lines, the thymus lobes are not always in the same plane of the transverse section. (C) Immunohistochemistry (IHC) was performed with antibodies selective for neural crest cell-derived mesenchymal cells, marking the thymus capsule (Pdgfra, red) and thymus vasculature (Pdgfrb, yellow), along with antibodies specific for thymus epithelial cells (EpCAM, green). Two examples of hypoplastic thymuses from Tbx1neo2/neo2 embryos are shown (e.g., 1 and 2). Bars are 50 μm. (D-E) Flow cytometric analyses of single cell suspensions reveal the percentages of (D) mesenchymal (Pdgfra+) and epithelial cells (EpCam+) and, (E) early thymus progenitors (ETPs) co-expressing CD117 (c-kit) and CD45. (F) The total thymus cell number and the specific numbers of mesenchymal cells and epithelial cells is enumerated from multiple Tbx1+/+, Tbx1+/neo2 and Tbx1neo2/neo2 embryos. In addition, the ratio of mesenchymal and TECs is shown (Mes/TECs). The Tbx1+/+ (n = 17), Tbx1+/neo2 (n = 32) and Tbx1neo2/neo2 (n = 28) genotyped embryos were used to determined cell numbers. (G) With the same thymus tissues characterized in (F), the percentages of mesenchymal, epithelial, and early thymocyte progenitors (ETPs) are shown. TEC and ETP percentages were determined from a smaller number of mice, Tbx1+/+ (n = 9-15), Tbx1+/neo2 (n = 9-20) and Tbx1neo2/neo2 (n = 6-17). Statistically significant differences were established by one-way ANOVA (Brown-Forsythe and Welch tests).
Fig. 2

A  E13-13.5 embryos

Tbx1\textsuperscript{+/+}  

Tbx1\textsuperscript{neo2/neo2}  

B  E13-13.5 embryos

Tbx1\textsuperscript{+/+}  

Tbx1\textsuperscript{neo2/neo2}  

C

Tbx1\textsuperscript{+/+}  

Tbx1\textsuperscript{+/neo2}  

Tbx1\textsuperscript{neo2/neo2 e.g.1}  

Tbx1\textsuperscript{neo2/neo2 e.g.2}  

Pdgfra = red; Pdgfrb = yellow; cytokeratin = green

D  Mesenchymal & epithelial cells

Tbx1\textsuperscript{+/+}  

Tbx1\textsuperscript{+/neo2}  

Tbx1\textsuperscript{neo2/neo2}  

E  Thymic progenitors

Tbx1\textsuperscript{+/+}  

Tbx1\textsuperscript{+/neo2}  

Tbx1\textsuperscript{neo2/neo2}  

F

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Figure 3. Hypoplastic fetal thymus lobes from 22q11.2DS mouse models have diminished thymopoiesis potential in culture. (A) Paired normal-sized (Tbx1+/+ or Tbx1+/neo2) and hypoplastic (Tbx1neo2/neo2) thymus lobes (e13-13.5) were cultured for 4- and 8- days. Live cell imaging revealed the changes in thymus size, which is limited in the Tbx1neo2/neo2 22q11.2DS mouse model. Yellow bar = 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 beta subunit. (C) Percentages of mesenchymal cells (Pdgfra+) and TECs (EpCAM+) was determined after 4- and 8-day cultures via flow cytometric analyses. (D) After 4 and 8 days of FTOC, thymus lobes were processed and total cell numbers along with the percentages of mesenchymal cells (Mes), TECs and DP thymocytes determined. Tbx1+/+ (n = 8), Tbx1+/neo2 (n = 14) and Tbx1neo2/neo2 (n = 12) embryonic thymuses were used. (E) Eight days post FTOC, total cell numbers and percentages of live cells, DP thymocytes and TECs were determined. Note that by day-8, relatively few Pdgfra-positive cells remain 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 one-way ANOVA (Brown-Forsythe and Welch tests).
Figure 4. Tissue expansion is restored for hypoplastic thymuses by replacement of Tbx1<sup>neo2/neo2</sup>-derived mesenchymal cells with normal controls. (A) Depiction of reaggregate thymic organ culture assays (RTOC) using flow sorted cells. Single cell suspensions from e13-13.5 fetal thymic lobes were prepared and mesenchymal cells (Pdgfra<sup>+</sup>), TECs (EpCam<sup>+</sup>) and the remaining unstained cells (Pdgfra<sup>-</sup>Epcam<sup>-</sup>; which includes ETPs, other hematopoietic cells and endothelial cells) are sorted by flow cytometry. These 3 subgroups were re-aggregated at cell ratios established with control fetal thymuses and placed onto membranes and cultured. A minimum of 30,000 cells/aggregate is needed to sustain RTOC growth with normal cells (Supplemental Figure S5). The aggregates appear as a small dot in the yellow circled area. Endothelial cell replacements required sorting CD31<sup>+</sup> cells from the remaining cell subsets prior to reaggregate culturing. (B) Live cell imaging was used to visualize RTOCs after 10-days of culture. Control corresponds to the 3 subgroups of cells from Tbx1<sup>+/+</sup>;+/neo2 thymus lobes. In the first column, control thymuses are a combination of cells from either Tbx1<sup>+/+</sup> and/or Tbx1<sup>+/neo2</sup> embryos. In the second column, 22q11.2DS hypoplastic thymuses were from Tbx1<sup>neo2/neo2</sup> embryos. In the third column, normal mesenchymal cells were used as substitutes for those in the 22q tissues (Sub Tbx1<sup>neo2/neo2</sup> Mes). In columns 4 and 5, normal TECs or endothelial cells were used as substitutes for Tbx1<sup>neo2/neo2</sup> TECs (Sub Tbx1<sup>neo2/neo2</sup> TECs) or endothelial cells (Sub Tbx1<sup>neo2/neo2</sup> Endo), respectively. Yellow bar = 1 mm. (C) Cell viability (upper row) and thymopoiesis (DN to DP and then SP progression, lower row) were 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 number is shown following 10-days of RTOC along with the cell viability and percentage of DP cells developing over this period. The number of experiments per group, represented in each column were 37, 28, 13, 8 and 5, respectively. Statistical analyses done with One-Way ANOVA (Brown-Forsythe and Welch tests).
Figure 5. Single cell RNA sequencing reveals distinct transcript levels in mesenchymal cells, TECs and endothelial cells in embryonic thymuses from control, Tbx1neo2/neo2 and Foxn1 mutant mouse models (A) Fetal thymuses, obtained from normal, Tbx1neo2/neo2 and Foxn11089/1089 e13-13.5 embryos, were used for single cell RNA sequencing. tSNE plots reveal 17 distinct cell subgroups for all 3 paired thymus lobes (Tbx1+/+, Tbx1neo2/neo2 and Foxn11089/1089 genotypes), with the relative percentages of these subgroups differing among the 3 genotypes. Five distinct mesenchymal cell clusters (M-1 to M-5), 6 epithelial groups (E-1 to E-6), an endothelial population (En-1), 4 hematopoietic cell types (H-1 to H-4), a red blood cell (U-1) and a mitochondrial signature are present in each of the thymuses. tSNE plot for the Foxn1 hypoplastic lobes (Foxn11089/1089) was generated by modifying the total number cells to 6000. (B) Transcripts that define cell subsets were compared among the 5 mesenchymal, 6 epithelial and 4 hematopoietic clusters. A dot plot comparison reveals key gene expression differences among the various populations. (C) A heat map reveals the differential expression of transcripts of biological importance for mesenchymal and epithelial clusters along with the one endothelial cluster, respectively. Regions boxed in red represent the Tbx1neo2/neo2 thymus. (D) Pathway enrichment analyses in mesenchymal cluster and Endothelial cluster (E) with the differentially expressed genes reveals key distinctions between control, Tbx1neo2/neo2 and Foxn11089/1089 fetal thymuses.
Figure 6. Elevated deposition of collagen is evident in hypoplastic thymuses from Tbx1\(^{neo2/neo2}\) embryos. (A) E13-13.5 thymuses from the indicated embryos were prepared for IHC. Staining is done with antibodies detecting Collagen I (green) and a combination of CD31 and endomucin (Red). Sections are prepared from Tbx1\(^{+/+}\), Tbx1\(^{+/neo2}\) and Tbx1\(^{neo2/neo2}\) embryonic thymuses. Two different Tbx1\(^{neo2/neo2}\) embryos are shown for comparative purposes. The merged image combines the collagen, CD31/endomucin and DAPI staining (nuclei). Blue arrow points to the thymus. (B) IHC was performed on embryos from the Tbx1\(^{+/+}\), Tbx1\(^{+/neo2}\) and Tbx1\(^{neo2/neo2}\) genotypes. Antibodies selective for Cspg4 (green), Mcam (purple) and CD31/endomucin are independently shown along with a merged image comprising all the stains. The blue arrow reveals the thymus, the yellow arrow the carotid artery and the light gray arrow the vagal trunk.
E13-13.5 Thymuses

Collagen = green; CD31 = Red, DAPI = blue

Csg4 = green; Mcam = purple, CD31/endomucin = red, DAPI = blue
Figure 7. The presence of minoxidil in RTOC cultures restores tissue growth for hypoplastic thymuses. Reaggregate thymic organ culture assays (RTOC) were performed using cell suspensions generated from e13-13.5 fetal thymic lobes. The cells from either normal or Tbx1neo2/neo2 thymuses were reaggregated with equivalent starting clusters of ~30,000 cells/group. Cultures were maintained in media alone or supplemented with 3 \( \mu \text{M} \) minoxidil. (A) Live cell imaging reveals cell expansion after 10-days of culture. (B) Thymopoiesis was compared using antibodies specific for CD4 and CD8. (C) Cell number, cell viability and the percentage of DP cells are shown. Note that the numbers of cells in Tbx1neo2/neo2 is severely limited, as established in Figure 4B, D-E. \( n = 10, 10, 3, 3 \) for the indicated groups from left to right in the columns. Statistical analyses done with One-Way ANOVA. (D) Control FTOC assays were cultured in the absence or presence of minoxidil. At 3- and 4-days post culture, the cells were processed for qRT-PCR using probes detecting two Plods and two Col1a genes, along with gapdh for normalization. Day 3, \( n = 5 \) and for day 4, \( n = 4 \). (E) Mesenchymal cells and TECs were flow sorted from e13-13.5 embryonic thymuses from Tbx1\(^{+/+}\) or Tbx1\(^{neo2/neo2}\) embryos. Mesenchymal sorted cells were grown in Mesencult\textsuperscript{TM} differentiation media. After 15-days of culture, the cells were fixed, and live cell images obtained. The well was from a 6-well tissue culture plate. Lower part, a representative cluster of cells was imaged following crystal violet staining. Bar is 0.5 mm in length. (F) The total number of pixels in the images shown in (E) in conjunction with 5 additional independent experiments was 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 added. This was compared with TECs grown in Epicult\textsuperscript{TM}. These cells were enumerated by cell counting, as shown. Statistical analyses done with Student’s T-test.
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*a* Uniquely elevated in Tbx1<sup>neo2/neo2</sup> thymus

*b* Uniquely reduced (E-3, E-5) or elevated (E-6) in Tbx1<sup>neo2/neo2</sup> versus Foxn1<sup>1089/1089</sup> hypoplastic thymus
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<sup>a</sup>Uniquely elevated (red)

<sup>b</sup>Uniquely reduced (blue)
Supplementary Materials

Methods

Supplemental Figure 1. Embryos from the Df1/+ mouse model of 22q11.2DS exhibit an infrequent hypoplasia of the thymus.

Supplemental Figure 2. Human thymuses from 22q11.2DS patients have variable phenotypes, with most having normal T cell development.

Supplemental Figure 3. Tbx1<sup>neo2/neo2</sup> hypoplastic lobes have diminished numbers of percentages of cells, including DP thymocytes and TECs after 8-days of FTOC.

Supplemental Figure 4. Normal thymus lobes that surround Tbx1<sup>neo2/neo2</sup> hypoplastic lobes do not enable tissue expansion to the small tissues.

Supplemental Figure 5. Reaggregate fetal thymic organ cultures require a minimum number of cells to support thymopoiesis.

Supplemental Figure 6. Mesenchymal cells from the Tbx1<sup>neo2/neo2</sup> embryonic thymuses do not antagonize normal thymus expansion in RTOC.

Supplemental Figure 7. Hypoplastic embryonic thymuses from Foxn1 knock-in mice are blocked in early T cell development with a significant reduction in early thymus progenitors.

Supplemental Figure 8. Mesenchymal cell transcript alterations in Tbx1<sup>neo2/neo2</sup> embryonic thymuses.

Supplemental Figure 9. Small thymuses from the Df1/+ mouse model of 22q11.2DS exhibit a distinct RNA signature compared to normal sized thymuses.

Supplemental Figure 10. Epithelial cell transcript alterations in Tbx1<sup>neo2/neo2</sup> embryonic thymuses.

Supplemental Figure 11. Endothelial cell transcript alterations in Tbx1<sup>neo2/neo2</sup> embryonic thymuses.
Supplemental Figure 12. Tbx1\textsuperscript{neo2/neo2} thymuses have a significantly increased percentages of Pdgfrb\textsuperscript{+}Pdgfra\textsuperscript{-/lo} mesenchymal cells.

Supplemental Figure 13. Human thymuses from 22q11.2DS patients have increased collagen levels relative to normal controls.

Supplemental Table 1. Comparative congenital malformations between human 22q11.2DS and mouse models.

Supplemental Table 2. Single cell RNA sequencing information with the indicated embryonic thymuses isolated at e13-13.5.

Supplemental Table 3. Top 20 gene identifiers for the 17 distinct cell clusters identified by scRNA sequencing.

Supplemental Table 4. Significantly altered pathways in mesenchymal subsets comparing Tbx1\textsuperscript{neo2/neo2} and Foxn1\textsuperscript{1089/1089} to wildtype controls

Supplemental Table 5. Key reagents, antibodies and oligonucleotides used in the study.

Excel Data File 1. Differentially expressed genes identified among all 17 cell subsets identified using scRNA comparing control, Tbx1\textsuperscript{neo2/neo2} and Foxn1\textsuperscript{1089/1089} embryonic day 13-13.5 thymuses.

Movie 1. The movie reveals the blood flow through the aorta with an interrupted aortic arch type B in one of the Tbx1\textsuperscript{neo2/neon2} embryos. The imaging required removal of the thymic lobes.

Supplemental References