Supplementary Information – Materials and Methods

Cell Isolation and Culture

Discarded thymus tissue from infant heart operations were mechanically minced into < 3mm fragments under sterile conditions. Tissue fragments were placed in 100 mm culture dishes (≈15 tissue fragments/dish) and submerged in MSC media (Dulbecco's modified Eagle medium, with high-glucose concentration, GLUTAMAX I, 10% heat inactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin, all from Gibco, Carlsbad, CA). Tissue fragments were incubated for 10 days prior to removal. MSCs that had migrated from the tissue explants were allowed to achieve 80% confluence prior to passaging with trypsin/EDTA (Gibco). Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 (both from Lonza, Basel, Switzerland) with growth factors under standard conditions, unless stated otherwise. Unless specified otherwise, all experiments were performed with cells from passages 3-9.

CFU-F Efficiency and Limiting Dilution Assay

For colony forming unit efficiency (CFE) determination, thymus MSCs were diluted in MSC medium in singe cell suspension and plated at about 100 cells per 10 cm tissue culture dishes (Falcon). After incubation with media changes every 2-3 days, cells were washed with PBS and fixed for 10 min with ice-cold 100% methanol, then stained with 0.5% Crystal Violet in methanol for 10 min at room temperature. Cells were washed with deionized water and colonies with >50 cells were counted and recorded.

Fibroblastic colony forming unit (CFU-F) prevalence was also estimated by a limiting dilution assay (LDA) [1]. Cells were seeded in a 96 well plate at 500, 250, 5 and 3 cells per well (24 replica for each condition). After 14 days of culture, thymus MSCs were washed with PBS and then stained with 0.1% crystal violet in methanol for 20 min at room temperature. The wells were then washed with deionized water and

then then inspected for the presence of colonies. CFU-F frequency was then calculated using L-Calc Software (STEMCELL Technologies, Vancouver, Canada).

Thymus MSC Growth Kinetics

Growth kinetics of thymus MSCs isolated from 4 patients were studied with a cumulative population doubling analysis. The MSCs obtained from the thymus tissue explant cultures were designated as passage 0 (P0). P0 thymus MSCs were counted by hemacytometer and replated at a 1:10 dilution. Cells were passaged and counted on a weekly basis and a subset replated at a 1:10 dilution. The average cell number (*C*) was calculated and recorded for each passage (*n*) and used to generate a growth curve. The population doubling level (*PDL*) and doubling time (*DT*) were calculated using the following equations:

$$PDL = 3.32\,(\log N - \log N_0)$$

and

$$DT = T\frac{\ln 2}{\ln\left({}^{N}/_{N_0}\right)}$$

where *N* is the final number of cells ($N = C\times10^{n}$) , N_0 is the initial number of cells seeded and *T* is the time in culture.

Multilineage Differentiation

The ability of thymus MSCs isolated from 3 patients to differentiate into osteogenic, adipogenic and chondrogenic lineages was investigated using the Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's directions. After osteogenic, adipogenic and chondrogenic differentiation, cells were stained for osteocalcin, Oil Red O and anti FABP-4 antibody, and anti-aggrecan antibody, respectively according to the manufacturer's

directions. Thymus MSCs and sections were then imaged using a NikonA-1 spectral confocal microscope (Nikon Instrument Inc., Japan).

Flow Cytometric Analysis

Surface markers of passage 4-9 thymus MSCs isolated from 6 patients were characterized by flow cytometry using fluorochrome conjugated anti-human CD29, CD44, CD45, CD90, CD105, CD73, CD166, CD49e, CD56, STRO-1, CD271, SSEA-4, HLA class I (ABC), HLA class II (DR) and nestin (all from BD Biosciences, San Jose, CA, except Stro1 which was purchased from BioLegend, San Diego, CA). The antibodies were incubated with thymus MSCs for 60 minutes at room temperature followed by three washes. Thymus MSCs were then analyzed using a MoFlo® Astrios™ flow cytometer (Beckman Coulter, Inc.) using the appropriate isotype-matched and unstained controls.

Pluripotency Gene Expression Analysis

Pluripotency gene expression was determined in thymus MSCs isolated from 5 neonates and human induced pluripotent stem cells (hiPSCs, generously supplied by Dr. Eric Devaney). Thymus MSCs from passage 5 or 6 and hiPSCs were harvested for total RNA isolation. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was carried out using the High capacity cDNA Reverse Transcription kit with random primers (Applied Biosystems, Invitrogen). Quantitative real-time polymerase chain reaction was performed in StepOne Plus Real time PCR system (Applied Biosystems) with a reaction mixture (10 µL) containing cDNA, forward and reverse primers (Supplemental Table 1), and 1× iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Expression of each gene was normalized to the expression of β-actin. Mean cycle threshold (Ct) value was calculated as the average of triplicates for each gene, and the fold change in gene expression was calculated based on $2^{-\Delta\Delta CT}$ method.

CD248 Expression

Thymus MSCs were cultured in 35 mm glass bottom culture dishes (MatTek Corporation, Ashland, MA). Cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) for 20 min, washed twice with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. Cells were then incubated overnight with anti-TEM/CD248 rabbit anti-human polyclonal, c-terminal antibody (LifeSpan BioSciences, Inc., Seattle, WA) at 4^{o}C. Cells were washed with PBS, incubated with goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR) while shielded from light for 2 hours and then counterstained with DAPI. Stained cells were then imaged with a confocal microscope.

2D Angiogenesis Assay

Thymus MSCs and HUVECs were plated in basal EGM-2 (no growth factors) in 12-well plates (3×10^4 cells per well, 1:3 ratio of thymus MSCs to HUVECs for combination group) coated with fibrin hydrogel. HUVECs were also plated on fibrin hydrogel with EGM-2 media supplemented with growth factors. Fibrin hydrogel was generated by adding 250 µL of thrombin (10 U/mL) and 100 µL of fibrinogen (20 mg/mL) (both from Sigma, St. Louis, MO) to each well of a 12-well plate and allowed to polymerize for 1 hour prior to the addition of cells. After selected time intervals, tube formation was examined with a phase-contrast microscope (Zeiss, Oberkochen, Germany).

Multicellular Spheroid Generation

Cell cultures were dissociated using trypsin/EDTA, centrifuged and resuspended in EGM-2 media containing 0.275% methylcellulose (Sigma). Cells in suspension were then seeded on the lid of a non-adhesive petri dish (20µL per drop) containing 600 cells/drop for the HUVEC group and 200 cells/drop for the thymus MSC groups. Unless otherwise indicated, HUVEC + thymus MSC group contained a total

of 800 cells/spheroid with a 1:3 thymus MSC to HUVECs composition. Hanging drops were then incubated overnight at 37°C and 5% CO_2 to allow for spheroid formation.

Angiogenic Gene Expression Analysis

To gain insight into the findings demonstrated by the spheroid and monolayer sprouting assays, we evaluated differential gene expression for VEGFA, b-FGF and HIF1-□ in the different groups and culture conditions using qPCR. After 48 hours of culture, fibrin gels containing spheroids or cell monolayers were washed with PBS and then digested with 500 □l of nattokinase (50 FU/ml, NSK-SD; Japan Bio Science Laboratory Co., Ltd) in PBS containing 1mM EDTA [2]. Fibrin gels were then incubated at 37°C for 30 min until completely digested. Digests were centrifuged and cell pellets were washed with PBS. For experimental details of qPCR, please see above for pluripotency gene analysis.

Vessel Density Determination

Construct and adjacent tissue vessel density quantification was performed in similar fashion to a method developed for intratumoral microvessel density determination [3]. Digital images were acquired from random hematoxylin and eosin sections (n=5/group) of vascular hotspots under 200x magnification. Blood vessels were identified by definitive lumens encompassing red blood cells.

Supplementary Information – Methods References

1. Schellenberg A, Hemeda H, Wagner W. Tracking of replicative senescence in mesenchymal stem cells by colony-forming unit frequency. **Methods in molecular biology**. 2013;976:143-154.
2. Carrion B, Janson IA, Kong YP et al. A Safe and Efficient Method to Retrieve Mesenchymal Stem Cells from Three-Dimensional Fibrin Gels. **Tissue Eng Part C-Me**. 2014;20:252-263.

3. Weidner N. Chapter 14. Measuring intratumoral microvessel density. **Methods in enzymology**. 2008;444:305-323.

Supplemental Figures and Table Legends

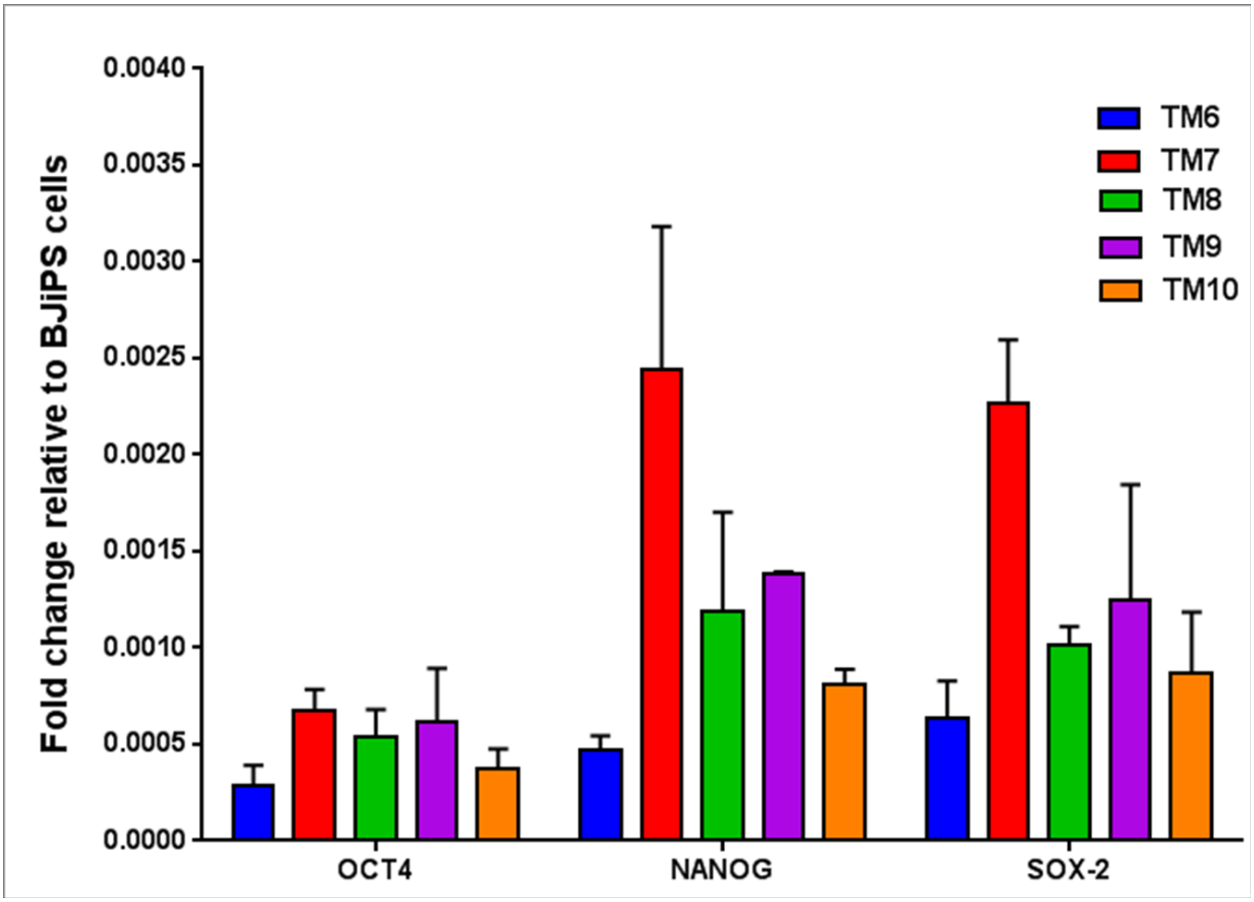

Figure S1. Neonatal human thymus MSCs express pluripotency-related genes. Low level expression of *Oct-4*, *Sox-2* and *Nanog* was demonstrated in thymus MSCs isolated by explant culture from 5 different patients.

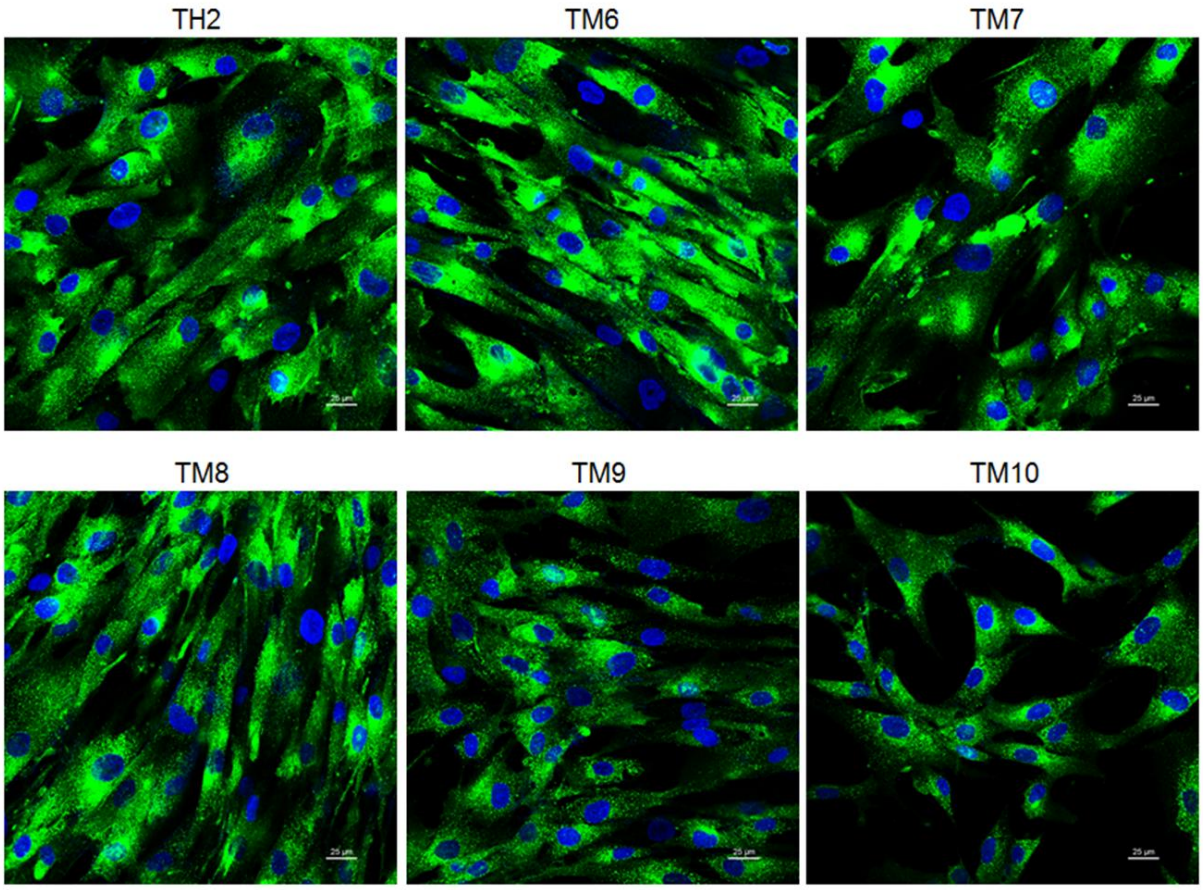

Figure S2. Neonatal human thymus MSCs express CD248. Immunofluorescence images of thymus MSCs isolated from 6 different patients using the explant culture technique demonstrates consistent expression of CD248 regardless of donor. Scale bar = 25 μm.

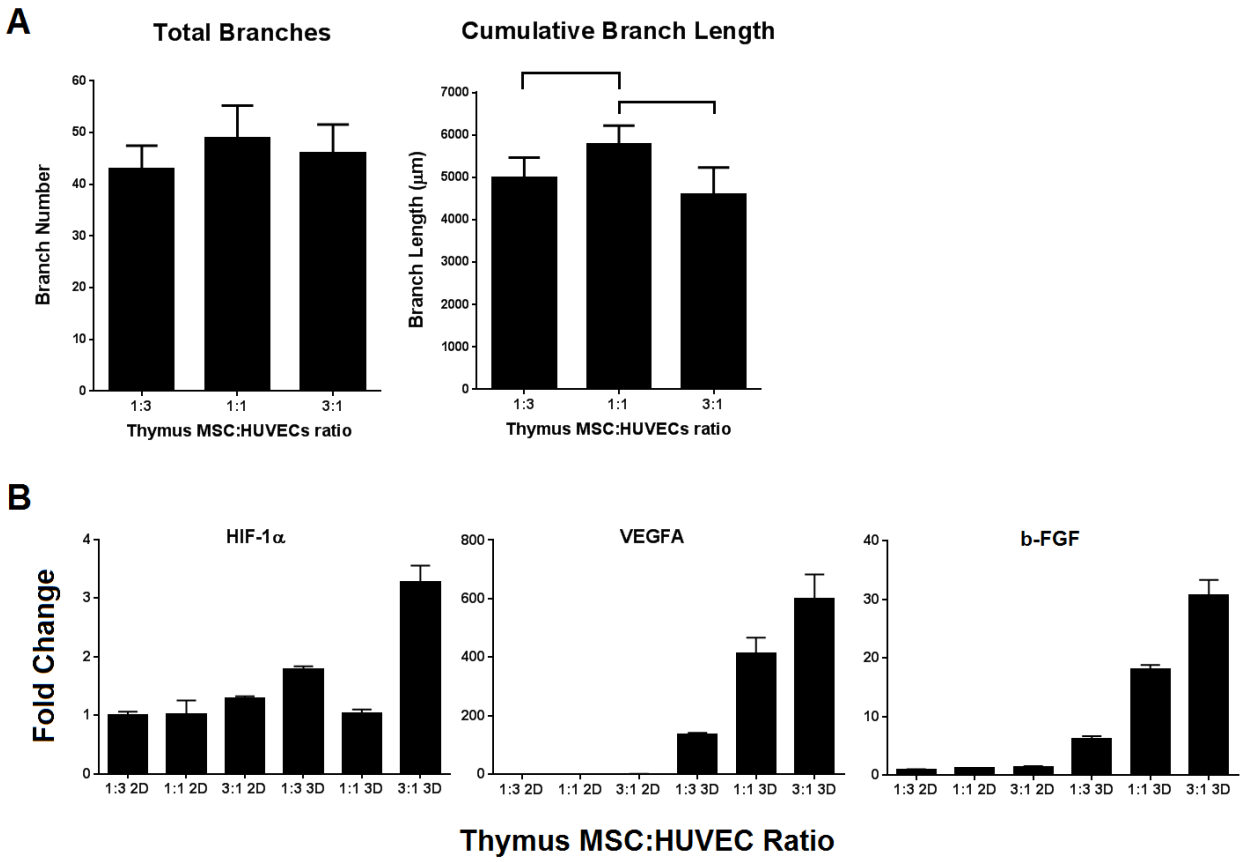

Figure S3. Effects of cellular composition on heterotypic spheroid sprouting and angiogenic gene expression. **(A):** Varying the thymus MSC:HUVEC ratio yielded no significant changes in branches but a 1:1 ratio yielded the greatest cumulative branch length. (B): Angiogenic gene expression was affected by 3D culture and by thymus MSC:HUVEC ratio. Spheroid (3D) groups with the highest thymus MSC ratio (3:1) had significantly higher expression of angiogenic genes as compared to other spheroid ratio and all 2D groups ($p<0.05$). All data are mean±SD and groups compared using two-way ANOVA with post hoc Tukey test.

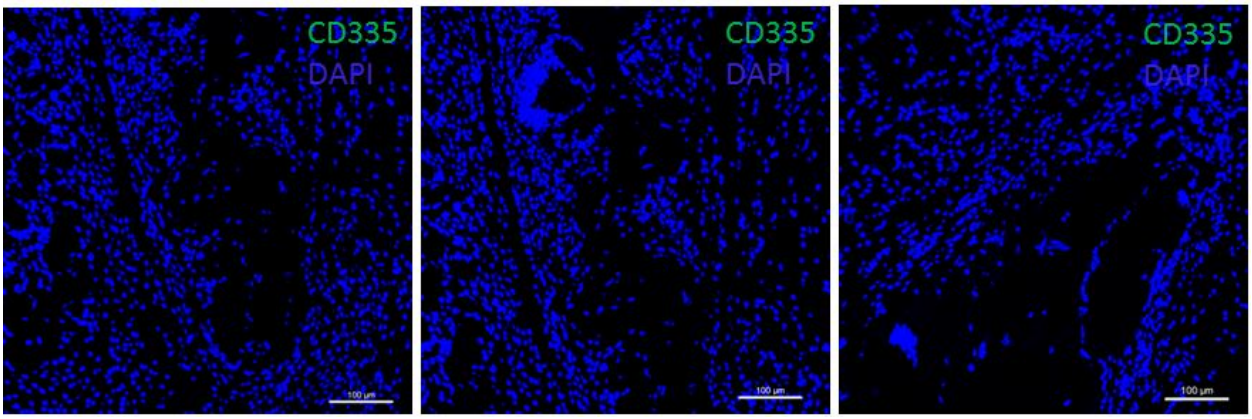

Figure S4. Evaluation of NK cells around and within constructs implanted subcutaneously in NOD SCID mice. Immunohistochemical studies with mouse-specific, FITC conjugated CD335 monoclonal antibody with DAPI counterstain of day 7 explants (n=3) containing fibrin gel constructs with HUVEC + thymus MSC yields no positive staining. Scale bar =100 μm.

Table S1. Primer sets used in qPCR.

Gene	Forward Primer	Reverse Primer
β-actin	TCCCTGGAGAAGAGCTACGA	AGCACTGTGTTGGCGTACAG
Oct-4	CGTGAAGCTGGAGAAGGAGA	CATCGGCCTGTGTATATCCC
Nanog	GATTTGTGGGCCTGAAGAAA	TTGGGACTGGTGGAAGAATC
Sox-2	GCGAACCATCTCTGTGGTCT	GGAAAGTTGGGATCGAACAA
HIF-1α	CAGCAACTTGAGGAAGTACC	CAGGGTCAGCACTACTTCG
VEGFA	GCCTTGCTGCTCTACCTCCA	ATGATTCTGCCCTCCTCCTTCT
b-FGF	GCTGGTGATGGGAGTTGTATTT	CTGCCGCCTAAAGCCATATT