Supplemental data

Reconstructing human brown fat developmental trajectory *in vitro*

Jyoti Rao[1,2,3,10], Yannis Djeffal[1,2,3], Jerome Chal[1,2,3], Fabio Marchianò[4], Chih-Hao Wang[5,11], Ziad Al Tanoury[1,2,3], Svetlana Gapon[1,2,3], Alicia Mayeuf-Louchart[6], Ian Glass[7], Elizabeth M. Sefton[8], Bianca Habermann[4], Gabrielle Kardon[8], Fiona M. Watt[9], Yu-Hua Tseng[5], Olivier Pourquié[1,2,3]

[1]Department of Pathology, Brigham and Women's Hospital, 60 Fenwood Road, Boston, MA 02115, USA.
[2]Department of Genetics, Harvard Medical School, 60 Fenwood Road, Boston, MA 02115, USA
[3]Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA
[4]Aix-Marseille University, CNRS, Institut de Biologie du Développement de Marseille UMR 7288, The Turing Center for Living Systems, 13009 Marseille, France
[5]Section on Integrative Physiology and Metabolism, Joslin Diabetes Center, Harvard Medical School, Boston, MA 02215, USA.
[6]Univ. Lille, INSERM, CHU Lille, Institut Pasteur de Lille, U1011-EGID, 59000 Lille, France
[7]Department of Pediatrics, University of Washington School of Medicine, Seattle, WA 98195, USA.
[8]Department of Human Genetics, University of Utah, Salt Lake City 84112, USA[9]King's College London Centre for Stem Cells and Regenerative Medicine, 28th Floor, Tower Wing, Guy's Campus, Great Maze Pond, London SE1 9RT, UK

[10]Current affiliation – Institute of Human Biology (IHB), Roche Pharma Research and Early Development, Roche Innovation Center Basel, Basel 4070, Switzerland
[11]Current affiliation - Graduate Institute of Biomedical Sciences, China Medical University, Taichung, 40402 Taiwan

*Lead contact – Olivier Pourquié
Lead contact email address - pourquie@genetics.med.harvard.edu

Figure S1

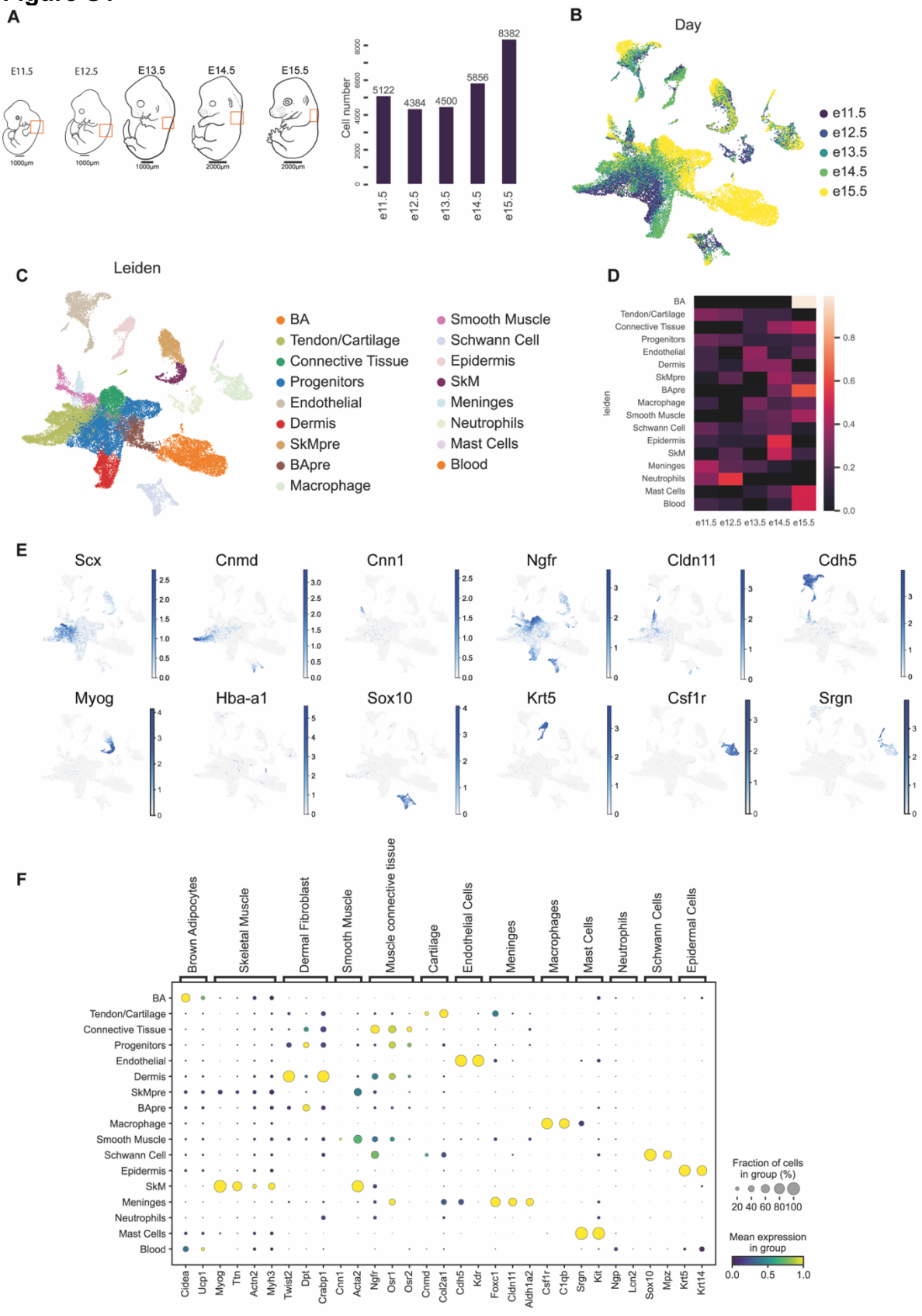

A
E11.5
E12.5
E13.5
E14.5
E15.5
1000μm
1000μm
1000μm
2000μm
2000μm
Cell number
5122
4384
4500
5856
8382
e11.5
e12.5
e13.5
e14.5
e15.5
B
Day
e11.5
e12.5
e13.5
e14.5
e15.5
C
Leiden
BA
Tendon/Cartilage
Connective Tissue
Progenitors
Endothelial
Dermis
SkMpre
BApre
Macrophage
Smooth Muscle
Schwann Cell
Epidermis
SkM
Meninges
Neutrophils
Mast Cells
Blood
D
leiden
e11.5 e12.5 e13.5 e14.5 e15.5
E
Scx
Cnmd
Cnn1
Ngfr
Cldn11
Cdh5
Myog
Hba-a1
Sox10
Krt5
Csf1r
Srgn
F
Brown Adipocytes
Skeletal Muscle
Dermal Fibroblast
Smooth Muscle
Muscle connective tissue
Cartilage
Endothelial Cells
Meninges
Macrophages
Mast Cells
Neutrophils
Schwann Cells
Epidermal Cells
Cidea
Ucp1
Myog
Ttn
Actn2
Myh3
Twist2
Dpt
Crabp1
Cnn1
Acta2
Ngfr
Osr1
Osr2
Cnmd
Col2a1
Cdh5
Kdr
Foxc1
Cldn11
Aldh1a2
Csf1r
C1qb
Srgn
Kit
Ngp
Lcn2
Sox10
Mpz
Krt5
Krt14
Fraction of cells in group (%)
20 40 60 80100
Mean expression in group
0.0 0.5 1.0

Figure S1 – Embryonic development of the mouse interscapular depot
Related to Figure 1 and Table S1

A – Schematic representation of cell isolation strategy for single cell RNA sequencing. Dorsal interscapular region was dissected out with intact dermis and epidermis for embryonic day (E) 13.5 and 14.5. For E15.5, skin and underlying dermis was removed before cell dissociation. For each stage, tissues were isolated from two embryos. Tissues were digested with enzymes followed by encapsulation using inDrops platform. Bar chart showing number of cells isolated from each stage from developing mouse embryos. The number represents sum of two replicates from each time point. E = embryonic day.
B – UMAP embedding of the single cells isolated from E11.5, E12.5, E13.5, E14.5 and E15.5 mouse embryos 50 PC dimensions, 28244 cells). Colors indicate embryonic days.
C – UMAP embedding showing cell clusters identified using Leiden based clustering on E11.5, E12.5, E13.5, E14.5, E15.5 mouse embryonic single cell data. Colors indicate identified cell cluster (BA=BAs, BApre=BA precursors, SkM=skeletal muscle, SkMpre=skeletal muscle precursors).
D – Normalized confusion matrix showing contribution of cells from different time point to identified clusters.
E – UMAP embedding showing expression of a curated list of cluster-specific genes. Scale represents log-normalized transcript counts.
F – Dotplot showing expression of a curated list of cluster-specific genes. Scale represents log-normalized transcript counts.

Figure S2

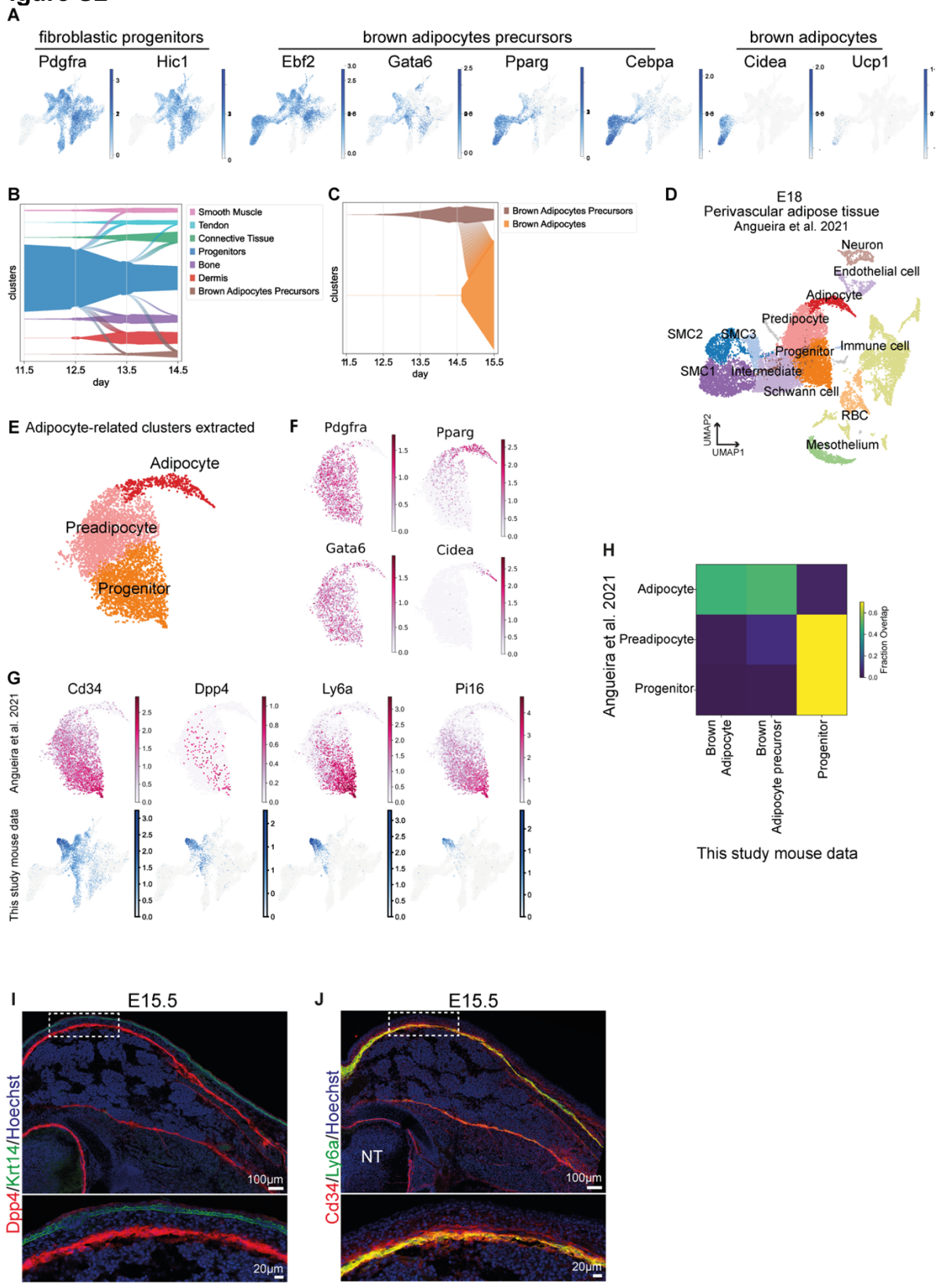

A
fibroblastic progenitors
brown adipocytes precursors
brown adipocytes
Pdgfra
Hic1
Ebf2
Gata6
Pparg
Cebpa
Cidea
Ucp1
B
clusters
day
Smooth Muscle
Tendon
Connective Tissue
Progenitors
Bone
Dermis
Brown Adipocytes Precursors
C
clusters
day
Brown Adipocytes Precursors
Brown Adipocytes
D
E18
Perivascular adipose tissue
Angueira et al. 2021
Neuron
Endothelial cell
Adipocyte
Predipocyte
SMC2
SMC3
Progenitor
Immune cell
SMC1
Intermediate
Schwann cell
RBC
Mesothelium
UMAP2
UMAP1
E Adipocyte-related clusters extracted
Adipocyte
Preadipocyte
Progenitor
F
Pdgfra
Pparg
Gata6
Cidea
G
Angueira et al. 2021
This study mouse data
Cd34
Dpp4
Ly6a
Pi16
H
Angueira et al. 2021
Adipocyte
Preadipocyte
Progenitor
Brown Adipocyte
Brown Adipocyte precurosr
Progenitor
Fraction Overlap
This study mouse data
I
E15.5
Dpp4/Krt14/Hoechst
100μm
20μm
J
E15.5
Cd34/Ly6a/Hoechst
NT
100μm
20μm

Figure S2 - Gata6 is expressed by developing BA precursors
Related to Figure 1 and Figure 2

A – UMAP embedding showing expression of a curated list of cluster-specific genes. UMAP plots are colored by log-normalized transcript counts.
B – Graph showing the probability mass flows from the progenitor cluster to the other indicated clusters as time increases based on waddington-OT transition matrix.
C – Graph showing how probability mass flows from the BAs precursors cluster to BA cluster as time increases based on waddington-OT transition matrix.
D – UMAP plot generated from mouse single cell transcriptomics data from the developing perivascular adipose tissue on embryonic day 18 described in Angueira et al. 2021. Cells were clustered using Leiden clustering.
E – UMAP plot generated from mouse single cell transcriptomics data from the developing mouse perivascular adipose tissue on embryonic day 18 described in Angueira et al. 2021. Cells were clustered using Leiden clustering and cell clusters representing the adipocyte lineage were selected.
F – UMAP plot showing gene expression patterns in adipogenic cells sub-setted from the single cell transcriptomics data from the developing perivascular adipose tissue on embryonic day 18 described in Angueira et al. 2021. Scale represents log-normalized transcript counts.
G – Heatmap showing gene expression patterns in adipogenic cells sub-setted from the single cell transcriptomics data from the developing perivascular adipose tissue on embryonic day 18 described in Angueira et al. 2021 and mouse datset generated in this study. Scale represents log-normalized transcript counts.
H – Classifier based machine-learning classification of perivascular adipose cells. A kNN-classifier trained on mouse single cell clusters was used to predict identities of cell clusters described in Angueira et al. 2021 study. Heatmaps depict fraction of mouse cluster assignments for adipocyte, preadipocytes and progenitors identified in the perivascular adipose tissue. BA= BAs and BApre=BA precursors identified in this study.
I – Transverse section at the forelimb level of a E15.5 embryo stained for Dpp4 and Keratin 14 (Krt14) antibody to detect cells from BApre cluster. NT= Neural tube, n≥4, scale bar=100μm (upper panel), scale bar=20μm (lower panel).
J –Transverse section from the interscapular region of a E15.5 embryo showing coexpression of Ly6a/Sca1 and Cd34 using antibody staining. NT= Neural tube, n≥4, scale bar=100μm (upper panel), scale bar=20μm (lower panel).

Figure S3

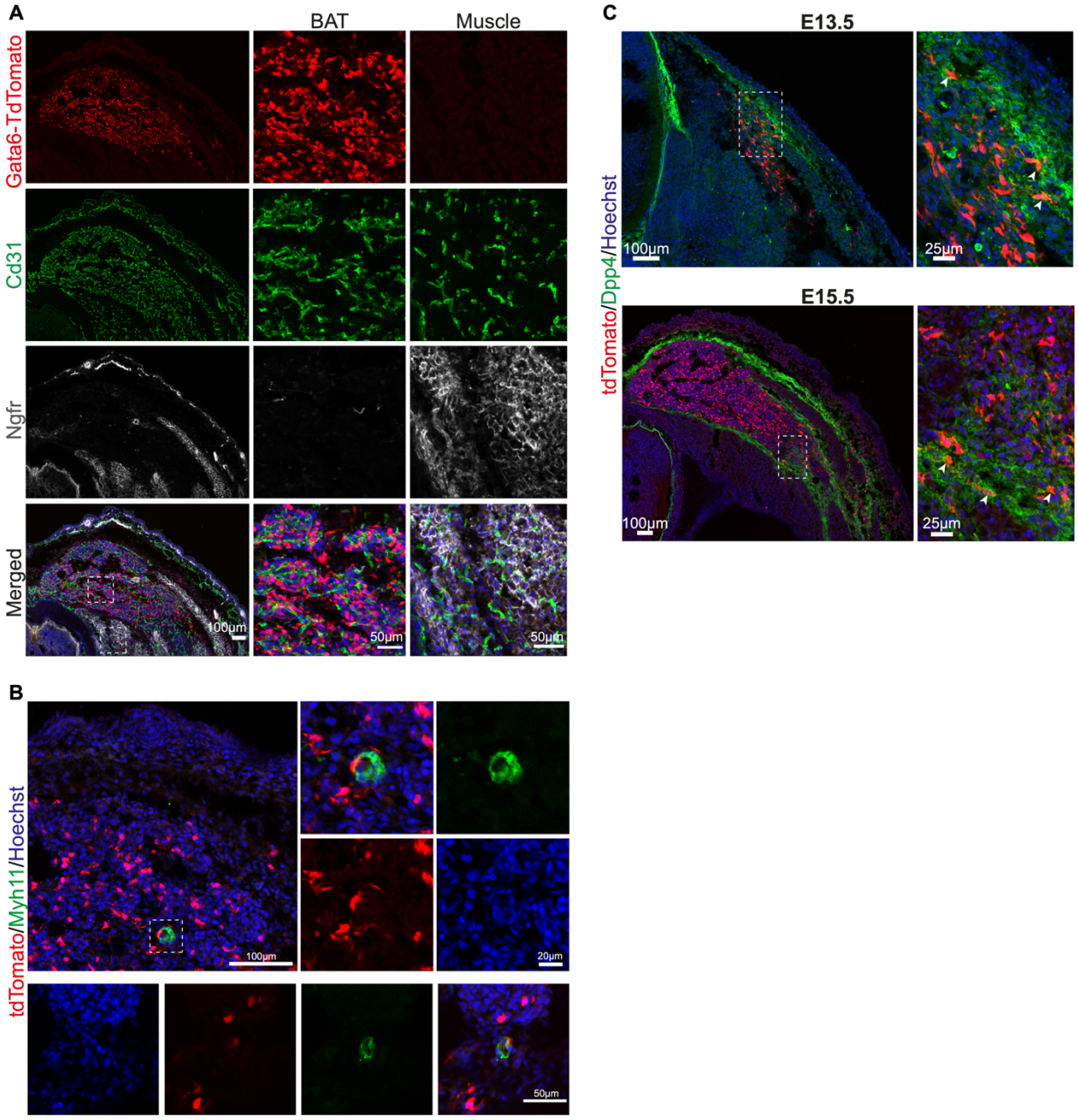

A
BAT
Muscle
Gata6-TdTomato
Cd31
Ngfr
Merged
100μm
50μm
50μm
B
tdTomato/Myh11/Hoechst
100μm
20μm
50μm
C
E13.5
tdTomato/Dpp4/Hoechst
100μm
25μm
E15.5
100μm
25μm

Figure S3 - Gata6 is expressed by developing BA precursors
Related to Figure 2

A – Transverse section of Gata6-CreERT2:Rosa26-tdTomato E15.5 embryo at the forelimb level showing contribution of Gata6-Cre tdTomato positive cells in brown fat and muscle area, n≥3, scale bar=100μm (left panel), scale bar=50μm (inset panel).
B – Two representative images of RFP positive Gata6-tdTomato and Myh11 positive smooth muscle cells in E15.5 embryo at the forelimb level, scale bar=100μm. Inset shows magnified image, scale bar=20μm, n≥3.
C – Transverse section of Gata6-CreERT2:Rosa26-tdTomato embryo at the forelimb level showing tdTomato and Dpp4 double positive cells at E13.5 and E15.5 n≥3, scale bar=100μm (left panel), scale bar=20μm (inset panel).

Figure S4

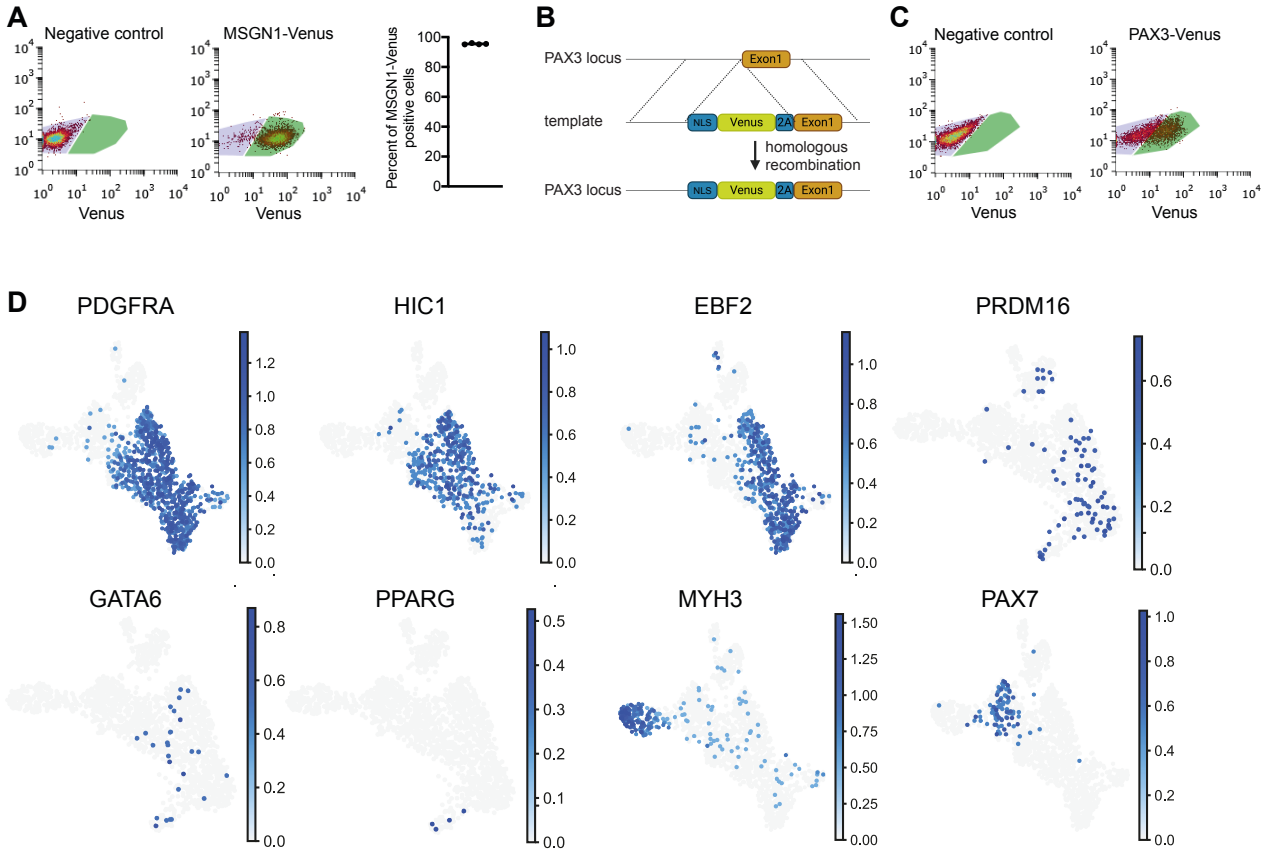

A
Negative control
MSGN1-Venus
Venus
Venus
Percent of MSGN1-Venus positive cells
B
PAX3 locus
Exon1
template
NLS
Venus
2A
Exon1
homologous recombination
PAX3 locus
C
Negative control
PAX3-Venus
Venus
Venus
D
PDGFRA
HIC1
EBF2
PRDM16
GATA6
PPARG
MYH3
PAX7

Figure S4 - Development of human BA precursors in vitro
Related to Figure 4

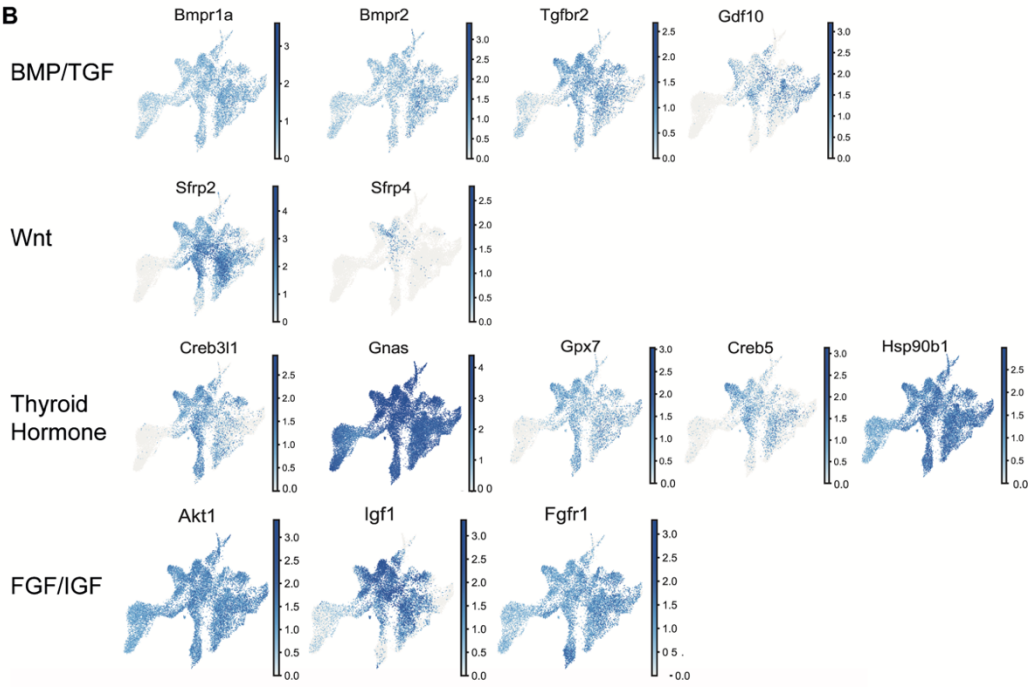
A – Flow cytometry analysis of day 2 of differentiating iPSC cultures to measure fraction of Mesogenin-Venus positive cells. Mean ± SD, n=4.
B – Schematic illustrating gene targeting strategy to generate PAX3-Venus knock-in iPSC line.
C – Representative flow cytometry plots showing gating strategy for quantifying PAX3-Venus positive cells on day 8 of differentiation.
D – UMAP embedding showing expression of a curated list of cluster-specific genes in human culture at day 20.

Figure S5

A

Term	P-value	Genes
ECM-receptor interaction	5.680E-10	ITGB1;TNXB;ITGB5;ITGB3;FN1;LAMB1;LAMC1;THBS2;THBS1;THBS3;COL1A1;COL1A2;COL6A2;COL6A1;COL6A3;COL6A6;CD47;CD44
Protein digestion and absorption	2.451E-07	COL16A1;COL14A1;ELN;COL12A1;DPP4;COL1A1;COL3A1;COL1A2;COL5A1;COL5A3;COL6A2;COL5A2;COL6A1;COL8A2;COL6A3;COL6A6
PI3K-Akt signaling pathway	3.281E-06	ITGB1;TNXB;CSF1;ITGB5;ITGB3;LPAR1;LPAR4;LAMC1;THBS2;THBS1;HSP90B1;THBS3;CCND3;CREB3L1;CREB3L2;AKT1;PDGFRA;FN1;LAMB1;IGF1;FGFR1;CREB5
Axon guidance	1.113E-03	SEMA5A;ITGB1;SEMA3C;RYK;NTN1;ENAH;EFNB1;CXCL12;RRAS;DPYSL2;ABL1;PLXNB2;FYN;EPHB2;EPHB3
Relaxin signaling pathway	6.399E-03	COL1A1;JUN;COL3A1;COL1A2;CREB3L1;MMP2;CREB3L2;GNAS;AKT1;TGFBR2;CREB5
Regulation of actin cytoskeleton	6.399E-03	ITGB1;PDGFRA;GSN;ITGB5;ARPC1B;ITGB3;LPAR1;FN1;LPAR4;ENAH;CXCL12;RRAS;TMSB4X;MYH10;FGFR1
Thyroid hormone synthesis	7.738E-03	HSPA5;GPX3;CREB3L1;CREB3L2;GNAS;GPX7;CREB5;HSP90B1
TGF-beta signaling pathway	2.749E-02	FST;NBL1;LTBP1;THBS1;DCN;THSD4;TGFBR2;FBN1
Rap1 signaling pathway	2.749E-02	ITGB1;PDGFRA;CSF1;ITGB3;LPAR1;LPAR4;IGF1;THBS1;ENAH;RRAS;GNAS;AKT1;FGFR1
Various types of N-glycan biosynthesis	2.863E-02	RPN2;MAN2A1;RPN1;STT3A;DDOST
Osteoclast differentiation	4.460E-02	JUN;JUND;CSF1;ITGB3;CTSK;AKT1;FYN;SQSTM1;TGFBR2
TNF signaling pathway	6.499E-02	JUN;MMP14;VCAM1;CSF1;CREB3L1;CREB3L2;AKT1;CREB5
N-Glycan biosynthesis	6.552E-02	RPN2;MAN2A1;RPN1;STT3A;DDOST
Wnt signaling pathway	7.123E-02	TCF7L2;CCND3;SFRP1;JUN;TCF7L1;SFRP2;FZD2;RYK;SERPINF1;DKK2
Glutathione metabolism	9.695E-02	GSTM1;GPX3;ANPEP;GPX7;GSTM5

B



C

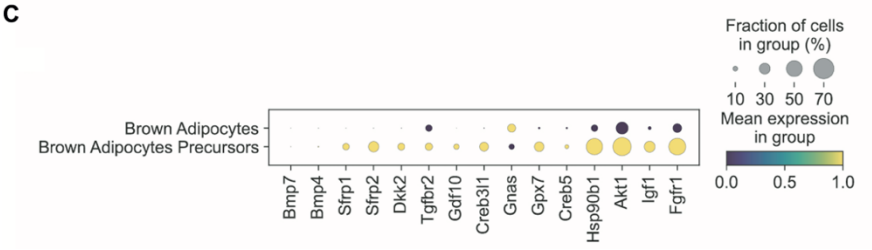


Figure S5 - Differentiation of human mature BA in vitro
Related to Figure 4

A – List of KEGG signaling pathway analysis using EnrichR. Differentially expressed genes in BApre cluster in mouse transcriptomics data were used as an input.
B – UMAP plots showing expression patterns of genes involved in BMP/Tgfb, Wnt, thyroid hormone and FGF/IGF signaling in mouse temporal single cell data. Scale represents log-normalized transcript counts.
C-Dot plots showing expression patterns of genes involved in BMP/Tgfb, Wnt, thyroid hormone and FGF/IGF signaling in mouse temporal single cell data.

Figure S6

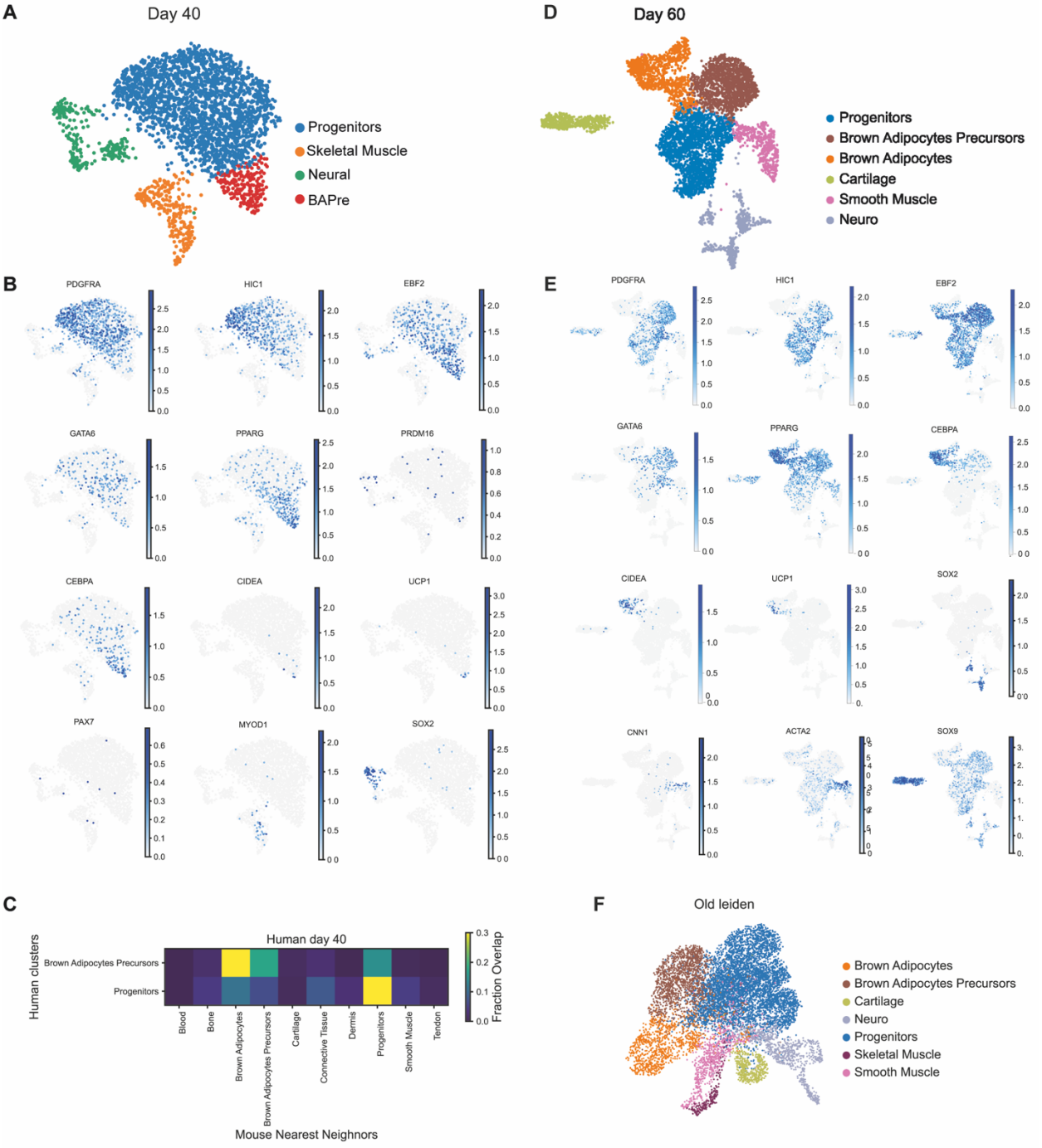

A
Day 40
Progenitors
Skeletal Muscle
Neural
BAPre
D
Day 60
Progenitors
Brown Adipocytes Precursors
Brown Adipocytes
Cartilage
Smooth Muscle
Neuro
B
PDGFRA
HIC1
EBF2
GATA6
PPARG
PRDM16
CEBPA
CIDEA
UCP1
PAX7
MYOD1
SOX2
E
PDGFRA
HIC1
EBF2
GATA6
PPARG
CEBPA
CIDEA
UCP1
SOX2
CNN1
ACTA2
SOX9
C
Human day 40
Human clusters
Brown Adipocytes Precursors
Progenitors
Blood
Bone
Brown Adipocytes
Brown Adipocytes Precursors
Cartilage
Connective Tissue
Dermis
Progenitors
Smooth Muscle
Tendon
Fraction Overlap
Mouse Nearest Neighnors
F
Old leiden
Brown Adipocytes
Brown Adipocytes Precursors
Cartilage
Neuro
Progenitors
Skeletal Muscle
Smooth Muscle

Figure S6 - ScRNAseq analysis of human BA generated in vitro
Related to Figure 6

A – UMAP embedding showing cell clusters identified using Leiden based clustering on human culture at d40. Colors indicate identified cell cluster.
B – UMAP embedding showing expression of a curated list of cluster-specific genes in human culture at d40.
C – Machine-learning classification of human in-vitro cultured cells and mouse embryo. A k-NN classifier trained on clusters of mouse clusters was used to predict identities of the human in-vitro cultured progenitors and BAs precursors.
D – UMAP embedding showing expression of a curated list of cluster-specific genes in human culture at d60.
E – Projection of the cell identities obtained from clustering analyses of individual time points onto the UMAP of the dataset combining the three time points.
F – UMAP embedding showing cell clusters identified using Leiden based clustering on the human in-vitro cultured cells at d20, 40 and 60 merged. Colors indicate identified cell cluster in individual time point analysis.

Figure S7

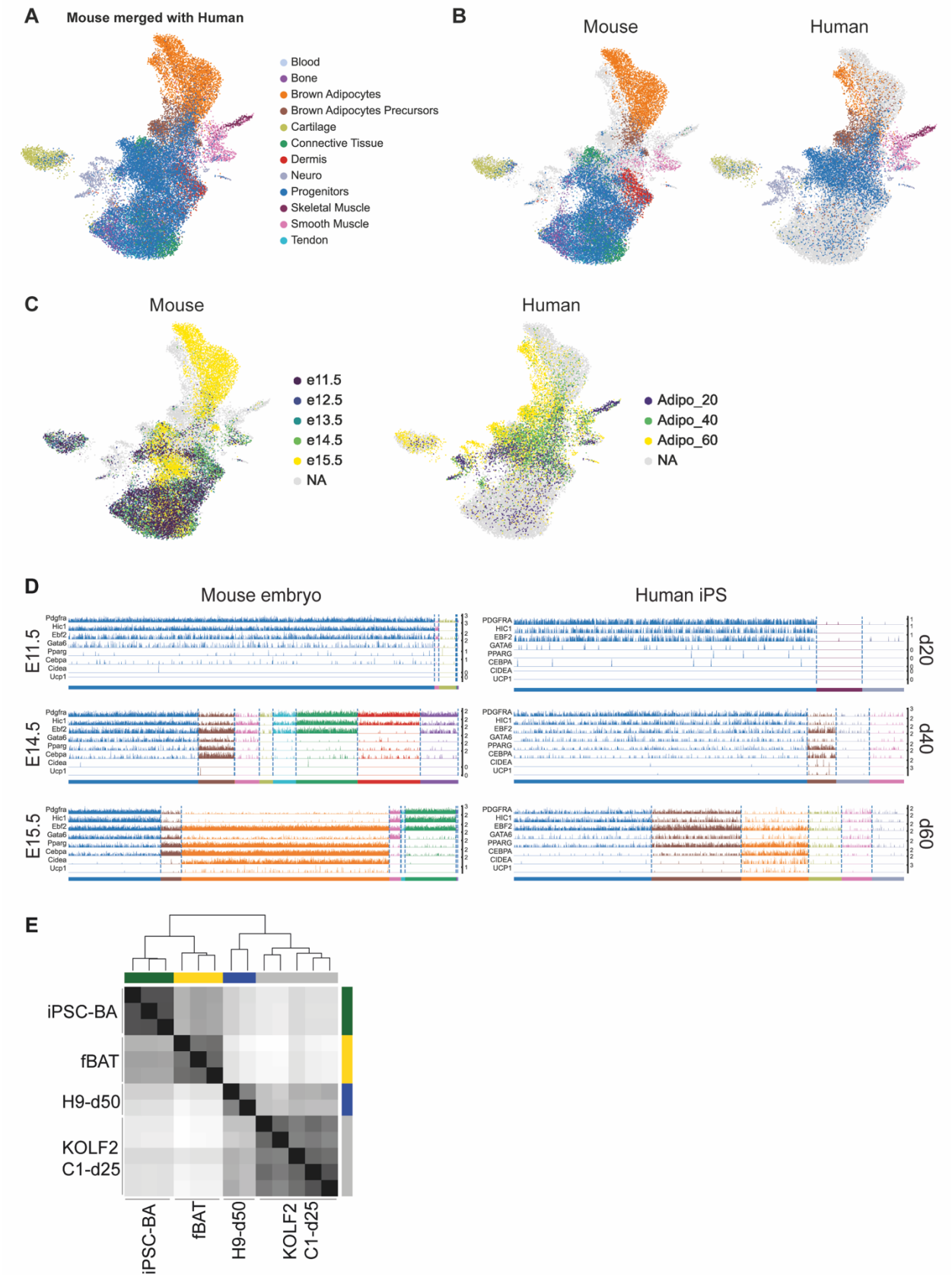

A
Mouse merged with Human
Blood
Bone
Brown Adipocytes
Brown Adipocytes Precursors
Cartilage
Connective Tissue
Dermis
Neuro
Progenitors
Skeletal Muscle
Smooth Muscle
Tendon
B
Mouse
Human
C
Mouse
Human
e11.5
e12.5
e13.5
e14.5
e15.5
NA
Adipo_20
Adipo_40
Adipo_60
NA
D
Mouse embryo
Human iPS
E11.5
E14.5
E15.5
d20
d40
d60
Pdgfra
Hic1
Ebf2
Gata6
Pparg
Cebpa
Cidea
Ucp1
PDGFRA
HIC1
EBF2
GATA6
PPARG
CEBPA
CIDEA
UCP1
E
iPSC-BA
fBAT
H9-d50
KOLF2
C1-d25

Figure S7 - Human BA generated *in vitro* are functional
Related to Figure 7

A – Merged UMAP embedding of the single cells isolated from day 20, 40 and 60 of human culture and E11.5, E12.5, E13.5, E14.5, E15.5 mouse embryos. Colors indicate both human and mouse clusters.
B – Merged UMAP embedding of the single cells isolated from day 20, 40 and 60 of human culture and E11.5, E12.5, E13.5, E14.5, E15.5 mouse embryos. Colors indicate mouse clusters (left) and human clusters (right).
C – Merged UMAP embedding of the single cells isolated from day 20, 40 and 60 of human culture and E11.5, E12.5, E13.5, E14.5, E15.5 mouse embryos. Colors indicate mouse embryonic days (left) and human culture days (right).
D – Comparison of tracksplots showing expression of marker genes of the BA lineage in the mouse embryo at different stages (left) and in human cultures at different days (right) in the clusters identified in Figure 1A and Figure 6C respectively.
E – Matrix plot showing transcriptional overlap between brown adipose specific genes in iPSC derived BAs (iPSC-BA), human fetal BAT (fBAT), human embryonic stem cell derived BAs in Zhang et al (H9-d50) and iPSC derived BAs in Carobbio et al (KOLF2-C1-d25).