Materials and Methods:

Human and mouse pluripotent stem cell culture

Human cell culture: Human iPS cells (NCRM-1) were grown on Matrigel (Corning CLS354277)-coated plates in mTeSR-Plus (STEMCELL Technologies 100-0276) or on a bed of irradiated mouse embryonic fibroblasts (Thermo Fisher A34180), and differentiated according to previously established protocols[20], without DAPT unless otherwise stated. For NKX2-2-CreERT2-dependent recombination, 200nM of 4-hydroxytamoxifen was added on day 9 and kept on for 48 hours. For cumulative BrdU assays, 10uM BrdU was added as early as day 9 and as late as day 19, kept on throughout the experiment and all harvested on day 21. For EdU/BrdU dual-labeling assays, 5uM EdU was added on day 13 for 24 hours, followed by 10uM BrdU which was kept on until the end of the experiment (day 16). DAPT was used at 10uM at various time points to drive progenitors out of the cell cycle.

Mouse cell culture: C57BL/6J mouse ES cells were grown on gelatin-coated plates in LIF+2i[49]-containing basal media, and differentiated according to previously established protocols[19]. For cumulative BrdU assays, 10uM BrdU was added as early as day 4 and as late as day 7, kept on throughout the experiment and all harvested on day 8.5.

To generate uniformly-sized embryoid bodies: 150 human or 200 mouse cells were seeded per ultra-low-adhesion round-bottom well (96-well format; Corning 7007) in day 0 differentiation media, and subsequent media changes were done individually for each well. In the case of human differentiations, embryoid bodies were transferred to larger vessels on day 16.

All cell culture experiments were repeated across at least three biological replicates, with the exception of in vitro differentiation for single-cell RNA-seq (two biological replicates per species).

Macaque iPSC culture and differentiation

Rhesus macaque iPS cells (RA-1; Salk Institute) were cultured on irradiated MEFs with mTESR Plus media (Stem Cell Technologies, 100-0276) and differentiated according to the same conditions as human.

Lentiviral barcoding and single-cell RNA-seq

Lentivirus was generated in HEK293T cells using a second-generation system with pMD2.G (Addgene 12259) and psPAX2 (Addgene 12260). Lentivirus harboring CAGGS-driven GFP with

unique 6bp barcodes ("timestamps") were used to transduce human iPS cells and mouse ES cells. 48-96 hours following transduction, GFP-positive cells were purified via FACS for continued culture and/or freeze-down. Each uniquely barcoded batch of cells was differentiated starting on different days, such that all timepoints could be harvested and processed for single-cell RNA-seq on the same day. Following differentiation, cells were dissociated, FACS-purified for GFP, pooled in equal parts across species and timepoints, and processed on a 10X Genomics V3 3'-end single-cell gene expression profiling platform at the Columbia University Sulzberger Genome Center. Single-cell RNA libraries were then sequenced on an Illumina Hi-seq 4000 at a depth of ~100,000 reads per cell.

Processing and analysis of in vitro single-cell RNA-seq data

Single-cell RNA-seq reads were demultiplexed, aligned to hg38 (Genome Reference Consortium Human Build 38) and mm10 (Genome Reference Consortium Mouse Build 38) and quantified (barcode and UMI) using CellRanger version 3.1.0 (10X genomics). Prior to alignment, the reference fastq files were supplemented with GFP and barcode sequences for lentiviral timestamp detection and quantification. Following quantification, cell profiles with fewer than 1500 unique genes detected were discarded, along with cell profiles that indicated a fibroblast-like (COL1A2-high) or V2 interneuron lineage (high VSX2 or IRX3) identity. In the mouse dataset, a V1 interneuron-like cluster (high EN1, PAX6) was also discarded. These cells were identified by preliminary clustering of each dataset individually, which made interneuron or fibroblast-like cells, if present, easily distinguishable, as they would each be found in a distinct cluster. Remaining cell profiles were processed and clustered (number of dimensions used: 8 for human or mouse, 10 for integrated, resolution parameter used: 0.5) using Seurat v4.0.6: cell profiles were integrated from across three 10X runs, and cell cycle-related gene expression signatures were regressed out.

Successfully timestamped cells were defined as those that showed detection of a single unique timestamp barcode. Only successfully timestamped cells were used for timestamp enrichment analysis **(Figure 1D)** and inferring gene expression dynamics. We did not use the timestamp information to infer pseudotime for the remaining, non-timestamped cells.

Integration of mouse and human datasets: Following independent clustering of human and mouse datasets separately, a list of 1:1 orthologous genes was generated using the Ensembl biomaRt homolog dataset to integrate the two datasets. This was then used to generate mouse and human

sub-datasets of the original single-cell RNA-seq UMI counts dataset, where rows are all ortholog-matched. Ortholog-matched datasets were subsequently integrated using Seurat v4.0.6. Following integration, mouse and human cells were clustered together to generate the set of combined clusters (Seurat v4.0.6).

Processing and analysis of embryonic human and mouse single-cell RNA-seq data

Cell profiles (Human Carnegie stage 12[2] and mouse E9.5 and 10.5[31]) were downloaded from EMBL's European Bioinformatics Institute (mouse; E-MTAB-7320) and Gene Expression Omnibus (human; GSE171890) and processed using Seurat v4.0.6. Individual replicates and/or timepoints were integrated within each species and cell profiles were clustered following removal of cell cycle-related gene expression signatures. Following clustering, individual clusters that displayed high expression of OLIG2, NKX2-2, ISL1, or MNX1, but low expression of IRX3 and PHOXA2 were then selected and clustered again separately to generate final cluster assignments. Integration of in vivo and in vitro datasets were performed in the same manner as detailed above for integration of human and mouse datasets.

Overlap between common and species clusters

The overlap between species cluster i and common cluster j was calculated as:

$$\frac{N_{ij}}{\sum_i N_{ij}}$$

, where N_{ij} is the number of cells with species cluster identity i and common cluster identity j. Given a common cluster's maximum overlap with human and mouse clusters (O_h and O_m, respectively) its degree of enrichment for human vs. mouse overlap was defined as:

$$\frac{O_h - O_m}{O_h + O_m}$$

Chi-square distance between human cluster x and mouse cluster y was calculated as:

$$\frac{1}{2}\sum_j \frac{\left\{H_{xj} - H_{xj} \cdot \frac{H_{xj} + M_{yj}}{\sum_j H_{xj}}\right\}^2}{\left\{H_{xj} \cdot \frac{H_{xj} + M_{yj}}{\sum_j H_{xj}}\right\}} + \frac{1}{2}\sum_j \frac{\left\{M_{yj} + M_{yj} \cdot \frac{H_{xj} + M_{yj}}{\sum_j M_{yj}}\right\}^2}{\left\{M_{yj} \cdot \frac{H_{yj} + M_{yj}}{\sum_{yj} M_{yj}}\right\}}$$

, where H_{xj} is the number of human cells with human cluster identity x and common cluster identity j, and M_{yj} is the number of mouse cells with mouse cluster identity y and common cluster identity j.

Human-based random forest classifier to predict cell categories

To test whether H3-5 are indeed human-specific, we trained a random forest classifier consisting of 500 trees, each grown with 50% of the input training data, using a misclassification cost of 1 (otherwise 0). As input training data we used the transcription factor (defined based on TcoF database; further filtered for those with 1:1 orthologous matches between human and mouse) expression profile of 1000 of the 3090 human cells, and classified them as the following: H0-2 (progenitors that don't express NKX2-2), H3-5 (mid-stage NKX2-2 and OLIG2 co-expressing, "human-specific" progenitors), H6 (late-stage NKX2-2 and OLIG2 co-expressing progenitor, OPC-like), or H7-9 (motor neuron). When we tested classifier performance using the remaining human cells, we found that for each human cluster, >80% of cells were correctly classified (Overlap is defined as the proportion of cells in each cluster that falls into a given classifier-predicted identity). Next, we input the mouse transcription factor expression data through the classifier, which revealed that cells in all mouse clusters predominantly classified as H0-2, H6 or H7-9, leaving the "human-specific" H3-5 category relatively void of classified mouse cells.

Differential gene expression analysis

DEsingle[50] v1.8.2 with standard parameters and default settings was used to generate a list of differentially expressed transcription factors in pair-wise comparisons between two sets of single-cell gene expression profiles, and their p-values. False discovery rate-adjusted q-values were obtained using the Benjamini-Hochberg method with a threshold value of 0.05. Single-cell gene expression profiles were first filtered for only transcription factors, and subsampled to have a maximum UMI of 2000 per cell. The same number of cells was randomly selected from each cluster (sampled number equal to the number of cells in smallest cluster) and pooled for the analysis.

Reactome pathway enrichment analysis

Pathways enriched in differentially expressed genes were identified using the standard web version of Reactome analysis v84 (reactome.org) [32]. Genes that were upregulated in vpMN relative to pMN with a q-value of less than 0.05 were used as input.

Immunostaining

For flow cytometry or FACS: Embryoid bodies (at least 50 EBs per sample) were pooled and dissociated using papain following the Neural Tissue Dissociation Kit – P (Miltenyi Biotec 130-092-628) and quenched with ovomucoid protease inhibitor (Worthington LK003182). Following dissociation, cells were washed twice with 1X PBS, fixed using 4% paraformaldehyde, washed with PBS, and then permeabilized with ice-cold methanol. Permeabilized cells were rehydrated with 1% BSA in PBS solution, and subsequently immunostained using a standard two-step immunofluorescence labeling method.

Sectioned embryoid bodies: Whole embryoid bodies were fixed in 4% paraformaldehyde, washed several times with PBS, then put through 30% sucrose prior to embedding in OCT and freezing. Frozen blocks were sectioned in a cryostat to 15um slices and mounted on microscope slides (Superfrost Plus, Fisher 12-550-15). Sections were rehydrated with staining solution (1% BSA, 0.1% Triton X-100 in PBS), then immunostained using a standard two-step immunofluorescence labeling method.

Primary Antibodies; host species and concentrations used: ISL1/2 (Mouse; 1:100; DSHB Cat# 39.4D5, RRID:AB_2314683), ISL1/2 (Goat; 1:5000; Neuromics Cat# GT15051-100, RRID:AB_2126323), MNX1 (Guinea pig; 1:100; from Jessell Lab), FOXP1 (Mouse; 1:400; Santa Cruz Cat# sc-398811), NKX2-2 (Mouse; 1:100; DSHB Cat# 74.5A5, RRID:AB_531794), BrdU (Rat; 1:400; Abcam Cat# ab6326, RRID:AB_305426), LHX1/2 (Mouse; 1:100; DSHB Cat# 4F2, RRID:AB_531784) , NeuN (Rabbit; 1:1000; Millipore Cat# ABN78, RRID:AB_10807945), OLIG2 (Guinea pig; 1:100; from Jessell Lab), Ki67 (Mouse; 1:100; BD Biosciences Cat# 550609, RRID:AB_393778), Cleaved Caspase3 (Rabbit; 1:200; Cell Signaling #9661), LHX3 (1:100 DSHB 67.4E12 supernatant)

EdU labeling: Click-iT Plus (Thermo Fisher C10640)

Imaging

Images were acquired with 20X objective using confocal laser scanning microscope (LSM Zeiss 780).

FACS and flow cytometry

BD FACSAria Cell Sorter equipped with 5 lasers (355nm, 405nm, 488nm, 561nm, 640nm) and 100um nozzle was used to acquire flow cytometry data (minimum 10,000 events per replicate; 50+ embryoid bodies were pooled for each sample) or FAC-sort cells (timestamped cells for scRNA-seq; 15+ embryoid bodies were pooled for each sample). Raw data was analyzed by custom MATLAB script. Gating to remove debris and cell clumps were done based on FSC-SSC and DAPI-height and width distributions, respectively. Positive immunofluorescence staining or positive levels of expressed fluorescence was determined by negative control samples: no-primary-antibody control or no-fluorescence control, respectively.

Estimation of cell cycle length based on EdU pulse

On day 14 of human culture, following 4OHT pulse between days 9 and 11, 5uM EdU was added to the culture media for 5-23 hours, after which embryoid bodies were dissociated and fixed with paraformaldehyde (4%). Following fixation, cells were permeabilized with ice-cold 100% methanol, then rehydrated with 1% BSA in PBS and immunolabeled for OLIG2. EdU was then labeled using click chemistry. Using flow cytometry, the proportion of EdU-positive cells among OLIG2-positive cells, or p, was measured for both RFP^+ and total populations. We then plotted p as a function of EdU duration, and used linear least squares regression to estimate the slope and y-intercept, from which we estimated the EdU duration t at which $p = 1$ (i.e., cell cycle length). Biological replicates (n = 3) were collected for each time point.

Imputing RFP^- population characteristics

Because tamoxifen-induced recombination is low-efficiency (<1% of the population is RFP^+ at day 11, even though 20-30% of cells are immunolabeled positively for NKX2-2), we imputed population characteristics using a conservative p($NKX2\text{-}2^+$) estimate of 0.2. Given the proportion of a certain phenotype (e.g., $BrdU^+$ or $FOXP1^+$) in the RFP^+ and total populations is p and q, respectively, its proportion in the $pMN^{IMPUTED}$ lineage population is:

$$\frac{q - 0.2 \cdot p}{0.8}$$

Sparse-labeling of human clones and whole-EB imaging

Following 4OHT treatment on day 9-11, NKX2-2-P2A-CreERT2 human cell cultures were transduced with a low-titer of lentivirus harboring CAGGS-driven GFP (titer calibrated to produce ~1 GFP+ clone per embryoid body). On day 19, whole embryoid bodies (EBs) were fixed with 4% PFA, washed with PBS, then put through 25% urea and 65% sucrose (in water) to clarify and allow for whole-EB imaging. EBs were then placed on a glass side, covered with mounting media and topped with a coverslip, allowing the weight of the coverslip to gently flatten the EBs. The slides were then sealed with clear nail polish and imaged on a confocal laser-scanning microscope with 5µm-interval z-stacks that span the entire EB to capture all cells in a given clone.

CRISPR-mediated knock-in of P2A-CreERT2 downstream of NKX2-2

A single cut in the NKX2.2 3'UTR was made using transient transfection (Lonza Human Stem Cell Nucleofector Kit VPH-5012) of with CAGGS::Cas9-mCherry and gRNA expression vector [51] (Addgene 41824) harboring guide RNA sequence: 5'-GGGGCCGCGAGTCTCGTTG-3' (PAM: GGG), along with linearized donor vector harboring P2A-CreERT2 sandwiched between 800-bp homology arms for NKX2-2 exon 2 and 3'UTR regions. Following transfection, cells were recovered, re-plated at clonal density on mouse embryonic fibroblasts (irradiated; Gibco A34180), after which individual clonal colonies were picked and genotyped. Genotyping primers: 5'-CGAAAGCCCCCAAAAACCTG-3', 5'-CGTAGAGTTCAGCCCTCTCC-3'. Induced pluripotent stem cell lines and DNA constructs will be made available to researchers upon request. More broadly, all renewable reagents including cDNA clones and cell lines are freely distributed to investigators at academic institutions for non-commercial research.

Knockout of NKX2-2 and co-culture with WT cells

Two cuts (one immediately upstream of NKX2-2 start and stop codon, respectively) were made using transient transfection (Lonza Human Stem Cell Nucleofector Kit VPH-5012) of with CAGGS::Cas9-mCherry and gRNA expression vector [51] (Addgene 41824) harboring guide RNA sequences: 5'-GTACCCGACAGCACACCCCC-3' (PAM: TGG), and 5'-TGGCGCTCCCCTGCCCCGG-3' (PAM: CGG). Following transfection, cells were recovered, re-plated at clonal density on mouse embryonic fibroblasts (irradiated; Gibco A34180), after which individual clonal colonies were picked and genotyped. Genotyping primers: 5'-

CTTGCTCTAGAGGGCCGTTG-3’ (specific to NKX2-2 coding region), 5’-GCGAACCTCATCACTCGTTG-3’ (specific to Cre).

For co-culture of NKX2-2 KO and WT cells, WT NKX2-2-p2A-CreERT2 cells were transduced with lentivirus harboring CAGGS-driven GFP. GFP-expressing cells were FACS purified and clonally selected for stable expression. Prior to differentiation, NKX2-2 KO and GFP-labeled WT cells were mixed at roughly 1:1 ratio, then seeded in microwell plates to ensure that each embryoid body had similar representation of KO and WT cells.