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Identification and comparison of orthologous cell types from primate embryoid bodies shows limits of marker gene transferability

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eLife Assessment

The authors have generated **important** resources such as a reference dataset of early primate development by utilizing single-cell transcriptomic technology together with induced pluripotent stem cells (iPSCs) from four primate species: humans, orangutans, cynomolgus macaques, and rhesus macaques. By analyzing marker gene expression and cell types across species during undirected differentiation of iPSCs, the authors provide **solid** evidence that the transferability of marker genes decreases as the evolutionary distance between species increases. This work demonstrates the extended usage of iPSCs for broader fields, which will benefit several scientific communities including anthropology, comparative biology, and evolutionary biology.

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Abstract

The identification of cell types remains a major challenge. Even after a decade of single-cell RNA sequencing (scRNA-seq), reasonable cell type annotations almost always include manual non-automated steps. The identification of orthologous cell types across species complicates matters even more, but at the same time strengthens the confidence in the assignment. Here, we generate and analyze a dataset consisting of embryoid bodies (EBs) derived from induced pluripotent stem cells (iPSCs) of four primate species: humans, orangutans, cynomolgus, and rhesus macaques. This kind of data includes a continuum of developmental cell types, multiple batch effects (i.e. species and individuals) and uneven cell type compositions and hence poses many challenges. We developed a semi-automated computational pipeline combining classification and marker based cluster annotation to identify orthologous cell types across primates. This approach enabled the investigation of cross-species conservation of gene expression. Consistent with previous studies, our data confirm that broadly expressed

genes are more conserved than cell type-specific genes, raising the question how conserved - inherently cell type-specific - marker genes are. Our analyses reveal that human marker genes are less effective in macaques and vice versa, highlighting the limited transferability of markers across species. Overall, our study advances the identification of orthologous cell types across species, provides a well-curated cell type reference for future *in vitro* studies and informs the transferability of marker genes across species.

Background

Cell types are a central concept for biology, but are - as other concepts like species - practically difficult to identify. Theoretically, one would consider all stable, irreversible states on a directed developmental trajectory as cell types. In practice, we are limited by our experimental possibilities. Historically, cell type definitions hinged on observations of cell morphology in a tissue context, which was later combined with immunofluorescence analyses of marker genes [1]. A lot of the functional knowledge that we have about cell types today is based on such visual and marker-based cell type definitions. With single cell-sequencing our capabilities to characterize and identify new cell types have radically changed [2, 3]. Clustering cells by their expression profiles enables a more systematic and higher-resolution identification of groups of cells that are then interpreted as cell types. However, distinguishing them from cell states or technical artifacts is not straight forward. A key criterion for defining a true cell type is its reproducibility across experiments, individuals, or even species.

Hence, identifying the same, i.e. orthologous, cell types across individuals and species is crucial. There are three principal strategies to match cell types from scRNA-seq data. 1) One is to integrate all cells prior to performing a cell type assignment on a shared embedding [4]. 2) The second approach is to consider cell types from one species as the reference and transfer these annotations to the other species using classification methods [5]. 3) The third strategy is to assign clusters and match them across species, which has the advantage of not requiring data integration of multiple species or an annotated reference [6, 7, 8].

Furthermore, established marker genes are still heavily used to validate and interpret clusters identified by scRNA-seq data [9, 10, 11]. Together with newly identified transcriptomic markers for human and mouse they are collected in databases [12, 13] and provide the basis for follow-up studies using spatial transcriptomics and/or immunofluorescence approaches. However, previous studies have shown that the same cell types may be defined by different marker genes in different species [14, 7]. For example, Krienen et al. [15] found that only a modest fraction of interneuron subtype-specific genes overlapped between primates and even less between primate and rodent species.

To better understand how gene expression in general and the expression of marker genes in particular evolves across closely related species, we used induced pluripotent stem cells (iPSCs) and their derived cell types from humans and non-human primates (NHP). One fairly straight forward way to obtain diverse cell types from iPSCs are embryoid bodies (EBs). EBs are the simplest type of iPSC-derived organoids, contain a dynamic mix of cell types from all three germ layers and result from spontaneous differentiation upon withdrawal of key pluripotency factors [16, 17, 18, 19, 20].

EBs and brain organoids from humans and chimpanzees have for example been used to infer human-specific gene regulation in brain organoids [21] or to investigate mechanisms of gene expression evolution [22].

Here we explore to what extent levels of cell type specificity of marker genes are conserved in primates. We generated scRNA-seq data of 8 and 16 day old EBs from human, orangutan (*Pongo abelii*), cynomolgus (*Macaca fascicularis*) and rhesus macaque (*Macaca mulatta*) iPSCs. Using this data, we established an analysis pipeline to identify and assign orthologous cell types. With this annotation we provide a well curated cell type reference for *in vitro* studies of early primate development. Moreover, it allowed us to asses the cell type-specificity and expression conservation of genes across species. We find that even though the cell type-specificity of a marker gene remains similar across species, its discriminatory power still decreases with phylogenetic distance.

Results

Generation of embryoid bodies from iPSCs of different primate species

We generated EBs from iPSCs across multiple primate species: two human iPSC clones (from two individuals), two orangutan clones (from one individual), three cynomolgus clones (from two individuals), and three rhesus clones (from one individual) [23, 24, 25]. To optimize conditions for generating a sufficient number of cells from all three germ layers across these four species, we tested combinations of two culturing media ("EB-medium" and "DFK20", see Methods) and two EB-differentiation conditions ("single-cell seeding" and "clump seeding", see Methods). After 7 days of differentiation, germ layer composition was analyzed by flow cytometry (**Supplementary Figure S1A,B,C**). Among the four tested protocols, culture in DFK20-medium with clump seeding resulted in the most balanced representation of all germ layers, yielding a substantial number of cells from each layer across all species (**Supplementary Figure S1D**).

Under these conditions, we established an EB formation protocol based on 8 days of floating culture in dishes, followed by 8 days of attached culture (**Figure 1A**). This results in the formation of cells from all three germ layers, as confirmed by immunofluorescence staining for AFP (endoderm), β-III-tubulin (ectoderm) and α-SMA (mesoderm) (**Figure 1B**). To generate scRNA-seq data, we dissociated 8 or 16 day old EBs into single cells and pooled cells from all four species to minimize batch effects (**Figure 1C**). We performed the experiment in three independent replicates, generating a total of four lanes and six lanes of 10x Genomics scRNA-seq at day 8 and day 16, respectively (**Supplementary Figure S2A**). This resulted in a dataset comprising over 85,000 cells after filtering and doublet removal, distributed fairly equally over time points, species and clones (**Supplementary Figure S2B-D**).

In agreement with the immunofluorescence staining, we detected well-established marker genes of pluripotent cells and of all three germ layers [26] in the scRNA-seq data: *SOX2*, *SOX10*, and *STMN4* expression was used to label ectodermal cells, *APOA1* and *EPCAM* for endodermal cells, *COL1A1* and *ACTA2* (α-SMA) for mesodermal cells, and *POU5F1* and *NANOG* for pluripotent cells (**Figure 1D**). Expression of these marker genes corresponded well with a classification based on a published scRNA-seq dataset from 21 day old human EBs [18]. This initial, rough germ layer assignment shows that our differentiation protocol generates EBs with the expected germ layers and cell type diversity from all four species (**Figure 1E**,**Supplementary Figure S3A**).

Assignment of orthologous cell types

Many integration methods encounter difficulties when they are applied to data from multiple species and uneven cell type compositions [4]. Indeed, when comparing clusters derived from an integrated embedding across all species [27, 28] to the aforementioned preliminary cell type assignments, we observed signs of overfitting. For instance, a cluster predominantly containing cells classified as neurons in humans, cynomolgus, and rhesus macaques consisted mainly of early ectoderm and mesoderm cells in orangutans (**Supplementary Figure S3B,C**). To

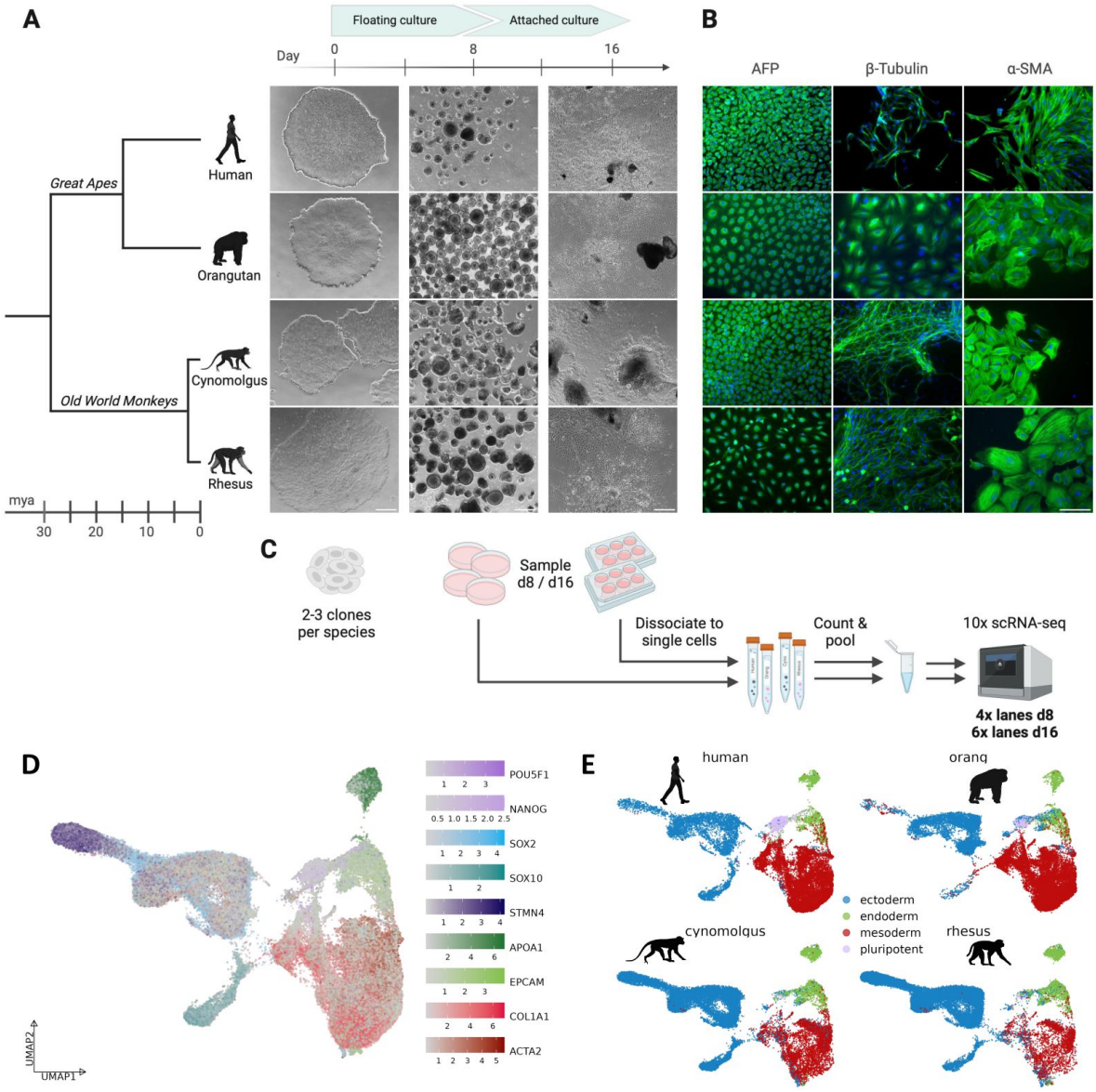

Figure 1.

Generation of primate embryoid bodies.

A) Overview about the EB differentiation workflow of the four primate species human (*Homo sapiens*), orangutan (*Pongo abelii*), cynomolgus (*Macaca fascicularis*) and rhesus (*Macaca mulatta*), including their phylogenetic relationship. Scale bar represents 500 µm. B) Immunofluorescence staining of day 16 EBs using *α*-fetoprotein (AFP), *β*-III-tubulin and *α*-smooth muscle actin (*α*-SMA). Scale bar represents 100 µm. C) Schematic overview of the sampling and processing steps prior to 10x scRNA-seq. D) UMAP representation of the whole scRNA-seq dataset, integrated across all four species with Harmony. Single cells are colored by the expression of known marker genes for the three germ layers and undifferentiated cells. E) UMAP representation, colored by assigned germ layers, split by species. Panels A-C created with *BioRender.com*.

address this issue, we developed an approach that assigns orthologous cell types without a common embedding space in an interactive shiny app (*https://shiny.bio.lmu.de/Cross_Species* CellType/; **Figure 2A, B**):

First, we assign cells to clusters separately for each species. To avoid losing rare cell types, we aim to obtain at least double as many high resolution clusters (HRCs) per species as expected cell types. We then use the HRCs of one species as a reference to classify the cells of the other species using SingleR [29]. These pair-wise comparisons are done reciprocally for each species and via a cross-validation approach also within each species (see Methods). For each comparison, we average the two values for the fraction of cells annotated as the other HRC. For example, a perfect "reciprocal best-hit" between HRC-A in human and HRC-B in rhesus would have all cells of HRC-B assigned to HRC-A when using the human as a reference and reciprocally all cells in HRC-A assigned to HRC-B when using the rhesus as a reference. Next, we used the resulting distance matrix as input for hierarchical clustering to find orthologous clusters across species and merge similar clusters within species. Here, the user can choose and adjust the final cell type cluster number. This allows us to identify orthologous cell type clusters (OCCs) across all four species, while retaining species-specific clusters when no matching cluster was identified.

In the last steps, OCCs are manually further refined by merging neighboring OCCs with similar marker gene and transcriptome profiles (see Methods). To avoid bias, we first identify marker genes independently for each species solely based on scRNA-seq expression data [30]. We then intersect those lists to identify the top ranking marker genes with consistently good specificity across all species. The final set of conserved marker genes then serves us to derive cell type labels by searching the literature as well as databases of known marker genes (**Figure 2E**). If the marker-gene based cell type assignment reveals cluster inconsistencies, they can be marked for further splitting. This feature is of particular importance for rare cell types. For example, we separated a cluster of early progenitor cells into iPSCs, cardiac progenitors, and early epithelial cells.

Suresh et al. [8] devised a conceptually similar approach to ours to identify orthologous cell types across species. The main difference is that they used scores from MetaNeighbor [6] where we use SingleR to measure distances between HRCs. However, in essence both scores are based on rank correlations and hence it may not be surprising that both scoring systems yield consistent cluster groupings that show high replicability across species. However, using our SingleR-based scores to compare OCCs across species may yield more clearly defined correspondences compared to MetaNeighbor scores (**Supplementary Figures S5** and **S4**).

Overall, we are confident that our approach yields meaningful orthologous cell type assignments, without requiring a prior annotation per species or a reference dataset. Moreover, the necessary fine tuning of the cell type clusters by the expert user is facilitated by an interactive app.

Cell type-specific genes have less conserved expression levels

Using the strategy described in the previous section, we detected a total of 15 reproducible cell types from the three germ layers, all of which were detected in at least 3 separate cell lines in 3 independent replicates. 9 of these were detected in at least 3 species, and 7 cell types were highly reproducibly detected in all four species (**Figure 2C, D**; **y Figure S6**). These 7 cell types consisted of iPSCs, two cell types representing ectoderm: early ectoderm and neural crest, two cell types of mesodermal origin: smooth muscle cells and cardiac fibroblasts and two endodermal cell types: epithelial cells and hepatocytes (**Figure 2C,E**). Based on the premise that it is not necessarily the expression level, but rather the expression breadth that determines expression conservation [31], we developed a method to call a gene 'expressed' or not that considers the expression variance across the cells of one type, which we then used to score cell type-specificity and expression conservation (**Figure 3B**); see Methods).

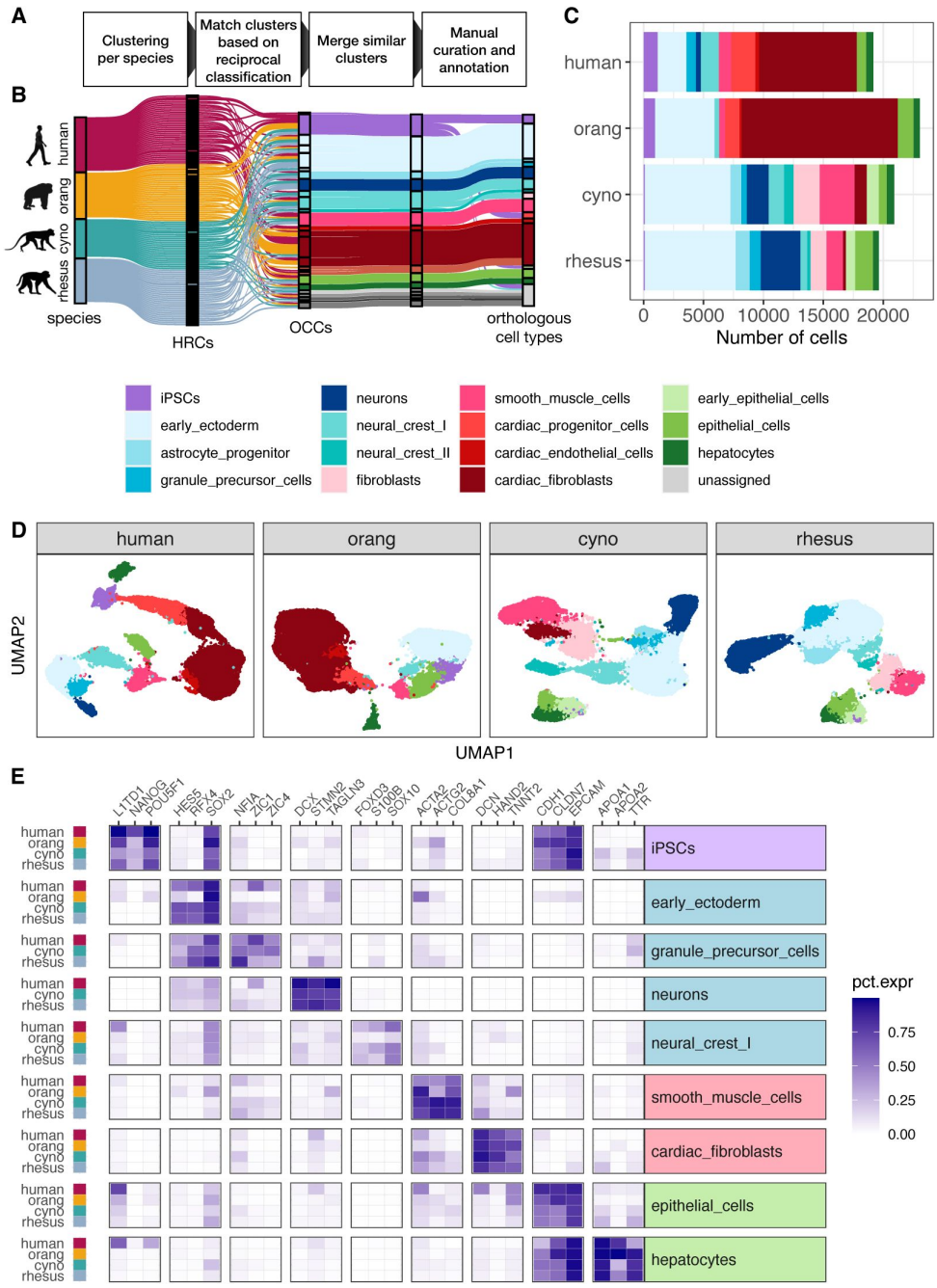

Figure 2.

Assignment of orthologous cell types across species.

A) Schematic overview of the pipeline to match clusters between species and assign orthologous cell types. B) Sankey plot visualizing the intermediate steps of the cell type assignment pipeline. Each line represents a cell which are colored by their species of origin on the left and by their current cell type assignment during the annotation procedure on the right. An initial set of 118 high resolution clusters (HRCs), 25-35 per species, was combined into 26 orthologous cell type clusters (OCCs). Similar cell type clusters were merged and after further manual refinement provided the basis for final orthologous cell type assignments. C) Fraction of annotated cell types per species. D) UMAPs for each species colored by cell type. E) To validate our cell type assignments, we selected three marker genes per cell type that exhibit a similar expression pattern across all four species and have been reported to be specific for this cell type in both human and mouse (**Supplementary Table S1**). The heatmap depicts the fraction of cells of a cell type in which the respective gene was detected for cell types present in at least three species.

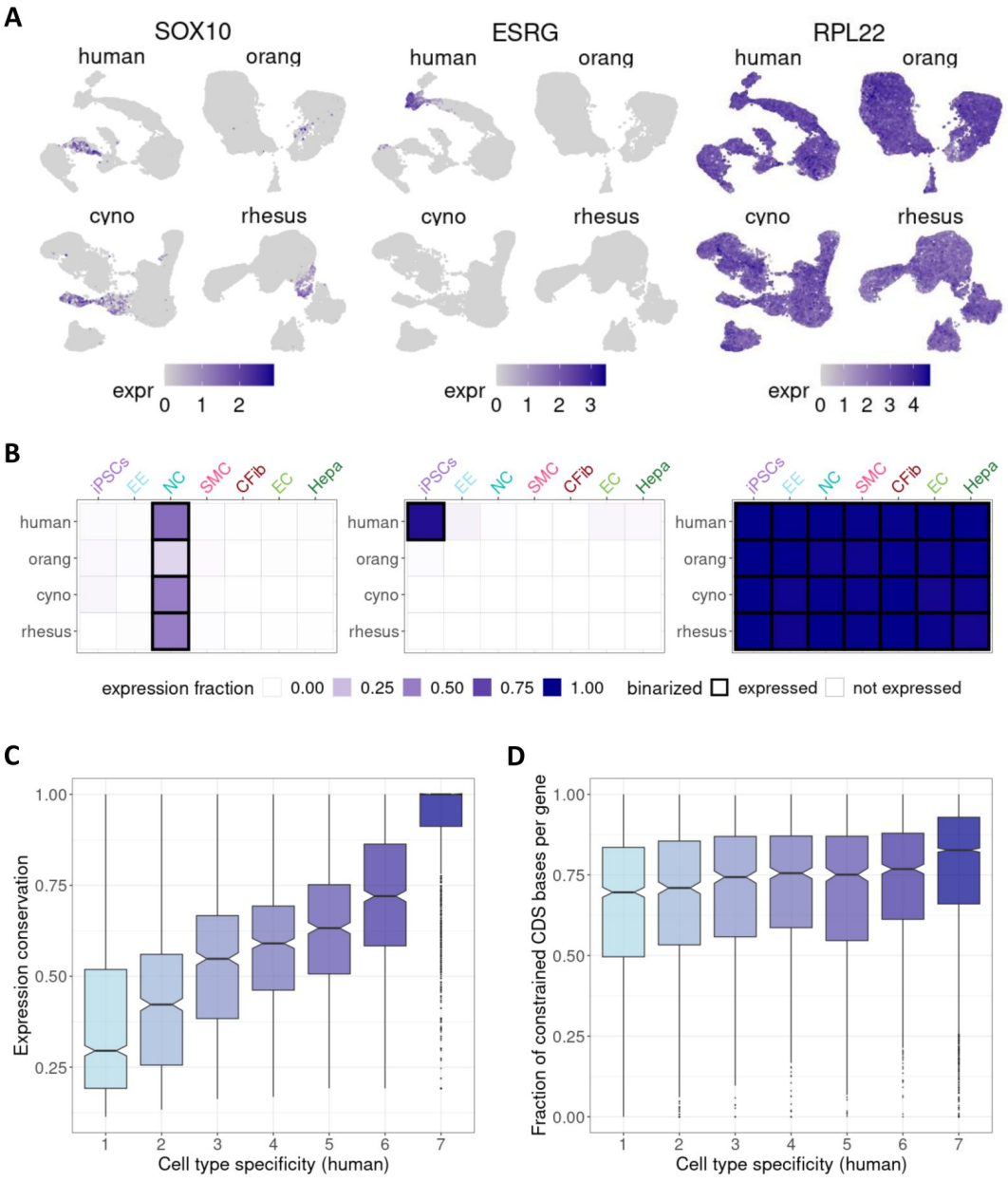

Figure 3.

Effect of cell type specificity on expression conservation.

A) UMAP visualizations depicting expression patterns of selected example genes: *SOX10* (conserved cell type-specific expression in neural crest cells), *ESRG* (species-specific and cell type-specific expression in human iPSCs), and *RPL22* (conserved, broad expression). B) For each gene, expression was summarized per species and cell type as the expression fraction and binarized into “not expressed”/”expressed” (black frame) based on cell type-specific thresholds. The same example genes as in A) are shown here. iPSCs: induced pluripotent stem cells, EE: early ectoderm, NC: neural crest, SMC: smooth muscle cells, CFib: cardiac fibroblasts, EC: epithelial cells, Hepa: hepatocytes. c) Boxplot of expression conservation of genes with different levels of cell type specificity in human. D) Boxplot of the fraction of coding sequence sites that were found to evolve under constraint based on a 43 primate phylogeny [34], stratified by human cell type specificity.

For example, we find that the neural crest-marker *SOX10* [32] is cell type-specific and conserved, the lncRNA *ESRG* is iPSC- and human-specific, in contrast *RPL22*, a gene that encodes a protein of the large ribosomal subunit, is broadly expressed and conserved (**Figure 3A**). Overall we find on average ~15% of genes to be cell type-specific, i.e. our score determined them to be expressed in only one cell type, while ~40% of genes were found to be broadly expressed in all seven cell types (**Supplementary Figure S7A**).

Additionally, we obtained a measure of expression conservation, which quantifies the consistency of the cell type expression score across species. We found that broadly expressed genes present in all cell types exhibited high expression conservation, whereas cell type-specific genes tended to be more species-specific (**Figure 3C**; **Supplementary Figure S7B**).

Unsurprisingly, broadly expressed genes also showed higher average expression levels [33] (**Supplementary Figure S7D**). To ensure that the observed relationship between expression breadth and conservation in our data is not solely due to expression level differences, we sub-sampled genes from all cell type-specificity levels for comparable mean expression. This did not change the pattern: also broadly expressed genes with a low mean expression level are highly conserved across species (**Supplementary Figure S7E,F**). Moreover, also the coding sequences of broadly expressed genes show higher levels of constraint than more cell type-specific genes, thus supporting the notion that also the higher conservation of the expression pattern that we observed here is due to evolutionary stable functional constraints on this set of genes (**Figure 3D**; **Supplementary Figure S7C**).

Marker gene conservation

Building on our previous observation that cell type-specific genes are less conserved across species, we investigated the conservation and transferability of marker genes, which are, by definition, cell type-specific, in greater detail. To this end, we call marker genes for all cell types and species, using a combination of differential expression analysis and a quantile rank-score based test for differential distribution detection[35]. Additionally, we define a good marker gene as one that is upregulated and expressed in a higher fraction of cells compared to the rest. To prioritize marker genes, we rank them based on the difference in the detection fraction: the proportion of cells of a given type in which a gene is detected compared to its detection rate in all other cells.

We found a low overlap of top marker genes among species, with a median of 15 of the top 100 ranked marker genes per cell type shared across all four species, while a larger proportion of markers was unique to individual species (**Figure 4A**). Notably, these species-specific markers often exhibited cell type-specific expression in only one species, with reduced or non-specific expression in others (**Figure 4B**; **Supplementary Figure S8**).

Given the special role of transcriptional regulators for the definition of a cell type [36] and the differences in conservation between protein-coding and non-coding RNAs [37], we analyzed the comparability of marker genes of different types. To this end, we assessed the concordance of the top 100 marker genes across species for protein-coding genes, lncRNAs, transcription factors (TFs) or all genes using rank biased overlap (RBO) scores [38]. We find that marker genes that are TFs have the highest concordance between species and that the two macaques species which are also phylogenetically most similar are also most similar in their ranked marker gene lists. In contrast, lncRNA markers show the lowest overlap between species. In fact, their cross-species conservation is so low that they also significantly reduce the performance if they are included together with protein-coding markers (**Figure 4C**).

To properly evaluate the performance of marker genes, it is essential to consider their ability to differentiate between cell types. This discriminatory power ultimately determines how well marker genes perform in cell type classification within and across species. To this end, we trained

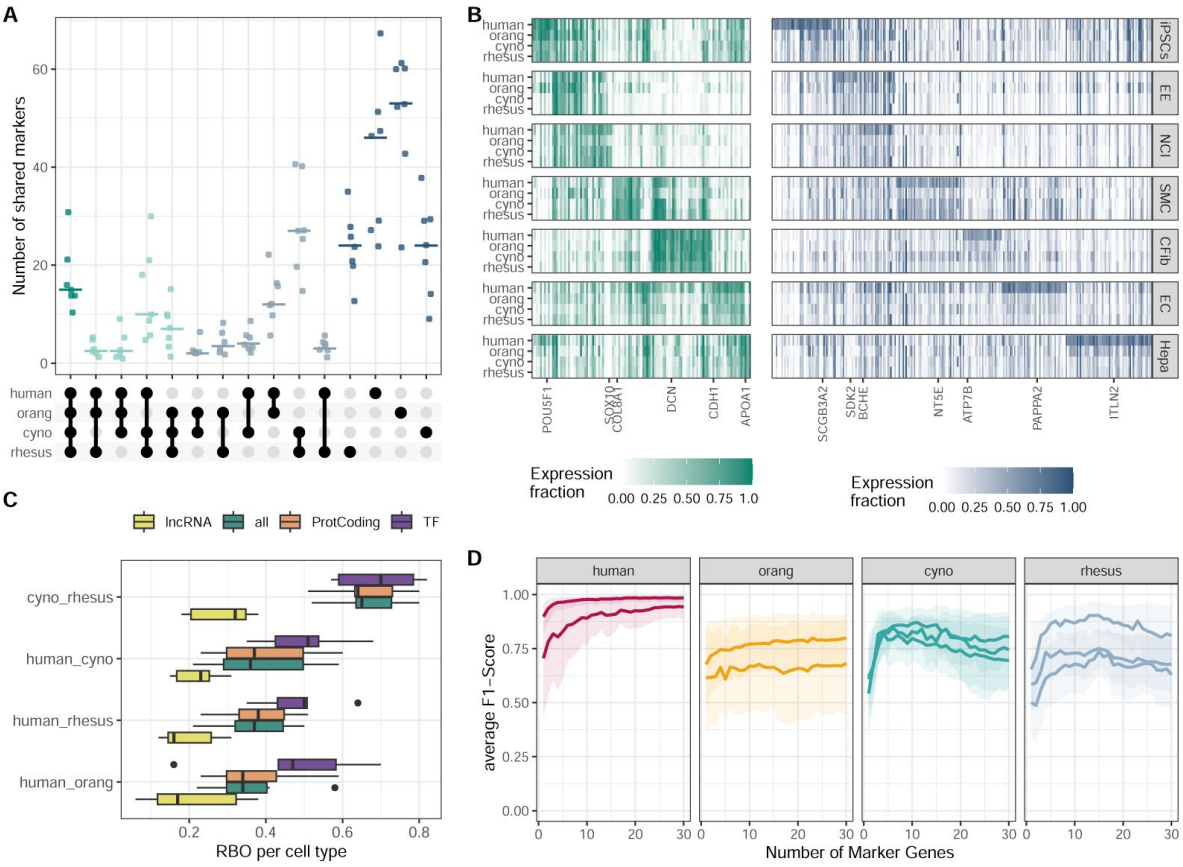

Figure 4.

Evaluation of marker gene conservation.

A) UpSet plot illustrating the overlap between species for the top 100 marker genes per cell type. B) Heatmap showing the expression fractions of marker genes: on the left, markers shared among all species, and on the right, markers unique to the human ranking. For each cell type, one representative gene is labeled and further detailed in **Supplementary Figure S8**. iPSCs: induced pluripotent stem cells, EE: early ectoderm, NC: neural crest, SMC: smooth muscle cells, CFib: cardiac fibroblasts, EC: epithelial cells, Hepa: hepatocytes. C) Rank-biased overlap (RBO) analysis comparing the concordance of gene rankings per cell type for lncRNAs, protein-coding genes and transcription factors. D) Average F1-score for a kNN-classifier trained in the human clone 29B5 to predict cell type identity based on the expression of 1-30 marker genes. Each line represents the performance in a different clone, with shaded areas indicating 95% bootstrap confidence intervals.

a k-nearest neighbors (kNN) classifier on varying numbers of marker genes per cell type in one human clone (29B5) and evaluated prediction performance using the average F1-score across cell types (**Supplementary Figure S9**). Again, we analyzed markers from a set of all protein-coding genes and TFs only and find that even though TFs appear to be more conserved across species, they do not discriminate cell types as well as the top protein-coding markers (**Supplementary Figure S10**). Using protein-coding marker genes only determined with 29B5 to classify the other human clone, we achieve good discriminatory power (F1 score > 0.9) with only 11 marker genes per cell type. In contrast, the classification performance for clones from the other species was substantially lower, failing to reach the performance levels observed in human clones even when using up to 30 marker genes (**Figure 4D**).

In summary, we find that lncRNA markers genes have low transferability between species, while protein-coding markers do reasonably well. However, the predictive value of marker genes decreases with increasing phylogenetic distance, requiring longer marker gene lists to achieve accurate cell type classification for more distantly related species.

Discussion

An essential criterion for a true cell type is reproducibility across experiments, individuals, or even species. This raises the question of how to reliably identify reproducible cell types across species. When cell types are annotated separately for each species, their reproducibility can be evaluated based on transcriptomic similarity [6, 39]. If integration-based methods are used to accomplish this task [22, 7], reproducibility not only depends on the similarity of the expression profiles but also on cell type composition. Integration works best when the cell type compositions are as similar as possible across experiments. This however is not the case for organoids, which often have highly heterogeneous cell type compositions [40] and our EB-data are no exception. Moreover, integration methods struggle with large and variable batch effects, which are expected due to the varying phylogenetic distances across species [4]. In contrast, classification methods such as SingleR [29] rely mainly on the similarity to a reference profile, which makes it less vulnerable to cell type composition and batch effects. Hence, in our pipeline to identify orthologous cell types we mainly rely on classification. We start with an unsupervised approach in that we identify cell clusters and then ensure reproducibility as well as comparability using a supervised approach with reciprocal classification of clusters across all species pairs.

Defining cell types in a developmental dataset is particularly challenging, and we do not believe that there is one perfect solution that would fit all cell types and samples. Therefore, we rely on an interactive approach that we implemented in a shiny app (*https://shiny.bio.lmu.de/Cross_Species_CellType/*) to facilitate the flexible choice of parameters for cluster matching, merging and inspection by visualizing marker genes. Suresh et al [8] employed a similar approach also requiring several manual parameter choices. This makes a formal comparison difficult. Generally both methods seem to agree well on the orthology assignments of cell type clusters (**Supplementary Figures S5** & **S4**).

Hence, the carefully annotated dataset presented here can serve as a valuable resource for future research. Non-human primate iPSCs are central to many studies focusing on evolutionary comparisons, and the pool of iPSC lines for these purposes is expected to grow, incorporating more species and individuals. In this context, the transcriptomic data we generated offer a reference dataset that can be used to verify the pluripotency and differentiation potential of non-human primate iPSC lines by examining gene expression during EB formation.

The set of shared cell types between all four primate species allowed us to evaluate the conservation and transferability of marker genes between species. To begin with, marker genes are by definition cell type-specific and also with this dataset, we can show that they are less

conserved than broadly expressed genes. Expression breadth can be interpreted as a sign of pleiotropy and hence higher functional constraint [41, 31]. Conversely, we expect cell type-specific marker genes to be among the least conserved genes. Indeed, we and others find that the overlap of marker genes across species is limited [14, 15, 7, 42]. Moreover, conservation varies significantly across gene biotypes. On the one hand, lncRNAs, which are often highly cell type-specific, exhibit lower cross-species conservation. Their low sequence conservation further complicates their utility for comparative studies [37]. On the other hand, TFs, which have been proposed as central elements of a Core Regulatory Complex (CoRC) that defines cell type identity [36], are among the most conserved markers across species. However, the power to distinguish cell types based solely on the expression of TF markers remains lower than when markers are selected from the broader set of all protein-coding genes (**Supplementary Figure S10**). Even though within species already a handful of marker genes can achieve remarkable accuracy, their discriminatory power remains lower for other species. Thus, whole transcriptome profiles offer a more comprehensive approach to cross-species cell type classification for single cell data.

This said, marker genes remain fundamental to most current cell type annotations. Moreover, marker genes will continue to be used to match cell types across modalities, as for example to validate cell type properties in experiments that are often based on immunofluorescence of individual markers or gene panels as used for spatial transcriptomics [43, 44]. To this end, we have refined the ranking of marker genes beyond differential expression analysis to focus on consistent differences in detection rate. Markers identified in this way are bound to translate better into protein-based validations than markers defined based on expression levels, due to the discrepancy of mRNA and protein expression [45]. Furthermore, the presence-absence signal is more robust against cross-species fluctuations in gene expression than measures based on expression level differences.

In conclusion, we present a robust reference dataset for early primate development alongside tools to identify and evaluate orthologous cell types. Our findings emphasize the need for caution when transferring marker genes for cell type annotation and characterization in cross-species studies.

Methods

EB differentiation method comparison

Four EB differentiation protocols are compared initially, which are combinations of two differentiation media (DFK20 and EB-medium) and two differentiation methods (dish and 96-well).

For single-cell differentiation in 96-well plates, primate iPSCs from one 80% confluent 6-well are washed with DPBS and incubated with Accumax (Sigma-Aldrich, SCR006) for 7 min at 37 °C. Afterwards, iPSCs are dissociated to single-cells, the enzymatic reaction is stopped by adding DPBS, and cells are counted and pelleted at 300 xg for 5 min. Single cells are resuspended in EB-medium consisting of StemFit Basic02 (Nippon Genetics, 3821.00) w/o bFGF or DFK20, both supplemented with 10 µM Y-27632 (Biozol, ESI-ST10019). The DFK20-medium consists of DMEM/F12 (Fisher Scientific, 15373541) with 20% KSR (Thermo Fisher Scientific, 10828-028), 1% MEM non-essential amino acids (Thermo Fisher Scientific, 11140-035), 1% Glutamax (Thermo Fisher Scientific, 35050038), 100 U/mL Penicillin, 100 µg/mL Streptomycin (Thermo Fisher Scientific, 15140122) and 0.1 mM 2-Mercaptoethanol (Thermo Fisher Scientific, M3148). Afterwards, 9,000 cells in 150 µl medium are seeded per well of a Nuclon Sphera 96-well plate (Fisher Scientific, 15396123) and cultured at 37 °C and 5% CO_2. A medium change with the corresponding EB differentiation medium w/o Rockinhibitor is performed every other day during the whole protocol. EBs are collected from the 96-well plate and subjected to flow cytometry after 7 days of differentiation.

For clump differentiation in culture dishes, primate iPSCs from one 80% confluent 12-well are washed with DPBS and incubated with 0.5 mM EDTA (Carl Roth, CN06.3) for 3-5 min at RT. The EDTA is removed, StemFit (Nippon Genetics, 3821.00) supplemented with 10 µM Y-27632 (Biozol, ESI-ST10019) is added and cells are dissociated to clumps of varying sizes. Subsequently, the clumps are transferred to sterile bacterial dishes with vents and cultured at 37 °C and 5% CO_2. After 24 h, the medium is exchanged by either EB-medium or DFK20 supplemented with 10 µM Y-27632 for additional 24 h, before changing the medium to EB-medium or DFK20. A medium change is performed every other day during the protocol from day 4 on. EBs are collected from the dishes and subjected to flow cytometry after 7 days of differentiation.

Flow cytometry

Flow cytometry is performed on day 7 of the differentiation protocol. Therefore, 1/10 of the EBs are collected, washed with DPBS, incubated with Accumax (Sigma-Aldrich, SCR006) for 10 min at 37 °C and dissociated to single cells. After washing, cells are incubated with the Viability Dye eFluor 780 (Thermo Fisher Scientific, 65-0865-18) diluted 1/1000 in PBS for 30 min at 4°C in the dark. The live/dead stain is quenched by the addition of Cell Staining Buffer (CSB) consisting of DPBS with 0.5% BSA (Sigma-Aldrich, A3059), 0.01% NaN_3 (Sigma-Aldrich, S2002) and 2 mM EDTA (Carl Roth, CN06.3). Subsequently, cells are pelleted and incubated with a mixture of the following antibodies diluted 1/200 in CSB for 1h at 4°C in the dark. The antibodies used are anti-TRA-1-60-AF488 (STEMCELL Technologies, 60064AD.1), anti-CXCR4-PE (BioLegend, 306505), anti-NCAM1-PE/Cy7 (BioLegend, 318317) and anti-PDGFR*α*-APC (BioLegend, 323511). After centrifugation, cells are resuspended in PBS containing 0.5% BSA, 0.01% NaN_3 and 1 µg/ml DNase I (STEMCELL Technologies, 07469), filtered through a strainer and analyzed using the BD FACSCanto Flow Cytometry System. Flow cytometry data are analyzed using FlowJo (V10.8.2).

In-vitro embryoid body differentiation

Two human, two orangutan, three cynomolgus and three rhesus iPSC lines are used for EB differentiation. The human and orangutan iPSCs are reprogrammed from urinary cells, while cynomolgus and rhesus iPSCs were reprogrammed from fibroblasts. All cell lines were characterized and validated previously and were tested negative for mycoplasma and SeV reprogramming vector integration [23, 24, 25].

For embryoid body formation prior to 10x scRNA-seq, the EB differentiation protocol using DFK20 medium in culture dishes is performed in duplicates for each clone. After 8 days of floating culture in dishes, EBs from both replicates are pooled and seeded into 6-wells coated with 0.2% gelatin (Sigma-Aldrich, G1890) for another 8 days of attached culture with subsequent medium changes every other day. In total, three replicates of EB formation are performed on different days, and each replicate includes cell lines from all four primate species.

scRNA-seq library generation and sequencing

EBs are sampled on day 8 and day 16 of the protocol. For dissociation, floating EBs are collected, while attached EBs are kept in their wells, washed with DPBS and incubated with Accumax (Sigma-Aldrich, SCR006) for 10-20 min at 37 °C. Afterwards, EBs are pipetted up and down with a p1000 pipette until they are completely dissociated. The enzymatic reaction is stopped by adding DFK20 medium, cells are pelleted at 300 xg for 5 min and resuspended in 1 mL DPBS. If cell clumps are observed, the liquid is filtered through a 40 µm strainer before counting them with a Countess II automated cell counter (Thermo Fisher Scientific, C10228). Equal cell numbers from each cell line are pooled, washed with DPBS + 0.04% BSA and resuspended in DPBS + 0.04% BSA aiming for a final concentration of 800 −1000 cells/µL. scRNA-seq libraries are generated using the 10x Genomics Chromium Next GEM Single Cell 3'Kit V3.1 workflow in three replicates. Each time, evenly pooled single cells from the different cell lines are loaded on 2 to 6 lanes of a 10x chip,

targeting 16,000 cells per lane. Libraries are sequenced on an Illumina NextSeq1000/1500 with an 100-cycle kit and the following sequencing setup: read 1 (28 bases), read 2 (10 bases), read 3 (10 bases) and read 4 (90 bases).

Alignment of scRNA-seq data

Reads are processed with Cell Ranger version 7.0.0. We map all reads to 4 reference genomes: *Homo sapiens* GRCh38 (GENCODE release 32), *Pongo abelii* Susie PABv2/ponAbe3, *Macaca fascicularis* macFas6 and *Macaca mulatta* rheMac10. The orangutan, cynomolgus macaque and rhesus macaque GTF files are created by transferring the hg38 annotation to the corresponding primate genomes via the tool Liftoff [46], followed by removal of transcripts with partial mapping (<50%), low sequence identity (<50%) or excessive length (>100 bp difference and >2 length ratio).

Species and individual demultiplexing

Since we pool cells from multiple species on each 10x lane, we use cellsnp-lite [47] version 1.2.0 and vireo [48] version 0.5.7 to assign single cells to their respective species. Initially, we obtain a list of 51000 informative variants (referred to as 'species vcf file') from a bulk RNA-seq experiment involving samples from *Homo sapiens*, *Pongo abelii* and *Macaca fascicularis*, mapped to the GRCh38 reference genome. We run cellsnp-lite in mode 2b for whole-chromosome pileup and filter for high-coverage homozygous variants to identify informative variants.

For the demultiplexing of species in the scRNA-seq data we employ a two step strategy:

1. Initial species assignment: Using the Cell Ranger output aligned to GRCh38, we genotype each single cell with cellsnp-lite providing the species vcf file as candidate SNPs and setting a minimum UMI count filter of 10. Subsequently we assign single cells to human, orangutan or macaque identity with vireo using again the species vcf file as the donor file.
2. Distinguishing macaque species: To differentiate between the two macaque species, *Macaca fascicularis* and *Macaca mulatta*, we use the Cell Ranger output aligned to rheMac10. After genotyping with cellsnp-lite we demultiplex with vireo specifying the number of donors to two, without providing a donor vcf file in this case. We assign the donor, for which the majority of distinguishing variants agreed with the rheMac10 reference alleles, to *Macaca mulatta* and the other donor to *Macaca fascicularis*.

To distinguish different human individuals pooled in the same experiment, we genotype single cells with cellsnp-lite with a candidate vcf file of 7.4 million common variants from the 1000 Genomes Project, demultiplexed with vireo specifying two donors and assign donors to individuals based on the intersection with variants from bulk RNA-seq data of the same individuals. To distinguish different cynomolgus individuals, we use a reference vcf with informative variants obtained from bulk RNA-seq data to genotype single cells and demultiplex the individuals.

Processing of scRNA-seq data

We remove background RNA with CellBender version 0.2.0 [49] at a false positive rate (FPR) of 0.01. After quality control we retain cells with more than 1000 detected genes and a mitochondrial fraction below 8%. We remove cross-species doublets based on the vireo assignments and intra-species doublets using scDblFinder version 1.6.0 [50], specifying the expected doublet rate based on the cross-species doublet fraction. For each species, we normalize the counts with scran version 1.28.2 [51] and integrated data from different experiments with scanorama [27]. UMAP dimensionality reductions are created with Seurat version 4.3.0 on the first 30 components of the scanorama corrected embedding per species. Besides the separate processing per species, we also

create an integrated dataset of all 4 species together using Harmony version 0.1.1 [28]. We identify clusters on the first 20 Harmony-integrated PCs with Seurat at a resolution of 0.1 (**Figure 1D,E**).

Reference based classification

To get an initial cell type annotation, we download a reference dataset of day 21 human EBs [18]. We normalize the count matrix with scran and intersect the genes between reference and our scRNA-seq dataset. Next, we train a SingleR version 2.0.0 [29] classifier for the broad cell type classes defined in **Figure 1G** of the original publication [18] using *trainSingleR* with pseudo-bulk aggregation. Cell type labels are transferred to cells of each species with *classifySingleR*.

Orthologous cell type annotation

To annotate orthologous cell types, we first perform high resolution clustering of the scRNA-seq data for each species separately. For this we take the first 20 components of the scanorama corrected embedding as input to perform clustering in Seurat with *FindNeighbors* and *FindClusters* at a resolution of 2 to obtain the initial high resolution clusters (HRCs).

Next, we score the similarity of all HRCs with an approach based on reciprocal classification. For each species, we train a SingleR classifier on all HRCs of a species. We then classify the cells of all other species with *classifySingleR*. In this way, we can calculate the similarity of each HRC in the target species to each HRC in the reference species as the fraction of cells of the target HRC classified as the reference HRC. To also obtain similarity scores between HRCs within a species, we split the data of each species into a reference set with 80% of cells and a test set with 20% of cells. Analogous to the cross-species classification scheme, we transfer HRC labels from the reference set to the test set and score the overlap of target and reference HRCs.

In the next step, we combine HRCs based on pairwise similarity scores. We average the bidirectional similarity scores for each HRC pair and construct a distance matrix with all HRCs. Subsequently, based on hierarchical clustering (hclust, average method) we define 26 initial orthologous cell type clusters (OCCs) based on the visual inspection of the distance matrix. In this way, we merge similar HRCs within species and match HRCs across species to obtain a set of OCCs.

OCCs with very similar expression and marker profiles can be further merged. Therefore, we create pseudobulk profiles for each OCC and calculate Spearman's ρ for all pair-wise comparisons within a species (s) based on the 2,000 most variable genes. We perform hierarchical clustering on $1 - \bar{\rho_s}$ and merge orthogolous clusters at a cut height of 0.1, that was interactively determined by also inspecting the similarity of the top marker genes as found by Seurat's *FindMarkers*. In the shiny app, we provide a list of OCC markers for each species separately, but also the intersection of conserved markers. Based on those marker combinations the user can then assign the cell types. If the marker gene distribution as visualized in UMAPs reveals overmerged OCCs, the user can split them interactively. Specifically, we separate merged OCC 4 into iPSCs, cardiac progenitor cells and early epithelial cells for the final assignment. We assign merged OCC 5 as neural crest I, but re-annotate a subcluster present only in cynomolgus and rhesus macaques as fibroblasts. Similarly, we re-annotate a subcluster of merged OCC 12 (granule precursor cells) as astrocyte progenitors in cynomolgus and rhesus macaque. Finally, we exclude OCCs with less than 800 cells that are only present in 1 or 2 species.

We assess the correspondence of the final cell type assignments across species with two approaches. For the scores shown in **Supplementary Figure S4** we apply the same reciprocal classification approach as described above providing cell type labels instead of HRCs as initial clusters. For the scores shown in **Supplementary Figure S5** we use the function *MetaNeighborUS* of MetaNeighbor version 1.18.0 to compare cell type labels across species.

Presence-absence scoring of expression

To determine when to define a gene as expressed in a certain cell type, we derive a lower limit of gene detection per cell type and species while accounting for noise and differences in power to detect expression. We first filter the count matrices for each clone, keeping only genes with at least 1% nonzero counts and cells within 3 median absolute deviations for number of UMIs and the number of genes with counts > 0 per cell type and species. These filtered matrices are then downsampled so that we keep the same number of cells in each species (n=18,800), while keeping the original cell type proportion. Next, per species, we estimate the following distributional characteristics per gene (i) across cell types (j): 1) the fraction of nonzero counts (f_{ij}), 2) the mean ($\mu ij \pm s.e.(\mu_{ij})$) and dispersion ($\theta i$) of the negative binomial distribution using glmgampoi v1.10.2 [52]. In the next step, we define a putative expression status per gene per cell type. 1) genes are detectable if their log mean expression $log(\mu_{ij})$ is above the fifth quantile of the $log(\mu)$ value distribution across all genes per cell type. 2) genes are reliably estimable if the ratio $log(\frac{s.e.(\mu_{ij})}{\mu_{ij}})$ is below the 90th quantile of $log(\frac{s.e.(\mu)}{\mu})$ value distribution. Only when both conditions are met is the expression status set to 1, otherwise 0. A binomial logistic regression model using Firth's bias reduction method as implemented in R package logistf (version 1.26.0) is then applied to derive the minimal gene detection needed to call a gene expressed, i.e. when P(Y=1) solve $log(\frac{p}{1-p}) = a + b * f_{ij}$ towards f_{ij}. To ensure consistency between species, we set the detection threshold for each cell type to the maximum threshold among all species.

Cell type specificity and expression conservation scores

To assess cell type specificity and expression conservation of genes across species, we first determine in which cell types a gene is expressed in a species, using the thresholds defined in the previous section. Thus we determine cell type specificity as the number of cell types in which a gene was found to be expressed. Here this score can be maximally 7, i.e. the gene is detected in all cell types that were found in all four species.

To evaluate expression conservation, we develop a phylogenetically weighted conservation score for each gene, reflecting the number of species in which the gene is expressed, weighted by the scaled phylogenetic distance as estimated in Bininda-Edmonds et al. [53]. For each gene, we calculate the expression conservation score as follows:

$$Expression\ conservation = \frac{1}{N_{ct}} \sum_{ct} \sum_{b \in detected} bl \qquad (1)$$

where N_{ct} is the number of cell types in which the gene is detected. We then simply sum the scaled branch lengths *bl* across all cell types (*ct*) and branches (*b*) on which we infer the gene to be expressed. Because we only have 4 species, we only have one internal branch, for which we infer expression if at least one great ape and one macaque species show expression in that cell type. The score ranges from 0.075 (detected only in cynomolgus or rhesus macaque) to 1 (detected in the same cell types in all 4 species).

Furthermore, we extract measures of sequence conservation for protein-coding genes from Supplementary Data S14 in the study by Sullivan et al. [34]. Here, we use the fraction of CDS bases with primate phastCons ¿= 0.96 as a gene-based measure of constraint.

Marker gene detection

We filter the count matrices for each clone to retain only genes with nonzero counts in one of the 7 cell types that were detected in all species. We then downsample these filtered matrices to equalize the number of cells across species, leaving us with ~11,600 cells per species. Furthermore, to mitigate differences in statistical power due to varying numbers of cells per cell

type, we perform testing on cell types with a minimum of 10 and a maximum of 250 cells for each pairwise comparison of 'self' versus 'other'. We identify marker genes using the p-values (p_{adj} < 0.1) determined by ZIQ-Rank [35] and use Seurat *FindMarkers* with logistic regression to identify the cell types for which the gene is a marker. Furthermore, the marker gene needs to be above the cell type's detection threshold (see above) and needs to be up-regulated in the cell type for which it is a marker (log fold change > 0.01). Finally, a marker gene must be detected in a larger proportion of cells for which it is a marker than in other cell types ($p_j - \bar{p}_{other} = \Delta > 0.01$). The detection proportion Δ is also used as to sort the lists of marker genes, deeming the genes with the largest Δ as the best marker genes. In order to also gauge within species variation in marker gene detection, we conducted the same analysis across clones instead of species. In order to compare cross-species reproducibility of different types of marker genes, i.e protein-coding, lncRNAs and transcriptional regulators, we wanted to compare the ranked lists of marker genes across species. To this end, we perform a concordance analysis using rank biased overlap (RBO) [38] on the top 100 marker genes (rbo R package version 0.0.1). For this part, a list of transcription factors were created by selecting genes with at least one annotated motif in the motif databases JASPAR 2022 vertebrate core [54], JASPAR 2022 vertebrate unvalidated [54] and IMAGE [55]. Annotations for protein-coding and lncRNA genes were extracted from the Ensembl GTF file provided with the human Cell Ranger reference dataset (GRCh38-2020-A). To assess the predictive performance of marker genes, we conduct a kNN classification (FNN R package version 1.1.4.1). We train a kNN classifier (k=3) on the log-normalized counts of the top 1-30 human markers per cell type in the human clone 29B5. We then predict the cell type identity in all clones and summarize classification performance per cell type with F1-scores, as well as the average F1-score across all seven cell types.

Declarations

Ethics approval and consent to participate

All procedures performed are approved by the responsible ethic committee on human experimentation (20-122, Ethikkommission LMU München). All experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

Code for analysis and figures is available on GitHub *https://github.com/Hellmann-Lab/EB-analyses*, and accompanying files are deposited in Zenodo (*https://doi.org/10.5281/zenodo.14198850*). All sequencing files were deposited in GEO (GSE280441).

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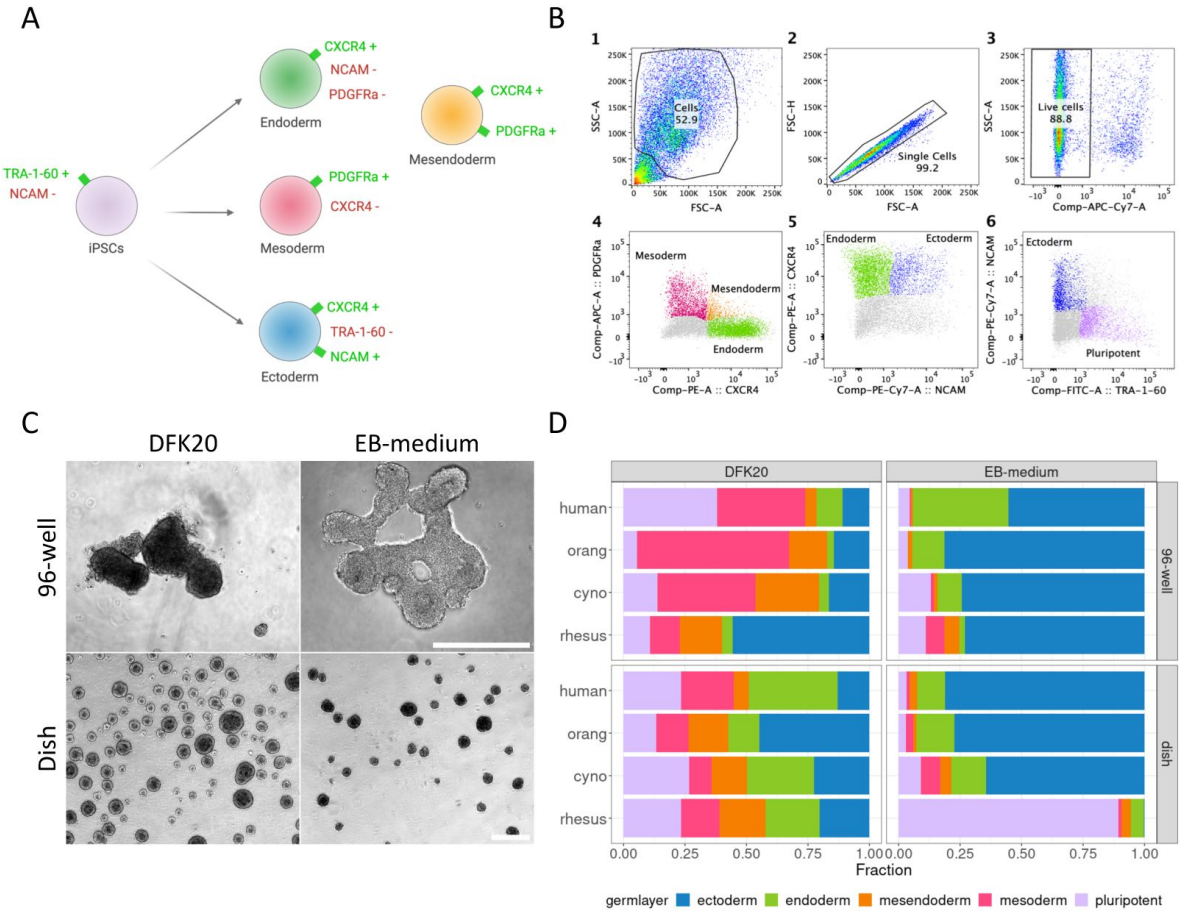
Author's contributions

WE and IH conceived the study. JJ optimized and conducted EB differentiation experiments and performed 10x scRNA-seq data generation with support of FCE. JG generated and provided human and orangutan iPSCs and supported optimization of EB differentiation protocols. PS established FACS analyses of EBs. PJ and JJ did primary data analysis. PJ did the pre-processing of the data, developed the pipeline for orthologous cell type assignment, and created the Shiny app. PJ and BV

performed the cell type specificity and marker gene conservation analysis. AT prepared reference genomes for non-human primates. TD supported cell type annotation. PJ, JJ and IH wrote the manuscript. All authors reviewed and edited the manuscript.

Supplementary Information



Supplementary Figure S1.

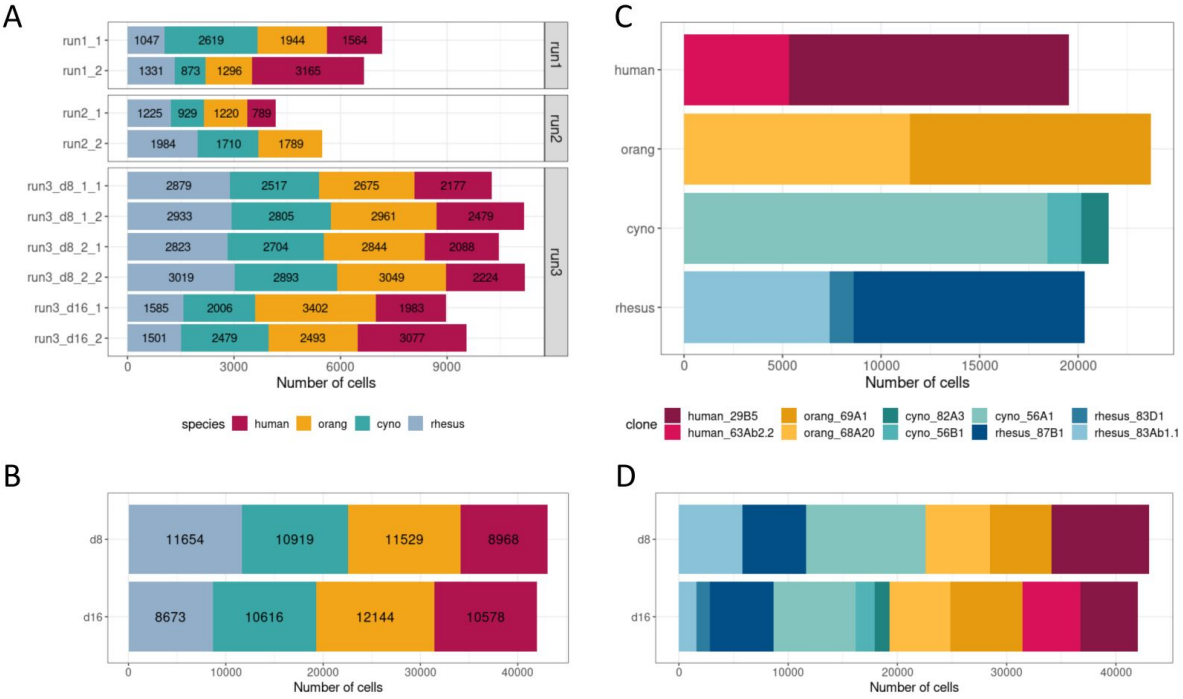
Comparison of EB differentiation protocols using flow cytometry.

A) Antibody combination to analyze iPSCs and cells of the three primary germ layers in a single sample. Created with *BioRender.com*. B) Flow cytometry gating overview using human EBs at day 7 of differentiation. 1. Gating of cell population. 2. Gating of single cell population. 3. Gating of live cell population. 4.-6. Gating of cells belonging to pluripotent or germ layer populations based on the antibody combination shown in S1A). C) Phase contrast images of orangutan EBs on day 6 of differentiation in 4 different culture conditions. Scale bar represents 250 µm. D) Barplot of pluripotency and germ layer proportions of day 7 EBs from human, orangutan, cynomolgus and rhesus in the 4 different culture conditions.

Supplementary Figure S2.

Total number of recovered cells.

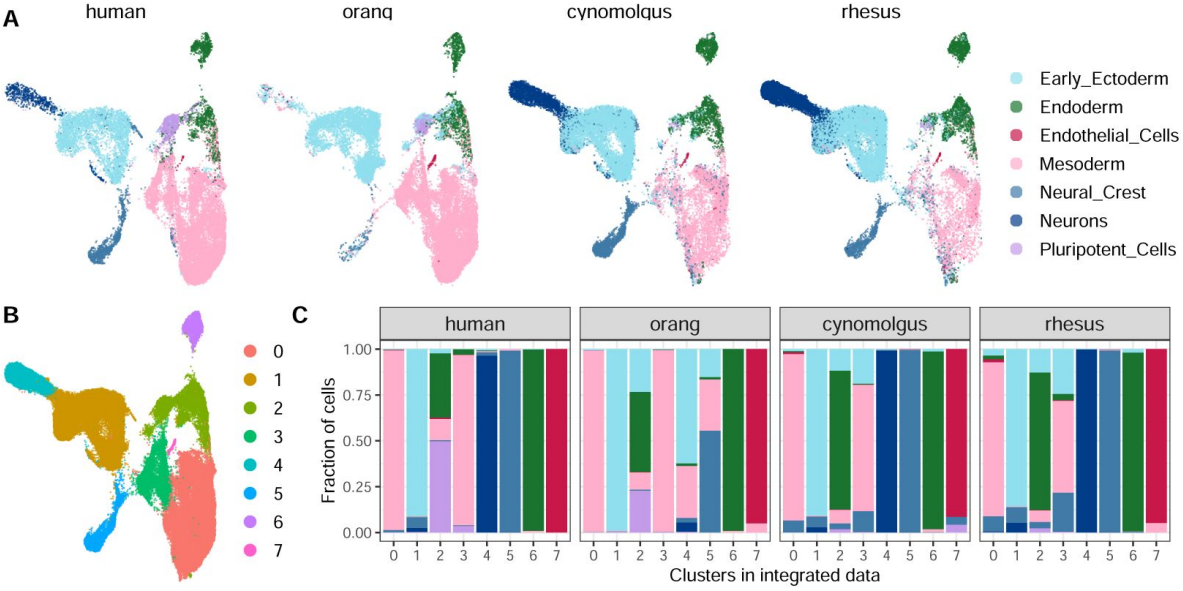
A) Barplot of cell numbers per species and experimental batch and 10x lane. B) Barplot of cell numbers per species and day of differentiation. C) Barplot of cell numbers per clone. D) Barplot of cell numbers per clone and day of differentiation.

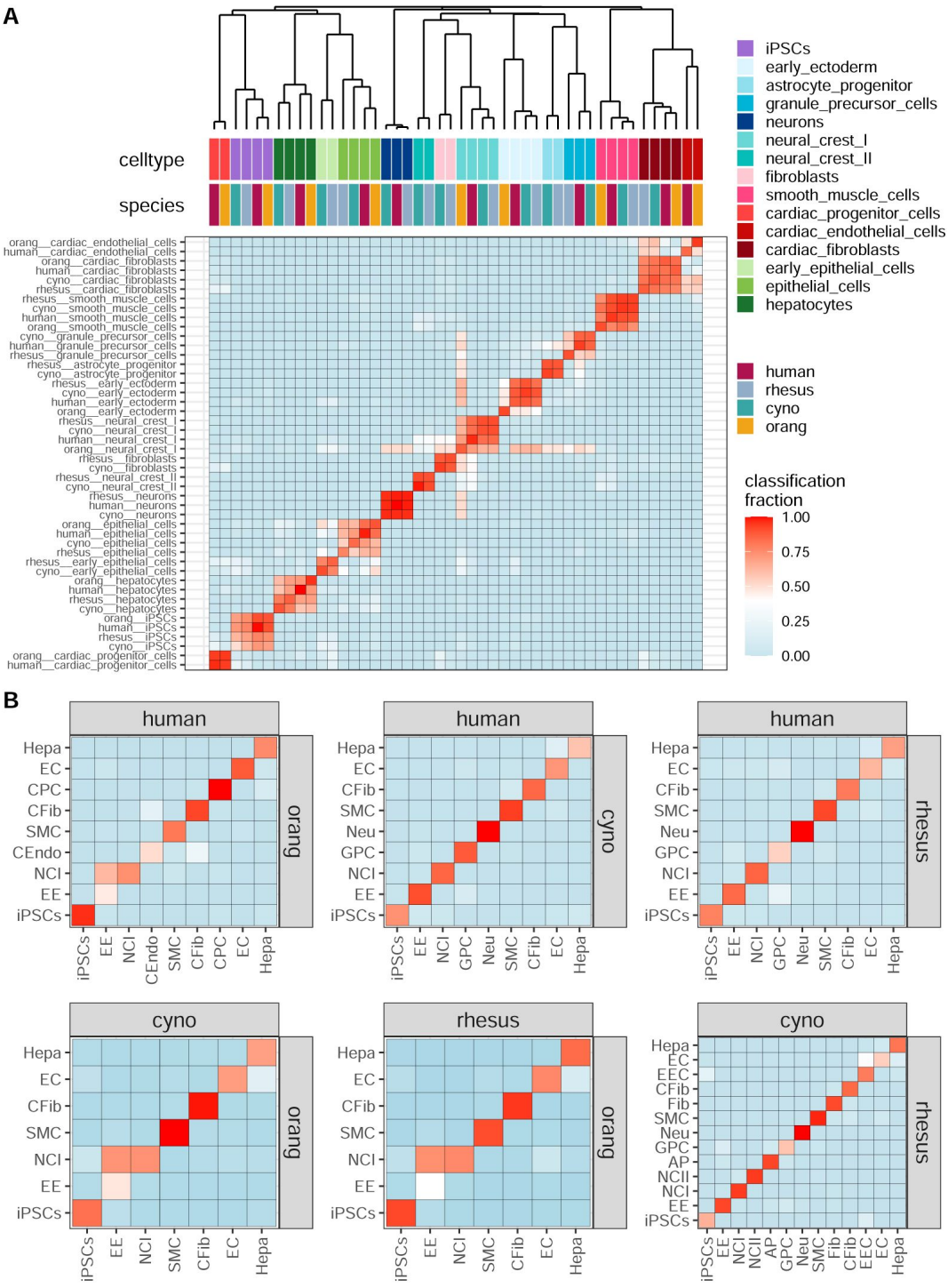


Supplementary Figure S3.

Reference based cell type classification.

A) UMAP representations colored by labels from a classification with a reference dataset of day 21 human embryoid bodies [18]. B) Single cell clusters in integrated data from all 4 species. C) Stacked bar plot of the proportions of predicted labels across clusters obtained in the integrated dataset.

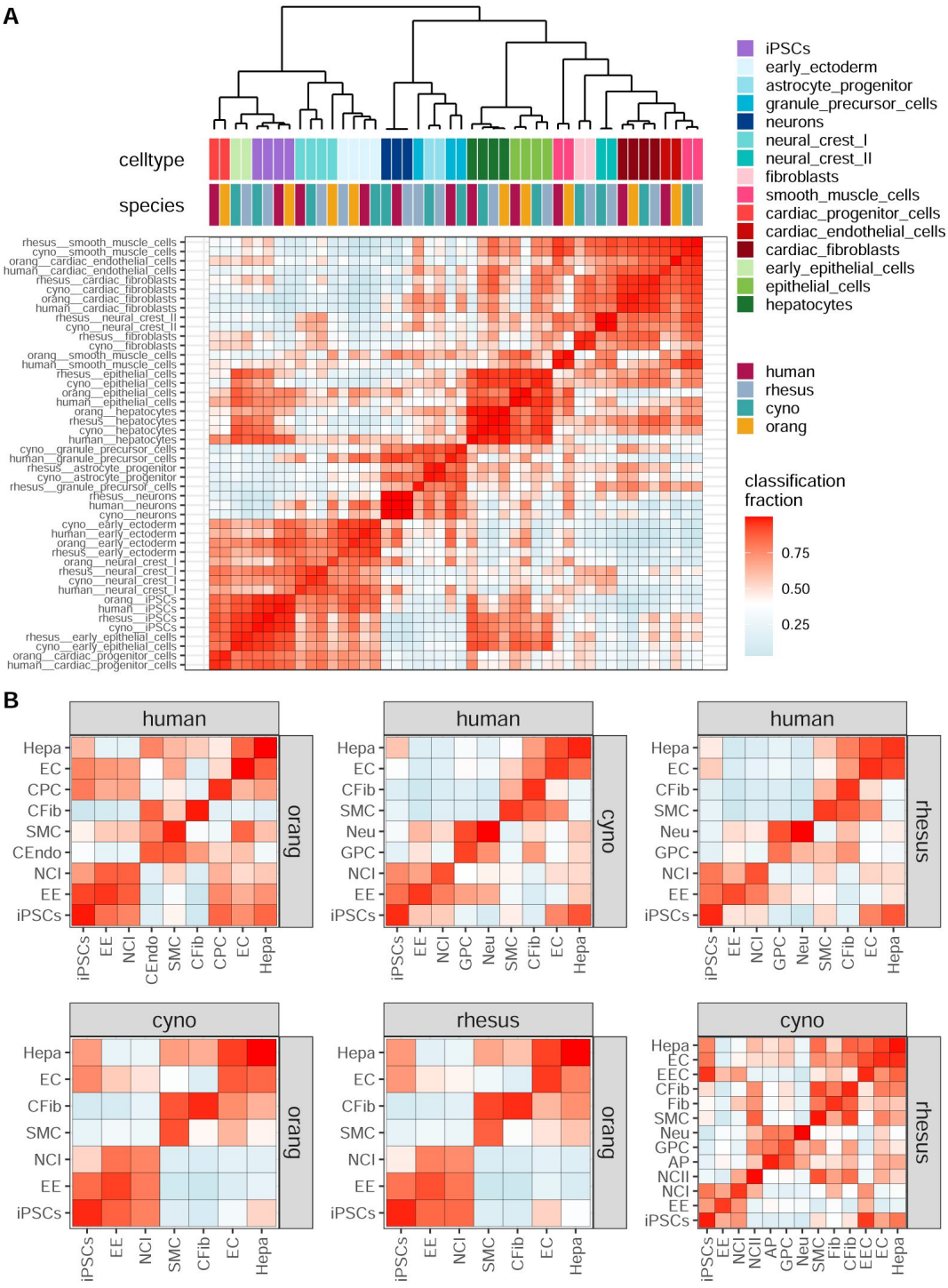




Supplementary Figure S4.

Replicability of cell types across species measured by reciprocal classification.

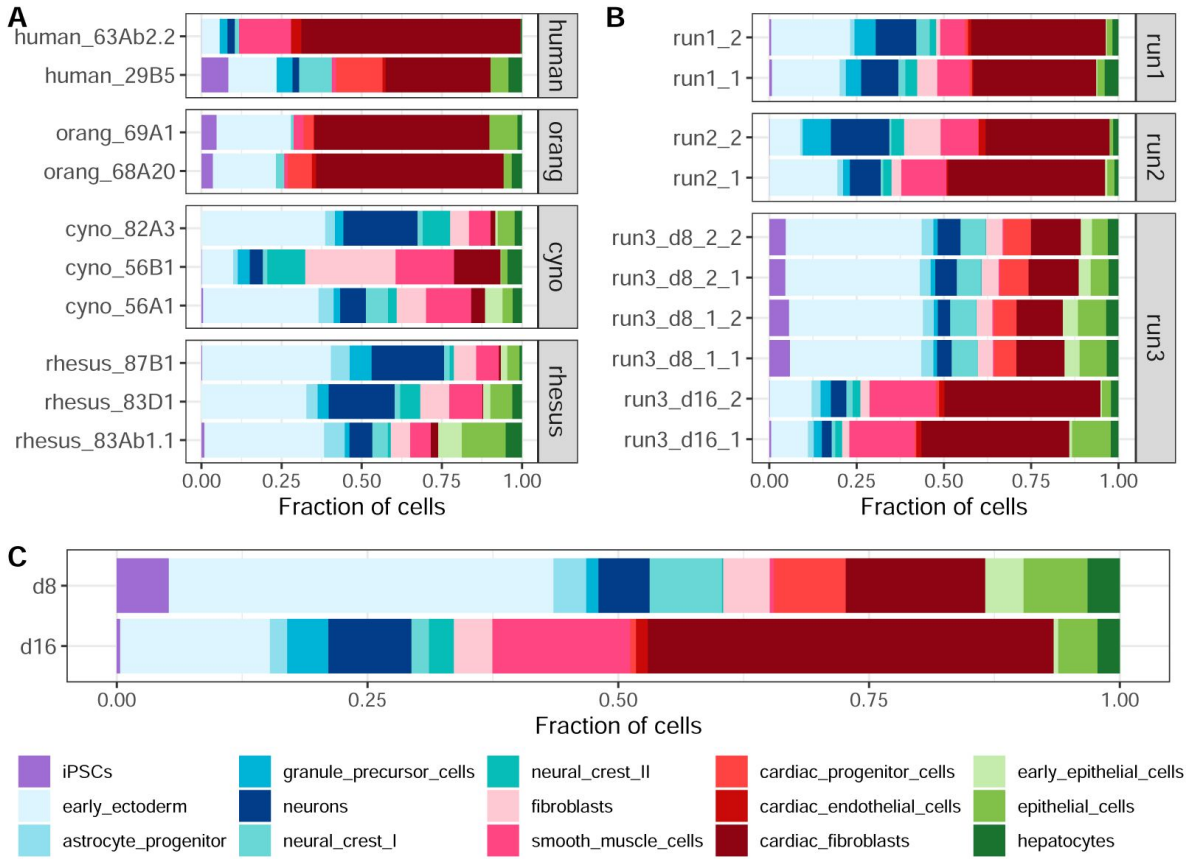
A) Heatmap illustrating 'all vs all' similarities of cell types from all four species. For each cell type pair the similarity represents the average classification fraction obtained through reciprocal classification between each species pair. B) Average classification fractions for cell types that are shared among each species pair. AP: astrocyte progenitor, CFib: cardiac fibroblasts, CEndo: cardiac endothelial cells, CPC: cardiac progenitor cells, EEC: early epithelial cells, EE: early ectoderm, EC: epithelial cells, Fib: fibroblasts, GPC: granule precursor cells, Hepa: hepatocytes, NCI: neural crest I, NCII: neural crest II, Neu: neurons, SMC: smooth muscle cells.



Supplementary Figure S5.

Replicability of cell types across species measured with MetaNeighbor.

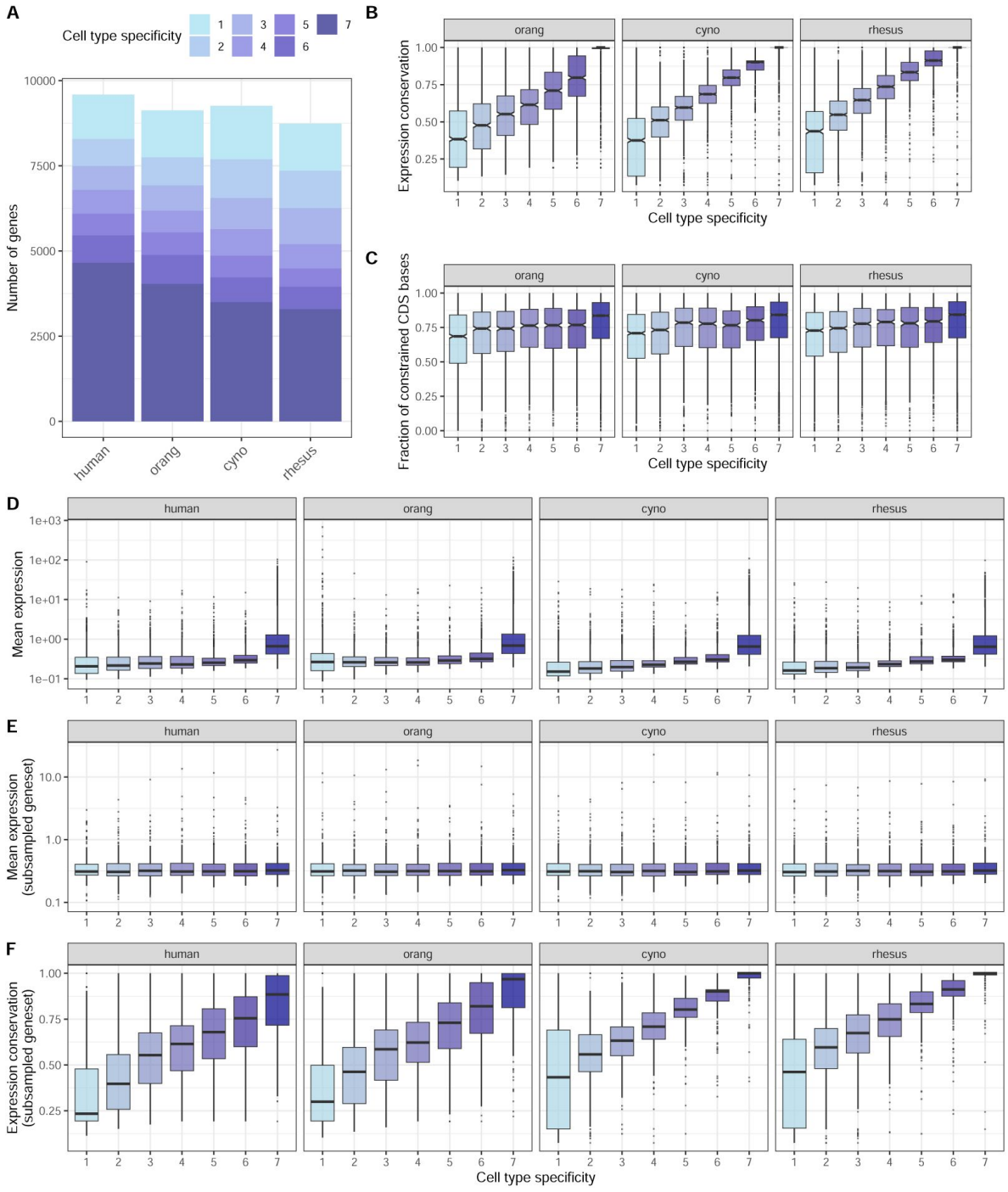
A) Heatmap illustrating 'all vs all' similarities of cell types from all four species. For each cell type pair the similarity represents area under the receiver operator characteristic curve (AUROC) scores obtained with MetaNeighbor [6] in unsupervised mode. B) AUROC scores for cell types that are shared among each species pair. AP: astrocyte progenitor, CFib: cardiac fibroblasts, CEndo: cardiac endothelial cells, CPC: cardiac progenitor cells, EEC: early epithelial cells, EE: early ectoderm, EC: epithelial cells, Fib: fibroblasts, GPC: granule precursor cells, Hepa: hepatocytes, NCI: neural crest I, NCII: neural crest II, Neu: neurons, SMC: smooth muscle cells.



Supplementary Figure S6.

Cell type annotation.

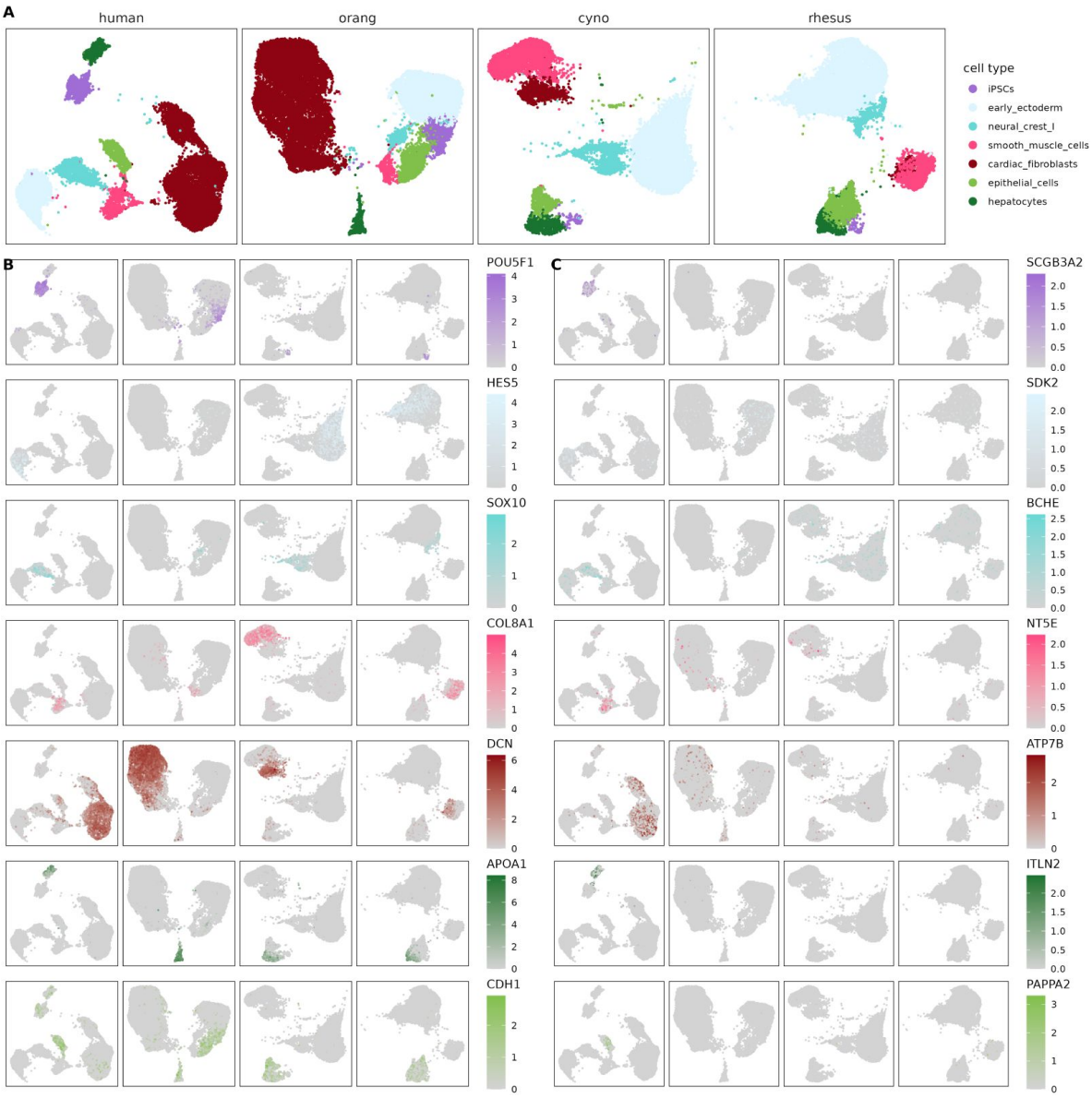
A) Barplot of cell type fractions per species and clone. B) Barplot of cell type fractions per experimental batch and 10x lane. C) Barplot of cell type fractions per day of differentiation.



Supplementary Figure S7.

Characteristics of genes with different levels of cell type-specific expression.

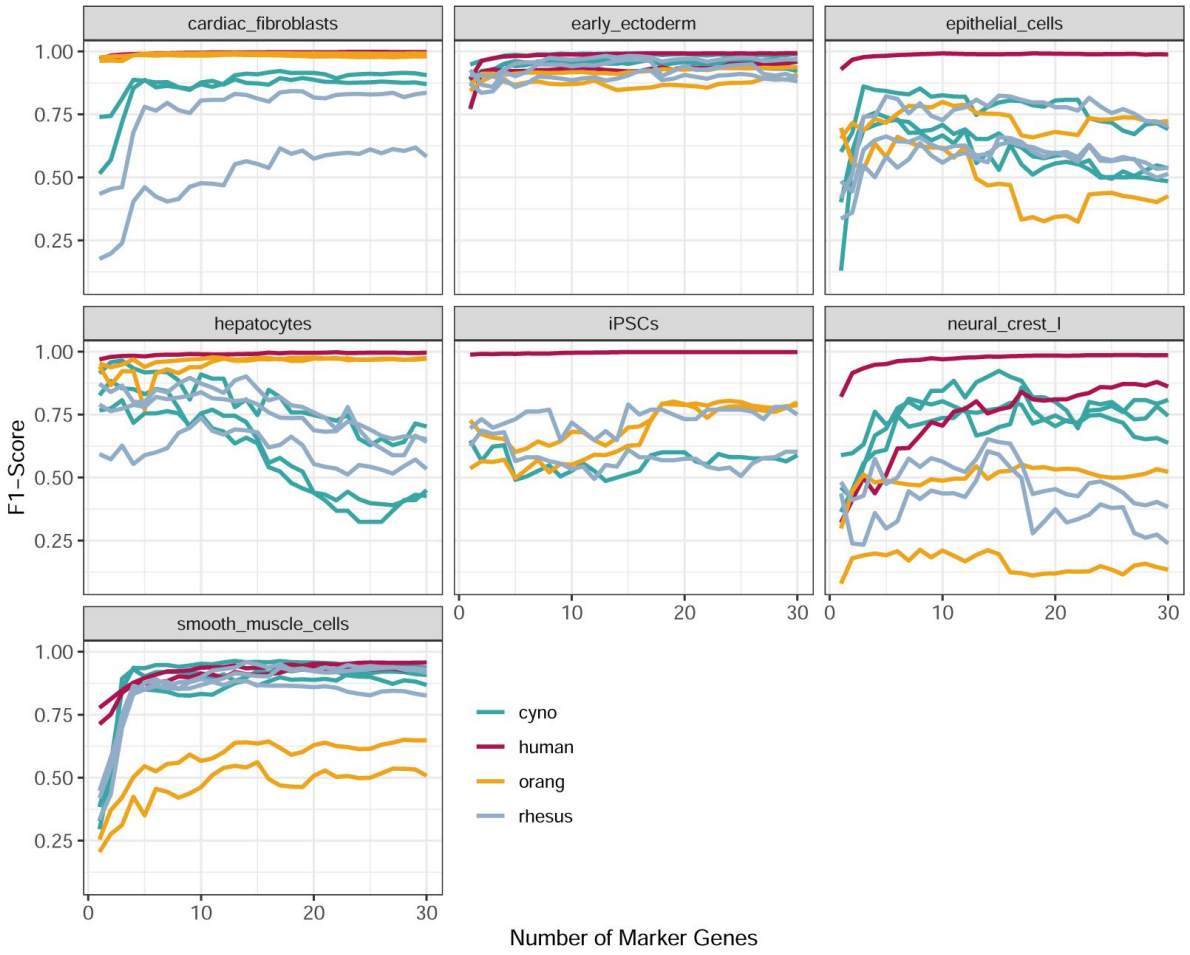
A) Stacked bar plot of the number of genes per cell type specificity level for different species. B) Boxplot of expression conservation of genes with different levels of cell type specificity in orangutan, cynomolgus and rhesus. C) Boxplot of gene-level constraint based on primate phastCons scores [34] for protein-coding genes. D) Boxplot of mean expression per cell type for genes with different levels of cell type specificity. E) Boxplot of mean expression per cell type for a subset of 236 genes per cell type specificity and species that were sampled to have a similar distribution of mean expression. F) Boxplot of expression conservation of the same subsampled genesets as in E).



Supplementary Figure S8.

Expression patterns of shared and human specific marker genes.

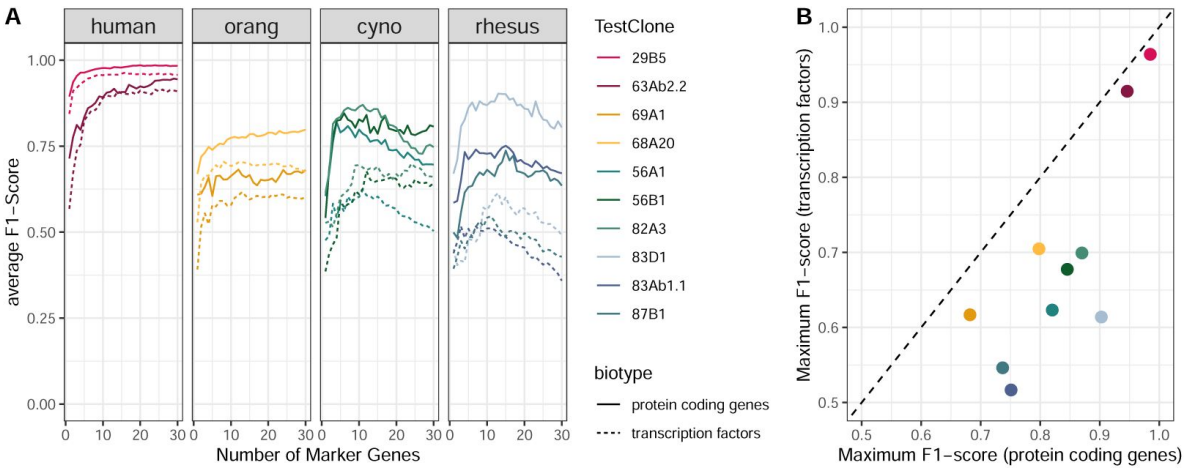
A) UMAP representation per species filtered for the 7 cell types that are present in all 4 species. B) UMAP representations colored by the log-normalized expression of 7 representative marker genes that are shared among the top100 marker genes per cell type in all 4 species. C) UMAP representations colored by the log-normalized expression of 7 representative marker genes that are only present in the human top100 marker gene ranking per cell type.



Supplementary Figure S9.

kNN classification performance per cell type.

F1-score per cell type for a kNN-classifier trained in the human clone 29B5 to predict cell type identity based on the expression of 1-30 protein-coding marker genes. Each line represents the performance in a different clone, colored by species identity.



Supplementary Figure S10.

kNN classification performance for transcription factors and protein coding marker genes.

A) Average F1-score for a kNN-classifier trained in the human clone 29B5 to predict cell type identity in the other clones. The classifier is trained on the expression of the top 1-30 protein coding markers (solid lines) or transcription factor markers (dashed lines). B) Comparison of the maximum average F1-score between transcription factors and protein coding markers for the classifications depicted in A).

Cell type	Marker gene	used in human	used in mouse
iPSCs	POU5F1	[56]	[57]
iPSCs	NANOG	[56]	[58]
iPSCs	L1TD1	[59]	[59]
early ectoderm	SOX2	[60]	[61]
early ectoderm	HES5	[62]	[63]
early ectoderm	RFX4	[62]	[64]
granule precursor cells	NFIA	[65]	[66]
granule precursor cells	ZIC1	[67]	[68]
granule precursor cells	ZIC4	[67]	[69]
neural crest	SOX10	[32]	[32, 70]
neural crest	FOXD3	[71]	[72]
neural crest	S100B	[73]	[74]
neurons	STMN2	[75]	[76, 77]
neurons	TAGLN3 (NP25)	[78]	[77]
neurons	DCX	[79]	[79]
smooth muscle cells	COL8A1	[80]	[81]
smooth muscle cells	ACTG2	[82]	[81]
smooth muscle cells	ACTA2	[80]	[81]
cardiac fibroblasts	TNNT2	[83]	[84]
cardiac fibroblasts	DCN	[85]	[86]
cardiac fibroblasts	HAND2	[83]	[87]
epithelial cells	CDH1	[88]	[89]
epithelial cells	EPCAM	[90]	[91]
epithelial cells	CLDN7	[92]	[93]
hepatocytes	TTR	[94]	[95]
hepatocytes	APOA1	[96]	[97]
hepatocytes	APOA2	[96]	[98]

Supplementary Table S1.

Marker genes.

Literature review for marker genes used in human and mouse / rodents to determine a specific cell type.

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Reviewer #1 (Public review):

Summary:

Jocher, Janssen, et al examine the robustness of comparative functional genomics studies in primates that make use of induced pluripotent stem cell-derived cells. Comparative studies in primates, especially amongst the great apes, are generally hindered by the very limited availability of samples, and iPSCs, which can be maintained in the laboratory indefinitely and defined into other cell types, have emerged as promising model systems because they allow the generation of data from tissues and cells that would otherwise be unobservable.

Undirected differentiation of iPSCs into many cell types at once, using a method known as embryoid body differentiation, requires researchers to manually assign all cell types in the dataset so they can be correctly analysed. Typically, this is done using marker genes associated with a specific cell type. These are defined a priori, and have historically tended to be characterised in mice and humans and then employed to annotate other species. Jocher, Janssen, et al ask if the marker genes and features used to define a given cell type in one species are suitable for use in a second species, and then quantify the degree of usefulness of these markers. They find that genes that are informative and cell type specific in a given species are less valuable for cell type identification in other species, and that this value, or transferability, drops off as the evolutionary distance between species increases.

This paper will help guide future comparative studies of gene expression in primates (and more broadly) as well as add to the growing literature on the broader challenges of selecting powerful and reliable marker genes for use in single-cell transcriptomics.

Strengths:

Marker gene selection and cell type annotation is a challenging problem in scRNA studies, and successful classification of cells often requires manual expert input. This can be hard to

reproduce across studies, as, despite general agreement on the identity of many cell types, different methods for identifying marker genes will return different sets of genes. The rise of comparative functional genomics complicates this even further, as a robust marker gene in one species need not always be as useful in a different taxon. The finding that so many marker genes have poor transferability is striking, and by interrogating the assumption of transferability in a thorough and systematic fashion, this paper reminds us of the importance of systematically validating analytical choices. The focus on identifying how transferability varies across different types of marker genes (especially when comparing TFs to lncRNAs), and on exploring different methods to identify marker genes, also suggests additional criteria by which future researchers could select robust marker genes in their own data.

The paper is built on a substantial amount of clearly reported and thoroughly considered data, including EBs and cells from four different primate species - humans, orangutans, and two macaque species. The authors go to great lengths to ensure the EBs are as comparable as possible across species, and take similar care with their computational analyses, always erring on the side of drawing conservative conclusions that are robustly supported by their data over more tenuously supported ones that could be impacted by data processing artefacts such as differences in mappability, etc. For example, I like the approach of using liftoff to robustly identify genes in non-human species that can be mapped to and compared across species confidently, rather than relying on the likely incomplete annotation of the non-human primate genomes. The authors also provide an interactive data visualisation website that allows users to explore the dataset in depth, examine expression patterns of their own favourite marker genes and perform the same kinds of analyses on their own data if desired, facilitating consistency between comparative primate studies.

Weaknesses and recommendations:

(1) Embryoid body generation is known to be highly variable from one replicate to the next for both technical and biological reasons, and the authors do their best to account for this, both by their testing of different ways of generating EBs, and by including multiple technical replicates/clones per species. However, there is still some variability that could be worth exploring in more depth. For example, the orangutan seems to have differentiated preferentially towards cardiac mesoderm whereas the other species seemed to prefer ectoderm fates, as shown in Figure 2C. Likewise, Supplementary Figure 2C suggests a significant unbalance in the contributions across replicates within a species, which is not surprising given the nature of EBs, while Supplementary Figure 6 suggests that despite including three different clones from a single rhesus macaque, most of the data came from a single clone. The manuscript would be strengthened by a more thorough exploration of the intra-species patterns of variability, especially for the taxa with multiple biological replicates, and how they impact the number of cell types detected across taxa, etc.

The same holds for the temporal aspect of the data, which is not really discussed in depth despite being a strength of the design. Instead, days 8 and 16 are analysed jointly, without much attention being paid to the possible differences between them. Are EBs at day 16 more variable between species than at day 8? Is day 8 too soon to do these kinds of analyses? Are markers for earlier developmental progenitors better/more transferable than those for more derived cell types?

(2) Closely tied to the point above, by necessity the authors collapse their data into seven fairly coarse cell types and then examine the performance of canonical marker genes (as well as those discovered de novo) across the species. However some of the clusters they use are somewhat broad, and so it is worth asking whether the lack of specificity exhibited by some marker genes and driving their conclusions is driven by inter-species heterogeneity within a given cluster.

https://doi.org/10.7554/eLife.105398.1.sa1

Reviewer #2 (Public review):

Summary:

The authors present an important study on identifying and comparing orthologous cell types across multiple species. This manuscript focuses on characterizing cell types in embryoid bodies (EBs) derived from induced pluripotent stem cells (iPSCs) of four primate species, humans, orangutans, cynomolgus macaques, and rhesus macaques, providing valuable insights into cross-species comparisons.

Strengths:

To achieve this, the authors developed a semi-automated computational pipeline that integrates classification and marker-based cluster annotation to identify orthologous cell types across primates. This study makes a significant contribution to the field by advancing cross-species cell type identification.

Weaknesses:

However, several critical points need to be addressed.

(1) Use of Liftoff for GTF Annotation

The authors used Liftoff to generate GTF files for Pongo abelii, Macaca fascicularis, and Macaca mulatta by transferring the hg38 annotation to the corresponding primate genomes. However, it is unclear why they did not use species-specific GTF files, as all these genomes have existing annotations. Why did the authors choose not to follow this approach?

(2) Transcript Filtering and Potential Biases

The authors excluded transcripts with partial mapping (<50%), low sequence identity (<50%), or excessive length differences (>100 bp and >2× length ratio). Such filtering may introduce biases in read alignment. Did the authors evaluate the impact of these filtering choices on alignment rates?

(3) Data Integration with Harmony

The methods section does not specify the parameters used for data integration with Harmony. Including these details would clarify how cross-species integration was performed.

https://doi.org/10.7554/eLife.105398.1.sa0

Author response:

> ***Reviewer #1 (Public review):***
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> *Summary:*
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> *This paper will help guide future comparative studies of gene expression in primates (and more broadly) as well as add to the growing literature on the broader challenges of selecting powerful and reliable marker genes for use in single-cell transcriptomics.*
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> *Strengths:*
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> *Marker gene selection and cell type annotation is a challenging problem in scRNA studies, and successful classification of cells often requires manual expert input. This can be hard to reproduce across studies, as, despite general agreement on the identity of many cell types, different methods for identifying marker genes will return different sets of genes. The rise of comparative functional genomics complicates this even further, as a robust marker gene in one species need not always be as useful in a different taxon. The finding that so many marker genes have poor transferability is striking, and by interrogating the assumption of transferability in a thorough and systematic fashion, this paper reminds us of the importance of systematically validating analytical choices. The focus on identifying how transferability varies across different types of marker genes (especially when comparing TFs to lncRNAs), and on exploring different methods to identify marker genes, also suggests additional criteria by which future researchers could select robust marker genes in their own data.*
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> *The paper is built on a substantial amount of clearly reported and thoroughly considered data, including EBs and cells from four different primate species - humans, orangutans, and two macaque species. The authors go to great lengths to ensure the EBs are as comparable as possible across species, and take similar care with their computational analyses, always erring on the side of drawing conservative conclusions that are robustly supported by their data over more tenuously supported ones that could be impacted by data processing artefacts such as differences in mappability, etc. For example, I like the approach of using liftoff to robustly identify genes in non-human species that can be mapped to and compared across species confidently, rather than relying on the likely incomplete annotation of the non-human primate genomes. The authors also provide an interactive data visualisation website that allows users to explore the dataset in depth, examine expression patterns of their own favourite marker genes and perform the same kinds of analyses on their own data if desired, facilitating consistency between comparative primate studies.*

We thank the Reviewer for their kind assessment of our work.

> *Weaknesses and recommendations:*
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> *that could be worth exploring in more depth. For example, the orangutan seems to have differentiated preferentially towards cardiac mesoderm whereas the other species seemed to prefer ectoderm fates, as shown in Figure 2C. Likewise, Supplementary Figure 2C suggests a significant unbalance in the contributions across replicates within a species, which is not surprising given the nature of EBs, while Supplementary Figure 6 suggests that despite including three different clones from a single rhesus macaque, most of the data came from a single clone. The manuscript would be strengthened by a more thorough exploration of the intra-species patterns of variability, especially for the taxa with multiple biological replicates, and how they impact the number of cell types detected across taxa, etc.*

You are absolutely correct in pointing out that the large clonal variability in cell type composition is a challenge for our analysis. We also noted the odd behavior of the orangutan EBs, and their underrepresentation of ectoderm. There are many possible sources for these variable differentiation propensities: clone, sample origin (in this case urine) and individual. However, unfortunately for the orangutan, we have only one individual and one sample origin and thus cannot say whether this germ layer preference says something about the species or is due to our specific sample.

Because of this high variability from multiple sources, getting enough cell types with an appreciable overlap between species was limiting to analyses. In order to be able to derive meaningful conclusions from intra-species analyses and the impact of different sources of variation on cell type propensity, we would need to sequence many more EBs with an experimental design that balances possible sources of variation. This would go beyond the scope of this study.

Instead, here we control for intra-species variation in our analyses as much as possible: For the analysis of cell type specificity and conservation the comparison is relative for the different specificity degrees (Figure 3C). For the analysis of marker gene conservation, we explicitly take intra-species variation into account (Figure 4D).

> *The same holds for the temporal aspect of the data, which is not really discussed in depth despite being a strength of the design. Instead, days 8 and 16 are analysed jointly, without much attention being paid to the possible differences between them.*

Concerning the temporal aspect, indeed we knowingly omitted to include an explicit comparison of day 8 and day 16 EBs, because we felt that it was not directly relevant to our main message. Our pseudotime analysis showed that the differences of the two time points were indeed a matter of degree and not so much of quality. All major lineages were already present at day 8 and even though day 8 cells had on average earlier pseudotimes, there was a large overlap in the pseudotime distributions between the two sampling time points (Author response image 1). That is why we decided to analyse the data together.

> *Are EBs at day 16 more variable between species than at day 8? Is day 8 too soon to do these kinds of analyses?*

When we started the experiment, we simply did not know what to expect. We were worried that cell types at day 8 might be too transient, but longer culture can also introduce biases. That is why we wanted to look at two time points, however as mentioned above the differences are in degree.

Concerning the cell type composition: yes, day 16 EBs are more heterogeneous than day 8 EBs. Firstly, older EBs have more distinguishable cell types and hence even if all EBs had identical composition, the sampling variance would be higher given that we sampled a similar number of cells from both time points. Secondly, in order to grow EBs for a longer

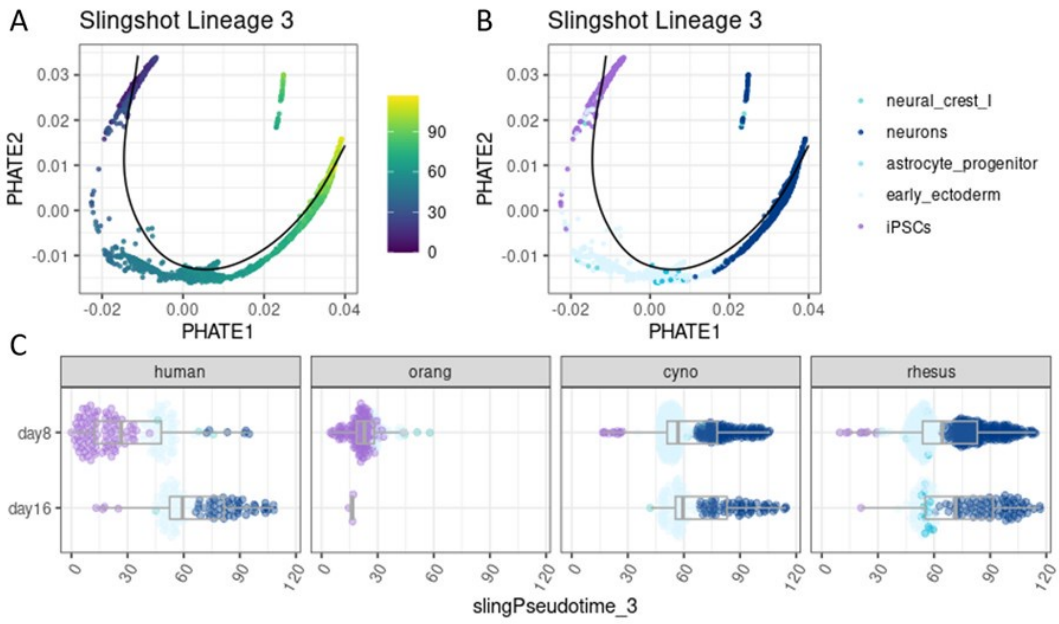
time, we moved them from floating to attached culture on day 8 and it is unclear how much variance is added by this extra handling step.

> *Are markers for earlier developmental progenitors better/more transferable than those for more derived cell types?*

We did not see any differences in the marker conservation between early and late cell types, but we have too little data to say whether this carries biological meaning.

Author response image 1.

Pseudotime analysis for a differentiation trajectory towards neurons. Single cells were first aggregated into metacells per species using SEACells (Persad et al. 2023). Pluripotent and ectoderm metacells were then integrated across all four species using Harmony and a combined pseudotime was inferred with Slingshot (Street et al. 2018), specifying iPSCs as the starting cluster. Here, lineage 3 is shown, illustrating a differentiation towards neurons. (A) PHATE embedding colored by pseudotime (Moon et al. 2019). (B) PHATE embedding colored by celltype. (C) Pseudotime distribution across the sampling timepoints (day 8 and day 16) in different species.



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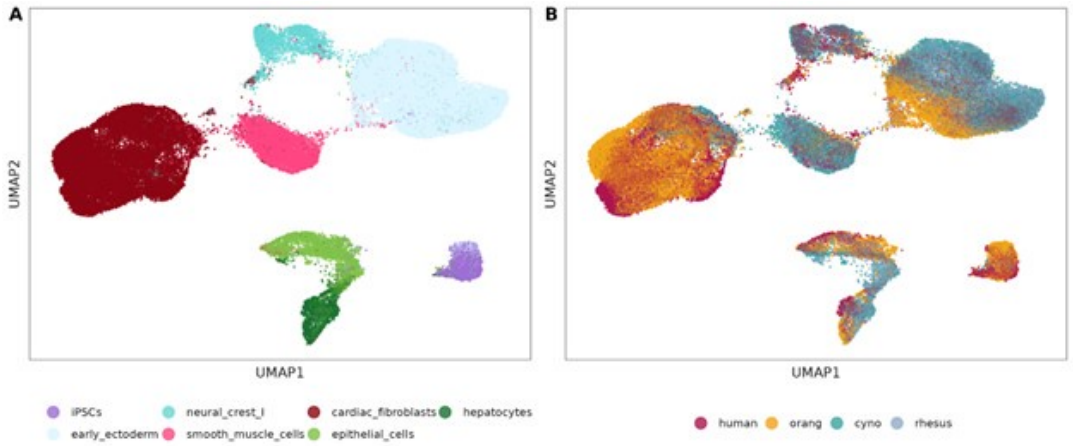
Author response image 2.

UMAP visualization for the Harmony-integrated dataset across all four species for the seven shared cell types, colored by cell type identity (A) and species (B).



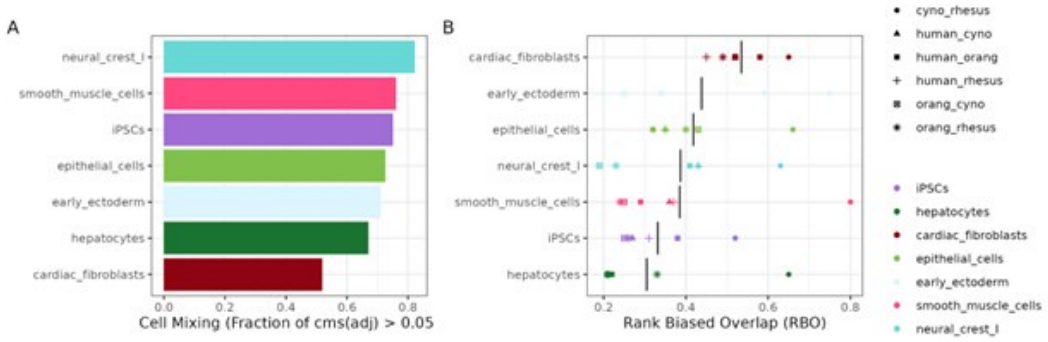
Good point, if we understand correctly, the concern is that in our relatively broadly defined cell types, species are not well mixed and that this in turn is partly responsible for marker gene divergence. This problem is indeed difficult to address, because most approaches to evaluate this require integration across species which might lead to questionable results (see our Discussion).

Nevertheless, we attempted an integration across all four species. To this end, we subset the cells for the 7 cell types that we found in all four species and visualized cell types and species in the UMAPs above (Author response image 2).

We see that cardiac fibroblasts appear poorly integrated in the UMAP, but they still have very transferable marker genes across species. We quantified integration quality using the cell-specific mixing score (cms) (Lütge et al. 2021) and indeed found that the proportion of well integrated cells is lowest for cardiac fibroblasts (Author response image 3A). On the other end of the cms spectrum, neural crest cells appear to have the best integration across species, but their marker transferability between species is rather worse than for cardiac fibroblasts (Supplementary Figure 9). Cell-type wise calculated rank-biased overlap scores that we use for marker gene conservation show the same trends (Author response image 3B) as the F1 scores for marker gene transferability. Hence, given our current dataset we do not see any indication that the low marker gene conservation is a result of too broadly defined cell types.

Author response image 3.

(A) Evaluation of species mixing per cell type in the Harmony-integrated dataset, quantified by the fraction of cells with an adjusted cell-specific mixing score (cms) above 0.05. (B) Summary of rank-biased overlap (RBO) scores per cell type to assess concordance of marker gene rankings for all species pairs.



> ***Reviewer #2 (Public review):***
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>
> *Strengths:*
>
> *To achieve this, the authors developed a semi-automated computational pipeline that integrates classification and marker-based cluster annotation to identify orthologous cell types across primates. This study makes a significant contribution to the field by advancing cross-species cell type identification.*

We thank the reviewer for their positive and thoughtful feedback.

> *Weaknesses:*
>
> *However, several critical points need to be addressed.*
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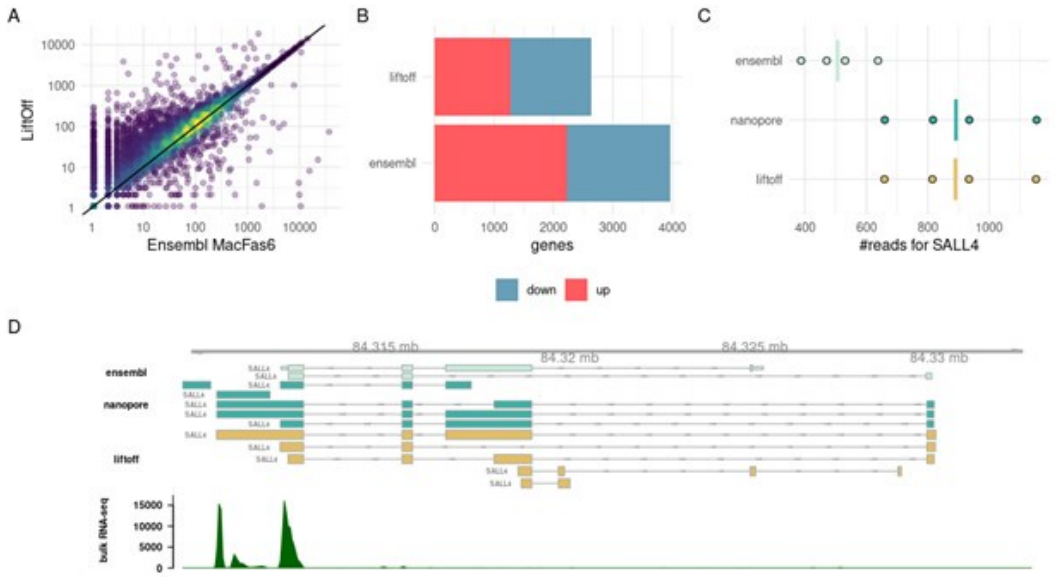
> *(1) Use of Liftoff for GTF Annotation*
>
> *The authors used Liftoff to generate GTF files for Pongo abelii, Macaca fascicularis, and Macaca mulatta by transferring the hg38 annotation to the corresponding primate genomes. However, it is unclear why they did not use species-specific GTF files, as all these genomes have existing annotations. Why did the authors choose not to follow this approach?*

As Reviewer 1 also points out, also we have observed that the annotation of non-human primates often has truncated 3'UTRs. This is especially problematic for 3' UMI transcriptome data as the ones in the 10x dataset that we present here. To illustrate this we compared the Liftoff annotation derived from Gencode v32, that we also used throughout our manuscript to the Ensembl gene annotation Macaca_fascicularis_6.0.111. We used transcriptomes from human and cynomolgus iPSC bulk RNAseq (Kliesmete et al. 2024) using the Prime-seq protocol (Janjic et al. 2022) which is very similar to 10x in that it also uses 3' UMIs. On average using Liftoff produces higher counts than the Ensembl annotation (Author response image 4A). Moreover, when comparing across species, using Ensembl for the macaque leads to an asymmetry in differentially expressed genes, with apparently many more up-regulated genes in humans. In contrast, when we use the Liftoff annotation, we detect fewer DE-genes and a similar number of genes is up-regulated in macaques as in humans (Author response image 4B). We think that the many more DE-genes are artifacts due to mismatched annotation in human and cynomolgus macaques. We illustrate this for the case of the transcription factor SALL4 in Author response image 4 C,D. The Ensembl annotation reports 2 transcripts, while Liftoff from Gencode v32 suggests 5 transcripts, one of which has a longer 3'UTR. This longer transcript is also supported by Nanopore data from macaque iPSCs. The truncation of the 3'UTR in this case leads to underestimation of the expression of SALL4 in macaques and hence SALL4 is detected as up-regulated in humans (DESeq2: LFC= 1.34, p-adj<2e-9). In contrast, when using the Liftoff annotation SALL4 does not appear to be DE between humans and macaques (LFC=0.33, p.adj=0.20).

Author response image 4.

(A) UMI-counts/ gene for the same cynomolgus macaque iPSC samples. On the x-axis the gtf file from Ensembl Macaca_fascicularis_6.0.111 was used to count and on the y-axis we used our filtered Liftoff annotation that transferred the human gene models from Gencode v32. (B) The # of DE-genes between human and cynomolgus iPSCs detected with DESeq2. In Liftoff, we counted human samples using Gencode v32 and compared it to the Liftoff annotation of the same human gene models to macFas6. In Ensembl, we use Gencode v32 for the human and Ensembl Macaca_fascicularis_6.0.111 for the Macaque. For both comparisons we subset the genes to only contain one to one orthologues as annotated in biomart. Up and down regulation is relative to human expression. C) Read counts for one example gene SALL4. Here we used in addition to the Liftoff and Ensembl annotation also transcripts derived from Nanopore cDNA sequencing of cynomolgus iPSCs. D) Gene models for SALL4 in the space of MacFas6 and a coverage for iPSC-Prime-seq bulk RNA-sequencing.



> *(2) Transcript Filtering and Potential Biases*
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> *The authors excluded transcripts with partial mapping (<50%), low sequence identity (<50%), or excessive length differences (>100 bp and >2× length ratio). Such filtering may introduce biases in read alignment. Did the authors evaluate the impact of these filtering choices on alignment rates?*

We excluded those transcripts from analysis in both species, because they present a convolution of sequence-annotation differences and expression. The focus in our study is on regulatory evolution and we knowingly omit marker differences that are due to a marker being mutated away, we will make this clearer in the text of a revised version.

> *(3) Data Integration with Harmony*
>
> *The methods section does not specify the parameters used for data integration with Harmony. Including these details would clarify how cross-species integration was performed.*

We want to stress that none of our conservation and marker gene analyses relies on cross-species integration. We only used the Harmony integrated data for visualisation in Figure 1 and the rough germ-layer check up in Supplementary Figure S3. We will add a better description in the revised version.

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