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# Species-specific developmental timing is maintained by pluripotent stem cells *ex utero*

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ABSTRACT

How species-specific developmental timing is controlled is largely unknown. By following human embryonic stem (ES) cell and mouse epiblast stem (EpiS) cell differentiation through detailed RNA-sequencing time courses, here we show that pluripotent stem cells closely retain *in vivo* species-specific developmental timing *in vitro*. In identical neural differentiation conditions *in vitro*, gene expression profiles are accelerated in mouse EpiS cells compared to human ES cells with relative rates of differentiation closely reflecting the rates of progression through the Carnegie stages *in utero*. Dynamic Time Warping analysis identified 3389 genes that were regulated more quickly in mouse EpiS cells and identified none that were regulated more quickly in human ES cells. Interestingly, we also find that human ES cells differentiated in teratomas maintain the same rate of differentiation observed *in vitro* in spite of being grown in a mouse host. These results suggest the existence of a cell autonomous, species-specific developmental clock that pluripotent stem cells maintain even out of context of an intact embryo.

## 1. Introduction

A central challenge for the human embryonic stem (ES) cell and induced pluripotent stem (iPS) cell field is that differentiation rates reflect a species with a nine month gestation period, with protocols often requiring several months (Shi et al., 2012b; Ebert et al., 2009; Espuny-Camacho et al., 2013; Krencik et al., 2011; Kriks et al., 2011). The extended time required for producing specific cell types and lack of physiological maturity are substantial obstacles to the clinical use of human pluripotent stem cells (Saha and Jaenisch, 2009; Broccoli et al., 2014). Understanding what controls species-specific rates of differentiation is essential for determining if and how these rates can be altered.

Although it is known that human pluripotent stem cell differentiation generally takes longer than mouse pluripotent stem cell differentiation, a detailed examination of these species-specific differentiation rates is lacking. In this study, we examined the differences in species-specific developmental timing by measuring rates of human and mouse pluripotent stem cell differentiation under identical anterior neural differentiation conditions *in vitro* through comprehensive RNA-sequencing (RNA-seq) time courses. When compared to Carnegie stage progression *in utero,* we found a remarkable similarity between *in utero* and *in vitro* rates of differentiation. Furthermore, we tested the autonomy of this timing by following human ES cell differentiation in teratomas by RNA-seq to assess if mouse host factors were capable of accelerating rates of development. We found that human timing was maintained in teratomas despite being exposed to murine host factors. These results provide evidence for an autonomous, species-specific developmental clock.

## 2. Results

### 2.1. *Species-specific timing is maintained during neural differentiation* in vitro

We first examined if species-specific developmental timing is maintained outside of the embryo (*ex utero)* by differentiating human

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and mouse pluripotent stem cells towards neural lineages under identical conditions *in vitro*. Mouse EpiS cells and human ES cells were compared because they represent closely related developmental stages and thus respond similarly to growth factors (Brons et al., 2007; Greber et al., 2010; Tesar et al., 2007; Rossant and Tam, 2017). A single defined neural differentiation medium was used throughout the time course to differentiate cells to the early forebrain and neocortex (see Section 4) (Espuny-Camacho et al., 2013; Levine and Brivanlou, 2007; Chambers et al., 2009). Enhanced green fluorescent protein (EGFP)-positive cell lines were used to remain consistent with the teratoma tracking studies later in this report. Mouse EpiS cells and human ES cells cultured in this defined neural differentiation medium induced similar homogeneous expression of neural marker PAX6 (Zhang et al., 2010), reaching maximal expression at day 3 in mouse and day 5 for human cells (Fig. S1).

Mouse and human cell differentiation were followed for 3 and 6 weeks, respectively, and RNA-seq was performed on samples taken every day for the first 8 days, then every other day for the remainder of the time course. PAX6-positive neural rosettes were detected by immunofluorescence at days 4 and 12 for mouse and human samples, respectively (Fig. 1A). Mouse cells expressed neocortex markers Ascl1 and Tbr1 after 6 days of differentiation, while human cells required 20 days to achieve similar marker expression and cell morphology (Fig. 1A). After 12 days for mouse and 38 days for human cells, neurons appeared elongated with bundles of cable-like projections characterized with axonogenic proteins BIII-TUB and DCX.

The RNA-seq time course revealed that key neural regulatory genes were upregulated in mouse cells before their human orthologs (Fig. 1B). Neuroectoderm gene expression (*e.g., PAX6, SOX1*) preceded neurogenic and embryonic neocortex markers (*e.g. FOXG1, BRN2, TBR1, NEUROG2, ASCL1* ), which is consistent with previous reports (Chambers et al., 2009; Espuny-Camacho et al., 2013; Gaspard et al., 2008; Shen et al., 2006; Shi et al., 2012b, Van De Leemput et al., 2014). Genes involved in axonogenesis (*MAP2, DCX, BIII-TUB*), neural stem cell expansion (*e.g. GLAST, OLIG2, GFAP* ), and neural maturation and synapse formation (*e.g. SYT4, SYT11, SNAP25* ) were all temporally upregulated in mouse EpiS cells before human ES cells (Fig. 1B). GABAergic and Glutamatergic-specific neural gene expression (*e.g. GABBR1, GAD1, VGLUT2,* and *GRIA2*), markers of the maturing synapses in the embryonic neocortex, were also expressed earlier in mouse cells. Thus, classic neural markers were expressed more quickly in mouse cells than human cells.

### 2.2. Temporal differences in gene expression across species identified by Dynamic Time Warping (DTW) and Pearson correlation analyses

Next, we broadly identified genes which were regulated significantly ($p < 0.01$) more quickly in one species compared to the other. Originally developed for speech pattern recognition (Sakoe, 1978), DTW is a powerful tool for comparing two independent time series that share similar patterns operating at different speeds. By calculating pattern matches, the algorithm locally compresses or stretches the time frame of one series to best fit a similar pattern in another and statistically assess differences in pattern velocity (Fig. 1C). DTW has previously been adapted to identify changes in gene expression in time series data from a single species (Aach and Church, 2001; Wexler et al., 2011), but here we employed the approach in a novel fashion to compare temporal expression data across two species during *in vitro* differentiation (see Section 4). After screening for genes that: (1) shared homologs in both humans and mice; (2) exhibited significant expression thresholds and dynamic ranges; and (3) demonstrated significant correlations of pattern similarities, we applied a DTW algorithm package (Giorgino, 2009) to the *in vitro* RNA-seq data. During neural differentiation under identical conditions, 3389 dynamic genes were identified as regulated more quickly in mouse cells than in human cells, and none were identified as slower in mouse cells than in human cells (Fig. 1D, Dataset S1). The top 2000 most significantly accelerated genes were screened for functional enrichment using DAVID Gene Ontology (GO) terms to elucidate the types of genes identified (Huang da et al., 2009a, 2009b). Several neural categories were significantly enriched, including the top 13 categories (Fig. 1E), illustrating that genes driving neural development are expressed more quickly in mouse cells than human cells during neocortex differentiation *in vitro*.

We also compared global rates of neural differentiation between mouse cells and human cells using sample correlation analyses of neural gene expression over time. Pearson correlation analyses were carried out using a list of 3061 neural genes accumulated from combining three separate neural GO gene lists (see Section 4). As expected, human *in vitro* samples correlated most strongly with other adjacent human *in vitro* time points (Fig. 2A), as did the mouse samples compared to other mouse time points (Fig. 2B). However, when human cells were correlated to mouse cells over time, a rather striking change in slope emerged (Fig. 2C). Mouse time points correlated most significantly with progressively later human samples; this distorted the stepwise slope expected if rates of differentiation were equivalent between species (Fig. 2C). For example, *in vitro* day 3 of mouse differentiation correlated most strongly with day 7 human, day 5 mouse with day 18 human, and day 7 mouse with day 34 human, indicating that mouse cells are differentiating more quickly than their human counterparts on a global level.

### 2.3. The rate of differentiation of human ES cells is maintained in teratomas

We next examined whether a mouse host would influence the timing of neural or other lineage differentiation of human ES cells in teratomas. EGFP-human ES (H1) cells or EGFP-mouse EpiS cells were injected intramuscularly into immunocompromised mice at day 0 (Fig. 3A). Mice were sacrificed at days 2, 8, 10, 11, 14, 16, 18, 21, 23, 28, 36, and 42 for human ES cells and at days 1, 2, 4, 8, 9, 10, 11, 14, 16 ,18, and 21 for mouse EpiS cell-derived teratomas. Teratomas were dissected from host tissues, digested into single cell suspensions, sorted for EGFP expression by fluorescence-activated cell sorting (FACS), and analyzed by RNA-seq (Fig. 3A).

Neurogenic genes were examined in order to compare rates of neural differentiation in teratomas with those observed *in vitro*. Despite the presence of mesoderm and endoderm lineages in the teratomas (which also peaked more quickly in mouse than human teratomas, Fig. S2), neural gene expression timing was remarkably similar to the timing observed *in vitro* (Fig. 3). When graphed together, temporal regulation of representative genes of early neural tube and forebrain development including *PAX6, ASCL1, BRN2, MEIS2, NEUROG1, and ATBF1* in teratomas paralleled those observed *in vitro* in a species-dependent manner (Fig. 3B). Subsequently, genes involved in both axono-/neurogenesis (*BIII-TUB, DCX, MAP2, DLK1)* and neural stem cell identity (*OLIG2, BLBP)* in teratomas also closely mirrored the temporal dynamics observed *in vitro* (Fig. 3C). Finally, genes involved in glutamatergic and GABAergic neuron identity and maturation as well as synapse function also followed similar expression timelines in teratomas and *in vitro* in a species-dependent fashion (Fig. 3D). Thus, the human-specific developmental timing observed *in vitro* was largely maintained in teratomas despite the presence of other germ layer lineages and despite being formed in a mouse host.

DTW analysis identified 3248 temporally regulated genes significantly accelerated ($p < 0.01$) in mouse teratomas compared to human teratomas and zero that were slower (Fig. S3A, Dataset S2). When the 2000 most significantly accelerated genes in teratomas were screened for function by the DAVID GO functional annotation tool, the list of terms involved in neurogenesis was enriched in the top 20 terms (Fig. S3B). Indeed, 15 out of the top 15 neural-specific GO terms enriched for genes accelerated *in vitro* were also significantly enriched for