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Species-specific developmental timing is associated with differences in protein stability in mouse and human

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Abstract

Although many molecular mechanisms controlling developmental processes are evolutionarily conserved, the speed at which the embryo develops can vary substantially between species. For example, the same genetic program, comprising sequential changes in transcriptional states, governs the differentiation of motor neurons in mouse and human, but the tempo at which it operates differs between species. Using in vitro directed differentiation of embryonic stem cells to motor neurons, we show that the program runs more than twice as fast in mouse as in human. This is not due to differences in signaling, nor the genomic sequence of genes or their regulatory elements. Instead, there is an approximately two-fold increase in protein stability and cell cycle duration in human cells compared to mouse. This can account for the slower pace of human development and suggests that differences in protein turnover play a role in interspecies differences in developmental tempo.

The events of embryonic development take place in a stereotypic sequence and at a characteristic tempo (1, 2). Although the order and underlying molecular mechanisms are often indistinguishable between different species, the timescale and pace at which they progress can differ substantially. For example, compared to their rodent counterparts, neural progenitors in the primate cortex progress more slowly through a temporal sequence of

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Author Contributions

T.R. and J.B. conceived the project, interpreted the data, and wrote the manuscript with input from all authors. T.R. designed and performed experiments and data analysis. D.S. designed and performed experiments and data analysis. R.P.C. performed theoretical modelling and data analysis. L.G.P. designed experiments and performed data analysis for smFISH. C.B. performed bioinformatic analysis. M.M. performed embryo work, generated and characterized the Ptch1::T2A-mKate2 mouse ES cell line. K.E. performed embryo work. J.L. analysed embryo data. E.M. and V.T. provided reagents and feedback.

Competing Interests

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neuronal subtype production (3, 4). Moreover, the duration of cortical progenitor expansion differs between species of primates, at least partly accounting for differences in brain size (5, 6). Even in more evolutionary conserved regions of the central nervous system (CNS) there are differences in tempo. The specification of neuronal subtype identity in the vertebrate spinal cord involves a well-defined gene regulatory program comprising a series of changes in transcriptional state as cells acquire specific identities as neural progenitors differentiate to post-mitotic neurons (7). The pace of this process differs between species, despite the similarity in the regulatory program and the structural and functional correspondence of the resulting spinal cords. The differentiation of motor neurons (MNs), a prominent neuronal subtype of the spinal cord, takes less than a day in zebrafish, 3-4 days in mouse, but ~2 weeks in human (8, 9). Moreover, differences in developmental tempo are not confined to the CNS. The oscillatory gene expression that regulates the sequential formation of vertebrate body segments – the segmentation clock – has a period that ranges from ~30mins in zebrafish, to 2-3h in mouse, and 5-6h in human (10–12). It is unclear as to what causes the interspecies differences in developmental tempo, termed developmental allochrony.

To address this question, we compared the generation of mouse and human MNs. Progenitors of the spinal cord initially express the transcription factors (TFs) Pax6 and Irx3 (13

and the shifts in gene expression are similar between mouse and human (Fig S1D). At their maximum extents, the OLIG2-expressing pMN domains comprise a large proportion of ventral progenitors, occupying approximately 30% of the DV length of the neural tube in mouse and a ~15% larger domain in human embryos (Fig. 1B, S1E). Consistent with this, there were more MN progenitors (pMN) in human but similar numbers of interneuron progenitors in mouse and human (Fig S1F). Over the following two days of mouse development, from E9.5 to E11.5, many post-mitotic MNs differentiate (Fig. 1C) resulting in a marked reduction in the size of the pMN domain (Fig. 1B), despite the continued proliferation of the progenitors (9). The proportion of neurons is higher in human compared to mouse (Fig S1B). By contrast, the pace of development is noticeably slower in human embryos. At Carnegie Stage (CS) 11 the pMN occupies a large proportion of the human neural tube, similar to the pMN in E9.0 mouse embryos. During the following 1-2 weeks of development (CS13-19, Fig. 1B), the size of the pMN decreases as MNs accumulate (Fig. 1C), but the rate of this change is slower than seen in mouse. MN production decreases at ~E11.5 in mouse whereas MN production continues to at least CS17 in human (Fig S1C), and glial progenitors, co-expressing SOX9 and NFIA, begin to arise in both species at these stages (Fig. 1D). Together, the data indicate an equivalent progression in neural tube development of mouse and human that lasts around 3 days in mouse and over a week in human (Fig. 1A).

differentiation of mouse and human pluripotent stem cells (29). To test whether the difference in tempo of mouse and human MN differentiation represented a global change in the rate of developmental progression we performed bulk transcriptomics. This revealed a similar pattern of gene expression changes in mouse and human but the changes occurred at a faster rate in mouse cells than human cells (Fig. 2H). Cross-species comparison of dynamic genes highly expressed across the differentiation showed a high degree of correlation although altered in time between mouse and human (Fig. 2I, S2D). Moreover, the relative difference in developmental tempo appears constant throughout the differentiation process suggesting a global temporal scaling – developmental allochrony – between mouse and human.

To relate the tempo of mouse and human MN differentiation, we estimated the global difference in the tempo of gene expression comparing the Pearson correlation coefficients from the transcriptome analysis of both species. This identified a scaling factor of 2.5 ± 0.2 (median ± sd, Fig. 2I). Additionally, we clustered gene expression profiles into sets of genes with similar dynamics during the time course and we measured the fold difference in the time of appearance of the clusters that contained Pax6, Irx3, Olig2, Nkx2.2, Isl1 and Tubb3 genes. This confirmed that a scaling factor of ~2.5 fit each of the gene expression clusters (Fig. 2J). Similarly, time factor measurements for individual genes identified a scaling factor between 2-3 (Fig. S2F,G). To test if the identified time factor could be extended to the whole transcriptome, we selected four cluster pairs comprising a high proportion of orthologous genes (Fig. S2E). A search for a scaling factor that accommodated the difference in the timing of expression in these groups indicated a factor of ~2.5 for each of the clusters (Fig. 2L). Together, these results suggest that MN differentiation can be recapitulated in vitro from mouse and human ESCs and results in a global 2.5- fold decrease in the rate at which gene expression programs advance in human compared to mouse.

Sonic Hedgehog Signalling Sensitivity Does Not Regulate Tempo

Having identified a global scaling factor for the GRN, we investigated the mechanism that sets the timescale. We reasoned that the mechanism was likely to be cell-autonomous since the temporal differences are observed between mouse and human cells grown in vitro, and it has been shown that in vitro differentiated cells transplanted to a host follow their own species-specific dynamics (30–32). Since the directed differentiation towards MNs occurs in response to Shh signalling, we hypothesized that the delay in the GRN in human compared to mouse could be a consequence of a reduced sensitivity to signalling. To test whether the human GRN could be sped up by higher levels of signalling, we differentiated human progenitors in the presence of increasing concentrations of SAG and in a combination of SAG and Purmorphamine (Pur), another smoothened agonist (Fig. 3A). Single cell measurements of NKX6.1, a GRN transcription factor induced by Shh in ventral progenitors, showed similar proportions and intensity of expression for all levels of signal at equivalent time-points (Fig. S3A,B). To test whether the competence of neural progenitors to respond to Shh was delayed in human compared to mouse, we delayed addition of SAG for 24h. A 24h delay in Shh addition resulted in higher initial levels of *IRX3*, as expected, but did not change the time of *NKX6.1, GLI1 or PTCH1* induction relative to the time of SAG

addition (Fig. 3C, S3D), corroborating that the onset of Shh responsiveness is acquired at neural induction in human as in mouse cells.

We then compared the kinetics of Shh signalling in mouse and human cells by assaying the response of Ptch1 and Gli1, two Shh pathway components that are Shh direct target genes

performed pulse-chase experiments labeling nascent proteins with AHA, conjugated labelled proteins to biotin, and pulled them down with streptavidin beads to purify. This revealed that pan-neural proteins SOX1 and SOX2 had longer lifetimes than OLIG2 and NKX6.1 proteins in both species (Fig. 6A, S6F,G). Moreover, human NKX6.1 and OLIG2 were ~2- fold more stable than their mouse homologues (mNKX6.1 ≈ 2.5h vs. hNKX6.1 ≈ 6h; mOLIG2 ≈ 3.5h, hOLIG2 ≈ 6.8h) (Fig. 6A, S6F,G). These results are consistent with the predictions of the model and the non-linear relationship between decay rates and tempo scaling.

The identification of a global increase in the lifetime of proteins in human compared to mouse neural progenitors raised the possibility that exogenous proteins would show species-specific stability. To this end, we generated Patched1::mKate2 reporter lines in mouse and human stem cells. In these lines, the monomeric far-red fluorescent protein Katushka-2 (mKate2) was fused to the C-terminus of endogenous Ptch1 via a self-cleaving peptide (Fig.

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Figure 1. Comparison of neural tube development in mouse and human embryos.

(**A**) Schema of mouse and human neural tube development (**B-D**). Immunofluorescence in transverse sections of mouse and human cervical neural tube from E9.0 to E11.5 in mouse and CS11 to CS17 in human embryos. (**B**) Expression of progenitor markers PAX6 (green), OLIG2 (magenta) and NKX2.2 (cyan). (**C**) Pan-neural progenitor marker SOX2 (blue), motor neuron markers ISL1 (magenta) and HB9/MNX1 (cyan) at neurogenic stages. (**D**) Ventral expression of gliogenic markers NFIA (red) and SOX9 (blue) in the neural tube can be detected from E10.5 in mouse and CS15 in human. NFIA also labels neurons, as indicated by TUBB3 (cyan) staining. Scale bars = 50 microns.

Figure 2. A global scaling factor for in vitro dn0r in vitiNy66.nacne2n of mouse and human MNs.

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expression but a different tempo (human n = 3 in triplicate, mouse n = 3 in triplicate). (**H**) Heatmap of RNA-seq data from mouse and human MN differentiation indicating the normalized expression of selected markers representative of neuromesodermal progenitors, neural progenitors, neurons, glia and mesoderm cell types (mouse n = 3, human n = 3). (**I**) Heatmap of the pair wise Pearson correlation coefficients of the transcriptomes of mouse (vertical) and human (horizontal) differentiation at the indicated time points. High positive correlation indicated by values close to 1 (red). White line shows a linear fit of the Pearson correlation with temporal scaling factor of 2.5 ± 0.2 (median ± std). (**J**) Scaling factor for transcriptome clusters that contain Pax6, Olig2, Nkx2.2, and Isl1. **(K)** Significant differences in the peak of gene expression in the RT-qPCR experiments between mouse (orange) and human (blue). (human n = 3 in triplicate, mouse n = 3 in triplicate). Two-way ANOVA with Tukey's multiple comparison post-hoc test *** adj p-value < 0.001. (**K**) Time factor estimations for cluster pairs with high proportion of orthologous genes.

Figure 3. Dynamics of Shh signalling in mouse and human neural progenitors.
(A) Flo

measured from the time of SAG addition (n = 3). **(D)** RT-qPCR data measured at 12h intervals reveal similar gene expression dynamics in mouse (orange) and human (blue) for Gli1, but distinct for Nkx6.1 (mouse n = 6, human n = 5). (a.u., arbitrary units).

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Figure 4. Temporal control of gene expression depends on the species cellular environment.

(A) Scatter plot with histograms of PAX6 and NKX6.1 intensity measured by FACS in NPs from wt (orange) and hChr21 (purple) mouse cells at Day 2. **(B)** RT-qPCR expression of Olig2 from the mouse (*mOlig2*) and human alleles (*hOLIG2*) (n = 9). **(C)** smFISH at Day 2 of differentiation in wt and hChr21 lines with probes for *mSox2*, and allele specific detection of *mOlig2* or human *OLIG2* (*hOLIG2*). Scale bars = 10 microns **(D)** smFISH in human NPs at Day 8 of differentiation for *hSOX2* and *hOLIG2*. Scale bars = 50 microns. **(E)** Boxplots and density distributions in wt and hChr21 cells of number of mRNA molecules per cell from Sox2, total Olig2 and human- and mouse- allele specific probes. The estimated mean difference in molecule number between hChr21 cells and mouse is 25.7 [22.3; 29.7] (mouse n=323, hChr21 n=337). **(F)** Boxplots and density distributions of the concentration (number of mRNA molecules per area unit) of Olig2 per cell in human NPs at Day 8, and mouse wt and hChr21 cells at Day 2. The estimated mean difference is 0.121 mRNAs/μm^2 [0.141; 0.101] between mouse and hChr21cells; and the mean difference is 0.157 mRNAs/μm^2 [0.175; 0.139] for human and hChr21 cells. Statistical significance (*) corresponds with <0.05 overlap between the distributions of mean estimations with a p-value for a two-sided permutation t-test < 0.001.(human n = 436, mouse n = 323, hChr21 n = 337).

Figure 5. Protein stability in the GRN corresponds to tempo differences between species.
(**A**) Normalized EU incorporation measurements to estimate mRNA half-life in mouse (orange) and human (blue) neural progenitors. Line and shadowed areas show best exponential fit and its 70% High Density Interval (HDI). (mouse Day 2 n = 5, human Day 4 n = 3, human Day 8 n = 5). **(B)** Half-life of the transcriptome in mouse neural progenitors at Day 2 (orange), and human neural progenitors at Day 4 (dark blue) and Day 8 (light blue). (**C**) Normalized AHA measurements of the proteome in mouse (orange) and human (blue) neural progenitors to estimate protein stability (mouse Day 2 n = 6, human Day 4 n = 4,

human Day 8 n = 4). **(D)** Global stability of the proteome in mouse neural progenitors at

Figure 6. Protein decay and cell cycle account for the speed differences between species.
(A) Normalised measurements of mouse and human NKX6.1, OLIG2, SOX1 and SOX2

n= 4, human Day 8 n = 5). **(E)** Cell cycle length estimations in mouse neural progenitors at Day 2, and human neural progenitors at Day 4 and Day 8. For all plots, mouse data is orange-colored, and human is blue. Statistical significance (**) corresponds with <0.01 overlap between the distributions of parameter estimations.

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