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SPOTLIGHT

On time: developmental timing within and across species

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ABSTRACT

Organisms across species differ in the relative size and complexity of their tissues to serve the specific purposes of the host. Correct timing is a crucial ingredient in the development of tissues, as reaching the right size and complexity requires a careful balance between cellular proliferation and differentiation. Premature or delayed differentiation, for instance, can result in tissue imbalance, malformation or malfunction. Despite seemingly rigid constraints on development, however, there is flexibility in both the timing and differentiation trajectories within and between species. In this Spotlight, we discuss how time is measured and regulated in development, and question whether developmental timing is in fact different between species.

KEY WORDS: Clock, Developmental timing, Differentiation, Heterochrony, Proliferation, Timekeeping

Introduction

Embryonic development appears to follow a highly optimized scheme for each species, yet the pace and trajectories of developmental events can, to some extent, be adjusted in response to the environment through cellular regulatory networks, which often exhibit convergent phenotypes. Classical embryology has laid the groundwork to reveal the pace and sequence of developmental processes and identify their determinants (Dollé et al., 1989; Maienschein, 2014; Palmeirim et al., 1997). Recent dynamic *in vitro* models and single-cell omics approaches promise to unveil the quantitative nature of developmental events (Azhar and Sonnen, 2021; Yu et al., 2021). The combination of classical and modern developmental biology allows development to be viewed as a collection of probabilistic outcomes, rather than a predetermined flow of events. This way, we can begin to truly understand what time means in development, and establish the relationship between chronological and biological time in developing organisms. In this Spotlight article, we discuss the known mechanisms that enable cells' progress in time through development, point to contributing factors in altered timing of events and propose a concept of biological time woven into a 'molecular fabric'.

Molecular mechanisms controlling the pace of development

Time spans vast stretches in biology, from evolutionary timescales down to biochemical reaction times (Fig. 1). This entire range of timescales is kneaded into the development of multicellular organisms, which is regulated by molecular reactions over seconds to hours that are sculpted by evolutionary forces over millions to billions of years. Development unleashes a seemingly irreversible sequence of events, which involves the decision of proliferation versus differentiation at every step of the way. The timing of the onset, pace and duration of these events are crucial for normal progression of development. This principle holds in simple and complex organisms alike, from bacterial sporulation to neurogenesis in the prefrontal cortex (Duboule, 2003; Fenlon, 2022; Otani et al., 2016; Simon et al., 1992).

Developmental timing is clearly optimized within each species, and is, at the chronological level, different between species. Which mechanisms then determine the pace, duration and order of developmental events within a species? The embryo relies on molecular circuits to time such events correctly, which we conceptualize in a 'molecular fabric' (see Box 1 and Fig. 2). These involve circuits dominated by a single gene, or entire gene regulatory networks collectively determining cellular outcomes (Exelby et al., 2021). The Hes/Her system is a prime example of a molecular circuit dominated by a single gene that determines the pace of differentiation. The master segmentation clock regulator Hes7 represses its own expression, and this repression is lifted when the protein gets degraded (Bessho et al., 2001). As has been shown extensively by mathematical models, such feedback loops ensure consistent and periodic activation in the form of oscillations (Lewis, 2003; Monk, 2003). Hes7 can thus be considered a molecular 'timekeeper' in somite development. Other timekeepers are known to underlie other periodic processes, such as the circadian clock and the cell cycle. The former is determined by the periodic expression of Per and Cry genes in a negative-feedback loop with the transcription factors (TFs) Clock and Bmal1 (Takahashi, 2017). The latter is controlled by periodic activation and depletion of cyclins, as well as dilution of the cell cycle inhibitor Rb by cell growth (Zatulovskiy et al., 2020).

Most efforts in modern developmental biology are directed to annotation of cell types and study of cellular diversity and heterogeneity based on (single-cell) transcriptomes. This approach is extremely useful to map differentiation trajectories and reveal the effects of genetic or environmental perturbations on the timing and complexity of development. However, it only provides a limited understanding of the underlying mechanisms. In general, mRNA levels can only explain about 40% of the variance in protein levels (Schwanhäusser et al., 2011). The selectivity and rate of protein synthesis and degradation, together with dynamic protein localization, play crucial roles in cell type commitment and differentiation, and, consequently, in developmental timing (Harnett et al., 2022). Thus, post-transcriptional mechanisms, together with transcription, enable highly complex, timed and localized regulatory activity within the cell.

It is important to mention that it is the local abundance of effectors that should matter (Kramer et al., 2022). The timing (initiation) of the first cell fate decision in early mouse embryos, namely the specification of the trophectoderm (TE), is dependent on the concentration of the TFs Tfap2c and Tead4, which progressively increase after zygotic genome activation at the two-cell stage (Zhu et al., 2020). However, these, only together with the activation of Rho GTPase, lead to apical-basal polarization and activation of the

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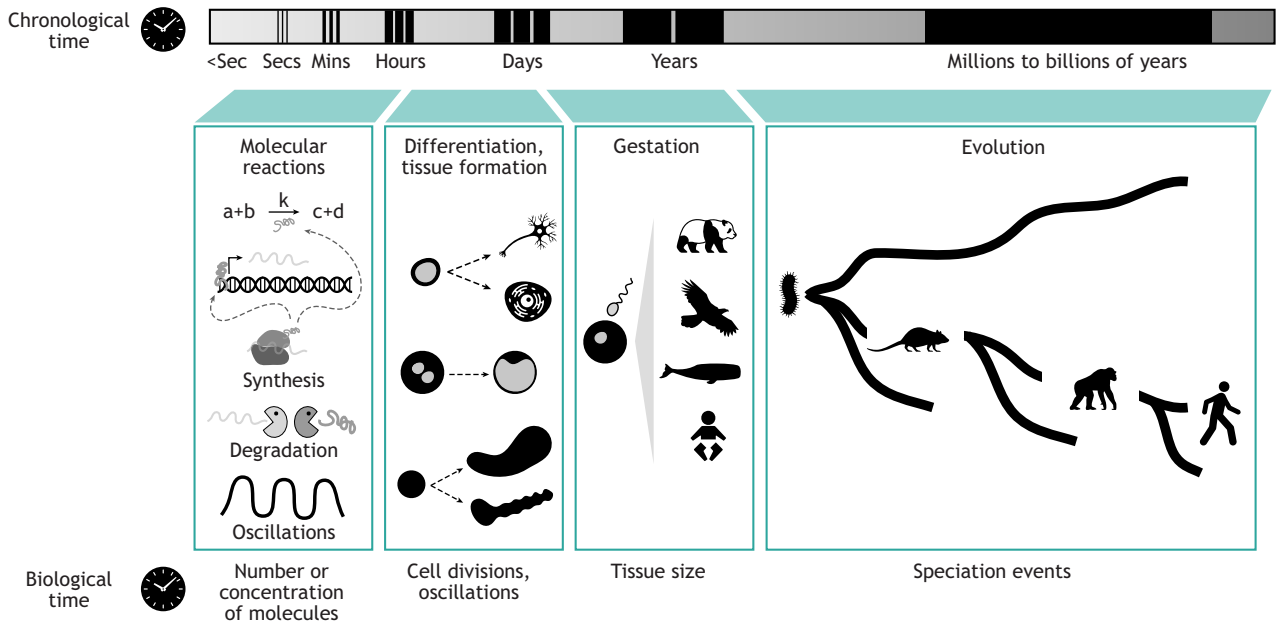

Fig. 1. Different timescales in biology. Embryonic development is sculpted by biomolecular reactions that span seconds to hours. These reactions provide the ground for cellular differentiation and tissue formation over hours to days. The whole gestational period spans days to multiple years in animals. All of the above steps are shaped by evolution over millions to billions of years. As chronological time is measured in duration, biological time can be measured in molecular units, such as outputs from biochemical reactions or the number of cell divisions.

TE program. Thus, local protein abundance is alterable not only by synthesis or degradation but also altered localization. In the same context of TE specification, although the transcriptional co-activators YAP (YAP1) and TAZ (WWTR1) are expressed in both inner and outer cells, only the outer cells acquire TE fate, which requires YAP/TAZ nuclear localization. In inner cells, YAP/TAZ remain cytoplasmic as a result of active upstream Hippo signaling, which inhibits their transport to the nucleus, and therefore the cells remain uncommitted (Nishioka et al., 2009). Among other processes, the circadian clock in fibroblast cells is also controlled by YAP/TAZ, with the circadian clock slowing down with more nuclear YAP accumulation (Abenza et al., 2023). Therefore, in addition to global abundance of effector proteins regulating developmental processes, their specific subcellular localization also dictates the onset of developmental events, affecting both timing and trajectories of development.

Even in the presence of signals inducing differentiation and downstream effectors, the onset of differentiation is subject to accessibility of DNA, particularly at regulatory elements. Epigenetic priming of regulatory elements, particularly enhancers, provides a framework for the orchestrated emergence of the three germ layers during gastrulation (Argelaguet et al., 2019). On a more global scale, the progressive transition from the open and flexible chromatin state of stem/progenitor cells to a less dynamic and more defined state limits the time window of differentiation, thereby allowing timed emergence of mature cells. Therefore, regulators of global chromatin accessibility and openness can define the timing of the onset and the duration of developmental events. An example is the high mobility group protein HMGA, which mediates the higher chromatin accessibility of neural precursor cells and allows neurogenesis (Kishi et al., 2012). Gradual loss of chromatin accessibility reduces the neurogenic potential of neural progenitor cells, thus limiting the generation of neural cells to a time window (Kishi et al., 2012). Although a subset of TFs with pioneering ability can actively alter their local chromatin environment, TF activity is canonically gated by DNA accessibility (Meers et al., 2019; Zaret, 2020). Interestingly, the protein synthesis capacity of embryonic stem cells determines chromatin openness, thereby potentially linking the growth rate to the timing of differentiation (Bulut-Karslioglu et al., 2018).

Cells are in constant contact with their environment, which, together with the intrinsic molecular mechanisms discussed above, shapes their cell fate decisions and eventual morphology and function. Self-organizing 3D cellular aggregates, such as organoids, present a tremendous opportunity to dissect the pace and ordering of developmental events under the influence of different culture conditions. From such experiments, we know that cells can uncouple the developmental gene expression programs from morphogenesis only to a certain extent. For example, somites in trunk-like structures or somitoids only take shape when cellular aggregates are transferred into Matrigel-containing media, which provides structural and chemical support, although somitic genes are expressed and a proper Hox code is in place in the absence of Matrigel in regular gastruloids (Beccari et al., 2018; Budjan et al., 2022; Miao et al., 2023; Sanaki-Matsumiya et al., 2022; Veenvliet et al., 2020). Thus, under these conditions, biological time advances without proper morphology. Yet, patterning of somites is only observed in morphologically organized somitic cells, indicating that further differentiation and maturation cannot be uncoupled from morphology, thus halting further progress in biological time. Similarly, although gastruloids contain a neuroectodermal compartment, spatial patterning of neural tissue does not happen without sonic hedgehog (Shh) secretion from notochordal cells (Ogura et al., 2018; Rito et al., 2023 preprint), again bringing neural development to a halt owing to the absence of a major signaling component. In addition to the morphological organization and signaling pathways, the nutrient, energy and oxygen levels in the cellular microenvironment also significantly affect the pace of development and cellular trajectories. For example, the efficiency of *in vitro* development of mouse embryos

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Box 1. The meaning and nature of biological time

Let's pause for a moment to reflect on a key question that begs consideration: what is time? From an Earthling's point of view, one year on Saturn is 29.4 Earth years. Yet, a Saturnian would experience roughly the same number of days during this period (10,475 versus 10,759 on Earth). To our human perception, timing across tissues and between species is different, because time is usually viewed as chronological and one-dimensional (Fig. 2A). In contrast to a fixed chronological clock, biological events feature internal timekeeping mechanisms, such as biochemical reaction rates and fluctuating protein concentrations. These internal timekeepers are often tissue and species specific because of their dependency on the rate of metabolism, rate of growth or metabolite availability. The discrepancy between the fixed chronological time and dynamic biological timekeepers creates a need to view time differently.

We propose viewing time as a molecular fabric woven by the key biomolecular reactions underlying each developmental decision (Fig. 2B). Such a fabric would be multi-dimensional because different developmental events are controlled by different molecular regulators. In this fabric, the differentiation trajectory that a cell takes would be influenced by the signals that it receives and its receptivity, defined by its molecular state (i.e. positioning in the fabric). In our view, this could be a meaningful template for understanding differentiation trajectories (and developmental outcomes) as a function of the cell's internal state.

The molecular fabric concept might also help us to understand the heterochronies that arise as a result of environmental or genetic perturbations, or species-specific regulation. For example, in diapause, inhibition of mTOR reduces overall cellular anabolism, including the rate of transcription and translation (Bulut-Karslioglu et al., 2016; Shao et al., 2022). As a result, the chronological time to undertake biomolecular reactions is vastly prolonged (the fabric is 'stretched'), although the cells do not advance significantly in development. Another example is the *in vitro* directed differentiation of mouse or human ESCs to motor neurons, which advance at different paces between the two species (mouse is ∼2.5 times faster) although the same molecular steps occur in both species (Rayon et al., 2020).

or embryo models significantly drops when exposed to non-physiological oxygen concentrations or in the absence of rat/human serum in culture (Aguilera-Castrejon et al., 2021; Tarazi et al., 2022). Adjusting environmental oxygen or oxygen-dependent cellular metabolism leads to different developmental outcomes (López-Anguita et al., 2022; Miyazawa et al., 2022; Oginuma et al., 2020). Heat is another parameter that plays a relevant role, by altering local reaction rates or affecting phase transitions (Rodenfels et al., 2019). Thus, cells, in interaction with their environment, determine the pace and course of development (Fig. 2).

Modulating biological time

Given that biological time, as a factor of molecular densities, is intricately linked to the anabolic rate of a cell, it is conceivable that altered anabolic rates will lead to altered developmental pace. Slow metabolizing conditions, arising from either genetic perturbations (such as in insulin pathway mutants) or environmental conditions (such as low temperature) slow down development and prolong the gestation period and lifespan in *Drosophila* (Cassidy et al., 2019). Furthermore, medaka (*Oryzias latipes*) embryos can fully develop in a wide range of temperatures (17-35°C), with variable developmental times (43 days at 17°C, 8 days at 30-35°C) (Vibe, 2020). Similarly, zebrafish embryos slow down their segmentation clock in colder temperatures (Schröter et al., 2008). In general, decreased metabolism slows down the pace of development within a species. However, metabolic pathways are multifaceted and should be interpreted with caution. For example, Diaz-Cuadros and colleagues showed that although respiration rate is higher in the mouse than in humans and inhibition of the electron transport chain slows down the segmentation clock, this is not due to reduced ATP production but rather to NAD+/NADH redox balance, and, further downstream, to reduced translation rates (Diaz-Cuadros et al., 2023). Correspondingly, overexpression of a bacterial NADH oxidase accelerated the segmentation clock by increasing the NAD+/NADH ratio and the translation rate (Diaz-Cuadros et al., 2023).

Translation and degradation rates determine the period of the segmentation clock by controlling the local concentration of its master regulator Hes7. The segmentation clock period scales with Hes7 kinetics across several species including mouse, human, marmoset and cattle, whereby slower Hes7 kinetics proportionally slow down the segmentation clock (Lázaro et al., 2023; Matsuda et al., 2020). In this example, fitting the experimental data to a mathematical model of the delayed auto-repression of Hes7 revealed that the timescale differences are due to variations in protein stability and in the delay time of Hes7 expression (Matsuda et al., 2020). General interference with the proteasome does not change the period of the oscillation clock, which does not scale with cellular metabolic rate (energy output) either, indicating that Hes7 is the specific and primary determinant of the clock period (Diaz-Cuadros et al., 2023; Lázaro et al., 2023). In line with these findings, inhibiting protein synthesis slows down the segmentation clock and accelerating Hes7 synthesis by removal of its introns speeds it up, resulting in smaller somites (Diaz-Cuadros et al., 2023; Harima et al., 2013). Similar regulatory principles determine the pace of development in other tissues and species as well. Motor neuron differentiation in the spinal cord takes more than twice as long in humans compared with mice, and the degradation rates of the corresponding regulators scale with the pace of differentiation (Rayon et al., 2020). In another example, growth temperature was shown to affect the development of zebrafish embryos in a tissue-specific manner, resulting from each tissue's ability to adjust its proteome, leading to asynchrony in development (Dorrity et al., 2022 preprint).

Although the development of mammals, unlike that of fishes, birds and invertebrates, is generally not permissive to extreme variations in its timing, mammalian development can be stopped or significantly slowed down during a specific time window prior to implantation (Renfree and Fenelon, 2017). This phenomenon, called embryonic diapause, equips over 130 known species with the ability to adjust the timing of birth or gestation in order to give the progeny the highest chances of survival. Embryonic diapause is triggered by a number of acute or periodic factors, such as nutrient scarcity or the photoperiod (Fenelon et al., 2014). Downstream of these factors, the pre-implantation, blastocyst-stage embryo enters dormancy and nearly eliminates all anabolic activity. Under these conditions, the pluripotent state of embryonic cells is preserved for days to months, depending on the species. Direct interference with growth pathways, most prominently via inhibition of mTOR activity, induces diapause *in vitro* in mouse and human (Bulut-Karslioglu et al., 2016; Iyer et al., 2023 preprint). Interestingly, well-known regulators of developmental timing originally discovered in *Caenorhabditis elegans*, such as LET-7 and DAF-16/Foxo1, also regulate diapause in mammals (Liu et al., 2020; Rougvie, 2001; van der Weijden et al., 2022 preprint). Complete shutdown of anabolism appears to be required for even longer dormancy periods, as in the case of oocytes, which disable protein synthesis by physically blocking ribosomes (Leesch et al., 2023). Such extreme alterations of developmental timing can even be found in simpler organisms, namely in bacterial sporulation,

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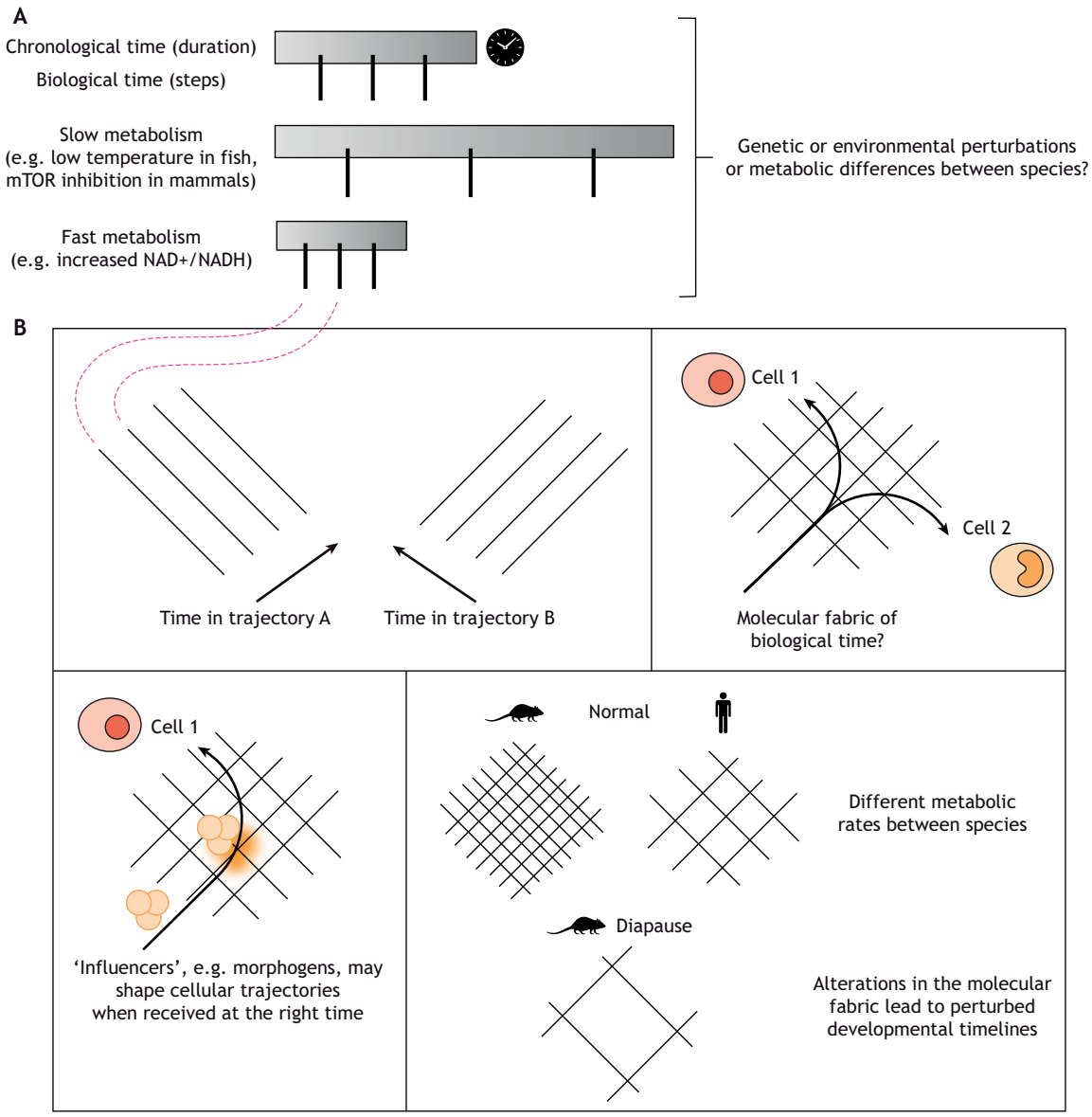

Fig. 2. The concept of biological time. (A) Whereas chronological time is measured in terms of the duration of biological events, biological time can be conceptualized as being made up of the events themselves. These may be biomolecular reactions with quantifiable outputs or the outcome of a collection of such reactions, e.g. oscillations, cell divisions, etc. Altered metabolic or reaction rates significantly alter the durations of such events, but biological time may remain roughly the same. (B) Executing a single event would take cells along a one-dimensional line in biological time. Considering multiple major events in a developmental process would instead create a multidimensional biological time (e.g. a 2D plane when two trajectories are considered), which can be conceptualized as a fabric in which the threads are the events themselves. The many differentiation trajectories that together make up ontogeny would weave a highly complex multidimensional fabric of biological time. Major influencers of cell fate decisions, such as morphogens, when received at the right biological time (i.e. when the cells are receptive to such signals) could divert cellular trajectories through biological time. Here, the orange circles depict influencers and the area of receptivity is highlighted by the orange cloud. The intervals in the landscape of biological time may differ between species or upon perturbations that alter cellular anabolism, such as diapause.

whereby stress induces metabolic arrest during arbitrarily long times. This behavior is key in the developmental cycle of bacterial biofilms (Claessen et al., 2014). Overall, the pace of developmental processes is dependent on the kinetics of the underlying molecular reactions and is, in principle, alterable across species. The examples presented above make the case for at least a set of core principles that determine the pace of development across vertebrates, including proteostasis and metabolic pathways. Species-specific and tissue-specific regulatory layers and constraints, in addition to these core principles, then results in an optimal developmental time for each species and condition.

Rewinding biological time

Biological time can be reversed either *in vivo* or in synthetic systems. Although mammals are largely incapable of fully regenerating lost body parts, with a few exceptions (Gawriluk et al., 2016; Han et al., 2003; Lehoczky and Tabin, 2015), many vertebrates, including fishes, amphibians and reptiles, are able to regenerate entirely upon injury (i.e. reparative regeneration). This type of regeneration involves dedifferentiating cells from the affected areas into multipotent blastema cells, followed by redifferentiation into mature tissues (Gerber et al., 2018; Morrison et al., 2006; Pfefferli and Jaźwińska, 2015). Biological time is

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evidently alterable in such cases *in vivo*. It is also possible to revert artificially to an earlier developmental state, a feat famously achieved by somatic cell nuclear transfer or by cellular reprogramming by overexpression of pioneer transcription factors (Gurdon, 1962; Takahashi and Yamanaka, 2006). Original cellular reprogramming methods were, however, extremely inefficient at overriding the existing robust transcriptional programs; only the cells surpassing a certain activation threshold became reprogrammed (depending on the delivery method, ∼0.001-0.0001% with a non-integrating vector) (Stadtfeld et al., 2008). Later, protocols were developed that increased reprogramming efficiency by lifting the regulatory layers that promote the robustness of gene expression programs, particularly via chromatin regulation and microRNAs (Anokye-Danso et al., 2011; Baumann et al., 2019; Onder et al., 2012; Singhal et al., 2010). These modifications increase the transcriptional noise and the proportion of cells that pass the activation threshold for dedifferentiation. The view that emerges from these examples is that biological time does not flow unidirectionally and can be reversed, given the right triggers. However, the more mature and invariable gene expression programs of differentiated cells appear to require additional interventions (e.g. at the chromatin level) for this to happen. In the next section, we focus on how gene expression noise and fluctuations contribute to progress through development, thus affecting both timing and trajectories of developmental events.

Precision, coordination and convergence: is timing as precise as we think?

Cells of the same type, growing *in vivo* or in culture, display asynchrony and heterogeneity, as revealed by numerous single-cell RNA-sequencing studies over the last decade (Cao et al., 2019). In fact, the abundance of relevant molecules within a cell determine the probability, not the certainty, of a cell committing to a certain fate. For instance, pluripotent human embryonic stem cells (ESCs) are more likely to commit to endoderm fate in G1 phase of the cell cycle owing to higher expression levels of Smad2/3 proteins in this phase (Pauklin and Vallier, 2013). In another example, different Sox2 levels lead to distinct interpretations of Wnt signals in mouse ESCs, with higher levels reinforcing pluripotency and lower levels allowing Wnt-induced mesodermal differentiation (Blassberg et al., 2022). A major mechanism underlying such a probabilistic regulation of cell fate and development is gene expression noise of individual genes, or the resulting collective stochastic fluctuations that gene regulatory networks can exhibit (Exelby et al., 2021). These are higher in undifferentiated cells and significantly contribute to the probability of activation of a developmental program (Kalmar et al., 2009). In that way, noise regulates the frequency of execution of a given developmental program within a population (Desai et al., 2021; Exelby et al., 2021), as shown by mathematical modeling (Süel et al., 2007). In addition to transcriptional noise, transcriptional oscillations and/or bursting also determine the cellular concentration of a given molecule at a given time and influence cell fate propensities (Chubb et al., 2006; Kobayashi et al., 2009; Lammers et al., 2020; Wee et al., 2012). These transcriptional fluctuations are often uneven in different cell cycle phases, suggesting that the cell cycle may be a major influencer of cell fate choices (Fischer et al., 2022; Sun et al., 2019). This calls for caution when regressing genes in transcriptome analyses, and particularly of single-cell RNA-sequencing datasets. In addition to cell fate propensities of individual cells, selection pressures within the cellular population, such as cellular competition, also contribute to final outcomes. Developing theoretical and mathematical models for quantitative single-cell measurements in developing tissues illuminates cellular differentiation outcomes within a population (Antebi et al., 2017; Exelby et al., 2021). These models point to developmental timing, particularly at cell fate/state transitions, as a factor of molecular concentrations of an effector and its receptors and the probabilistic outcome of their interaction (Saiz et al., 2020).

Timing differences across species

Are there in fact species-specific clocks, or does embryonic development follow the same biological time across species, at least in mammals? The species-level specificity (or lack thereof) of molecular clocks has been under the spotlight for the last two decades. These studies have revealed a clear anti-correlation between the body mass of an organism and its metabolic rate (West and Brown, 2004). When normalized to body mass, many features of the adult body, such as brain and bone size, appear to follow a common blueprint (Campione and Evans, 2012; Smaers et al., 2021). This blueprint includes non-morphological characteristics such as lifespan, which is inversely correlated with body mass and organismal basal metabolic rate (Atanasov, 2007). This phenomenon, called allometric scaling, suggests a constant biological time (Box 1) between adult organisms of different species. Whether embryonic development involves similar allometric scaling with respect to cellular (instead of organismal) metabolic rates is an exciting open question in developmental biology. Recently, several groups started to investigate this question by measuring the biochemical reaction speeds (e.g. protein synthesis and decay rates) and cellular metabolic rates (energy output) in the segmentation clock system as well as during spinal cord neurogenesis. The first insights revealed that biochemical reaction speeds scale with the segmentation clock period, and that this in turn scales with embryogenesis length in diverse mammalian species (Diaz-Cuadros et al., 2023; Lázaro et al., 2023). However, Hes7 reaction kinetics scale with neither cellular metabolic rates nor body size (Lázaro et al., 2023). Similar to the segmentation clock period, neurogenesis duration in the spinal cord scales with protein degradation rates (Rayon et al., 2020). Thus, even though these developmental processes differ in absolute time, they appear to scale with the reaction kinetics of their molecular regulators. However, a universal concept of allometric scaling with respect to body size or organismal metabolic rate does not seem to apply to all developmental processes. In addition to differences in their absolute timing, the order of events can also be remarkably flexible across species (i.e. sequence heterochrony). For example, marsupials grow the upper body and craniofacial muscles earlier than eutherians, to be able to use these body parts to climb up to the pouch and suckle after birth (Nunn and Smith, 1998). As such, sequence heterochrony is perhaps one of the best embodiments of the vast timescales involved in development – in this case, evolutionary forces shaping the order of developmental events and altering their timing entirely.

Perspectives

We have known for centuries that animals take variable times to generate progeny, yet we are only now beginning to understand the inner workings of these differences. The examples highlighted in this Spotlight show that, at least for some developmental processes, diverse species follow a similar biological time, even though the chronological durations differ. Evidence from classical embryology studies, as well as recent, more dynamic models of embryo development, suggest that development can be more easily slowed down than sped up in mammals without compromising original

tissue parameters. When the pace is made faster, albeit incrementally, it usually leads to smaller tissues. This would imply that biological time is optimized to its maximum capacity for each species.

We are only beginning to understand the many regulators of developmental timing across species. Only precise and multifaceted data can allow us to construct models that reveal the quantitative nature of gene regulation, which determines pace and trajectories in development. When studying regulatory mechanisms, collecting precise data on concentrations of effector molecules would greatly improve our understanding of biological time in development. When aiming to identify temporally dynamic gene regulatory mechanisms on a broader scale, the next frontier is to quantitatively measure synthesis and decay rates at both RNA and protein levels over time. Applying mathematical modeling and statistical analyses to quantitative longitudinal datasets, which can now be generated using highly sensitive methods and embryo models, will sharpen our understanding of molecular time across species.

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