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Current Opinion in Systems Biology

Control of tissue dimensions in the developing neural tube and somites

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Abstract

Despite its fundamental importance for development, the question of how organs achieve their correct size and shape is poorly understood. This complex process requires coordination between the generation of cell mass and the morphogenetic mechanisms that sculpt tissues. These processes are regulated by morphogen signalling pathways and mechanical forces. Yet, in many systems, it is unclear how biochemical and mechanical signalling are quantitatively interpreted to determine the behaviours of individual cells and how they contribute to growth and morphogenesis at the tissue scale. In this review, we discuss the development of the vertebrate neural tube and somites as an example of the state of knowledge, as well as the challenges in understanding the mechanisms of tissue size control in vertebrate organogenesis. We highlight how the recent advances in stem cell differentiation and organoid approaches can be harnessed to provide new insights into this question.

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Current Opinion in Systems Biology 2023, **35:**100459

This review comes from a themed issue on **Stem cells, development & differentiation (2022)**

Edited by **Andrew Oates**, **Marcos Gonzalez-Gaitan** & **Jose Negrete**

For complete overview of the section, please refer the article collection - Stem cells, development & differentiation (2022)

Available online 17 May 2023

https://doi.org/10.1016/j.coisb.2023.100459

The control of cell number in the developing neural tube and somites

Development of the body plan in vertebrates involves progressive anterior to posterior elongation and growth of the neural tube and somites. These tissues arise from common neuromesodermal progenitor cells (NMPs) specified at gastrulation in a neuromesodermal competent region in the caudal-lateral epiblast and node-streak border (Figure 1a) [1]. After the ~30 somite stage in mouse, NMPs become incorporated into the tailbud [2–4]. While NMPs in amniotes proliferate to maintain the NMP niche, they also continuously differentiate into presomitic mesoderm (PSM) and neural tissues. Thus, the number of cells initially incorporated into the PSM and neural tube depends on a dynamic balance between the proliferation and differentiation rates of the NMP pool.

The proliferative capacity of NMPs differs between species [4,6]. For example, in zebrafish, the proliferation of NMPs is limited from the tailbud stage onward (corresponding to ~12 somite stage) [7]. By contrast, in chick and mouse, NMPs remain highly proliferative in the tailbud and for much of the duration of axis extension [2,8]. In chick, cell divisions of NMPs occur with a cell cycle length of approximately 4.5 h, thereby amplifying the NMP population from 50 cells at stage 4 to 550 cells at the 30-somite stage [8]. In mouse, the NMP population grows from approximately 1000 to 2300 cells between E8.5 and E9.5 [9].

The size of the NMP pool is controlled by a gene regulatory network that determines the probabilities of NMPs differentiating into mesoderm or neural fates, while at the same time also controlling NMP specification and proliferation [10]. Current understanding of this network suggests that it integrates Wnt, Fgf and RA signalling through a transcriptional network that includes Sox2, T/Bra, Cdx genes, and Tbx6 [11–13]. The signalling molecules form interdependent gradients along the anterior-posterior (AP) axis and are essential for the formation of the posterior body plan. For instance, loss of Wnt/beta-catenin and Fgf activity results in truncations of the body axis [14,15]. Nevertheless, it is challenging to distinguish how signalling is interpreted to control each individual process (differentiation, proliferation or cell loss). Current evidence suggests that Wnt signalling promotes mesoderm formation and is required for the amplification of the NMP population in mice and zebrafish [9,16,17]. By contrast, Fgf signalling has been suggested to regulate the cell survival of NMPs [14,18] and inhibit differentiation of NMPs into the neural lineage [19,20]. Later in development, Gdf11 signalling promotes a trunk-to-tail transition by inhibiting Nr6a1 expression [21] and controls the size of the NMP pool in the tailbud. Gdf11

inhibits cell proliferation and biases cells towards neural fates, hence Gdf11 mutant mice have expanded neural tubes [22].

After they differentiate from the NMP pool, neural and mesodermal progenitors in amniotes continue to be highly proliferative. PSM cells divide with a cell cycle length that differs between species, from ~34 h on average in the corn snake to ~9 h in chick and quail, and ~7 h in zebrafish [23,24]. The mitotic index of the PSM and newly formed somites depends on the developmental stage (somite number) and decreases approximately 2-fold over time [25]. The mechanisms that regulate the proliferation of paraxial mesoderm cells are poorly understood.

Similar to the paraxial mesoderm, neural progenitors in the neural plate proliferate rapidly (with a cell cycle of ~8–9 h in mouse) and with a uniform proliferation rate in space [26,27]. In both mouse and chick, the proliferation rate of neural progenitors declines over time [27] via a heterogenous lengthening of the G1 phase [28]. As the cell cycle slows from E9.5 onwards, neural progenitors begin to terminally differentiate into mature neurons in a cell-type specific manner. Existing evidence suggests that proliferation, neuronal differentiation and cell survival of neural progenitors are in some way regulated by the Shh, BMP and Wnt signalling pathways, among others (reviewed in Ref. [29]). These pathways are also involved in dorsoventral pattern formation in the neural tube. Perturbations of these signalling pathways result in changes in tissue size. In some cases, these alterations have been attributed to specific processes: for instance, Shh is required for cell survival, while BMP signalling alters the cell division mode [30,31]. However, how individual cells interpret and integrate signalling and how this gives rise to the rates of cell proliferation, cell cycle exit and apoptosis across the tissue over time is still poorly understood.

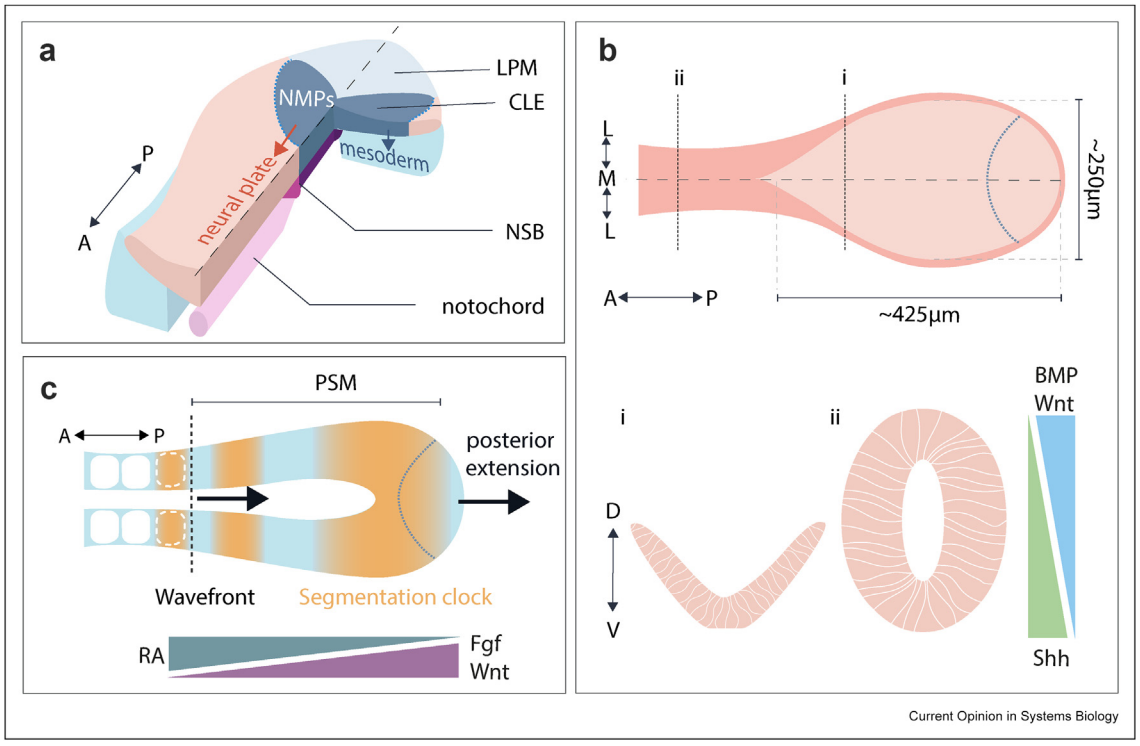
Shaping cells into tissues

The generation of tissues with defined dimensions requires morphogenetic mechanisms to organise and distribute the available cells in space (neural tube and somite morphogenesis has been extensively reviewed in Refs. [32–34]). Morphogenesis is dependent on the mechanical forces generated by tissue interactions [35], and is also influenced by the mechanisms that control tissue growth. An example of this has been observed in the neural plate (Figure 1b). In the neural plate and closed neural tube, there is no preferred orientation of cell divisions within the plane of the epithelium [36–38]. However, neural progenitors exchange neighbours and rearrange in an oriented manner. In the mouse neural plate, planar cell polarity signalling directs basolateral protrusive activity and mediolateral cell intercalations, resulting in axial elongation [37,39]. The extent of cell rearrangements within the neuroepithelium declines over time due to a decrease in the proliferation rate of neural progenitors [5]. The rate of cell proliferation also affects the shape of the mouse neural plate. Transient inhibition of cell proliferation leads to a reduction in the mediolateral width of the neural plate, while its AP length remains unaffected [5]. This can be explained by the external mechanical forces that constrain AP growth being larger than the constraint on the dorsal-ventral (DV) axis. In the presence of anisotropic mechanical constraints, the rate of tissue growth determines the resulting anisotropy of tissue shape within a given time interval [38].

In the paraxial mesoderm, AP dimensions are defined by the distinct segmentation of this tissue. In this system, oscillations of gene expression within the PSM (known as the “somite clock”) give rise to a periodic pattern that underlies the formation of somites (Figure 1c). Segments are determined by the readout of the clock at a particular position in the PSM known as the ‘wavefront’, so that cells anterior to the wavefront form newly determined segments (reviewed in Ref. [40]). As the body axis extends in the posterior direction, the wavefront moves posteriorly. At the same time, newly specified mesoderm progenitors are incorporated from the NMP pool into the PSM. The AP length of the PSM is therefore determined by the wavefront velocity and the rate of posterior extension of the PSM. These processes are regulated by signalling as well as morphogenetic mechanisms.

The wavefront position depends on the signalling gradients of Fgf, Wnt and RA along the AP axis [40]. How exactly the signalling gradients influence the clock and wavefront is still incompletely understood. Different models suggest that the spatial fold-change in Erk activity [41], the phase difference in oscillations of Wnt and Notch signalling [42], and Wnt signalling relay [43] are interpreted to define the segmentation front. In addition to the wavefront, PSM length depends on factors that influence posterior elongation, such as cell flow [44], volumetric growth driven by ECM production [45], and tissue material properties [46]. The contributions of these processes to posterior elongation differ between species. For example, volumetric growth, which is partially dependent on increasing cell numbers, contributes little to axis extension in zebrafish, while its contribution is larger and occurs at earlier developmental stages in chick and mouse [7,33]. Consistent with this, perturbations of cell proliferation in zebrafish have a mild effect on axis elongation [47]. By contrast, in mice, compensatory proliferation resulting from transient induction of cell death in early embryogenesis is accompanied by corresponding changes in PSM and somite sizes [25]. The exact contribution of cell

Figure 1



Formation of the neural tube and somites. (**a**) 3D diagram of the caudal region of the mouse embryo at E8.5 showing the approximate position of the NMP (neuro-mesodermal progenitor) pool located in the caudal-lateral epiblast (CLE, dark blue) and the border between node (magenta) and primitive streak (purple) (NSB). At later stages, NMPs are located in the chordo-neural hinge within the tailbud. NMPs self-renew and differentiate into neural epithelium (red) and presomitic mesoderm (blue). LPM indicates lateral plate mesoderm. (**b**) Dorsal view of the neural plate and neural tube (top). Dashed line indicates the midline. Transverse sections at the indicated positions (bottom): i, open neural plate, ii, closed neural tube. Approximate dimensions of the neural plate at the 6 somite stage in mouse are indicated as measured in Ref. [5]. The neural tube is patterned along the dorsal ventral axis by gradients of BMP/Wnt and Shh signalling. Region occupied by CLE and LPM is outlined with blue dotted line in b and c. (**c**) Mesoderm layer. Somites (white) are generated from the presomitic mesoderm (PSM). Cells in the PSM undergo oscillations in gene expression (segmentation clock), which give rise to a periodic pattern (orange). According to the clock and wavefront model, segments are determined when cells in an "ON" phase of the clock come in contact with the wavefront (dashed line). Thus, the wavefront separates the determined segments from the unsegmented PSM. The wavefront moves posteriorly in the direction of axis extension and its position is dependent on the anterior-posterior signalling gradients of Fgf, Wnt and RA. A, anterior; P, posterior; M, medial; L, lateral; D, dorsal; V, ventral.

proliferation to the kinetics of axis elongation and PSM size in mice remains an open question.

Both the PSM length and the length of newly formed segments change during development and have been shown to scale with each other at mid-late somitogenesis stages [24,25,41,48]. This scaling has been suggested to result from scaling of the Fgf activity gradient with PSM length [48,49]. Although the mechanisms underlying gradient scaling are unclear, this observation illustrates that tissue size feeds back into morphogen gradient formation and the determination of somites. Besides the AP signalling gradients, somite clock periodicity is also key for somite size determination. Clock oscillations depend on multiple factors [32,40], including cell cycle dynamics [50]. Furthermore, somite size has recently been shown to be fine-tuned by surface tension, which helps achieve precise coordination between the left and right sides of the embryo [51]. Although the clock and wavefront model is the most widely supported view of somitogenesis, the formation of self-organised somite-like structures in primitive streak explants has led to the proposal that segment size is intrinsically determined by cell communication rather than long-range signalling [52].

In summary, studies are beginning to dissect the processes that control the dimensions of the neural tube and somites. Key signalling pathways and morphogenetic mechanisms that regulate dimensions have been identified. However, how mechanical and chemical signalling is interpreted at the cellular level and how cellular mechanisms give rise to tissue-scale regulation is still incompletely understood.

Organoid models of trunk development

In vitro models based on directed embryonic stem cell (ESC) differentiation (here, we will refer to these as

"organoids") have become a powerful tool to test developmental principles. Directed differentiation offers a natural platform to study responses to signalling and has so far been most extensively used for unravelling the regulatory networks that underlie cell fate decisions. In the context of trunk development, milestone studies have, for instance, helped to establish that spinal neural progenitors derive from NMPs, and to decipher the regulatory logic of posterior neural and mesodermal differentiation [12,53,54]. Since then, the number of *in vitro* systems that model some aspect of trunk development (NMP, neural tube, somitogenesis) has rapidly expanded, encompassing a diverse range of culture protocols and species (Table 1).

Although the use of organoid systems in studying growth control mechanisms is rudimentary, it is a promising future research direction. This is perhaps best illustrated by 2D differentiation systems, in which monolayer differentiation protocols allow live imaging of cell movements and cell cycle dynamics [78], as well as quantitative imaging of morphogen signalling levels [79]. The possibility of combining such systems with microfluidics offers a powerful way to manipulate signalling spatially as well as temporally. This has been demonstrated in differentiated mouse neural progenitor cells, where DV and AP patterning could be recapitulated using engineered signalling gradients [63,64]. Temporal control of signalling molecule delivery with microfluidics also allowed manipulating the period of the somite clock and studying the responses of ESCs to TGF-beta [42,80,81].

Micropatterns and microfluidic control reduce the heterogeneity typically associated with organoid systems. The variability in responses, organoid sizes and morphologies have been key challenges, but continuous improvement of protocols, as well as developments of geometric constraints, are beginning to remedy these shortcomings [82,75]. Controlling the geometric and mechanical conditions of organoid growth provides the means to understand the influence of these factors on growth and cell fate decisions. For example, neural organoids have stereotypic growth curves that depend on the mechanical properties of the matrix and the application of external mechanical forces [58,60]. A micropattern-based system for 3D neural differentiation was used to show that the width of the neural tissue influences folding morphogenesis [62].

Despite the heterogeneity, the developmental time and length scales of *in vitro* generated tissues can display

Table 1

Selected examples of organoid systems that model amniote trunk development. List of abbreviations: Dim (dimensionality), IC (initial conditions), m (mouse), h (human), sc (single cells), agg (aggregate). The smallest approximate AP dimension of somites is given.

Type of organoid	Species	Dim	IC	Matrix	Characteristics	Reference
Neural						
self-elongating organoid	m	3D	sc	matrigel	dorsal interneurons; hindbrain to lumbar spinal cord	[55]
dorsal neural organoid	h	3D	agg	free floating	dorsal interneurons; cervical spinal cord	[56]
dorsal neural organoid	m	2D/3D	agg	adherent	dorsal progenitors/interneurons; brachial spinal cord	[57]
human neural tube organoids	h	3D	sc	PEG/geltrex	(DV) size ~ 60 μm (day 5); floor plate formation, DV patterning; hindbrain	[58,59]
mouse neural tube organoid	m	3D	sc	matrigel/PEG	DV length 80–290 μm (day 6); cervical spinal cord;	[60,61]
neural tube on chip	h	3D	sc	matrigel + micropattern	folding morphogenesis in 3D; forebrain	[62]
microfluidic neural patterning	m	3D	sc	matrigel/geltrex	opposing and orthogonal morphogen gradients	[63,64]
Paraxial mesoderm						
2D clock models	m/h	2D/3D	sc/agg	free floating	clock period: ~5 h (human), ~2.5 h (mouse)	[65–68]
somitoid (non-elongating)	h	3D	agg	free floating	clustered non-sequential segmentation	[69]
somitoid (elongating)	h	3D	agg	matrigel	mean segment size: 110 μm, clock period: ~5 h	[70]
segmentoid	h	3D	agg	matrigel	clock period: 5 h	[71]
axioloid	h	3D	agg	matrigel	segment size: 50–180 μm, clock period: ~5 h	[72]
Mixed						
gastruloid	m	3D	agg	matrigel	segment size: ~50 μm, clock period: ~2 h	[73,74]
coupled organoid	h	3D	sc	matrigel + micropattern	segment size: 30–70 μm, clock period: ~4.5 h	[75,76]
neuromuscular organoid	h	3D	agg	free floating	neuromuscular junctions	[77]

striking similarities to their *in vivo* counterparts (Table 1). For instance, the period of the somite clock *in vitro* is very similar to the one *in vivo*, and species-specific differences in the oscillation period are also conserved (~2.5 h in mouse and ~5 h in human) [65–68,83]. Similarly, the species differences in the timing of neuronal differentiation are also reproducible *in vitro* [84]. This has led to the suggestion that the slower temporal progression in human compared to mouse is associated with increased protein stability and slower biochemical reactions [67,84,85].

Organoid systems are also beginning to capture some of the typical developmental length scales. For instance, human paraxial mesoderm organoids develop clusters of "somite-like" structures that are similar in size to human somites at Carnegie stage 11 [69]. Mouse neuroepithelial cysts range in diameter from ~80 to ~290 μm at Day 6 [61], while the mouse neural tube increases in DV length from 150 to 300 μm in the corresponding time period of development (between E8.5 and E9.5) [27]. Length scales appear to be recapitulated better in 3D organoid models comprising multiple tissues (e.g. gastruloids and related systems). This is perhaps unsurprising, given that many of these systems form signalling gradients and recapitulate axis elongation with considerable anisotropic growth to approximately 0.7–1.0 mm in length (Table 1). Several of these human organoid systems generate segments of approximately 100 μm length, which is comparable to *in vivo* human segment size at analogous developmental stages (60–120 μm for CS10) [70,72].

Studies in organoid systems are beginning to test and build on the knowledge of how signalling gradients are interpreted in somite formation and neurogenesis. As expected, Wnt overactivation disturbs the balance of neuromesodermal differentiation, yielding excess mesoderm and loss of neural tissue [73]. Similar to *in vivo*, Fgf and Wnt signalling affects the PSM length *in vitro* [75,76]. Fgf signalling has also been shown to regulate the phase and period of oscillations in mouse and human *in vitro* systems [66,76]. Investigation of somitogenesis in human organoids demonstrated that the Fgf gradient controls the propagation of clock waves and the movement of the somite differentiation front, thus ensuring sequential segmentation, while Wnt and Fgf drive axis elongation [76]. Furthermore, organoid systems of somite formation are beginning to contribute new knowledge of the cell behaviours that contribute to somite morphogenesis. For instance, recent studies implied a new role for RA in somite epithelialisation [72], as well as the involvement of cell sorting in establishing AP segment patterning [71].

Finally, multi-tissue organoids offer opportunities to study the functional interactions between tissues. For example, trunk neuromesodermal organoids cultured over a long period develop neuronal circuits and skeletal muscle [77]. In these organoids, neural and mesodermal tissues interact throughout time and self-organise to form neuromuscular junctions.

Conclusion

The recent advances in organoid technologies have contributed to our knowledge of *in vivo* embryo development and advanced our understanding of the gene regulatory networks and signalling pathways that regulate cell differentiation, cell cycle progression and cell motility. The imaging and quantitative analysis in organoid systems continue to advance, as do the versatility of cell behaviours and mechanochemical responses studied in these systems. The potential of organoid systems can be harnessed to address key challenges in understanding growth control. Combining quantitative measurements, controlled, versatile manipulations, and multiscale theoretical frameworks that bridge the behaviours of individual cells with tissue-scale effects will be key for further progress in this field.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

We thank J. Briscoe for comments on the manuscript. Work in the AK lab is supported by ISTA, the European Research Council under Horizon Europe: grant 101044579, and Austrian Science Fund (FWF): F78 (Stem Cell Modulation). SR is supported by Gesellschaft für Forschungsförderung Niederösterreich m.b.H. fellowship SC19-011.

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