Sonic hedgehog in temporal control of somite formation

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Vertebrate embryo somite formation is temporally controlled by the cyclic expression of somitogenesis clock genes in the pre-somatic mesoderm (PSM). The somitogenesis clock is believed to be an intrinsic property of this tissue, operating independently of embryonic midline structures and the signaling molecules produced therein, namely Sonic hedgehog (Shh). This work revisits the notochord signaling contribution to temporal control of PSM segmentation by assessing the rate and number of somites formed and somitogenesis molecular clock gene expression oscillations upon notochord ablation. The absence of the notochord causes a delay in somite formation, accompanied by an increase in the period of molecular clock oscillations. Shh is the notochord-derived signal responsible for this effect, as these alterations are recapitulated by Shh signaling inhibitors and rescued by an external Shh supply. We have characterized chick smoothened expression pattern and have found that the PSM expresses both patched1 and patched2 and smoothened Shh signal transducers. Upon notochord ablation, patched1, gil1, and fgf8 are down-regulated, whereas gil2 and gil3 are overexpressed. Strikingly, notochord-deprived PSM segmentation rate recovers over time, concomitant with raldh2 overexpression. Accordingly, exogenous RA supplement rescues notochord ablation effects on somite formation. A model is presented in which Shh and RA pathways converge to inhibit PSM Gli activity, ensuring timely somite formation. Altogether, our data provide evidence that a balance between different pathways ensures the robustness of timely somite formation and that notochord-derived Shh is a component of the molecular network regulating the pace of the somitogenesis clock.

Results

Timely Somite Segmentation of Undetermined PSM Tissue Requires Presence of Notochord. To evaluate the role of the notochord in PSM segmentation, the posterior part of chicken embryos was cut along the embryo midline, generating two embryo explants (Fig. 1A): a control explant containing half of the neural tube, the floor plate, and the notochord (No+), and an experimental explant containing half of the neural tube but without the floor plate and notochord (No-). Explant pairs were cultured separately for different time periods (4.5, 7.5, 9, and 12 h), and the number of somites formed in culture was assessed (Fig. 1B and C). The number of somites formed was significantly lower in the experimental explants than in the control explants, indicating that the notochord is necessary for timely somite formation.


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References

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presence or absence of notochord on the control and experimental explants was randomly confirmed by in situ hybridization using a sonic hedgehog (shh) antisense RNA probe (Fig. 1B).

Explants incubated for 4.5 h formed three somites at the expected rate of one somite/90 min, even in the absence of the notochord (n = 68) (Fig. 1B and C). Surprisingly, for greater incubation periods of 7.5 h (n = 33) and 9 h (n = 100), the PSM tissue did not segment to the same extent in the absence of notochord as in control explants (Fig. 1B and C). In fact, somite formation considerably slowed down from 4.5 h to 9 h of incubation. Both PSM cell apoptosis and proliferation levels were assessed, and showed that the results obtained were not caused by alterations in either of these cellular processes (Fig. S1). Strikingly, from 9 h to 12 h (n = 25), an average of two new somites was formed even in the absence of the notochord, suggesting a recovery of the system (Fig. 1B and C). Our experimental approach was reproduced in ovo; upon 9 h (n = 25) or 15 h (n = 24) of reincubation, the results obtained recapitulate those observed in explant cultures: the side without notochord consistently formed less somites than the control part (Fig. 2 and Movie S1). Moreover, somite formation rate is recovered over time and, accordingly, a difference of two somites in control and experimental sides is maintained after 9, 12, or 15 h of incubation (Figs. 1C and 2C). In some embryos the size of the slit allowed to observe a delay in somite segmentation in the PSM facing the incision, whereas timely bilateral somite formation occurred caudal to the slit, leaving a rostral gap of unsegmented tissue with approximately two somites length (Fig. 2D). Because somite formation periodicity in the absence of the notochord is already reestablished at 15 h (Figs. 1C and 2C), the No− tissue will undergo further segmentation in a timely fashion, concomitantly with the generation of new somites caudal to the slit. Accordingly, after a 24 h incubation, both control and experimental PSMs are completely segmented indicating that notochord deprivation does not impair, but transiently delays somite formation (Fig. S2A and B).

Altogether, we show that the undetermined PSM tissue depends on the presence of the notochord for timely somite segmentation. Moreover, as long as the PSM cells retain contact with the notochord, they preserve autonomous correct temporal information.

PSM rostral to the determination front no longer requires the notochord to segment at normal rate.

Notochord Ablation Alters Clock and Determination Front Gene Expression. Because notochord ablation affected temporal control of morphological somite formation, we evaluated the expression of genes involved in the somitogenesis clock and determination front establishment. Explants such as those previously described (No− and No+) were incubated for 4.5 or 9 h and hybridized with probes for the clock genes hairy2 and lunatic fringe (lfng), and determination front genes fgf8 and radhh (Fig. 3). Molecular clock gene expression was significantly altered in the absence of the notochord both after 4.5 h (fgf8, n = 4/4; hairy2, n = 8/11) and 9 h (lfng, n = 19/22; hairy2, n = 15/18) (Fig. 3). The same could be observed upon ovo microsurgery (Fig. S2C). Furthermore, in notochord-deprived explants the expression domains of both fgf8 (Fig. 3) and its downstream target sprout1 (Fig. S3) were shifted caudally. However, this was not the primary cause for somite formation delay, because FGF8 supplementation did not rescue somite formation in No− explants and inhibition of FGF signaling in No− explants did not mimic Shh deprivation (Fig. S4). Raldh2 expression was unaltered by notochord ablation upon 4.5 h of incubation (n = 9/10) but was highly overexpressed after 9 h (n = 4/5) (Fig. 3). The expression of meis2, a RA downstream target, further confirms these results and was already slightly up-regulated after 4.5 h (Fig. S3). As a control, two equivalent No− explants were generated by splitting the notochord in half, and no alterations in segmentation rate or gene expression were observed (Fig. S5). In conclusion, somite formation delay in the absence of the notochord is accompanied by alterations in both molecular clock and determination front gene expression.

Ectopic Shh Replaces Notochord in Temporal Control of Gene Expression and Somite Formation. Shh is one of the most well-studied signaling molecules produced by the notochord. Shh-producing (QT6-Shh) or control (QT6-ctrl) QT6 cells were juxtaposed to No− explants which were cultured for 9 h in parallel to their No− counterpart (Fig. 4A). No−/QT6-ctrl (n = 43) failed to form the expected number of somites and presented gene expression perturbations, similarly to what had been previously observed for the No−
Notochord ablation in ovo delays somite segmentation of the undetermined PSM. (A) Schematic representation of embryo microsurgical operation. Only one of the embryo’s PSMs (PM) was left in contact with the notochord (No). HN, Hensen’s node; FP, floor plate; NP, neural plate. (B) Representative results obtained in operated embryos after a reincubation period of 9 or 15 h. (C) Graphic representation of the mean number of somites formed after 9 or 15 h. Data are mean ± SD. **P < 0.01. (D) Operated embryo reincubated for 15 h where a gap in somite formation is observed, resulting from the absence of notochord (No) in the PSM tissue juxtaposed to the surgical slit, whereas reestablished contact of the posterior PSM with the notochord reinstates timely somite formation. Presence (No+) or absence (No−) of notochord is indicated. Black arrowheads indicate somites formed before the surgical intervention; white arrowheads indicate somites formed during reincubation (New so).

Notochord ablation alters somitogenesis clock and determination front gene expression. Expression patterns of hairy2, fgf8, and raldh2, evaluated by in situ hybridization, are similar in explants cultured for 4.5 or 9 h. Presence (No+) or absence (No−) of the notochord is indicated. Black arrowheads indicate somites formed before culture; white arrowheads indicate somites formed during incubation (New so).

Retinoic acid is able to recover somite formation rate in the absence of notochord. Notochord-deprived PSMs present delayed somite formation from 4.5 h of incubation onward, accompanied by a downregulation of smoothened (Smo) and a lack of retinoic acid (RA) signaling. Shh inhibition, however, does not halt molecular clock oscillations: cyclopamine-treated explants incubated for time periods differing by 15 min (6 h and 6.5 h) presented dissimilar phases of molecular clock gene expression (Fig. 5D). In accordance to the observed delay in somite formation, Shh-inhibited explants incubated for time periods differing by 90 min (6 h and 7.5 h) differed in the phases of molecular clock gene expression (Fig. 5E).

To evaluate the delay in the molecular clock, we cultured pairs of Shh-inhibited explants for time periods differing by 1 h 10 min, 2 h, 2 h 15 min, or 2.5 h. Explant pairs presented different phases of expression of the clock and the same number of somites. When explants were incubated with a time difference of 2.5 h, 27% of the cases presented similar phases of expression of molecular clock genes but already presented different molecular clock patterns. These results indicate that clock oscillations are severely delayed in the absence of Shh, with a new period between 2 h 45 min and 3 h. The imprecision in the determination of the new oscillation period could be due to differing levels of perturbation of the system imposed by Shh deprivation, which can be dependent on the phase of the molecular clock at the moment of notochord ablation. In conclusion, Shh ensures timely somite segmentation by setting the pace of the somitogenesis molecular clock.
culbated for 14 h, with or without the axial structures, formed the same number of somites (7, 8), suggesting that these structures do not contribute to somite formation. However, the rate of PSM segmentation during the incubation period was not evaluated. When the molecular clock genes were described, their expression oscillations were also shown to occur independently of axial structures (2, 17, 18). Nevertheless, these studies were performed only up to 4.5 h of incubation, and we now know that this accounts only for determined PSM segmentation (6). Only one report has suggested that contact with tissues near the midline influences somite formation (9).

We have revisited this issue by evaluating somite formation rate over time in the presence or absence of the notochord. The temporal framework used allowed us to assess segmentation of both determined and undetermined PSM. Our results clearly show that the anterior third of the PSM does not require the notochord for timely segmentation, indicating that this tissue is determined not only spatially (6) but also temporally (this work). Segmentation of the undetermined PSM, however, is significantly delayed in the absence of the notochord. This is particularly evident for the first three undetermined somites, which resume proper segmentation only after 9–12 h of incubation. Hence, although the absence of the notochord does not impair segmentation (this work; 7, 8), we show that the strict, characteristic rate of embryo somite formation is dependent upon a signal from the notochord.

**Shh Produced by Embryo Midline Structures Ensures Somite Formation Rate.** Shh signaling pathway is not classically associated with somite segmentation. In fact, neither the Shh downstream Gli effectors nor the pathway’s membrane receptors have been reported to be expressed throughout the PSM (19, 20). We show that *pcth1* is expressed in the medial PSM tissue and that *smo* is ubiquitously present in the PSM, creating the conditions for Shh signaling readout in this tissue.

Embryo midline structures produce diffusible Shh. Our results indicate that the notochord role on somite formation rate may be the production of a Shh signal delivered to the adjacent PSM. In fact, exogenous supply of this protein rescued timely somite formation in the presence of the notochord. Nevertheless, the presence of canonical Shh inhibitors somite formation rate was perturbed to the same extent as when the notochord was removed. These experiments strongly suggest that embryo midline structures ensure strict control of somite formation rate through the supply of canonical Shh signal to the PSM.

**Somitogenesis Clock Periodicity Is Modulated by Shh Signaling.** Could a perturbation in the clock period underlie the delay in somite formation? A clump (pink) of control QT6 fibroblasts (QT6-ctrl) or QT6-producing Shh cells (QT6-Shh) juxtaposed to the neural tube (yellow) of explants with tissues near the midline in incubation (New so).
expression patterns are significantly different from those obtained in control conditions. Moreover, an exogenous Shh supply to notochord-ablated explants recovered molecular clock expression patterns. Hence, we could detect a clear correlation between the presence of Shh and both timely somite formation and molecular clock gene oscillations. Our group has previously shown that only the medial-most PSM (M-PSM) cells possess intrinsic information for both somite formation and molecular clock gene expression (21). The study suggested that a signal supplied by M-PSM travels along the medial-lateral PSM axis, ensuring both timely segmentation and cyclic clock expression. However, the nature of this signal was not identified (21). The results obtained throughout the present work suggest that M-PSM cells are exposed to notochord-derived Shh signaling, as evidenced by ptc1/2 restricted expression, providing them crucial information for timely clock oscillations and somite formation. M-PSM cells are then capable of instructing and recruiting their lateral neighbors for segmentation. This hypothesis is supported by the observation that somites formed in the absence of Shh are smaller along the medial-lateral (M-L) axis (Fig. S2B). How this is achieved in vivo is subject of ongoing studies in the laboratory.

Inhibition of Wnt signaling has also been described to perturb the molecular clock (22). We found that axin2 expression is unaltered in 60% of the notochord-ablated explants (Fig. S8) and the other 40% are only slightly down-regulated after 4.5 h. Because clock expression is perturbed in a much higher percentage of cases (80%), somite formation delay in the absence of Shh should not be due primarily to an impairment of Wnt signaling. Moreover, Wnt does not seem to be involved in the recovery of timely segmentation, as axin2 is clearly down-regulated after 9 h (Fig. S8).

Somitogenesis clock gene expression perturbation in the absence of Shh signaling was not due to a halt in gene oscillations, but to an alteration of the clock period. In fact, the period of hairy2 expression cycles is considerably enlarged in the absence of Shh signaling, from 1.5 h to a time point between 2 h 45 min and 3 h. Significantly, somite formation in these conditions is also greatly delayed, reinforcing the dependence of timely somite formation on the phase of clock oscillations. Interestingly, somite formation delay observed upon Shh inhibition was dose dependent, as fewer somites were formed with increased inhibitor concentrations (Fig. S9). In situ hybridization on sections suggests the existence of a ptc1 expression gradient, with higher levels in the determined PSM (Fig. S6D). It is tempting to postulate that an anterior–posterior gradient of Shh activity could account for the different segmentation rates observed for rostral (early) and caudal (late) somites. Shh knock-out mice present a striking absence of the entire spinal column and most of the ribs (14). Our data suggest that a perturbation in somite segmentation could be contributing to this phenotype.

Shh and RA Pathways Interplay with the Molecular Clock to Ensure Timely Somite Formation. This work shows that Shh produced by the notochord/floor plate is required to maintain timely operation of the molecular clock and the correct pace of somite formation (Fig. 7C, Left). An important consequence of PSM isolation from its source of Shh is the up-regulation of gli1 and gli3 (Fig. 7C, Middle), which corroborates previous reports (19). This strongly suggests that low gli levels in the PSM are not due to the lack of Shh signaling readout, as is commonly believed, but rather are actively restricted by notochord-derived Shh. Another striking molecular consequence of notochord ablation is the down-regulation of fgf8 expression in the PSM, showing that Shh signaling is required to preserve the fgf8 gradient underlying the determination front. These molecular changes, observed in the PSM after 4.5 h of incubation in the absence of Shh, most probably account for the subsequent delay in somite formation rate and molecular clock oscillations.

The PSM fgf8 gradient counteracts an opposing RA gradient (5). Hence, the reduction of fgf8 levels in the absence of Shh could allow the observed up-regulation of Raldh2 after 9 h of incubation (Fig. 7C, Right). Under these molecular conditions, there is a recovery of somite formation rate. Accordingly, we show that an external RA supply is able to rescue timely somite formation in the absence of Shh (Fig. 7A and B). Although RA has been shown to...
inhibit gli2 expression (23), this does not occur within our experimental time frame. Because RA also modulates Gli activity (24), our results suggest that raldh2 overexpression after 9 h of notochord ablation rescues somite formation through an inhibition of Gli2/3 activity in the PSM (Fig. 7C, Right). It is plausible that somite formation recovery may further involve the inhibition of gli2 expression levels later in time. Our work strongly suggests that Shh and RA pathways interplay in the PSM to ensure timely somite formation and positions Gli2/3 at the intersection of these two pathways. Gli2 was recently reported to exert direct transcriptional regulation on the murine hes gene in retinal explants (25). In our experimental setup, gli2 overexpression was concomitant with a deregulation in the tempo of hair2 transcriptional bursts, suggesting that in the PSM the same transcriptional regulation could take place. Hence, Shh-mediated restriction of Gli activity in the PSM seems to be essential to ensure the pace of both molecular clock oscillations and somite formation. Interestingly, Shh has been recently described to be essential for proper timing of neural progenitor differentiation, and RA was proposed to compensate for Shh deficiency in this developmental system (26).

Somitogenesis is a robust developmental mechanism. This can be achieved only by the integration of multiple signaling pathways. In fact, each Wnt, Fgf, and RA signaling pathway operates in the PSM molecular clock and/or determination front (3, 4, 6). In the present work, we provide conclusive evidence that Shh signaling is a component of the intricate molecular machinery responsible for temporal control of somite formation.

Multiple congenital vertebral malformations have been associated with mutations in somitogenesis clock genes (1, 3). Furthermore, there are reports associating the Shh signaling pathway with congenital vertebral anomalies (27, 28). We report a previously uncharacterized association between these two molecular systems operating in the formation of early embryonic vertebral precursor structures. A deeper understanding of the multiple molecular interactions occurring during the somitogenesis process is crucial for an adequate scientific contribution to the medical community, striving to better comprehend and treat such human malformations.

Materials and Methods

Detailed materials and methods can be found in SI Materials and Methods. Chick embryos were staged according to Hamburger and Hamilton (HH) (29), and somites were considered to be formed when a definitive cleft was observed (30). Antisense digoxigenin-labeled RNA probes were produced as described: shh (31), hair2 (17), lunatic fringe (32), fgf8 (33), raldh2 (34), patched-1 (22), gii1 and gii2 (35), and gii3 (19). A smoothed-specific probe was generated. For explant cultures, ventrally positioned embryos were divided into two halves by cutting across the three germ layers at the midline level, either parallel to the embryonic axis or bisecting the axial structures. In Shh-graft experiments, clumps of either QTF-ctl or QTF-Shh cells (36) were juxtaposed to the No° experimental explants. For chemical treatments, 15 μM of RA (Sigma), 10 μg/mL cyclopamine (Calbiochem), 25 μg/mL forskolin (Sigma), or DMSO was added to the culture medium. In situ hybridization was performed as previously described (37).

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9. Packard DS, Jr (1980) Somitogenesis is a robust developmental mechanism. This can be achieved only by the integration of multiple signaling pathways. In fact, each Wnt, Fgf, and RA signaling pathway operates in the PSM molecular clock and/or determination front (3, 4, 6)