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Heterochrony in somitogenesis rate in a model marsupial, *Monodelphis domestica*

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SUMMARY Marsupial newborns are highly altricial and also show a wide array of shifts in the rate or timing of developmental events so that certain neonatal structures are quite mature. One particularly notable feature is the steep gradient in development along the anterior–posterior axis such that anterior structures are generally well developed relative to posterior ones. Here, we study somitogenesis in the marsupial, *Monodelphis domestica*, and document two heterochronies that may be important in generating the unusual body plan of the newborn marsupial. First, we demonstrate a 4-fold change in somitogenesis rate along the anterior–posterior axis, which appears to be due to somitogenesis slowing posteriorly. Second, we show that somitogenesis, particularly in the cervical region, initiates earlier in *Monodelphis* relative to other developmental events in the embryo. The early initiation of somitogenesis may contribute to the early development of the cervical region and forelimbs. Other elements of somitogenesis appear to be conserved. When compared to mouse, we see similar expression of genes involved in the clock and wavefront, and genes of the Wnt, Notch, and fibroblast growth factor (FGF) pathways also cycle in *Monodelphis*. Further, we could not discern differences in somite maturation rate along the anterior–posterior axis in *Monodelphis*, and thus rate of maturation of the somites does not appear to contribute to the steep anterior–posterior gradient.

INTRODUCTION

Marsupial mammals exhibit a unique mode of reproduction in which young are born after a short intrauterine period and complete their development nursing. Newborns are highly altricial, and in addition show a wide array of shifts in developmental timing, or heterochronies, so that certain neonatal structures are quite mature. Advanced structures are believed to be adaptive for the altricial neonate, allowing it to survive in an essentially embryonic state outside of its mother's womb (Hughes and Hall 1988). One particularly notable heterochrony is the steep gradient in development along the anterior–posterior axis such that anterior structures are generally well developed relative to posterior ones. For example, the forelimbs are well differentiated and relatively large and are used to crawl to the teat immediately after birth. In contrast, the hind limbs are much smaller, relatively underdeveloped, and not yet functional. The steep anterior–posterior developmental gradient can also be seen in the degree of chondrification of the axial skeleton. A comparison of skeletal preparations of model marsupial and eutherian mammals shows that vertebrae at cervical and upper thoracic levels are well chondrified at birth in an opossum, while in posterior regions structures are relatively undifferentiated. In contrast, a mouse at a similar stage of development exhibits much more uniform chondrification along the anterior–posterior axis (Fig. 1) (Smith 2003). This extreme developmental gradient has been known to exist for some time, and authors have proposed that during the short period of gestation available to marsupials, an energy tradeoff allocates limited cellular resources to structures required at birth at the expense of others (Müller 1967; Smith 1997 Weisbecker et al. 2008).

Many of the structures discussed here are derived partially or in whole from the somites, transient structures in vertebrate embryos that are the first morphological sign of segmentation. Somites give rise to much of the axial skeleton and all postcranial skeletal muscles, including limb muscles. In addition, the tongue muscle precursors in mammals migrate from the anterior-most somites. Therefore, in order to determine the origins of the heterochronies observed along the body axis, it is critical to document potential heterochronies in somite formation in marsupials.

A study of heterochrony in somite formation is also of interest given the nature of somite formation. Somites bud off in pairs, one on either side of the neural tube, from the rostral end of the presomitic mesoderm (PSM). Somite formation is frequently described as "clock-like," and most studies do not report rate variation in a given species during development. However, most somitogenesis studies focus on a small window during developmental time, and when the entire course of somitogenesis has been examined, changes

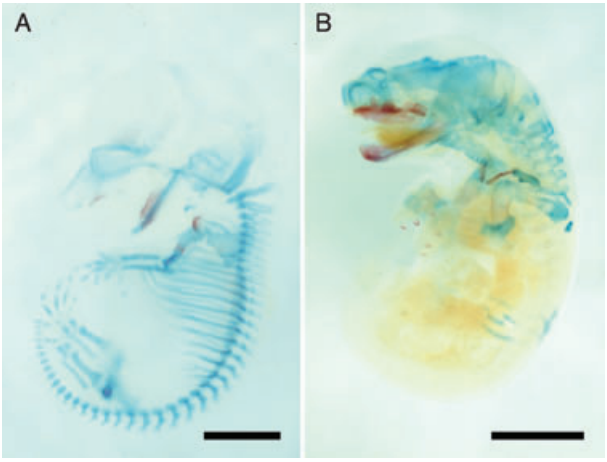

Fig. 1. Alcian blue and alizarin red skeletal preparations of an E14 mouse (A) and a 2-day old opossum (B). Bone is stained red and cartilage is blue. Scale bars are 0.25 cm.

in somitogenesis rate during the course of development have been documented (Tam 1981; Schroter et al. 2008).

The mechanism of somitogenesis appears to operate via a "clock and wavefront" model (Cooke and Zeeman 1976; Pourquie 2003; Aulehla and Herrmann 2004; Dequeant and Pourquie 2008; Gomez and Pourquie 2009). The model posits that each cell in the PSM has its own internal clock, which oscillates between permissive and nonpermissive states for formation of a segment boundary; cells are coupled so that oscillations within the PSM are synchronized. A wavefront travels rostro-caudal through the PSM, and after it has passed, cells are competent to form a segment boundary. In this fashion, a segment boundary is formed when the clock is in the permissive state and wherever the wavefront happens to be at that time (Dubrulle and Pourquie 2004b). A large number of studies have detailed the specific molecular components of the clock and wavefront; these include members of the Notch, FGF, and Wnt signaling pathways (Aulehla and Herrmann 2004; Dequeant and Pourquie 2008; and references therein).

In this study, we examine the timing and pattern of somitogenesis in marsupials relative to eutherian mammals and other amniotes. Our goal is to identify specific developmental changes that have led to the unique configuration of the marsupial newborn. Specific questions we address include the following. Does the steep anterior–posterior gradient in axial development arise from changes in the rate of generation of somites, or later events in somite maturation? If the rate or timing of somitogenesis is changed, what are the specific patterns of heterochrony in somite generation in marsupials relative to other amniotes? Are there major pauses or shifts in the rate of somitogenesis, or is there a gradual change in rate? Are there changes in elements of the somite clock and wavefront at the molecular level or is this mechanism is conserved in marsupials as well as more common model organisms? In order to answer these questions, we detail the development of somites along the body axis in the opossum, *Monodelphis domestica*. We examine the spatiotemporal pattern of expression of major genes implicated in the somite clock and wavefront. Finally, we examine genes downstream of somitogenesis involved in somite maturation in order to differentiate heterochronies in somite formation and heterochronies in later somite maturation. In all cases, we compare patterns observed in *M. domestica* to those reported in mice, and where available, other amniotes.

METHODS

Embryos and staging

All work was conducted according to protocols approved by Duke University IACUC. Opossum embryos were obtained from a breeding colony of *M. domestica* located at Duke University. The colony was composed primarily of descendants of individuals from the Southwest Foundation for Biomedical Research (San Antonio, TX, USA). Protocols for animal husbandry, breeding, and embryo collection were followed as described in previous publications (Keyte and Smith 2009). Matings were videotaped in order to determine the exact time of copulation so that embryos of known age could be collected. Embryos were staged according to McCrady (1938) with modifications by Mate et al. (1994).

Probe construction and whole-mount in situ hybridization

Total RNA was extracted from freshly dissected stage 24, 25, and 29 embryos using the Aurum Total RNA kit (Bio-Rad, Hercules, CA, USA, cat no. 732–6820). The reverse transcription (RT) reaction was carried out using the Omniscript RT kit at 42°C (Qiagen, Valencia, CA, USA, cat no. 205111). Segments of *Wnt3a*, *Lnfg*, and *Dll1* coding regions were all amplified by a two-step, nested polymerase chain reaction (PCR) with primers from Gomez et al. (2008). *Snail*, *Fgf8*, *Axin2*, *Myf5*, and *Uncx4.1* segments were each amplified in one reaction with primers designed either from *M. domestica* genome sequence or to conserved nucleotide sequences within amniote alignments constructed using the program Se-Al (Rambaut, A. 1996. Se-Al: Sequence Alignment Editor. Available at http://evolve.zoo.ox.ac.uk/). PCR products were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA, cat no. K4600) and sequenced. Gene identity was confirmed by BLAST to the *M. domestica* genome and other mammals (Altschul et al. 1990). Sequences have been deposited into GenBank under accession numbers GU647075-GU647085. Antisense and sense riboprobes were synthesized using the DIG RNA Labeling Kit (Roche, Penzberg, Germany, cat no. 1175025) and either linearized plasmid or PCR product as template. Whole-mount in situ hybridization was done as described (Keyte and Smith

2009). For each of the four cyclic genes (*Dll1*, *Axin2*, *Snai1*, and *Lfng*), in situs were performed on a large number of litter mates with the same number of somites, so as to identify different phases of cyclic expression.

Immunohistochemistry and somite counts in older embryos

In older embryos, the most anterior somites have already begun to dissociate, while somitogenesis continues posteriorly. In order to obtain accurate somite counts, we established the identity of anterior somites using spinal nerves marked by an antibody to neurofilament protein (2H3, developed by T. M. Jessell and J. Dodd and obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Immunohistochemistry was done as described in previous papers (Hanken et al. 1992; Smith 1994). At stage 28, the third cervical somite (C3) lies at or just within the anterior boundary of the forelimb bud. In older embryos where the most anterior somites are less visible, somite counts began with somite C3.

Analysis of developmental sequence

Shifts in the timing of specific developmental events were identified in *M. domestica* by comparing the sequence of development in mice and *M. domestica* (Nunn and Smith 1998; Smith 2001). We assembled a data set consisting of 50 developmental events from all areas of the embryo and the stages at which these events occurred in mouse and *M. domestica* (Table 1). The data set included the timing of appearance of the first cervical, thoracic, lumbar, and sacral somite pairs. Mice and opossums have the same number of presacral vertebrae at each anatomical level. The sequence of events in the two species was compared to reveal which events appear relatively early in the sequence. A measure of the degree of the shift in relative timing of the onset of somitogenesis in each vertebral region was calculated using a resampling method (jackknife) as follows: A subset of 10 unique events was randomly chosen from the total data set. The relative timing (early, simultaneous, or late in *M. domestica* compared to mouse) of onset of somitogenesis in each somite region was determined. This random resampling was performed 1000 times. The percentage of times an event was determined to be early, simultaneous, or late was calculated. This percentage is the jackknife score, which is essentially a confidence measure (Keyte and Smith 2010).

Throughout this article we discuss changes in the relative rate of developmental events as well as changes in sequence, and use the term *heterochrony* for these changes. Properly heterochrony refers to a change in timing relative to a primitive condition, but is more commonly used, as we use it, in reference to changes in the relative timing of events in two or more taxa or clades. When only two taxa are compared, neither the primitive condition nor directionality can be established. Therefore, one may say that taxon A is accelerated relative to taxon B or that B is delayed relative to A. Where data exist for other taxa, that information is included to help determine polarity. Where it is not, we recognize

Table 1. All events data set

Event ID	Event	Stage	
		M. domestica	*M. musculus*
1	Neural groove begins to form	21	11
2	Notochord begins to form	21	11
3	First arch crest begins migration	22	12
4	First cervical somite pair	23	12
5	Otic sulcus	22	12
6	Preotic sulcus	22	12
7	Optic pit	23	12
8	Heart tubes	24	12
9	Otic placode appears	24	12
10	Rhombomeres start to appear	24	12
11	First aortic arch formed	25	12
12	Neural folds first beginning to fuse	25	12
13	Endocardial tubes start to fuse	26	12
14	First thoracic somite pair	25	13
15	Liver diverticulum appears	28	13
16	Oropharygeal membrane becomes perforated	29	14
17	Wolffian duct appears as a thickening with no lumen	24	14
18	Forelimb condensations	24	14
19	Trigeminal ganglion (of cranial nerve V) becomes distinct as cellular aggregate	24	14
20	Otic placode depressed (otic pit)	25	14
21	Thyroid (or endostyle) depression appears in floor of pharynx	27	14
22	Anterior neuropore closed	28	14
23	First lumbar somite pair	29	15
24	Lens placode appears	28	15
25	Otocyst closed but still connected with surface ectoderm	28	15
26	Otocyst detached from ectoderm	28	15
27	Dorsal pancreas beginning as a diverticulum	29	15
28	Endocardial cushions of atrioventricular canal just beginning	30	15
29	Gall bladder beginning as a diverticulum	30	15
30	Liver cords forming	30	15
31	Ventral pancreas beginning as a diverticulum	30	15

Table 1. Continued

Event ID	Event	Stage	
		M. domestica	*M. musculus*
32	Laryngotracheal (part of the median pharyngeal) groove indicated	25	15
33	First myocyte migration from somite into forelimb	27	15
34	Forelimb bud	27	15
35	Nasal placodes depressed (formation of olfactory pit)	28	16
36	Endolymphatic appendage appears	29	16
37	Externally visible tailbud beginning	29	16
38	First sacral somite pair	30	16
39	Lens placode depressed	29	16
40	Hind limb bud	30	16
41	Posterior neuropore closed	30	16
42	Lung buds as distinct evaginations	27	16
43	Septum primum of atrium just beginning	30	17
44	Ureteric bud just forming from Wolffian duct	31	17
45	Spleen anlage beginning as thickening of peritoneal epithelium	33	18
46	Lens vesicle pinches off from surface ectoderm	30	19

that directionality is not clear, timing is discussed in a relative sense, and the primitive condition cannot be resolved.

Determination of somitogenesis rate

Litters of embryos were collected at various ages from 10 to 12.3 days post mating (240–295 h), corresponding to stages 22–31, which covered the formation of the first somites (occipital 4/5) to the first caudal somites. A total of 217 embryos were sampled from 27 litters, including 9 full litters and 18 partial litters. Embryos that were severely retarded or displayed developmental defects were not included in the counts. An in situ probe to *Uncx4.1*, which marks the caudal compartment of each somite (Mansouri et al. 2000), was used to visualize somites. The first somite stained by *Uncx4.1* is the fourth or fifth occipital somite (O4 or O5). By stage 29, *Uncx4.1* expression is diminished in the occipital and most anterior cervical somites. In order to obtain accurate somite counts for older embryos, an antibody to neurofilament protein was used to visualize spinal nerves in the segments and determine axial level as previously described (Keyte and Smith 2010). Within a litter, the number of somites per embryo did not vary by more than plus or minus one somite, except for the oldest litter collected. In this litter, somite number ranged from 28 to 32 (Table S1). In order to maximize the sampling while making best use of the limited embryos available, representative embryos were selected for in situ hybridization using *Uncx4.1* for some litters, rather than using the entire litter. The number of somites per embryo was plotted versus age in hours (time from copulation to collection). Somitogenesis rate was calculated for each individual axial level (cervical, thoracic, lumbar, and sacral/caudal) via two methods. First, a straight line was fit locally to the data points within each axial level, and rate was computed by taking the inverse of the slope of this line. Second, a quadratic polynomial was fit to the data set as a whole (global fit), and the tangent line taken to the curve at the midpoint of each anatomical level. Rate was calculated by taking the inverse of the slope of the tangent line. *Monodelphis domestica* has only two sacral segments, so sacral and caudal levels were combined.

When calculating embryo age, we do not know when fertilization occurs or development begins; we only know when mating takes place. It is known that in *M. domestica*, there are generally about 24 h between mating and the commencement of development (Mate et al. 1994). In addition, development moves very quickly through stages 22–25; these four stages span only about 18 h. Thus, slight variation in the 24-h interval between mating and development will yield significant variation in embryo stage over this period. There was a large amount of variation in stage, and therefore somite number, between litters collected before 10.5 days (approximately 250 h). Stages ranged from 22 to 25, and we collected a number of litters that were much more developed than we expected based on previous collections around 10 days in our lab. These atypical litters were included in the data analysis, but may skew the somitogenesis curve upward for these earliest stages. Initial somitogenesis rate should therefore be interpreted as a minimum of the actual rate.

RESULTS

Somitogenesis rate

Rates for each of the axial levels (cervical, thoracic, lumbar, and sacral/caudal) calculated locally differed substantially from rates calculated from the globally fit polynomial at lumbar and sacral/caudal levels (Table 2). This difference is most

Table 2. Somitogenesis rates expressed as number of hours to form a somite pair. See Methods for a description of the derivation of each rate type (global vs. local) in *Monodelphis domestica*.

	Cervical	Thoracic	Lumbar	Sacral/caudal
M. domestica (local)	1.69	2.36	9.47	7.06
M. domestica (global)	1.22	1.65	2.39	4.60
M. musculus (Tam 1981)	1			2.5

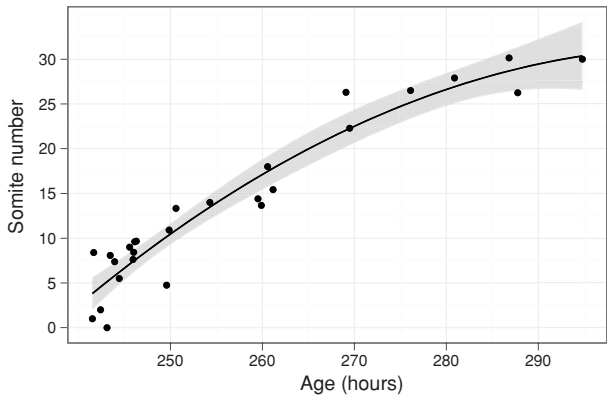

Fig. 2. Number of somites versus age in hours. Each dot represents one litter; number of somites was averaged per litter. Curve is fit globally to the entire data set. Shaded area represents the 0.95 confidence interval. "Global" somitogenesis rates were calculated as follows: A tangent line was drawn at the midpoint of each anatomical level (cervical, thoracic, lumbar, sacral/caudal) and the slope of the tangent was calculated. Somitogenesis rate for each axial level expressed as number of hours to form a somite pair is the inverse of this slope.

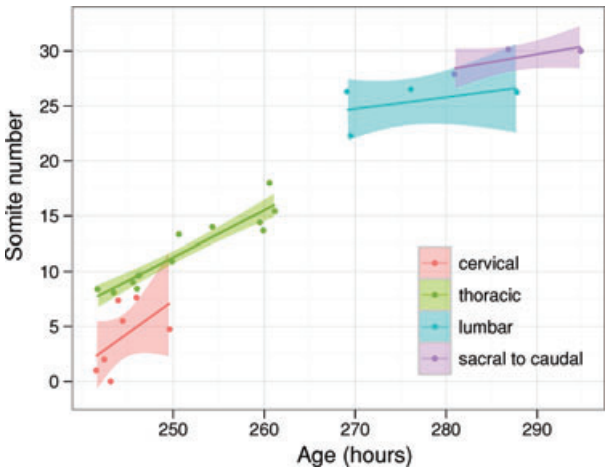

Fig. 3. Number of somites versus age in hours. Each dot represents one litter; number of somites was averaged per litter. Trend lines are fit locally to each anatomical level. Shaded areas represent the 0.95 confidence interval. "Local" somitogenesis rates were calculated by taking the inverse slope of the trend line.

likely due to the fact that sampling of litters was limited for older embryos. For the purposes of discussion and comparison with mouse rates, we use the rates calculated from the global polynomial fit, as these are more conservative.

Somites in *M. domestica* initially form about one pair every hour. Somitogenesis rate slows to one pair every four and a half hours by caudal levels (Figs. 2 and 3; Table 2). The change in rate along the vertebral axis appears to be gradual, without any major pauses or shifts in rate.

Developmental sequence

Mouse and *M. domestica* have the same number of presacral somites and vertebrae in each anatomical region, which allows us to directly compare the timing of appearance of the first cervical, thoracic, lumbar, and sacral somite pairs. Developmental sequence analysis indicates that these somite pairs appear early in opossum relative to other developmental events when compared to mouse (Fig. 4). However, support for these heterochronic shifts is not as strong as for other events that are known to be early in marsupials; the jackknife scores are not as high for the onset of somitogenesis in the aforementioned axial regions as they are for the forelimbs, lungs, and nasal placodes. In addition, when the stages for these somitic events are changed to one stage later (e.g., stage 24 to 25), only the first cervical somites still appear to differentiate early relative to other structures. This simple test indicates that differences in staging resolution between mouse and opossum may cause the appearance of an early heterochronic shift, where in fact there is none (see Keyte 2010 for more details).

Clock and wavefront gene expression

We documented the expression in *M. domestica* of a number of genes shown to participate in somitogenesis in other amniotes and compared expression patterns to those reported for mouse (Figs. 5 and 6). Table 3 lists the gene expression examined, the role of these genes in somitogenesis, and any differences from mouse expression. Within the formed and forming somites, expression patterns in *Dll1* and *Axin2* are slightly different in opossum compared to mouse, but cyclic expression of these genes within the PSM appears similar. *Fgf8* and *Wnt3a*, partly responsible for positioning the determination front or wavefront, are also similar (Fig. 5). Each putative cyclic gene (*Axin2*, *Dll1*, *Lfng*, *Snai1*) was expressed in the PSM in a variety of patterns in different embryos of the same stage, strongly suggesting that these genes are expressed cyclically in opossum as they are in mouse (Fig. 6; Table 3). However, we cannot say whether the cyclic genes oscillate in the same respective phases, as this was not investigated. Aside from the differences noted in Table 3, the expression in opossum for these genes is virtually identical to mouse except for differences in morphology between the two species.

Somite maturation gene expression

It is possible that the anterior–posterior gradient in marsupials is generated by changes in the rate of maturation of somites. We studied expression patterns of several genes involved in somite maturation to determine whether somites

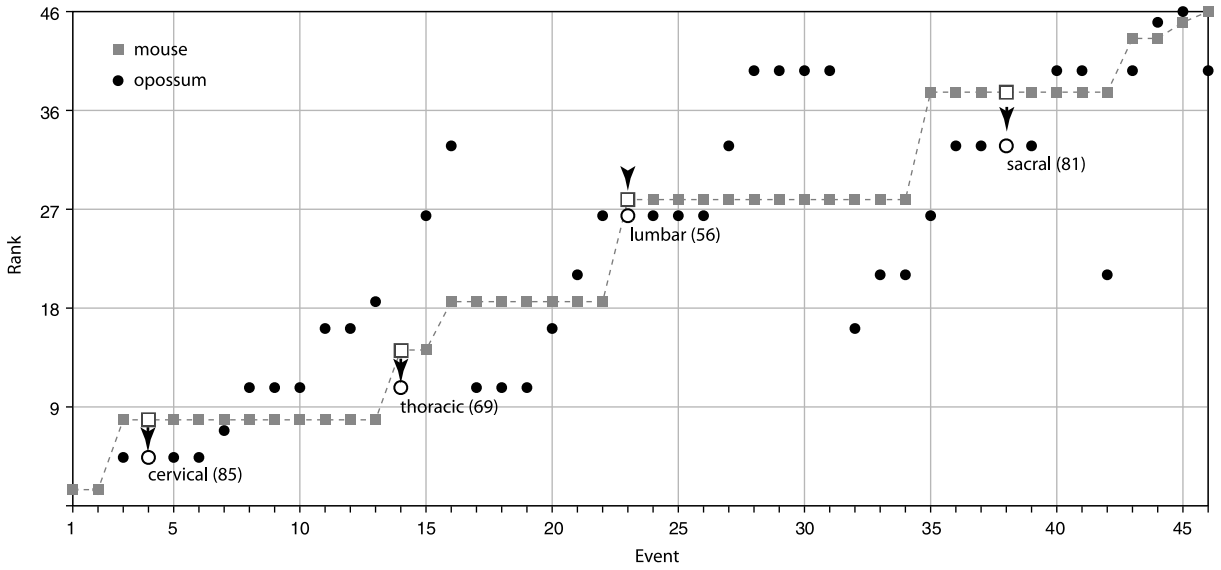

Fig. 4. Plot of relative timing of developmental events in opossum and mouse. Heterochronic shifts in the timing of onset of somitogenesis at different axial levels were identified in opossum by comparing the sequence of development in mouse and opossum. Jackknife scores are indicated in parentheses. (See Methods for a description of the jackknife score.) Open circles and squares indicate somitic events.

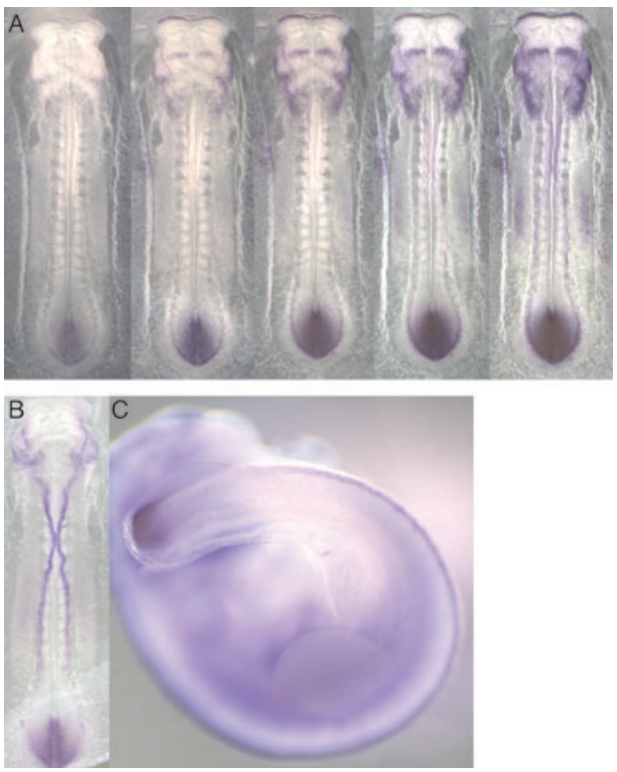

Fig. 5. (A) *Fgf8* expression gradient in the presomitic mesoderm (PSM). This stage 25 embryo was photographed at successive time points in the color reaction. (B and C) *Wnt3a* expression in the PSM at (B) stage 25 and tailbud at (C) stage 28. Anterior is oriented to the top.

mature more or less slowly at different axial levels in opossum. These genes and their functions are listed in Table 3. We noted the position of expression relative to the last formed somite (somite I) in embryos of different ages. Expression was studied in embryos ranging from the formation of the most anterior somites to the formation of the first caudal somites (Fig. 7). We measured relative expression times by examining the distance (in number of somite lengths) between the most recently formed somite and the onset of expression of a maturation gene. If the distance is different in embryos of different ages, then the rate of maturation was assumed to be different for different axial levels. *Mesp2* was expressed in one or rarely two bands in the rostral PSM at a distance of about one somite length from the most recently formed somite regardless of the age of the embryo. *Myf5* was expressed broadly in the PSM, but immediately after somite formation expression narrowed to a subdomain of the newly formed somite, and this was consistent for embryos of different ages. (We did not take into account timing of expression in different somitic compartments, such as epaxial vs. hypaxial dermomyotome, because of differences in expression domains present at different axial levels.) *Pax3* expression in stage 22–23 was confounded by overlying expression in the neural plate border, but at all later stages expression clearly extended one to two somite lengths beyond the newest somite into the PSM. Thus, for the three genes examined here, there do not appear to be differences in the rate of somite maturation at different anterior–posterior levels, at least through the first caudal somites.

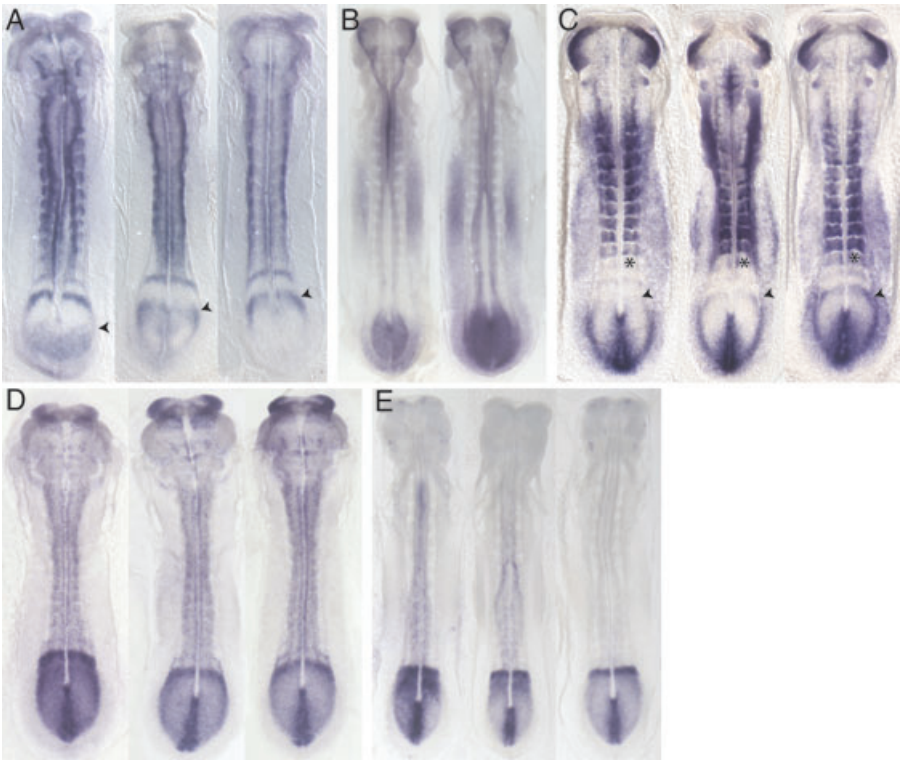

Fig. 6. Cyclic gene expression in opossum litter mates. (A) *Lfng* expression in stage 24 embryos. Arrowhead indicates peak of expression in PSM. (B) *Axin2* expression in stage 25 embryos. (C) *Snai1* expression in stage 24 embryos. Arrowheads indicate a band of expression appearing in the rostral PSM. Asterisks indicate the most posterior somite in the process of forming. (D and E) *Dll1* expression in stage 24 (D) and stage 25 (E) embryos. Anterior is oriented to the top.

DISCUSSION

Patterns in the evolution of the vertebral column have long been of interest and are increasingly an important topic in the study of the relation between developmental and evolutionary changes. Previous studies include the role of *hox* genes in generating regional differentiation (Burke et al. 1995; Cohn and Tickle 1999; Burke and Nowicki 2001), the potential developmental basis for the extraordinary conservation of mammalian vertebral regionalization (Sanchez-Villagra et al. 2007; Hautier et al. 2010; Müller et al. 2010), and the mechanisms for changing the number of vertebrae in organisms such as snakes (Gomez et al. 2008). Most previous studies have examined the changing regional boundaries of the vertebral column or the number of vertebrae. In this article, we are taking a different approach to the question and examine changes in the rate of somitogenesis during development, both at the genetic and morphological level to understand the specific evolutionary changes we observe in marsupial mammals.

In most previous studies, somite formation has been studied over a limited span of the vertebral column, and reports have been limited to an average rate. Reported average somite formation rates for zebrafish, frog, and chick at standard temperatures are one pair every 30, 60, and 90 min, respectively (Romanoff 1960; Pearson and Elsdale 1979; Schroter et al. 2008). Tam (1981) has studied somitogenesis in mice along the entire column, and has reported that the maximum rate of somitogenesis in anterior regions is approximately one somite pair per hour. In caudal regions, somitogenesis is reported to slow by a factor of approximately 2.5. In anterior regions in *M. domestica*, one pair of somites appears every 60 min, but somitogenesis slows by a factor of at least 4. This change in rate in marsupials appears to be gradual without pauses or sudden shifts in rate, as has been observed in mouse (Tam 1981). The rate of somitogenesis in posterior regions of *M. domestica*, at least 4 h per somite pair, is one of the slowest somitogenesis rates so far reported for any vertebrate. Additionally, the relative rate (a 4-fold slowing) of somitogenesis in posterior regions of *M. domestica* is far slower than has been previously reported for other vertebrates. Therefore, we conclude that the steep anterior–posterior gradient in axial structures in *M. domestica* arises primarily through changes in somitogenesis rate along the vertebral axis, and it appears that the 4-fold rate change seen in opossum is due to somitogenesis slowing posteriorly.

In addition, somitogenesis begins early relative to other developmental events in *M. domestica* compared to mouse. When the first cervical somites appear in mouse at E8.0, the neural folds have begun to close and are apposed, the optic pit is in the early stage of differentiation, and the heart tube has formed (Kaufman 1992). In contrast, when the first cervical somites appear in the stage 22 opossum embryo, none of the aforementioned events have yet occurred

Table 3. Genes examined in this study

Gene	Role in somitogenesis and mouse expression pattern	*M. domestica* expression
Fgf8	Posterior–anterior RNA gradient in PSM, helps define position of determination front (Dubrulle et al. 2001; Sawada et al. 2001; Dubrulle and Pourquie 2004a)	Same as mouse
Wnt3a	RNA expressed in tail bud and posterior PSM, Wnt signaling gradient in posterior PSM parallel to FGF gradient (Takada et al. 1994; Aulehla et al. 2003, 2008)	Same as mouse
Dll1	Member of the Notch pathway, cyclic in mouse PSM, required for maintenance of somite borders (Bettenhausen et al. 1995; Hrabe de Angelis et al. 1997; Maruhashi et al. 2005)	Also cyclic in PSM as in mouse, but expression not restricted to caudal portion of formed somites
Lfng	Cyclic expression, inhibits Notch (Forsberg et al. 1998; McGrew et al. 1998; Aulehla and Johnson 1999; Dale et al. 2003)	Same as mouse
Snai1	Cyclic FGF target (Dale et al. 2006; Niwa et al. 2007)	Same as mouse
Axin2	Cyclic, negative feedback inhibitor of Wnt pathway (Aulehla et al. 2003)	Differs from mouse in that there is no stripe of expression in the caudal half of presomite 0 (S0), but cyclic intensity in PSM same
Mesp2	Defines future segmental domain by suppressing Notch (Saga et al. 1997; Takahashi et al. 2000; Morimoto et al. 2005)	Same as mouse
Myf5	Muscle maturation (Ott et al. 1991; Tajbakhsh and Buckingham 1994; Summerbell et al. 2000)	Same as mouse
Pax3	Muscle maturation, required for limb muscle precursor migration (Bober et al. 1994; Daston et al. 1996)	Same as mouse

PSM, presomitic mesoderm.

(McCrady 1938; Mate et al. 1994) and the embryo is little more than a flat featureless plate. Without a broader phylogenetic treatment, it is impossible to determine the polarity of this change—it is possible that somitogenesis is delayed in mice and other eutherians. Nonetheless, the first somites appear in marsupials when there is little or no differentiation of other axial structures.

Our observations suggest that the pattern observed in marsupials is due to two different heterochronies. First, somitogenesis as a whole is advanced relative to other developmental events. The early initiation of somitogenesis may contribute to the early development of the cervical region and forelimbs. Second, the rate of somitogenesis slows to a greater extent in marsupials than has been previously observed in other amniotes with similar overall rates of development, contributing to the steep anterior–posterior gradient.

In contrast, other elements of somitogenesis appear to be conserved. We see similar expression of genes involved in the clock and wavefront, and genes of the Wnt, Notch, and FGF pathways are also cyclic in opossum. Further, we could not discern differences in the relative rate of somite maturation along the anterior–posterior axis in opossum. Therefore, it is unlikely that changes in the rate of maturation contribute to the steep anterior–posterior gradient.

It is likely that early initiation of somitogenesis anteriorly is critical for timely development of the forelimb musculature and somite-derived supporting structures. This hypothesis is supported by a number of observations. First, limb muscle develops from myocytes that migrate from limb-level somites. In *M. domestica*, forelimb specification, outgrowth, and patterning all occur early relative to other developmental events (Keyte and Smith 2010). Myocyte migration is induced by FGF signaling from the apical ectodermal ridge (AER) (Alvares et al. 2003). Relative to other events in the *M. domestica* embryo, the appearance of *Fgf8* in the AER is early, and we have shown that relative to other developmental events myocytes migrate early (Keyte and Smith 2010). Second, in *M. domestica*, the anterior–posterior developmental gradient can be observed even within the population of migrating forelimb myocytes. Myocytes originating from more anterior somites begin migration from the somites earlier than myocytes from more posterior somites (Fig. 7T). In mouse, there is no discernible gradient in timing of anterior to posterior myocyte migration at forelimb level (Bober et al. 1994). The presence of this gradient in *M. domestica* suggests that myocytes migrate as soon as they are mature enough to do so; there exists a constraint on the timing of myocyte maturation and migration. Third, Weisbecker et al. have demonstrated early ossification of anterior axial skeletal elements, including cervical vertebrae, scapula, and ribs—all somite-derived structures and points of attachment for limb muscles (Huang et al. 2006). For these reasons, early initiation of somitogenesis anteriorly likely provides for timely development of the

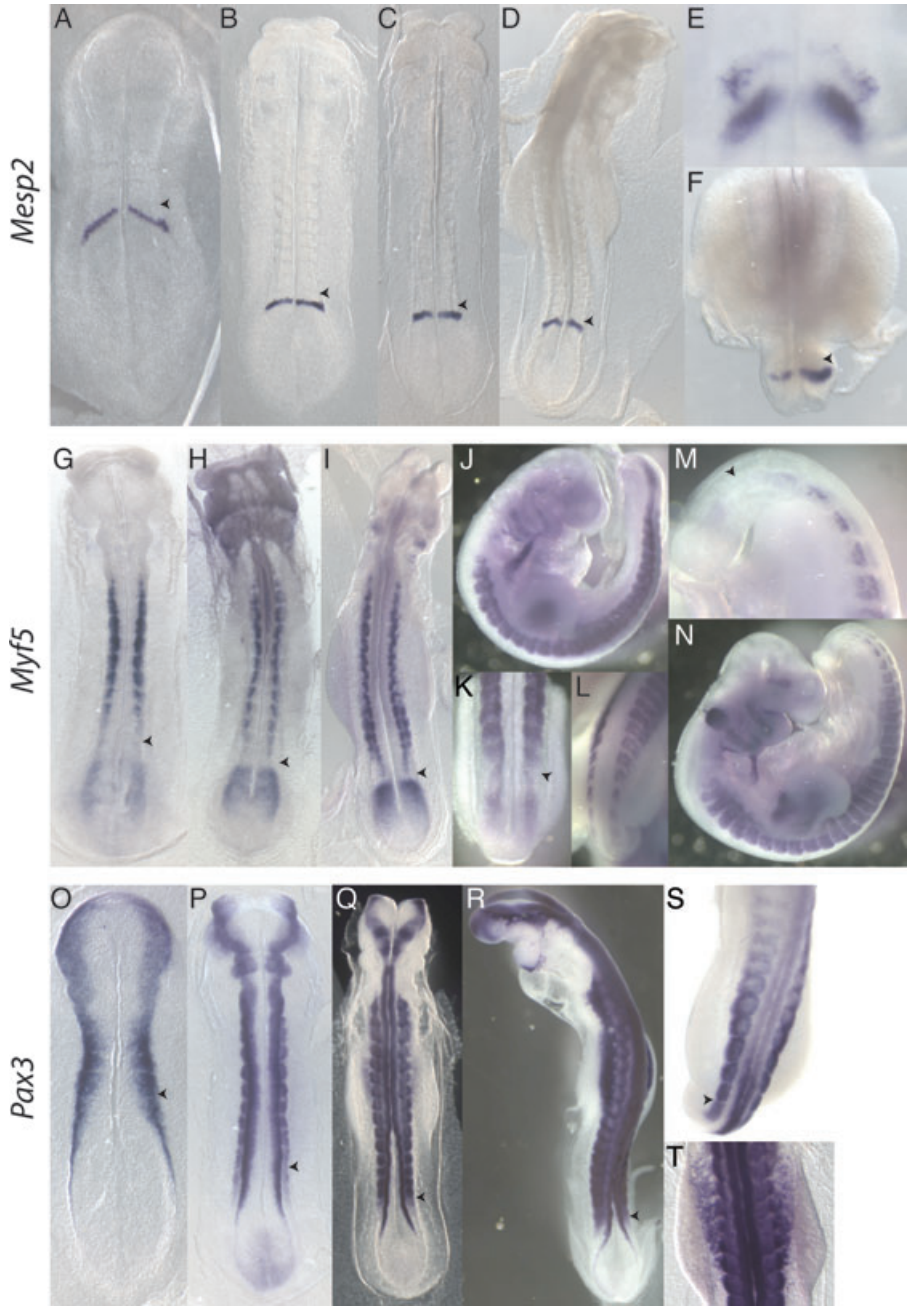

Fig. 7. Expression of genes involved in somite maturation. (A–F) *Mesp2* expression in (A) stage 22–23, (B) stage 24, (C) stage 25, (D and E) stage 27, and (F) stage 29. Arrowheads indicate the caudal-most somite boundary forming. A double stripe of expression is seen in (E). (G–N) *Myf5* expression in (G) stage 24, (H) stage 25, (I) stage 27, (J and K) stage 28, and (L–N) stage 29. (K) is a dorsal, close-up view of the embryo in (J). (L) and (M) are, respectively, dorsal and lateral close-ups of the embryo in (N). (O–T) *Pax3* expression in (O) stage 22–23, (P) stage 24, (Q) stage 26, (R) stage 27, (S) stage 29, and (T) stage 27. Anterior is oriented to the top

forelimb musculature and somite-derived supporting structures.

At posterior levels, structures are generally rudimentary at birth. Given the demands for elaborated anterior structures during the short period from primitive streak to birth (approximately 4.5 days in *M. domestica*), other authors have proposed that an energy trade-off exists to emphasize structures required at birth and de-emphasize nonnecessary posterior structures, such as the hind limbs (Müller 1967; Smith 1997; Weisbecker et al. 2008). Here, we observe a slowing of somitogenesis posteriorly relative to mouse. With somitogenesis occurring more slowly, the maturation of posterior somites will be delayed, possibly freeing resources for other structures in the embryo. Newborns of different marsupial species with differing lengths of gestation tend to display greater or lesser degrees of steepness to their anterior–posterior gradient (Hughes and Hall 1988; Smith 2006; Fig. 1), supporting the energy trade-off hypothesis. For example, *Dasyurus viverrinus* (eastern quoll) is ultra-altricial at birth, undergoing organogenesis in just 2.5 days and weighing only 10–15 mg (Tyndale-Biscoe and Renfree 1987). *Dasyurus viverrinus* and other dasyurid neonates exhibit the

steepest anterior–posterior gradients among marsupial newborns, most obvious in the huge difference in the relative degree of development of the fore- and hind limbs (Hill and Hill 1955). At the other extreme are the macropodid marsupials (kangaroos and wallabies). *Macropus eugenii* (Tammar wallaby) goes from primitive streak to birth in approximately 10 days, neonates weigh roughly 370 mg, and the forelimb to hind limb difference is much less pronounced.

While the slowing of somitogenesis in marsupials contributes to the delay in maturation of posterior neonatal structures, other developmental elements are likely involved that we do not address here. For example, in a previous paper, we demonstrated a delay in hind limb outgrowth in *M. domestica* (Keyte and Smith 2010). Limb outgrowth begins with proliferation of the lateral plate mesoderm, and only later do myocytes emigrate from the somites into the limb bud. There is, apparently, a delay not only within the somitic mesoderm, but also within the lateral plate mesoderm. Generation of the posterior axis at the streak and tailbud may link the delay in maturation of posterior structures of varied mesodermal origin, and remains to be investigated further.

It will be necessary to look upstream of the somite clock for the origin(s) of the steep anterior–posterior gradient. The identity of the somite clock pacesetter is unknown, as well as what causes variation in clock rate from anterior to posterior (Aulehla and Pourquie 2008). New advances in our understanding of somitogenesis in the next few years will hopefully shed light on the underlying cause of clock slowing in marsupials. Formation of the steep anterior–posterior gradient also likely involves regulation of the length of the PSM. As the somite clock slows, movement of the determination front must also slow in order to maintain consistent somite size. (If the clock were to slow with no compensatory change in determination front progression, somite size would increase.) Progression of the determination front depends primarily on the rate of addition of new cells to the end of the embryonic axis (Dubrulle and Pourquie 2004a). As the growth of the axis slows and the length of the PSM decreases, movement of the determination front also slows. It is unknown if PSM length decreases precociously in marsupials or to a greater extent than in eutherians. Given how dramatically the somite clock slows with no obvious increase in caudal somite size, we predict that PSM length would diminish precociously in marsupials.

Here, we have documented two changes in the relative timing of somitogenesis in *M.* domestica relative to the mouse, which we believe contribute to the characteristic configuration of the marsupial neonate. We document a change in the rate of somitogenesis along the anterior–posterior axis of a marsupial. Somitogenesis slows posteriorly to a degree not previously reported. Additionally, we show that somitogenesis initiates early relative to other developmental events, providing somitic derivatives at an earlier stage of development. Other elements of somitogenesis appear to be conserved—gene expression of clock and wavefront components as well as rate of somite maturation along the anterior–posterior axis are consistent with those reported for mouse.

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References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215: 403–410.

Alvares, L. E., et al. 2003. Intrinsic, Hox-dependent cues determine the fate of skeletal muscle precursors. *Dev. Cell.* 5: 379–390.

Aulehla, A., and Herrmann, B. G. 2004. Segmentation in vertebrates: clock and gradient finally joined. *Genes Dev.* 18: 2060–2067.

Aulehla, A., and Johnson, R. L. 1999. Dynamic expression of lunatic fringe suggests a link between notch signaling and an autonomous cellular oscillator driving somite segmentation. *Dev. Biol.* 207: 49–61.

Aulehla, A., and Pourquie, O. 2008. Oscillating signaling pathways during embryonic development. *Curr. Opin. Cell Biol.* 20: 632–637.

Aulehla, A., et al. 2003. Wnt3a plays a major role in the segmentation clock controlling somitogenesis. *Dev. Cell.* 4: 395–406.

Aulehla, A., et al. 2008. A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. *Nat. Cell Biol.* 10: 186–193.

Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J. L., and Gossler, A. 1995. Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to *Drosophila* Delta. *Development* 121: 2407–2418.

Bober, E., Franz, T., Arnold, H. H., Gruss, P., and Tremblay, P. 1994. Pax-3 is required for the development of limb muscles—a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* 120: 603–612.

Burke, A. C., Nelson, C. E., Morgan, B. A., and Tabin, C. 1995. Hox genes and the evolution of vertebrate axial morphology. *Development* 121: 333–346.

Burke, A. C., and Nowicki, J. L. 2001. Hox genes and axial specification in vertebrates. *Am. Zool.* 41: 687–697.

Cohn, M. J., and Tickle, C. 1999. Developmental basis of limblessness and axial patterning in snakes. *Nature* 399: 474–479.

Cooke, J., and Zeeman, E. C. 1976. A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. *J. Theor. Biol.* 58: 455–476.

Dale, J. K., et al. 2006. Oscillations of the snail genes in the presomitic mesoderm coordinate segmental patterning and morphogenesis in vertebrate somitogenesis. *Dev. Cell* 10: 355–366.

Dale, J. K., Maroto, M., Dequeant, M. L., Malapert, P., McGrew, M., and Pourquie, O. 2003. Periodic notch inhibition by lunatic fringe underlies the chick segmentation clock. *Nature* 421: 275–278.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. 1996. Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development* 122: 1017–1027.

Dequeant, M. L., and Pourquie, O. 2008. Segmental patterning of the vertebrate embryonic axis. *Nat. Rev. Genet.* 9: 370–382.

Dubrulle, J., McGrew, M. J., and Pourquie, O. 2001. FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* 106: 219–232.

Dubrulle, J., and Pourquie, O. 2004a. Coupling segmentation to axis formation. *Development* 131: 5783–5793.

Dubrulle, J., and Pourquie, O. 2004b. fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* 427: 419–422.
Forsberg, H., Crozet, F., and Brown, N. A. 1998. Waves of mouse Lunatic fringe expression, in four-hour cycles at two-hour intervals, precede somite boundary formation. *Curr. Biol.* 8: 1027–1030.
Gomez, C., Ozbudak, E. M., Wunderlich, J., Baumann, D., Lewis, J., and Pourquie, O. 2008. Control of segment number in vertebrate embryos. *Nature* 454: 335–339.
Gomez, C., and Pourquie, O. 2009. Developmental control of segment numbers in vertebrates. *J. Exp. Zool. B Mol. Dev. Evol.* 312: 533–544.
Hanken, J., Klymkowsky, M. W., Summers, C. H., Seufert, D. W., and Ingebrigtsen, N. 1992. Cranial ontogeny in the direct-developing frog, *Eleutherodactylus coqui* (Anura: Leptodactylidae), analyzed using whole-mount immunohistochemistry. *J. Morphol.* 211: 95–118.
Hautier, L., Weisbecker, V., Sánchez-Villagra, M. R., Goswami, A., and Asher, R. J. 2010. Skeletal development in sloths and the evolution of mammalian vertebral patterning. *Proc. Natl. Acad. Sci. USA.* 107: 18903–18908.
Hill, J. P., and Hill, W. C. O. 1955. The growth stages of the pouch young of the native cat (*Dasyurus viverrinus*) together with observations on the anatomy of the new born young. *Trans. Zool. Soc. London.* 28: 349–453
Hrabe de Angelis, M., McIntyre, J., 2nd, and Gossler, A. 1997. Maintenance of somite borders in mice requires the Delta homologue DII1. *Nature* 386: 717–721.
Huang, R., Christ, B., and Patel, K. 2006. Regulation of scapula development. *Anat. Embryol. (Berl).* 211(Suppl 1): 65–71.
Hughes, R. L., and Hall, L. S. 1988. Structural adaptations of the newborn marsupial. In H. Tyndale-Biscoe and P. A. Janssens (eds.). *The Developing Marsupial*. Springer-Verlag, New York, pp. 8–27.
Kaufman, M. H. 1992. *The Atlas of Mouse Development*. Academic Press, Inc, San Diego.
Keyte, A. 2010. The development of limbs and somites in the opossum, Monodelphis domestica: A study of heterochrony, heterotopy and the origins of neonatal adaptation. Biology, Duke University, North Carolina, United States 113 pp.
Keyte, A. L., and Smith, K. K. 2009. Opossum (*Monodelphis domestica*): a marsupial developmental model. In D. A. Crotty and A. Gann (eds.). *Emerging Model Organisms: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 557–576.
Keyte, A. L., and Smith, K. K. 2010. Developmental origins of precocial forelimbs in marsupial neonates. *Development* 137: 4283–4294.
Mansouri, A., Voss, A. K., Thomas, T., Yokota, Y., and Gruss, P. 2000. Uncx4.1 is required for the formation of the pedicles and proximal ribs and acts upstream of Pax9. *Development* 127: 2251–2258.
Maruhashi, M., Van De Putte, T., Huylebroeck, D., Kondoh, H., and Higashi, Y. 2005. Involvement of SIP1 in positioning of somite boundaries in the mouse embryo. *Dev. Dyn.* 234: 332–328.
Mate, K. E., Robinson, E. S., Vandeberg, J. L., and Pedersen, R. A. 1994. Timetable of in vivo embryonic development in the grey short-tailed opossum (*Monodelphis domestica*). *Mol. Reprod. Dev.* 39: 365–374.
McCrady, E. 1938. *The Embryology of the Opossum*. Wistar Institute of Anatomy and Biology, Philadelphia.
McGrew, M. J., Dale, J. K., Fraboulet, S., and Pourquie, O. 1998. The lunatic fringe gene is a target of the molecular clock linked to somite segmentation in avian embryos. *Curr. Biol.* 8: 979–982.
Morimoto, M., Takahashi, Y., Endo, M., and Saga, Y. 2005. The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity. *Nature* 435: 354–359.
Muller, F. 1967. Zum Vergleich der Ontogenesen van Didelphis virginiana und Mesocricetus auratus. *Revue Suisse de Zoologie* 74: 607–613.
Müller, J., et al. 2010. Homeotic effects, somitogenesis and the evolution of vertebral numbers in recent and fossil amniotes. *Proc. Natl. Acad. Sci. USA.* 107: 2118–2123.
Niwa, Y., Masamizu, Y., Liu, T., Nakayama, R., Deng, C. X., and Kageyama, R. 2007. The initiation and propagation of Hes7 oscillation are cooperatively regulated by Fgf and Notch signaling in the somite segmentation clock. *Dev. Cell.* 13: 298–304.
Nunn, C. L., and Smith, K. K. 1998. Statistical analyses of developmental sequences: the craniofacial region in marsupial and placental mammals. *Am. Nat.* 152: 82–101.
Ott, M. O., Bober, E., Lyons, G., Arnold, H., and Buckingham, M. 1991. Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development* 111: 1097–1107.
Pearson, M., and Elsdale, T. 1979. Somitogenesis in amphibian embryos. I. Experimental evidence for an interaction between two temporal factors in the specification of somite pattern. *J. Embryol. Exp. Morphol.* 51: 27–50.
Pourquie, O. 2003. The segmentation clock: converting embryonic time into spatial pattern. *Science* 301: 328–330.
Romanoff, A. L. 1960. *The Avian Embryo: Structural and Functional Development*. Macmillan, New York.
Saga, Y., Hata, N., Koseki, H., and Taketo, M. M. 1997. Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. *Genes Dev.* 11: 1827–1839.
Sanchez-Villagra, M. R., Narita, Y., and Kuratani, S. 2007. Thoracolumbar vertebral number: the first skeletal synapomorphy for afrotherian mammals. *Syst. Biodivers.* 5: 1–7.
Sawada, A., Shinya, M., Jiang, Y. J., Kawakami, A., Kuroiwa, A., and Takeda, H. 2001. Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. *Development* 128: 4873–4880.
Schroter, C., Herrgen, L., Cardona, A., Brouhard, G. J., Feldman, B., and Oates, A. C. 2008. Dynamics of zebrafish somitogenesis. *Dev. Dyn.* 237: 545–553.
Smith, K. K. 1994. Development of craniofacial musculature in *Monodelphis domestica* (Marsupialia, Didelphidae). *J. Morphol.* 222: 149–173.
Smith, K. K. 1997. Comparative patterns of craniofacial development in eutherian and metatherian mammals. *Evolution* 51(5): 1663–1678.
Smith, K. K. 2001. Heterochrony revisited: the evolution of developmental sequences. *Biol. J. Linn. Soc.* 73: 169–186.
Smith, K. K. 2003. Time's arrow: heterochrony and the evolution of development. *Int. J. Dev. Biol.* 47: 613–621.
Smith, K. K. 2006. Craniofacial development in marsupial mammals: developmental origins of evolutionary change. *Dev. Dyn.* 235: 1181–1193.
Summerbell, D., Ashby, P. R., Coutelle, O., Cox, D., Yee, S., and Rigby, P. W. 2000. The expression of Myf5 in the developing mouse embryo is controlled by discrete and dispersed enhancers specific for particular populations of skeletal muscle precursors. *Development* 127: 3745–3457.
Tajbakhsh, S., and Buckingham, M. E. 1994. Mouse limb muscle is determined in the absence of the earliest myogenic factor myf-5. *Proc. Natl. Acad. Sci. USA.* 91: 747–751.
Takada, S., Stark, K. L., Shea, M. J., Vassileva, G., McMahon, J. A., and McMahon, A. P. 1994. Wnt-3a regulates somite and tailbud formation in the mouse embryo. *Genes Dev.* 8: 174–189.
Takahashi, Y., et al. 2000. Mesp2 initiates somite segmentation through the Notch signalling pathway. *Nat. Genet.* 25: 390–396.
Tam, P. P. 1981. The control of somitogenesis in mouse embryos. *J. Embryol. Exp. Morphol.* 65(Suppl): 103–128.
Tyndale-Biscoe, H., and Renfree, M. B. 1987. *Reproductive Physiology of Marsupials*. Cambridge University Press, Cambridge.
Weisbecker, V., Goswami, A., Wroe, S., and Sanchez-Villagra, M. R. 2008. Ossification heterochrony in the therian postcranial skeleton and the marsupial-placental dichotomy. *Evolution* 62: 2027–2041.

Supporting Information

Additional Supporting Information may be found online on Wiley Online Library:

Table S1. Somite counts.

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