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Developmental origins of precocial forelimbs in marsupial neonates

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SUMMARY

Marsupial mammals are born in an embryonic state, as compared with their eutherian counterparts, yet certain features are accelerated. The most conspicuous of these features are the precocial forelimbs, which the newborns use to climb unaided from the opening of the birth canal to the teat. The developmental mechanisms that produce this acceleration are unknown. Here we show that heterochronic and heterotopic changes early in limb development contribute to forelimb acceleration. Using *Tbx5* and *Tbx4* as fore- and hindlimb field markers, respectively, we have found that, compared with mouse, both limb fields arise notably early during opossum development. Patterning of the forelimb buds is also accelerated, as *Shh* expression appears early relative to the outgrowth of the bud itself. In addition, the forelimb fields and forelimb myocyte allocation are increased in size and number, respectively, and migration of the spinal nerves into the forelimb bud has been modified. This shift in the extent of the forelimb field is accompanied by shifts in Hox gene expression along the anterior-posterior axis. Furthermore, we found that both fore- and hindlimb fields arise gradually during gastrulation and extension of the embryonic axis, in contrast to the appearance of the limb fields in their entirety in all other known cases. Our results show a surprising evolutionary flexibility in the early limb development program of amniotes and rule out the induction of the limb fields by mature structures such as the somites or mesonephros.

KEY WORDS: Limb field, Heterochrony, Heterotopy, Gastrulation

INTRODUCTION

Marsupial and eutherian mammals have long been known to have different reproductive and life history patterns. Eutherian mammals generally exhibit a long period of maternal nutrition of the embryo via a placenta, whereas marsupial mammals always have a relatively short placental period and a long period of nutrition via lactation. In marsupials, the neonate is always highly altricial compared with any newborn eutherian. The marsupial neonate ranges from 4 to 830 mg; it is blind and has a poorly developed brain. Although embryonic, it is characterized by a complex series of heterochronies in the craniofacial, axial and limb skeleton (Smith, 2006; Tyndale-Biscoe, 2005) (Fig. 1). Astonishingly, the newborn crawls from the opening of the birth canal to the teat without any direct assistance from the mother. Precocial forelimbs have long been considered to be essential for this journey (Gemmell et al., 2002).

We sought to identify changes in the marsupial limb development program that contribute to the forelimb heterochrony using the opossum, *Monodelphis domestica*, as a model marsupial (Keyte and Smith, 2009). We focus on early events, namely limb field specification and the initial outgrowth and patterning of the limb buds.

A variety of developmental mechanisms could contribute to the relative advancement of the forelimb over the hindlimb in the marsupial embryo, including timing shifts in limb field specification or limb field outgrowth. For example, forelimb field specification could occur early, hindlimb field specification could occur late, or there could be a combination of both. Additionally, outgrowth or patterning could initiate early for the forelimb, late for the hindlimb, or any combination thereof. Finally, differences in the amount of material allocated to either limb field could also contribute to the differences observed at birth. A larger limb field, for instance, could allow the forelimb to reach a given developmental stage earlier.

In order to distinguish among the above mechanisms, we analyzed the relative timing of several key molecular and morphological events in limb development in the context of the broader timing of developmental events in *M. domestica* as compared with the mouse, a representative amniote. We examined the timing of limb field specification using T-box 5 (*Tbx5*) and T-box 4 (*Tbx4*) gene expression as markers for the fore- and hindlimb fields, respectively. *Tbx5* is the first gene currently known to be expressed in the prospective forelimb field within the lateral plate mesoderm (Gibson-Brown et al., 1996). Paired-like homeodomain transcription factor 1 (*Pitx1*) is upstream of *Tbx4* in the hindlimb (Logan and Tabin, 1999); however, its domain of expression is much broader than the hindlimb itself (Lanctot et al., 1997), making *Tbx4* a more specific marker for the hindlimb field. Expression and knockdown/knockout studies in diverse vertebrates indicate that these genes play a key conserved role in early limb development (Agarwal et al., 2003; Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Khan et al., 2002; Naiche and Papaioannou, 2003; Takeuchi et al., 1999; Takeuchi et al., 2003; Tamura et al., 1999; Tanaka et al., 2002). We also investigated the expression of two Hox genes that are thought to be involved in limb positioning: homeobox B5 (*Hoxb5*) and homeobox C6 (*Hoxc6*) (Burke et al., 1995; Cohn and Tickle, 1999; Rancourt et al., 1995). The expression of fibroblast growth factor 10 (*Fgf10*) and 8 (*Fgf8*), two genes important in initiating limb outgrowth

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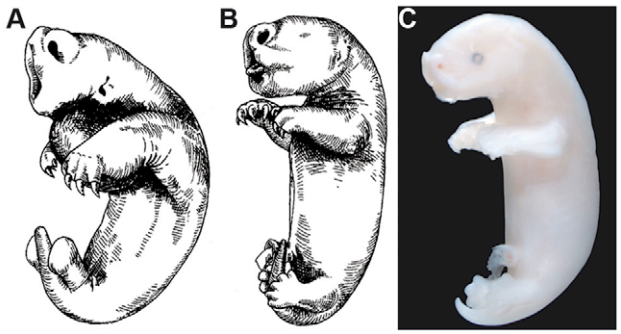

Fig. 1. Marsupial neonates at birth. Illustrations of (**A**) *Dasyurus viverrinus* (see Hill and Hill, 1955) and (**B**) *Trichosurus vulpecula* (see Klima and Bangmann, 1987) and whole-mount image of (**C**) *M. domestica*, reflecting the variation in the degree of altriciality of newborn marsupials. Illustrations are not to scale. *D. viverrinus* is ~6 mm in length, *T. vulpecula* ~15 mm and *M. domestica* ~10 mm.

downstream of *Tbx5* and *Tbx4* (Ohuchi et al., 1997; Takeuchi et al., 2003), was examined. Finally, sonic hedgehog (*Shh*), a patterning gene indicative of the presence of the zone of polarizing activity (ZPA) and the initiation of anterior-posterior patterning in the bud (Echelard et al., 1993), was also examined.

The extreme heterochrony in marsupial mammals provides us with a unique opportunity to examine limb field specification and positioning in a developmental context that is different from that of the more typical model organisms. As we will demonstrate, this comparison provided unique insights into the mechanism of vertebrate limb specification. This study further provides important data on the question of the developmental origins of one of the most noted heterochronies observed in amniote vertebrates.

MATERIALS AND METHODS

Embryos and staging

Opossum embryos were obtained from a breeding colony of *M. domestica* located at Duke University. Protocols for animal husbandry, breeding and embryo collection were followed as described (Keyte and Smith, 2009) and were conducted according to protocols approved by Duke University IACUC. Embryos were staged according to McCrady (McCrady, 1938) with modifications by Mate et al. (Mate et al., 1994) and our laboratory (see http://www.biology.duke.edu/kksmithlab for staging series). CD1 mice were bred to collect embryos.

Isolation of *M. domestica* genes

Total RNA was extracted from freshly dissected stage 24 and 25 embryos. Primers for PCR amplification were designed from *M. domestica* genome sequence or to conserved nucleotide sequences within amniote alignments (Table 1). *M. domestica* gene fragments were isolated by RT-PCR, cloned and sequenced. Gene identity was confirmed by BLAST (Altschul et al., 1997) to the *M. domestica* genome and that of other mammals. Sequences were deposited into GenBank under accession numbers GQ240843 and GU593349-GU593354.

Whole-mount in situ hybridization and histology

Whole-mount in situ hybridization and histology were performed as previously described (Keyte and Smith, 2009). Even though expression patterns for all the genes in this study have been published for mouse, we repeated many of these whole-mount in situ hybridizations using the same protocol as for opossum to ensure that our assessments of the time of first expression were comparable between the two species. In situ hybridizations were also performed on the stage preceding that of first expression to verify the lack of expression in the tissue of interest. These in situ hybridizations were left to develop for several days or weeks at 4°C. Paraffin sections were cut from whole-mount in situs.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Smith, 1994) with the following modifications: embryos were fixed overnight in 4% paraformaldehyde at 4°C, transferred into methanol and stored at –20°C until use. Specimens were bleached in 1:2 30% H_2O_2:Dent's fixative for 15-60 minutes, depending on the size of the embryo.

Identification of axial level

From its formation and throughout embryonic development, the root of the first cervical nerve is significantly smaller than that of the second and all caudal spinal nerves. Just rostral to the first cervical spinal nerve is the 'Y-shaped' hypoglossal nerve; the arc of the cranial accessory nerve is just rostral to the hypoglossal. These landmarks were used to identify the first cervical spinal nerve and the first cervical somite in opossum embryos stained with the 2H3 (neurofilament) antibody (Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences). At stage 28, the third cervical somite lies at or just within the anterior boundary of the forelimb bud. Paired box 3 (*Pax3*) expression at stage 28 shows that there are three somites rostral to somite C3 (O5, C1 and C2), plus the remnant of another occipital somite, O4. By following these somites back through consecutive stages, we were able to identify somites and the axial level in stage 26 and 27 Hox in situ hybridizations, and thus determine the absolute level of anterior expression boundaries (Fig. 2).

In mouse embryos the somitic level was identified according to Sporle and Schughart (Sporle and Schughart, 1997). At E9.5 the forelimb bud is adjacent to somites 7-12 (Chan et al., 2004). The seventh somite is the C4 somite. C1 can also be identified by its characteristic shape in the middle of a somite triad.

Analysis of the sequence of developmental events

We compiled a list of 64 developmental events from the literature (Kaufman, 1992; Mate et al., 1994; McCrady, 1938; Theiler, 1989) and from our own observations (see Table 4). The timing of many of the opossum morphological events was inferred from published accounts of *Didelphis virginiana* (McCrady, 1938), whose developmental sequence matches that of *M. domestica*. Events were placed in an ordered sequence

Table 1. Primers (5′ to 3′) used in this study

Gene	Forward	Reverse
Tbx5	GATGAGGAGTGTTCCACCACTGAC	TGGTACTGATGAGGAGAGAGGGTTCGAGGTA
Tbx4	GACTTGCCCTGCAAACGCTCCTATCTGGAA	CCAGTTCTCCACAGTCCCCATTCCTGA
Fgf8	GAGCAGAGCCTGGTGACRGATCAGC	CYTTGCGSGTRAAAGCCATGTACC
Fgf10	TGGATACTGACACATGGTGCCTCA	CCATTCAATGCCACATACATCTGTCTCC
Pitx1	AGGAACGGAGCGGAGAAACC	CGACATGGCCGAGTTGAGTG
Pax3	TGTCCACTCCTTTAGGGCAGGGTAG	CTCTCTGGATTGGATGGTAGCGTACTTTG
Hoxb5	TGAAGAGTATGAGTTTGGCTACTGC	CAGGCCCCCACCTAGACC
Hoxc6	TTCCACCGCCTATGATCCAG	CCATAATGACCCCCGAGGA
Nkx2.5	AAGGAGCCCAAAGCCGACAAG	CCTGCATTGCTCTGCGGAAT
Shh	CACGGCCCTCATAGTGTAGC	GATCCGCGTACAAGCAGTTT

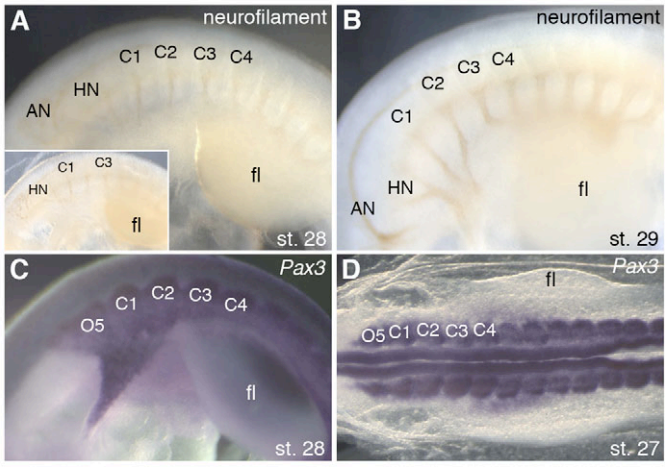

Fig. 2. Identification of the axial level in opossum. (**A**,**B**) An antibody to neurofilament (2H3) highlights nerves, allowing identification of the axial level of specific somites. Inset in A is the same embryo with slight changes in illumination to highlight nerves. (**C**,**D**) *Pax3* expression shows somites. Anterior is to the left. AN, accessory nerve; HN, hypoglossal nerve; fl, forelimb; C1-4, cervical nerves/somites 1-4; O5, occipital somite 5.

independently for opossum and mouse, and a rank was assigned to each event in each species based on the sequence of occurrence of events. The ranks of each event were compared (Smith, 2001). Events in which the opossum rank was lower than the mouse rank occur early in opossum relative to mouse. Our results agree with those published for a similar data set using other analysis approaches (Bininda-Emonds et al., 2003).

In order to measure the degree of heterochronic shift in each event, the data set of 64 events was resampled without replacement 1000 times with a resample size of ten events. Each time a random group of ten events was chosen, the relative timing (early, simultaneous or late) of each limb event was determined. A jackknife score was constructed by calculating the percentage of times an event was determined to be early, simultaneous or late out of the 1000 trials. A subset size of ten events was chosen because it gave the greatest spread of jackknife scores for the limb events, and therefore facilitated comparison between them (A. Keyte, PhD thesis, Duke University, 2010).

RESULTS

Gastrulation and axis extension in opossum

Gastrulation in *M. domestica* has been described by Mate et al. (Mate et al., 1994) and is very similar in topology to that of the flat chick embryo. The primitive streak and Hensen's node first appear in the stage 18 embryo, at the beginning of day 10 of gestation. This embryo is ~2.2 mm in length and is a tear-drop-shaped bilamminar blastodisc sitting on a fluid-filled vesicle. The embryo and the primitive streak elongate during the next 2 days. The notochord first appears in the stage 21 embryo and the first somites in the stage 22 embryo (day 10.25). Gastrulation continues in the posterior regions of the embryo until approximately stage 27, when the primitive streak disappears. After this time, axis extension continues from the tailbud region (see Table 2 for the average age in days of the different *M. domestica* stages).

Forelimb field specification, patterning and outgrowth

In *M. domestica* we observed *Tbx5* expression in the heart, optic vesicle, forelimb bud, and body wall of the thorax, consistent with previous reports from mouse (Gibson-Brown et al., 1996) (Fig. 3A-D,G). *Tbx5* is first expressed in the prospective forelimb field in *M. domestica* at stage 22 (0-2 somites) (Fig. 3A). At this stage, the expression of a heart field marker, NK2 transcription factor related 5 (*Nkx2.5*) (Komuro and Izumo, 1993; Lints et al., 1993), is restricted to an anterior portion of the *Tbx5* domain, allowing us to distinguish the limb and heart fields (Fig. 3A,E). Notably, in half of the embryos (5/10), *Tbx5* expression at stage 22 extended all the way to the posterior end of the axis, directly adjacent to the primitive streak. This has not been reported for any other vertebrate (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Khan et

Table 2. Age/stage relationship in *M. domestica*

McCrady stage	Average age (days)
22	10.25
23	10.3
24	10.4
25	10.5
26	10.75
27	10.9
28	11.3
29	11.7
Birth	14.5

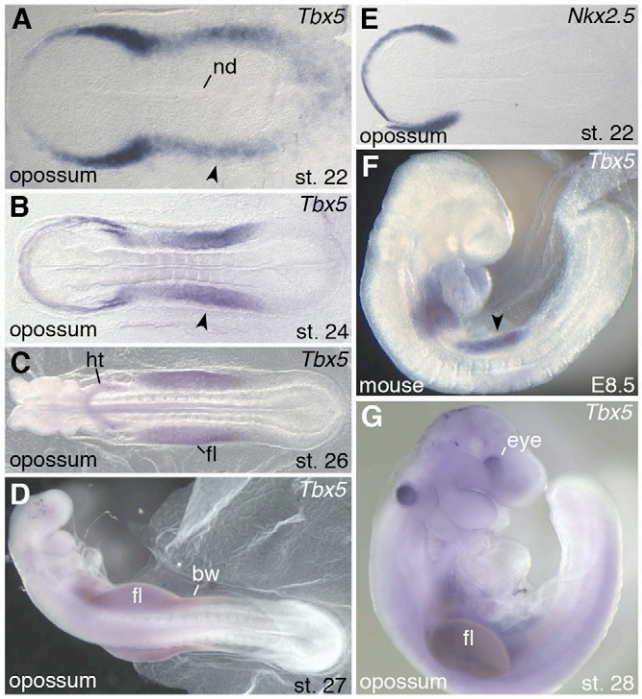

Fig. 3. Expression of *Tbx5* in opossum and mouse. (**A-D**,**G**) *Tbx5* expression in opossum embryos. Views are dorsal (A-C) or dorsolateral (D) with anterior to the left, or lateral with anterior to the top left (G). *Tbx5* is first expressed in the presumptive forelimb field in opossum at the neural plate stage with little differentiation of other structures. (**E**) *Nkx2.5* expression marks the heart field of stage 22 opossum. Dorsal view, anterior to left. As *Tbx5* is expressed in both limb and heart field, subtraction of *Nkx2.5* expression allows identification of presumptive forelimb field. (**F**) *Tbx5* expression in the presumptive forelimb field first occurs in mouse at E8.5. Lateral view, anterior to top left. Limb-associated *Tbx5* is expressed early in the development of the opossum relative to that in mouse (A versus F). Arrowheads point to what is presumed to be forelimb field expression. fl, (morphological) forelimb; nd, node; ht, inflow region of heart; bw, bodywall.

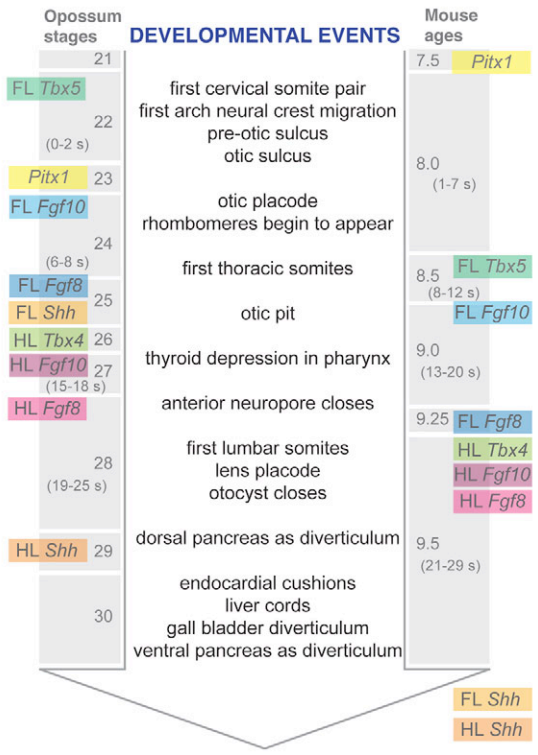

Fig. 4. Comparison of timing of limb events in opossum and mouse relative to other, non-limb developmental events. The center column shows non-limb developmental events that occur in mouse and opossum in the same sequence. The sequence runs from top to bottom. The timing of limb events is mapped onto this sequence for mouse (right) and opossum (left). Note that relative to the non-limb events, the majority of limb events occur earlier in opossum than in mouse. Somite numbers for some stages are given in parentheses. FL, forelimb; HL, hindlimb.

al., 2002; Tamura et al., 1999; Tanaka et al., 2002). This observation indicates that anterior-posterior positional information that is sufficient to place the forelimbs is present in cells as soon as they have exited the streak.

Tbx5 is first expressed in the mouse prospective forelimb field at embryonic day (E) 8.5 (Gibson-Brown et al., 1996) (Fig. 3F), when the embryo has at least eight somites, the neural tube has begun to close, the otic pit is visible, the heart has begun to pump, and the second brachial arch is evident (Kaufman, 1992). At the time of first *Tbx5* expression in the opossum forelimb field, none of these developmental events has been achieved (Fig. 3A versus 3F and Fig. 4). In combination with developmental sequence analysis of *Tbx5* forelimb expression (Fig. 5; Table 4), these observations confirm that the forelimb field of the opossum arises much earlier relative to other developmental events than the mouse forelimb field.

In vertebrates, *Fgf10* and *Fgf8* are downstream of *Tbx5* in the forelimb and are thought to form a positive-feedback loop that drives limb outgrowth (Ohuchi et al., 1997; Takeuchi et al., 2003). In *M. domestica Fgf10* expression was observed in the limb lateral plate mesoderm, intermediate mesoderm, otic placodes, nasal placodes, developing brain (Fig. 6A-F) and lungs (data not shown), in agreement with previous observations of other vertebrates (de Maximy et al., 1999; Ohuchi et al., 1997; Yamasaki et al., 1996). It is first expressed in the prospective opossum forelimb at stage 24 and in mouse at E9.0 (Xu et al., 1998) (Fig. 6A versus 6G). Opossum *Fgf8* expression was seen in the forebrain, midbrain-hindbrain boundary (isthmus), pharyngeal arches, somites, caudal presomitic mesoderm, streak, tail bud, apical ectodermal ridge (AER) of each limb bud (Fig. 6J-P) and in the nasal placodes (data not shown), consistent with previous reports (Mahmood et al., 1995; Vogel et al., 1996). It is first expressed in the opossum forelimb at stage 25 and in mouse at E9.25 (17 somites) (Fig. 6K versus 6Q) (Mahmood et al., 1995; Xu et al., 1998). Analysis of developmental sequences (Fig. 5) indicates that both *Fgf10* and *Fgf8* are expressed early in opossum relative to mouse forelimb. Developmental sequence analysis also shows that the forelimb bud begins outgrowth earlier in opossum, in agreement with previous reports for a variety of marsupial species (Bininda-Emonds et al., 2003; McCrady, 1938; Sears, 2009).

Shh marks the ZPA, a signaling center located in the posterior limb bud that is responsible for patterning the anterior-posterior axis of the developing bud (Echelard et al., 1993). We observed *Shh* expression in the notochord, the floor plate of the neural tube and the posterior limb buds, as described for mouse (Fig. 7) (Echelard et al., 1993). In opossum, *Shh* is first expressed in the posterior forelimb field late in stage 25, before there is much overt outgrowth of the bud (Fig. 7A). In the mouse forelimb, *Shh* appears at E9.75, only after the forelimb bud is relatively well defined (Charite et al., 2000; Echelard et al., 1993). Opossum forelimb *Shh* expression at stage 27 (Fig. 7B) resembles the posterior band of expression in the E10.5 mouse forelimb; however, the opossum forelimb has not yet elongated distally to the extent of the mouse forelimb. *Shh* expression in the opossum forelimb weakens and then disappears in stages 29 and 30, when the forelimb has formed a paddle, much like the cessation of expression seen in the mouse. Thus, compared with mouse, *Shh* expression in the opossum forelimb occurs early relative to the outgrowth of the bud and to other developmental events in the embryo (Fig. 5). Taken together with the *Tbx5* and *Fgf10/8* expression described above, these results demonstrate that an early heterochronic shift in forelimb specification, outgrowth and patterning has accelerated forelimb development in marsupials.

Table 3. Forelimb field size in various amniotes

Species	Somites medial to forelimb bud	Reference	Embryo stage
Gallus gallus (chick)	6	Bellairs and Osmund, 1998	HH stage 18
Monodelphis domestica (opossum)	8	This study	McCrady stage 27
Homo sapiens (human)	5-6	Blechschmidt, 1961	4.2 mm
Sus scrofa (pig)	5-6	Patten, 1948	5 mm
Mus musculus (mouse)	6	Kaufman, 1992; Theiler, 1989	E9.5

The opossum forelimb field has expanded its axial position relative to mouse and other amniotes. Note that although the opossum somites medial to the forelimb level may be smaller, and one might argue that this is therefore not a good measure of relative size, the edges of the forelimb have expanded along the embryonic axis, reflecting an underlying genetic change; it is this change in which we are interested.

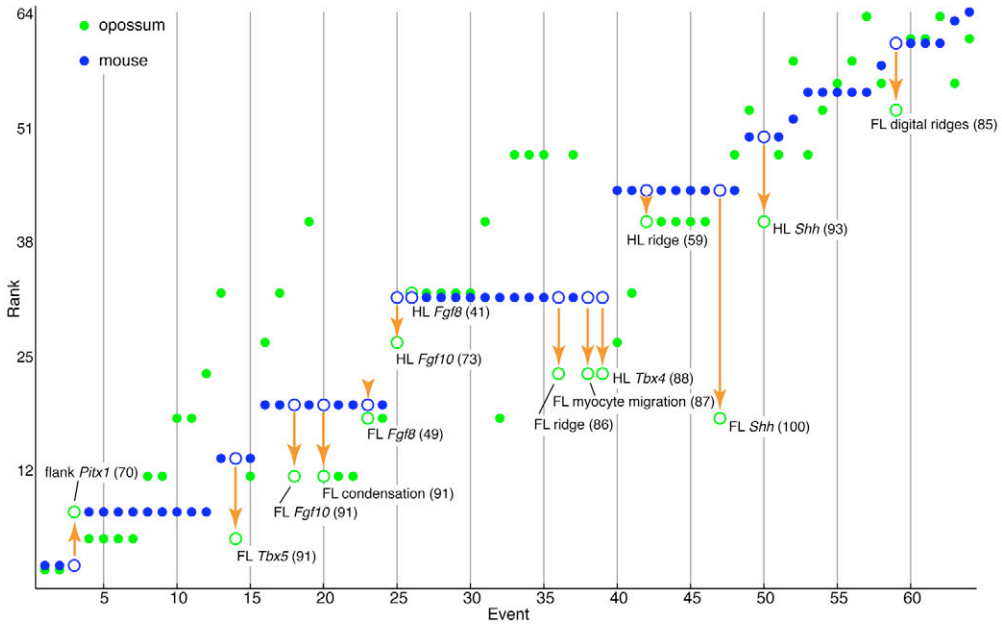

Fig. 5. Developmental sequence analysis of opossum and mouse events indicates that most limb events occur early in opossum relative to mouse development. Events are arranged on the *y*-axis by the rank or order of occurrence of events in mouse. If an event has a higher rank in the opossum then the event occurs later in development in opossum relative to mouse. A lower rank indicates that it occurs early in opossum. 'Flank *Pitx1*' shows a late heterochronic shift in opossum relative to mouse. 'HL *Fgf8*' shows no evidence of a heterochronic shift. All other limb events shown are early in opossum. Mouse events are blue, opossum events green. Limb events are indicated by open circles, non-limb events by solid circles. Orange arrows indicate the direction of heterochronic shift in opossum (early or late). Jackknife scores (see Materials and methods) are indicated in parentheses. Indices of events on the *x*-axis correspond to column 1 of Table 4. FL, forelimb; HL, hindlimb.

Hindlimb field specification and outgrowth

Tbx4 was expressed in the allantois, hindlimb buds, a restricted area of the forelimb bud, sinus venosus, and the body wall of the thorax in *M. domestica*, as previously described for mouse (Fig. 8A-F) (Gibson-Brown et al., 1996). At stage 26 (13-15 somites), *Tbx4* was expressed in a dot at the posterior end of the primitive streak (Fig. 8C). Subsequently, expression expanded into a crescent and then split into two separate limb fields at stage 28 (19-25 somites). Transverse sections showed *Tbx4*-positive cells exiting the streak (Fig. 8J,K). This pattern of hindlimb field appearance has not been reported in any other vertebrate (Gibson-Brown et al., 1996; Gibson-Brown et al., 1998; Khan et al., 2002; Tamura et al., 1999; Tanaka et al., 2002); in all known instances, *Tbx4* expression appears as two separate limb fields that are positioned some distance from the end of the embryonic axis. As with the *Tbx5* expression data described above, these results suggest that significant anterior-posterior positional information is present at the most posterior and immature end of the embryonic axis.

Tbx4 is first expressed in the mouse prospective hindlimb field at E9.5 (Gibson-Brown et al., 1996; Naiche and Papaioannou, 2003) (Fig. 8G). At that time, the embryo has 21-29 somites, the rostral neuropore and otocyst have closed and the forelimb has reached the bud stage (Kaufman, 1992). By contrast, a stage 26 opossum has only 13-15 somites and the aforementioned events have not yet occurred (Fig. 8C versus 8G). These results indicate that the hindlimb fields arise earlier in opossum than in mouse.

In opossum, *Fgf10* is restricted to the prospective hindlimb late in stage 27 and in mouse at E9.5 [a stage earlier than previously reported (Xu et al., 1998)] (Fig. 6D,E versus 6H,I). *Fgf8* appears in the opossum hindlimb at stage 28 and in mouse at E9.5 (Fig. 6O,P versus 6Q,R). Analysis of developmental sequences indicated that hindlimb *Fgf10* expression, as well as hindlimb outgrowth, are slightly early in opossum relative to mouse, whereas hindlimb *Fgf8* expression does not show evidence of a heterochronic shift (Fig. 5). Hindlimb *Shh* expression is evident in opossum at stage 29 (Fig. 7D,E) and in mouse at E10.5 (Echelard et al., 1993); this is a considerable early shift in opossum relative to mouse.

In opossum, *Pitx1* is expressed in the lateral plate mesoderm, hindlimb, posterior extraembryonic mesoderm and first pharyngeal arch, in agreement with previously published results (Fig. 9A-F) (Lanctot et al., 1997; Logan et al., 1998; Szeto et al., 1996). It is upstream of hindlimb Tbx4 (Logan and Tabin, 1999), but its broad lateral plate mesoderm expression does not make it a good hindlimb marker; the domain of expression in the lateral plate mesoderm extends from the umbilical stalk up to, but not including, the forelimb buds (Lanctot et al., 1997). Even though the pattern of *Pitx1* expression is uninformative for identifying the hindlimb fields, its timing does provide an indication of where the limb heterochrony arises in the limb gene network. Lateral plate mesoderm expression of *Pitx1* is first seen in opossum at stage 23 (Fig. 9B) and in mouse at E7.5 (Lanctot et al., 1997), which is late in opossum relative to mouse (Fig. 5) but still before *Tbx4* expression in the presumptive hindlimb field.

Forelimb heterotopy

We also examined the relative size of the forelimb field by documenting the number of medial somites and their axial level. An increase in the size of the specified field would potentially provide a greater amount of cellular material to the developing bud. At the time at which the bud can first be clearly delineated (stage 27), the opossum forelimb is lateral to about eight somites, compared with six in an E9.5 mouse (Kaufman, 1992; Theiler, 1989), and has thus expanded its axial position relative to mouse (Fig. 10A versus 10B) and a variety of other amniotes (Table 3).

Table 4. Developmental event data set used in the sequence analysis

Event		Stage		Jackknife score		
Index	Name	*M. domestica*	*M. musculus*	Early	Same	Late
1	Neural groove begins to form	21	11	15.2	84.8	0.0
2	Notochord begins to form	21	11	14.8	85.2	0.0
3	Flank Pitx1 expression (not allantois)	23	11	0.0	30.1	69.9
4	First arch crest begins migration	22	12	59.8	35.2	5.0
5	First cervical somite pair	22	12	63.9	31.7	4.4
6	Otic sulcus	22	12	64.9	30.2	4.9
7	Pre-otic sulcus	22	12	57.2	37.1	5.7
8	Otic placode appears	24	12	10.3	19.2	70.5
9	Rhombomeres start to appear	24	12	10.0	20.8	69.2
10	First aortic arch formed	25	12	1.4	4.7	93.9
11	Neural folds first beginning to fuse	25	12	0.6	6.4	93.0
12	Endocardial tubes start to fuse	26	12	0.0	0.7	99.3
13	Liver diverticulum appears	28	13	0.0	0.3	99.7
14	First presumptive FL field Tbx5 expression	22	13	90.7	9.3	0.0
15	First thoracic somite pair	24	13	49.4	25.4	25.2
16	Thyroid (or endostyle) depression appears in floor of pharynx	27	14	6.9	13.2	79.9
17	Anterior neuropore closed	28	14	0.6	3.3	96.1
18	FL Fgf10 expression appears	24	14	90.5	9.5	0.0
19	Oropharygeal membrane becomes perforated	29	14	0.0	0.4	99.6
20	FL condensations	24	14	90.5	9.5	0.0
21	Wolffian duct appears as a thickening with no lumen	24	14	91.2	8.8	0.0
22	Trigeminal ganglion (of cranial nerve V) becomes distinct as cellular aggregate	24	14	89.3	10.7	0.0
23	FL Fgf8 expression appears	25	14	48.4	23.6	28.0
24	Otic placode depressed (formation of otic pit)	25	14	46.3	25.3	28.4
25	HL Fgf10 expression appears	27	15	72.8	13.0	14.2
26	HL Fgf8 expression appears	28	15	36.8	22.2	41.0
27	First lumbar somite pair	28	15	35.4	21.9	42.7
28	Lens placode appears	28	15	36.9	19.5	43.6
29	Otocyst closed but still connected with surface ectoderm	28	15	36.4	21.8	41.8
30	Otocyst detached from ectoderm	28	15	34.0	23.9	42.1
31	Dorsal pancreas beginning as a diverticulum	29	15	4.2	7.9	87.9
32	Laryngotracheal (part of the median pharyngeal) groove indicated	25	15	98.1	1.6	0.3
33	Endocardial cushions of atrioventricular canal just beginning	30	15	0.0	0.5	99.5
34	Gall bladder beginning as a diverticulum	30	15	0.0	1.0	99.0
35	Liver cords forming	30	15	0.0	0.9	99.1
36	FL: stage 1* (limb bud first visible)	26	15	86.2	8.9	4.9
37	Ventral pancreas beginning as a diverticulum	30	15	0.0	0.7	99.3
38	First myocyte migration from somite into FL	26	15	87.0	8.3	4.7
39	First presumptive HL field Tbx4 expression	26	15	87.7	7.0	5.3
40	Lung buds as distinct evaginations	27	16	98.6	1.3	0.1
41	Nasal placodes depressed (formation of olfactory pit)	28	16	95.7	3.2	1.1
42	HL: stage 1* (limb bud first visible)	29	16	59.1	21.8	19.1
43	Endolymphatic appendage appears	29	16	58.1	22.8	19.1
44	Externally visible tailbud beginning	29	16	57.0	23.0	20.0
45	First sacral somite pair	29	16	60.2	21.8	18.0
46	Lens placode depressed (formation of optic pit)	29	16	57.8	24.7	17.5
47	FL Shh in ZPA	25	16	99.9	0.1	0.0
48	Posterior neuropore closed	30	16	9.8	19.4	70.8
49	Ureteric bud just forming from Wolffian duct	31	17	0.0	40.9	59.1
50	HL Shh in ZPA	29	17	92.9	7.1	0.0
51	Septum primum of atrium just beginning	30	17	54.5	33.1	12.4
52	Spleen anlage beginning as mesenchymal proliferation	33	18	0.0	25.5	74.5
53	Lens vesicle pinches off from surface ectoderm	30	19	89.7	10.3	0.0
54	Auricular tubercles (hillocks) become distinct	31	19	48.9	36.4	14.7
55	Vertebral bodies are mesenchymal condensations around notochord	32	19	23.7	34.1	42.2
56	Jacobson's organ beginning as a diverticulum	33	19	11.4	29.8	58.8
57	Retinal pigmentation beginning	35	19	0.0	12.4	87.6
58	Septum primum broken through (foramen ovale/secundum)	32	20	45.6	36.5	17.9
59	FL: stage 8* (initial indentations between digits)	31	21	84.9	15.1	0.0
60	Cloacal partition just completed	34	21	20.8	48.1	31.1
61	Semilunar (aortic and pulmonary) valves first appearing	34	21	21.0	46.6	32.4
62	Anlagen of metanephric tubules	35	21	7.2	36.5	56.3
63	Eyelid anlage appears	32	22	76.0	24.0	0.0
64	Umbilical hernia totally reduced	34	25	50.6	49.4	0.0

M. domestica events are listed by their McCrady stages; mouse events are listed by Theiler stages.
FL, forelimb; HL, hindlimb; ZPA, zone of polarizing activity.
*Wanek et al. (Wanek et al., 1989).

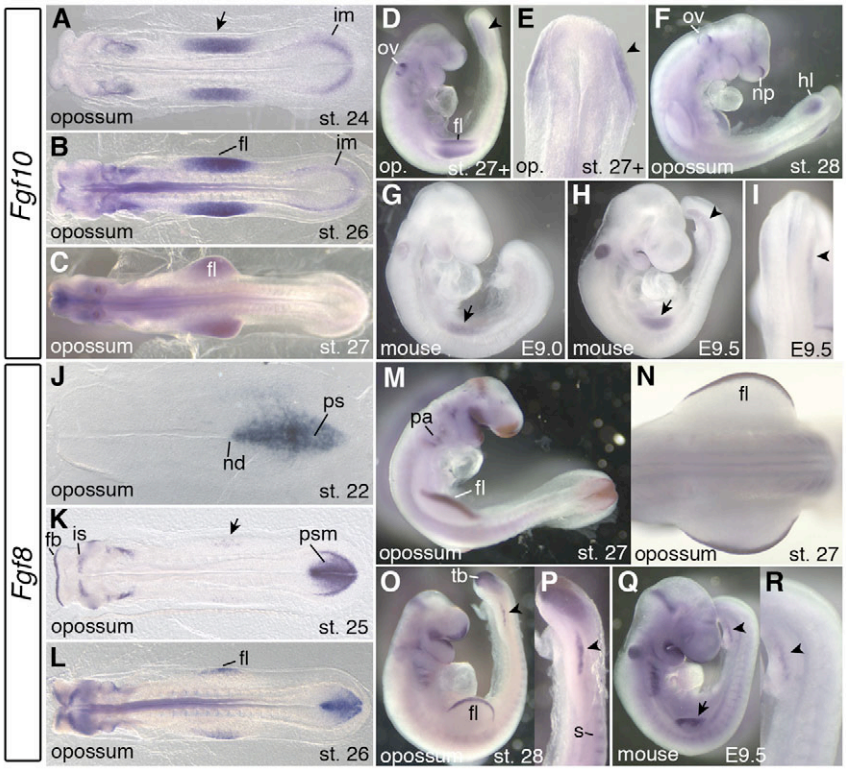

Fig. 6. Expression of *Fgf10* and *Fgf8* in opossum and mouse. (**A-I**) *Fgf10* expression in opossum (A-F) and mouse (G-I). (A-C) Dorsal views, anterior to left. (E) Dorsal view of posterior end of embryo in D. (I) Dorsal view of posterior end of embryo in H. *Fgf10* expression in the presumptive forelimb first occurs at stage 24 in opossum and is early relative to mouse expression at E9.0 (A versus G). Expression in the presumptive hindlimb first occurs at stage 27+ in opossum and is slightly early relative to mouse expression at E9.5 (D,E versus H,I). (**J-R**) *Fgf8* expression in opossum (J-P) and mouse (Q,R). (J-L) Dorsal views, anterior to left. (N) Dorsal view, anterior to left. (R) Magnified view of embryo in Q. *Fgf8* forelimb expression first occurs at stage 25 in opossum and is early relative to mouse forelimb expression at E9.5 (K versus Q). Hindlimb expression first occurs at stage 28 in opossum (O,P) and at E9.5 in mouse (Q,R), with no evidence of a heterochronic shift. Arrows indicate presumptive forelimb expression and arrowheads indicate presumptive hindlimb expression. fl and hl refer to (morphological) forelimb and hindlimb, respectively. im, intermediate mesoderm; ov, otic vesicle; np, nasal placode; n, node; ps, primitive streak; psm, presomitic mesoderm; fb, forebrain; is, isthmus; pa, pharyngeal arch; tb, tail bud; s, somite.

Because the opossum forelimb field appears to occupy an expanded position along the axis, we examined the expression of Hox genes thought to be involved in limb positioning. Previous studies have shown that *Hoxb5* mouse mutants show a slight positional shift in the shoulder girdle (Rancourt et al., 1995) and *Hoxc6* expression correlates with the cervical-thoracic transition in a wide variety of vertebrates with differing cervical counts (Burke et al., 1995). In *M. domestica*, *Hoxc6* and *Hoxb5* show a rostral shift in their anterior expression boundaries in the lateral plate mesoderm that coincides with the rostral expansion of the forelimb bud (Fig. 10C-H). Furthermore, *Hoxc6* is shifted posteriorly within the paraxial mesoderm from C7/T1 in mouse to T2 in opossum. This correlates with the posterior expansion of the limb bud and, notably in *M. domestica*, the boundary of *Hoxc6* expression in the paraxial mesoderm is no longer at the cervical-thoracic transition. It is unclear whether or not there has also been a change in *Hoxb5* paraxial mesoderm expression, as somitic expression in mouse is very weak and a range of anterior expression boundaries (from C1 to C3) have been reported (Rancourt et al., 1995; Sharpe et al., 1988; Wall et al., 1992). In *M. domestica*, the anterior expression boundary of *Hoxb5* is at somites C2/C3.

Myocyte migration into the heterotopic forelimb bud

Previous studies have shown that only limb lateral plate mesoderm is able to induce muscle cell precursor (myocyte) migration from the somites (Hayashi and Ozawa, 1995). As the opossum forelimb field is expanded, we predicted that the number of somites contributing myocytes is also increased. We used *Pax3* as a myocyte marker and counted the total number of somites from which *Pax3*-positive cells appeared to emigrate into the forelimb (Fig. 11A-C). Determining the exact contribution of somites would require in vivo labeling and tracing; however, a comparison of *Pax3* expression in mouse and opossum does allow an assessment of which regions appear to contribute myocytes. In *M. domestica*, myocytes appeared to emigrate from somite levels C3 through T3, a total of eight somites, compared with only 5-6 somites reported for mouse (Bober et al., 1994). Thus, forelimb myocyte allocation appears to have increased with increased forelimb field size. Additionally, myocytes are first seen in the adjacent forelimb lateral plate mesoderm at stage 26 (~14 somites) in opossum and at E9.5 (21-29 somites) in mouse (Bober et al., 1994), suggesting that migration of myocytes to the limb bud is early in opossum relative to mouse (Fig. 5).

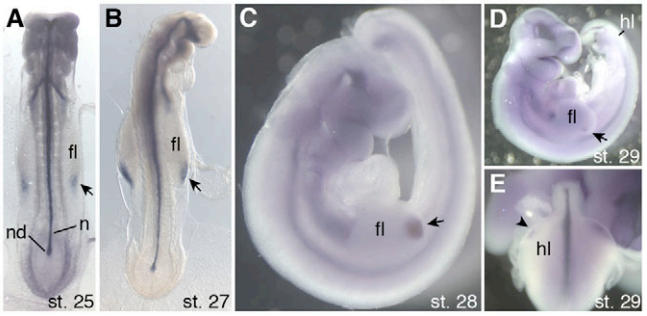

Fig. 7. Expression of *Shh* in opossum. (**A**) First expression of *Shh* in opossum forelimb at stage 25 is early relative to outgrowth of the limb bud, as well as to expression reported for mouse at E9.75 (Echelard et al., 1993). Dorsal view, anterior to top. (**B**) Dorsolateral view of a stage 27 embryo, anterior to top. (**C**,**D**) Lateral views of stage 28 and 29 embryos, anterior to top left. (**E**) First expression of *Shh* in opossum forelimb at stage 29 is early relative to that reported for mouse at E10.5 (Echelard et al., 1993). Dorsal view, anterior to bottom. Arrows indicate forelimb expression and arrowheads indicate hindlimb expression. n, notochord; nd, node; fl, forelimb; hl, hindlimb.

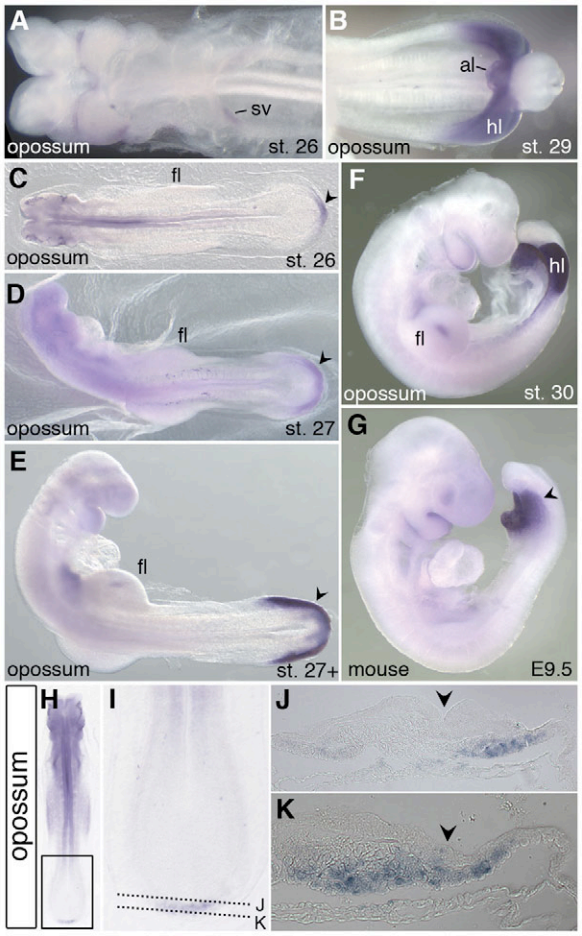

Fig. 8. Expression of *Tbx4* in opossum and mouse embryos. (**A**,**B**) Ventral views of *Tbx4* expression in opossum. Anterior is to the left. (**C**) Hindlimb-associated expression of *Tbx4* first occurs in stage 26 opossum in a single posterior domain. Dorsal view, anterior to the left. (**D-F**) Dorsolateral (D,E) and lateral (F) views of opossum *Tbx4* expression. Anterior is to the top left. (**G**) In mouse, first expression of *Tbx4* in the hindlimb field occurs at E9.5 in two separate domains. Lateral view, anterior to top left. The expression of *Tbx4* in the hindlimb is early in opossum relative to mouse. Arrowheads (C-G) point to what is presumed to be hindlimb field expression (hl refers to morphological hindlimb). (**H**) Representative stage 26 *Tbx4* opossum embryo used for sectioning through the streak. (**I**) Magnified view of boxed region in H. Dotted lines indicate the section planes of J,K. Anterior is to the top in H,I. (**J**,**K**) Paraffin transverse sections through the primitive streak (arrowheads). *Tbx4* staining is evident in cells exiting the streak (K). fl, forelimb; hl, hindlimb; sv, sinus venosus; al, allantois.

Spinal nerve migration into the heterotopic forelimb bud

In experiments in the chick, ectopic growths of the limb buds along the anterior-posterior axis are innervated by spinal nerves from flank/non-limb levels (Turney et al., 2003). The expansion of the opossum forelimb is analogous to these ectopic regions, and we examined whether spinal nerve outgrowth is also expanded. Initially, spinal nerves growing into the forelimb bud include cervical nerves 3-8 (C3-C8) and thoracic nerves 1-2 (T1-T2) (Fig. 11D). The contribution from the third cervical nerve is reduced through stages 30 and 31 (Fig. 11E,F); in an adult opossum (and in mammals in general) there is no connection from C3 to the brachial

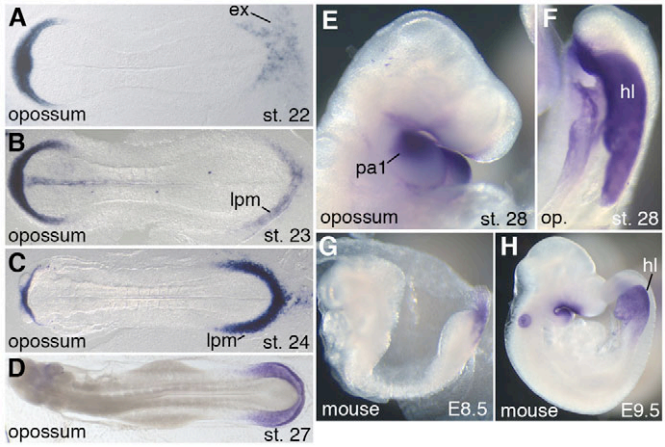

Fig. 9. Expression of *Pitx1* in opossum and mouse. (**A-H**) First flank *Pitx1* expression occurs in opossum at stage 23 (B) and is late relative to first flank *Pitx1* expression in mouse at E7.5 (Lanctot et al., 1997). Anterior is to the left (A-D), to the top (E,G,H) or to the bottom (F). ex, extraembryonic expression; lpm, lateral plate mesoderm; pa1, pharyngeal arch 1; hl, hindlimb.

plexus (Harris, 1939) (verified for *M. domestica* by our laboratory, data not shown). In mouse, C3 never grows towards or into the brachial plexus during embryonic development (Nakao and Ishizawa, 1994). Thus, although the anterior expansion of the forelimb bud seems to have impacted early spinal nerve growth into the limb, this is corrected as development proceeds so that in the adult a typical mammalian pattern is observed. Spinal nerve T3 never grows towards the plexus, even though the limb bud has expanded posteriorly as well as anteriorly. However, owing to the steep anterior-posterior developmental gradient within the embryo as a whole, the T3 spinal nerve does not begin outgrowth until after the limb has reached the paddle stage, and by this time T3 is no longer medial to the limb.

DISCUSSION

Development in the marsupial forelimb is accelerated through a combination of early field specification, early initiation of outgrowth and patterning and greater tissue allocation. Concomitant with forelimb field expansion and early specification, the number of somites contributing myocytes appears to be greater than in other vertebrates and the migration of myocytes into the forelimb bud occurs earlier. The expression of Hox genes, which are hypothesized to be involved in limb positioning, has also shifted along the anterior-posterior axis in a way that correlates with increased limb field size. Therefore, the acceleration of forelimb development in *M. domestica* is produced by multiple changes in early limb development.

Although we see changes in multiple aspects of limb development in marsupials relative to eutherians, because the limb development program is so tightly integrated this cascade of events may arise from two simple shifts in development: first, an early initiation of the forelimb field, or heterochrony; and second, a shift in the area of the early limb field, or heterotopy. Once the forelimb field is specified early and *Tbx5* expression is initiated early, *Fgf10* and then *Fgf8* expression would also occur early, as *Fgf10* is a direct target of *Tbx5* (Agarwal et al., 2003) and *Fgf8* is a target of *Fgf10* (Xu et al., 1998). Because limb lateral plate mesoderm induces myocyte migration from the somites (Hayashi and Ozawa,

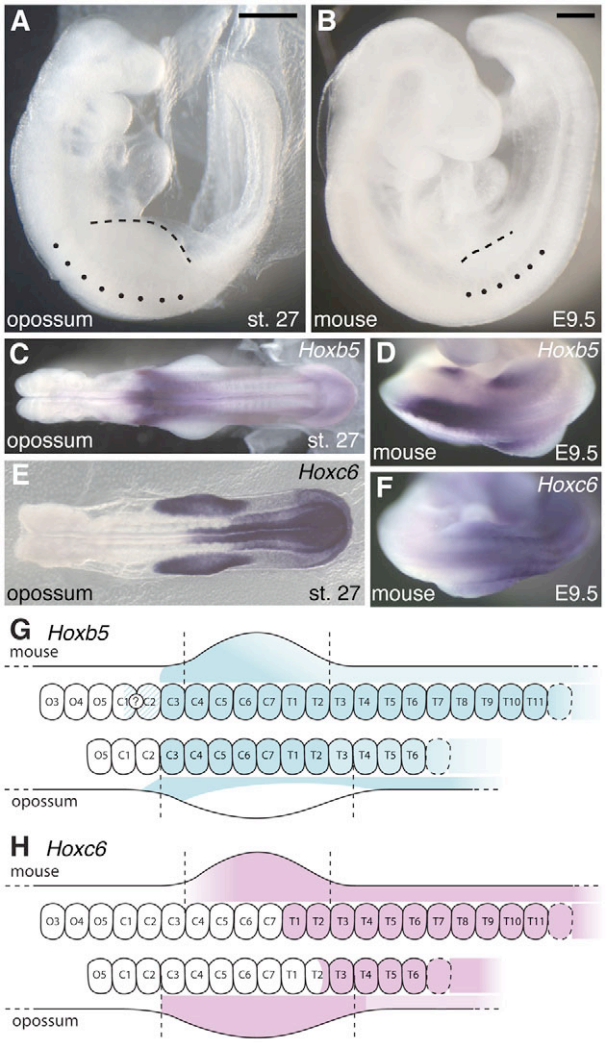

Fig. 10. Hox expression in opossum and mouse correlates with the forelimb heterotopy. (**A**,**B**) Eight somites are medial to the opossum forelimb (A) and six in mouse (B). The dashed line outlines the edge of the forelimb bud and dots mark somites adjacent to the bud. Lateral views, with anterior to the top left. (**C-F**) *Hoxb5* (C,D) and *Hoxc6* (E,F) expression in stage 27 opossum (C,E) and E9.5 mouse (D,F). Dorsal views, anterior is to the left. (**G**,**H**) Schematic contrasting opossum and mouse *Hoxb5* (G) and *Hoxc6* (H) expression in the somites and lateral plate mesoderm. Note the anterior shift in *Hoxb5* and *Hoxc6* expression in the lateral plate mesoderm of opossum relative to mouse, as well as the posterior shift in *Hoxc6* expression in the somites of opossum relative to mouse. Vertical dashed lines indicate the anterior (left) and posterior (right) extent of the limb buds. Scale bars: 0.5 mm.

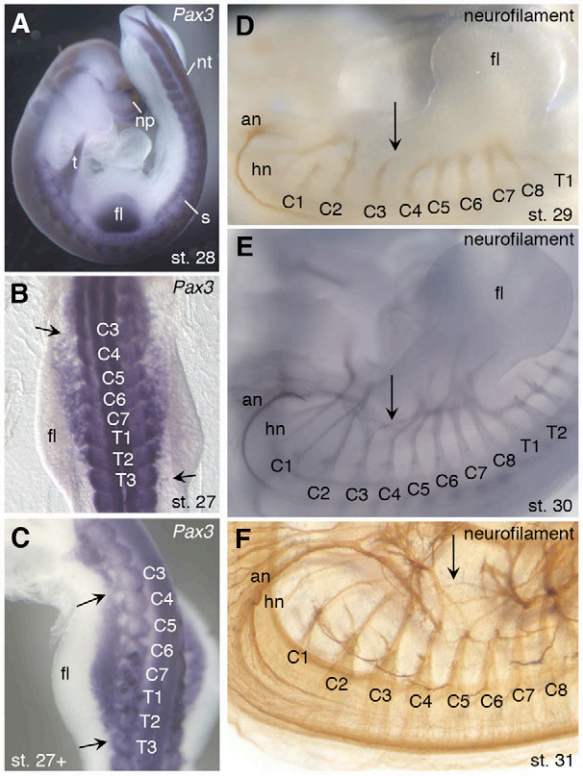

Fig. 11. Compared with mouse, myocyte and spinal nerve migration into the opossum forelimb bud appear to be modified. (**A-C**) In situ hybridization for *Pax3*. In opossum, *Pax3* is expressed in the dorsal neural groove and neural tube, somites, nasal pit, and tongue and limb muscle precursors, consistent with observations in mouse (Bober et al., 1994; Goulding et al., 1991). Arrows (B,C) point to the most anterior and posterior myocytes that appear to be migrating towards the forelimb bud. Eight somites just medial to these myocytes are labeled (B,C). Five to six somites are reported for mouse (Bober et al., 1994). Anterior is to the top; views are lateral (A), dorsal (B) or dorsolateral (C). (**D-F**) An antibody to neurofilament highlights nerves. Anterior is to the left; lateral views. The C3 spinal nerve near the anterior border of the forelimb can be seen growing towards the forelimb in stage 29 (D, arrow). At stage 30, a branch from the C3 spinal nerve grows into the brachial plexus (E, arrow). By stage 31, the branch from C3 into the brachial plexus is greatly diminished (F, arrow). In mouse, C3 never grows towards the brachial plexus (Nakao and Ishizawa, 1994). nt, neural tube; np, nasal pit; t, tongue muscle precursors; fl, forelimb bud; s, somite; an, accessory nerve; hn, hypoglossal nerve.

1995) and the migration of brachial plexus spinal nerves (Turney et al., 2003) to the limb area, shifts in the timing and extent of these latter events are likely to be a consequence of the early and expanded forelimb field. The forelimb in marsupials is thus a good example of an integrated evolutionary and developmental module. One heterochronic and one heterotopic change in early limb development may lead to an entire series of shifts in gene expression and cellular behavior.

The considerable early shift in both fore- and hindlimb *Shh* expression indicates an acceleration of patterning within the limbs in addition to the acceleration of specification. The appearance of *Shh* in the limb buds in *M. domestica* shows a greater heterochronic shift than any of the other limb development events examined here, and *Shh* expression is early not only in relation to non-limb events in the embryo, but also to events within the limb buds themselves.

Contrary to most predictions, the hindlimb field is also specified and patterned early in marsupials, despite the fact that the hindlimb is small and not functional at birth. It is possible that a common developmental mechanism in anterior-posterior patterning couples expression of the T-box genes: once *Tbx5* is induced early, early expression of *Tbx4* follows. Additionally, *Shh* and *Fgf10* are

expressed early in the hindlimb in *M. domestica*, as would be expected given the model of a tightly integrated developmental module. However, unlike the forelimb, there is no expansion in the relative size of the field. When *Tbx4* is first expressed, little mesoderm has accumulated in the posterior end of the body and *Tbx4* is expressed as cells leave the primitive streak (Fig. 8C,K). We do not observe outgrowth of the hindlimb until later, after additional differentiation of the somitic and lateral plate mesoderm.

Pitx1 is upstream of *Tbx4* in the hindlimb (Logan and Tabin, 1999) and its expression in opossum at stage 23 precedes that of *Tbx4* at stage 26. Relative to the mouse, *Pitx1* expression in opossum is late, but its expression appears to be delayed because of the steep anterior-posterior developmental gradient within the embryo as a whole. Like *Tbx4*, *Pitx1* appears to be expressed as soon as the appropriate segmental level is generated.

We have used Tbx expression as a proxy for limb field specification, but this is an inexact proxy. Studies in other taxa have shown that the limb fields are specified prior to Tbx expression in the field itself (Saito et al., 2006), and the Tbx expression domain in the lateral plate mesoderm extends slightly beyond the limbs (e.g. Fig. 3D). It is possible that the *Tbx4* expression seen at stage 26 is not part of the hindlimb field. This cannot be definitively assessed without lineage tracing of this cell population. However, at stage 27, a large portion of the *Tbx4* domain in the lateral plate mesoderm is laid down and, based on in situ analysis of expression at later stages, some of this domain must contain a portion of the hindlimb fields. If, for the analysis of developmental sequences, we assume that *Tbx4* expression in the lateral plate mesoderm first occurs at stage 27, rather than at stage 26, expression would still be early in opossum, with a jackknife score of 73. Furthermore, our conclusions regarding the gradual appearance of the hindlimb fields with elongation of the embryonic axis are unchanged, i.e. the limb fields in opossum do not appear in their entirety.

These results suggest that the disparity in the size and stage of development of the opossum forelimb and hindlimb at birth is not due to changes in the timing of hindlimb specification, but of later events in hindlimb development. It appears that the hindlimb is first specified when there is little cellular material available to build the limb bud and produce subsequent outgrowth. The fact that hindlimb *Fgf8* expression is delayed in the opossum (not early, relative to mouse) suggests a pause in development after limb field specification. Other studies have also hypothesized a period of developmental 'dormancy' at much later stages (Bininda-Emonds et al., 2007; Weisbecker et al., 2008). From an evolutionary perspective, it has been suggested that the delay in hindlimb development is an energy allocation trade-off that provides more resources to the growing forelimb and other structures during the short period of gestation (Müller, 1967; Weisbecker et al., 2008). A similar trade-off has been hypothesized in the head, where CNS development is delayed in favor of precocial migration of the neural crest in marsupials (Smith, 1997; Smith, 2006). Neural crest derivatives contribute to much of the craniofacial apparatus that is required for suckling in the altricial neonate. Our results thus corroborate the hypothesis that elements crucial for development and survival immediately after birth develop early, whereas other elements are delayed.

Models of vertebrate limb initiation

Previous studies of limb development have hypothesized that structures medial to the lateral plate mesoderm, such as the intermediate mesoderm, mesonephros, node, somites and segmental plate, produce an axial cue that is required for limb induction (Capdevila and Belmonte, 2001; Crossley et al., 1996; Geduspan and Solursh, 1992; Logan, 2003; Martin, 1998; Saito et al., 2006; Stephens and McNulty, 1981; Strecker and Stephens, 1983). We show that in marsupials both limb fields are specified, as signaled by Tbx expression, before any such medial axial structures exist. We observe cells exiting the primitive streak that express *Tbx4*. No medial structures are present at this end of the axis that could produce a hypothetical cue for limb induction. We therefore conclude that limb field induction does not require a signal from any of the above structures. This conclusion is supported by experiments in chick, in which the mesonephros was removed and limb development proceeded normally (Fernandez-Teran et al., 1997), and in mouse, in which a hypothesized inducer (*Fgf8*) from the intermediate mesoderm was eliminated and limb bud initiation was unaffected (Boulet et al., 2004). More recently, it has been proposed that retinoic acid in the body axis provides an environment permissive for forelimb induction by inhibiting *Fgf8* in the developing trunk (Gibert et al., 2006; Mic et al., 2004; Niederreither et al., 2002; Zhao et al., 2009). However, in the opossum primitive streak, *Fgf8* expression extends anterior into axial levels of *Tbx5* expression, contrary to this proposed mechanism (Fig. 3A and Fig. 6J). Our results do not preclude the existence of early axial cues, such as induction by the Hox genes, or later reinforcement of correct limb placement by medial axial structures. Work by Iimura and Pourquie has shown that the temporal collinear activation of Hoxb genes in precursors located in the chick epiblast is linked to mesoderm cell ingression through the primitive streak (Iimura and Pourquie, 2006). Activation of the Tbx genes in the cells of their respective limb fields might be directly downstream of Hox activation. In the absence of inductive signals from mature axial structures, limb field placement might initially be under the control of general anterior-posterior patterning. Such a mechanism would account for our observation of Tbx expression in cells exiting the streak.

Limb development is highly conserved across vertebrates and the changes seen in marsupial development appear to be largely limited to shifts in timing and changes in location. In *M. domestica* we see the same basic mechanism of limb development as in mouse, with the same genes and same order of expression. However, because limb specification appears before any other axial structures are in place, we can infer that these structures are not necessary to initiate the limb development program in vertebrates. In this way, marsupials act as a natural model, allowing us to test the generality of commonly held hypotheses.

The use of non-traditional models, such as marsupials, is an important addition to the typical experimental approach in developmental biology of focusing on a particular set of model organisms. In the case of marsupials, diverse systems are either accelerated or delayed during development as a result of their distinct life history adaptations. These shifts in timing and pattern allow us to test mechanistic hypotheses for both validity and generality. Examination of limb development in a marsupial has provided a window on a developmental mechanism in a new context. Because the developmental timing has changed in marsupials, the developmental conditions have changed as well. The limbs develop before many structures of the embryonic axis are present and we can observe the limb fields emerging, patterned from the streak. No mutant or experimental model provides the same context.

The oversized precocial forelimbs seen in marsupial neonates derive from multiple shifts in development: an extreme advancement of specification, outgrowth and patterning, as well as

an expansion of the region contributing to the bud. Our study demonstrates an extraordinary flexibility in early development and suggests that the mechanisms of limb field specification and positioning are yet to be fully understood.

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Competing interests statement
The authors declare no competing financial interests.

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