A dual fate of the hindlimb muscle mass: cloacal/perineal musculature develops from leg muscle cells

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Summary

The cloaca serves as a common opening to the urinary and digestive systems. In most mammals, the cloaca is present only during embryogenesis, after which it undergoes a series of septation events leading to the formation of the anal canal and parts of the urogenital tract. During embryogenesis it is surrounded by skeletal muscle. The origin and the mechanisms regulating the development of these muscles have never been determined. Here, we show that the cloacal muscles of the chick originate from somites 30-34, which overlap the domain that gives rise to leg muscles (somites 26-33). Using molecular and cell labelling protocols, we have determined the aetiology of cloacal muscles. Surprisingly, we found that chick cloacal myoblasts first migrate into the developing leg bud and then extend out of the ventral muscle mass towards the cloacal tubercle. The development of homologous cloacal/perineal muscles was also examined in the mouse. Concordant with the results in birds, we found that perineal muscles in mammals also develop from the ventral muscle mass of the hindlimb. We provide genetic evidence that the perineal muscles are migratory, like limb muscles, by showing that they are absent in *met*$^{d/d}$ mutants. Using experimental embryological procedures (in chick) and genetic models (in chick and mouse), we show that the development of the cloacal musculature is dependent on proximal leg field formation. Thus, we have discovered a novel developmental mechanism in vertebrates whereby muscle cells first migrate from axially located somites to the pelvic limb, then extend towards the midline and only then differentiate into the single cloacal/perineal muscles.

Key words: Striated sphincter muscles, Cloaca, Perineum, Chick/quail chimera, Somite, Segmental origin, *limbless*, *MyoD*, *Pax3*, *Pax7*, *Met*, *Lbx1*, *Meox2*

Introduction

During embryogenesis, the cloaca serves as a common opening to the digestive, urinary and reproductive systems. Voluntary control of this passage is maintained by skeletal cloacal muscles. Whereas birds maintain a single cloacal opening throughout their lives, most mammals develop two or three openings: anal, urethral and vaginal in females. Therefore the muscles of the cloacal sphincter specialise into the perineal muscles in mammals (Gegenbaur, 1883; Popowsky, 1899; Nishi, 1938). The origin and development of the early common cloacal muscles has never been determined.

The degree of evolutionary homology of muscles in birds and mammals has facilitated the use of the avian model to decipher the origin and formation of distinct muscles in mammals. In particular, the chick/quail chimeras (Le Douarin, 1969) have been used to determine the origin of a plethora of tissues. Furthermore this technique has been used to determine the axial origin of numerous muscles. These studies have shown that skeletal muscles of the head originate from the non-segmented head mesoderm (Noden, 1983; Wachtler et al., 1984), whereas all skeletal muscles of the body originate from the somites (Christ and Ordahl, 1995).

Epaxial musculature of the back develops locally from the dorsomedial part of the somites (Brand-Saberi et al., 1996a) whereas the lateral aspect of the somites form the hypaxial musculature that comprises the ventral body wall muscle and those found in the limbs. Hypaxial muscle forms by two distinct mechanisms: (1) extension of the myotomal sheet for the body wall; or (2) migration of individual muscle precursors for limb muscles (Dietrich et al., 1998). The migratory mechanism is also deployed for the formation of the tongue and the diaphragm muscles of mammals (Dietrich, 1999). Migration of muscle precursors is dependent upon Scatter Factor/Hepatocyte Growth Factor (Heymann et al., 1996; Brand-Saberi et al., 1996b), which acts on its receptor, Met, which is expressed by migrating myogenic cells. Genetic deletion of either molecule results in complete absence of migratory muscles (Bladt et al., 1995; Dietrich et al., 1999). During migration, the muscle precursors express transcription factors *Pax3* and *Pax7*, and only initiate the expression of *MyoD* having once reached their final location (Amthor et al., 1998). By contrast, myotomal extension for the body wall musculature involves *MyoD*-expressing cells from the very beginning.

In this study, we have determined the axial origin of the cloacal skeletal muscles and investigated the mechanism of how these muscles develop both in birds and mammals. Using two independent labelling techniques (chick/quail chimeras and retroviral labelling of somitic cells), we have established the somitic origin of the cloacal striated muscles in chick. Surprisingly, the cloacal muscles derive in large part from the same somites as those that give rise to leg muscles. A study of the early muscle development in chick using a panel of markers, including *MyoD*, showed that cloacal muscles were derived from the ventral muscle mass of the leg. Homologous perineal muscles in mouse were also found to develop from the ventral muscle mass of the hindlimb. We provide evidence that the perineal muscles are migratory, in the same way as the muscles of the limbs, as they all are absent in the $met^{d/d}$ mutant embryo. Furthermore, we show through the use of surgical procedures and genetic mutants that the development of the cloacal muscles is dependent on the development of the limbs.

The results from this study show that the cloacal muscles develop via novel mechanism that involves an initial phase of myogenic precursors migration from the somites into the limb. These precursors then extend out from the limbs towards the ventral midline and the process is subsequently followed by differentiation into individual muscles. The knowledge of the early development of these muscles will allow us to understand the aetiology, or at least the consequences, of a variety of human conditions that affect the caudal part of the body. These include imperforate anus (Tichy et al., 1998), persistent cloaca, caudal dysgenesis (Bohring et al., 1999; Inomata et al., 1989) and body wall closure anomalies (Hartwig et al., 1991).

Materials and methods

Chick/quail chimeras

Fertilised White Leghorn chick eggs and Japanese quail eggs were incubated at 38°C and 80% humidity. The five most newly formed somites [I-V (Christ and Ordahl, 1995)] with the overlying ectoderm were cut out from quail donor embryos [stage HH17-18 (Hamburger and Hamilton, 1951)] using electrolytically sharpened tungsten needles (Dossel, 1958), then separated into single segments and used for several non-orthotopic transplantations.

Each chick recipient embryo was manipulated in ovo at somite stages 29-36 (HH17-19). After windowing the eggshell and the subjacent shell membrane, the embryo was floated with sterile PBS/antibiotics to facilitate the operation procedure (Hara, 1971). The most newly formed somite (I) was removed along with its surface ectoderm using a tungsten needle. Remaining cells were removed with a microcapillary. Viral marking of a more cranial interlimb somite was carried out at this stage (see below). The donor quail somite was transferred to the chick surgery site with a thin glass Pasteur pipette and positioned using drawn glass needles. The surface ectoderm allowed orientation of the graft. Albumin (1-2 ml) was removed, the egg was closed with a surgical tape and re-incubated for 6-7 days until HH35-36.

Orthotopical transplantations of quail leg bud was carried out at stage HH23 after ablation of the chick leg. The proximal leg field of the chick was left intact. Retention of the transplanted limb was achieved by closure of the amnion. Embryos were reincubated until HH35-36.

Tissue fixation and processing

Embryos at stage HH35-36 were decapitated and cut transversally below the thorax. The caudal part of the embryo was fixed in Serra's fixative (Serra, 1946), then dehydrated in ethanol and processed for paraffin embedding using CNP30 and Fibrowax. Serial 10 μm transverse sections were mounted on albumen-glycerin coated slides. After de-paraffinisation, sections were treated in PBS with 0.1% Tween, rinsed in PBS and pre-incubated in 1% dried skimmed milk powder in PBS. QCPN anti-quail monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa) was applied in the spin-cleared milk solution over night at 4°C. Secondary Goat anti-mouse antibody conjugated to alkaline phosphatase (DAKO) was used 1:1000 in PBS for 1 hour. Blue colour AP substrates (Roche) in NTMT buffer revealed the presence of quail nuclei in the host tissue. After eosin counterstaining, sections were dehydrated and mounted in DPX.

Somite counting and segmental level determination

One or more interlimb somite (20-26) was marked at the time of surgery at a known distance from the operated somite (Fig. 1A). This allowed us to locate exactly at the end of the experimental period which caudal somite was operated with respect to the reference point. Somites were marked by injecting a small volume of avian retrovirus coding for heat resistant alkaline phosphatase RCAS(A)-AP (titre ca. 10^8IU/ml with 0.1% Fast green) using a hand held microcapillary. At time of fixation the thoracic region was fixed in 4%PFA washed in PBS, followed by heat inactivation of endogenous alkaline phosphatase. The enzymatic colour reaction in the infected intercostal muscle (Fig. 1B) was carried out as for in situ whole mounts with AP substrates. Only embryos with identifiable normal seven ribs were included in the analysis.

Retroviral marking

Retroviral marking of the somites (Rees et al., 2003; Evans, 2003), a system that avoids excessive surgical artefacts, was also used for somatic fate mapping. Briefly, a small volume of replication incompetent retrovirus, containing the *lacZ* gene, was pressure injected into somites 29-35 of chick embryos at stage I-XIV using a glass micropipette. For each batch of injections, several embryos received sham injections, which contained only the vehicle medium. All embryos were re-incubated until HH36. X-Gal staining was performed after brief fixation in 2% paraformaldehyde on unskinned decapitated embryos. A similar protocol was used to label cells at hindlimb cells at HH25.

Whole-mount in situ hybridisation

In situ hybridisation was performed according to Nieto et al. (Nieto et al., 1996). Briefly, chick embryos HH17-36 and mouse embryos (C57BL6) E11.5-E15.5 were fixed in 4%PFA/PBS/0.1%Triton, dehydrated in methanol, re-hydrated, treated with proteinase K and re-fixed. For good quality in situ hybridisation, chicken HH32 and mouse E14.5 and older embryos were skinned while in methanol at this stage. Antisense RNA probes of chicken *MyoD* (a 1518 bp probe, Dr Bruce Paterson), mouse *MyoD* (*Myod1* – Mouse Genome Informatics), *Meox2* (1833 bp, Dr Baljinder Mankoo), chicken *Pax3* (645 bp, Dr Martin Goulding) and *Pax7* (582 bp, Dr Susanne Dietrich) were labelled with digoxigenin. Fab fragments of sheep antibody to digoxigenin conjugated to alkaline phosphatase mediated the visualisation (1:5000, Roche).

Chick legless models

Unilateral legless chicks were produced by ablating the limb anlage (ranging from lateral somatopleura to a limb bud, HH16-23) either with tungsten needles or with ophthalmology scissors. On HH34, the embryos were processed for *MyoD* whole-mount in situ hybridisation. Spontaneously amelic chicken embryos *limbless* were kindly provided by Professor John Fallon (University of Wisconsin, USA) (Prahlad et al., 1979; Fallon et al., 1983).

Mouse models

To partially rescue placental phenotype of *met*$^{d/d}$ mutant embryos (Maina et al., 1996), heterozygous males on a mixed C57BL/6x129/sv background were inter-crossed with heterozygous females on outbred strain CD1 (Maina et al., 2001). Mice null for *p63*, *Meox2* and *Lbx1* were produced by simple heterozygous breeding programmes. Mouse mutants were embedded in tissue freezing medium (Leica) by slow freezing in isopentane on liquid nitrogen. Embryos were sectioned parallel to the axis of rectum. Serial cryosections (16 μm) were re-hydrated in PBS, fixed in 4%PFA/PBS for 3 minutes, rinsed in PBS and preblocked in 10% heat-inactivated goat serum in PBS. Sections were incubated for 2 hours with biotinylated mouse monoclonal antibody against myosin heavy chain (DSHB A41025, 1:1000), which was developed with ABC streptavidin/peroxidase kit and DAB staining (Vectorlabs). Sections were counterstained with Haematoxylin and Alcian Blue.

Anatomy and terminology

We use the descriptive terms cranial/caudal rather than anterior/posterior in order to avoid confusion with their usage in human anatomy (Baumel et al., 1993). The normal anatomy of the pelvic region was determined by studying serial 10 μm paraffin wax embedded sections of ED10 chick and 16 μm serial cryosections of mouse embryos. Sections were stained by a myosin heavy chain monoclonal antibody. Photographs of serial sections were imported into Microsoft Powerpoint, which enabled movie-like scanning and identification of single muscles. *MyoD* in situ hybridisation wholemounts were used for gaining a three-dimensional concept. Photographs were taken on a stereomicroscope with a digital camera (Nikon). Images for Fig. 2D and Fig. 4B were taken on Olympus SZX-12. Photos of sections were taken on Nikon Eclipse 400. Image processing was carried out using Adobe Photoshop 5.0LE.

Results

Anatomy and nomenclature of avian cloacal and mouse perineal muscles

Caudal region of chick has three groups of muscles: (1) cloacal, (2) pelvicaudal and (3) axial muscles (Fig. 1C,D) (P.V. and K.P., unpublished) (Baumel et al., 1993; Nickel et al., 1986; Vanden Berge, 1975). The cloacal muscles comprise *m. sphincter, transversus* and *levator cloacae*. The pelvicaudal muscles are *m. pubocaudalis externus* (*PCE*) and *internus* (*PCI*). The axial muscles are associated with the tail vertebral column, i.e. *m. levator caudae*, *m. interspinales*, *m. lateralis caudae* and *m. depressor caudae*.

We next identified the homologous perineal muscles in mouse using sectioning and whole-mount in situ hybridisation with *MyoD* probe. The single muscles are recognisable from E15.5 (Fig. 1E,F). Similar to the chick, we could discern three groups of muscles in the pelvic outlet.

(1) The perineal muscle group comprises of external anal sphincter, external urethro-vaginal sphincter, levator ani, bulbospongiosus and ischiocavernosus muscles (Greene,

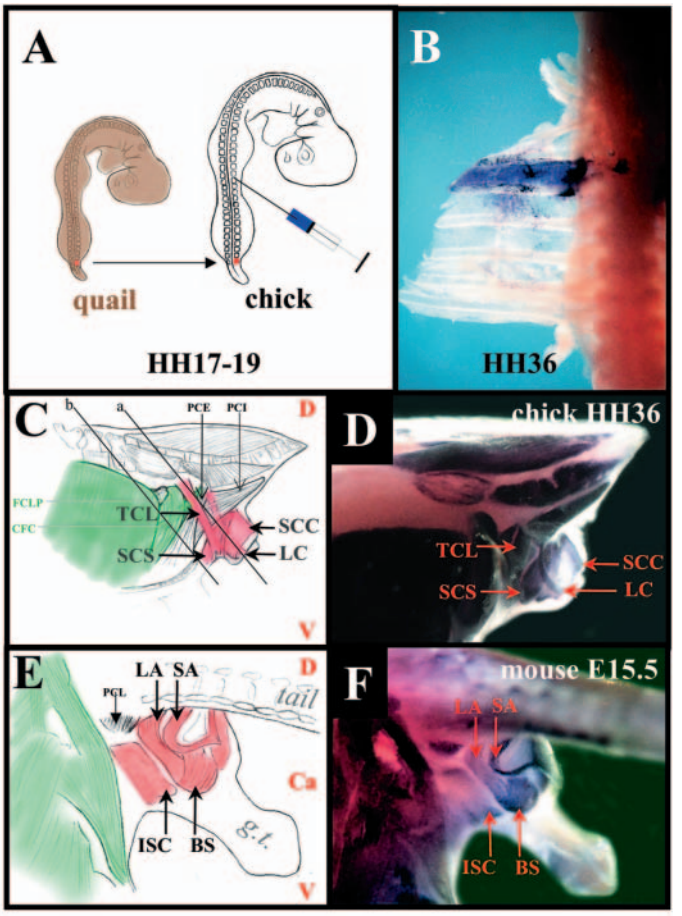

Fig. 1. Anatomy of cloacal and perineal muscles. (A) Experimental procedures using newly formed quail somites for heterotopic single somite grafting into the chick recipient. Viral injection into interlimb somite was carried out for future exact determination of the operated segmental level. (B) Result of the single somite marking by retroviral alkaline phosphatase transfection. The second intercostal muscle is a derivative of somite 21 (Evans, 2003). Operated somite was 10 somites further caudally, therefore somite 31 was transplanted. (C) Lateral view of a chick HH36 with cloacal muscles (red), two leg muscles (green) and the tail region. Lines 'a' and 'b' show the approximate level of sections in Fig. 2A and 2B respectively. Orientation is marked: V, ventral; D, dorsal; Ca, caudal. The *m. sphincter cloacae* has 2 parts: *m. sphincter* circular (SCC) and *m. sphincter* sling-shaped (SCS). The sling region originates in the supracloacal septum and on the apex of pubis and forms a slightly dorsally positioned sling around the cloacal tubercle. These layers merge in the dorsocaudal aspect. The *m. transversus cloacae* (TCL) in the adult is a very prominent superficial muscle and with its almost transverse course it forms the boundary between the abdomen and the tail. The *m. levator cloacae* (*m. retractor phalli caudalis*) (LC) is a long narrow muscle arising from the underside of feather bulge of the lateral rectrices, inserting near the midline of the cloacal tubercle – phallus. Sexual dimorphism was not evident at the stages examined. Because of close topographical relationship to the cloacal muscles, we also focus on two hindlimb muscles. CFC begins from the proximal half of the femur and inserts on the ventral side of pygostyl on the fascia of *m. depressor caudae* in the vicinity of the rectrices. FCLP is the caudal-most muscle of the leg (C,D). (D) Lateral view of a skinned HH36 chick after in situ hybridisation with *MyoD*. (E) Caudolateral view of a mouse E15.5 with perineal muscles (red), leg muscles (green) and the tail region. (F) Caudolateral view of a skinned mouse E15.5 after in situ hybridisation with *MyoD*. *g.t.*, genital tubercle; BS, *m. bulbospongiosus*; CFC, *M. caudofemoralis* (*pars caudofemoralis* of *m. caudo-ilio-femoralis*); FCLP, *M. flexor cruris lateralis – pars pelvica*; ISC, *m. ischiocavernosus*; LA, *m. levator ani*; LC, *levator cloacae*; PCE, *m. pubocaudalis externus*; PCI, *m. pubocaudalis internus*; SA, *m. sphincter ani externus*; SCC, *m. sphincter* circular; SCS, *m. sphincter* sling-shaped; TCL, *m. transversus cloacae.*

1935). [In female newborn mouse, the urethral sphincter encompasses both the urethra and vagina (data not shown).]

(2) The pelvicaudal muscles consist of *m. pelvicaudalis medialis* and *lateralis* (C. P. Wendell-Smith, PhD Thesis, University of London, 1967).

(3) The axial muscles are muscles associated with the tail vertebral column.

Cloacal muscles develop predominantly from hindlimb somites

To identify the somites from which the cloacal muscles arise, we first used chick/quail somitic chimera approach. Among the 116 chimeras, 76 survived until stage HH36, and 71 had unequivocal reference intercostal AP marking. Fifty-four chimeras had quail tissue, and 30 had hypaxial musculature derived from the quail tissue. The heterotopic transplants of quail somites I-V were adequate for fate mapping, as we found no differences between their muscle derivatives. Using this approach, we found that somites 30-34 were the source of myogenic cells of the cloacal sphincter muscle complex (asterisks in Table 1). Remarkably, this contribution domain was extremely precise and restricted, as both adjacent somites 29 and 35 did not contribute to the formation of these muscles. Each of the four cloacal muscles had a contribution from at least three somites. However, the four cloacal muscles were not supplied in the same proportion by the five somites. Fig. 2A,B show an example of contribution of quail-derived cells from somite 31 to the formation of cloacal and leg muscles. In addition, as previously described (Lance-Jones, 1988; Rees et al., 2003), we found that leg muscles originate from somites 26-33 (Fig. 2B; Table 1). Interestingly, these transplantation experiments revealed that whenever a cloacal muscle developed that contained quail cells from somites 30-33, there were also quail cells integrated within the leg muscles (see Fig. 2B). This was not the case for somite 34, which gave rise to cloacal muscle cells but not to cells within leg muscles.

To confirm the fate-mapping data, we injected replication deficient retrovirus containing the *lacZ* gene (indicated by a dagger in Table 1). Single somites of 50 embryos were injected at the levels 29-35 with small volumes of retrovirus and 40 embryos resulted in β-galactosidase-positive staining (see Fig. 2D). Data from these experiments also showed that somites 30-34 contributed to the cloacal muscles and confirmed the results from the chick/quail experiments.

Taken together with the leg muscles somitic origin from somites 26-33 and the fact that somites 30-33 gave rise to both groups of muscles, we suggest that cloacal muscles develop together with leg muscles.

Cloacal muscle precursors migrate from the somites into the developing leg bud

Having established that individual somites provide cellular contribution for both leg and cloacal muscles, we next focused on how these two different muscle regions recruit their myogenic content. Migration of single muscle precursors from the somite would be characterised by *Pax3* and *Pax7* expression, without *MyoD*. However, myotomal extension presents as an elongation of a process continuous with the myotome. It expresses not only the Pax genes but also *MyoD* (Amthor et al., 1998).

In the first instance, we studied the expression of *Pax3* at the levels of somites 30-34. We detected cells leaving these somites between HH17-20, which subsequently populated the hindlimb bud as we previously described (Amthor et al., 1998). We were unable to detect migration of cells from the somites after stage HH20 using in situ hybridisation. To confirm this molecular observation, we labelled the ventrolateral region of the somites after HH20 with the *lacZ* expressing reterovirus

Table 1. Fate mapping of the cloacal musculature using chick/quail chimeras* and viral somite labelling†

Somite	29	30	31	32	33	34	35
Chimeras/injected embryos (*n*)	2/9	4/5	3/3	4/4	7/4	6/2	4/2
Cloacal muscles‡							
Sphincter cloacae sling (SCS)		*,†	*,†	*	*,†	*	
Sphincter cloacae circular (SCC)		*,†	*,†	*,†	*,†	*,†	
Levator cloacae (LC)		*,†	*,†	*	*	*	
Transversus cloacae (TCL)		*,†	*,†	*,†	*,†		
Selected thigh muscles§							
Dorsal							
Iliotibialis cranialis (IC)	*,†						
Femorotibialis medius (FTM)	*,†	*,†	*				
Iliotrochantericus cranialis (ITCR)	*,†	†					
Iliotrochantericus caudalis (ITC)	*,†	*,†	*,†	*,†	†		
Iliofemoralis externus (IFE)	*	*,†	*,†	*,†	*,†		
Iliofibularis (IF)		*,†	*,†	*,†	*,†		
Ventral							
Obturatorius (OBT)		*,†	†				
Flexor cruris medialis (FCM)	*,†	*,†	*,†				
Flexor cruris lateralis pars pelvica (FCLP)	†	*,†	*,†	*,†	*,†		
Caudofemoralis pars caudalis (CFC)	*,†	*,†	*	*	*		
Selected shank muscles§							
Tibialis cranialis (TC)	†	*,†	*,†	*,†			
Fibularis longus (FL)		*,†	*,†	*,†	*		

‡Contribution of single somites 29-35 to the cloacal muscles are shown in the context of selected muscles of the leg§.

Adjacent somites 29 and 35 never contributed to the cloacal muscles. Positive results mean that at least 25% of the examined embryos for a given segmental level contributed their labelled somitic cells to the particular muscle.

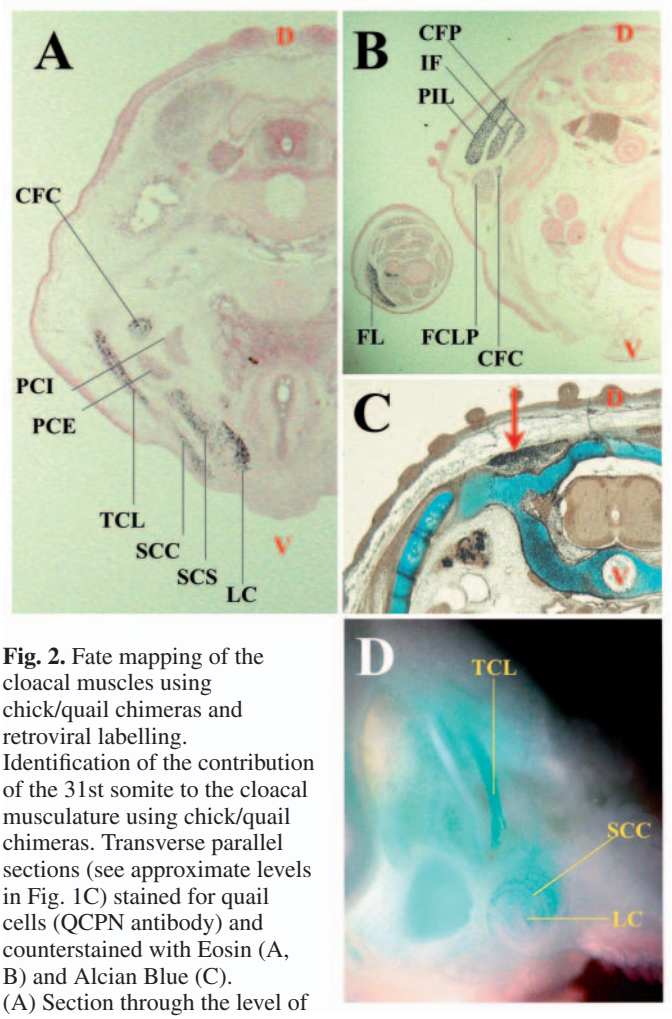

Fig. 2. Fate mapping of the cloacal muscles using chick/quail chimeras and retroviral labelling. Identification of the contribution of the 31st somite to the cloacal musculature using chick/quail chimeras. Transverse parallel sections (see approximate levels in Fig. 1C) stained for quail cells (QCPN antibody) and counterstained with Eosin (A, B) and Alcian Blue (C). (A) Section through the level of the cloaca, showing all four cloacal muscles to be populated with quail cells. (B) Section through the level of the thigh and shank, showing quail derived cells from the somite 31 in some leg muscles. (C) Contribution of somite 31 to the hemivertebra and the adjacent epaxial muscle (arrow). (D) Detail of cloacal region of a chick embryo after marking somite 30 (at HH18) with *lacZ*-encoding retrovirus and X-gal whole-mount staining at HH36. X-Gal staining was detected in cloacal and leg muscles, see Table 1. Orientation is marked: V, ventral; D, dorsal. CFC, *M. caudofemoralis* (*pars caudofemoralis* of *m. caudo-ilio-femoralis*); CFP, *m. caudofemoralis – pars pelvica*; FCLP, *M. flexor cruris lateralis – pars pelvica*; FL, *fibularis luingus*; IF, *iliofibularis*; LC, *levator cloacae*; PCE, *m. pubocaudalis externus*; PCI, *m. pubocaudalis internus*; PIL, *m. iliotibialis lateralis pre-acta bularis*; SCC, *m. sphincter* circular; SCS, *m. sphincter* sling-shaped; TCL, *m. transversus cloacae.*

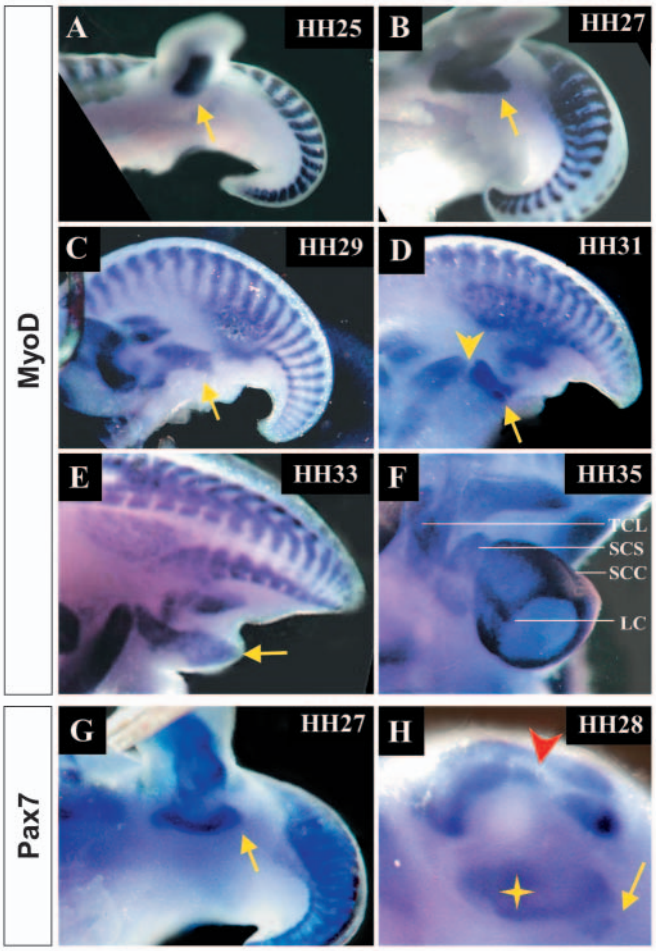

Fig. 3. Molecular analysis of chick cloacal muscle development. (A-F) Ventrolateral views of whole-mount in situ for *MyoD* on chick embryos at stages HH25-35 show the development of the cloacal muscles from the ventral muscle mass of the leg. (A) Ventral muscle mass (arrow) of the hindlimb at HH25. (B) Elongation of ventral muscle (arrow) mass ventrocaudally towards the cloaca at HH27. (C) Further elongation of cloacal anlage (arrow) at HH29; the cleavage of leg muscle mass has started. (D) Cloacal anlage arrives to the vicinity of cloacal tubercle (arrow) at HH31, note the discontinuity with the anlage of FCLP and CFC muscles of the leg (arrowhead). (E) Cloacal anlage extends around the tubercle at HH33 (arrow). (F) Single muscles of the cloacal complex are discernible at HH35. (G) *Pax7* expression at HH27 (arrow). (H) Details of the *Pax7* expression of the proximal limb anlage at HH28, limb has been removed. Cloacal anlage (arrow) extends from the ventral muscle mass (cross). Dorsal muscle mass (red arrowhead) began to split into single muscles. LC, *levator cloacae*; SCC, *m. sphincter* circular; SCS, *m. sphincter* sling-shaped; TCL, *m. transversus cloacae.*

but were never able to detect any cells the limbs (n=5, data not shown). Furthermore, we were never able to detect a population of migrating cells moving from the somites towards the developing cloaca using either molecular or cellular techniques (n>50, data not shown). We then investigated the development of myotomal extension from the somites of interest using in situ hybridisation with *MyoD* (Amthor et al., 1998) or virally labelled cells. We were unable to find any elongation of myotomes from the somites 30-34 towards the developing cloaca (data not shown; see also Fig. 3A,B).

Thus, using molecular and cell labelling techniques, we were only able to detect the displacement of somitic cells in one event. This displacement occurred between HH17 and HH20, and involved the migration of muscle precursors into the hindlimb buds.

Cloacal muscles develop from the ventral muscle mass of the hindlimb

To our surprise, we had only detected one wave of migration from somites 30-34. However these cells had all entered the

developing leg bud. However, our fate-mapping studies had shown that these somites contributed to cloacal muscles. We therefore followed the myogenic events of the leg at later stages. The ventral and dorsal muscle masses of the hindlimb begin to express *MyoD* at stage ~HH23. The masses expand within the limb bud until HH25. At this stage there were still no myogenic cells in the cloacal region (Fig. 3A). At stage HH27, when the cleavage of ventral and dorsal masses into muscle groups is initiated (Schroeter and Tosney, 1991), we observed a ventrocaudal elongation of the ventral muscle mass towards the cloaca (Fig. 3B). The elongation of the muscle mass continues (Fig. 3C) until HH31, when the cloacal anlage reaches the vicinity of cloacal tubercle (arrow in Fig. 3D) and separates at the other end from the future *flexor cruris lateralis* (FCLP) and *caudofemoralis* (CFC) muscles (arrowhead in Fig. 3D). The cloacal anlage at HH33 extends around the tubercle (Fig. 3E). At HH35, the single muscles of cloaca are discernible (Fig. 3F). Thus, from a molecular perspective, cells from the ventral muscle mass exit the developing limb bud and form the muscles of the cloaca. To investigate this at the cellular level, we first determined that cloacal muscle precursors do reside at some point in the leg bud. To this end, we replaced homotopically chick leg bud with a quail leg bud at stage HH23 when migration from somites is completed. We replaced only part of leg bud containing muscle masses, leaving the proximal limb field intact. The resulting HH35 chimera showed all leg and cloacal muscles to be of quail origin, while all material of pelvic bones was formed by the chick recipient (Fig. 4A). Having established that cloacal muscle precursors temporarily reside in the limb, we investigated their distribution within the bud. To this end, we virally labelled cells in one of four regions of the leg bud (proximoventral, proximodorsal, mid-length and distal) and then determined which site(s) contributed to cloacal musculature. Eleven labelling procedures were performed and we were only able to detect a contribution of the ventral proximal of the limb bud to the formation of cloacal muscles (Fig. 4B and Table 2).

These results show that at HH27, the myoblasts of the proximal hindlimb ventral muscle mass contains two populations. One population remains within the leg, while the second leaves the limb and elongates ventrocaudally to colonise the cloaca.

Cloacal muscle anlage translocation resembles myotomal extension

In order to analyse the mechanism of the cloacal anlage translocation from the leg bud to the cloaca, we deployed again the comparison of *Pax3/7* and *MyoD* gene expression. The presence of population of cells expressing only *Pax3/7* in front of the *MyoD* domain would suggest involvement of precursor migration. On the other hand, co-expression of the Pax genes and *MyoD* throughout the translocation would indicate a mechanism akin to myotomal extension.

Data from the expression of *Pax3* was difficult to interpret at these later stages as this gene became also expressed in non myogenic tissues, including the ectoderm of the cloacal cleft (data not shown). We therefore concentrated on *Pax7*, which remains more specific for the myogenic lineage. We found that *Pax7* was expressed by the ventrocaudal elongation (Fig. 3G,H) of the ventral limb muscle mass directed towards the cloaca. When we compared the expression domains of *Pax7* (Fig. 3G) with that of *MyoD* (Fig. 3B) at stage HH27 we found that they were identical. Therefore, we were unable to detect a population of only *Pax7*-positive cells in more

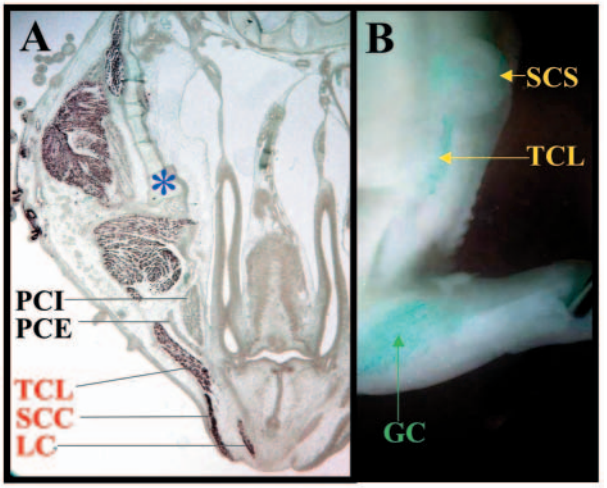

Fig. 4. Cloacal muscle precursors reside in the hindlimb. (A) Quail leg bud transplanted onto chick hindlimb stump at HH23 results in cloacal muscles consisting of quail cells at HH36. Proximal skeletal elements are of only chick origin (asterisk). (B) Injection of retrovirus into the proximoventral region of the leg bud at stage HH25 gives rise to cloacal muscles (TCL and SCS) labelled with *lacZ*. There is concomitant signal in the leg muscles. GC, gastrocnemius muscle; LC, *levator cloacae*; PCE, *m. pubocaudalis externus*; PCI, *m. pubocaudalis internus*; SCC, *m. sphincter* circular; SCS, *m. sphincter* sling-shaped; TCL, *m. transversus cloacae.*

Table 2. Retroviral labelling of leg bud at stage HH25

Leg bud labelled at HH25 in locations	Proximal ventral	Proximal dorsal	Mid-length	Distal
Injected embryos (*n*)	4	4	3	4
Cloacal muscles				
Sphincter cloacae sling (SCS)	2	0	0	0
Sphincter cloacae circular (SCC)	2	0	0	0
Levator cloacae (LC)	1	0	0	0
Transversus cloacae (TCL)	3	0	0	0
Thigh muscles	4	3	2	0
Shank muscles	2	2	3	4
Foot muscles	0	0	1	2

Injections were performed into one of four regions: proximoventral, proximodorsal, mid-length and distal. Only labelling of the proximoventral region gave rise to marked cloacal muscles.

advanced positions compared with the domain expressing *MyoD*.

In summary, during the initial migration period, cells express *Pax7* and *Pax3* on route to the limb bud. These cells start to express also *MyoD* only once they populate the developing limb. This co-expression of the Pax and *MyoD* genes then persists during the extension of the muscle mass towards the cloaca and thus resembles a myotomal extension.

Mouse perineal muscles develop from the ventral muscle mass of the hindlimb

To establish whether this mechanism of cloacal muscle anlage formation is conserved also in mammals, we followed the development of the cloacal/perineal muscles using *MyoD* expression in mouse embryos between E11.5 and E15.5. Similar to the chick, although at E11.5 we only observed *MyoD*-expressing cells in the ventral muscle mass of hindlimb (Fig. 5A), at E12.5 the ventrocaudal edge of the mass began to extend towards the genital tubercle (Fig. 5B). The direction of this extension is mainly ventral, without the pronounced caudal direction as in chick. At E13.0, this extension already reached the base of the tubercle (Fig. 5C), and further at E13.5 began to split into future single muscles (Fig. 5D). At later stage E14.5, it is easy to see how the common cloacal/perineal anlage differentiates into separate muscle branches around the openings (Fig. 5E) of the already septated cloaca. Thus, there is never a common cloacal sphincter, because the muscles arrive after the septation has occurred.

These results showed that, similar to the chick, the mouse perineal muscles develop from the ventral muscle mass of leg. The same developmental mechanism thus confirms the homology of cloacal muscles in birds and perineal muscles in mammals.

Development of the perineal muscles in *met*$^{d/d}$, *Lbx1* and *Meox2* null embryos

It has been shown that the receptor Met is expressed in all migratory myoblast precursors and is required for myoblast migration to colonise limbs, diaphragm and tongue (Bladt et al., 1995). As we found the intimate relationship between the development of cloacal/perineal and leg muscles, we expected the perineal muscles to be absent in *met*$^{d/d}$ mutant embryos. Serial transverse cryosections of E15.0 homozygous *met*$^{d/d}$ embryos (Maina et al., 1996) were compared with the wild-type siblings. Sections were stained for striated skeletal muscle (MyHC). Fig. 5G shows that all perineal muscles were completely absent in all examined embryos (three embryos from three different litters). Consistent with results obtained with *legless* mutant chick embryos (see below), the only muscles present in the pelvic region were *m. pelvicaudalis lateralis* (Fig. 5G) and *medialis* (not shown). The subcutaneous muscle of the panniculus carnosus was completely absent (data not shown). As previously reported, all hindlimb muscles were absent. By contrast, abdominal muscles were normal, as were the axial muscles of the tail.

These results demonstrate that the development of the perineal muscles in mammals is dependent on the migration of muscle precursors and thus that these muscles belong to the group of hypaxial migratory musculature.

Our molecular analysis showed that cells migrated as single entities into the limb and then moved towards the genital tubercle as a sheet. To achieve this, we studied the development of perineal muscle in the *Lbx1* null mouse. This mutant is

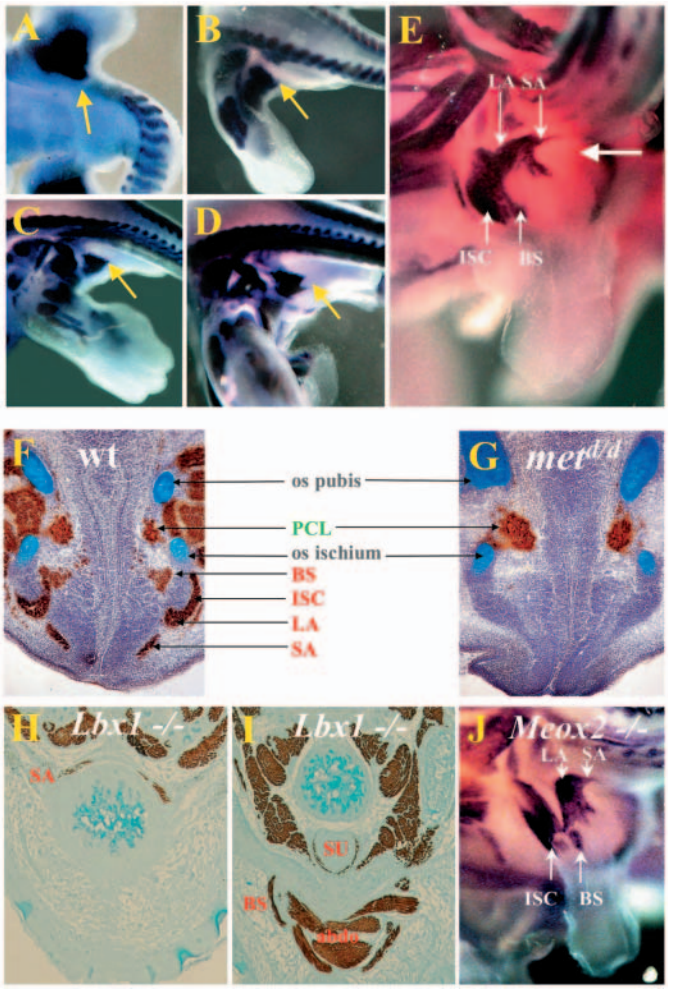

Fig. 5. Molecular analysis of mouse perineal muscle development. (A-E) Whole mount in situ *MyoD* hybridisation on mouse embryos showing the development of perineal muscles from the ventral muscle mass of the leg. (A) E11.5 ventral view showing ventral muscle mass. (B) E12.5 caudal view showing extension in ventral direction. (C) E13.0 caudolateral view of the perineal anlage separating from the ventral leg muscle mass. (D) E13.5 caudolateral view of the anlage extending to the base of the genital tubercle. (E) E14.5 caudolateral view of perineal anlage forming individual muscles around the already septated cloaca, before they merge with the contralateral counterpart. Anal opening – arrow. (F,G) Muscle primordia in the pelvis of E15.0 wild type (F) and *met*$^{d/d}$ mutant (G) embryos. Cryosections in the axis of rectum, stained for muscle (anti-MyHC) and counterstained by Haematoxylin and Alcian Blue. All perineal muscles are absent in *met*$^{d/d}$ mutants mouse. Note the only muscles present in *met*$^{d/d}$ mutants are PCL and *medialis* (not shown). E19.5 *Lbx1* null mutant stained for MyHC show presence of the external anal sphincter (SA in Fig. H) and of bulbospongiosus and external urethral sphincter muscles (BS and SU respectively in Fig. I). (J) *Meox2* null mutant E15.5 shows full complement of perineal muscles as detected by whole-mount in situ hybridisation using *MyoD*. abdo, abdomen; BS, *m. bulbospongiosus*; CFC, *M. caudofemoralis* (*pars caudofemoralis* of *m. caudo-ilio-femoralis*); ISC, *m. ischiocavernosus*; LA, *m. levator ani*; PCL, *m. pelvicaudalis lateralis*; SA, *m. sphincter ani externus*; SU, external urethral sphincter muscle.

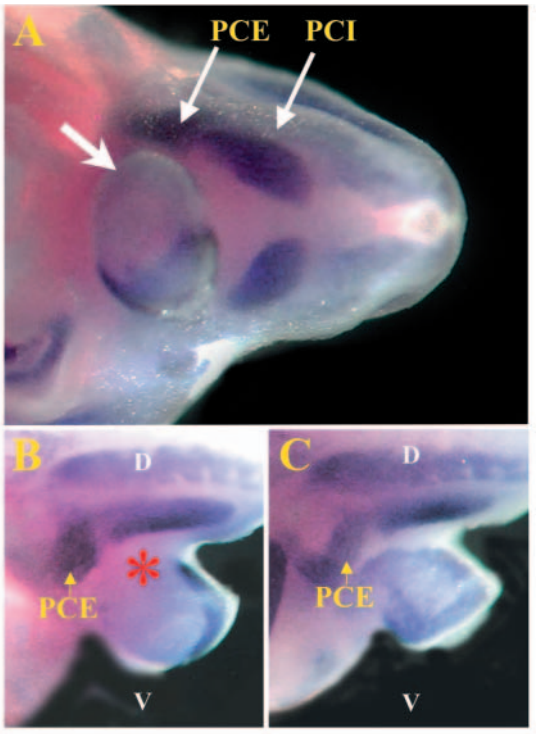

Fig. 6. Experimental evidence for cloacal muscle dependency on leg muscle development. (A) Whole-mount in situ hybridisation with *MyoD* probe on chick embryo at stage HH34 after surgical leg bud ablation at HH18. Ventral view of the cloacal region shows the unilateral absence of all cloacal muscles on the operated side (arrow). (B) Left operated side, lateral view; (C) right control side, lateral view (mirror image). Unilateral absence of all cloacal muscles on the operated side (asterisk in B) and normal pubocaudal and tail muscles are shown. Orientation is marked: V, ventral; D, dorsal. PCE, *m. pubocaudalis externus*; PCI, *m. pubocaudalis internus.*

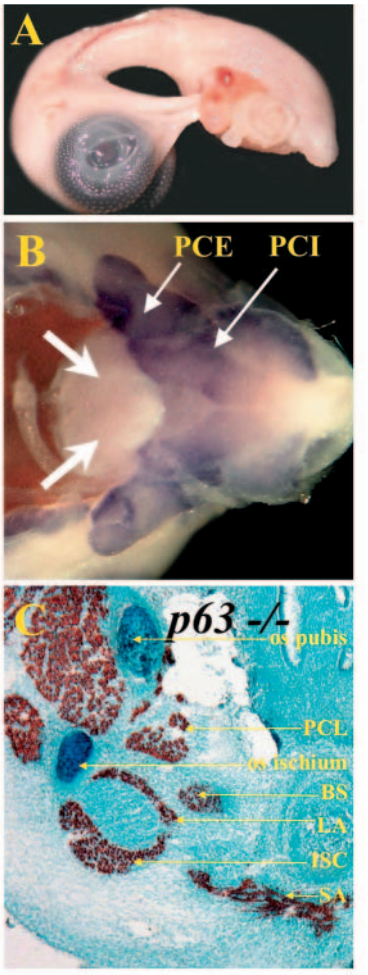

Fig. 7. Genetic evidence for the development of cloacal/perineal muscles depending on leg field. (A) *limbless* chick mutant HH36 before skinning for hybridisation. (B) Ventral view of the cloaca of *limbless* chick mutant after *MyoD* hybridisation reveals complete bilateral (arrows) absence of all cloacal muscles. Pubocaudal (PCI and PCE) and tail muscles are again normal. (C) *p63*-null E16.5 mutant mouse shows the presence of all perineal muscles. BS, *m. bulbospongiosus*; ISC, *m. ischiocavernosus*; LA, *m. levator ani*; PCE, *m. pubocaudalis externus*; PCI, *m. pubocaudalis internus*; PCL, *m. pelvicaudalis lateralis*; SA, *m. sphincter ani externus.*

interesting since although muscle cells migrate into the hindlimb, they are unable to migrate any further and results in a hindlimb that has no distal muscle. We found that even though there are no distal limb muscles, *Lbx1* null embryos showed perineal musculature development (Fig. 5H,I).

We also examined the development of the perineal muscles in the *Meox2*-null mouse embryos. These embryos display an abnormal limb muscle patterning that has been proposed to reflect alterations to the instructive signals. Although we are able to detect changes in the limb muscle pattern, the muscles of the perineal region were normally formed (Fig. 5J).

Proximal leg development is required for cloacal muscles

Our molecular analysis showed that the development of cloacal muscles is intimately linked to the development of the hindlimb muscle mass. We next wanted to test the role of limb development and verify to what extent cloacal muscle formation depends on the hindlimb.

To address this point, unilateral hindlimb primordium surgical ablations on chick embryos were performed between stages HH16 and HH23. These resulted in unilateral absence of the leg and ipsilateral absence of all cloacal muscles (Fig. 6A,B). Neither *mm. pubocaudales* nor tail muscles were affected (Fig. 6A-C), and there were no malformations of the cloacal and tail regions. The extent of the limb ablation correlated inversely to the development of cloacal muscles. Thus, whenever we observed some remnants of the proximal leg muscles reaching the vicinity of the cloacal tubercle, the sphincter complex did develop (data not shown).

Having established that total absence of the hindlimb field results in complete absence of cloacal muscles, we next examined the relationship between limb development and cloacal muscle formation in genetic models, which are free from operational artefacts.

First, we examined chicken *limbless* autosomal recessive mutants (Prahlad et al., 1979). These mutants form no visible limbs (Fig. 7A), owing to the failure of apical ectodermal ridge (AER) formation (Fallon et al., 1983). Limb-bud development is, however, initiated and parts of the pectoral and pelvic girdles form (Prahlad et al., 1979), as do a few muscles of the

proximal hindlimb (data not shown). However, we found that all cloacal muscles were completely absent (Fig. 7B) in *limbless*. These results show that cloacal muscles do not develop when the limb development programme is severely curtailed.

Next, we investigated the relationship between the formation of the cloacal muscles and the extent of limb development by studying the *p63*-null mutant mouse. Although like *limbless* this mutant lacks all stylopod, zeugopod and autopod elements, it retains the ability to form a pelvic girdle, which is considerably more developed (Mills et al., 1999) than that of *limbless*. Upon examination of the muscles with myosin heavy chain antibody in sections, the proximal thigh and the cloacal/perineal muscles were fully formed (Fig. 7C).

In conclusion, both surgical procedures and genetic mutants showed that the development of the cloacal muscles is dependent on the degree of growth of the proximal hindlimb.

Discussion

The chick/quail chimera system has served as an excellent technique with which to determine the origins of skeletal muscles. Furthermore, as many of the avian skeletal muscles have homologous structures in other species, these studies have greatly increased our understanding of the origin of mammalian muscles. Fate mapping of skeletal muscles has been performed in both the head and trunk of the organism. However, the caudal end has been neglected, partly because these tissues are not as readily amenable to experimentation. This has led to the cloacal muscles being regarded in the past as 'a special group of muscles' owing largely to the lack of information on their early development. Nevertheless in a classical comparative study, Nishi (Nishi, 1938) postulated that they probably originated from a limb muscle. In this study, we have identified the origin of the cloacal muscles and have also investigated the mechanism by which they develop. We show that cloacal and hindlimb muscles originate to a great extent from the same somites. Furthermore, we show that the cloacal muscle precursors invade the hindlimb mesenchyme through a process of cell migration before exiting the limb and then taking up their final position. Using a combination of surgical procedures and genetic mutants we have discovered a novel mechanism that shows that development of the cloacal muscles is dependent on normal proximal leg field formation.

Cloacal muscles are homologous to perineal muscles

This is the first study to confirm that the avian cloacal muscles are homologous to the perineal muscles of mammals. Homologies between species in comparative anatomy are based on anatomical resemblance and innervation, but mainly on the same developmental mechanism. We show both in chick and mouse that the early anlagen of the cloacal and perineal muscles derive from the ventral muscle mass of the hindlimb. Subsequently, these give rise to all the muscles around the cloaca the chick and the perineum in mouse. It follows that the situation in humans is very likely to be similar.

It has been previously suggested that the perineal muscles develop from a common precursor – a circular cloacal sphincter (Popowsky, 1899). We show that this is not the case, because the muscle arrives to the vicinity of the orifices at embryonic day 14. The cloacal membrane perforates in mouse by E14.0 and the gross septation of the cloaca is completed by this stage (Hynes and Fraher, 2004). Thus, the connection with its contraleteral counterpart occurs after splitting into the single muscular primordia around the orifices.

Multiple fate of somitic cells and dual fate of hindlimb muscle mass cells

Our somitic fate-mapping studies have determined that the four cloacal muscles originate from somites 30-34. Previous work, which is confirmed in this investigation, has shown that leg muscles originate from somites 26-33 (Lance-Jones, 1988; Rees et al., 2003). Therefore, cells from somites 26-34 have a number of developmental possibilities: to give rise to interlimb musculature (somites 26-29), to give rise to limb muscle (somites 26-33), to form both cloacal and limb muscle (somites 30-33), or to contribute only to cloacal muscle (somite 34). Furthermore, we know that the pelvicaudal muscles arise from somites 33 and caudally (P.V. and K.P., unpublished). Therefore, a major issue is how the same somites give rise to the musculature of two different structures.

Skeletal muscle development at hypaxial sites can occur through two mechanisms. Myogenic precursors can move as single migrating cells following the epithelio-mesenchymal transition from the dermomyotome or form part of an extension of the myotome (reviewed by Galis, 2001). The most parsimonious means of generating two separated populations of cells (one for limb and one for cloacal) would be either: (1) using different mechanisms of muscle development – migration being used for limb and myotomal extension for cloacal muscle; or (2) having two separate waves of migrating myogenic cells. Using a panel of genes and cell-labelling techniques we have not detected the presence of myotomal extension from somites 30-34 directed towards the cloacal tubercle. Furthermore, we have shown that the development of the cloacal/perineal muscles is dependent on cell migration by demonstrating that they do not develop in *met*$^{d/d}$ mutant embryos. Therefore, we can now state that the development of cloacal/perineal muscles is dependent on cell migration. The second possibility can also be discounted as our somite labelling studies have detected only one wave of cells delaminating from the somites and entering the hindlimb bud. Therefore, myogenic cells of the hindlimb and the cloaca migrate from the somites at the same time.

The two mechanisms postulated above for separating cloacal and limb muscle precursors can therefore be discounted. Segregation may instead be achieved through tissue patterning. From the results of our study, we suggest that myogenic cells enter the limb and coalesce in a single entity before dividing into the dorsal and ventral muscle masses (Amthor et al., 1998). All the cells of somite 34 would be part of the latter group. Cells of the ventral muscle mass are then further divided into those that will remain in the limb and those that will form cloacal muscle. Again, all the cells from somite 34 are destined to leave the limb. Cells from somites 26-29 will be distributed between the dorsal and ventral muscle mass but never allowed to participate in cloacal muscle development. Recent studies have shown that muscle precursors have intrinsic patterning properties and therefore it is possible that the fate and behaviour of the cloacal muscle cells is established prior to migration (Alvares et al., 2003; Mootoosamy and Dietrich, 2002).

Development of cloacal muscles from the ventral limb muscle mass

The whole process of cloacal muscle formation can be described in three steps: (1) migration of somitic myogenic cells into the ventral limb muscle mass; (2) translocation of the cloacal subpopulation from the limb to the cloaca; and (3) differentiation into four individual muscles.

It has been proved that myogenic cells migrate from the somites into the leg bud (Chevallier et al., 1977; Jacob et al., 1979). We have detected single spindle shaped cells that express *Pax3* but not *MyoD* during this initial phase of movement (Amthor et al., 1998). However, the situation is different as the cloacal anlage extends from the ventral muscle mass towards the cloacal tubercle. *Pax3*, *Pax7* and *MyoD* are expressed at the same level both at the leading and trailing edges of the cloacal anlage and thus resemble a myotomal extension. This situation is similar to the mode of development displayed by the pectoral muscles, which extend to gain attachment to the ventral midline.

The deployment of an extension mechanism as opposed to migration of individual precursors is supported by our observations of *SF/HGF* expression during this process (data not shown). *SF/HGF* expression in the early limb bud is well documented (Thery et al., 1995; Heymann et al., 1996; Scaal et al., 1999) and is responsible for the entry of *met*-expressing myogenic cells from the somites into the limb. The expression of *SF/HGF* is subsequently downregulated and is not present at all by the time of the movement of the cloacal anlage from the ventral muscle mass. Therefore, we can conclude that *SF/HGF* does not play a role in the cloacal muscle anlage extension to the cloacal tubercle.

Interestingly, the perineal muscles develop relatively normally in the *Lbx1* mouse mutant, whereas the migratory muscles of the distal hindlimb are absent. One interpretation of this phenotype is that the cloacal muscle progenitors use different mechanism for translocation compared with those that migrate to populate the distal leg. We propose that this occurs through mechanism resembling myotomal extension and is substantiated by the co-expression of *MyoD* and the Pax genes.

Formation of cloacal muscles is dependent on initiation and continued development of proximal limb field

We have demonstrated that unilateral ablation of the lateral plate mesoderm that forms leg results in the absence of ipsilateral cloacal muscles. These results, combined with the phenotype of the $met^{d/d}$ mutant embryos, suggest that the initiation of migration is an absolute requirement for the generation of precursors that will eventually develop into the cloacal muscles. Lateral plate mesoderm ablation experiments also show that muscle cells from the unoperated side do not cross over the midline to populate the cloacal region on the operated side.

The complete absence of cloacal muscles in the *limbless* chick mutant shows that not only the initiation, but also the subsequent early formation of the proximal limb field is required for the normal development of these muscles. Previous works have suggested that the limb development programme in these mutants is inhibited because of defects in dorsoventral patterning that prevent the induction of the apical ectodermal ridge (AER). These results stem from the finding that markers of dorsal identity extend into the ventral regions. In addition, application of a bead soaked in FGF results in the rescued outgrowth of a limb that has double-dorsal characteristics of the feathers (Ros et al., 1996; Grieshammer et al., 1996). These suggestions have been questioned by Laufer et al. (Laufer et al., 1997) who have demonstrated that the AER forms independently of dorsoventral patterning. In addition, our observations of the normal ventral pectoral muscles show that the dorsoventral patterning is not impaired at least in the proximal region. Therefore, although at present we do not know the mechanism behind the development of the *limbless* phenotype (McQueeney et al., 2002), it is beyond doubt that it does not develop an AER. The limb phenotype of *limbless* is much more severe than any produced by experimental ablation of the AER. Even the removal of AER precursors leads to the development of the proximal aspect of the femur (Rowe and Fallon, 1982). We have been able to repeat these findings and have found that even the removal of AER precursors from the developing limb bud does not prevent the development of the cloacal muscles (P.V. and K.P., unpublished).

The cloacal muscle phenotype of *limbless* could be explained by the absence of precursor migration from the somites. However, we show that this is not the case, as we show both dorsal and ventral muscle develop and are well formed. Yet there is no cloacal muscle development in *limbless*. As the cloacal musculature develops from the ventral muscle mass, loss of cloacal muscle could be explained by dorsalisation of all muscles. The presence of near-perfect pectoral muscle (ventral in identity) argues against this scenario. In order to understand why the cloacal muscles fail to form in *limbless* but are present in chicks after limb truncations, it is necessary to keep in mind the developmental kinetics of the anlagen that form these muscles. Our cell labelling studies of the leg bud have shown that cloacal muscles develop from the ventroproximal region of the muscle mass. The extension occurs after the base of the limb bud has expanded in all directions including towards the cloacal tubercle. After AER removal, the stump of the leg develops normally and therefore expands as usual. However, in the case of *limbless*, the expansion of the proximal hindlimb field is defective. This is not the case in the *p63* null mice where the proximal limb develops to a greater extent than in *limbless* and leads to the development of the perineal muscles. We therefore suggest the limb growth is required to bring the ventral muscle mass into range of signals that cause it to expand into the developing cloacal region. Therefore, the initiation of limb development, together with subsequent early growth, is required for cloacal muscle formation.

Thalidomide, AER removal, hindlimb and cloacal muscles development

Limb outgrowth can also be modulated through the use of pharmacological compounds or by tissue manipulations. Thalidomide was prescribed in the 20th century to alleviate the symptoms of morning sickness during pregnancy. However, it impaired limb development and babies born to mothers who took the drug displayed proximodistal truncations. Nevertheless, in all cases there is some degree of limb outgrowth. Furthermore, there are no reports of perineal muscle defects in individuals exposed to Thalidomide during

embryogenesis. In concordance with these observations, we have effected leg truncation through the removal of the AER. Leg AER or even apical ectoderm removal (precursor of AER) is not sufficient to stop the development of all limb elements. We have found normal cloacal muscle development in all cases of AER removal (P.V. and K.P., unpublished).

Concluding remarks

We show that the formation of cloacal sphincter muscle is dependent on limb development. Phylogenetically, however, cloaca appeared before limbs. Therefore, it will be intriguing to determine the mechanism used to form the cloacal muscles in pre-limb animals (as well as highly evolved limbless animals). One possibility is that they developed from local myotomal extensions, similarly to the rectus abdominis muscle of higher vertebrates. A more radical proposition would be that migration predates limb development. The combination of migration and differential growth has been also described (Neyt et al., 2000). We are currently investigating these possibilities.

Despite the derivation of cloacal/perineal muscles from the limb muscle mass, one should be aware of some important differences between the former and leg muscles. The motoneurons that innervate the cloacal/perineal muscles originate from the ventral horns of the spinal cord and the cell bodies are located in a specific region called Onuf's nucleus X. Their cells bodies show characteristic morphology (smaller than common motoneurons) and afferent connections. Furthermore, in pathological conditions such as in amyotrophic lateral sclerosis (ALS) or following poliomyelitis virus infection motoneurons of skeletal muscles are affected but the motoneurons of perineal muscles spared. However, they are strongly affected in a group of diseases that involve selectively the autonomic visceromotoneurons such as Shy-Drager syndrome (Schroder, 1985; Mannen, 2000). This might be a reflection of the mixed voluntary and involuntary nerve control mechanisms of the sphincter, but the underlying developmental cues remain to be elucidated.

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