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Dual origin and segmental organisation of the avian scapula

Ruijin Huang[1], Qixia Zhi[1], Ketan Patel[2], Jörg Wilting[1] and Bodo Christ[1,*]

[1]Anatomisches Institut der Universität Freiburg, Albertstrasse 17, 79104 Freiburg, Germany
[2]University of Reading, Zoology Division, Whiteknights, PO Box 228, Reading RG66AJ, UK
*Author for correspondence (e-mail: christb@uni-freiburg.de)

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SUMMARY

Bones of the postcranial skeleton of higher vertebrates originate from either somitic mesoderm or somatopleural layer of the lateral plate mesoderm. Controversy surrounds the origin of the scapula, a major component of the shoulder girdle, with both somitic and lateral plate origins being proposed. Abnormal scapular development has been described in the naturally occurring *undulated* series of mouse mutants, which has implicated *Pax1* in the formation of this bone. Here we addressed the development of the scapula, firstly, by analysing the relationship between *Pax1* expression and chondrogenesis and, secondly, by determining the developmental origin of the scapula using chick quail chimeric analysis. We show the following. (1) The scapula develops in a rostral-to-caudal direction and overt chondrification is preceded by an accumulation of *Pax1*-expressing cells. (2) The scapular head and neck are of lateral plate mesodermal origin. (3) In contrast, the scapular blade is composed of somitic cells. (4) Unlike the *Pax1*-positive cells of the vertebral column, which are of sclerotomal origin, the *Pax1*-positive cells of the scapular blade originate from the dermomyotome. (5) Finally, we show that cells of the scapular blade are organised into spatially restricted domains along its rostrocaudal axis in the same order as the somites from which they originated. Our results imply that the scapular blade is an ossifying muscular insertion rather than an original skeletal element, and that the scapular head and neck are homologous to the 'true coracoid' of higher vertebrates.

Key words: Scapula, Somite, Pax1, Sclerotome, Dermomyotome, Somatopleura

INTRODUCTION

The shoulder girdle has a double function. It provides stability for the upper limb, articulating with the humerus in the glenoid fossa. Secondly, it provides flexibility, which is of major importance for the bipode species with free upper limbs. Whereas, the pelvic girdle is a closed osseous circle, the shoulder girdle is an incomplete circle of skeletal elements, closed dorsally by skeletal muscle. In the human, the shoulder girdle is made up of the scapula and the clavicula, extending ventrally towards the sternum. The scapula forms laterally the glenoid fossa and the acromion, and ventrally the carocoid process. It develops through indirect ossification, which means that it is preformed by cartilage. In contrast, the clavicle is a dermal bone, developing by direct mesenchymal ossification.

The shoulder girdle of birds differs from the human in many aspects, due to the fact that birds need very large ventral pectoral muscles for flight. The major differences reside in the fact that the clavicle does not directly articulate with the sternum, and that an additional ventral bone is present spanning between the scapula and the sternum. This bone is commonly called the coracoid (Hamilton, 1952; Nickel et al., 1992). However, it is not homologous with the coracoid process of the human, but with the coracoid plate of fishes, and has therefore been called 'anterior coracoid' or 'procoracoid' (Romer, 1951). In fishes, birds, reptiles and monotremes, it serves as attachment for ventral muscles (Romer, 1951). We will therefore retain the term procoracoid. The procoracoid is the strongest bone in the avian shoulder girdle. It forms the major part of the glenoid fossa and articulates with the scapula, which only contributes to a small part of the glenoid fossa. The scapula is fixed to the vertebral column by rhomboid muscles and serves as attachment for dorsal muscles acting on the humerus. The scapula is a long thin bone (scapular blade), spanning the proximal shaft of all ribs. Its cranial end is slightly thickened and forms the acromion, the scapular head, which contributes to the glenoid fossa, and the coracoid tubercle, which connects the scapula with the procarocoid. Scapular head and scapular blade are connected by a thin part, the collum (neck) of the scapula (Baumel and Witmer, 1993).

The development of the scapula in mice is dependent on the function of the *Pax1* gene. *Pax1* is one of the nine members of a family of genes that contain the conserved sequence motif of the *paired-box* (Noll, 1993). *Pax1* is expressed in the sclerotomes and the chondrifying vertebral column, and also in the shoulder girdle (Deutsch et al., 1988; Timmons et al., 1994). In *undulated* mice in which the *Pax1* gene is mutated (Grüneberg, 1950; Balling et al., 1988), severe malformations of the vertebral column and the scapula have been observed (Wallin et al., 1994; Timmons et al., 1994). However, it is not known whether in mice the *Pax1*-expressing cells of the shoulder girdle are of somitic or of somatopleural origin.

Previous studies have demonstrated different origins of scapula-forming cells in lower and higher vertebrates. In the salamander *Ambystoma maculatum*, the extirpation of brachial somites results in total depletion of epaxial muscles, but leaves the scapula unaffected (Burke, 1991a). This suggests a somatopleural origin of the scapula. In contrast, in birds, the scapula has been demonstrated to be of somitic origin (Chevallier, 1977). This was shown by grafting experiments employing the quail-chick chimera technique (Le Douarin, 1969). According to Chevallier (1977), the scapula is derived from somites 15-24, whereas the clavicle, procoracoid, sternum and pelvic girdle are of somatopleural origin.

We have studied the development of the scapula of birds by various techniques. We have performed skeletal preparations to visualize the developing elements of the shoulder girdle. The expression of *Pax1* and *Pax3* was studied by double whole-mount in situ hybridization. The origin of the scapula-forming cells was investigated by grafting of single somites and somatopleura. More detailed studies were performed by grafting single sclerotomes and single dermomyotomes. Our results demonstrate that the scapula has a dual origin. The scapular blade is formed by cells from the dermomyotomes of somites 17-24. The neck and the head of the scapula are derived from the somatopleura. Irrespective of their origin, the scapula-forming cells express *Pax1*. Head and neck of the scapula may be homologous to the 'true coracoid', which replaces the procoracoid in higher vertebrates.

MATERIALS AND METHODS

Embryos

Fertilized eggs of the White Leghorn chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix*) were incubated at 80% relative humidity and 37.8°C. The embryos were staged according to Hamburger and Hamilton (1951).

Skeletal preparations

To investigate the chondrification of the scapula, whole embryos ranging from day 4 to day 9 were stained with Alcian blue as described by Wallin et al. (1994). Briefly, embryos were first skinned, fixed in 100% ethanol for 24 hours and then kept in acetone for another 24 hours. Incubation in the staining solution was performed for 3 days at 37°C. Then the specimens were rinsed in water and then in 20% glycerol in 1% KOH. They were then dehydrated in 50% and 80% glycerol, and finally stored in 100% glycerol.

Whole-mount in situ hybridization

All chick embryos were rinsed in phosphate-buffered saline (PBS) and then fixed overnight in 4% paraformaldehyde at 4°C. Then they were rinsed in PBS, transferred into 100% methanol and stored at –20°C. Sense and antisense *Pax1* RNA probes were labelled with digoxigenin-UTP as described previously (Wilting et al., 1995). For double in situ hybridization with *Pax1* and *Pax3* probes (a 645 bp fragment corresponding to nucleotides 468-1113 was a generous gift from Dr Martin Goulding), *Pax1* RNA was labelled with digoxigenin-UTP and *Pax3* with fluorescein-UTP, and whole-mount in situ hybridization was performed as described by Nieto et al. (1996). Shortly, after hybridization, embryos were first incubated with an alkaline phosphatase (AP)-conjugated anti-fluorescein antibody (Boehringer Mannheim, Mannheim, Germany) and the colour reaction was developed with NBT/BCIP (Boehringer Mannheim) to receive a blue signal. After inactivation of the AP by treatment with 30% acetic acid in methanol, the embryos were rehydrated in AP buffer, and the *Pax1* probe was detected with an anti-digoxigenin-AP antibody and Fast-red as substrate (Boehringer Mannheim).

Grafting procedure

To investigate the origin of the scapula, we carried out four series of experiments including 61 successfully operated embryos: transplantation of (1) somatopleura (6 experiments), (2) single somites (45 experiments), (3) single sclerotomes (4 experiments), and (4) single dermomyotomes (6 experiments).

Series 1

Transplantaion of somatopleura: the grafting procedure was performed on HH stage 12-13 quail and chick embryos in the prospective wing bud region. A strip of somatopleura was isolated together with its surface ectoderm using an electrolytically sharpened tungsten needle. The first cut was laterally from the Wolffian duct at the level of somites 15-21. Thereby the coelom was opened. Then the 2nd and 3rd cuts were performed transversally at the level of somites 15 and 21, from the medial to the lateral border of the coelom. Finally the somatopleura was completely isolated through a longitudinal cut along the lateral border of the coelom. It was then transferred to the chick host by means of a Spemann pipette, and implanted in its original orientation making use of Nile-blue labelling of the medial edge and the surface ectoderm of the grafts. The chick hosts were reincubated for 5-6 days.

Series 2

Transplantation of single somites: single somites of HH stages 13-15 quail embryos, ranging from somite 14 to somite 26, were grafted homotopically into chick hosts of the same stages. An average of three experiments was performed for each somite. Details of the grafting procedure were described in our previous studies (Huang et al., 1996; Zhi et al., 1996). The host embryos were reincubated for 6-8 days.

Series 3 and 4

Transplantation of single sclerotomes and dermomyotomes: dermomyotomes and sclerotomes of one somite (somite 22) were grafted in the same way as described previously (Huang et al., 2000). The host embryos were reincubated for 5-6 days.

Immunohistochemistry

The chimeras were fixed in Serra's fixative (Serra, 1946), dehydrated, embedded in paraffin and sectioned serially in transverse and coronal planes, at 8 μm. Quail cells were detected by an anti-quail antibody (QCPN) (dilution 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA) as a primary antibody, and an alkaline phosphatase-conjugated goat anti-mouse antibody (dilution 1:1000; DAKO, Hamburg, Germany) as a secondary antibody. Nitroblue tetrazolium (NBT) and X-phosphate (Boehringer Mannheim) were used as chromogens to reveal a blue signal. In the same sections, muscle cells were identified by a polyclonal anti-desmin antibody (dilution 1:400; Sigma, Deisenhofen, Germany) as a primary antibody, and peroxidase-conjugated goat anti-rabbit antibody (dilution 1:300; Sigma) as a secondary antibody. 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen to yield a brown signal. Double labelling was performed as described in our previous studies (Huang et al., 2000).

RESULTS

Pax1 expression and chondrification of the scapula

To demonstrate the molecular characteristics of cells that form the avian scapula, we determined the expression of *Pax1* and *Pax3* by two-colour whole-mount in situ hybridisation in the pectoral region and correlated this to cartilage condensation in

corresponding embryos. On day 4, *Pax1* expression in the scapula-forming mesenchyme is extremely faint, but there is a significant expression in the sclerotomes, the visceral arches and the anteroproximal border of the limbs (Fig. 1A). At stage 26 HH (day 5), a stripe of *Pax1* expression is located in the mesenchyme adjacent to somites 17-20 (Fig. 1B). Transverse sections show that these *Pax1*- positive cells (with no evidence of *Pax3* expression) are located in the mesenchyme close to the ectoderm and adjacent to the ventro-lateral lip of the myotome (Fig. 1C). In older embryos, the stripe of *Pax1*-expressing cells extends further caudally (Fig. 1D), so that at stage 30 HH (day 6.5) a stripe of *Pax1*-positive cells (distinct cranially, faint caudally) spans the entire thoracic region (Fig. 1E).

Skeletal preparations were performed by staining embryos with Alcian blue. On day 4, no chondrification can be detected in the scapula anlagen, whereas on day 5 the anlagen of the scapula, humerus, radius and ulnar are visible (Fig. 1F). The most cranial part of the scapula, the head, is located at the level of the most caudal cervical vertebrae (C13-C14). Whereas the chondrification process of the stylopod and zeugopod elements is fairly advanced, the programme of cartilage development in the scapula is still at an early stage and suggests that the proximal-to-distal development of cartilage does not include the shoulder elements. Studies on older embryos show that the scapula grows in a caudal direction. On day 6, the developing scapular blade spans the first two ribs (Fig. 1G), on day 7, the first three ribs (Fig. 1H), on day 8 the first four ribs (Fig. 1I), and on day 9 the first five ribs (Fig. 1J). In the adult chick, the scapular blade reaches the seventh rib. Therefore cranial-to-caudal chondrification of the scapula is preceded by the expression of *Pax1* in the corresponding region.

Grafting experiments

The origin of the scapula-forming cells was studied by grafting somatopleura and individual somites, from day 2 quail embryos homotopically into chick embryos. The host embryos were reincubated until days 7-10, and the quail cells were detected with the QCPN antibody, which specifically stains a nuclear epitope of quail cells (Wilting et al., 1995; Selleck and Bronner-Fraser, 1995). In contrast to previous studies (Chevallier, 1977), we observed a contribution of somatopleural cells to the scapula. This contribution is confined to the most cranial parts of the scapula, the head with its glenoidal part (Fig. 2A) and the neck (Fig. 2B). In addition, the somatopleure gives rise to the procoracoid and the humerus, and to connective tissue of the body wall and the limbs, but not to skeletal muscle (Fig. 2A,B). These results show the somatopleural cells contribution to the scapula is limited to the head and the neck, and there is no contribution to the scapular blade.

Since only the rostral portion of the scapula is of lateral plate mesodermal origin, we investigated the somitic contribution to the structure by homotopically grafting single quail somites into chick embryos. We observed that somites 14-16 did not contribute to the scapula. The most rostral somite to contribute to the formation of the scapula is somite 17. However cells from this somite only contribute to the rostral portion of the scapular blade and not to the head or the neck of the scapula. Single somite grafts show that somites 17-24 all contribute to the scapular blade. Derivatives of somite 25 are not found in the scapula. Therefore the scapula has a dual origin, with the head and neck being formed from cells of the somatopleure and the scapular blade composed of somitic cells. We determined the distribution of somitic cells in the scapula anlagen and found that the distribution of the cells in the scapula along its rostrocaudal axis is the identical to the order of the somites from which they have originated. Hence, cells from the rostral contributing somites are in the rostral portion of the scapular blade (Fig. 3A) and cells from somite 24 contribute only to the distal tip of the scapula (Fig. 3B). Furthermore almost all cells from individual somites are concentrated to unique regions along the rostrocaudal axis of the scapular blade (Fig. 3A,B). Sharp rostral and caudal boundaries (Fig. 3A) demarcate the zone of cells, with very few cells found outside their respective zone. Thus cells of the scapular blade not only maintain the positional order of the somites from which they originate but, by limiting mixing between somitic populations, the scapular blade develops into a segmentally organised structure. The results are schematically illustrated in Fig. 3C. The segmental organisation not only involves the scapula proper, but also the inserting muscles, which are mainly derived from the corresponding segment (Fig. 3B).

We determined the specific somitic compartment from which the scapular blade cells originate. The epithelial somites give rise to the dermomyotomes and the sclerotomes (Christ and Ordahl, 1995). We demonstrate that the early scapula anlagen expresses *Pax1* and previous work has shown that this gene is expressed in the sclerotome (Deutsch et al., 1988). To study which of the two compartments is the source of scapula-forming cells, we first grafted individual somites composed of sclerotome of quail origin with the dermomytome of chick. The sclerotome cells form the meninges, vertebral body, neural arch and connective tissue, but there is no contribution to the scapula (Fig. 2E). We subsequently grafted the somite at the same level composed of a sclerotome from chick and the dermomyotome from quail. This procedure not only reveals quail cells in the scapula but also in adjacent skeletal muscle and connective tissue (Fig. 2F). These results represent the first evidence of a dermomyotomal contribution to the development of bone.

DISCUSSION

The development of the scapula has been a matter of debate, because different results have been obtained in experimental studies on chick, turtle and salamander embryos (Yntema 1970; Chevallier, 1977; Burke, 1991a,b). Somite-grafting experiments have revealed the origin of the chick scapula from somites 15-24, but it has not been determined which of the somitic compartments are the source of scapula-forming cells (Chevallier, 1977). Ablation of brachial somites in the salamander *Ambystoma maculatum* produced severe defects of the epaxial muscles, but left the scapula unaffected (Burke, 1991a). This could mean that there the scapula is of somatopleural origin, or that cervical somites are the source of scapula-forming cells. The latter has been observed in the turtle (Yntema, 1970; Burke, 1991b). Extirpation of cervical somites has resulted in the depletion of the scapular blade. At least in amniotes (turtle, chick), there has been conformity that the scapula is of cervical and/or brachial somitic origin. Our

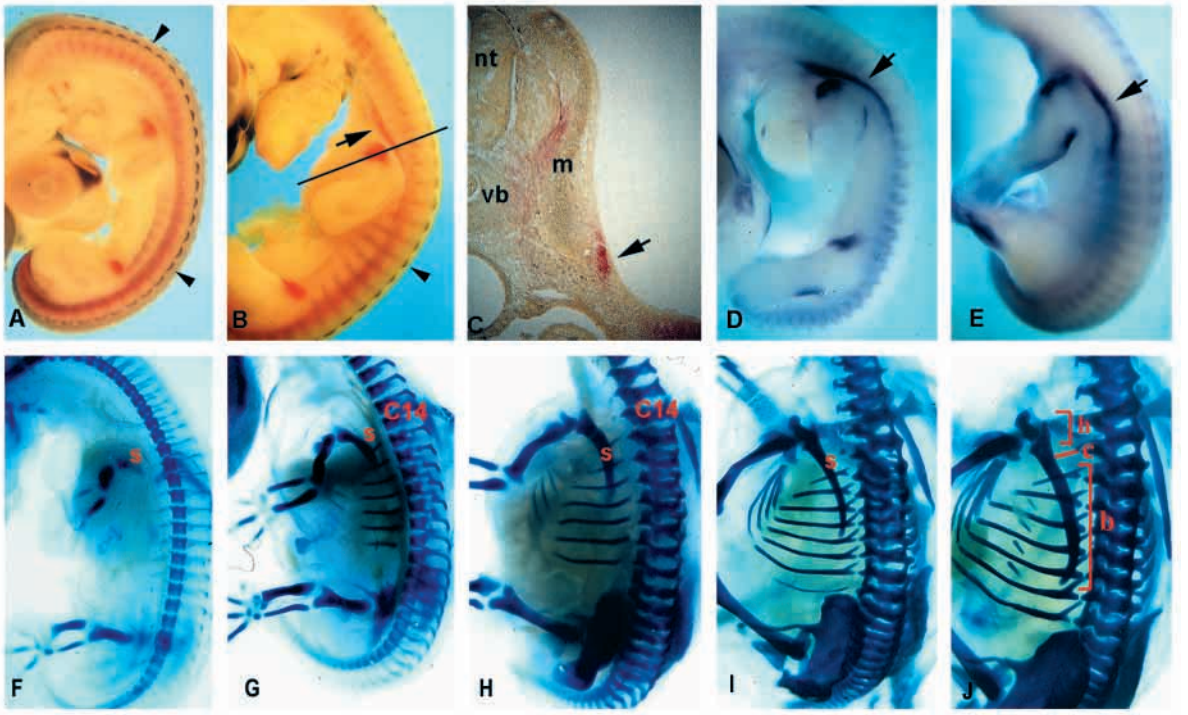

Fig. 1. Expression of *Pax* genes and chondrogenesis of the scapula. (A) Stage 24 HH (day 4) embryo shows *Pax3* (blue) staining in the myotomes (arrowhead), and *Pax1* (red) in the sclerotomes, pharyngeal arches and anteroproximal borders of the limb buds. The scapula-forming region is hardly visible. (B) Stage 26 HH (day 5) embryo showing *Pax3* expression (blue) in developing muscles (arrowhead), and *Pax1* (red) in the scapula-forming region (arrow). (C) Section of the specimen shown in B. The anlage of the scapula expressing *Pax1* (arrow) is located beneath the ectoderm near the *Pax3*-expressing ventrolateral lip of the myotome (m). nt, neural tube; vb, vertebral body. (D) Stage 27 HH (day 5.5) embryo. The *Pax1*-expressing anlage (blue) of the scapula (arrow) has extended caudally. (E) Stage 30 HH (day 6.5) embryo. The *Pax1*-positive anlage of the scapula (arrow) spans the whole thoracic region. Alcian-blue staining of whole embryos reveals the chondrifying skeletal elements of day 5 (F), day 6 (G), day 7 (H), day 8 (I) and day 9 (J) chick embryos. The anlage of the scapula (s) is visible from day 5 onwards. The cranial end is located in the region of the 13th to 14th cervical vertebrae (C14). The scapular blade grows in a caudal direction, spanning the proximal shafts of the ribs. b, scapular blade; c, scapular collum; h, scapular head.

studies on chick embryos now show that the scapula has a dual origin. The cranial part, head and neck, are of somatopleural origin, whereas the major part, the scapular blade, is derived from somites 17-24. The slight discrepancy to the findings of Chevallier (1977) may be due to different grafting techniques. Whereas we have grafted single somites, Chevallier (1977) has grafted blocks of segmental plate mesoderm.

Our results illustrating the capacity of the dermomyotome to form bone are consistent with the findings of Tajbakhsh et al. (1996) who demonstrated that preventing somitic muscle progenitors from executing their myogenic programme results in them responding to positional information from their local environment and adopting non-muscle fates, including cartilage. Results from this and previous work can be integrated into a mechanism that elucidates the development of the scapular blade from dermomyotomes at a precise axial level. Previous work has shown that migrating *Pax3*-expressing cells of somitic origin are only detected at the level of the tongue and limb levels and go on to execute a myogenic programme of differentiation. However, cells in the scapula-forming region are *Pax1* rather than *Pax3* positive and this gene specifies somitic cells to a cartilage lineage. Classical experiments from Kieny et al. (1972) have shown that cervical somites transplanted into the thoracic region did not form ribs or scapula whereas transplantation of cervical presomitic mesoderm into the same region resulted in the loss of rib formation but caused a normal scapula to develop. These results suggest that the sclerotome is determined prior to segmentation, whereas the scapular blade/nonscapular blade fate is determined by local cues after segmentation. In the same study, transplantation of thoracic somites into the cervical region resulted in development of ectopic ribs, but scapula development was not seen. Again, environmental cues are likely to be important in the development of the scapular blade from thoracic somites. We suggest that the development of the scapular blade is dependent not only on the presence of an inductive signal from the lateral plate but also upon the competence of somites to respond to this signal. Since the hypaxial domain of the dermomyotome normally expresses *Pax3*, lateral plate signals in the scapular-blade-forming region may instruct adjacent somitic cells to downregulate this gene and suppress their myogenic fate. Subsequently, switching on the expression of *Pax1* would allow them to acquire a new developmental programme. The development of a skeletal element (the scapular blade) from a compartment (the dermomyotome) that gives rise to all of the skeletal muscle of the trunk (Christ et al., 1977; Christ and Wilting, 1992) implies that the scapular blade is not a skeletal element proper but an ossifying muscle attachment. The segmental organisation of the scapula could be generated by positional information

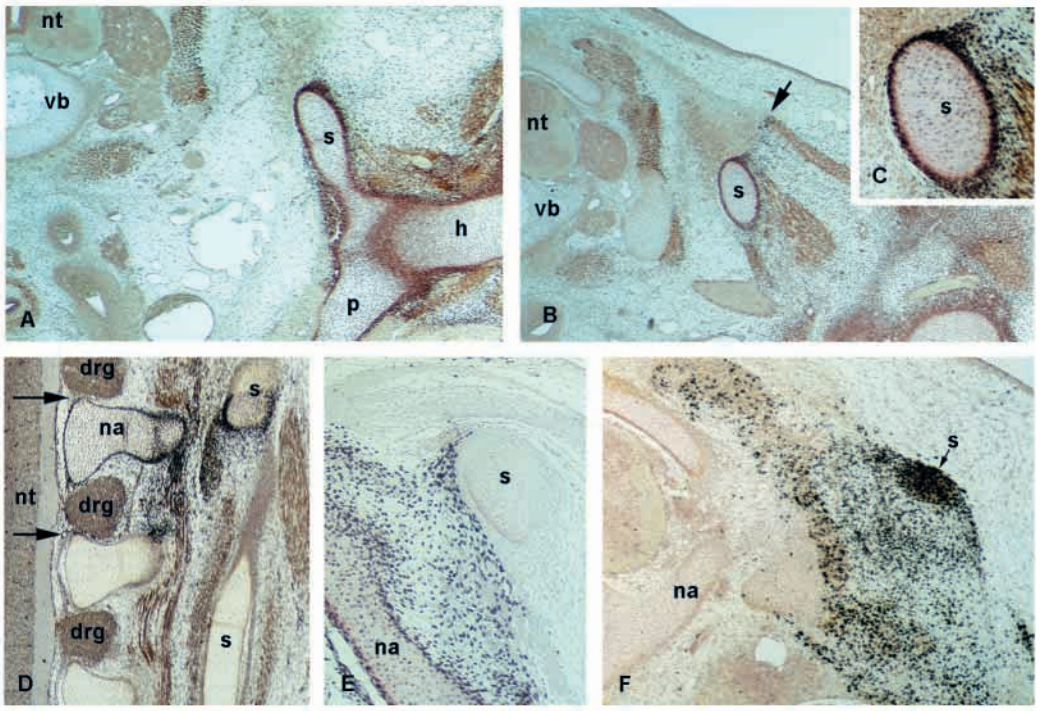

Fig. 2. Origin of the scapular head and contribution of the sclerotome and dermomytome to the scapular blade. Staining of quail cells with the QCPN antibody (A-F) and of skeletal muscle with an anti-desmin antibody (A-D) in quail-chick chimeras. (A-C) Chick embryo which received a quail somatopleura graft. Reincubation 6 days. (A) Transverse section showing quail origin of the scapular head (s), the procoracoid (p) and the humerus (h). nt, neural tube; vb, vertebral body. (B) Transverse section showing quail origin of the neck of the scapula (s). Arrow, border between somitic and somatopleural region. (C) Higher magnification of B, showing quail cells (blue) in the scapular neck. (D) Representative coronal section showing the results after grafting of somites 22 from a quail into a chick embryo. Reincubation 7 days. The lamina of one neural arch (na) and the adjacent segment of the scapular blade (s) are made up of quail cells. The scapular blade is sectioned twice because of its curved shape. drg, dorsal root ganglion. Arrows, borders of one somitic segment. (E) Homotopical grafting of the sclerotome of somite 22. Reincubation 6 days. The neural arch (na) and adjacent connective tissue are of quail cells, the scapular blade (s) is formed by chick cells. (F) Homotopical grafting of the dermomyotome of somite 22. Reincubation 5 days. The scapular blade (s) is formed by quail cells, the neural arch (na) by chick cells.

possibly by utilising unique combinations of *Hox* gene expression for each somite (Kessel and Gruss, 1991) contributing to the scapular blade and we note that somites 17-24 are rich in *Hox* expression boundaries (Burke et al., 1995; Gaunt et al., 1999).

There are remarkable similarities between the segmental organisation of the scapula and the segmental organisation of the neural-crest-derived cranial skeleton (Köntges and Lumsden, 1996). Firstly, whereas the progenitors arise from segmentally organised tissue (scapular blade from somites and neural crest from rhombomeres) the final structures are not segmental. Secondly, in both sites, the order of cells in the differentiated tissues is the same as the order of the progenitors. Thirdly, cells with differing fates originating from the same segment execute their ontogenic programme as an integrated unit. Thus, in both cases, connective tissues derived from a specific segment attaches to skeletal elements originating from the same region. Our results suggest that, in the case of the scapular blade, not only does the bone and connective tissues for each scapular segment originate from an individual somite but so does the attaching muscle. Therefore, solely permitting interactions between tissues with similar positional information would insure precise connections between muscle, connective tissue and bone along the scapular blade.

The results in this study have evolutionary implications regarding the development of the shoulder girdle**.** The endoskeletal shoulder girdle carries the limb, which articulates in the glenoid fossa; this is formed by a dorsal skeletal element,

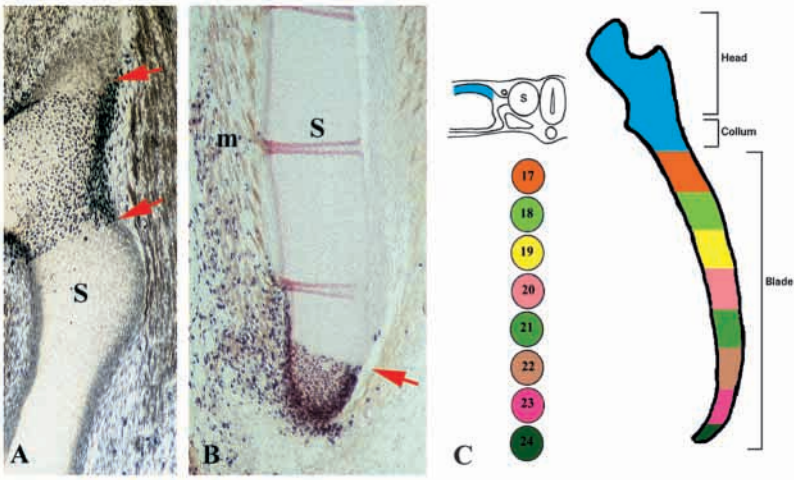

Fig. 3. Segmental organisation of the scapular blade. Staining of quail cells with the QCPN antibody showing (A) the segmentally organised contribution of somite 21 and (B) somite 24 to the scapular blade. Note the tight boundaries between labelled and unlabelled tissues (red arrows). In addition, B shows the co-ordinated distribution of muscle, connective tissue and cartilage from an individual somite. (C) Schematic representation of the origin of the scapula. The somatopleure layer of the lateral plate mesoderm is represented in blue. Coloured circles represent somites with numbers referring to their axial position.

the scapular blade, and a ventral element, the coracoid plate also termed ‘anterior coracoid’ or ‘procoracoid’ (Romer, 1951). During the development of terrestrial life, there was an obvious need to stabilise the glenoid fossa. In mammal-like reptiles and in primitive mammals, the monotremes, a third endoskeletal element is present, the ‘true coracoid’ (Kardong, 1995). This forms much of the glenoid fossa and successively replaces the procoracoid. An ossification centre has been observed in all three elements. In birds, however, the endoskeletal shoulder girdle has only two ossification centres, one in the scapular blade and one in the procoracoid (Hamilton, 1952). We have now shown that the scapula has a dual origin. The scapular blade is derived from the dermomyotomes, whereas the parts that participate in the formation of the glenoid fossa, the head and neck of the scapula, are of somatopleural origin. The separate origin of this part of the scapula suggests that it is the anlage of a separate skeletal element, located in the position of the true coracoid of reptiles. We therefore suggest that the anlage of a true coracoid is present in the chick, but has not been detected, because it does not possess an ossification centre.

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