Dual origin and segmental organisation of the avian scapula

Ruijin Huang¹, Qixia Zhi¹, Ketan Patel², Jörg Wiiting¹ and Bodo Christ¹,*

¹Anatomisches Institut der Universität Freiburg, Albertstrasse 17, 79104 Freiburg, Germany
²University of Reading, Zoology Division, Whiteknights, PO Box 228, Reading RG66AJ, UK

*Author for correspondence (e-mail: christb@uni-freiburg.de)

Accepted 8 June; published on WWW 9 August 2000

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 vertebrae.

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 coracoid 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 procoracoid. 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 goat anti-mouse antibody (dilution 1:1000; DAKO, Hamburg, Germany) as a secondary antibody, and peroxidase-conjugated goat anti-rabbit antibody (dilution 1:1000; Sigma, Deisenhofen, Germany) as a primary antibody, and 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).

**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**

Transplantation 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 a chromogen 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.

**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 anteroposterior 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). 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 somatopleural origin, or that cervical somites are the source of scapula-forming cells. The latter has been observed in the turtle (Yntema, 1970; Chevallier, 1977; Burke, 1991a,b). 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

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 dermomyotome 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
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 Kiency 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/non-scapular 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 Willting, 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
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,
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 monotrems, 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.

We are indebted to Dr Martin Cohn for helpful discussion and comments. We thank Mrs S. Antoni, Mrs E. Gimbel, Mrs L. Koschny, Mrs U. Pein, Mrs M. Schüttoff and Mr G. Frank for their excellent technical assistance, and Mrs U. Uhl for typing of the manuscript. The QCNP antibody was obtained from the Developmental Studies Hybridoma Bank, under contract NO1-HD-6-2915. This study was supported by grants from the Deutsche Forschungsgemeinschaft (R. H., B. C.) and the Wellcome Trust (K. P.).

REFERENCES


