The formation of the avian scapula blade takes place in the hypaxial domain of the somites and requires somatopleure-derived BMP signals

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Abstract

The avian scapula is a long bone located dorsally on the thorax. The cranial part that articulates with the upper limb is derived from the somatopleure of the forelimb field, while the caudal part, the scapula blade, originates from the dermomyotomes of brachial and thoracic somites. In previous studies, we have shown that scapula blade formation is intrinsically controlled by segment-specific information as well as extrinsically by ectoderm-derived signals. Here, we addressed the role of signals derived from the lateral plate mesoderm on scapula development. Chick–quail chimera experiments revealed that scapula precursor cells are located within the hypaxial domain of the dermomyotome adjacent to somatopleural cells. Barrier implantation between these two cell populations inhibited scapula blade formation. Furthermore, we identified BMPs as scapula-inducing signals from the somatopleure using injection of Noggin-producing cells into the hypaxial domain of scapula-forming dermomyotomes. We found that inhibition of BMP activity interfered with scapula-specific Pax1 expression and scapula blade formation. Taken together, we demonstrate that the scapula-forming cells located within the hypaxial somitic domain require BMP signals derived from the somatopleure for their specification and differentiation.

Keywords: Chick; Embryo; Chick–quail chimera; Somite; Dermomyotome; Somatopleure; Scapula; BMP

Introduction

Somites are the first visible segmental embryonic structures, located on both sides of the axial organs, the neural tube and notochord. They arise from the unsegmented paraxial mesoderm as epithelial spheres and include cells of chondrogenic, myogenic, dermogenic and angiogenic potential. Each somite becomes subdivided into a dorsal compartment, the dermomyotome, and a ventral one, the sclerotome. In most of the somites, compartmentalization leads to a restriction of chondrogenic cells to the sclerotome (reviewed in Christ et al., 2000, 2004; Huang et al., 2000). In brachial and thoracic somites, however, dermomyotomes contain not only angiogenic, dermogenic and myogenic cells (for review see Christ and Ordahl, 1995; Christ et al., 2000; Scaal and Christ, 2004), but also chondrogenic cells for the scapula (Huang et al., 2000).

The avian scapula is a long, rod-shaped bone extending craniocaudally and spanning the dorsal aspect of the thorax. According to its function and origin, the avian scapula can be subdivided into a cranial and a caudal part. The cranial part, forming the acromion, the glenoid fossa and the coracoid tubercle, is derived from the somatopleure, while the caudal part, the long scapula blade, which is fixed to the vertebral column by the rhomboid muscles and serves as attachment for muscles moving the humerus, originates from the dermomyotomes (Huang et al., 2000). The scapula anlagen express Pax1 and Sox9 (Ebensperger et al., 1995; Hofmann et al., 1998; LeClair et al., 1999; Healy et al., 1999). This expression and the following chondrification proceed in a cranio-to-caudal direction (Huang et al., 2000).

The intrinsic scapula-forming potential of dermomyotomal cells has been investigated in a previous study (Ehehalt et al., 2004). After heterotopic transplantation of dermomyotome
anlagen, we observed scapula-specific Hox gene expression and an additional scapula-specific cartilage originating from the grafted dermomyotome anlagen. These results indicate that the scapula-forming potential of dermomyotomal cells coded by Hox genes is a prerequisite of scapula blade formation in the chick embryo. Hoxb5 homozygous mouse mutants display an anterior shift of the scapula (Rancourt et al., 1995).

Since the scapula and the forelimb are functionally inseparable, the scapula can be incorporated into the hypaxial structures in terms of its function. However, scapula and wing develop independently. This is demonstrated clearly in limbless chick mutants which display severe limb truncation but normal scapula formation (Pralhad et al., 1979). Furthermore, scapula development seems to be independent of axial signaling which has been shown after surgical ablation of more, scapula development seems to be independent of axial signaling in the hypaxial domain of the somite.

Signals originating from ectoderm and somatopleure are required for the differentiation of hypaxial muscles (Pourquie et al., 1996; Dietrich et al., 1998). Moreover, ectodermal signals have been shown to be involved in scapula blade formation. Without surface ectoderm, the scapula-specific Pax1 expression is not switched on and the scapula blade as well as its associated muscles are not formed (Prols et al., 2004; Ehhardt et al., 2004). In this study, we investigated the role of non-ectodermal hypaxial signals on scapula development, namely signals from the lateral plate mesoderm.

Using chick–quail chimeras, homotopic transplantation experiments were performed to determine the localization of scapula precursor cells within the epithelial somite. Implantation of a physical barrier and application of the BMP-antagonist Noggin were performed to investigate the influence of signals from the somatopleure on dermomyotomal cells with respect to scapula blade formation. Our results demonstrate that the scapula precursor cells are located within the hypaxial domain of the somites, where they receive BMP signals emanating from the adjacent somatopleure, that are required for their Pax1 expression and subsequent chondrogenic differentiation.

Materials and methods

Embryos

Fertilized White Leghorn chick (Gallus gallus) and Japanese quail (Coturnix coturnix) eggs were incubated at 80% relative humidity and 37.8°C. The embryos were staged according to Hamburger and Hamilton (1992), henceforth referred to as “HH-stage”.

Microsurgeries

Homotopic transplantation

Dorsomedial and dorsolateral quarters of one or two consecutive epithelial scapula-forming somites (somite 19 and 20, somite stage II–III) were removed in a chick host at HH-stage 14. The corresponding somite quarters of a stage-matched quail embryo (donor) were transferred to the chick host and placed into the prepared gap. After 2 to 6 days of reincubation, the chimeras were harvested, transversally sectioned and analyzed with immunohistochemistry.

Barrier implantation between matured scapula-forming somites and somatopleure

For this operation, chick embryos were incubated to HH-stage 16–20. At scapula-forming level (Somites 17–24), a tungsten needle was pushed through the ectoderm and somatopleure into the coelom and a longitudinal cut was set in the most medial edge of the coelom without damaging the ventral visceropleura. An aluminium foil was inserted into the prepared slit. The operated embryos were reincubated for 3–6 days.

Injection of Noggin-expressing cells

Noggin-expressing CHO B3 cells were a kind gift of Professor Richard Harland, University of California, Berkeley. Cells were cultured and harvested as described in our previous study (Nimmagadda et al., 2005). The cell injection was undertaken at HH-stage 20–22 in chick embryos. Cells were injected into the subectodermal mesenchyme over the lateral part of dermomyotome in the scapula-forming region at the level of somites 16–19 and somites 19–23, respectively. Before injection, an anterior–posterior tunnel was made in the subectodermal mesenchyme. Thereafter, concentrated cell suspensions were injected into the tunnel using a mouth controlled micropipette with about 50 μm in diameter. Thus, injected cells formed a cell-rod (about 50 μm in diameter and 4–5 segments in length) in the subectodermal mesenchyme. After the injection, the embryos were reincubated for 2–4 days for in situ hybridization and skeletal staining.

Whole-mount in situ hybridization

Whole-mount in situ hybridization with cPax1 (Ebensperger et al., 1995) was performed as described by Nieto et al. (1996) with modifications described by Stolle et al. (2002).

Skeletal preparations

Whole-mount Alcian blue staining was used to investigate the skeletal pattern of the shoulder girdle. Specimens were stained with 0.015% Alcian blue in 80% ethanol and 20% acetic acid for one to several days, fixed and dehydrated in ethanol for 1 day, cleared and stored with 100% methylsalicylate (Kant and Goldstein, 1999). This method provides the possibility of consecutive paraffin sectioning followed by immunohistochemistry.

Immunohistochemistry

Immunohistochemistry of histological sections was performed as described previously (Huang et al., 2003). Quail cells were detected with a monoclonal QCPN-antibody (Developmental studies Hybridoma Bank, Iowa City, IA, USA). A polyclonal anti-desmin-antibody (Sigma, Deisenhofen, Germany) was used for identification of muscle cells. Choice of second antibodies and color reactions lead to a brown signal for desmin including cells and a blue signal for nuclei of quail cells. Ultimately, sections were counterstained with Nuclear Fast Red (Sigma, Deisenhofen, Germany).

Results

The scapula blade is derived from dorsolateral somite quarters

According to its derivatives and its exposition to inductive signals, the somite can be subdivided along a mediolateral axis into an epaxial (medial half) and a hypaxial (lateral half) compartment. To determine whether the scapula is derived from either the epaxial or the hypaxial compartment, we produced chick–quail chimeras in which individual somite parts of the chick were replaced with equivalent quail tissue. Since the epaxial dermomyotome has been found to be derived from the dorsomedial somite quarter and the hypaxial dermomyotome from the dorsolateral quarter (Ordahl and Le Douarin, 1992), dorsomedial or dorsolateral quarters of one or two epithelial thoracic somites were homotopically
transplanted from 2-day quail embryos into stage-matched chick embryos (Fig. 1A).

After 2 days of reincubation, quail–chick chimeras were sectioned transversally. In all cases, quail cells from the dorsolateral somite quarter were found to be located in the hypaxial domain contributing to the subectodermal mesenchyme, myotome and ventrolateral dermomyotomal lip (n = 6). The subectodermal mesenchyme made up of grafted quail cells was restricted to a very narrow area located at the boundary between trunk and ventrolateral body wall (Fig. 1B).

After 6 days of reincubation, chimeras were analyzed to establish whether quail cells derived from either dorsomedial or dorsolateral somite quarter grafts contribute to the scapula blade. Transplantation of the dorsomedial somite quarter never gave rise to scapula cartilage (n = 6, data not shown). However, we did detect a few quail cells in the scapula perichondrium in two specimens (data not shown—see Discussion). After transplantation of the dorsolateral somite quarter, quail cells generated cartilage and perichondrium of the scapula blade (n = 7, Figs. 1C, D). In addition, intercostal muscles, limb muscles and part of scapula associated muscles were found to consist of grafted quail cells. We could not observe any quail cells in the vertebrae.

Taken together, these results indicate that the scapula blade-forming cells are located in the dorsolateral somite quarter. Thus, we consider the scapula blade to be a hypaxial derivative of the somite.

**Scapula blade formation was affected by barrier implantation between matured somites and somatopleure**

Signals emanating from the somatopleure have been shown to be important in hypaxial myogenesis (Pourquie et al., 1996; Dietrich et al., 1998). To investigate the role of the somatopleure in scapula blade formation, we implanted a barrier between mature scapula-forming dermomyotomes and the somatopleure at HH-stage 16–19 (n = 20; Fig. 2A).

In six cases, the barrier kept its mediolateral position for the duration of the reincubation period (6 days). In these specimens, we found that the scapula blade was absent medial to the barrier (Fig. 2C). In all cases, the distal parts of ribs were lacking, which is consistent with the observations made by Sudo et al. (2001). The wing skeleton was present without malformations in three specimens (data not shown). Three specimens showed limb truncation in addition to the scapula blade defects. Vertebræ development was undisturbed in all cases.

Transverse sections of the successfully operated specimens showed that implantation of the lateral barrier had a specific effect on scapula chondrogenesis. Cartilage of the scapula blade was not formed on the operated side. Although the hypaxial muscles were reduced, they developed in normal positions (Fig. 2D). Thus, the operation did not hinder the scapula-attaching muscles from finding their final position, but rather prevented differentiation of the scapula progenitor cells.

The other specimens showed a laterally displaced barrier during the reincubation period and, consequently, no developmental defects in the scapula blade were observed in these samples (data not shown).

**Scapula-specific Pax1 expression and scapula blade formation were affected by injection of Noggin-expressing cells**

BMPs are important inductive signals emanating from the somatopleure required for the lateralization of the epithelial somites and formation of hypaxial muscles. To test the function of BMP signals on scapula formation, we blocked lateral BMP signaling using Noggin-expressing cells injected into the scapula-forming region at HH-stage 20–22 in 4-day-old chick embryos (Fig. 3A). The operated embryos were reincubated for 2 days for in situ hybridization with Pax1, which is considered as an early marker for cells that differentiate into scapula blade cartilage. It is expressed in scapula-forming cells shortly before chondrification occurs (Huang et al., 2000). Scapula formation was analyzed in the operated embryos after a reincubation period of 4–6 days following alcian blue staining of cartilage.

The normal scapula-specific Pax1 profile resembles a long and narrow rod-like zone. After Noggin-expressing cell injection into the subectodermal mesenchyme at the level of somites 16–19, scapula-specific Pax1 expression was not...
detected at limb level (n = 30, Figs. 3B, C). If the cells were injected at the level of somites 19–23, the expression domain of Pax1 was truncated (n = 16, Figs. 3D–F). Injection of control cells did not change the scapula-specific Pax1 expression (n = 15, data not shown). These results reflect the segmental organization of the scapula blade (Huang et al., 2000).

Transverse sections of 8-day-old operated embryos demonstrated that scapula cartilage failed to form on the operated side. It is very interesting to notice that only the serratus muscle, one of the scapula muscles, was missing. However, other hypaxial muscles were not affected by Noggin-producing cell injection (Figs. 3J, K).

Taken together, after blockage of BMP signaling by Noggin, the scapula-specific Pax1 expression was down-regulated and the scapula blade cartilage was missing. These results indicate that BMP signaling is required for the scapula-specific Pax1 expression and scapula blade formation.

Discussion

In this study, we investigated the origin of the scapula blade precursor cells and the effects of signals emanating from the somatopleure on scapula formation. Our results show that the scapula-forming cells are localized in the dorsolateral somite quarters and the induction of Pax-1 as a prelude to cartilage development requires BMP signals that originate from the somatopleure.

Hypaxial location of scapula precursor cells within the somite

The scapula is a part of the shoulder girdle that serves for the stability and dynamics of the limb. Hence, the scapula may be regarded as a hypaxial skeletal element. The human scapula is a triangular flat bone. In birds, the scapula is a long bone, consisting of a cranial and a caudal part. The caudal part, the scapula blade, has been shown to be of somitic origin, and develops uniquely from the dermomyotome (Huang et al., 2000). The first part of this study was aimed at determining whether the scapula progenitors originate either from the epaxial or hypaxial domain of the dermomyotome.

Myogenic cells derived from the somite form muscles of the back, ventral body wall and limbs (reviewed in Christ and Ordahl, 1995). According to the innervation and topography, the back muscles can be described as epaxial muscle and the ventral and limb muscles as hypaxial muscle. Epaxial and hypaxial muscles originate from different progenitors within the somite. Using the chick–quail nucleolar marker system and microsurgery, Ordahl and Le Douarin (1992) demonstrated that epaxial muscle originates from the medial somite half, and that limb muscle is derived from the lateral half. In terms of muscle development, the medial somite half can be considered as epaxial and the lateral half as hypaxial domain of the somite.

In this study, we determined whether the scapula blade is derived from the medial or lateral somite half. Since the dorsal somite half is the precursor of the dermomyotome, we only grafted either the dorsomedial or dorsolateral somite quarters, which develop to medial and lateral dermomyotome, respectively. The results showed that the scapula blade was derived from the grafted dorsolateral part of the somite. This means that the scapula precursors are located within the lateral dermo-
Fig. 3. Blockage of BMP signals using Noggin-cell injection interferes with scapula blade formation. (A) Operation schema. Noggin-producing cells (red circle) were injected into subectodermal mesenchyme (sem) at the lateral edge of the myotome (m). (B, C) Pax1-expression of an embryo after Noggin-cell injection at the level of somites 16–19 and 2 days reincubation. Scapula-specific Pax1 expression was absent at the levels of somites 16–19 (S19) at the injection side (C). The remaining Pax1 expression was significantly weaker than that on the control side (B). (D–F) Pax1-expression of an operated embryo after Noggin-cell injection at the level of somites 19–23 and 1.5 day reincubation. The scapula-specific Pax1-expression extends at this stage to the level of somite 20 (S20) on the control side (D), while this reach only to the level of somite 18 to 19 on the operated side (E). Two white arrows indicate the extension of scapula-specific Pax1-expression. (F) Cross-section through the level of somite 19. The scapula-specific Pax1-expression (red arrows) cannot be detected on the operated (right) side. (G–K) An operated embryo after a reincubation period of 4 days. (G) Noggin-cells were injected into the somites 19–23. The scapula cartilage (between white arrows) extends from the level of the last cervical vertebra to the level of the fourth rib on the control side (left), while the cranial part of the scapula blade is missing between two white arrows on the operated side (right). (H, I) An operated embryo after Noggin-cell injection at the level of somites 19–23. (H) The normal extension of the scapula at the control side (left side) was pointed by two white arrows. (I) Two white arrows indicate the remaining cranial part of the scapula. (J, K) Cross-section through the first thorax vertebra of the embryo in panel G. The control side (J) shows the normal development of the scapula blade (S) as well as both epaxial (ep) and hypaxial (lat, ser) muscles. On the operated side (K), the scapula blade and an attaching muscle (ser) are missing. The epaxial and other hypaxial muscles are completely normal. h: humerus, nc: notochord, lat: M. latissimus dorsi, nt: neural tube, scl: scleromtome, ser: M. serratus, vb: vertebral body.
myotome. The observation that a few cells from the grafted dorsomedial somite part occasionally contributed to the perichondrium of the scapula blade might indicate that the scapula precursor cells are located within the dorsolateral somite quarter in close vicinity to the dorsomedial quarter. In other words, the precursors must be located just laterally adjacent to the midline separating the somite into a medial and lateral half (Fig. 4).

After a short reincubation period, we observed that quail cells from the dorsolateral somite part formed the lateral part of the myotome and the lateral dermomyotome lip. A part of the subectodermal mesenchyme located at the lateral somitic border originated also from the lateral somite half. The scapula precursor cells detected by Pax1 expression are located between ectoderm and myotome at the lateral somitic border (Huang et al., 2000). Hence, the scapula progenitors must be located within this subectodermal mesenchyme.

Based on their lateral location, the scapula precursor cells are likely to receive signals from the lateral mesoderm at a short distance, whereas they are at a considerable distance from the influence of axial signals. In Shh mouse mutants, the scapula develops completely normal (Chiang et al., 1996). Microsurgical ablation of axial organs did not affect the scapula formation in the chick (Teillet et al., 1998). Furthermore, after barrier implantation between axial organs and somites, the scapula developed to a nearly normal shape and size (Ehehalt, 2003). The independence of scapula formation from axial signals supports our finding that the scapula is not only functionally but also developmentally a derivative of the hypaxial domain of the somite.

**Dependence of scapula blade formation on BMPs emanating from the somatopleure**

As assayed above, the scapula blade precursor cells differentiate medially adjacent to the lateral plate mesoderm. Due to this position, scapula blade formation is likely to be influenced by lateral signals. Our results show that isolation of scapula-forming somites from the somatopleure results in an absence of the scapula blade medial to the barrier. This indicates that without signals from the somatopleure, somites cannot form the scapula blade. BMP is a somatopleure-derived signal, participating in somite lateralization and hypaxial muscle formation (Pourquie et al., 1996; Dietrich et al., 1998). In this study, inhibition of BMP activity by Noggin resulted in the down-regulation of the scapula-specific Pax1 expression. Subsequently, the scapula blade could not form. The results lead us to conclude that the scapula-specific Pax1 expression and subsequently the scapula formation are induced directly or indirectly by BMP signals emanated from the somatopleure.

This is a very interesting paradox: BMP signals induce Pax1 expression in the scapula-forming cells within the dorsal somitic compartment, the dermomyotome, whereas they inhibit this in the ventral somitic compartment, the sclerotome (McMahon et al., 1998). The explanation of this paradox remains to be resolved.

In this study, hypaxial muscle formation was not interrupted by either barrier implantation between somite and somatopleure or the introduction of Noggin-producing cells at stages when the experiments were performed in this study. However, previous work has shown that BMPs do influence hypaxial muscle development (Pourquie et al., 1996). It is important to note that this mechanism is liable at stages much earlier than those investigated in this study.

Taken together, our results imply that the scapula-forming cells and the myogenic cells within the hypaxial domain are induced by the same signals, but the induction of these two different cell types occurs at different stages. The myogenic differentiation can be detected by myogenic regulation factors, like MyoD, Myf5, in 4-day-old chick embryos (Dietrich et al., 1998; Teillet et al., 1998), while expression of early markers of cartilage-forming cells begins in 5-day-old chick embryos (Healy et al., 1999; Huang et al., 2000). Furthermore, we observed that scapula formation was disrupted if Noggin-producing cells were injected directly into that site of the subectodermal mesenchyme from which the scapula blade arises. This observation suggests a temporal control mechanism on the differentiation of different cell lines within the hypaxial dermomyotome.

**A model of dermomyotomal chondrogenesis**

Heterotopic transplantations of presumptive dermomyotomes of the scapula-forming somites have suggested that...
dermomyotomal cells have got the intrinsic segment-specific potential to form cartilage during somite formation (Ehehalt et al., 2004). Furthermore, the ability of somite cells to form scapula and ribs is not restricted to either dorsal or ventral somite cells, respectively. After either dorsoventral rotation of segmental plate or exchange of dorsoventral position of somite halves, ventral cells that were experimentally displaced into the dorsal compartment were able to form scapula cartilage, while dorsal cells that were dissociated into the ventral compartment formed ribs (Dieuguie Fomenou et al., 2005). This indicates that all somite cells at the thoracic level have got the potential to form cartilage of either scapula or ribs during somite formation, irrespective of their position in the somite. Whether they differentiate into scapula or rib is determined by local environmental signals during somite maturation.

Our recent work and this study have shown that ectodermal and somatopleural signals are among the inductive signals required for scapula blade formation (Proksch et al., 2004; Ehehalt et al., 2004). Results of ablation of the surface ectoderm at different stages have shown that ectoderm over newly formed somite regulates the development of all dermomyotomal derivatives, while ectoderm over mature somites specifically influences scapula formation (Ehehalt et al., 2004). Hence, the role of ectodermal signals is likely to depend on the developmental stage.

Among ectodermal signals are Wnt5 and Wnt-antagonists (reviewed by Roelink, 1996; Baranski et al., 2000; Cauwenberghs et al., 2001). Wnt6 is expressed in the ectoderm over epithelial somites and the hypaxial domain of the developing dermomyotome (Rodriguez-Niedenfuhr et al., 2003). A recent study has shown that when Wnt signaling is enhanced, somites up-regulate Pax3-expression and the epithelial state of the somite and the dermomyotome is maintained (Schmidt et al., 2004). By overexpression of Wnt signals, cells in the scapula-forming region fail to express Pax1 and as a long-term effect, the scapula blade is absent (Moeller et al., 2003). These observations indicate that an epithelial-to-mesenchymal transition is essential for the execution of the cartilage-forming process. The epithelial organization of the somite and dermomyotome might be broken down by the Wnt-antagonist, Frzb-1, that is expressed in the surface ectoderm overlying the scapula-forming region (Baranski et al., 2000). After an epithelial-to-mesenchymal transition, the scapula-forming cells become mesenchymal and translocate into the subectodermal space. BMP signals induce these cells to express Pax1 transcripts. It remains to be resolved whether ectodermal and somatopleural signals act synergistically to control scapula formation.

In summary, we propose the following model for scapula blade formation: In a first step, somite cells in the brachial and thoracic region gain scapula-forming potential by Hox gene expression during somite formation (Ehehalt et al., 2004; Dieuguie Fomenou et al., 2005). In a second step, cells emigrate from the dermomyotome dorsally to form subectodermal mesenchyme. In a third step, cells of the lateral cell population of this mesenchyme will be activated to realize their intrinsic scapula-forming program by signals from the somato-pleure (Fig. 4) and the surface ectoderm. This process considered as dermomyotomal chondrogenesis appears different from other somitic chondrogenic processes occurring in sclerotome derivatives.

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