Neural crest contribution to the avian shoulder girdle and implications to girdle evolution in vertebrates

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Abstract

Neural crest (NC) is an established source for many endochondral and intramembranous bones in the skull and postcranial skeleton in vertebrates. Neural crest cells also contribute to the trapezius/cleidohyoideus muscle attachment sites on the shoulder girdle of the mouse, where they are found in the scapula, clavicle, and sternum. In the avian shoulder girdle, NC cells from the level of the first two cranial−most somites were only found so far in the clavicle, while in the axolotl, the NC contribution to the shoulder girdle was not found at all. In this study we aimed to determine whether NC cells caudal to the level of the second somite contribute to the cartilaginous shoulder girdle in birds and to analyse the phylogenetic distribution of NC cells in the vertebrate shoulder girdle. Homotopic quail to chick embryos and GFP+ to wild type chick embryos transplantations of the neural tube including presumptive NC, as well as immunohistochemical detection of NC markers, such as HNK-1 and PDGFRα revealed no contribution of NC cells from somite levels 3−27 to the skeletal elements of the shoulder girdle, including, but not restricting to muscle attachment sites, despite abundance of other NC derivatives. Thus, in birds, NC does not contribute to the formation of the cartilaginous shoulder girdle. The negative result is discussed in a broad evolutionary aspect. It supports the notion of the uniqueness of NC contribution to the variety of endochondral bones in mice (or mammals). In other vertebrates, including birds, only the cells of the cranial NC seem to migrate to the shoulder girdle and contribute to the intramembranous clavicles and/or interclavicle. We critically evaluate the existing hypotheses on evolution of NC contribution to the shoulder girdle in vertebrates.

Keywords: neural crest, shoulder girdle, clavicle, scapula, evolution, chick, vertebrates, chimeras, HNK-1, PDGFRα

Introduction

The shoulder girdle of vertebrates consists of endochondral and intramembranous bones of various embryonic origin (Figure 1). Osteostraci,the most advanced group of jawless vertebrates, where elements of the shoulder girdle are already present, the skeleton of the forelimbs and the endoskeletal shoulder girdle is attached to the head shield composed of dermal (exoskeletal) elements (Coates 1994). Therefore it was suggested that the dermal shoulder girdle of vertebrates probably originated from the head bony shields of ostracoderms (McGonnell 2001). The neural crest (NC), a unique character of vertebrates was suggested to play a significant role in head evolution (Gans and Northcut 1983), and the skull itself (particularly, neurocranium) is a mixture of intramembranous and endochondral bones, which are often, NC and mesodermal in origin, respectively

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(Gross and Hanken 2008). Dual origin of the shoulder girdle, from NC and from mesoderm has been, therefore, expected to reflect the evolution of the head-trunk interface once occupied by the head shield. This interpretation considers the exoskeletal part of the shoulder girdle as a hind portion of the ancestral head bony shield.

This traditional view has been challenged in recent years with the development of a number of transgenic model systems, demonstrating explicitly that the NC-mesoderm interface and the intramembranous bone–endochondral bone division do not coincide in the skull and in the shoulder girdle (Matsuoka et al. 2005; Valasek et al. 2010; Davidian and Malashichev 2013) — see Fig. 1 for a schematic mapping. In the mouse, cells from post-otic NC were found in the neck connective tissue and skeletal elements of the shoulder girdle—scapular spine, coracoid process, acromion, both the intramembranous and the endochondral part of the clavicle, as well as in the manubrium of the sternum (Matsuoka et al. 2005; Valasek et al. 2010). In contrast, there is no evidence for a NC contribution to the cleithrum (dermal shoulder girdle) in zebrafish, suggesting a mesodermal origin (Kague et al. 2012). Most recently, the cartilaginous viscerocranium of amphibians, which for a long time was considered as originating exclusively from the hyoid and pharyngeal streams of the head NC, has been shown to be of dual origin, with some midline elements developing from the lateral plate head mesoderm in contrast to NC-derived homologous structures of fish, birds, and mammals (Davidian and Malashichev 2013). Hence, there is no reason anymore to assume that neural crest and mesoderm have different skeletogenic capacities in that they preferably form skeletal elements of one type, and vice versa, that endochondral or intramembranous mode of development of a skeletal element is a reliable marker of its embryonic origin.

Matsuoka and co-authors (Matsuoka et al. 2005), suggested a "muscle scaffold" model, which would predict the embryonic origin of skeletal elements in the shoulder girdle based on the embryonic origin of connective tissue of the muscles entering to a particular skeletal element, and which, in principle, could replace the classical views, which are lacking support from contemporary experimental evidence. According to the model, the pattern of attachment of muscles to the shoulder girdle in vertebrates is more conserved than the mode of ossification of the underlying skeletal elements. Therefore, the attachments of cucullaris/trapezius/cleidohyoid muscles, whose connective tissue originate from NC can serve as predictors of the corresponding embryonic origin of the skeletal element to which the muscle attaches. For example the cleithrum of lower vertebrates evolved to the scapular spine in mammals. This idea actually dates back to the very end of XIX century and is based on some early observations of the ossification of the scapula in marsupials (Broom 1900), later not supported, and accepted neither for marsupials (Sanchez-Villagra and Maier 2003), nor for monotremes (Klima et al. 1973).

The "muscle scaffold" model, and particularly, the idea of homology of cleithrum and the scapular spine, as a consequence, had provoked discussion from the point of view of comparative anatomy and palaeontology (Sánchez-Villagra and Maier 2006; Epperlein et al. 2012). Particularly, it was further pointed out to an inconsistency between the variability of the trapezius muscle attachment sites across gnathostome taxa and the conservatism of its attachments postulated by the model, etc. (Sánchez-Villagra and Maier 2006). More recently, the lack of NC contribution to the entire shoulder girdle or connective tissue of attaching muscles of the axolotl was shown by transplantation of neural folds from GFP+ to white embryos, suggesting that at least to some species the "muscle scaffold" model is not applicable (Epperlein et al. 2012). The latter evidence was more in agreement to the idea of uniqueness of mammals, possessing apomorphic shoulder girdle elements partially built with NC cells (Sanchez-Villagra and Maier 2003; Sánchez-Villagra and Maier 2006). Finally, the most recent discovery that in zebrafish, the cleithrum — a central element of the "muscle scaffold" model — is unlikely to originate from NC (Kague et al. 2012), casts even more doubts on this evolutionary scenario.

Nonetheless, the discussion of the evolution of the NC within the shoulder girdle is far from resolved since the actual distribution of the NC cells in the intramembranous and endochondral shoulder elements of various vertebrate taxa remains understudied. Little is known about the NC contribution to the shoulder girdle even in birds, let alone diverse taxa of fish, amphibians, and reptiles. The early work on embryonic origin of the shoulder elements of the chick, based on quail to chick transplantation technique, has suggested only mesodermal contribution (Chevallier 1977), although at that time a potential NC contribution was not considered. Later, a small population of cranial NC cells migrating from the neural tube at the level of the first two (cranial) somites to the region of the developing furcula was found by expression of NC marker *Mafb* and long-term cell labelling techniques (McGonnell et al. 2001). A part of those migrating NC cells differentiate later to intramembranous bone. Furthermore, it was shown that trunk NC in birds has also certain skeletogenic potential in an appropriate environment (Nakamura and Ayer-le Lievre 1982; McGonnell and Graham 2002), although there was a lack of experimental evidence that this potential can be realized in formation of any parts of the postcranial skeleton, and particularly, in the shoulder girdle.

To address the existing gap in the knowledge of distribution of NC cells in the skeletal tissues of the avian

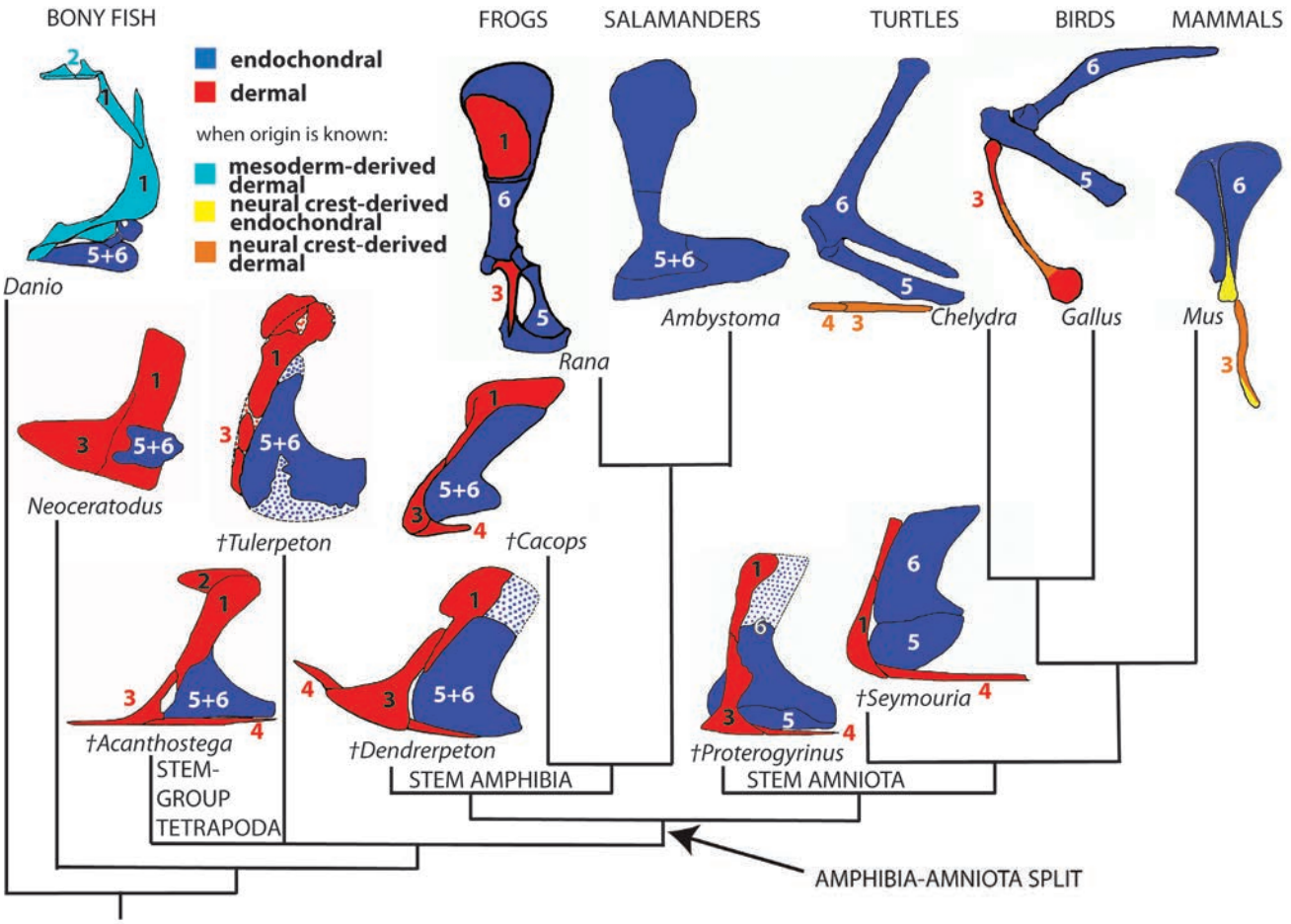

Fig. 1. Current view of shoulder girdle evolution and NC/Mesoderm contribution to bony and cartilaginous elements.

In bony fish (Grandel and Schulte-Merker 1998; Johanson et al. 2004) and stem-group tetrapods (Heatwole and Carroll 2000; Lebedev and Coates 2008) (left and lower parts of the cladogram) the dermal [red and sky blue] elements predominate. They are composed generally of a series of cleithra [1] and connect the endochondral [deep blue] scapulocoracoid [2] to the skull. This dermal connection has been gradually reduced in clades including modern amphibians and amniotes, so that the endochondral elements predominate in the shoulder girdle (Coates et al. 2008). Among modern tetrapods only frogs possess cleithrum. In amniotes only dermal clavicles [3] and an interclavicle [4] may be present. Urodelan amphibians (e.g., *Ambystoma*) have lost dermal bones completely. The endochondral scapulocoracoid of tetrapods develops a coracoid plate or coracoid [5] and an extended scapular blade or scapula [6]. This development of the high scapular blade has already took place in stem-group amphibians and stem-group amniotes (Heatwole and Carroll 2000). Stem-group tetrapods usually had no developed scapula blade, a more pronounced one was found in *Tulerpeton* (Lebedev and Coates 2008).

It is generally assumed that the tetrapod endochondral limb girdles develop from the lateral plate mesoderm and mesodermal somites, whereas dermal bone may probably arise as a neural crest derivative. These were commonly accepted hypotheses, which have been rarely experimentally verified. In the mouse, the neural crest-derived cells have been shown as participants of all muscle attachment points for neck and shoulder muscles connecting the shoulder girdle to the skull (Matsuoka et al. 2005). The neural crest appeared to contribute not only to the dermal bone (e.g., dermal part of the clavicle, as in birds (McGonnell, McKay, and Graham 2001), but to a number of endochondral elements of the mammalian shoulder girdle, e.g., manubrium sterni, scapular spine, endochondral part of the clavicle. In frames of the "muscle scaffold theory" (see text for more detail) the fish and amphibian dermal bone, the cleithrum, was homologized to the discovered neural crest population within the endochondral scapula of the mouse (Matsuoka et al. 2005). This generalization, however, is not fully correct since completely endochondral shoulder girdle and muscle attachments in the shoulder-neck region of *Ambystoma* do not include contributions from neural crest (Epperlein et al. 2012). This evidence is further complemented by information from fish (Kague et al. 2012; Mongera et al. 2013). In *Danio*, the dermal cleithral series of bones was found to be of mesodermal, rather than neural crest origin. Hence, the neural crest population of cells, which take part in building in muscle-skeletal connections in the mouse shoulder girdle may be a synapomorphy of mammals or autapomorphy of the mouse. These neural crest derived elements in the shoulder girdle of the mouse, cleithra of fish and amphibians might be not homologues.

†, extinct groups; shoulder girdle models shown from a lateral view with head to the left, not to scale.

shoulder girdle, we made an effort to identify both the cranial and the trunk NC cells (with special reference to levels caudal to somite 2, which were not addressed by previous work — see McGonnell, McKay, and Graham, 2001) within the avian endochondral shoulder girdle by applying two experimental approaches. First, we performed homotopic and homochronous transplantations of the pieces of the neural tube from quail donor embryos to chick hosts, and from GFP+ to white chick embryos, followed by immunofluorescent visualization of the transplanted tissues. Second, by using NC markers (Human Natural Killer-1) HNK-1 and (Platelet Derived

Growth Factor — Receptor alfa) PDGFRα, suitable for the chick model, we followed NC cells at different stages of development of the chick embryo, in order to find any transient populations of the NC (either cranial or trunk) in the developing cartilaginous shoulder girdle. Despite an extensive labelling of the NC, we revealed no evidence of NC contribution to any part of the avian shoulder girdle. These data are discussed in the framework of existing hypotheses on the evolution of the NC and the shoulder girdle in vertebrates.

Materials and Methods

Generation of quail/chick and transgenic GFP+/white chick chimeras

Fertilized eggs of white domestic chick (*Gallus gallus domesticus*); and Japanese quail (*Coturnix japonica*) were obtained from the local suppliers (Plemreproductor "Nasia" and Leningrad Zoo respectively). Eggs were incubated at 38° C and 80–85% humidity.

The general approach to the transplantation of quail/chick and transgenic/non transgenic chick embryos to produce chimeras was the same with the only difference that in the former we transplanted both the left and the right halves of the neural tube fragments, while in the latter only the right side half of each neural tube segment was transplanted. Grafting experiments from quail to chick embryos were performed at somite levels 1 to 27. Neural tube fragments corresponding to approximately four somite lengths were transplanted. Firstly, the neural tube fragment was cut out from the chick embryo with tungsten needles and was replaced with the same part of the neural tube isolated from a quail embryo. The graft was transferred to the chick host with a Pasteur pipette. All transplantations were performed with stage-matched quail and chick embryos. For GFP+/white chick chimeras we used transgenic chick embryos expressing cytoplasmic GFP under control of the beta-actin promoter (kind gift of Dr H. Sang, Roslin Institute, UK) (McGrew et al. 2004). Surgery was carried out at 8–15 HH stages of development, depending on the axial level of grafting (Table 1). After the transplantations, the chick chimeras were reincubated until 4 to 9 days of development.

Embryo processing and immunostaining

The embryos were staged according to Hamburger and Hamilton normal tables (Hamburger and Hamilton 1951). Embryos of quail-chick chimeras and chick embryos from 4 to 7 days of development were fixed in 4% PFA at 4°C overnight. After washing in PBS (two times, each for 2h) embryos were embedded in gelatin (20%)/sucrose (15%) solution and were frozen on dry ice. Embryos were stored at -80°C. 15μm transverse and frontal sections were made through the region of the shoulder girdle with a cryostat Leica CM3050S.

On serial cryosections of quail-to-chick chimeras, which were analysed independently by two researchers, we followed all the cartilaginous elements of the shoulder girdle, paying special attention to the cranial aspects of each element and muscle attachment sites, where NC cells were ever found by other investigators in chick or mouse (McGonnell, McKay, and Graham 2001; Matsuoka et al. 2005). The transgenic chimeras were embedded into paraffin. Paraffin sections of 10 μm thickness were prepared as standard procedure and were analysed independently by three researchers.

To visualize transplanted NC in quail-to-chick chimeras we used the monoclonal QCPN antibodies against quail antigens (Developmental Studies Hybridoma Bank, USA) with secondary goat-anti-mouse antibodies conjugated to Alexa 488 fluorochrome (ab150113, ThermoFisher Scientific, dilution 1:500). For transgenic chimeras we used the natural GFP fluorescence enhanced with anti-GFP primary polyclonal antibodies (A11122, Invitrogen, dilution 1:300) with secondary goat-anti-rabbit antibodies conjugated to Alexa 488 fluorochrome (A-11034, Molecular Probes, dilution 1:300) oranti-GFP antibodies conjugated to Alexa 488 fluorochrome (A21311, Invitrogen, dilution 1:300). Additionally, we used rhodamine-conjugated anti-Myosin heavy chain antibodies (clone 4A. 1025, a kind gift from Simon Hughes, Kings College, London) to visualize skeletal muscles on some sections. To visualize NC marker molecule expression in chick embryos we used monoclonal anti-HNK-1 (ab8597, Abcam, dilution 1:200) and polyclonal anti-PDGFRα primary antibodies (ab61219, Abcam, dilution 1:200) with secondary goat-anti-mouse antibodies conjugated to Alexa 488 (A11001, Molecular Probes, dilution 1:300), and secondary goat-anti-rabbit antibodies conjugated with Cy3 (111–165–008, Dianova, dilution 1:300) or Alexa 555 (A21428, Molecular Probes, dilution 1:300) fluorochromes, respectively. High resolution panorama images were prepared with a PTGui v.9.1.16 Pro software (New House Internet Services B. V., Rotterdam, The Netherlands).

Ethics statement

This study included the study of chick embryos, but only involved grafting experiments done in early embryos, collection of tissues for fixation, and histological and anatomical analyses; hence this work was done using widely approved methods for treating chick embryos to reduce suffering and thus does not require any formal approval by an ethics committee. The European Directive 86/609/EEC states that fetal animals in the third trimester of development are protected by law. This di-

rective, however, does not apply to our study, because the embryos we used for transplantation experiments were early neurula to somitogenesis stage embryos (days 1–3 of development), whereas we followed up the neural crest cells in chick or chimeric embryos until 9 days of incubation. Since in the chick the embryos develop 21 days, all the embryos treated or sacrificed for evaluation of the experiments had far from reached the protected development stages and passed not more than half of their embryogenesis.

Results

Quail-to-chick and transgenic chick chimeras do not reveal NC cells in the avian shoulder girdle

In 23 out of 36 survived quail-to-chick chimeras (Table 1), the neural tube transplants were successfully incorporated to the host neural tube and the coloured quail feathers containing donor's melanocytes were visible, since the recipient chick embryos were a white line. Apart from the transplant itself, donor quail cells formed characteristic NC derivatives such as spinal ganglia, and myelinated sheaths of nerves (Figure 2A).

The previous work revealed presence of cranial NC cells from the level of somites 1 and 2 in the medial aspect of the avian furcula (McGonnell, McKay, and Graham 2001). On the other hand, we did not find any cells of NC origin in the furcula either in its medial or lateral parts even in chimeras (N = 3), in which the transplantation has been made relatively cranial, at the level of somite 3–6 (Fig 2B). In other 20 chimeric embryos, in which operations were made more caudally, we had the same result.

We have further considered scapula and sternum as potential places where NC cells could hypothetically form neck muscle attachment sites, since they were previously found in acromion and coracoid process, as well as in the cranial aspect of the sternum in the mouse (Matsuoka et al. 2005). Cartilage and surrounding connective tissue of these elements were carefully investigated on serial sections in all the 23 chimeric embryos from the cranial to the most caudal aspect and we did not find a single cell of NC origin (Fig. 2C-F). The sternum in birds is a large skeletal element, to which neck musculature inserts and the flight muscles originate from. Despite this, we found no NC contribution to the muscle attachment sites or cartilage in this element including keel (Fig. 2E, F), as well as to the joints between the sternum and coracoids (Fig. 2E), or the sternum and ribs (data not shown). The same is true for coracoids (exemplified at two different levels in Fig. 1C, E; other levels — data not shown). To summarize, on serial sections through all cartilaginous elements of the shoulder girdle in 23 quail-to-chick chimeras, we did not find any cells originating from the donor NC.

To ensure that the negative result obtained with the method of quail-to-chick chimeras, is not an artefact due to transplantation or donor cell visualization techniques used in the study, we performed an additional series of transplantations using a modern chimerization model: GFP+ transgenic chick to white chick embryonic tissue transplantation (see Methods).

As expected, the GFP+ cells were present in the grafts, central nervous system (data not shown), as well as in the adjacent spinal ganglia and myelinated sheaths of spinal nerves (Fig. 3A). However, despite modifications in techniques, the analysis of paraffin histological sections again revealed no NC cells in the cartilaginous scapula (Fig. 3A, B) and coracoid (data not shown), as well as anlagen of other elements of the girdle and the connective tissues (data not shown) in eight transgenic chimeras, which were younger than eight days of incubation (Table 2).

Table 1. The level of neural tube (NT) transplantation and survival rate in quail-chick chimeras

Somitic level of the NT graft	Stage HH	No. of somites formed	No. grafts	No. of survived embryos	Number of embryos with NT graft preserved
< 3	< 8	< 4	23	0*	–
3–6	8–9	5–7	14	5	3
7–10	9–10	7–10	13	8	5
11–14	11–12	10–13	11	7	4
15–18	12–13	16–19	13	5	5
19–22	13–14	19–22	10	5	3
23–27	15	24–27	13	6	3
At all levels			97	36	23

* Zero survival rates for the embryos operated at cranial levels may be due to a greater developed neural tube at those levels at the time of surgery, which is therefore more prone to damage during transplantation of relatively large pieces of neural tissue.

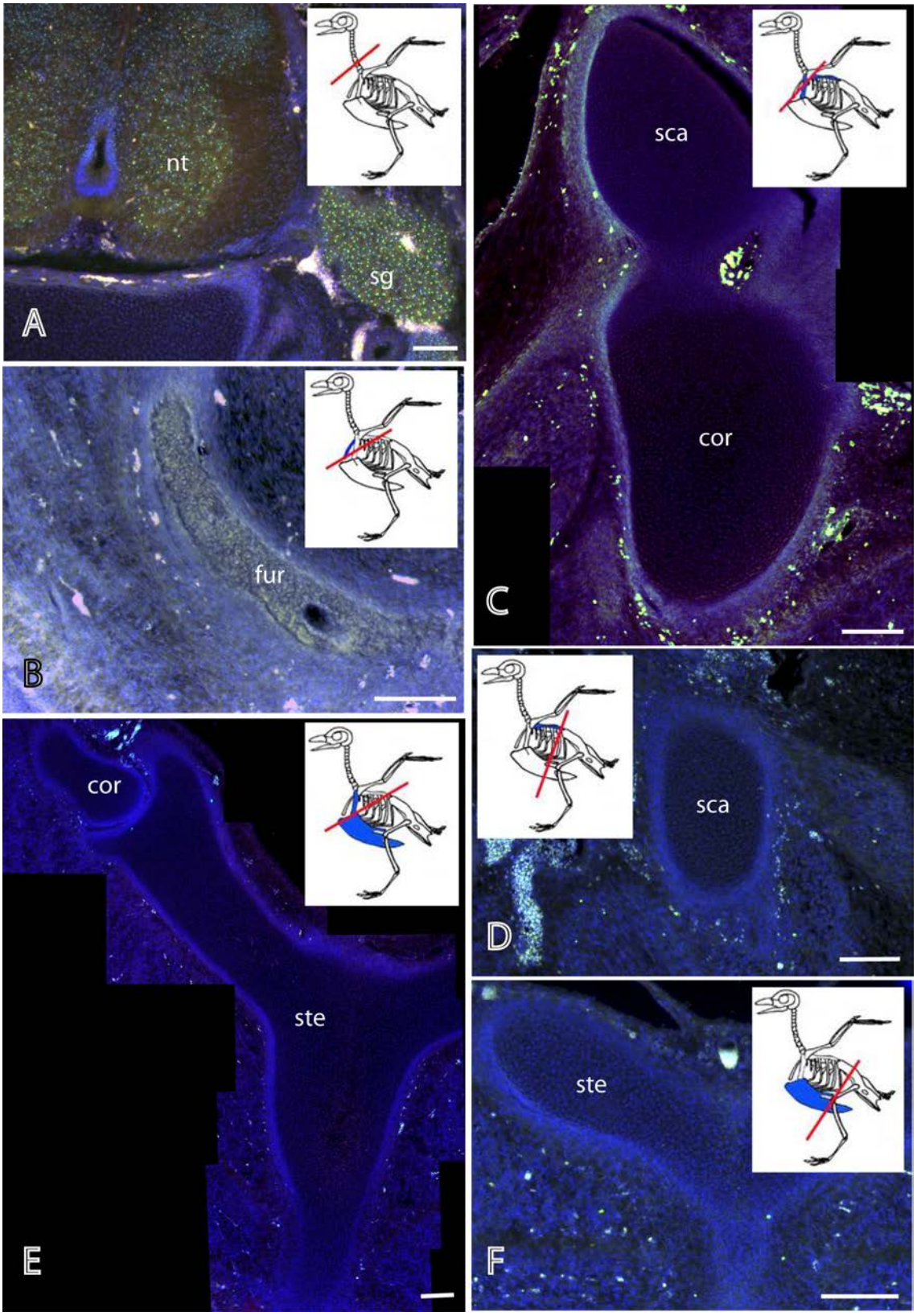

Fig. 2. Results of quail to chick grafting experiments

Transverse representative cryosections through day 9 chimeric embryos showing (A) the neural tube quail transplant and quail NC cells revealed with anti QCPN antibodies (green) in spinal ganglia (sg). Counterstaining with DAPI (blue). The shoulder girdle elements are shown at different anterior-posterior levels (B-F). No detectable quail cells are seen in shoulder cartilage, perichondrium, or bone. Scattered green cells in surrounding tissues represent mostly cut nerves or those with rose or white spots — auto fluorescence of erythrocytes. Skeleton schemes are inserted to show the level of each section, the skeletal element of the shoulder girdle represented on the photo is filled in blue in the schemes. Other abbreviations: fur — furcula, cor — coracoid, nt — neural tube, sca — scapula, ste — sternum. Scale bar — 100 μm.

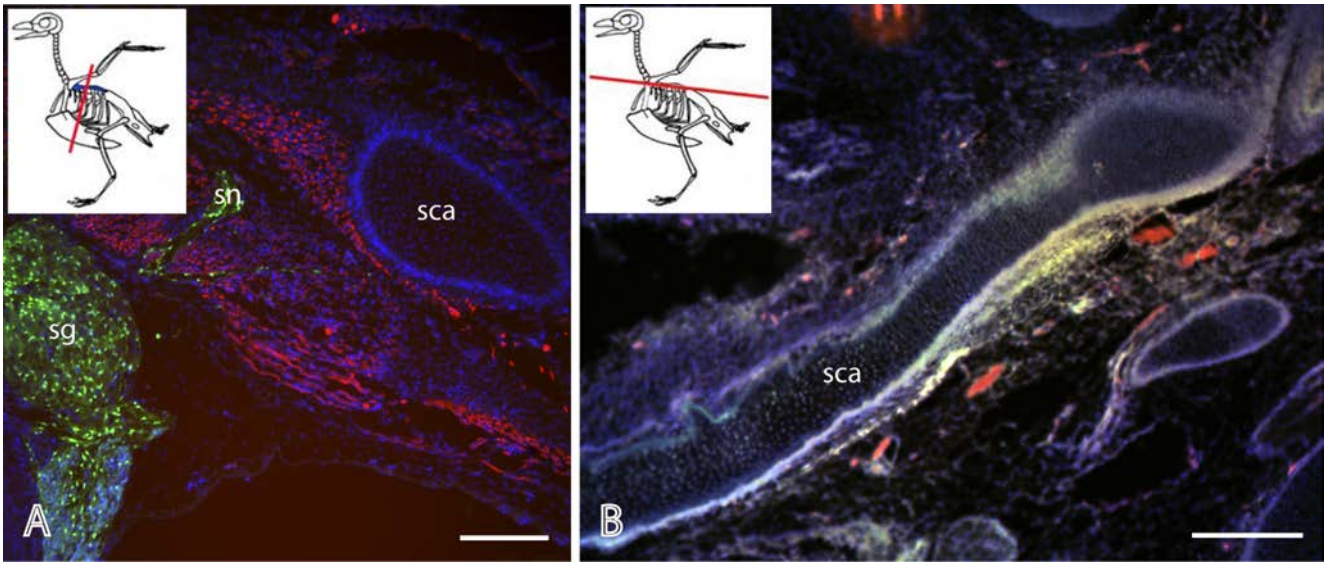

Fig. 3 – Results of GFP+ to white chick grafting experiments

Two representative paraffin sections, transverse (A) and frontal (B) through scapular region (sca) of the shoulder girdle exemplifying marking of NC derivatives in transgenic chimeras at day 7 of incubation. GFP+ cells (green) are localized in the region exclusively in spinal ganglia (sg) and spinal nerves (sn). Counterstaining with DAPI (blue) and anti-myosin heavy chain for muscles (red). Scale bars — 100 μm.

Table 2. The level of neural tube (NT) transplantation and survival rate in GFP+/white chick chimeras

Somitic level of the NT graft	Stage HH	No. of somites formed	No. of grafts	No. of survived embryos	Number of embryos with NT graft preserved
< 3*	8–9	3–9	11	4**	3
3–6	9–10	6–8	4	1	1
7–10	10–11	9–12	4	3	2
11–14	11–12	14–17	5	3	3
15–20	12–13	17–21	7	4	4
21–25	14 donor/15 host	21 donor/25 host	1	1	0
At all levels			32	16	13

* Some embryos in this series carried longer transplants extending to the level of somite 5, but all of those died. In other series some grafts were longer than the length of 4 somites; hence, those embryos were classified as belonging to the somitic level of the major part of the graft.

** Due to a low survival rates all the survived transgenic chimeras were fixed at age not exceeding eight days of incubation, i.e. before furcula formation and ossification of the rest of the shoulder girdle, therefore we were unable to analyse NC contribution to dermal furcula or the ossified endochondral shoulder girdle in transgenic chimeras.

NC markers HNK-1 and PDGFRα do not express in shoulder girdle anlagen

In order to corroborate our data with a method independent of manipulation with its possible artefacts we used NC markers, e.g., HNK-1 and PDGFRα that were used previously in detecting NC cell populations in the developing skeleton of turtles (Cebra-Thomas et al. 2007; Gilbert et al. 2007). Although these markers are not universal and the work on turtles has questioned their reliability for this purpose (Nagashima, Shibata, et al. 2014), they are quite usual markers employed in the chick model to reveal NC (Rickmann et al. 1985; Bronner-Fraser 1986).

In our experiments HNK-1 epitope was found in chick embryos of days 4 to 7 of development, but only in cells of the neural tube, spinal ganglia, roots of the spinal nerves, and some peripheral neurons, what corresponds to the normal pattern of its distribution in neural and NC-derived tissues (Fig. 4A). Presence of PDGFRα on sections was restricted to days 4 to 6 and was not found in day 7 embryos. This NC-marker was found only in somitic sclerotomal cells, spinal ganglia, and in the myelinated sheaths of spinal nerves, as expected (Fig. 4B). However, neither in the mature coracoid, nor in the scapula, nor skeletogenic mesenchyme, corresponding to other developing elements of the shoulder girdle, we did not find any cells expressing these factors

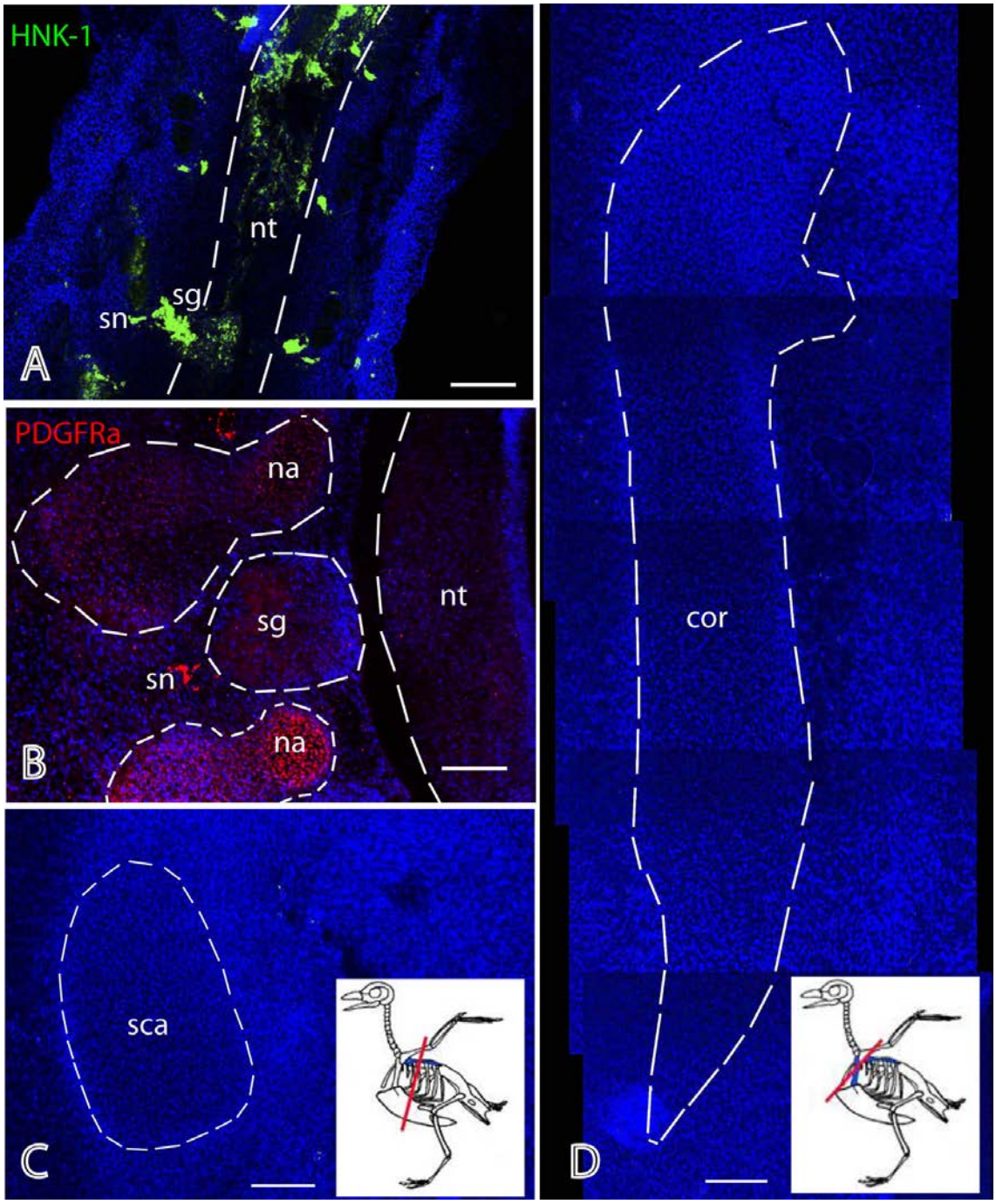

Fig. 4. Expression of NC-marker molecules

HNK-1 (A, green) and PDGFR-α (B, red) marker molecules were found in the neural tube (nt), spinal ganglia and spinal nerves, as well as neural arches (na) and other sclerotome derivatives (PDGFR-α), but not in any part of the cartilaginous shoulder girdle (C, D). Counterstaining with DAPI (blue). Other abbreviations — as on Figures 1 and 2. Scale bars — 100 μm.

(Fig. 4C, D). Thus, in the period between the 4th and the 7th day of development no cells of NC origin, which bear molecular markers HNK-1 or PDGFRα, were found in the mesenchymal anlagen and cartilaginous elements of the chick shoulder girdle.

Discussion

In our work we used two different types of avian chimerization: interspecific quail to chick, and intraspecific transgenic GFP+ chick embryo to white chick embryo transplantations of pieces of the neural tube containing presumptive NC. With either method we did not find

NC cells in the cartilaginous shoulder girdle and surrounding connective tissue at muscle attachment sites, at least until incubation day 9, when all the cartilaginous elements of the girdle are well formed. Embryonic tissue transplantation is a reliable method of marking cell populations in vivo, and given the variation in the techniques used (different in vivo systems, laboratories, operators, ways of cell visualization, etc.) we think that possible artefacts or mistakes were compensated for. The absence of the NC cells in the chick endochondral shoulder girdle was further confirmed with a different methodological approach, i.e. immunohistochemical visualization of cells bearing molecular markers of NC fate, such as HNK-1 and PDGFRα between days 4 and 7 of incubation. HNK-1 is a "standard" general marker of NC cells in birds (Tucker et al. 1984; Rickmann, Fawcett, and Keynes 1985; Bronner-Fraser 1986). It is a carbohydrate epitope of several glycoproteins and proteoglycans, with a role in cell-cell and cell-matrix adhesion. PDGFRα is usually considered as a marker of skeletogenic NC (Schatteman et al. 1992; Ho et al. 1994). Although these two markers are not strictly specific to NC cells, it was previously possible to identify some cell populations in the bony plastron of turtles expressing these factors (Cebra-Thomas et al. 2007). This result was confirmed by Nagashima, Shibata, et al. (2014), but the correspondence of the cells to NC was questioned due to a lack of evidence of marker molecule expression in the early stages between the initial NC cell migration and the time of osteogenesis of skeletal elements. However, a possible correspondence of the same cell population to NC derivatives was supported by additional evidence of p75 and FoxD3 marker expression (Gilbert et al. 2007). Therefore, although discrepancies in interpretation of the finding exist, it is equally probable that the two markers do not match the NC populations of cells at some late stages or, alternatively, that they are not expressed in NC derivatives at all stages. Anyway, this discrepancy does not apply to our negative results, suggesting that there is no marker molecule expression, i.e. neither the physiological state of any cells is comparable to that found in NC cells, nor any NC derivatives is likely found in the cartilaginous shoulder girdle of the chick embryo. Thus, each technique employed in the study failed to show a NC contribution to the avian cartilaginous shoulder girdle. In addition, grafting of NC from the occipital somites level made by others did not reveal any NC contribution to the cartilaginous shoulder girdle (R. Huang, personal communication). Therefore, we can confidently conclude, that NC is unlikely to participate in formation of the endochondral shoulder girdle in the chick, while in the intramembranous component (the furcula) only a small population of NC cells described previously has a contribution (McGonnell, McKay, and Graham 2001).

So far, a NC-derived cell population contributing to the chick shoulder girdle emigrating very late from the neural tube at the level of somites 1 and 2 was found in the intramembranous furcula (McGonnell, McKay, and Graham 2001). These cells were localized in the medial part of the furcula (hypocleideum) in its cranial periosteum and attachment site of the cleidohyoid muscle. This evidence was supported both by analysis of quail-to-chick chimeras and expression of basic leucin rich transcription factor *Mafb*, a specific postotic cranial (level of somites 1 and 2) NC marker. In our work we used transplantation of pieces of neural tube containing the presumptive NC from the levels of somites 1 to 27, but the majority of embryos at the most cranial levels died (see Methods). Since the above mentioned late emigrating population of the NC cells was not in the focus of our attention, the NC cells were found neither in the furcula, nor in the rest of the girdle in the current study. Although we further used two molecular markers of the NC, our analysis was not extended in this case to later developmental stages, when furcula develops.

Another point in the shoulder girdle where contribution of the NC could be expected, is the place of insertion of the musculus cucullaris (= m. trapezius) on the scapula. In chick, this muscle, which connects the shoulder girdle to the skull, originates from lateral plate mesoderm, unlike most trunk muscles (Theis et al. 2010). This muscle did not express *Pax3* during its development, but contained *Pax3*+ endomysial connective tissue of NC origin (Theis et al. 2010). In the cited work, the NC cells for the connective tissue within the cucullaris muscle originated from neural tube at the level of somites 2 to 4. In our work we partially labelled tissue from this level (level of somite 3); moreover, the molecular markers we used (HNK-1 and PDGFRα) are expressed just at the time of scapula and coracoid development. Nonetheless, neither in the previous reports (Theis 2010; Theis et al. 2010), nor in the current study, were NC cells found in the cartilage or perichondrium of the scapula (also R. Huang, personal communication), suggesting that a particular embryonic origin of the connective tissue in the muscle and that of the underlying shoulder girdle element proper may be different. Similarly, we have found no NC cells in any other location throughout the entire shoulder girdle (including coracoid and sternum), although we cannot completely rule out very late migration of NC cells from some more cranial levels of the neural tube. Despite the acromion and coracoid process of the scapula of mammals are sometimes considered as partial homologues of the avian coracoid (Vickaryous and Hall 2006),(—but see alternative interpretations of the homology of the shoulder elements (Nagashima, Fumiaki, et al. 2014)), and that they have partial NC-origin in the mouse (Matsuoka et al. 2005), we did not find evidence of any contribution of the NC to the avian

coracoid. Therefore, it is probable that the cranial NC-derived cell population originating at the level of somites 1 and 2 and building the cleidohyoid muscle attachment site in the furcula (McGonnell, McKay, and Graham 2001) — is unique in that it makes contribution to the avian shoulder girdle.

The avian furcula is one of few intramembranous bones present in the amniotic shoulder girdle. It is either interpreted as fused clavicles, or interclavicle, or both (Parker 1868; Vickaryous and Hall 2010; Tschopp and Mateus 2013). NC contribution to the homologue intramembranous bones of the shoulder girdle was previously suggested not only for birds, but also for other amniotic lineages. In turtles it was suggested that epiplastra and entoplastron might be NC-derived elements, which are considered to be homologues of, respectively, the clavicle and interclavicle of other vertebrates (Clark et al. 2001; Cebra-Thomas et al. 2007). Both endochondral and dermal parts of the mammalian clavicle also contain cells of NC origin. McGonnell and colleagues (McGonnell 2001; McGonnell, McKay, and Graham 2001) proposed a hypothesis that NC-contribution to the clavicle and interclavicle in vertebrates appeared de novo when the shoulder girdle lost its connection to the skull in land-dwelling vertebrates that received new opportunities to move the head in comparison to fish. This evolutionary scenario has a weakness, since the actual embryonic origin of clavicles/interclavicles or the composition of the connective tissue of neck muscles in most anamniotes is not known and requires direct experimental investigation. It is equally probable that NC contribution to the bony clavicles/interclavicles is a vertebrate basic and quite ancient characteristic, and hence it is probably present even in basal fish. This NC-derived cell population may, however, be lost in certain lineages along with the corresponding bones themselves, for example, in teleost fishes, crocodiles, and salamanders.

Most NC-derivatives, which have ever been found, are present in the shoulder girdle of the mouse (Matsuoka et al. 2005). Here, the NC cells were found in intramembranous and endochondral parts of the clavicles, coracoid process of the scapula, acromion, scapular spine, and manubrium of the sternum. Matsuoka and colleagues (Matsuoka et al. 2005) suggested that this contribution of the NC in the mouse shoulder girdle is a reflection of the NC origin of connective tissue of the neck musculature (trapezius/cleidohyoideus). Furthermore, they hypothesised that insertion of the trapezius muscle, whose connective tissue is NC-derived, into the scapular ridge indicates homology of this part of the shoulder girdle in the mouse to the cleithrum of amphibians and primitive amniotes. Hence, both shoulder girdle elements are NC-derived. However, this evolutionary proposition is not sufficiently supported with the evidence from other model systems. For instance, even 12 years after the original paper was published, we do not know, whether cleithrum of anamniotes indeed originates from NC. This seems not to be the case for zebrafish as the cleithrum, as well as other dermal shoulder elements in this species appear to develop from mesoderm (Kague et al. 2012). Previously we have also shown, that NC-derived skeletal elements or muscle connective tissue cannot be found in the cartilaginous shoulder girdle of the axolotl (*Ambystoma mexicanum*) (Epperlein et al. 2012). Our current results in the chick further confirm our suggestion (Epperlein et al. 2012) that NC has rarely played considerable part in connecting the shoulder girdle to the skull and building in muscle attachment sites or inner parts in the majority of shoulder elements. Altogether, these facts indicate so far that with the obvious exception for the intramembranous (parts of) clavicles, NC contribution to the rest of the shoulder girdle is a unique trait of mammals, marking mammalian skeletal synapomorphies (Sánchez-Villagra and Maier 2006; Epperlein et al. 2012) or even more strictly indicating to an autapomorphy of the mouse. Moreover, the mouse model and particularly the Wnt1-Cre line used by Matsuoka et al. 2005, is not the best one to reveal NC progenitors for several reasons, e.g., Wnt1 expression is not restricted to the neural crest but rather marks dorsal neural stem cells and activates Wnt signalling in ectopic locations, particularly in mid brain (Barriga et al. 2015). Hence, this method much like PDGFRα and HNK-1 expression may indicate more the physiological state of the cells rather than the real mother-daughter relationships and continuity of NC cell populations as revealed by cell-lineage tracing techniques in non-mouse models.

Interestingly, skeletal elements may develop either from NC or mesoderm, and there is a possibility for compensatory replacement of one tissue with the other in evolution (Davidian and Malashichev 2013; Hall and Gillis 2013), and development (Schneider 1999; Murdoch et al. 2012). A most striking example is development of basibranchial elements of the hyobranchial apparatus from mesoderm in amphibians (Davidian and Malashichev 2013), while it is originating from NC in fish (Schilling and Kimmel 1994; Kague et al. 2012; Mongera et al. 2013) and amniotes (Chai et al. 2000; Matsuoka et al. 2005; Gross and Hanken 2008). This represents a special difficulty in interpreting homologies and predicting the embryonic origin of a particular skeletal element.

Nevertheless, there are continuing attempts to interpret homology of shoulder elements based on the pattern of attaching muscles. It was recently suggested that nuchal plate in the carapace of turtles is homologous with paired cleithra (Lyson et al. 2013). Indeed, nuchal plate usually develops initially also as paired anlagen, which may fuse into a single bone (Cherepanov 1997),

and which position in the shell and relation to the trapezius muscle may in principle resemble that of the cleithra (Lyson et al. 2013). However, previously nuchal bone was homologized based on anatomy and position also to supracleithra (Vallen 1942), while cleithra were actually described in an extinct turtle *Kayentachelys aprix* as paired dermal elements separate from the nuchal bone (Joyce et al. 2006). Epiplastral processes of primitive turtles were interpreted sometimes as cleithra that are fused with epiplastra (clavicles) during ontogeny (Joyce, Jenkins, and Rowe 2006). Furthermore, nuchal plate strongly expresses HNK-1 and PDGFRα markers of the neural crest in *Trachemys scripta* (Gilbert et al. 2007). However, the use of HNK-1 antibodies is questioned in models other than chick, e.g., in turtles, since it does not seem to mark all neural crest derivatives at all stages (Nagashima, Shibata, et al. 2014). Again, the bone in focus for the comparison, the cleithrum, together with other dermal shoulder elements in zebrafish are not NC-derived, and likely develop from mesoderm (Kague et al. 2012). This adds difficulty for interpretation of the nuchal bone in turtles as having cleithrum as an evolutionary predecessor (Lyson et al. 2013). In comparison, such a difficulty seems not to arise when interpreting the epiplastra of turtles as clavicles because clavicles of all studied vertebrate species have a NC component. Finally, interpretation of the NC-derived nuchal bone in turtles as cleithrum mainly on the basis of muscle attachments is obviously contradictory to the fact that a homologous cucullaris muscle with NC-derived connective tissue is attached in the chick to entirely mesodermal scapula, which one would hardly ever name cleithrum, and which does not form a specific structure like a scapular spine of mammals. All these comparisons and examples clearly show that one should be very cautious in establishing homologies between the parts of the shoulder girdle based solely on pattern of muscle attachment sites or their embryonic origin.

To date we do not have a clear-cut picture of the evolution of NC and mesoderm within the shoulder girdle of vertebrates despite there being four model species (zebrafish (Kague et al. 2012), axolotl (Epperlein et al. 2012), chick (this study and (McGonnell, McKay, and Graham 2001)), and mouse (Matsuoka et al. 2005)), in which the relative contribution of these embryonic tissues are comprehensively described. The issue deserves further intensive work because our knowledge in the field is still obviously insufficient. Therefore, we suggest turning from attempts of creating "single fact/single species" models of shoulder girdle evolution to collecting more facts on the embryonic origin of skeletal elements, particularly of cleithra, in a variety of taxa, from basal fish to diverse groups of reptiles.

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