Histol Histopathol (2007) 22: 51-60

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Development of the mouse mandibles and clavicles in the absence of skeletal myogenesis

I. Rot-Nikcevic[1], K.J. Downing[1,2], B.K. Hall[2] and B. Kablar[1]

[1]Department of Anatomy and Neurobiology, Halifax NS, Canada and

[2]Department of Biology, Faculty of Science, Halifax NS, Canada

Summary. In this report we employed double-knock-out mouse embryos and fetuses (designated as *Myf5*$^{-/-}$:*MyoD*$^{-/-}$ that completely lacked striated musculature to study bone development in the absence of mechanical stimuli from the musculature and to distinguish between the effects that static loading and weight-bearing exhibit on embryonic development of skeletal system. We concentrated on development of the mandibles (= dentary) and clavicles because their formation is characterized by intramembranous and endochondral ossification via formation of secondary cartilage that is dependent on mechanical stimuli from the adjacent musculature. We employed morphometry and morphology at different embryonic stages and compared bone development in double-mutant and control embryos and fetuses. Our findings can be summarized as follows: a) the examined mutant bones had significantly altered shape and size that we described morphometrically, b) the effects of muscle absence varied depending on the bone (clavicles being more dependent than mandibles) and even within the same bone (e.g., the mandible), and c) we further supported the notion that, from the evolutionary point of view, mammalian clavicles arise under different influences from those that initiate the furcula (wishbone) in birds. Together, our data show that the development of secondary cartilage, and in turn the development of the final shape and size of the bones, is strongly influenced by mechanical cues from the skeletal musculature.

Key words: Mouse embryos, Skeletal muscle, Secondary cartilage, Mandible, Clavicle

Offprint requests to: Dr. Boris Kablar, Department of Anatomy and Neurobiology, Faculty of Medicine, Dalhousie University, 5850 College Street, Halifax, NS, Canada B3H 1X5. e-mail: bkablar@dal.ca

Introduction

In vertebrates, during embryonic development and after birth, skeletogenesis and myogenesis are closely related processes. Dependence of bone development on muscles has been mostly studied in birds (reviewed by Hall and Herring, 1990) and mice (Herring and Lakars, 1982; Hall, 2001) and the effects that muscles have on bones are generally viewed as mechanical (reviewed by Herring, 1994). The main body of research done so far has been performed on paralyzed chicken embryos (reviewed by Hall and Herring, 1990) and mutant mouse embryos with *muscular dysgenesis* (Herring and Lakars, 1982). In both of these models the muscles are still present and although weight-bearing (i.e., compressive or bending loads of an active muscle) is eliminated muscles still put significant loads on the developing bones in the form of static loading. It has been shown that muscle loading alone (without weight-bearing) is sufficient to mold the skeleton (reviewed in Herring, 1994). In our recent study (Rot-Nikcevic et al., 2006) we described skeletal anomalies found in mutant mouse embryos completely lacking striated muscle. Double knock-out mouse fetuses, carrying null mutations in both Myf5 and MyoD genes (designated as *Myf5-/-:MyoD-/-* or amyogenic), completely lack differentiated myoblasts and skeletal muscle (Rudnicki et al., 1993; Kablar et al., 1999, 2003) and therefore are the only *in vivo* model system available in which to study skeletogenesis in the complete absence of mechanical stimuli from the muscles (Rot-Nikcevic et al., 2006). The mandibles and clavicles of amyogenic mouse fetuses were significantly affected, showing altered shape and reduced size (Rot-Nikcevic et al., 2006).

The mandibles and clavicles of mammals and birds are the first bones to ossify and are among the skeletal elements most dependent upon mechanical stimulation for their growth (Hall, 2001, 2005). Bone is formed by two different processes: a) endochondral or indirect

ossification is first seen as a mesenchymal condensation that gives rise to a cartilaginous template (i.e., the primary cartilage) eventually replaced by the bone tissue (e.g., mostly long bones in the limbs), and b) membranous or direct ossification is characterized by the bone formation directly from a mesenchymal condensation, where the mesenchymal cells differentiate directly into osteoblasts (e.g., usually in the flat bones) (Kaufmann, 1999; Moore and Persaud, 2003). However, early evidence of a cartilaginous model is seen in some bones that are supposed to exclusively form by intramembranous ossification. In mammals, the clavicles show a combination of intramembranous and endochondral ossification (Hall, 2001). A single center of ossification is present in the avian clavicle, and two centers of ossification are visible in the mouse clavicle (Hall, 2001). In the mandibles, the body and the base of the processes form by intramembranous ossification, spreading from ramus, whereas secondary cartilage of the condylar, angular and coronoid processes appears in the periosteum and then is replaced endochondrally. However, in rats the cartilage arises as a sesamoid and then fuses to the membrane bone of the processes (reviewed in Fang and Hall, 1997; Kaufmann, 1999; Hall, 2005).

Morphogenetic events that require muscle activity often involve secondary cartilage, whose formation is apparently dependent on mechanical stimulation from the skeletal muscle (reviewed in Herring, 1994). Secondary cartilage is tissue that provides growth sites and articulations during the subsequent steps of bone and joint morphogenesis and therefore is critical for later growth and shaping of bones and joints (reviewed in Herring, 1994). Considering that the mandibles and clavicles appeared abnormal in *Myf5*$^{-/-}$:*MyoD*$^{-/-}$ embryos (Rot-Nikcevic et al., 2006), our analysis focused on the initiation vs. maintenance of the secondary cartilage in double-mutant embryos and fetuses. Obtaining an answer to this question is important from the evolutionary point of view. As it appears, the initiation vs. maintenance of secondary cartilage differs between birds and mammals, suggesting that secondary cartilage in birds has different mode of formation and/or has separate evolution from secondary cartilage in mammals (Hall, 2000). In mice and humans, secondary cartilage is seen in all three mandibular processes, i.e., in the condylar, coronoid and angular processes (Fang and Hall, 1997) and on the acromial end of the clavicles (reviewed by Hall, 2001), while in rats it is a sesamoid (Hall, 2005). So far, using different approaches, it has been found that secondary cartilage in mice can be initiated without biomechanical stimuli (Herring and Lakars, 1982), but cannot be maintained (Tran and Hall, 1989). By contrast, in birds, secondary cartilage initiation and maintenance are both dependent on mechanical stimuli (Fang and Hall, 1997). However, the studies so far have only been performed *in vitro* or on either paralyzed chick embryos (Hall, 1986; Hall and Herring, 1990) or mice with muscular dysgenesis (Pai, 1965; Herring and Lakers, 1982). In both *in vivo* cases, static loading from muscles remains as a possible critical effect influencing secondary cartilage development.

Our model aims to test the hypothesis that secondary cartilage formation depends on mechanical stimuli by studying development of the mandibles and clavicles in mutant mouse embryos that completely lack skeletal musculature. (N.B. Throughout the text we use the term "mandible" when speaking of the bone alone, i.e., the dentary portion of the mandible. Therefore, we are not referring to the entire low jaw that would include the Meckel's cartilage, dentary and muscles.) The goal of our research was to: a) morphometrically describe altered clavicular and mandibular size and shape in mice in isolation from muscular activity, and b) analyze secondary cartilage development (initiation vs. maintenance) on the two bones in amyogenic embryos and fetuses.

We report that the mandibles and clavicles in double-mutant mouse fetuses were significantly altered in terms of shape and size and we describe these changes in detail. In addition, we find that the secondary cartilage formation in amyogenic embryos was significantly affected, and was not maintained in the double-mutant clavicles. Together, our data show that the development of secondary cartilage, and in turn the development of the final shape and size of the bone, is strongly influenced by mechanical cues from the skeletal musculature.

Materials and methods

Interbreeding and collection of fetuses

Embryos and fetuses lacking both *Myf5* and *MyoD* were derived by a two generation breeding scheme. First, *MyoD-/-* mice were bred with *Myf5+/-* mice to generate *Myf5+/-:MyoD+/-* mice. Second, *Myf5+/-:MyoD+/-* mice were interbred to obtain embryos and fetuses of 9 different genotypes including *Myf5-/-:MyoD-/-* (designated as double-mutant) that appeared with the incidence of 1:16 (Rudnicki et al., 1993; Kablar et al., 2003).

Tissue processing and genotyping

Fetuses and the fetal portions of the placenta were collected by Cesarean section on the required embryonic day (E16.5-E18.5), and tissues were prepared for whole-mount alizarin red/alcian blue staining and hematoxylin/eosin (H&E) staining. Differential staining of cartilage and bone in whole mouse fetuses was performed using alizarin red and alcian blue following standard methods (Patton and Kaufman, 1995). Skin was removed and embryos were fixed in 95% ethanol for 7 days, and then placed in acetone for 2 days (7 days for E16.5 embryos) to remove fat. Fetuses were stained with alizarin red and alcian blue at 37°C for 3 days and subsequently cleared using 1% KOH for 24 hours. The

specimens were stored in glycerol. Under a stereomicroscope, we isolated mandibular (wild-type, n=3; *Myf5*$^{-/-}$:MyoD$^{-/-}$, n=3) and clavicular (wild-type, n=4; *Myf5*$^{-/-}$:*MyoD*$^{-/-}$, n=4) tissues for further analysis. All bones were sectioned to produce sagittal serial sections. Genomic DNA was isolated from the fetal portion of the placenta using the procedure of Laird et al. (1991). Fetuses were genotyped by Southern analysis (Sambrook et al., 1989) of placental DNA using Myf5 and MyoD specific probes as described previously (Rudnicki et al., 1993).

Photography and morphometry

Digital photos (Nikon 4500) were obtained using a stereomicroscope (Leica MZ6) or a compound light microscope (Zeiss Axioplan 2), and panels were generated in Adobe Photoshop 7.0. Mandibular (wild-type, n=3; *Myf5-/-*:*MyoD-/-*, n=3) and clavicular (wild-type, n=4; *Myf5-/-*:*MyoD-/-*, n=4) morphometry was obtained using a compass and a metric ruler under the stereomicroscope at 40x magnification. For the morphometric analysis of the mandibles 12 landmarks were used according to Atchley et al. (1992). The resulting set of polygons was analyzed using NIH Image program. Lengths between landmarks, areas and angles were compared using two sample t-test (α=0.05). Data were presented as means ± standard deviation (SD).

Results

Mandibular and clavicular phenotypes reveal the dependence of bone shape and size development on the presence of the musculature

Previously, we reported various abnormalities of the skeleton that develops in the complete absence of skeletal musculature (Rot-Nikcevic, et al., 2006). Here, to study more closely the alterations of bone shape and size in the absence of skeletal musculature, we concentrated on the mandibles and the clavicles. The final shape of these bones depends on the development of secondary cartilage, whose formation is in turn dependent on mechanical stimuli from the adjacent skeletal musculature (Hall, 1978; Atchley and Hall, 1991; Hall, 2005).

The mandibles in E18.5 double-mutant fetuses were significantly altered in shape when compared to wild-type (Fig. 1A,B). Condylar process, the attachment site of the pterygoideus externus muscle, was slender and the

Table 1. Morphometric analysis of the mandibles in wild-type (WT) and double-mutant (DM) E18.5 mouse fetuses.

TRAIT	GENOTYPE		
	WT (N=3)	DM (N=3)	P
Anterior mandible length (Euclidean distance from points 2-5)	2.35 ± 0.21	1.87 ± 0.18	0.001*
Posterior (molar) height of mandible (Euclidean distance from points 1-2)	1.22 ± 0.04	1.00 ± 0.05	<0.001*
Height at incisor region (Euclidean distance from points 2-12)	1.16 ± 0.06	0.98 ± 0.08	0.006*
Condylar process width (Euclidean distance from points 6-7)	0.72 ± 0.11	0.42 ± 0.12	<0.001*
Condylar process area (Area defined by the polygon 5-6-7-8)	0.43 ± 0.04	0.23 ± 0.09	<0.001*
Coronoid process area (Area defined by the triangle 8-9-10)	0.12 ± 0.01	Not visible	N/A
Angular process area (Area defined by the triangle 3-4-5)	0.34 ± 0.07	0.14 ± 0.05	<0.001*
Incisor area (Area defined by the triangle 1-2-12)	0.74 ± 0.09	0.58 ± 0.12	0.004*
Molar area (Area defined by the polygon 2-3-10-11)	1.90 ± 0.13	1.61 ± 0.16	0.001*
Tooth-bearing area (Area defined by the points 1, 2, 3, 10, 11, 12)	2.64 ± 0.23	2.19 ± 0.19	0.004*
Process area (Area defined by the points 3, 4, 5, 6, 7, 8, 9, 10)	0.89 ± 0.10	0.37 ± 0.09	<0.001*
Anterior mandibular angle (Angle formed by points 1, 2, 3)	139.39 ± 2.97	116.31 ± 4.18	<0.001*

Values are means ± SD. Linear measurements are given in mm, the angle is given in degrees. N, sample size; P, significance of the two tailed t-test; *, significantly different values.

angular process, the attachment site of the masseter muscle, was significantly reduced or absent. Surprisingly, we also noticed unusually large ligaments (as if compensatory for the absence of the musculature) that in same cases completely wrapped around the condylar process of the mandible, but also in other locations of the body (not shown). In the ligaments adjacent to the condylar process osteoclasts were easily noticeable, indicating that the ligament is in tight connection to the bone. Finally, the coronoid process, an attachment site for the temporalis muscle, was not visible in whole-mount preparations (Fig. 1B), but it was apparent in histological sections (Fig. 2D). The overall shape was consistent with that of the *muscular dysgenesis* (Herring and Lakers, 1982). The posterior portion of the double-mutant mandibles was displaced dorsally and posteriorly in comparison to the wild-type mandibles, resulting in noticeable retrognathia. In addition to the obvious morphological alterations of the double-mutant mandibles, we employed a complex morphometric analysis (Atchley et al., 1992). Landmarks on the mouse mandibles used for morphometric analysis are shown in Fig. 1A, B. Mandibles in double-mutant fetuses showed significantly smaller values for all the traits measured (Table 1). The mandibles were also more sharply bent, approximately at the anterior limit of the molar region. In summary, even though all the measured traits were

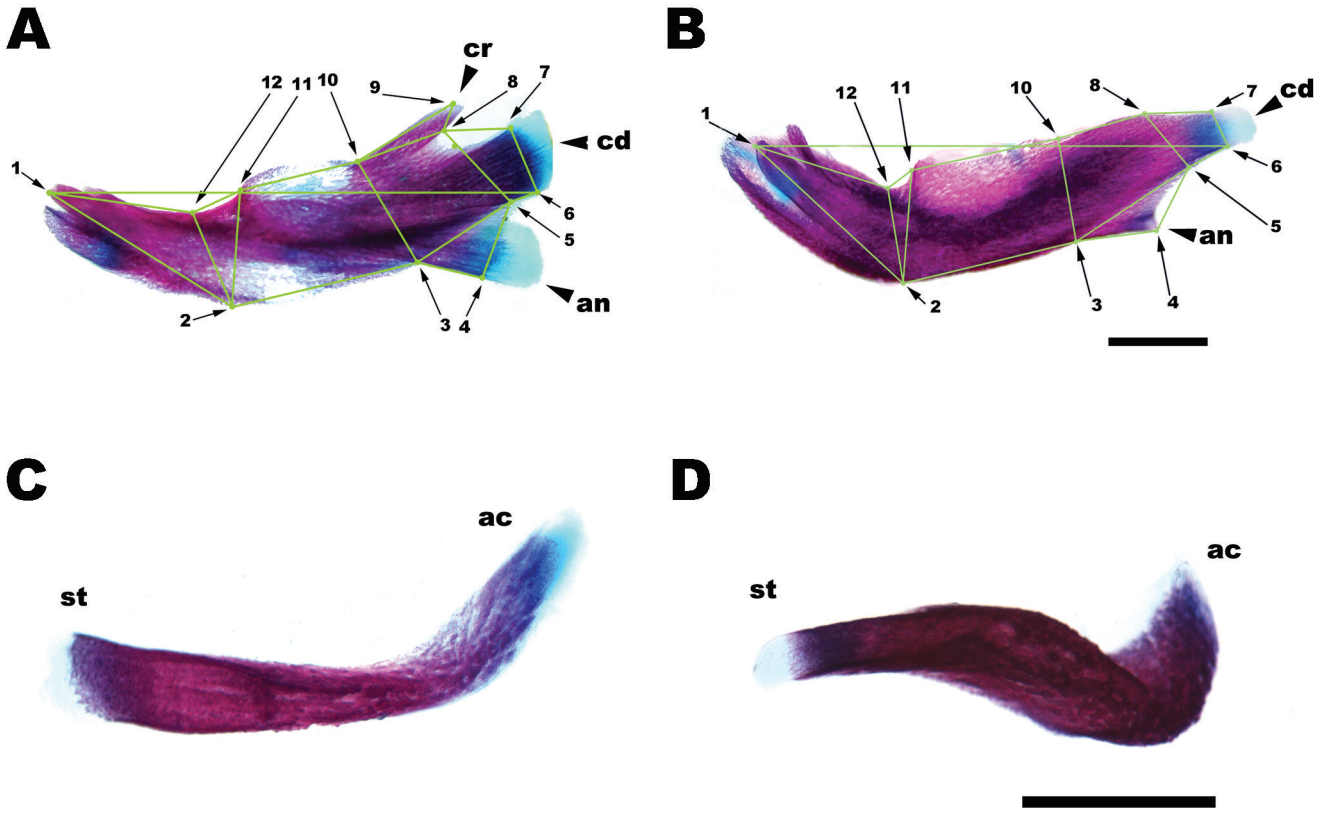

Fig. 1. Mandibular and clavicular phenotypes in double-mutant fetuses. Mandibles (**A, B**) and clavicles (**C, D**) of wild-type (**A, C**) and double-mutant (**B, D**) E18.5 fetuses are stained with alcian blue and alizarin red to show ossified (red) and cartilaginous (blue) parts. The wild-type mandible (**A**) and the double-mutant mandible (**B**) are also showing the 12 landmarks used for the morphometric analysis performed according to the previously established criteria (Atchley et al., 1992). The wild-type mandible has well developed coronoid (cr in **A**), condylar (cd in **A**) and angular (an in **A**) processes, while the double-mutant mandible lacks the coronoid process (no cr in **B**), has significantly reduced condylar process (cd in **B**) and a very reduced angular process (an in **B**) (in fact, some double-mutant mandibles do not have the angular process at all). The four triangular and three polygonal finite elements that were used for comparisons are also shown: 1, most ventral ossified point of alveolus at incisor; 2, most inferior point on corpus; 3, deepest point on angular notch; 4, most inferior point on angular process; 5, intersection point between angular and condylar processes; 6, most posterior/inferior point of condylar process (and farthest ossified point from 1); 7, most superior ossified point of condylar process; 8, intersection between condylar and coronoid processes; 9, most superior point of coronoid process; 10, most inferior point immediately posterior to molar row; 11, point of inflection on edge of molar region; 12, deepest point in incisor notch. In addition, the wild-type clavicle **(C)** and the double-mutant clavicle **(D)** are shown. Note the difference in clavicular shape and angle between the two genotypes. Abbreviations: cr, coronoid process; cd, condylar process; an, angular process; st, sternal end; ac, acromial end. Scale bars: 1 mm.

significantly affected, our data show that different regions of the mandible were altered differently. For instance, the posterior muscle attachment areas of the mandible were more strongly affected in comparison to the tooth-bearing regions.

Furthermore, the clavicular shape was also significantly affected in E18.5 double-mutant fetuses (Fig. 1C, D). The clavicles were more bent acquiring the "S" shape as opposed to the "L" shape observed in wild-type fetuses. The acromial (or lateral) end of the clavicles, normally pointed in wild-type fetuses, was wider in the double-mutant clavicles. The sternal (or medial) end of the clavicles, normally expanded, was slender in double-mutants. Overall, the double-mutant clavicles seemed to be more condensed in shape. Due to the specific three-dimensional characteristics of the clavicles, i.e., its curved shape in comparison to the more linear mandibular shape, we were unable to perform analogous morphometric analysis as described for the mandibles. Therefore, only the maximum width and the surface area were measured (Table 2). The clavicles in double-mutant fetuses had significantly smaller surface area compared to the wild-type.

Table 2. Morphometric analysis of the clavicles in wild-type (WT) and double-mutant (DM) E18.5 mouse fetuses.

	GENOTYPE		
TRAIT	WT (N=4)	DM (N=4)	P
Maximum width	0.34 ± 0.02	0.32 ± 0.06	0.540
Surface area	0.91 ± 0.06	0.73 ± 0.04	0.002*

Values are means ± SD. Linear measurements are given in mm, surface area in mm2. N, sample size; P, significance of the two-tailed t-test; *, significantly different values.

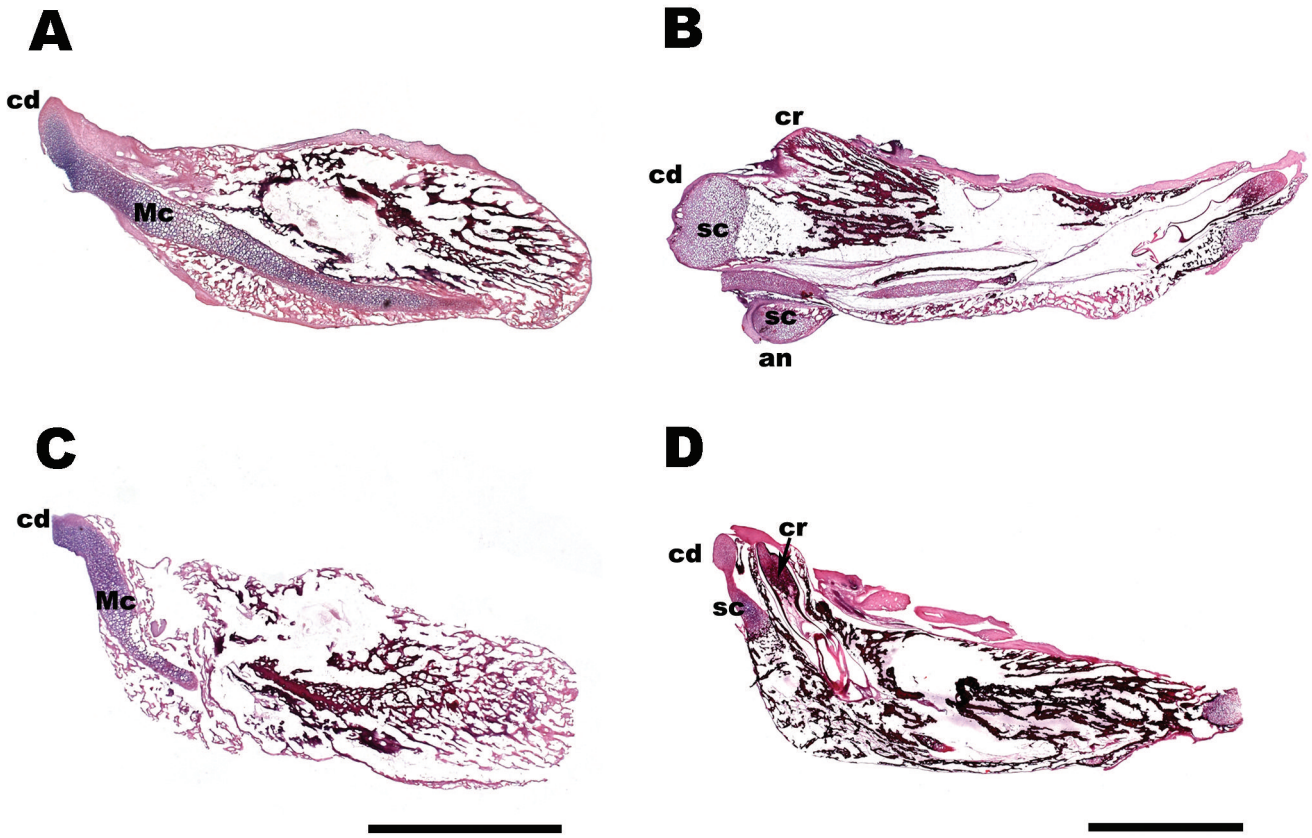

Fig. 2. The initiation and maintenance of secondary cartilage in the mouse mandibles. Sagittal sections through the mandibles of E16.5 (**A**) and E18.5 (**B**) wild-types and E16.5 (**C**) and E18.5 (**D**) double-mutants. Condylar process (cd in **A** and **C**) is the only process visible in both genotypes at E16.5. At E18.5, secondary cartilage (sc in **B** and **D**) is maintained in the condylar process (cd in **B** and **D**) in both genotypes. Note the elongated shape of secondary cartilage in the double-mutant condylar process (cd in **D**). Secondary cartilage in the angular process does not form in double-mutants (no an in **D**) and is clearly visible in wild-types (an in **B**). Finally, secondary cartilage in the coronoid process forms in double-mutants (cr in **D**). Histologically, secondary cartilage in all three mandibular processes differs from the Meckel's cartilage, that appears much denser packed with almost no extracellular spaces between the chondrocytes and unusually long, that might be a mouse strain-specific trait. Abbreviations: sc, secondary cartilage; cd, condylar process; cr, coronoid process; an, angular process; Mc, Meckel's cartilage. Scale bars: 1 mm.

Together, our data suggest that the mechanical forces from the adjacent skeletal muscles play an essential role in the fine tuning of the mandibular and clavicular shapes and sizes.

The development of secondary cartilages is differently affected in the mandibles vs. the clavicles

As mentioned earlier, the final shape and size of the mandibles and clavicles apparently depends on the formation of the secondary cartilage and mechanical stimuli from the adjacent musculature (Hall, 1978, 2001). To study the dependence of initiation vs. maintenance of the secondary cartilage formation on the mechanical stimuli from the musculature, we analyzed different stages of the mandibular and clavicular development.

The formation of the secondary cartilage-derived condylar process was observed in the mandibles of both wild-type and double-mutant E16.5 embryos (Fig. 2A,C). E16.5 is considered to be the stage in which secondary cartilage starts to form in mice (Tran and Hall, 1989). The condylar process in E16.5 double-mutants was the only visible process at this stage (Kaufmann, 1999) and it was similarly present in wild-type embryos. In the mandibles of E18.5 fetuses, secondary cartilage was maintained in wild-type, but absent on some processes of the double-mutant mandibles (Fig. 2B,D). While the maintained secondary cartilage was clearly visible in the condylar and angular processes of the wild-type mandibles, only the condylar process of the double-mutant mandibles contained some secondary cartilage. The angular process in double-mutants was significantly reduced or absent and did not contain any secondary cartilage. The shape of the secondary cartilage in the double-mutant condylar process was very different from the control. Finally, even though the coronoid process probably has the largest muscle attachment and so should be the most dependent on muscle action for its maintenance (Atchley et al., 1985), our data show that, in histological sections, the coronoid process actually maintains secondary cartilage. These data show that the maintenance of the double-mutant mandibular secondary cartilage is possible in the condylar process, the one that articulates with the temporal bone, and to some extent in the coronoid process, but is not possible in the angular process (as is not its initiation) that serves as a muscle attachment only.

In the clavicles, secondary cartilage is normally visible only in the acromial ends and it starts to be apparent at E16.5 (Tran and Hall, 1989). In our experiments, well-developed secondary cartilage was visible in E16.5 wild-type clavicles, while significantly

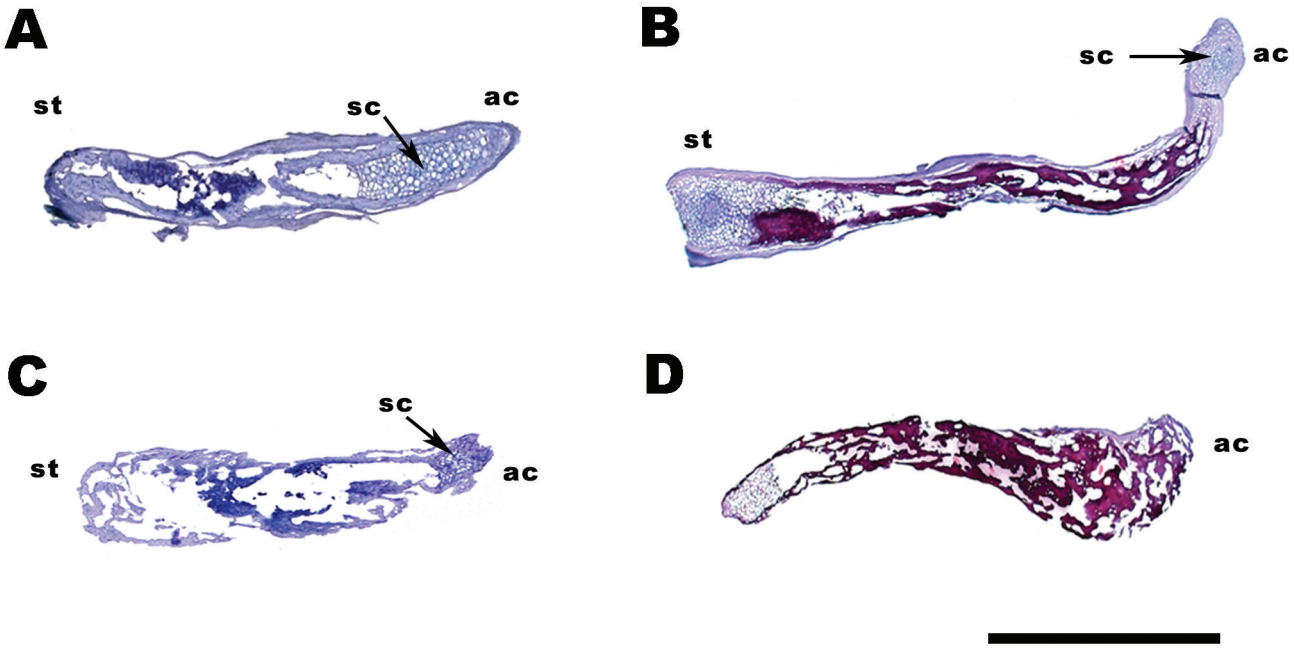

Fig. 3. The initiation and maintenance of secondary cartilage in the mouse clavicles. Longitudinal section through the clavicles of E16.5 (**A**) and E18.5 (**B**) wild-types and E16.5 (**C**) and E18.5 (**D**) double-mutants. Secondary cartilage (sc in **A** and **C**) is visible in the acromial (ac) end in both genotypes at E16.5. Note reduced secondary cartilage in the double-mutants (sc in **C**) in comparison to the control (sc in **A**). At E18.5, secondary cartilage (sc in **B** and **D**) is maintained at the acromial (ac) end in wild-types only (sc in **A**). Note complete absence of secondary cartilage at the acromial (ac) end of the double-mutant clavicle (**D**). Abbreviations: sc, secondary cartilage; st, sternal end; ac, acromial end. Scale bars: 1 mm.

Table 3. Summary of the findings regarding the initiation vs. maintenance of the secondary cartilage formation in the mandibles and clavicles of mouse embryos and fetuses.

LOCATION	SECONDARY CARTILAGE	
	INITIATION	MAINTENANCE
Mandible (N=3)	+ condylar process	+ condylar process (reduced)
	+ coronoid process (reduced)	+ coronoid process (reduced)
	- angular process	- angular process
Clavicle (N=4)	+ (reduced)	-

+/-, indicates the presence/absence of the secondary cartilage.

reduced, but still present, secondary cartilage was visible in E16.5 double-mutants (Fig. 3A, C). Thus, the initiation of secondary cartilage is possible in the complete absence of skeletal musculature, but to a lesser extent. In fact, the avian clavicle develops secondary cartilage in paralyzed embryos probably due to the rhythmic and continuous contractions of the amnion, and therefore the threshold for mechanical action is much lower in clavicles than in mandibular membrane bone (Hall, 1986). At E18.5, secondary cartilage was observed to be maintained in the clavicles of wild-type fetuses (Fig. 3B), but no secondary cartilage was observed in the acromial ends of the clavicles in double-mutants (Fig. 3D). These results suggest that the maintenance of the secondary cartilage in the clavicles is not possible without mechanical stimuli from the adjacent muscles.

In summary, our data show that the initiation of secondary cartilage formation in the absence of the adjacent skeletal musculature (i.e., mechanical stimuli from the muscles) is possible in both the mandibles and clavicles (to a lesser extent), while the maintenance of secondary cartilage is not possible in the clavicles and only attainable in the condylar process of the mandibles (Table 3).

Discussion

Double-mutant mouse embryos and fetuses, completely lacking the striated musculature, allowed us to study bone development in the absence of mechanical stimuli from the musculature and to distinguish between the effects that static loading and weight-bearing exhibit on embryonic development of skeletal system. In this report, we concentrated on the development of the mandibles and clavicles because of the following reasons: a) the two bones had reduced size and altered shape in double-mutant fetuses (Rot-Nikcevic et al., 2006), b) the formation of the two bones is characterized by both intramembranous and endochondral ossification, the endochondral ossification arising from secondary cartilage (Fang and Hall, 1997; Kaufmann, 1999; Hall, 2001), and c) the formation of secondary cartilage in the two bones is dependent on mechanical stimuli from the adjacent musculature (Herring, 1994; Fang and Hall, 1997; Hall, 2001).

First, we analyzed the development of the mandibles. As previously reported the mandibles were significantly altered in double-mutant fetuses (Rot-Nikcevic et al., 2006). Here, we performed detailed morphometric analysis of the mandibles at E18.5 and showed the details of the mandibular size and shape alterations, in comparison to the wild-type control. The three mandibular processes are common muscle attachment sites (e.g., condylar for pterygoideus externus, coronoid for temporalis and angular for masseter), have secondary cartilage and in the complete absence of myogenesis their development is significantly affected. However, each process seems to be affected at a different level of severity. The condylar process was found the least affected, followed by the coronoid process, that was significantly reduced in size (in fact, it was not visible in the whole-mount preparations), and finally the angular process, that was significantly reduced or completely absent in the examined fetuses. The coronoid process, including its secondary cartilage, develops within the temporalis muscle and is largely dependent upon muscular stimulation for its development (Avis, 1961; Sperber, 2001). In fact, it has been demonstrated that the muscle attachment is necessary to sustain this process (Avis, 1961). Therefore, our whole-mount data further support this finding and even imply that coronoid process may have the embryonic origin within the myogenic lineage. Consistently, dermomyotomal embryonic origin has been suggested for the avian scapula blade (Huang et al., 2000). By contrast, our histological findings show that coronoid process is actually partially maintained in the complete absence of musculature and appears to be hidden inside the mandible. Importantly, goosecoid and transforming growth factor beta (TGFß-2) knock-outs fail to form very particular morphogenetic units of the mandible (Hall, 2003, 2005). For instance, the angular and coronoid processes are absent in *goosecoid*$^{-/-}$ (Rivera-Perez et al., 1999), while all three processes are absent in *TGFß-2*$^{-/-}$ (Martin et al., 1995). Therefore, it appears that our double-mutant mandibular phenotype partially phenocopy goosecoid-/- phenotype, indicating that both mechanical and genetic factors are necessary for the maintenance of a complete appearance of the coronoid process. Furthermore, it could be that the mechanical stimuli from the musculature are transmitted via yet unknown mechanochemical signal transduction pathways that may involve goosecoid and *TGFß-2*, as analogously suggested for the lungs whose development heavily depends on mechanical factors (reviewed in Inanlou et al., 2005). The condylar process, in addition to being a muscle attachment site, also articulates with the temporal bone and is a part of the temporomandibular joint (TMJ). Therefore, in comparison to the angular process (the most dependent

upon local muscle action; Hall, 2005), it might receive additional mechanical stimulation from its bone-to-bone articulation within the TMJ. This may account for the condylar process being the least affected in double-mutant mouse embryos and fetuses. Together, our data suggest that the differences between the three mandibular processes in their response to the absence of the adjacent musculature may be dependent on their embryonic origin and the specific gene expression patterns (for the coronoid process) as well as on their anatomical location and function (for the condylar and angular processes). Finally, even though all the examined morphometric traits were altered in the double-mutant mandibles, the most affected part of the mandibles was its posterior or the processes-bearing portion. Thus, the influence of the musculature on the tooth-bearing part of the mandible is significantly smaller in comparison to its processes-bearing area. In fact, there is virtually no muscle insertion on the tooth-bearing region (also known as alveolar and ramal morphological unit of the dentary), hence it is independent of muscle action (Atchley and Hall, 1991; Hall, 2005).

The shape and the size of the clavicles also significantly changed in double-mutants in comparison to wild-type embryos. The double-mutant clavicles were smaller and more bent. The sternal end that articulates with sternum and is also the site of muscle attachments was slenderer in double-mutants when compared to the control. In fact, double-mutant fetuses have been shown to exhibit sternal cleft (Rot-Nikcevic et al., 2006), which may account for the altered joint between the two bones and the resulting smaller clavicular sternal end. The acromial end, normally pointed in wild-type embryos, was wider in double-mutant clavicles. It appears that, in the absence of mechanical stimulation from the adjacent muscles, the clavicles are not pulled or stretched along their longitudinal axes. In addition, it appears that the growth and development of the clavicles are dependent on muscular action, since without the movements by the skeletal musculature the stimulus for the clavicular growth was decreased. In fact, clavicular hypoplasia is also reported in mice carrying the *muscular dysgenesis* (*mdg*) gene (Pai, 1965).

In mammals and birds, the clavicles and the mandibles are the first bones to ossify (Kaufmann, 1999). During the normal process of endochondral ossification, like in the long bones, the forming cartilage (i.e., the primary cartilage) forms from the mesenchyme. By contrast, chondrocytes in secondary cartilage (i.e., in the processes of the mandible and the clavicle in mammals) are derived from the cells in the periosteum, because this cartilage appears after bone is formed (reviewed by Fang and Hall, 1997). Indeed, it has been shown that mechanical loading stimulates rapid changes in periosteal gene expression leading to increased periosteal cell proliferation (i.e., increased levels of proto-oncogene c-fos), decline in bone matrix proteins (i.e., alkaline phosphatase, osteopontin, osteocalcin and cytoskeletal protein beta-actin) and, finally, induction of TGFß-2 and insulin-like growth factor I (IGF-I) synthesis (Raab-Cullen et al., 1994). Secondary cartilage develops on craniofacial membrane bones of birds and mammals (Hall, 1970, 1978; Beresford, 1981) as well as on clavicles in chick (Hall, 1986) and mice (Tran and Hall, 1989). It develops in response to mechanical factors in both the embryonic chick (Hall, 1986) and mouse (Tran and Hall, 1989). In paralyzed chick, secondary cartilage only develops in clavicles, but not in mandibles (Hall, 1986). By contrast, in the complete absence of the musculature, we found that the initiation (at E16.5) of secondary cartilage formation was possible in the both bones, while the maintenance (at E18.5) of secondary cartilage was only somewhat possible in the condylar and coronoid processes of the double-mutant mandibles. Accordingly, Herring and Lakars (1982) reported that secondary cartilage in mice can be initiated without biomechanical stimuli. This was found in mdg mice, suggesting that the static loading alone, i.e., the presence of the muscular tissue alone provides enough stimuli to initiate the formation of secondary cartilage. In our model, the myogenic specification does not occur, but double-mutant embryos contain some myogenic precursor cells that will never differentiate into skeletal myoblasts (Kablar et al., 1999; 2003). Even though these cells eventually undergo apoptosis (Kablar et al., 2003), their presence may be sufficient to initiate the formation of secondary cartilage and therefore our model at an earlier stage of development may be more similar to mdg. In fact, when the clavicles are cultured *in vitro* and therefore without any static loading, secondary cartilage does not form (Tran and Hall, 1989).

Taken together, our results show that different bones respond differently to the mechanical stimuli from the adjacent musculature. For instance, in double-mutant embryos and fetuses, the initiated clavicular secondary cartilage is reduced, while the mandibular is of normal size. Similarly, the maintained secondary cartilage is only somewhat visible in the condylar and coronoid processes of the mandible, but is completely absent in the clavicles. The difference in the initiation and maintenance of secondary cartilage between the clavicles and mandibles in double-mutant embryos could be due to the different levels of dependence on muscular activity for growth and development in these two bones. For example, in chick embryos, the clavicles are more dependent upon muscular action for their growth than any other bone (Hall, 1986; Tran and Hall, 1989) and it might be the case with the mammalian clavicles as well. In fact, that is even further supported by the observations from *Myf5*$^{-/-}$ mutants that, because of the rib cage defect (Braun et al., 1992) and impaired respiratory and other movements (Inanlou et al., 2005), had reduced secondary cartilage in the clavicles but normal mandibles (Rot-Nikcevic and Kablar, unpublished data). However, the fact that secondary cartilage on the chick clavicles forms *in vitro*, but fails to form on the mouse clavicles (Tran and Hall, 1989), implies that the

dependency of secondary chondrogenesis in the murine clavicles is significantly higher than that in the chick (Hall, 1986). As pointed out earlier, obtaining an answer to this question was important from the evolutionary point of view. Indeed, the initiation vs. maintenance of secondary cartilage differed in double-mutant mouse embryos and fetuses in comparison to the birds. Therefore, our new data confirm the previous suggestion that secondary cartilage in birds has a separate evolutionary history from secondary cartilage in mammals (Hall, 2000; reviewed in Hall, 2001).

In conclusion, the results obtained on double-mutant mouse embryos and fetuses suggest that skeletogenesis is highly dependent on myogenesis, i.e., that mechanical impulses from the muscles significantly affect the shape and size of the developing bones. Moreover, we show that the effect varies depending on the bone (the clavicles being more dependent on mechanical stimulation than the mandibles) and even within the same bone. Finally, we further support the notion that, from the evolutionary point of view, the mammalian clavicles have different origin from the furcula or wishbone in birds.

Acknowledgments. We thank Anne C. Belliveau for expert technical assistance and Tyler Reddy for literature review. This work was supported by Nova Scotia Health Research Foundation (NSHRF), Grant Number: 2004-2013 (Med-Project); Natural Sciences and Engineering Research Council of Canada (NSERC), Grant Number: 238726-01 to B.K. and A5056 to B.K.H. K.J.D. is recipient of an NSERC Undergraduate Student Research Award.

References

Atchley W.R. and Hall B.K. (1991). A model for development and evolution of complex morphological structures. Biol. Rev. Camb. Philos. Soc. 66, 101-157.

Atchley W.R., Plummer A.A. and Riska B. (1985). Genetics of mandible form in the mouse. Genetics 111, 555-577.

Atchley W.R., Cowley D.E., Vogl C. and McLellan T. (1992). Evolutionary divergence, shape change, and genetic correlation structure in the rodent mandible. Syst. Biol. 41, 196-221.

Avis V. (1961). The significance of the angle of the mandible: an experimental and comparative study. Am. J. Phys. Anthrop. 17, 99-104.

Beresford W.A. (1981). Chondroid bone, secondary cartilage and metaplasia. Urban & Schwarzenberg, Munich.

Braun T., Rudnicki M.A., Arnold H.H. and Jaenisch R. (1992). Targeted inactivation of the muscle regulatory gene myf-5 results in abnormal rib development and perinatal death. Cell 71, 369-382.

Fang J. and Hall B.K. (1997). Chondrogenic cell differentiation from membrane bone periostea. Anat. Embryol. 196, 349-362.

Hall B.K. (1970). Cellular differentiation in skeletal tissues. Biol. Rev. Camb. Phil. Soc. 45, 455-484.

Hall B.K. (1978). Developmental and cellular skeletal biology. Academic Press. New York.

Hall B.K. (1986). The role of movement and tissue interactions in the development and growth of bone and secondary cartilage in the clavicle of the embryonic chick. J. Embryol. Exp. Morphol. 93, 133-152.

Hall B.K. (2000). The evolution of the neural crest in vertebrates. In: Regulatory processes in development. Olsson L. and Jacobson, C.-O. (eds). Wenner-Gren International Series. Volume 76. The Portland Press. London. pp 101-113.

Hall B.K. (2001). Development of the clavicles in birds and mammals. J. Exp. Zool. 289, 153-161.

Hall B.K. (2005). Bones and Cartilage: Developmental and evolutionary skeletal biology. Elsevier Academic Press. London.

Hall B.K. (2003). Unlocking the black box between genotype and phenotype: cell condensations and morphogenetic (modular) units. Biol. Phil. 18, 219-247.

Hall B.K. and Herring S.W. (1990). Paralysis and growth of the musculoskeletal system in the embryonic chick. J. Morphol. 206, 45-56.

Herring S.W. (1994). Development of functional interactions between skeletal and muscular systems. In: Bone: differentiation and morphogenesis of bone. Volume 9. Hall B.K. (ed). CRC. Boca Raton. pp 165-191.

Herring S.W. and Lakars T.C. (1982). Craniofacial development in the absence of muscle contraction. J. Craniofac. Genet. Dev. Biol. 1, 341-357.

Huang R., Zhi Q., Patel K., Wilting J. and Christ B. (2000). Dual origin and segmental organization of the avian scapula. Development 127, 3789-3794.

Inanlou M.R. and Kablar B. (2005). Abnormal development of the intercostal muscles and the rib cage in *Myf5-/-* embryos leads to pulmonary hypoplasia. Dev. Dyn. 232, 43-54.

Inanlou M.R., Baguma-Nibasheka M. and Kablar B. (2005). The role of fetal breathing-like movements in lung organogenesis. Histol. Histopathol. 20, 1261-1266.

Kablar B., Krastel K., Tajbakhsh S. and Rudnicki M.A. (2003). Myf5 and MyoD activation define independent myogenic compartments during embryonic development. Dev. Biol. 258, 307-318.

Kablar B., Krastel K., Ying C., Tapscott S.J., Goldhamer D.J. and Rudnicki M.A. (1999). Myogenic determination occurs independently in somites and limb buds. Dev. Biol. 206, 219-231.

Kaufmann M.H. (1999). The atlas of mouse development. Academic, San Diego.

Laird P.W., Zijderveld A., Linders K., Rudnicki M.A., Jaenish R. and Berns A. (1991). Simplified mammalian DNA isolation procedure. Nucl. Acids. Res. 19, 4293.

Martin J.F., Bradley A. and Olson E.N. (1995). The paired-like homeobox gene MHox is required for early events of skeletogenesis in multiple lineages. Genes. Dev. 9, 1237-1249.

Moore K.L. and Persaud T.V.N. (2003). The Developing Human. Clinically oriented embryology. 7th ed, WB Saunders, Philadelphia.

Pai A.C. (1965). Developmental genetics of a lethal mutation, muscular dysgenesis (Mdg) in the mouse. I. Genetic analysis and gross morphology. Dev. Biol. 11, 82-92.

Patton J. and Kaufman H. (1995). Timing of ossification of limb bones and growth rates of various long bones of the fore and hind limbs of the prenatal and early postnatal laboratory mouse. J. Anat. 186, 175-85.

Raab-Cullen D.M., Thiede M.A., Petersen D.N., Kimmel D.B. and Recker R.R. (1994). Mechanical loading stimulates rapid changes in periosteal gene expression. Calcif. Tissue Int. 55, 473-478.

Rivera-Pérez J.A., Wakamiya M. and Behringer R.R. (1999). Goosecoid acts cell autonomously in mesenchyme-derived tissue during craniofacial development. Development 126, 3811-3821.

Rot-Nikcevic I., Reddy T., Downing K.J., Belliveau A.C., Hallgrimsson B., Hall B.K. and Kablar B. (2006). *Myf5*$^{-/-}$*:MyoD*$^{-/-}$ amyogenic fetuses reveal the importance of early contraction and static loading by striated muscle in mouse skeletogenesis. Dev. Genes. Evol. 216, 1-9.

Rudnicki M.A., Schnegelsberg P.N., Stead R.H., Braun T., Arnold H.H. and Jaenisch R. (1993). MyoD or Myf5 is required for the formation of skeletal muscle. Cell 75, 1351-1359.

Sambrook J., Fritsch E.F. and Maniatis T. (1989). Molecular cloning: a laboratory manual. Nolan C. (ed). Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Sperber G.H. (2001). Craniofacial development. BC Decker, Hamilton.

Tran S. and Hall B.K. (1989). Growth of the clavicle and development of clavicular secondary cartilage in the embryonic mouse. Acta Anat. 135, 200-207.

Accepted July 19, 2006