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Growth characteristics underlying lack of a chin in pigs: a histomorphometric study

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Abstract

Objectives—Despite similar mandibular growth to that of humans, pigs lack a chin projection as shown in most humans. To understand whether this divergence is contributed to differences in local symphyseal growth, this project characterized bone modeling activities at the symphyseal surfaces of juvenile pigs.

Material and Methods—Symphyseal specimens from 2 age groups (4- and 6-month-old, n=10) were processed into histological sections with and without decalcification, which were assessed for surface mineral apposition and bone resorption, respectively. In a blinded fashion, measurements of four parameters (MAR: mineral apposition rate, MAZ: mineral apposition zone; ES/BS: eroded surface; OC.N/BS: osteoclast number) were obtained and tested by a multivariate two-way mixed-model analyses of variance (MANOVA) for the differences between symphyseal regions and ages.

Results—Qualitatively, pig symphyseal labial and lingual surfaces were horizontally oriented and characterized by mineral apposition and bone resorption, respectively. Quantitatively, labial mineral apposition tended to be greater rostrally than caudally at 4 months, which became greater caudally than rostrally at 6 months (region/age interactions: $p=0.127$ for MAR, $p=0.012$ for MAZ). Lingual bone resorption tended to be greater caudally than rostrally, but only ES/BS measurements was significant ($p=0.039$) regardless of age while OC.N/BS measurements varied with ages and regions (age/region interaction, $p=0.087$).

Conclusions—Insufficient differential in symphyseal surface modeling between the labial-caudal and labial-rostral regions contributes to the lack of chin projection in the pig.

Keywords

Bone formation; Chin; Histology; Osteogenesis; *Sus scrofa*

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INTRODUCTION

In humans, a proper chin projection is important for facial harmony and esthetics. Although relatively broad and narrow chins are often considered more attractive for men (1), and women (2), respectively, deficient chin projections undermine facial beauty regardless of gender. Generally resulting from growth rotation of the entire mandible and local bone modeling at the symphyseal surfaces, chin formation varies substantially among individuals in the specific processes (3–5). First, although bone resorption and apposition consistently characterize the anterior and posterior ramal surfaces, respectively (6), which produce anterior displacement of the mandibular body, the overall rotation of the mandible can still be either forward or backward depending on the directions of condylar growth (3, 5). Next, bone modeling at the symphyseal surfaces is not invariable. On one hand, Enlow stated that the lingual symphyseal surface is appositional and the labial surface is divided by a reversal line into superior and inferior regions, which are characterized by bone resorption and apposition, respectively (6). On the other hand, Björk and coworker's implant studies demonstrated certain subjects could deviate from "labial-superior resorption" norm and exhibited labial-superior apposition (4); Buschang and coworker's cephalometric studies revealed that the labial-inferior region (also termed mental protuberance) remained unchanged in males but moved slightly lingually in females during puberty, suggesting that this region is also likely to be inactive or even mildly resorptive (7).

Regardless of variations of the processes, deficient chins in human patients are currently treated by one of three options: 1) orthognathic surgeries aimed at advancing the mandible or inducing mandibular forward rotation, 2) direct symphyseal surgeries aimed at repositioning the mental protuberance forward using alloplastic implants or osteotomy (genioplasty) (8) and, 3) subcutaneous injections of dermal filler to the chin area. Though effective, these options are rather invasive, costly and susceptible to complications (9). With recent advancement in tissue engineering techniques (10), a potentially less invasive growth-modification approach entailing stem cells or growth factors may be developed, for which a reasonable preclinical animal model is needed.

Compared to humans, pigs (11), monkeys (12), dogs (13) and rats (14) all have similar mandibular growth patterns at the ramus, and yet none of them possesses a prominent chin. In fact, no mammals except for humans and elephants show a prominent chin (6). Pigs are strongly similar to humans in mandibular anatomy and function (15, 16) and have been used commonly for craniofacial bone regeneration studies (17, 18). Mechanical strains at the pig symphyses have also been investigated previously (19, 20). The bone modeling processes underlying lack of chin projections in pigs, especially whether they are different from those of human symphyses, are currently unclear.

To better understand bone growth of pig symphysis and assess the advantages and limitations of using pigs as animal models to investigate growth-modification approaches in treating deficient chin projections, this study characterized bone modeling at the symphyseal surfaces of juvenile pigs.

MATERIALS & METHODS

Animals and Sample Size

Cadaver mandibles were obtained from 2-age groups of juvenile (4-month-old and 6-month-old) female domestic pigs (*Sus scrofa*), which were used in a previous tooth extraction study (21) and a distraction osteogenesis study, respectively (22). All live animal procedures were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Fluorescent labeling for bone mineralization, specimen collection and processing

Before sacrifice, all 10 pigs used in this study received fluorescent dyes for labeling of bone mineralization as previously described (21, 22). More specifically, 12.5 mg/kg (body weight) Calcein and Alizarin-3-methyliminodiacetic acid (Sigma-Aldrich, St. Louis, MO) were dissolved in normal saline to make 5 mg/ml solutions, adjusted for pH to 7.3–7.4 and filtered through a 0.22 μ m Millipore membrane filter, then injected intravenously to the pig at 10 and 3 days, respectively, before euthanasia. The injections were conducted with the pigs under general anesthesia (6 mg/kg Telazol, IM; maintained by 2–3% isoflurane with 2–5% oxygen through a mask). For euthanasia, the pigs were placed under general anesthesia followed by 125 mg/ml potassium chloride through IV injection.

After euthanasia, specimens containing the symphyseal area with attached periosteum were harvested, then divided into right and left halves, and each half was further divided into rostral and caudal regions. Specimen blocks from the right and left halves were processed for decalcified and undecalcified histology, respectively. For undecalcified histology, the specimens were fixed with Prefer (Anatech LTD, Battle Creek, MI) for a week, dehydrated with an ethanol series and infiltrated with MicroBed resin solution (Electron Microscopy Sciences, Fort Washington, PA). The resin was then cured under 365 nm ultraviolet light for 3–4 days in an ice box. The embedded specimens were then cut into 50 μ m thick labial-lingual sections using a diamond circular microtome (Leica SP1600, Leica Microsystems Inc, Buffalo Grove, IL), and the sections were flattened, mounted on slides and cover-slipped with VectaMount (Vector Laboratories, Inc., Burlingame, CA).

For decalcified histology, the specimens were fixed in Prefer for two weeks and decalcified using 10% disodium ethylenediamine tetraacetic acid (EDTA) solution in PBS at a pH of 7.2–7.4. Once decalcified, the specimens were dehydrated and embedded in paraffin, cut into 5 μ m thick labial-lingual sections using a rotary microtome. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin and mounted on glass slides.

Assessment of bone formation and resorption

Undecalcified bone sections were used to assess bone formation. First, under a fluorescent microscope (Axioplane 400 Zeiss, Carl Zeiss MicroImaging, NY), the presence/absence of mineral apposition at the labial and lingual surfaces was observed, with positive mineral apposition characterized by clearly separated layers of fluorescent labels (Fig. 1A–B). Subsequently, images containing the labial and lingual surfaces were captured for quantification. Under 100 $\times$ magnification, from each labial or lingual surface (typically

constituted 5–6 consecutive non-overlapping images), 2 images were selected following a random table and saved as .tif files. For each animal, a total of 6 sections (3 each from the rostral and caudal regions) and 4 images/section (2 each from the labial and lingual surfaces) were analyzed.

The captured images were then used to quantify mineral apposition by one trained rater (J.P.) who was blinded to the identity of images using the ImageJ software (NIH, Bethesda, MD). As previously described (21), two linear parameters reflecting new bone growth termed mineral apposition rate (MAR) and mineral apposition zone (MAZ) were quantified using the digital measurement tool of ImageJ. To determine the locations of measurements, 6 equally-spaced horizontal grid lines were superimposed to the image at random offsets by ImageJ (Fig. 1A–B), and measurements were made at the line-surface intersections.

Hematoxylin-eosin (H&E) stained decalcified sections were used to assess bone resorption. First, under a light microscope (Olympus BX51, Olympus America Inc, Center Valley, PA), they were viewed for the presence/absence of bone resorption at the labial and lingual surfaces. Positive resorption was characterized by presence of resorptive concavities and osteoclasts. Then, because bone resorption was only present at the lingual but not the labial surface, images containing the lingual surface were captured for quantification of bone resorption. More specifically, low power (100X) images were captured from 3 randomly selected areas (using a random table) of the lingual surface followed by 2 more high power (400X) images captured from each area.

The captured images were analyzed by the same rater (J.P.) who was blinded to image identity. Two parameters reflecting bone resorption: eroded surface (ES/BS) and osteoclast number (OC.N/BS) (Fig. 1C–D) were quantified by using low and high power images, respectively. Osteoclasts were distinguished from osteoblasts and bone lining cells by a relatively large size, 2 or more nuclei, and being present in resorptive concavities.

Statistical analysis

All quantitative measurements were first tested for normal distribution using Shapiro-Wilk tests. Then, mineral apposition measurements (MAR and MAZ) from lingual and labial surface, bone resorption measurements (OC.N/BS and ES/BS) from the lingual surface were tested by a single mixed-model multivariate analysis of variance method (MANOVA) to analyze the differences between regions (rostral vs. caudal, within factor) and ages (4-month vs. 6-month, between factor). A $p < 0.05$ was considered statistically significant.

RESULTS

Qualitatively, the labial surface was characterized by abundant mineral apposition showing separated layers of fluorescent labels. In contrast, mineral apposition at the lingual surface was minimal in the rostral region and negligible in the caudal region (Fig. 2A-a,b). H&E stained sections showed that the lingual side mainly consisted of relatively mature cortical bone with resorptive concavities (Fig. 2B-a) and osteoclasts at the surface, while the labial sides mainly consisted of plexiform woven bone strands and the surface was lined by

osteoblasts (Fig. 2B-b). The general features of bone apposition and resorption at the labial and lingual surfaces, respectively, appeared uniform.

Quantitative measurements of mineral apposition at both surfaces are summarized in Fig. 3. On the lingual side, the measurements confirmed qualitative observation that there was very minimal apposition present and only at the rostral region. Because of the absence of mineral apposition at multiple areas which lead to “0” measurements, this set of data was not normally distributed and was not included in the MANOVA test. Measurements of labial mineral apposition were more substantial and normally distributed, and were included in a MANOVA test (Fig. 3C–D). For MAR, neither the main effects of the region and age factors alone ($p=0.846$ and $p=0.744$, respectively), nor their interaction ($p=0.127$) were significant. For MAZ measurements, the main effects of neither region nor age factor were significant ($p=0.677$ and $p=0.411$, respectively), but their interaction was significant ($p=0.012$).

Bone resorption measurements from the lingual surface are summarized in Fig. 4. For OC.N/BS, the main effects of regions and ages were insignificant ($p=0.995$ and $p=0.845$, respectively), but their interaction was nearly significant ($p=0.087$). For ES/BS measurements, only the main effect of regions was statistically significant, being greater caudally than rostrally ($p=0.039$) regardless of age.

DISCUSSION

Morphologically, compared to the human counterpart, the pig’s symphysis is more horizontally oriented and rostro-caudally elongated. This orientation and shape have been found to be compatible with the pig’s mechanical environment during chewing (19). Interestingly, when incisor occlusion is naturally missing in Yucatan swine (19, 23) or artificially deprived using posterior bite blocks, the symphysis tends to become more vertical. While the former may be attributed to a more forward rotation of the entire mandible, the latter is very likely a phenomenon of local symphyseal adaptation to loss of incisor function since posterior bite blocks induce backward rotation of the mandible.

Our finding that the entire labial and lingual symphyseal surface in pigs are appositional and resorptive, respectively (Fig. 2), also closely reflects masticatory mechanics at these surfaces (19). During mastication, the entire labial surface undergoes tension while the lingual surface is compressed (19). Notably, this pattern of labial surface apposition is also present in monkeys (*Macaca mulatta*), an anthropoid primate that also lacks chin projection (24), but not typically present in humans. In fact, according to histological findings made by Enlow (6), symphyseal bone modeling in most humans demonstrates apposition of the entire lingual surface and resorption of the labial surface except for the labial-inferior region. It is important to note, however, exceptions do exist as labial-superior apposition were found in certain human subjects (3, 4), and inactivity or mild resorption may also mark the labial-inferior surface (7).

Therefore, compared to that in humans, local growth at the symphyseal surfaces in pigs shows a simpler and more consistent pattern. To further understand the mechanism

underlying this pattern, we assessed bone modeling at lower incisor alveolar processes of 6 pigs, which were of similar ages to those used in this study. The summarized data and a representative image showing mineral apposition of the lower incisor alveolar process are presented in Fig. 5. Overall, there is abundant mineral apposition at the labial alveolar process, which is continuous with modeling of the entire labial symphyseal surface, but differs from a common pattern observed from the human labial alveolar counterpart which is characterized by resorption at the labial-superior region (6).

Based on our current findings of pig symphyses and previous findings of the human counterpart (3, 4, 6, 7), we illustrate in a diagram the modeling activities at the different regions and surfaces of these two species (Fig. 5C). Bone modeling features at the labial alveolar process and superior-symphyseal regions in both species very likely reflect the movements of the incisors in the sagittal plane, i.e., labial proclination in pigs whereas commonly lingual retroclination in humans (3–6, 25). Notably, in human subjects who showed an atypical symphyseal modeling pattern, i.e., apposition rather than resorption in the labial-superior region as illustrated in Cases #1, 3, 20 in the Björk and Skieller study (4), their lower incisors demonstrated proclination rather than retroclination, which further supports this notion. In addition, compared to the human counterpart, the pig's symphysis is more horizontally oriented in space, which makes its labial and lingual surfaces the dorsal and ventral surfaces, respectively. Thus, the pig's tongue directly rests on the symphysis, which may mechanically further procline the incisors labially and prevent uprighing of the symphysis.

Extrapolating these data to humans, at the local symphyseal level, a large differential of modeling activities between the labial-superior and labial-inferior regions would increase the prominence of chin projection, which is well reflected in subjects with age-associated lower incisor retroclination (4). Although previous reports on modeling at the human labial-inferior symphyseal surface are inconsistent (5, 6), which suggest that apposition, inactivity and slight resorption are all possible, a strong resorption at the labial-superior symphyseal surface and alveolar process is still able to make the labial-inferior symphyseal region prominent relative to the labial-superior region. On the other hand, in human subjects with age-associated lower incisor proclination (4), because the labial-superior region undergoes apposition rather than resorption, the differential between the labial-superior and labial-inferior regions is small, hence decreasing chin prominence. This pattern is similar to what was found from the pigs in this study. Specifically, we found that the pig labial-caudal region grew less than the labial-rostral region at 4 months, which was reversed at 6 months. Although the latter finding suggests that the labial-caudal region is likely to grow more than the labial-rostral region with age, thus making the labial-caudal region more prominent and the entire symphysis more vertical, our data are incapable of predicting that this growth pattern retains with age. Mechanically, it has been found that there was no consistent rostro-caudal gradient of masticatory strain in 1.5–6 months old pigs (19), indirectly suggesting that the caudal-rostral increases in bone modeling observed at certain ages may be transient. This notion was further supported by that the pig's symphysis remains fairly horizontal and lacks a distinguishable chin projection regardless of age.

Conceivably then, to make a more prominent chin in the pig, one may enhance bone apposition at the labial-caudal region to make it substantially thicker than the labial-rostral region. For humans with age-associated lower incisor proclination and deficient chins, this type of approach would also be effective at the local symphyseal level because it can enhance the differential in modeling between the labial-superior and labial-inferior regions in the sagittal plane. In this sense, the pig is a relevant model for investigating local growth modification approaches to address human chin deficiency. In addition to well-described mechanical environment (19) and local bone modeling revealed in this study, the pig's symphysis also resembles the human counterpart better than those of rodent models in size, time of fusion of the symphysis and lack of evergrowing incisors, which offer additional advantages to use this model.

As with many animal models, however, because of the differences between pigs and humans in symphyseal orientation and local bone modeling, several cautions are warranted when using this animal model. First, a prominent chin projection in humans is largely contributed by mandibular forward growth and rotation (5, 6), local growth modification at the symphysis may only address a relatively small part of the problem. Next, modeling activities at the labial-inferior region of human chins may vary among inactivity, apposition, and mild resorption, which differs from consistent apposition at the labial-caudal region of pigs. Thus conditions of pig periosteum at the labial-caudal region may not fully represent the complexity of the periosteum of the human counterpart. Finally, our present study included a relatively narrow age window (4–6 months) of pigs. While this window is within the accelerated growth period of juvenile pigs, it does not adequately account for the entire adolescence phase, nor does it reflect the status of adulthood, which in humans is the age when some patients seeking treatment for deficient chins.

This study also has two additional limitations. First, the pigs underwent previous tooth extraction (4-month-old group) (21) or mandibular distraction osteogenesis (6-month-old group) (22), which may affect symphyseal growth. Reassuringly, however, neither procedure directly involved the symphysis, and previous studies have found that mechanical strain at the extraction site was comparable to the contralateral control site (21), and masticatory strains at the symphyses were not changed by mandibular distraction osteogenesis (19). Therefore, the confounding effects are probably minimal. The other limitation is that identification of osteoclasts could have been confirmed by tartrate-resistant acid phosphatase (TRAP) staining, which was attempted but unsuccessful probably due to lengthy decalcification of our relatively large specimens.

Despite these limitations, in general our data revealed some histomorphometric features of symphyseal surface modeling in juvenile pigs, which adds to the understanding of bone modeling activities underlying the lack of chin projections in this species. The entire labial surface from the alveolar crest to the caudal border is horizontally oriented in space and undergoes apposition, and lacks a persistent rostral-caudal increase in apposition, which differs from symphyseal bone modeling of most growing humans with age-associated lower incisor retroclination (6), but may resemble those with age-associated lower incisor proclination and chin-deficiency (3, 4). These modeling characteristics suggest that juvenile pigs may be used to investigate local cell or growth factor-based growth modification

approaches by enhancing apposition at the labial-caudal region to make chin projection more prominent. When translating these approaches to human patients, however, the extent of improvement in chin projection may be constrained by the overall mandibular forward growth and rotation as well as the variability of bone modeling at human chins.

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Clinical Relevance

Adequate chin projection is an important component of facial attractiveness. Deficient chin projection is a common growth-related problem seen in some patients, but currently not treated by any symphyseal growth modification approaches. Developing such an approach using recent advances in tissue-engineering technologies is a plausible idea with a reasonable animal model. This study characterized bone modeling of the symphysis of juvenile pigs. Our study first clarified the differences and similarities of symphyseal bone modeling between pigs and humans, then discussed the relevance and limitations of using this animal model for future investigations on symphyseal growth modifications.

Figure 1.

Methods for histomorphometric analyses. (A–B) Quantification of mineral apposition. Fluorescent images were opened in ImageJ. Using the Analyze-Grid module, 6 equally-spaced, randomly-positioned horizontal test lines were superimposed perpendicularly to the surface. Where the lines intersect the surface, linear distances perpendicular to bone surfaces between labels were collected using the Measurement module. Mineral apposition rate (MAR) was defined as the average distance between calcein (green label, broken line b) and alizarin-3-methyliminodiacetic acid (red label, broken line a) fronts, divided by 7 days (time interval between bone label injections). Mineral apposition zone (MAZ) was defined as the average distance between old (broken line c) and new bone fronts (broken line a). Note the images were oriented vertically for easier completion of the measurements, which did not represent the true orientation of the symphyseal surface in space (Fig. 5C). (C–D) Quantification of bone resorption. H&E images of the lingual surface were analyzed for two parameters of bone resorption. Eroded surface (ES/BS) was defined as the length of the surface contour (dotted line) divided by its straight length (solid line). Resorptive regions were marked by arrow heads. Osteoclast number (OC.N/BS) was defined as the number of osteoclasts (arrows) divided by the straight length of the surface.

Figure 2.

Representative fluorescent (A) and H&E (B) images of symphyseal surfaces. Images a (lingual surface) and b (labial surface) are magnified images from areas marked rectangles shown in A and B. Note only minimal apposition was shown at the lingual surface (A-a), whereas abundant mineral apposition was present at the labial surface (A-b) as multiple layers of fluorescent labels. In contrast, bone resorption characterized by resorptive concavities and osteoclasts (arrow head) was abundant at the lingual surface (B-a) but not at

the labial surface (B-b), which was characterized by newly formed woven bone strands lined by osteoblasts (arrow) instead.

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Figure 3.

Mean mineral apposition measurements from the lingual (A,B) and labial surfaces (C,D).

Error bar, standard error. (A–B) At the lingual surface, overall MAR and MAZ

measurements were minimal at the rostral region and near 0 at the caudal region. (C–D) At

the labial surface, all measurements of mineral apposition were substantial and there was a

trend of interaction, suggesting that as age increased, mineral apposition increased at the

caudal region and decreased at the rostral region.

Figure 4.

Mean bone resorption measurements from the lingual surface. Error bar, standard error. (A) For OC.N/BS measurements, a trend of interaction between the two factors was shown. As age increased, OC.N/BS became greater at the caudal region and smaller at the rostral region. (B) For ES/BS measurements, regardless of age, ES/BS at the caudal region was greater than that at the rostral region.

Figure 5.

Comparison between the symphyses of humans and pigs. (A) A representative image showing all three regions of the pig symphyseal bone modeling. Note the entire labial surface was undergoing active apposition, whereas on the lingual surface there was minimal apposition in the alveolar process region and none in the rostral and caudal symphyseal regions. (B) Mean mineral apposition measurements of lower incisor alveolar bone surfaces from 6 pigs in the same age range as those used in this study. Error bar, standard error. Note that labial surface has significantly more bone formation than the lingual surface (Repeated measures MANOVA test, $p < 0.05$). (C) Schematic representation of human and pig symphyseal growth. +, apposition; -, resorption, () atypical modeling pattern. Typical pattern data were from Enlow (6), Björk and Skieller (4) and Buschang et al. (5), atypical pattern data were from Björk and Skieller (4); pig growth data were from this study. Note the growth of labial surface in the pig differs from the typical modeling pattern in humans but resembles the atypical pattern in humans. Lacking substantial caudal-rostral differential in pigs (or substantial superior-inferior differential in humans) of labial symphyseal surface modeling activities may contribute to the lack of chin projections.