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The potential for vertical bone regeneration via maxillary periosteal elevation

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Abstract

Background: While many studies have been performed on the characteristics and regenerative capacity of long bone periosteum, the craniofacial periosteum remains poorly understood.

Aim: The aim of this study was to investigate the potential for a maxillary periosteum tunnelling procedure to induce vertical alveolar bone regeneration.

Materials and Methods: We employed a murine injury model that activates skeletal stem cells in the periosteum without overtly damaging the underlying cortical bone, preserving the integrity of the long bone and maxilla, and avoiding the introduction of pathological motion at the injury site. Further, we introduced a collagen sponge to serve as a scaffold, providing the necessary space for vertical bone regeneration.

Results: Periosteal elevation alone resulted in bone formation in the tibia and delayed bone resorption in the maxilla. With the presence of the collagen sponge, new bone formation occurred in the maxilla.

Conclusions: Periosteal response to injury varies with anatomical location, so conclusions from long bone studies should not be extrapolated for craniofacial applications. Murine maxillary periosteum has the osteogenic potential to induce vertical alveolar bone regeneration.

The periosteum is a specialized, vascularized connective tissue anchored to the surface of bone, and is generally considered a reservoir of undifferentiated, multi-potent mesenchymal cells. The periosteum has

two distinct layers: the outer fibrous layer containing fibroblasts, nerves, vessels, and Sharpey's fibres (Simon et al. 2003, Al-Qtaitat et al. 2010) and the inner cambium layer containing the osteoprogenitors (Siems et al. 2012). In vivo, these periosteal stem/progenitor cells are able to differentiate into chondrogenic and osteogenic lineages; in vitro, they can be induced to differentiate into adipogenic and myogenic lineages as well (Malizos & Papatheodorou 2005, Siems et al. 2012).

Duhamel's 1739 "silver ring experiment" was considered the first scientific demonstration of the osteogenic capacity of the periosteum (Macewen 1907). Since those experiments in the early 18th century, the periosteum has been under continued investigation, with the goal being to harness its bone-forming potential. For example, in several surgical orthopaedic techniques, including distraction osteogenesis, the periosteum exhibits regenerative potential; likewise, periosteal grafts have been used to treat bone fractures

and cartilage injuries (Baums et al. 2007, Giannoudis et al. 2011, Li et al. 2012, Soldado et al. 2012). Periosteal grafts are not commonly employed in oral surgical procedures, and in periodontology, it is thought that osteogenic cells are derived from undifferentiated perivascular connective tissue cells or pericytes (Davies 2003). In fact, most assumptions about bone healing are extrapolated from experimental models of skeletal repair in long bones, like that of Duhamel's early periosteal studies.

Because of their histological similarities and equivalent mineral densities, most investigators presuppose that the periosteum of long bones and of craniofacial bones is analogous. A number of unique features, however, distinguish the appendicular skeleton and its associated periosteum from the craniofacial skeleton and its periosteum. For example, craniofacial and appendicular periostea are differentially affected by anabolic agents such as bisphosphonates (Adamo et al. 2008, Reid 2009, Silverman & Landesberg 2009, Knight et al. 2010). Leucht et al. (2008) used a lineage labelling strategy to demonstrate that craniofacial and long bone periostea contribute differently to bone repair: in long bones, periosteal stem/progenitor cells are derived from mesoderm, whereas in craniofacial bones, the periosteal stem/progenitor populations are derived from the neural crests (Leucht et al. 2008).

Here, we sought to expand our understanding of the differences between craniofacial and long bone periostea. We investigated the characteristics and regenerative capacity of the maxillary periosteum, using the long bone periosteum as a control. Bone loss, especially vertical bone loss, in edentulous ridges of partially dentate patients constitutes one of the major surgical challenges in oral surgery (McAllister & Gaffaney 2003, Rocchietta et al. 2008, Esposito et al. 2009), so a deeper understanding of the maxillary periosteum and its management in surgery could allow us to harness its regenerative potential for improvement of osseointegration and vertical bone regeneration in oral surgery.

Materials and Methods

Animal care

All procedures followed protocols approved by the Stanford Committee on Animal Research. Animals were housed in a temperature-controlled environment with 12-h light/dark cycles and were given soft diet food (Bio Serv product #S3472) and water ad libitum. No antibiotics were given to the animals and there was no evidence of infection or prolonged inflammation at the surgical site.

Periosteal surgery

Adult wild-type mice (males, between 3 and 5 months old) were anaesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (16 mg/kg). The mouth was rinsed using a povidone-iodine solution for 1 min.

In cases where the tunnel technique was used, a small incision was made behind the maxillary incisor, perpendicular to the crest. Subsequently, a periosteal was gently inserted into the incision, with bone contact, raising the periosteum in full thickness along the crest, toward the first molar (in tunnel technique) ($N = 17$). An analogous injury was made in the tibia, with a small incision made on the proximal tibia, perpendicular to the crest, followed by insertion of a periosteal, raising the periosteum distally, in full thickness along the medial surface ($N = 15$). In cases to evaluate the potential for vertical maxillary bone regeneration, an absorbable collagen sponge (HELISTAT, ref.1690-ZZ Integra LifeSciences Corporation) was inserted into the tunnel ($N = 5$).

The surgical site was carefully rinsed with 0.9% sodium chloride, and the wound was sutured closed with non-absorbable single interrupted sutures (Ethilon monofilament 9-0, Johnson & Johnson Medical, New Brunswick, New Jersey, USA). Following surgery, clinical examinations were performed and the mice received subcutaneous injections of buprenorphine (0.05–0.1 mg/kg) for analgesia each day for 3 days. Surgeries on the left sides of the mice were performed 1 week after the right sides to obtain two different time points per mouse. Mice were sacrificed in order to obtain each of the following time points: 1, 3, 7, 14, 21, and 28 days post-surgery.

Sample preparation, processing, and histology

Maxillae and tibiae were harvested, the skin and outer layers of muscle were removed, and tissues were fixed in 4% paraformaldehyde overnight at 4°C. The samples were decalcified in a heat-controlled microwave in 19% EDTA for 2 weeks. After demineralization, specimens were dehydrated through an ascending ethanol series prior to paraffin embedding. Eight-micron-thick longitudinal sections were cut and collected on Superfrost-plus slides for histology including Movat's pentachrome, Aniline blue, H&E, Safranin-O, and Picro sirius red staining.

Cellular assays

Alkaline phosphatase (ALP) activity was detected by incubation in nitro blue tetrazolium chloride (NBT; Roche, Indianapolis, Indiana, USA), 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche), and NTM buffer (100 mM NaCl, 100 mM Tris, pH 9.5, 5 mM MgCl). Tartrate-resistant acid phosphatase (TRAP) activity was observed using a leukocyte acid phosphatase staining kit (Sigma). After developing, the slides were dehydrated in a series of ethanol and xylene and subsequently cover-slipped with Permount mounting media.

For terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining, sections were incubated in proteinase K buffer (20 μg/ml in 10 mM Tris, pH 7.5), applied to a TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche), and mounted with DAPI mounting medium (Vector Laboratories). Slides were viewed under an epifluorescence microscope.

Immunohistochemistry

Tissue sections were deparaffinized following standard procedures. Endogenous peroxidase activity was

quenched by 3% hydrogen peroxide for 5 min, and then slides were washed in PBS. Slides were then immunostained using a proliferating cell nuclear antigen (PCNA) staining kit (Invitrogen, Frederick, MD, USA, Ref: 93-1143), following manufacturer's protocol.

Results

Periostea differ by anatomic location

Although maxillary and mandibular vertical bone loss is a major issue in ageing patients with missing teeth (Cawood & Howell 1988), periostea of these craniofacial bones have largely escaped analysis. As the periosteum is a source of stem cells (Malizos & Papatheodorou 2005), we focused our analyses on the periostea associated with these bones, and compared these results with analogous data from the tibia.

By histological analyses, the first obvious distinction between the maxilla and tibia was the thickness of the periosteum: the tibial periosteum was thick and the ratio of cells/stroma was high (Fig. 1a). In contrast, the oral maxillary periosteum was thin and the cells/stroma ratio was considerably lower (Fig. 1b). Clinicians appreciate that the tibial periosteum is loosely adherent to the surrounding muscle tissue, which allows for easier movement of the periosteum, while the maxillary periosteum is tightly bound to the surrounding connective tissue, restricting movement (Shaly Bothla: *Periodontics Revisited* 2011). Cell proliferation was another distinction between the periosteal. Cells immuno-positive for proliferating cell nuclear antigen (PCNA) were found throughout the tibia periosteum (Fig. 1g), while the maxillary periosteum was devoid of PCNA[+ve] cells (Fig. 1h). Nearby gingival epithelial cells were PCNA[+ve], showing that the immunostaining was successful (not shown). Collectively, these data indicated the intact tibial periosteum was more proliferative than its maxillary counterpart, resulting in a thicker, more densely cellular tissue.

We evaluated alkaline phosphatase (ALP) activity as a measure of mineralization (Gerstenfeld et al. 1987) and found in the tibia that
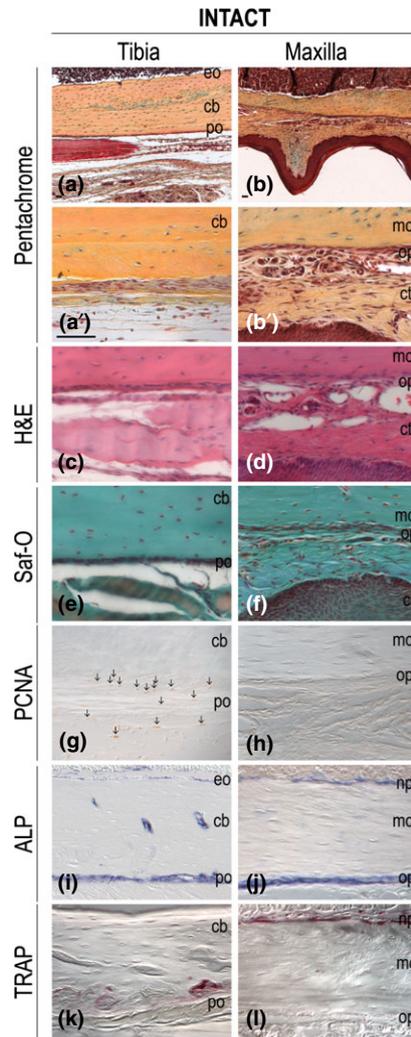

Fig. 1. A histological and cellular comparison of tibial and maxillary periostea. Histological staining of representative longitudinal tissue sections through the tibia and the maxillary crest (a–b′) pentachrome staining, (c, d) H&E, (e, f) Safranin-O (Saf-O). Proliferating cell nuclear antigen (PCNA) immunostaining is present in (g) the tibial periosteum and (h) is undetectable in the maxillary periosteum. Alkaline phosphatase (ALP) activity is detectable in osteoblasts of (i) the periosteal surface of the tibia and (j) the oral surface of the maxillary crest. Tartrate-resistance acid phosphatase (TRAP) staining is detectable in osteoclasts (k) periosteal surface of the tibia and (l) lining the nasal surface of the maxillary crest. Scale bars: (a–l) 50 μm. Abbreviations: eo, endosteum; cb, cortical bone; po, periosteum; oe, oral epithelium; mc, maxillary crest; ct, connective tissue; g, gingiva; op, oral periosteum; np, nasal periosteum.

activity was high in the periosteum and low in the endosteum (Fig. 1i). In the maxilla, ALP activity was high on the oral surface low on the nasal surface of the periosteum (Fig. 1j). Using tartrate-resistant acid phosphatase (TRAP) staining to detect osteoclast activity (Minkin 1982), we found that in the tibia, TRAP activity was limited to the periosteum and was undetectable in the endosteum (Fig. 1k). In the maxilla, TRAP activity was limited to the nasal periosteum, with no detectable activity in the oral periosteum (Fig. 1l). These observations suggest that in the intact bone, mineralization predominates on the oral surface of the intact maxilla, whereas resorption predominates on the nasal surface. This is in agreement with published reports (Atherton et al. 1974). Collectively, these data indicate that the morphology and cellular activity of the tibial and maxillary periostea are markedly different. This conclusion supports the widely established conclusion that periostea differ by anatomical location (Chang & Knothe Tate 2012).

A surgical model of mechanical stimulation to the periosteum

Post-development, the bone-forming potential of the periosteum is reactivated by trauma, infection, and in some cases, growing tumours (Malizos & Papatheodorou 2005). To evaluate the bone-forming potential of the maxillary periosteum, we developed an animal model in which a standard, minimally invasive technique was used to elevate the periosteum away from the underlying bone. The general organization of the maxillary periosteum relative to alveolar bone, its overlying connective tissue and gingiva, as well as the olfactory epithelium were determined (Fig. 2a). The surgical approach began with an incision down to the alveolar bone followed by elevation of the tissue with a blunt periosteal elevator (Neubauer et al. 2011, Masic et al. 2012), resulting in a tunnel between the connective tissue/periosteum and the periosteum/bone surface (Fig. 2b–e).

We evaluated periosteal and connective tissues immediately after the tunnelling procedure. We noted the stabilization of a clot within the tunnel (dotted lines), with little evidence
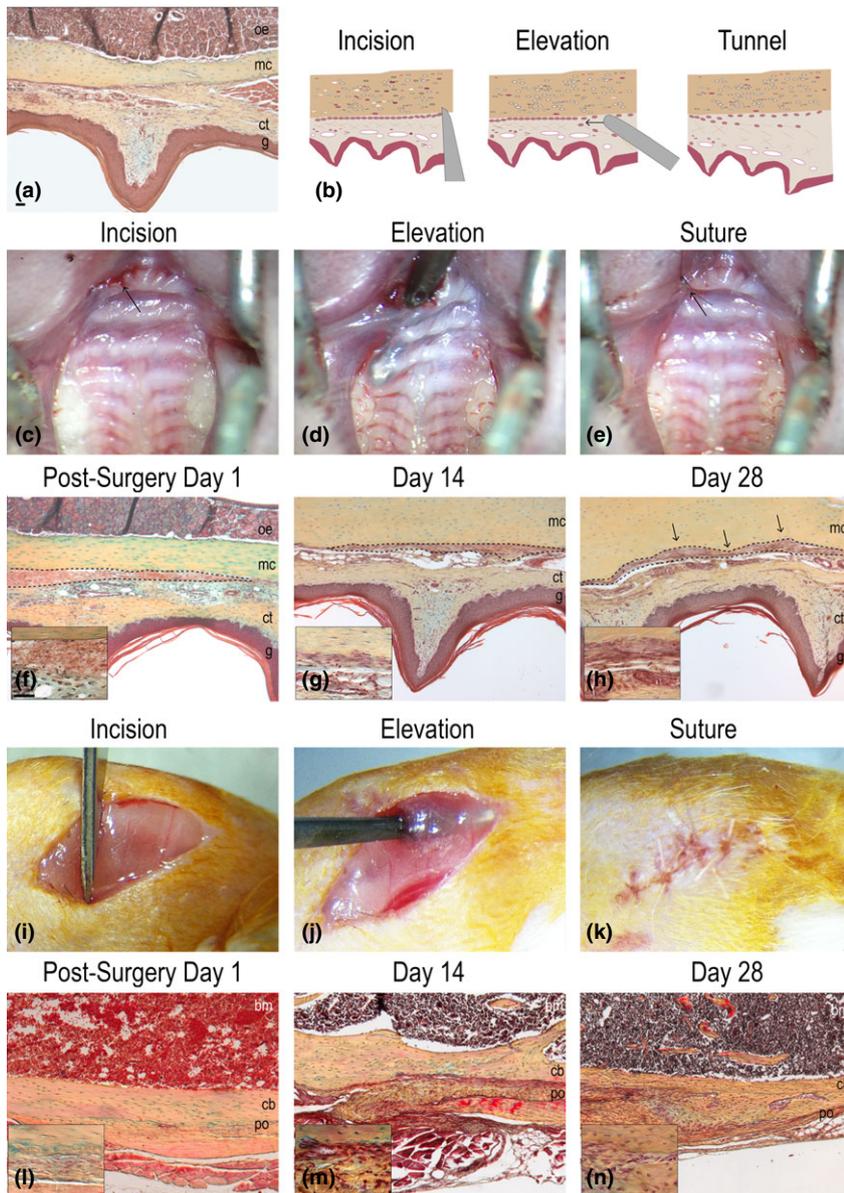
Fig. 2. Periosteal tunnelling technique. (a) Pentachrome staining of a representative longitudinal tissue section through the maxillary crest, pre-operatively. (b) Schematic illustration of the incision site and elevation of a full-thickness periosteal flap using a tunnelling procedure. Intraoperative photographs of the maxillary tunnelling procedure include (c) the incision site, (d) the tunnelling and elevation, and (e) the site of suturing. Pentachrome staining of representative longitudinal tissue sections through the maxillary crest on (f) post-surgical day (PSD) 1 illustrating the tunnel filled with a blood clot (dotted line, and inset); note the preserved architecture of the surrounding connective tissues. (g) On PSD14, illustrating the tunnel occupied by a population of dense, flattened cells (dotted line, and inset) and loosely organized connective tissue. (h) On PSD28, the neo-periosteum covers the partially resorbed oral surface of the maxillary crest (dotted line and inset). Analogously, intraoperative photographs of the tibial tunnelling procedure include (i) the incision site, (j) the tunnelling and elevation, and (k) the site of suturing. Pentachrome staining of representative longitudinal tissue sections through the tibia on (l) PSD1. (m) On PSD14. (n) On PSD28. Scale bars: (a, f–h, l, m) 50 μm. Abbreviations: oe, olfactory epithelium; mc, maxillary crest; ct, connective tissue; g, gingiva; cb, cortical bone; po, periosteum; bm, bone marrow.

of inflammation (Fig. 2f). On post-surgery day 14, a well-organized, densely cellular fibrous tissue occupied the tunnel (dotted lines, Fig. 2g). The connective tissue immediately above the tunnel appeared normal, without

evidence of an inflammatory infiltrate (Fig. 2g). By post-surgery day 28, cells within the tunnel were further organized and areas of bone resorption under the tunnel were observed (arrows, Fig. 2h).

An analogous procedure was performed in the tibia, with a bone depth incision followed by periosteal elevation that resulted in a periosteal tunnel (Fig. 2i,k). On post-surgery day 1, histological analyses showed a densely cellular response associated with slight periosteal thickening (Fig. 2l). By post-surgical day 14, further periosteal thickening occurred (Fig. 2m), and by day 28, areas of new woven bone were observed (Fig. 2n). Thus, the same injury performed in maxilla and in tibia resulted in radically different responses. In the maxilla, bone resorption ultimately occurred in response to the tunnelling procedure while in tibia, the response to tunnelling was bone formation.

Relative to the tibia, the maxillary periosteum exhibits little bone-forming potential

At post-surgical day 3, histological analyses revealed robust and densely cellular soft tissue response to periosteal elevation in tibia and a minimal cellular response tunnel in maxilla (Fig. 3a–f). PCNA staining showed a strong proliferative response in the tibial periosteum (Fig. 3g), but a minimal response in the maxillary periosteum (Fig. 3h). Minimal cell death was detected in response to the tunnelling procedure in the tibia (Fig. 3i), whereas marked cell death was evident in the maxilla (Fig. 3j). Analyses of osteoclast activity revealed that TRAP staining was virtually undetectable in the tibia (Fig. 3k). In contrast, TRAP^{+ve} cells were evident throughout the maxillary periosteum (Fig. 3l). These observations suggest that the tibia displayed a stronger repair response to the tunnelling procedure than those observed in the maxillary periosteum.

At post-surgical day 7, mineralized tissue had formed at the site of the tunnelling procedure in the tibia (Fig. 3m,m′; see also Fig. 3o,q). The response in the maxillary periosteum remained unremarkable (Fig. 3n,n′; see also Fig. 3p,r). PCNA staining
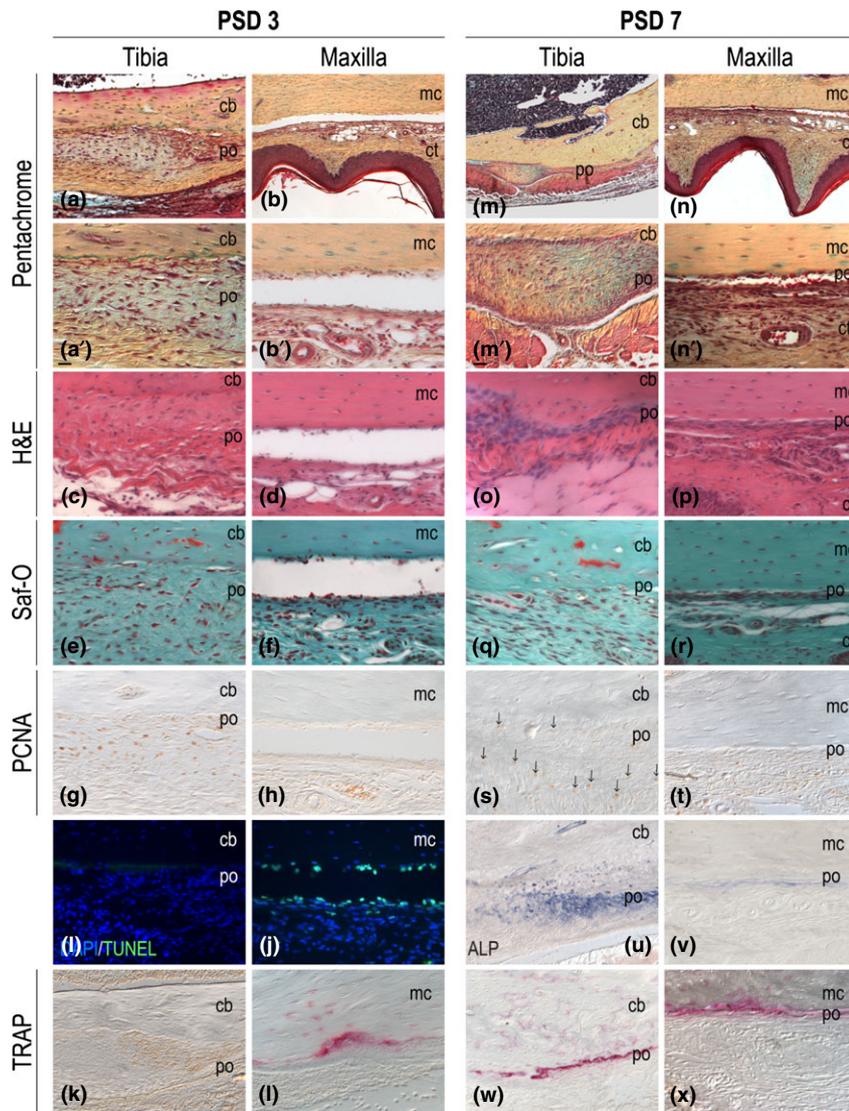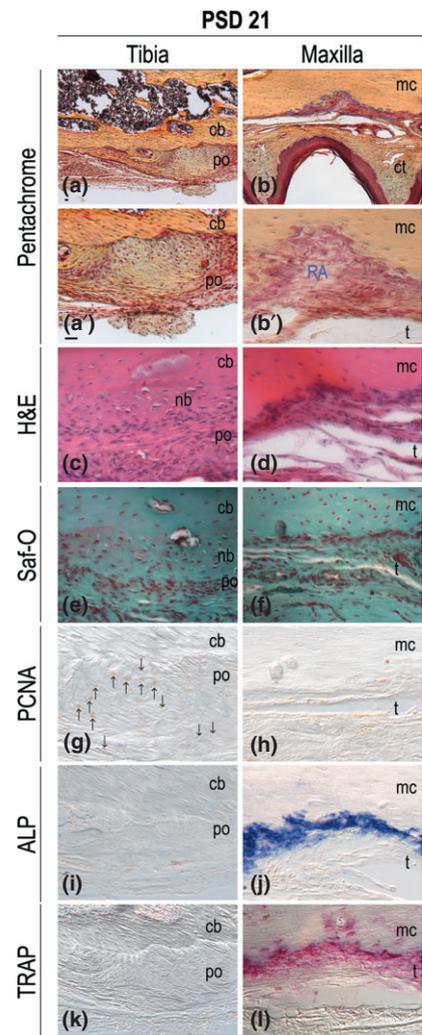
Fig. 3. Early tibial and maxillary response to injury. At post-surgical day (PSD) 3, histological analyses of representative longitudinal tibial and maxillary tissue sections stained with (a–b′) pentachrome, (c, d) H&E, and (e, f) Saf-O. PCNA immunostaining showing proliferating cells in (g) the tibia and (h) the maxilla. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining of (i) tibial and (j) maxillary response showing apoptotic cells in green and nuclei in blue. TRAP staining of (k) tibial and (l) maxillary response. At PSD7, histological analyses of tibial and maxillary responses stained with (m–n′) pentachrome, (o, p) H&E, and (q, r) Saf-O. PCNA staining of (s) proliferating cells in the tibia and (t) maxilla. ALP staining of osteoblast activity (u) in the tibia and (v) in the maxilla. TRAP staining in both the (w) tibia and (x) maxilla. Scale bars: (a–x) 50 μm. Abbreviations: cb, cortical bone; po, periosteum; mc, maxillary crest; ct, connective tissue.

Fig. 4. Periosteal tunnelling induces osteogenesis in the tibia and bone resorption in the maxilla. At PSD 21, histological analyses of representative longitudinal tissue sections through the injured tibia and maxillary crest, stained with (a–b′) pentachrome, (c, d) H&E, and (e, f) Saf-O. PCNA staining of (g) the tibial response, and (h) the maxillary response. ALP activity in (i) the tibial response and (j) the maxillary response. TRAP staining of (k) the tibial response and (l) the maxillary response. Scale bars: (a–l) 50 μm. Abbreviations: cb, cortical bone; po, periosteum; mc, maxillary crest; ct, connective tissue; t, tunnel; RA, resorptive area.

showed continued proliferation in tibial periosteum (Fig. 3s); there was no detectable proliferation in maxillary periosteum (Fig. 3t). ALP staining was robust in the tibia (Fig. 3u), but remained at baseline in maxilla (Fig. 3v). TRAP staining for osteoclast activity was notable in both tibial (Fig. 3w) and maxillary (Fig. 3x) responses.

The maxillary periosteum has a delayed cellular response to the tunnelling procedure

At post-surgery day 21, histological analyses showed new woven bone formation in tibia (Fig. 4a,a′; see also Fig. 4c,e) and bone resorption (resorptive area; RA) in maxilla (Fig. 4b,b′; see also Fig. 4d,f). PCNA staining was still detectable

in the tibia (Fig. 4g). Proliferation remained virtually undetectable in the maxilla (Fig. 4h). Overall, ALP activity was diminished compared to earlier time points (Fig. 4i), suggesting that the mineralization phase was largely complete in the tibia. For the first time since the injury,

strong ALP activity was evident in maxilla (Fig. 4j). Bone remodelling was largely complete in the tibia, as indicated by minimal TRAP staining (Fig. 4k). In contrast, TRAP staining of osteoclast activity was robust in the maxilla (Fig. 4l). Results from these late-stage analyses suggest that relative to the tibia, the maxillary periosteum has a delayed and resorptive response to the tunnelling procedure.

Insertion of collagen sponge into the periosteal tunnel induces vertical bone regeneration in the maxilla

In order to investigate the potential for the maxillary periosteum to regenerate bone, we inserted a collagen sponge into the periosteal tunnel (Fig. 5a), effectively creating space in which bone could form. Indeed, in clinical practice, achieving vertical bone regeneration is extremely difficult due to the collapse of available space for the new osteoid tissue. By inserting a collagen sponge into the periosteal tunnel, we provided the scaffolding necessary to maintain the space in which new bone could form.

By 28 days after the procedure, we noted an area of robust new bone formation (black dashed line, Fig. 5b). We analysed two areas of interest: the area near the collagen sponge (red line, Fig. 5b,c–h) and the site of bone regeneration (black line, Fig. 5b,i–n). Histological analyses revealed an area where the collagen sponge remained without new bone formation (Fig. 5c–e), and DAPI nuclear staining showed little cellular infiltrate into this area (Fig. 5f). Within the collagen sponge, ALP activity and TRAP staining were restricted to the alveolar bone surface (Fig. 5g,h), similar to the analogous intact counterpart. Histological analyses revealed an area of new, woven bony regenerate (Fig. 5i–k). Picro sirius red staining of the bony regenerate revealed orientation of collagen fibres parallel to the tension vector caused by the tenting of the periosteum with the collagen sponge (Fig. 5l). Within the bone regeneration site, high ALP activity and TRAP staining were observed in the newly formed matrix (Fig. 5 m and n), indicating healthy bone turnover throughout the vertically regenerated bone. Immunohistochemical analyses of proliferation in the two areas revealed little to no proliferation in both areas (data not shown), suggesting that cells in the new bone area likely migrate into the collagen sponge, colonizing and mineralizing the scaffold. In addition, the major proliferation burst likely occurred before post-surgery day 28.

Discussion

Periosteal characteristics differ with anatomical location

The physical and cellular characteristics of periostea differ with anatomical location (Leucht et al. 2008). In clinical practice, the tibial periosteum is loosely attached to overlying muscle, while the maxillary periosteum is tightly adherent to overlying connective tissue (Popowics et al. 2002). In the intact mouse tibia, we observe that much of the endoge-
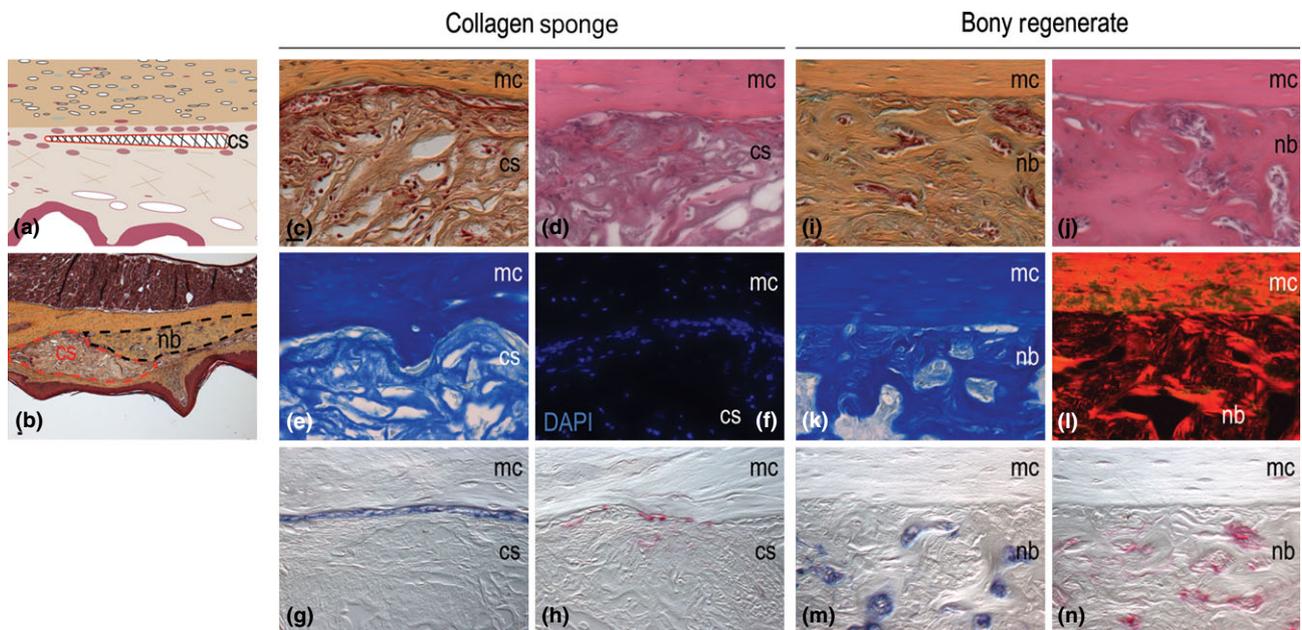
Fig. 5. Vertical maxillary bone regeneration occurs with insertion of collagen sponge into maxillary periosteal tunnel. (a) Schematic of injury model with collagen sponge inserted into periosteal tunnel, between elevated periosteum and maxillary crest. Representative longitudinal tissue sections through the injured maxillary crest on PSD28, stained with (b) pentachrome showing un-resorbed collagen sponge (red dotted line) and new bone (black dotted line). The un-resorbed collagen sponge stained with (c) pentachrome, (d) H&E, (e) aniline blue, and (f) DAPI. Both (g) ALP and (h) TRAP staining restricted to the surface of the maxillary crest. The new bony regenerate stained with (i) pentachrome, (j) H&E, and (k) aniline blue, all showing areas of newly deposited woven bone. (l) Picro sirius red staining shows orientation of new collagen fibres parallel to direction of periosteal tenting. Both (m) ALP and (n) TRAP staining is robust within the area of new woven bone. Scale bars: (b–n) 50 μm. Abbreviations: cs, collagen sponge; nb, new bone; mc, maxillary crest.

nous bone remodelling activity is found on the periosteal surface (Fig. 1 and see Leucht et al. 2007), whereas in the intact mouse maxilla, bone deposition occurs along the oral periosteum and bone resorption along the nasal periosteum (Fig. 1). This physical separation between sites of bone deposition and bone resorption suggests that the size of the sinus changes with growth (Atherton et al. 1974).

Molecular, cellular, and genetic studies demonstrate that long bone periostea are more osteogenic than flat bone periostea (Chang & Knothe Tate 2012), but that craniofacial periostea enclose cells with greater multi-potency (Leucht et al. 2008). These apparent differences highlight the need for an in-depth understanding of the bone-forming potential of oral periostea, which has direct relevance for bone reconstructive procedures in oral surgery. Stimulation of long bone periostea induces an osteogenic, regenerative response (Goldman & Smukler 1978), but here we show that the maxillary periosteum has a delayed, attenuated, or opposite response to the same injury. For example, the periosteal tunnelling procedure can induce robust bone formation in the tibia while the very same procedure can induce bone resorption in the maxilla (Figs 3 and 4).

Maxillary periosteal elevation alone did not result in vertical bone regeneration (Fig. 4). We used a collagen sponge to provide scaffolding and to maintain space, as both functions are essential for successful tissue regeneration (Sturm et al. 2010, Chen et al. 2011). When the periosteal tunnel architecture is maintained and the periosteum tented away from the maxillary crest with a collagen sponge, bone formation can occur (Fig. 5). The highly specific organization of the new collagen fibres parallel to the direction of the periosteal tenting suggests that the introduction of a collagen sponge provided the necessary mechanical environment for bone regeneration to occur. There is, however, a caveat: despite the fact that successful bone regeneration was observed in this model system, it is important to note that mice regenerate much faster than humans (Auer et al. 2007). In addition, the experiments

shown here were performed in young animals. In humans, it is likely that a material such as the collagen sponge would resorb much faster than the human tissue would regenerate, and thus the mechanical stability provided by this scaffold would be lost before the pro-osteogenic periosteal tissue would occupy the site.

Collectively, our data here suggest that regenerative techniques that utilize long bone periosteum cannot simply be extrapolated to craniofacial applications. Further study of the anatomically specific cellular and molecular characteristics of various periostea is needed in order to harness the full regenerative capacity of the oral periosteum.

Acknowledgements

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Clinical Relevance

Scientific rationale for the study: In the ageing population, there is a clinical need for vertical alveolar bone regeneration. As a source of osteogenic cells, the maxillary periosteum might be a useful tool to address this need.

Principal findings: Using periosteal tunnelling procedures in maxilla and tibia, it was demonstrated that long bone and craniofacial periostea respond differently to injury and that, in the appropriate mechanical environment, maxillary periosteum can induce vertical alveolar bone regeneration.

Practical implications: Results suggest a potentially useful method for surgical management and manipulation of the maxillary periosteum to achieve vertical bone regeneration.