Proximate mechanisms involved in the formation of Secondary Osteon Morphotypes

Important considerations and a putative role of primary cilia of osteoblasts and osteocytes

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Keywords

Osteocyte, osteoblast, osteons, osteon morphotypes, cilia, bone

Summary

Proximate mechanisms involved in forming extracellular matrix (ECM) variations within and between bones are not yet clear. Deficiencies in the collective understanding of details required to illuminate the process that forms a highly ordered ECM are exposed when considering that there is still significant debate as to the importance of cellular control in the assembly of the ECM vs. the observation of collagen fibrillar "self-assembly" (i.e., occurring devoid of cells). We examined data and opinions with respect to possible mechanisms involved in the formation of distinctly different ECM patterns of secondary osteon morphotypes (SOMs). Important considerations include: (1) stretch within the osteoid during fibrillogenesis, (2) various mechanotransduction mechanisms, and (3) whether or not the formation of regional variations in osteonal ECMs requires osteoblast alignment and/or rotation and migration. We propose that primary cilia of osteoblasts and osteocytes have an important role in their perception of variant-related (vectorial) stimuli, which is deemed essential in the genesis of distinctive and mechanically relevant ECM patterns of SOMs.

Schlüsselwörter

Osteozyt, Osteoblast, Osteone, Osteon-Morphotypen, Zilien, Knochen

Zusammenfassung

Die proximaten Vorgänge, die an der Entstehung von Variationen extrazellulärer Matrix in und zwischen Knochen beteiligt sind, sind noch nicht abschließend geklärt. Es bestehen nach wie vor Defizite im kollektiven Verständnis der Details, die für das Entstehen einer hoch geordneten extrazellulären Matrix erforderlich sind. Das zeigen die nach wie vor erheblichen Diskussionen, die über die Bedeutung zellulärer Kontrolle in der Anordnung der extrazellulären Matrix gegenüber der Beobachtung einer Selbstorganisation der Kollagenfasern (d.h. ohne Zellbeteiligung) geführt werden. Wir untersuchen Daten und Auffassungen im Hinblick auf die Mechanismen, die an der Bildung von unterschiedlichen Mustern extrazellulärer Matrix von Morphotypen sekundärer Osteone beteiligt sind. Wichtige Betrachtungen umfassen: (1) ein Dehnen innerhalb des Osteoids während der Fibrillogenese, (2) verschiedene Mechanismen der Mechanotransduktion, und (3) die Frage, ob die Bildung regionaler Variationen in der osteonalen extrazellulären Matrix eine Ausrichtung der Osteoblasten erfordert und/oder eine Rotation und Migration. Wir schlagen vor, dass primäre Zilien der Osteoblasten und Osteozyten eine bedeutende Rolle bei der Wahrnehmung veränderlicher (vektorieller) Stimuli innehaben, was als wesentlich erachtet wird in der Entstehung charakteristischer und mechanisch relevanter Muster der extrazellulären Matrix von Morphotypen sekundärer Osteone.

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Proximate Mechanismen bei der Bildung von Morphotypen sekundärer Osteone
Wesentliche Überlegungen und die mutmaßliche Rolle der primären Zilien von Osteoblasten und Osteozyten
Osteologie 2016; 25: 101–112
received: February 23, 2016
accepted after revision: March 31, 2016

Secondary Osteon Morphotypes (SOMs) and their biomechanical relevance

All osteons referred to herein are secondary osteons (Haversian systems), which are formed by osteoclasts and osteoblasts. Osteons often have distinctive lamellar and/or collagen fiber orientation (CFO) patterns of extracellular matrix (ECM) organization, which are called secondary osteon morphotypes (SOMs) (▶Fig. 1) (1–3). Nearly all secondary osteons can be designated as being in a specific SOM category (usually scored as 0, 1, 2, 3, 4, or 5). Expansion of these categories might be necessary in cases where the osteons are "hybrids" (not uncommon) – having varying amounts of highly birefringent (brighter) lamellae (layers or bands) within their

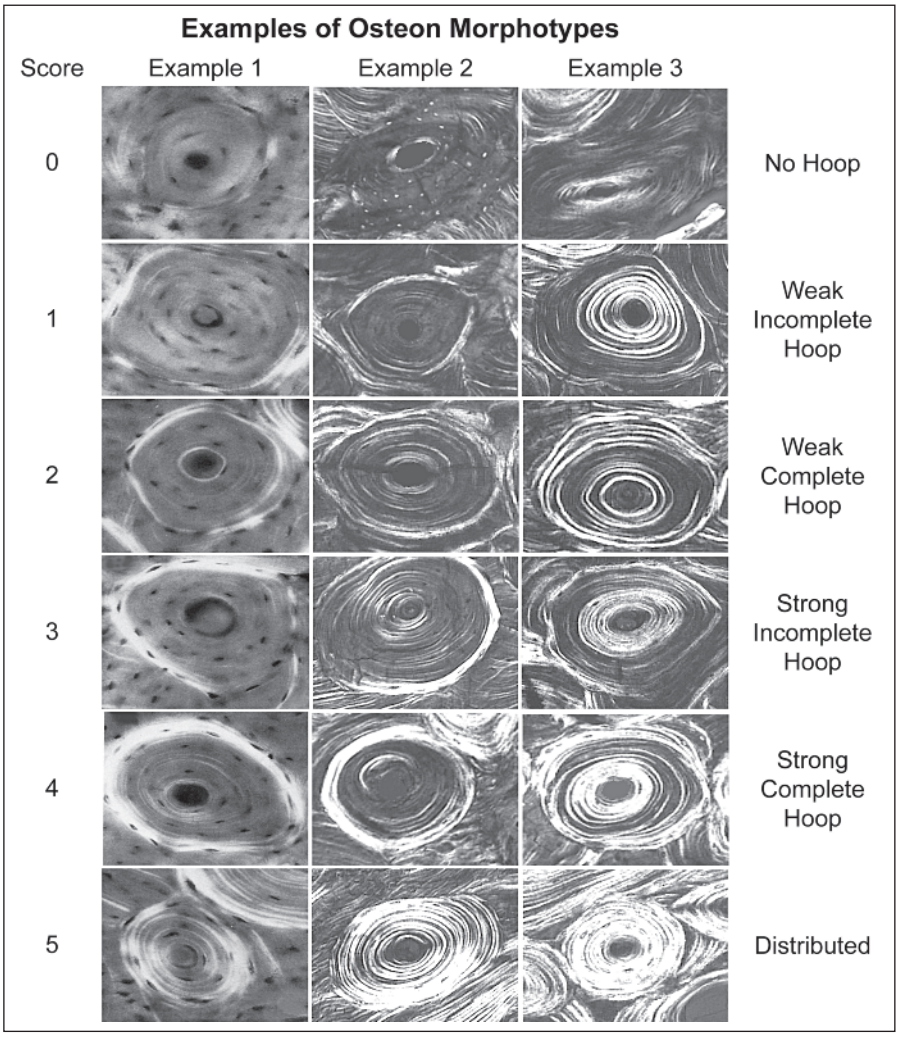


Fig. 1 Examples of the six "types" of SOMs, each transversely sectioned. The images at the far right are hybrid SOMs except for the "bright osteon" at the bottom right (which is still scored as #5). The numerical values of each of the six SOMs are used to calculate the osteon morphotype score of entire microscopic images that contain many osteons (1). Four of the six numerical scores shown include consideration of the completeness and birefringence strength (brightness) of the peripheral ring "O" or hoop (W = weak; I = incomplete): 0 = category N, a dark osteon with no birefringent lamellae; 1 = category OWI; 2 = category OW; 3 = category OI; 4 = category O (strongly birefringent peripheral lamellae); 5 = category D, birefringent lamellae are distributed throughout the wall of the osteon ("distributed" osteon group).

Key Points

- Key-variant (vectorial) related stimuli are likely perceived by osteoblasts/osteocytes.
- Primary cilia of these bone cells are likely important in perceiving variant stimuli.
- Adapting to different variant stimuli (tension – compression) enhances bone toughness.
- Highly oriented bone extracellular matrix likely requires primary ciliary functions.
- Formation of osteon/Haversian morphotypes is likely enabled by primary cilia function.

Kernbotschaft

- Veränderliche (vektorielle) Reize können wahrscheinlich von Osteoblasten und Osteozyten detektiert werden.
- Die primären Zilien dieser Knochenzellen sind wahrscheinlich von Bedeutung für die Wahrnehmung veränderlicher Reize.
- Die Anpassung an unterschiedliche veränderliche Reize (Zug – Druck) verbessert die Knochenzähigkeit.
- Funktionen primärer Zilien werden wahrscheinlich für die starke Ausrichtung der extrazellulären Knochenmatrix benötigt.
- Die Ausbildung verschiedener Havers-Kanal-/Osteon-Morphotypen wird wahrscheinlich durch Funktionen primärer Zilien ermöglicht.

walls (e.g., images in the central portions of the middle and right columns of ▶Fig. 1) (2).

To identify a specific SOM, the specimen must be sectioned transverse to the bone's or osteon's long axis, ground and polished to a thin wafer (50–100 µm thick), and viewed in circularly polarized light (CPL). Dark gray levels in CPL images represent collagen fibers that are aligned roughly along the osteon's long axis, and the brighter/whiter gray levels represent fibers that are aligned obliquely or transversely to the long axis. An average "osteon morphotype score" correlates strongly with predominant CFO, which is often interpreted as being an adaptation for a predominant/prevalent strain mode (tension, compression, or shear) (1, 4–7). These studies show that:

1. darker SOMs (with varying intensity/completeness of birefringent peripheral "hoops") are adaptations for habitual tension loading, and
2. brighter SOMs are adaptations for habitual compression loading (▶Fig. 2).

Monikers for these osteons include:

1. for dark osteons, "longitudinal," "parallel-fibered," and "tension-adapted" osteons, and
2. for bright osteons, "transverse," "intermediate," "distributed," "lamellar," "alternating," and "compression-adapted" osteons (8–11). Hybrid osteons are likely more prevalent in regions that habitually receive shear (e.g., from torsional loading) (2).

Many studies have examined the composition and structure of the ECM of osteons. The past 10 years have been especially fruitful because of the use of advanced technol-

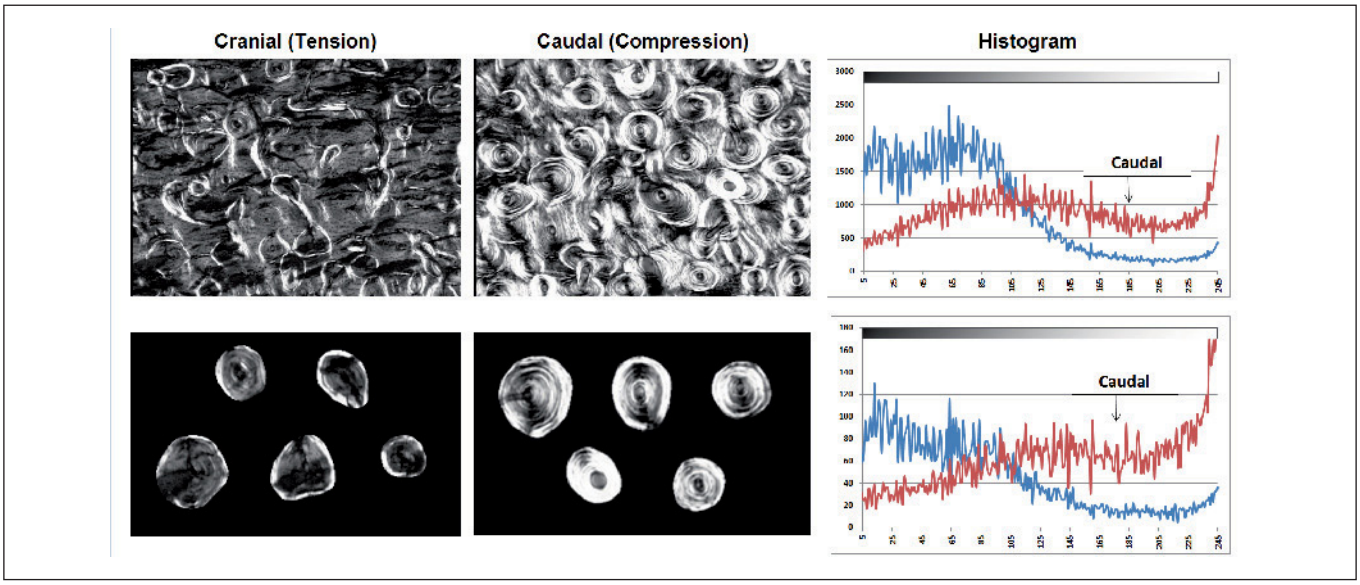


Fig. 2 Circularly polarized light (CPL) images of a transversely sectioned mid-shaft horse radius (adult animal) and gray level profiles from all pixels of each image are shown at right (at top). At bottom are five osteons that were isolated from each image, and their gray level profiles are at right. The images were obtained at the same magnification and illumination and the tissue was not decalcified or stained. The dark-to-bright gradient over each of the gray level profiles show how the gray levels change from left to right on the x-axis. Darker gray levels, which are seen more so in the left images, represent relatively more longitudinally oriented collagen. This is considered an adaptation for habitual tension in the cranial cortex. Oblique-to-transverse collagen fibers that represent adaptation for habitual compression are seen as increased overall brightness/whiteness in the images on the right (caudal cortex).

ogies (12–15). However, these studies often examine one or only a few osteons in addition to considering them as "generic," usually having dark and bright lamellar patterns that are "alternating" or "intermediate" osteons. This is unfortunate because the ECM organization of this SOM, though common, is not found in the majority of the walls of the other non-hybrid SOMs.

The notion of a "generic osteon" can be highly misleading, as shown by dramatic differences between cranial-caudal cortices of equine radii where there are numerous darker SOMs in the cranial cortex (habitual tension loading) versus bright (few) and alternating (many) osteons (SOM #5) in the caudal cortex (habitual compression loading) (▶Fig. 2) (6, 7, 16). Although regional differences are less distinct in cases of increased load complexity, regional differences in biomechanically relevant SOMs is a common finding because bending in a preferred direction is typical of a large majority of bones (9). SOMs help adapt the bone (especially its toughness) for the potentially deleterious consequences of regional variations in strain mode produced by bending – failure is more likely in shear and tension, than compression (9).

Steps, events, and considerations in the formation of SOMs

See (17–20) for discussions of mechanisms and processes that are likely involved in the formation of the SOMs. van Oers et al. (21) emphasize tissue stretch in the osteon wall as being an important proximate stimulus in guiding their various CFO/lamellar patterns (▶Fig. 3). But the proposed mechanisms for transducing specific substrate strain characteristics (e.g., stretch direction) and other physical stimulation at the cell level (e.g., lacunar-canalicular fluid flow) seem lacking because they are insufficient for enabling cellular interpretation of vectorial (variant) information. Below we argue that formation of the often highly oriented and strain-mode-related ECM of the various SOMs requires the recognition of vectorial information and this is accomplished by fluid-flow-induced bending of the primary cilia of osteoblasts/osteocytes.

Notably, van Oers et al. (21) focused on the formation of SOMs that are not common (i.e., dark/longitudinal and bright types) (▶Fig. 3). In our histomorphological study of mature chimpanzee femora (~10,400 osteons), we calculated the percent prevalence of the osteons with the #5 SOM score: the bright SOM had 3.9% prevalence and the alternating type had 27.4% prevalence (hybrid osteons were included in the alternating osteon group). In addition to being both scored as #5, the bright (uncommon) and alternating (very common) SOMs are both considered as adaptations for habitual compression loading in the various habitually bent bones that we have studied (2). When considering the very common alternating SOM, van Oers et al. (21) only mentioned the possible influence of shear in explaining its ECM organization but offered no details other than referring to our study where we also mention the association with shear but provided no mechanistic basis for it (3).

Ascenzi and Bonucci (22, 23) were the first to find correlations between the mechanical properties of individual osteons and their birefringence patterns: dark osteons had greater strength in tension than those having bright or "alternating lamellae." Following the interpretation of lamellar structure postulated by von Ebner (24) and Gebhardt (25), Ascenzi and Bonucci assumed that osteons with alternating lamellae, as seen when viewed between crossed polar-

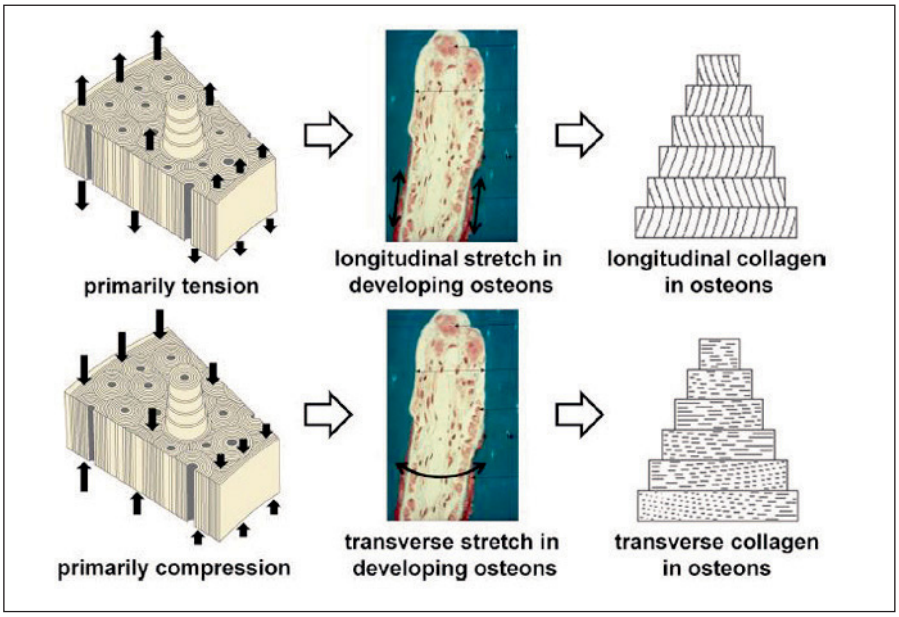


Fig. 3 Hypothetical explanation of observed differences in preferential collagen fiber orientation (CFO) that are caused by stretch in the osteon resulting from loading mode (tension or compression) during osteon formation (21).

izers, have lamellae in which the collagen fibers alternate between longitudinal and transverse directions (▶Fig. 4). Osteons appearing entirely bright or dark were assumed to have mostly transversely or longitudinally oriented fibers, respectively (11).

Here we focus on the formation of these SOMs:

1. dark/longitudinal with variable presence of the high birefringent peripheral "hoop" (scores 0–4 in ▶Fig. 1),
2. bright (lower right in ▶Fig. 2),
3. alternating (middle lower in ▶Fig. 2), and
4. "hybrids."

After osteoclasts make the resorption cavity the cement line region forms

Osteoblasts form the cement line, which is thought to be collagen deficient (26), and this region is enriched with noncollagenous proteins including osteopontin which likely act as an interfacial adhesion promoter bonding dissimilar tissues and maintaining the overall integrity of bone during the remodeling process (27). The outermost lamella of osteons often has very oblique-to-transverse collagen of a generic twisted plywood design (TPD) (described below) that might attenuate the propagation microcracks from adjacent tissue regions (13).

Lamellae are formed, but what are they?

The layers in the layered appearance of osteons are typically called lamellae (singular lamella). There is no consensus on what a lamella actually is. For example, Varga et al. (13) states, "bone lamellae can be defined as repeated orientation patterns." This definition does not explicitly consider whether or not these repeated orientation patterns, also termed "periods," are produced by what could be called a single phase of ECM synthesis vs. a fraction of one of such so-called synthesis phases (28). The definition of lamella can also be influenced by the resolution or magnification of the imaging technology that is being used (29).

Osteon ECM designs include orthogonal plywood, twisted plywood, oscillating plywood, irregular oscillatory plywood, and other staggered arrays (e. g. [12, 30])

Among these various ECM designs (▶Fig. 4, ▶Fig. 5, ▶Fig. 6), orthogonal and TPD are likely the most common. Using transmission electron microscopy, Giraud-Guille (31) was the first to provide rigorous evidence that a TPD is a common finding in osteons, being characteristic of the alternating SOM (▶Fig. 4, ▶Fig. 5). She also showed that:

1. the "orthogonal plywood" design was consistent with the classical description of osteons that are bright or dark (▶Fig. 4a, b), and
2. the existence of a design that resembles orthogonal plywood can be adjacent to the TPD within the same osteon (▶Fig. 5c).

Giraud-Guille's work helps to clarify the physical bases for differences in ECM patterns of the various SOMs. Studies that seem to refute the existence and/or high prevalence of the TPD are typically fraught with methodological limitations, including inadequate image resolution (e. g., confocal microscopy) or artifacts of specimen preparation (e. g., excessive acidic etching of the specimen surface) (29, 32–36).

Yamamoto and co-workers (33) discuss some of these limitations and provide photographs of three-dimensional models made of drinking straws that greatly help visualize how the TPD can produce the dark/bright banded pattern of the alternating SOM. Varga et al. (13) advanced this work by using synchrotron X-ray phase nano-tomography to study the ECM organization of osteons. They examined osteons that were probably hybrids of the longitudinal and alternating types because the walls of the osteons had features of what they considered to be oscillating plywood, TPD, and irregular oscillatory plywood (▶Fig. 7).

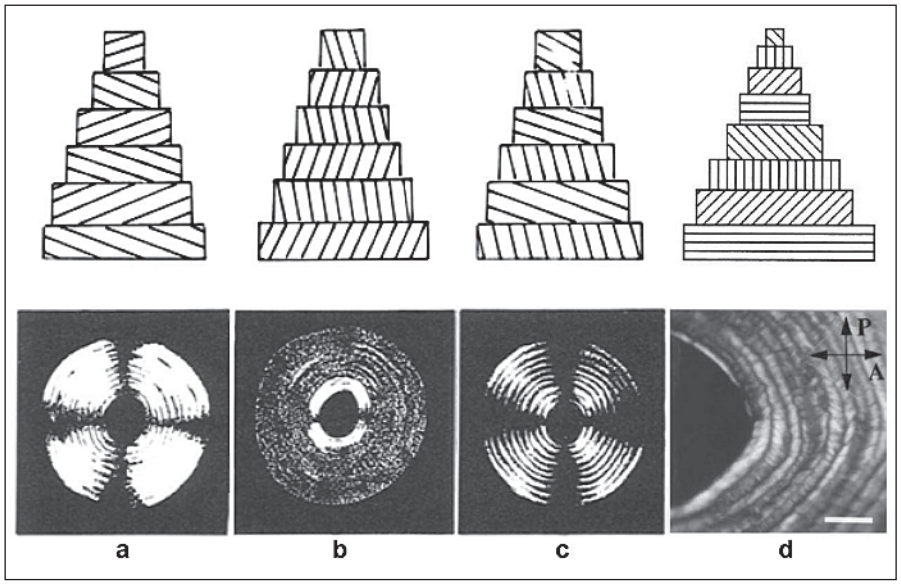
Successive osteoblast synthesis phases make the osteon wall, but do these cells rotate and/or migrate?

The idea that cell orientation is essential in the formation of highly ordered ECMs is pervasive in studies of connective tissues. For example, in studies of fibroblasts during tendon formation, Donnelly et al. (37) suggest that the orientation of the ECM starts with fibroblast orientation. With respect to SOM formation, important questions include:

1. does the osteoblast align first and then collagen aligns, or vice versa and
2. can the ECM self-assemble, largely independent of cellular control?

During the formation of the osteon wall, it is generally accepted that osteoblasts move radially (i.e., from outside to inside) as the osteon fills in. Parfitt (38) has stated that: "Because the osteoclasts move forward but the osteoblasts remain in the same cross-sectional location, each successive ring of new osteoblasts lining the circumference of the cavity can be regarded as belonging to one remodeling cycle." Jones et al. (39) appear to be the first to suggest that cell alignment might be essential for creating a highly oriented ECM in bone. They noted that in parietal (skull) specimens from rats, 80% of the osteoblasts were parallel to the underlying CFO to within 15°, and 93% were parallel to within 30°. In 57 osteoblast specimens from Rhesus monkey parietal bones, the orientation of the cells was parallel to within 15° of the underlying CFO in 53% of the sample, and to within 30° in 74% of the sample. They concluded that these results are indicative of a strong correlation between cell and CFO in the samples studied. The cells that did not fit were mostly those at the periphery of the patch of cells where presumably the process of change from the formation of one domain to the next was in progress. They favoured the possibility but could not prove that the cells determined the orientation of the collagen rather than the other way around. They foreshadowed cilia-based mechanisms offered below when stating, "if cell orientation controls collagen direction, what controls the former?"

More recently, in a study of rat lamellar bone, Yamamoto et al. (40) suggest that osteoblasts move in a group, migrating in successive preferred directions, which they explicitly suggest is necessary for the construction of the TPD. To us it seems unlikely that this cell migration occurs during osteon formation because the osteoblasts are spatially constrained in circular/cylindrical groups when they form osteon lamellae. In a study of trabecular bone formation in embryonic chick calvaria, Hosaki-Takamiya et al. (41) stated that "in order to form the lamellar structure, the group movement of the osteoblasts would be important." However, they did not detect evidence of significant cell movements.



The figures and figure legend (with modifications) are from Giraud-Guille et al. (31, 70) with permission of the senior author and Springer-Verlag

Fig. 4 Three osteon types (a, b, c), observed in polarized light microscopy between crossed polarizers, from the model of Ascenzi and Bonucci (23) (reproduced from Giraud-Guille [31]). (a and b) Two osteons with orthogonal plywood designs that have alternating fibril directions (but are not considered "alternating osteons") that give rise to either bright (a) or dark (b) type osteons, which are the result of their marked oblique-to-transverse orientation (bright, also see lower right image in ▶Fig. 1) or marked longitudinal orientation (dark, also see upper right image in ▶Fig. 1). The bright osteon occurs when the angular shift of the fibers between the layers is greater than 90° with respect to the osteon's long axis. The dark osteon shown occurs when the angular shift of the fibers between layers is much less than 90° with respect to the osteon's long axis. (c) This is an "alternating" or "intermediate" SOM. In accordance with the classical interpretation of the orthogonal plywood design, lamellae in alternating osteons have alternating directions of predominant CFO that intersect with angles that are greater than those seen between lamellae of the osteons in (a) and (b). In contrast to this interpretation for the differences in predominant CFO between the lamellae of the alternating SOM, Giraud-Guille re-interpreted the physical basis for the alternating pattern as a "twisted plywood design" (TPD) where the fibers exhibit regular small and constantly angular changes (bar = 5 microns in d). However, "regular changing fibrils" are not depicted well in the layers in the drawing shown in (d) where it is considered to be a TPD in Giraud-Guille et al. (70); here only a few discrete layers are shown. In contrast to (c) and (d) that are in fact comprised of the TPD or some modification of it with CFO in progressively staggered arrays, the layered structures in (a) and (b) are more accurate depictions of two general orthogonal plywood designs that comprise these two different SOMs.

The primary cilium of osteoblasts is a strong candidate for the cellular apparatus that provides vectorial information for the construction of different SOMs. In a review of the non-cilia mechanosensation/mechanotransduction mechanisms, Vatsa et al. (42) state "matrix strain-derived interstitial fluid flow imparts shearing forces on osteocytes thereby mechanically stimulating them to orchestrate bone remodeling by modulating the activity of osteoblasts and/or osteoclasts…. The actin-rich cell processes possess a strain amplification mechanism, which might play a key role in mechanosensing. This hypothesis is not extended to the osteocyte cell bodies as the fluid flow-mediated shear forces on the osteocyte cell bodies are too small to be sensed…. However, recently we have shown that the osteocyte cell body exhibits integrin-mediated mechanosensing after localized mechanical loading, which accentuates the possibility of direct mechanosensing of matrix strains by osteocyte cell bodies…. Hence, during bone formation, it is likely that the osteocytes, which are simultaneously being embedded in the matrix (or osteoid), perform direct mechanosensing of matrix (or osteoid) strains via the

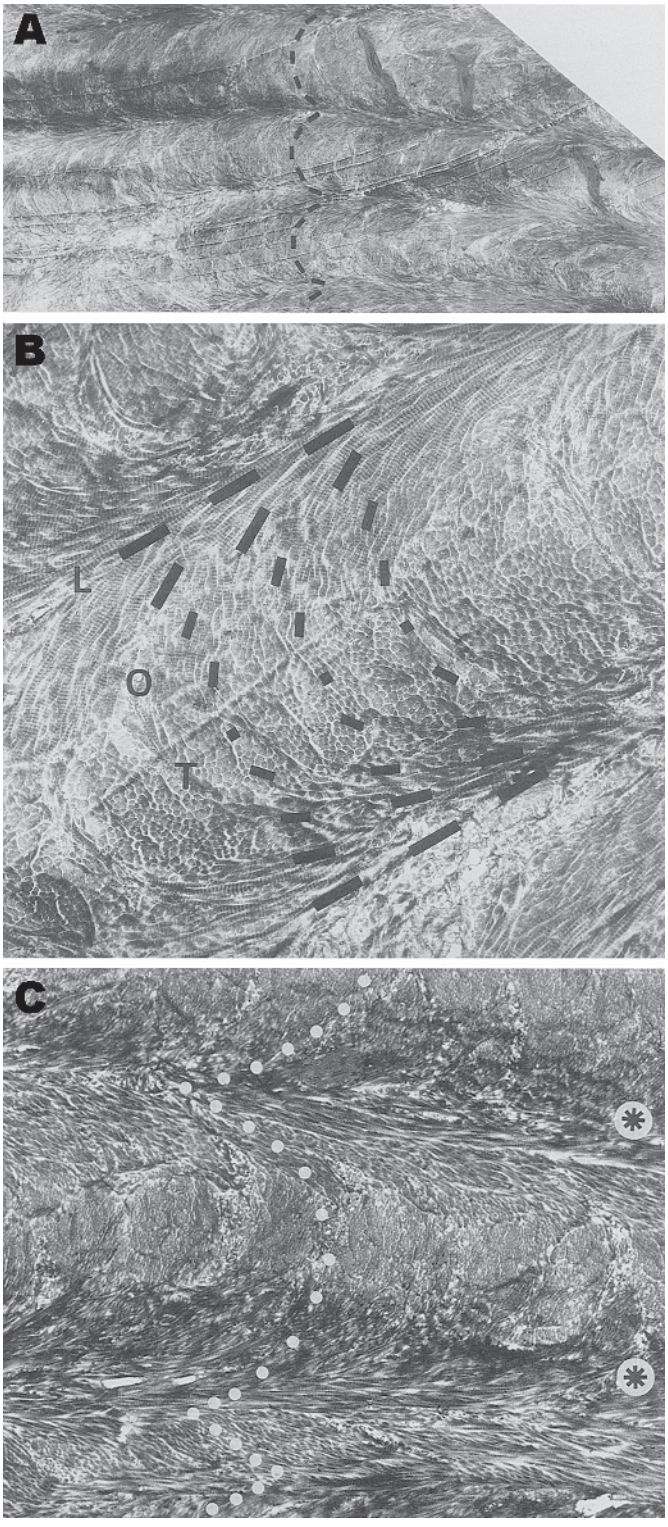


Fig. 5
Microscopic images of decalcified human compact bone showing a TPD (top and middle images, and upper three-fourths of lower image) and an orthogonal plywood design (lower one-fourth of lower image). (A) Nested arcs, representing several lamellae, in ultrathin oblique sections of decalcified human compact bone (2,700 x). The hatched line indicates three periods (lamellae) of the arced pattern. (B) Arced patterns, over one lamella, formed by collagen fibrils in ultrathin oblique sections of decalcified human compact bone. The successive directions of fibrils, longitudinal (L), oblique (O), and transverse (T), respectively, are indicated by the black bars (15,750 x). (C) The transition can be observed between TPD and orthogonal plywood designs. The corresponding arcs and herringbone pattern (at bottom) are indicated by the dotted line (7,650 x).

cell bodies and align themselves parallel to the principle mechanical loading direction for efficient load bearing."

It is notable that the primary cilium is now considered as one of the top three-most important mechanisms (▶Fig. 8). Primary cilia (one per cell) are: (1) a few microns in length, (2) intrinsically non-motile (but can be moved by an external force like fluid flow), (3) microtubule-based (43, 44), and (4) common, having been described as potentially serving mechanosensation/mechanotransduction and/or chemosensation in all cell types (but not universally in all cells; either they are not present or do not persist) (43, 45). See http://www.bowserlab.org/primarycilia/cilialist.html for a list of cells containing primary cilia.

Ciliary mechanosensation seems likely when considering that osteocyte cell processes are estimated as being 10 times more sensitive to mechanical loading than the cell body (46) and fluid-flow-induced membrane strains are likely greatest where the cilium merges with the cell body (47–49). A video in the online supplement of (44) shows that osteoblast cilia can bend substantially under physiologic fluid-flow rates. To our knowledge, physiological fluid-flow rates have not been measured within the lumen of forming osteons. However, they have been measured in vivo in lacunar-canalicular spaces (50) and are sufficient for activating cell-deformation mechanisms that are important for osteocyte physiology.

It has been suggested that fluid-flow induced bending of osteocyte cilia produce paracrine factors that have downstream signaling effects on nearby osteoblasts (hence physical cell-cell communication is not critical) (51). Membrane deformation associated with ciliary bending might also involve integrin-ECM interactions (52, 53). Compelling findings of the analytical model of Vaughan et al. (48) of fluid-structure interactions that characterize the deformation of integrin- and primary cilia-based mechanosensors in bone cells under fluid-flow simulation include:

1. a short free-standing cilium (as in ▶Fig. 8c) could not effectively fulfill a flow sensing role in vivo;
2. when a primary cilium is attached to the lacunar wall it can be highly stimulated, suggesting that cilia mechanotransduction continues after osteoblast burial.

However, the extremely small distance between the osteocyte cell body and lacunar wall (likely <50 nm) seems insufficient (54) for functionality that was possible during osteon formation when the cilium extended into the lumen of the Haversian canal. Perhaps the cilia of osteocytes are actually less functional because they are tuck-

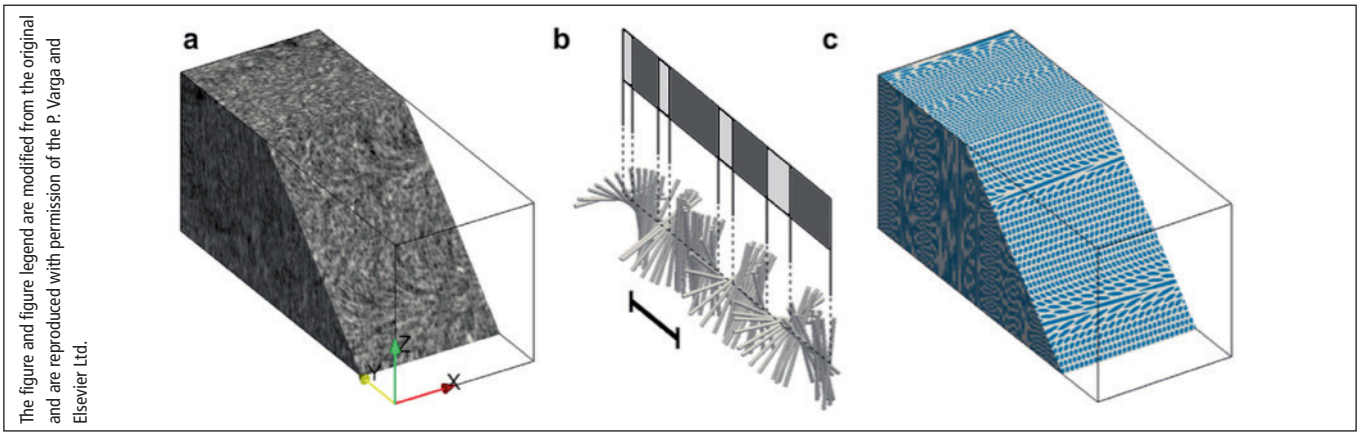


Fig. 6 Comparison of the raw image data and the derived fibril orientation from a human secondary osteon from Varga et al. (13). (a) A volume of interest (VOI) cropped with an oblique plane. The gray scale was inverted to enhance the visualization of collagen. Note the arc pattern in the cut plane (see online version of Varga et al. for best resolution). (b) The corresponding mineralized collagen fibril orientations are illustrated as cylinders. In contrast to the original drawing, here the gray scale shown above the staggered array of fibers is not inverted; these gray levels depict what would be seen if the specimen was viewed from above with polarized light passing vertically through the specimen. This depiction corrects an error in the original version (P. Varga personal communication). Each line represents a 600 nm thick region. The orientation-based definition of thick (or dark, with $-45^\circ \leq \theta \leq 45^\circ$) and thin (or bright, with $-45^\circ > \theta > -180^\circ$ or $45^\circ < \theta < 180^\circ$) lamellae are shown as dark and light gray regions, respectively. The black scale bar represents 10 µm. (c) Virtual lamellar bone model showing fibrils idealized as cylinders (in blue) embedded in extra-fibrillar matrix (white); note the similarity to (a).

ed up against the cell body as shown in (55).

We could not locate studies implicating primary cilia as being important in SOM formation. In tendon formation, Donnelly et al. (37) suggested that the primary cilia of fibroblasts align with the collagen fibers due to the tensile loading environment. They also suggest that orientation of the cilia (we suggest fluid-flow-mediated bending) plays a role in determining ECM orientation in other connective tissues with highly ordered CFO patterns. Although mechanosomes, focal adhesions, and other similar structures (►Fig. 8a, b) can assess substrate stretch/strain, we suspect that they are probably not important during SOM formation because they are not likely well attached between osteoblasts and the forming matrix.

Some ideas for how fluid-flow dynamics cause preferential/vectorial ciliary motions that result in different SOMs.

►Figure 9 shows our simplified ideas of how stereotypical loading conditions, ranging from roughly unidirectional bending (►Fig. 9a, b) to varied/multi-directional (►Fig. 9c), that a bone region experiences might produce different fluid-flow-induced cilia bending patterns/directions that trigger different synthesis pathways that lead to highly ordered, and often strain-mode-related, ECMs in SOMs. Close examination of data and concepts reviewed in (49, 56) support the plausibility that the basic flow patterns illustrated in ►Figure 9 exist and lead to differences in cell responses. Adaptive modification of cilia structure might be possible as a means for adjusting functionality, for example, by changing their length with respect to the local ambient strain mode or magnitude. This was suggested by Mathieu et al. (57), but their finite element analysis leads to the conclusion that ciliary orientation is much more important.

Diurnal variations in fluid-flow dynamics or other rhythmic- or circadian-related factors might influence the formation of ordered/periodic ECMs in bone and teeth (58, 59). Cyclic sleep and wake periods in humans are associated with changes in fluid flux and interstitial fluid pressures that can significantly affect bone physiology (60). We think that these potential influences have little impact on the emergence of the lamellar patterns in osteons, as revealed by Parfitt's data for bone remodeling parameters (Table 7 in

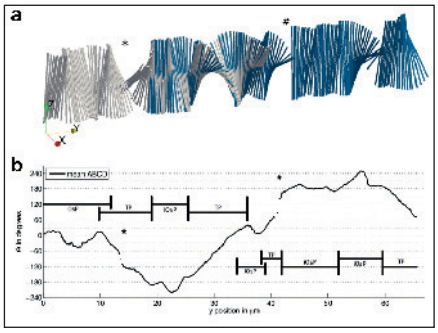


Fig. 7 In-plane fibril orientation and tissue mass density of the four volumes of interest (VOIs) from an osteon wall examined by Varga et al (13). (a) Quasi 3-D illustration of the fibril orientation for VOIs A (white) and C (blue). One rod represents a 300 nm thick region (5 image slices). (b) The y-axis represents the distance along the osteon wall; for details see (13). The horizontal thick lines in (b) represent a possible, idealized separation of the different plywood patterns: oscillating plywood (OsP), irregular oscillating plywood (iOsP), and twisted plywood (TP = TPD). The asterisk represents the location of a microcrack. To the left of the asterisk the tissue is mostly an oscillating plywood design. Between the asterisk and "#" the tissue includes both irregular plywood and TPDs.

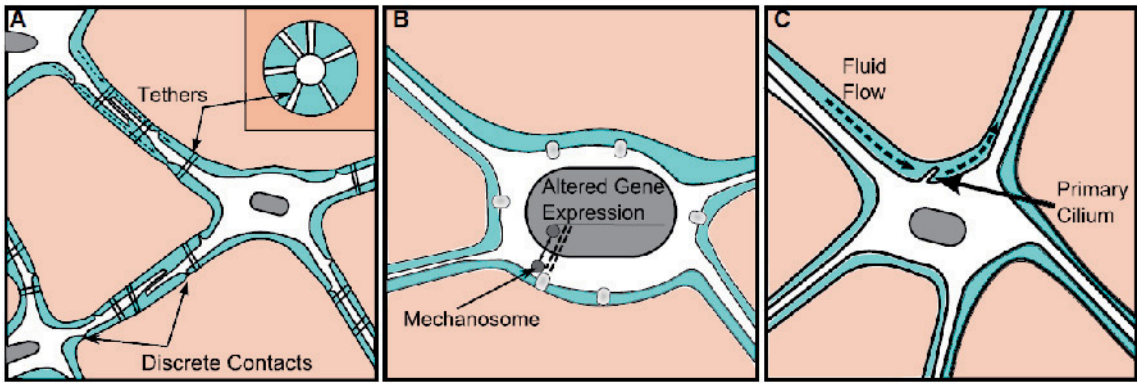


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Fig. 8 Osteocyte mechanosensing theories can be divided into three main groups: (A) dendritic-process focal-adhesion sensing where strain and fluid flow initiate a cellular response; (B) cell body focal-adhesion sensing where lacunar matrix strain is detected; and (C) direct fluid flow sensing, such as where the primary cilium detects lacunar fluid flow.

[61]). This includes mineral apposition rates in forming human osteons (0.8–1.0 µm/24 hours); hence, it would take about 94 days to make a 75 µm thick osteon wall. The mean number of lamellae in adult human osteons (tibiae, SEM images, Na_3PO_4 etching) is about 8–9/osteon, with rarely more than 12 lamellae in large osteons (28). Therefore, one bright or dark lamella in an alternating osteon cannot be attributed to day (awake/active) and night (asleep/inactive) cycles. There remains the possibility that periodic ECM synthesis follows some other biological oscillation where cell division, matrix formation, and quiescence are rhythmic, but not clearly circadian (58).

An important question is: what is the baseline, or default, program in the synthesis of the ECM of an osteon? One study favors the idea that the baseline process results in generally oblique-to-transverse CFO (increased birefringence in polarized light) across the osteon wall. Mehraban Alvandi, Schaffler, and co-workers (62) examined polarized images of transversely sectioned fourth metacarpals of dogs. Some animals were subjected to 12 months of forelimb immobilization (63). Osteons that formed during immobilization showed overall brighter birefringence when compared to control animals (normal loading). The authors concluded that "osteoblasts appear to use information from mechanical loading to orient bone collagen in a more longitudinal direction (darker osteons in the loaded group), but in the absence of mechanical loading cross-ply lamellar bone seems to be the 'default' pattern for bone collagen orientation." Consequently, if the TPD or some other staggered CFO/plywood design is the physical basis for the increased birefringence that they found, then this could be a default program. This would not be surprising because staggered ECM/CFO designs (e.g., TPDs) are common in other tissues, including in non-vertebrate organisms.

Is one period of the orthogonal plywood design a truncated portion of the TPD?

Here we assume that osteons being considered have similar size so that the issue of different infilling rates is inconsequential (64, 65). As shown in ▶Figure 4b, a typical lamella of a dark (tension adapted) SOM has a range of CFOs that deviate <~45° with respect to the long axis of the osteon. Consequently, the angle formed between the predominant CFOs of this lamella and its adjacent "backflip" lamella is <90° (see [15, 66] for more discussion regarding backflip lamellae). By contrast, a typical lamella of a bright SOM has CFO that is relatively closer to perpendicular to the osteon axis (▶Fig. 4a). Consequently, the predominant CFOs of one lamella and its adjacent (backflip) lamella of this SOM form angles that are >90° with respect to the osteon's long axis. The paired lamellae of these two SOMs are characteristic of the orthogonal plywood design, exhibiting a herringbone pattern when sectioned obliquely (31). Could this design be a portion of the full period of the TPD or is it made by a different synthetic program?

If paired lamellae of orthogonal plywood osteons are a portion of the twisted plywood synthesis program, then:

1. Does one full synthesis period of a group of osteoblasts represent one or both lamellae of the orthogonal plywood design? and
2. Is the duration of tissue deposition of more acute CFO angles (dark osteons) or obtuse angles (bright osteons) the same duration of the full 0° to 180° twist (i.e., full period) of the TPD (which is seen as three lamellae, as shown in [67])?

These questions are aimed at determining if one synthetic period of a group of osteoblasts forms a layer of tissue of equivalent thickness regardless of the type of morphotype formed. If this is the case then the formation of a pair of orthogonal plywood lamellae (possibly one period), when compared to the three "lamellae" (one period; or two according to [13]) of the TPD, would likely have relatively more collagen fibers per distance across each lamella with orientations that are either acute or obtuse (i.e., dark or bright osteons, respectively). This result is shown diagrammatically in ▶Figure 9a and b as two to three rows of collagen fibers having the same orientation. This can be compared to ▶Figure 9c which shows one discrete CFO per row in the TPD (see also ▶Fig. 9d). But the lower portion of image "C" in ▶Figure 5 shows that a lamellar pair of an orthogonal plywood design is narrower than the typical arc (one period) of the adjacent TPD. Weiner et al. (66) describe differences in la-

mellar thickness in rat tibia bone: "about 60% of the lamellar unit is composed of thick lamella (i.e., about 24 collagen fibril layers). The 120° 'back-flip' fibril array comprises about 25% of the average lamellar unit." Additional high-resolution imaging studies of lamellar thickness and ECM periodicity in various SOMs as well as advanced in vivo imaging studies (68) are needed to resolve these issues. Perhaps the preferred ciliary motions that we suggest are involved in making dark vs. bright SOMs control the orientation of the fibers only in so far as keeping them confined either <90° between lamellae (dark osteons) or >90° between lamellae (bright osteons), but do not control the program where the osteoblasts switch into an apparent oscillation that makes the orthogonal plywood design. This clearly differs from the continuous deposition process that makes the TPD, which we described above

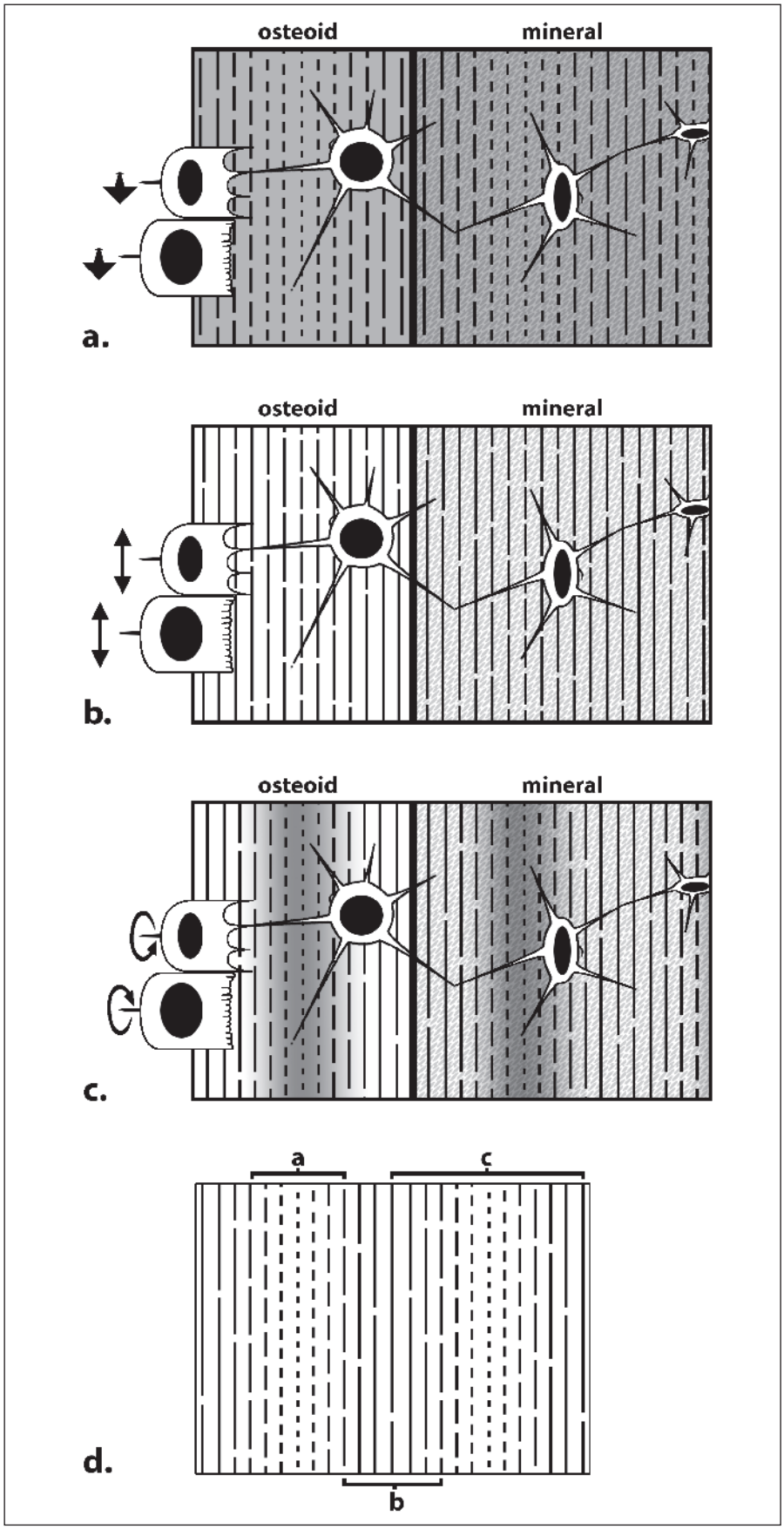

Fig. 9 Hypothesized general bending patterns of cilia caused by load-induced fluid flow. In each drawing is a small portion of an osteon wall that is transversely sectioned; two osteoblasts are shown at the left and there are three osteocytes (one within osteoid and two within mineralized bone). (a) The arrows show longitudinal ciliary bending (approximately orthogonal to the plane of the drawing) that leads to the formation of a dark SOM, which is comprised of the longitudinal CFO portion of the TPD; hence the fibers are roughly aligned along the osteon's longitudinal axis (the tissue is darker in CPL). (b) Oblique-to-transverse ciliary bending (approximately in the plane of the drawing) leads to the formation of a bright SOM, which is comprised of the oblique-to-transverse CFO portion of the TPD; hence the fibers are relatively more oblique-to-transverse to the osteon's longitudinal axis (the tissue is brighter in CPL). (c) Circular arrows indicate varying directions of ciliary bending that produces the alternating SOM, which is comprised of what we consider to be "full periods" (CFO from 0° to 180°) of a TPD. This results in fibers that range from longitudinal to transverse; hence the tissue would have alternating dark and bright layered appearance as is also shown in ▶ Figure 6b. (d) Shows two entire periods of an idealized TPD (one period = CFO from 0° to 180°) in 15° CFO increments (resembling ▶ Figure 9b in [31]); the brackets indicate the longitudinal CFO portion used in (a), the oblique-to-transverse CFO portion used in (b), and all increments used in (c).

as "the" default program. But can there be two (or more) basic default programs?

Cilia might be important upstream regulators of the type 2 osteoblast processes, which control alignments of intra-cellular pre-collagen molecules, the fibropositors that release them, and the collagen fibers that form just outside the osteoblast

Osteoblasts and osteocytes have two types of cell processes:

1. type 1 are the most well known and these radiate from osteocyte cell bodies into the canaliculi and form connections with other cells, and
2. type 2 processes are less well known and are more closely apposed to the surface of osteoblasts that are forming bone.

Yamamoto et al. (40) state: "In the early genesis of tendons, corneas, and cellular cementum (in teeth), fibroblasts (tendons and corneas) and cementoblasts (cementum) have been shown to secrete fibrils alongside finger-like (type 2) processes, thus controlling the fibril arrangement. Extrapolating these findings to bone would support our proposal that the fibril arrangement is controlled by osteoblasts."

Based on observations made using transmission electron microscopy, they also noted that where:

1. transverse lamellae were forming, type 2 processes were also transverse, and
2. longitudinal lamellae were forming, type 2 processes also paralleled the longitudinal collagen fibrils.

To achieve the TPD, they suggest that osteoblasts:

1. migrate regularly, secreting collagen fibrils, and
2. move the type 2 processes synchronously and periodically to create the alternating changes in TPD.

But how do connections between type 1 osteoblast processes change as the pattern is created? We could not find any evidence that osteoblasts or very recently formed osteocytes can form connections transiently, detach, and move their cell processes. We suggest that bending of primary cilia of osteoblasts activates the cellular machinery that functions in orientating the forming collagen molecules (without cell rotation/migration) via alignment of the type 2 processes. One observation that also makes it unlikely that type 1 processes control predominant CFO is that the processes are perpendicular within the lamellae of alternating SOMs regardless of the lamella being dark or bright (27, 69).

We also suggest that pre-collagen molecules that are in a liquid crystalline phase (70) begin to orient within the osteoblast (41, 71) in accordance with patterns of ciliary motion (▶Fig. 9) prior to their evagination by fibropositors. As fibrillogenesis continues, type 2 processes beneath the osteoblast continue the assembly process. This idea is derived in part from Donnelly et al. (37) who stated that "ciliary orientation may play a role in determining the orientation of the surrounding ECM. Directionality of collagen secretion arises from an intracellular alignment of collagen fibrils within fibropositors prior to secretion." What then follows appears to be a strongly genetically and evolutionarily conserved synthesis program(s) that produces repeating periods of staggered CFO designs.

CFO provides cell "contact guidance" that restricts significant influences of substrate stretch on cell orientation/rotation

Cyclic stretching experiments of cultured fibroblasts, osteoblasts, embryonic myocytes, and arterial smooth muscle and endothelial cells have shown various responses: increases in cell proliferation, alterations in mRNA levels and protein synthesis, and modifications of the actin cytoskeleton (72, 73). Cell culture experiments have also shown that the cells orient away from the stretching direction. This "cell orientation response" is governed by the strain (or stretch) beneath a cell and along its long axis (73). While in vitro data show that fibroblasts (and osteoblasts) orient away from the direction of stretching (away from excessive deformation), the in vivo situation is quite different – fibroblasts align along the collagen fibers, hence in the stretch direction. The inability of fibroblasts to change orientations in vivo likely results from contact guidance provided by collagen fibers (73). We suggest that preferred CFO (governed by type 2 processes) provides contact guidance that restricts the position of osteoblasts despite substrate stretching (challenging mechanisms suggested in ▶Figure 3).

The aforementioned experiments used relatively low cell densities in order to avoid confounding effects of cell-cell contact. In reality, cells are more closely apposed during bone formation, as was recognized long ago (39). Cell-cell contacts that are important in tendon formation (74) are also critically important in osteon formation (75). Planar cell polarity (coordinated alignment of cell polarity across the tissue plane) (76), might also be involved in osteon formation. However, although cilia appear to be important in establishing planar cell polarity in some cases (76), there are data that diminish this possibility in some tissues (77, 78).

"Collagen self-assembly" is a misnomer in the context of in vivo ECM formation

Prominent investigators (27) have stated "a debate still exists as to whether the 3D organization of collagen fibrils is cell-driven or a result of self-assembly, thus the influence of the arrangement of the cells on the final structure of bone is still an open question." Although Kadler et al. (79) concluded that there is "unequivocal proof that collagen fibril formation can occur readily in the absence of cells," they emphasized that collagen "self-assembly" only occurs in vitro. Hence we strongly assert that is a misnomer in the context of in vivo bone formation. Kadler et al. (79) make this clear in noting that the high number of binding partners (~50) that fibrillar collagens have in vivo is required to generate the diversity of fibril patterns, which range from parallel bundles in tendon and ligament, to orthogonal lattices in cornea, and interlocking weaves in blood vessels, skin, and bone. In turn they argue that "a large number of binding partners can spell disaster for a protein self-assembly/polymerization pro-

cess. Purified collagen spontaneously assembles into fibrils in vitro because collagen molecules are free to bind to other collagen molecules, and only collagen molecules. In vivo, however, the situation is very different. Faced with so many potential-binding partners, collagen molecules might easily be sequestered into dead-end molecular interactions, which would lower the effective concentration of collagen monomers available to form fibrils.... The cells [likely] use collagen V and XI to nucleate collagen fibrils, and fibronectin (FN) and integrins to specify their site of assembly. By localizing fibril formation to the plasma membrane the cell maintains tight regulatory control of collagen fibrillogenesis, which is clearly essential for the formation of long-range packing assemblies of collagen fibrils in different tissues."

Conclusions

We propose that fluid-flow within the lumen of a forming osteon causes preferential bending patterns of osteoblast cilia, which process vectorial (variant) stimuli that ultimately activate pathways that synthesize highly ordered, and often strain-mode-related, ECMs in SOMs. Cilia-based mechanisms are also likely at work in the ECM formation of hemiosteons of cancellous bone (80) and in bones that do not form or have few secondary osteons.

Acknowledgements

The authors are grateful for the criticisms of the manuscript provided by René van Oers and Peter Varga.

Conflict of interest

The authors report no conflict of interest.

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