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PERSPECTIVES

TGFβ and Runx2 Calibration of Bone Extracellular Matrix Quality for Tissue-Specific Function

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Abstract

Each extracellular matrix (ECM) throughout the body has specific physical properties. However, very little is known about how tissue-specific physical properties such as ECM elastic modulus are established or maintained. The fine regulation of ECM elastic modulus is particularly apparent in the skeleton where the material properties of bone are anatomically distinct, developmentally regulated, and evolutionarily conserved. Consequently, the skeleton has served as an excellent model system in which to study mechanisms that define tissue-specific material properties and couple them to tissue-specific function. Using this approach, we found that the uniquely hard ECM of cochlear bone is regulated through the activity of the lineage-specific transcription factor Runx2 via the same TGFβ-dependent pathway that controls osteoblast differentiation and ECM protein expression. Furthermore, this regulation is essential for tissue-specific function in hearing. While the mechanisms by which TGFβ regulates ECM material properties are currently most well-defined, they likely serve as an example for other signaling pathways. It is possible that similar mechanisms are active in non-skeletal tissues, such that growth factors target lineage-specific transcription factors to define ECM material properties of other tissues. By examining the role of bone quality in hearing, this article will explore mechanisms by which tissue-specific material properties are established and linked to normal tissue function. *IBMS BoneKEy*. 2011 August;8(8):370-380.

Introduction

What is the hardest bone in the body? A search through the literature for the answer leads to fascinating studies of bones from wallabies to whales (1). Using machined specimens of mineralized tissues from many species, Currey performed three-point bending to determine the hardness and elastic modulus of each. Hardness and elastic modulus are two of many material properties, which are independent of sample size or geometry. Elastic modulus reflects the ability of tissue to resist deformation. Currey's studies reveal a surprising diversity in bone elastic modulus within an individual organism, let alone across the animal kingdom (Table 1) (1). Taking advantage of nanoindentation to measure the elastic modulus of much smaller bones, we found that mouse bone also has a range of material properties. For example, the elastic modulus of the cochlear bone extracellular matrix (30 GPa) is more than twice that of the nearby calvarial bone (14 GPa) (Fig. 1) (2). This result was satisfyingly consistent with Currey's finding that the ear bone of the fin whale had the highest elastic modulus (34 GPa), as well as many clinical claims that cochlear bone is harder than any other (3).

Rather than satisfy our curiosity, these findings made clear that the elastic modulus of bone is carefully specified in a manner that is developmentally regulated, anatomically distinct, and evolutionarily conserved (1;2). However, very little is known about the mechanisms by which bone matrix material properties are regulated. Building on what we learned about the uniquely hard bone of the cochlea,

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this article seeks to explore two compelling questions. First, how are the anatomically distinct material properties of bone, or any tissue, specified and maintained? Second, are the precisely calibrated material properties of a specific bone required for its function?

Table 1. The diversity in bone elastic modulus within individual organisms.

Species and tissue	*E* (GPa)
Polar bear (3 months), femur	6.7
Red deer, mature antler	7.2
Red deer, immature antler	10.0
Narwhal, tusk dentine	10.3
Polar bear (9 months), femur	11.2
Donkey, radius	15.3
Polar bear (3 years, femur)	16.5
Human (adult), femur	16.7
Sarus crane, ossified tendon	17.7
Roe deer, femur	18.4
Polar bear (3.5 years), femur	18.5
Cow, tibia	19.7
Wallaby, femur	21.8
Polar bear, femur	22.2
King penguin, humerus	22.8
King penguin, ulna	22.9
Sarus crane, tarsometatarsus	23.1
Sarus crane, tibiotarsus	23.5
Horse, femur	24.5
Wallaby, tibia	25.4
Fallow deer, radius	25.5
Cow, femur	26.1
Fallow deer, tibia	26.8
Flamingo, tibiotarsus	28.2
Axis deer, femur	31.6
Fin whale, ear bone	34.1

E, Young's modulus of elasticity. All values are the mean of the values of several specimens. Reprinted from (1) with permission from Elsevier.

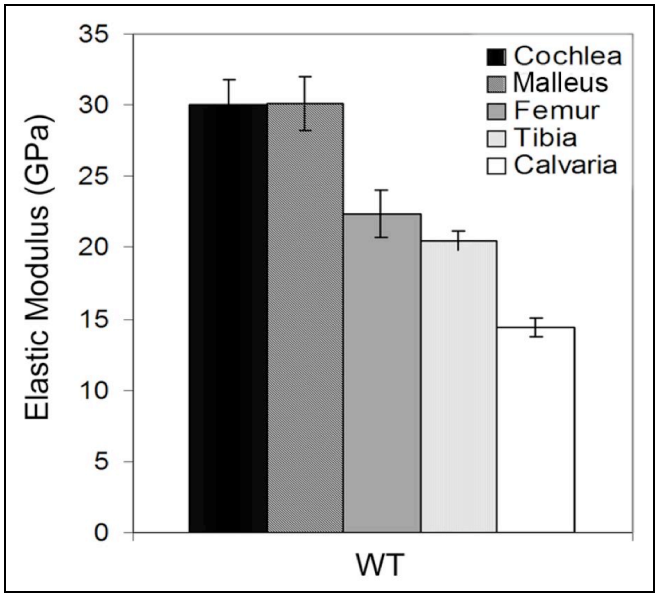

Fig. 1. Nanoindentation analyses of several mouse bones affirms that material properties are anatomically distinct. Reprinted from (2).

Material Properties of Extracellular Matrices Are Carefully Regulated

The material properties of tissues are largely defined by each tissue's unique extracellular matrix (ECM). Tissue-specific ECM composition and organization confer a wide range of elastic moduli that spans several orders of magnitude, from compliant nerve and lung tissue to stiffer cartilage, bone and enamel (4). While hydroxyapatite is responsible for the high elastic modulus of mineralized tissues (5), the organic components of the extracellular matrix also play a critical but less well-defined role in defining material properties (6-9). Despite this extraordinary range of elastic moduli, ECM material properties are finely tuned throughout development to support the evolving mechanical requirements of the tissue (4;5). For example, the stiffness of mammary tissue increases throughout pregnancy to facilitate the demands of lactation (4). ECM material properties also change in disease. ECM elastic modulus is increased by fibrosis and malignancy, resulting in palpable "lumps" (4), whereas it is decreased in degenerating osteoarthritic cartilage (10;11). Much remains to be learned about the mechanisms responsible for the developmental or pathological changes in ECM material properties.

In addition to their structural role, ECM material properties provide critical cues that direct cellular function. Through a process termed mechanoreciprocity, cells sense and respond to ECM elastic modulus using mechanoreceptors, integrins, actin, myosin and other cytoskeletal proteins (4). The cell translates these physical cues into biochemical signals that lead to specific changes in cell proliferation, lineage selection, differentiation, migration and transformation (12-14). Accordingly, disruption of ECM material properties can exacerbate disease progression (12). Therefore, understanding the mechanisms by which ECM material properties are established, maintained, and disrupted is critical to our understanding of development and disease.

TGFβ Regulation of ECM Elastic Modulus

The first identified biological regulators of ECM elastic modulus, TGFβ and glucocorticoids, were found by studying the skeleton (6;15). Using pharmacologic antagonists of TGFβ signaling and several genetically-modified mouse lines, we found that TGFβ regulates bone matrix elastic modulus through a pathway that includes the TGFβ type I and type II receptors and the downstream transcriptional effector, Smad3 (6;16). Bone ECM elastic modulus was reduced by high levels of TGFβ activity but was increased by partial TGFβ inhibition. Remarkably, the same trend was observed in dentin and skin (17;18). The possibility that TGFβ regulates ECM material properties in multiple tissue types is consistent with its well-known role in the regulation of ECM protein synthesis.

We sought to identify downstream effectors of the TGFβ pathway in the control of bone ECM elastic modulus. *In vitro*, a downstream target of TGFβ is the osteogenic transcription factor Runx2. Runx2 integrates signals from TGFβ and other bone-metabolic pathways to direct osteoblast gene expression (19). TGFβ represses Runx2 function and inhibits terminal osteoblast differentiation through a Smad3- and class II histone deacetylase-dependent pathway (20;21). We hypothesized that Runx2 was also a target of TGFβ repression in the control of ECM elastic modulus. Since Runx2-deficient mice are not viable, this hypothesis was tested in *Runx2*(+/-) mice.

Runx2-Dependent Regulation of Bone ECM Elastic Modulus

Studies of *Runx2*(+/-) mouse bone revealed that ECM elastic modulus is calibrated by this lineage-specific transcription factor (2). Furthermore, TGFβ represses Runx2 function to control ECM elastic modulus just as it does to control osteoblast differentiation. While retrospectively intuitive, this was the first observation that the physical properties of an ECM are regulated through the same growth factor/transcription factor pathway that controls the expression

of genes encoding tissue-specific ECM proteins such as osteocalcin (OCN) (20).

What is the target of TGFβ/Runx2 in the control of ECM elastic modulus? TGFβ and Runx2 co-regulate the expression of several osteoblast-secreted proteins including osteopontin (OPN), matrix metalloproteinase-13 (MMP-13), RANKL and others (22). Some of these are already known to impact the material properties of bone matrix directly or indirectly. For example, OPN and OCN can regulate the size, shape, and rate of mineral crystal formation *in vitro* and can impact the mineral composition or organization of bone matrix *in vivo* (7;8;23;24). MMP13 may affect material properties by degrading the organic components of bone matrix, whereas RANKL may alter material properties by accelerating osteoclast activity and bone turnover (25). We and others have observed that ablation of either OPN or OCN in mice affects bone ECM material properties (7;8). However, the role of these factors as downstream targets of TGFβ and Runx2 in the control of bone ECM material properties is either not supported (OPN) or not yet known (OCN). The role of MMPs in the control of ECM material properties is under investigation. MMP-2 was recently shown to modulate bone matrix material properties (26). Likely, a combination of extracellular proteins is co-regulated by glucocorticoids, TGFβ, Runx2, and other factors to confer anatomically distinct ECM material properties. Although the mechanisms of specificity are not yet known, we do know that the material properties of bone matrix are not simply a product of the extent of osteoblast differentiation. None of the markers of osteoblast differentiation that we examined were expressed in a gradient that corresponded to the gradient of ECM elastic modulus (cochlea > femur > tibia > calvaria) (unpublished observations).

A General Mechanism for the Regulation of ECM Material Properties?

The mechanisms identified in bone, in which TGFβ targets the lineage-specific transcription factor Runx2 to in some way control ECM elastic modulus, may be relevant in multiple tissue types. TGFβ or other regulators may target lineage-specific transcription factors in other tissues to control ECM protein expression, ECM composition and organization, and consequently, ECM material properties. Already, TGFβ is known to control the activity of c/EBP-β in adipocytes (27), MyoD in myoblasts (28), and Sox9 in chondrocytes (29). The extent to which this hypothesis can be validated in tissues other than bone remains an area of active investigation.

Regulation of ECM Material Properties Is Essential for Tissue-Specific Function

The fact that bone matrix material properties are anatomically distinct and evolutionarily conserved suggests that they may also be functionally essential (1;2). However, discrimination of the functional contribution of ECM material properties in long bone is difficult because of the many factors that influence fracture resistance. The ability of bone to resist fracture depends on bone mass and bone quality. In addition to ECM material properties, bone quality is the result of multiple parameters including trabecular architecture, microdamage, and geometry (30). Furthermore, experimental manipulations that alter ECM elastic modulus (TGFβ, glucocorticoids, or Runx2 function) simultaneously affect many aspects of bone mass and quality (16;31;32).

In contrast, the uniquely hard cochlear bone afforded the opportunity to examine the contribution of ECM elastic modulus to a different functional outcome – hearing. If ECM material properties are calibrated for tissue-specific function, then loss of this regulation would be expected to impair tissue function. Aside from the experimental advantages, this hypothesis was supported by clinical observations. Humans with the genetic bone syndrome cleidocranial dysplasia (CCD), due to heterozygous loss of Runx2 function, have hearing loss (33;34).

In normal sound conduction, sound waves travel down the external auditory canal, vibrate the tympanic membrane, and propagate via the ossicular bones of the middle ear to reach the cochlea or inner ear

(Fig. 2). Within the bony cochlear capsule, a traveling wave causes deflection of the organ of Corti in a frequency-specific manner, exciting sensory hair cells and allowing the sound signal to be propagated via the auditory nerve to the central nervous system. Hearing loss can take on several forms and is classified using clinical definitions based upon where in this pathway a pathologic process occurs. Conductive hearing loss is caused by pathology preventing sound from reaching the inner ear, and includes defects in the outer ear canal, tympanic membrane, or middle ear space and ossicles. In contrast, sensorineural hearing loss classically refers to hearing loss resulting from defects of the cochlea or auditory nerve.

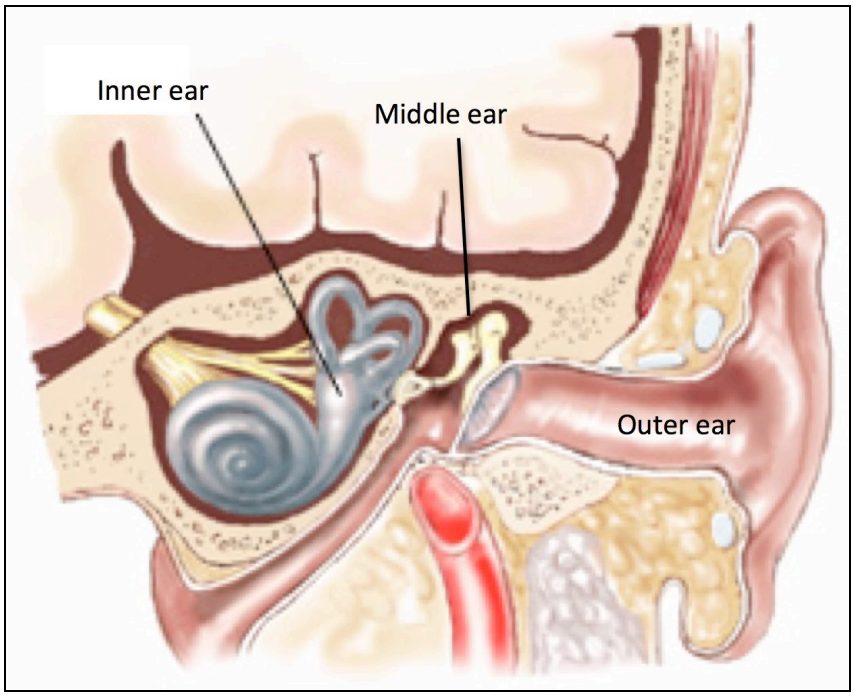

Fig. 2. Diagram of the ear showing the bony structures of the outer, middle, and inner ear, including the ear canal, ossicles, and cochlea, respectively.

Unexplained Sensorineural Hearing Loss Accompanies Many Bone Syndromes

In many bone diseases, these classical distinctions blur. Well-known examples include fibrous dysplasia and cochlear otosclerosis (35-38), where some patients exhibit sensorineural hearing loss in the absence of obvious pathology in the organ of Corti or auditory nerve. Humans with CCD can have sensorineural, conductive, or mixed types of hearing loss. Case studies have suggested external ear canal abnormalities, ossicular malformations, eustachian tube dysfunction, or otitis media as possible causes for conductive hearing loss in CCD (33;34). Less clear is the cause of sensorineural hearing loss in CCD and in several other bone disorders, including Paget's disease, osteogenesis imperfecta tarda, and Camurati-Engelmann disease (39-41). Although these diseases highlight that bone is critical for normal cochlear function, how bony abnormalities can cause sensorineural hearing loss is still unknown.

We tested the hypothesis that sensorineural hearing loss in CCD resulted from disruption of mechanisms that establish the unique material properties of cochlear bone ECM. We examined cochlear bone ECM elastic modulus and hearing in *Runx2*(+/-) mice and in "D4" mice that overexpressed an active form of TGFβ under control of the OCN promoter, both of which showed classical hallmarks of CCD (dysplastic clavicles and patent cranial sutures) (2;42-44). In both mouse models, cochlear bone ECM elastic modulus was reduced (2). The cochlear bone elastic modulus and hardness in these

mice were closer to the material properties of the wild-type tibia. In addition, both mouse models of CCD had hearing loss. Furthermore, rescue of the cochlear bone ECM material properties by inhibition of TGFβ signaling in osteoblasts also rescued hearing loss. These data strongly suggest that the sensorineural hearing loss accompanying CCD is due in part to loss of the distinctive hardness of the cochlear capsule. This is the first demonstration that the carefully defined material properties of bone matrix are essential for tissue-specific function, much as Currey had postulated based on his studies of whale ear bone and other mineralized tissues (1;5).

It is possible that these findings could be extrapolated to the hearing loss in Camurati-Engelmann disease, osteogenesis imperfecta, and Paget's disease (Table 2). While CCD results from mutations in *Runx2* (45;46), Camurati-Engelmann disease results from mutations that impact TGFβ activity (47;48), both of which regulate bone matrix material properties (2;6). In osteogenesis imperfecta, collagen mutations compromise bone matrix material properties (9). In Paget's disease, cochlear bone mineral density is reduced in patients with sensorineural hearing loss (39). The undermineralized Pagetic bone, like *Runx2*(+/-) and TGFβ-overexpressing bone matrix, likely has impaired material properties. The sensorineural hearing loss in each of these syndromes, therefore, might result from impaired bone matrix material properties.

Table 2. Bone diseases with associated hearing loss and defective bone matrix material properties.

Bone Disease with Associated Hearing Loss	Mutations or Defects	Hearing Loss Reference	Bone Quality Reference
Cleidocranial dysplasia	Runx2	Visosky (33), Cooper (34)	Chang (2)
Osteogenesis imperfecta	Type I collagen	Hartikka (40)	Kozloff (9)
Camurati-Engelmann disease	TGFβ	Higashi (41)	Balooch (6), Mohammad (16)
Paget's disease	Mineralization defects	Monsell (39)	Roschger (55)
Otosclerosis	Bone turnover, TGFβ	Chole (38), Thys (57)	Balooch (6), Mohammad (16)

Cochlear Bone Is Protected from Osteoclast-Mediated Bone Remodeling

Another highly unique feature of cochlear bone is its protected status from bone remodeling. Unlike other bones that are continually remodeled to accommodate endocrine or nutritional changes, mechanical load or injury, the cochlea undergoes very little if any remodeling postnatally (38;49;50). This "protected status" is enabled by the elevated cochlear expression of osteoprotegerin (OPG), an antagonist of osteoclast differentiation and function. Cochlear expression of OPG far surpasses that detected in other bones (51). It is perhaps then not surprising that in OPG-deficient mice, the cochlear bone matrix is remodeled and the ossicles are severely eroded and grossly deformed (52;53). Administration of bisphosphonates to OPG-deficient animals ameliorates the excessive remodeling of ear bone and hearing loss (54). The cause of hearing loss in OPG-deficient mice (20-30 dBSPL) is distinct from that in TGFβ-overexpressing and *Runx2*(+/-) mice (approximately 15 dBSPL), in which no apparent defect in ossicular structure was observed. Therefore, a relatively subtle defect in the specific material property signature of ear bone matrix impairs hearing nearly as much as gross defects in ear bone structure.

Despite the differences in mechanisms responsible for hearing loss, the effect of bone remodeling on bone matrix properties

remains an important area of investigation. Inhibition of bone remodeling with bisphosphonates has been shown to increase bone ECM mineral content and elastic modulus (55). Consequently, the uniquely hard cochlear bone may result from the lack of bone remodeling at this site. Furthermore, TGFβ and Runx2 regulate osteoclast activity, in part through regulation of OPG and RANKL expression within osteoblasts (56). Whether TGFβ and Runx2 exert some of their effects on bone ECM material properties via the OPG/RANKL axis is an area of active research that is currently unresolved. Interestingly, a human TGFβ allele is protective for otosclerosis (57), in which hearing loss results from aberrant osteoclast function. Though an association between hearing loss and bone fragility, osteoporosis or bisphosphonate use has not yet been reported, the relationship between hearing, bone remodeling, and ECM material properties is an area that deserves additional investigation.

Mechanisms by Which Bone Quality Contributes to Hearing

Although bone ECM material properties are clearly important for cochlear function, the mechanism by which they contribute to normal hearing remains unclear. The combination of three functional hearing tests (auditory brainstem response, distortion product otoacoustic emissions, and compound action potential) localizes the hearing defect to the peripheral auditory system and shows that conductive hearing remains relatively intact. As suggested by Monsell, loss of cochlear bone quality may cause absorption of acoustic energy, resulting in lower amplitude displacement of the basilar membrane in response to sound (39). Another possibility is that defects in cochlear bone quality impair sensorineural development. Crosstalk between bone and neural structures is critical during development. Abnormalities in the development of either structure can compromise the development of the other. Our histological analyses showed intact organ of Corti with normal numbers and organization of ciliated hair cells in *Runx2*(+/-) and TGFβ-overexpressing mice (2). Nonetheless, physical cues provided by the bone ECM may yet contribute to the crosstalk between bone and sensorineural structures in development or postnatally. Further study is needed to better understand the mechanisms by which bone contributes to the sensorineural function of the cochlea.

Summary

In conclusion, ECM material properties are carefully defined throughout the body through mechanisms that have only recently been discovered. A biological pathway already implicated in the control of cell differentiation and ECM protein expression is essential for the calibration of bone ECM elastic modulus. Specifically, TGFβ defines bone ECM elastic modulus through a Runx2-dependent mechanism. This pathway serves as a model that other growth factors and hormones likely employ. The ability of a lineage-specific transcription factor to control ECM material properties provides cells with a streamlined way to control the biological and physical features of the cellular microenvironment – both of which can, in turn, influence cell behavior. Loss of this regulation in bone compromises the uniquely hard cochlear bone matrix resulting in hearing loss, demonstrating the functional significance of the pathways regulating ECM material properties. Developing a better understanding of the mechanisms by which material properties are defined and maintained will elucidate the role of matrix quality in normal tissue function, development and disease.

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References

1. Currey JD. Mechanical properties of bone tissues with greatly differing functions. *J Biomech*. 1979;12(4):313-9.

2. Chang JL, Brauer DS, Johnson J, Chen CG, Akil O, Balooch G, Humphrey MB, Chin EN, Porter AE, Butcher K, Ritchie RO, Schneider RA, Lalwani A, Derynck R, Marshall GW, Marshall SJ, Lustig L, Alliston T. Tissue-specific calibration of extracellular matrix material properties by transforming growth factor-β and

Runx2 in bone is required for hearing. *EMBO Rep*. 2010 Oct;11(10):765-71.

3. Cummings CW, Haughey BH, Thomas JR, Harker LA, Flint PW. *Cummings Otolaryngology – Head & Neck Surgery*. 4th ed. Philadelphia, PA: Mosby; 2004.

4. Butcher DT, Alliston T, Weaver VM. A tense situation: forcing tumour progression. *Nat Rev Cancer*. 2009 Feb;9(2):108-22.

5. Currey JD. The design of mineralised hard tissues for their mechanical functions. *J Exp Biol*. 1999 Dec;202(Pt 23):3285-94.

6. Balooch G, Balooch M, Nalla RK, Schilling S, Filvaroff EH, Marshall GW, Marshall SJ, Ritchie RO, Derynck R, Alliston T. TGF-beta regulates the mechanical properties and composition of bone matrix. *Proc Natl Acad Sci U S A*. 2005 Dec 27;102(52):18813-8.

7. Thurner PJ, Chen CG, Ionova-Martin S, Sun L, Harman A, Porter A, Ager JW 3rd, Ritchie RO, Alliston T. Osteopontin deficiency increases bone fragility but preserves bone mass. *Bone*. 2010 Jun;46(6):1564-73.

8. Kavukcuoglu NB, Patterson-Buckendahl P, Mann AB. Effect of osteocalcin deficiency on the nanomechanics and chemistry of mouse bones. *J Mech Behav Biomed Mater*. 2009 Aug;2(4): 348-54.

9. Kozloff KM, Carden A, Bergwitz C, Forlino A, Uveges TE, Morris MD, Marini JC, Goldstein SA. Brittle IV mouse model for osteogenesis imperfecta IV demonstrates postpubertal adaptations to improve whole bone strength. *J Bone Miner Res*. 2004 Apr;19(4):614-22.

10. Kleemann RU, Krocker D, Cedraro A, Tuischer J, Duda GN. Altered cartilage mechanics and histology in knee osteoarthritis: relation to clinical assessment (ICRS Grade). *Osteoarthritis Cartilage*. 2005 Nov;13(11):958-63.

11. Silver FH, Bradica G, Tria A. Elastic energy storage in human articular cartilage: estimation of the elastic modulus for type II collagen and changes associated with osteoarthritis. *Matrix Biol*. 2002 Mar;21(2):129-37.

12. Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA, Weaver VM. Tensional homeostasis and the malignant phenotype. *Cancer Cell*. 2005 Sep;8(3):241-54.

13. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell*. 2006 Aug 25;126(4):677-89.

14. Wang HB, Dembo M, Wang YL. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. *Am J Physiol Cell Physiol*. 2000 Nov;279(5):C1345-50.

15. Lane NE, Yao W, Balooch M, Nalla RK, Balooch G, Habelitz S, Kinney JH, Bonewald LF. Glucocorticoid-treated mice have localized changes in trabecular bone material properties and osteocyte lacunar size that are not observed in placebo-treated or estrogen-deficient mice. *J Bone Miner Res*. 2006 Mar;21(3):466-76.

16. Mohammad KS, Chen CG, Balooch G, Stebbins E, McKenna CR, Davis H, Niewolna M, Peng XH, Nguyen DH, Ionova-Martin SS, Bracey JW, Hogue WR, Wong DH, Ritchie RO, Suva LJ, Derynck R, Guise TA, Alliston T. Pharmacologic inhibition of the TGF-beta type I receptor kinase has anabolic and anti-catabolic effects on bone. *PLoS One*. 2009;4(4):e5275.

17. Saeki K, Hilton JF, Alliston T, Habelitz S, Marshall SJ, Marshall GW, Denbesten P. Elevated TGF-beta2 signaling in dentin results in sex related enamel defects. *Arch Oral Biol*. 2007 Sep;52(9):814-21.

18. Arany PR, Flanders KC, Kobayashi T, Kuo CK, Stuelten C, Desai KV, Tuan R, Rennard SI, Roberts AB. Smad3 deficiency alters key structural elements of the extracellular matrix and mechanotransduction of wound closure. *Proc Natl Acad Sci U S A*. 2006 Jun 13;103(24):9250-5.

19. Franceschi RT, Xiao G, Jiang D, Gopalakrishnan R, Yang S, Reith E. Multiple signaling pathways converge on the Cbfa1/Runx2 transcription factor to regulate osteoblast differentiation. *Connect Tissue Res*. 2003;44 Suppl 1:109-16.

20. Alliston T, Choy L, Ducy P, Karsenty G, Derynck R. TGF-beta-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J*. 2001 May 1;20(9):2254-72.

21. Kang JS, Alliston T, Delston R, Derynck R. Repression of Runx2 function by TGF-beta through recruitment of class II histone deacetylases by Smad3. *EMBO J*. 2005 Jul 20;24(14):2543-55.

22. Alliston T, Piek E, Derynck R. TGF-β family signaling in sketetal development, maintenance, and disease. In: Derynck R, Miyazono K, eds. *The TGF-β Family*. Woodbury, NY: Cold Spring Harbor Press; 2008:667-723.

23. Hunter GK, Hauschka PV, Poole AR, Rosenberg LC, Goldberg HA. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem J*. 1996 Jul 1;317(Pt 1): 59-64.

24. Kavukcuoglu NB, Denhardt DT, Guzelsu N, Mann AB. Osteopontin deficiency and aging on nanomechanics of mouse bone. *J Biomed Mater Res A*. 2007 Oct;83(1):136-44.

25. Busse B, Hahn M, Soltau M, Zustin J, Püschel K, Duda GN, Amling M. Increased calcium content and inhomogeneity of mineralization render bone toughness in osteoporosis: mineralization, morphology and biomechanics of human single trabeculae. *Bone*. 2009 Dec; 45(6):1034-43.

26. Nyman JS, Lynch CC, Perrien DS, Thiolloy S, O'Quinn EC, Patil CA, Bi X, Pharr GM, Mahadevan-Jansen A, Mundy GR. Differential effects between the loss of MMP-2 and MMP-9 on structural and tissue-level properties of bone. *J Bone Miner Res*. 2011 Jun;26(6):1252-60.

27. Choy L, Derynck R. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *J Biol Chem*. 2003 Mar 14;278(11):9609-19.

28. Liu D, Black BL, Derynck R. TGF-beta inhibits muscle differentiation through functional repression of myogenic transcription factors by Smad3. *Genes Dev*. 2001 Nov 15;15(22):2950-66.

29. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. *J Biol Chem*. 2005 Mar 4;280(9):8343-50.

30. Hernandez CJ, Keaveny TM. A biomechanical perspective on bone quality. *Bone*. 2006 Dec;39(6):1173-81.

31. Xiao Z, Awad HA, Liu S, Mahlios J, Zhang S, Guilak F, Mayo MS, Quarles LD. Selective Runx2-II deficiency leads to low-turnover osteopenia in adult mice. *Dev Biol*. 2005 Jul 15;283(2):345-56.

32. Rehman Q, Lane NE. Effect of glucocorticoids on bone density. *Med Pediatr Oncol*. 2003 Sep;41(3):212-6.

33. Visosky AM, Johnson J, Bingea B, Gurney T, Lalwani AK. Otolaryngological manifestations of cleidocranial dysplasia, concentrating on

audiological findings. *Laryngoscope*. 2003 Sep;113(9):1508-14.

34. Cooper SC, Flaitz CM, Johnston DA, Lee B, Hecht JT. A natural history of cleidocranial dysplasia. *Am J Med Genet*. 2001 Nov 15;104(1):1-6.

35. Megerian CA, Busaba NY, McKenna MJ, Ojemann RG. Teflon granuloma presenting as an enlarging, gadolinium enhancing, posterior fossa mass with progressive hearing loss following microvascular decompression. *Am J Otol*. 1995 Nov;16(6):783-6.

36. Govaerts PJ, Casselman J, Daemers K, De Ceulaer G, Somers T, Offeciers FE. Audiological findings in large vestibular aqueduct syndrome. *Int J Pediatr Otorhinolaryngol*. 1999 Dec 15; 51(3):157-64.

37. Halmagyi GM, Aw ST, McGarvie LA, Todd MJ, Bradshaw A, Yavor RA, Fagan PA. Superior semicircular canal dehiscence simulating otosclerosis. *J Laryngol Otol*. 2003 Jul;117(7):553-7.

38. Chole RA, McKenna M. Pathophysiology of otosclerosis. *Otol Neurotol*. 2001 Mar;22(2):249-57.

39. Monsell EM. The mechanism of hearing loss in Paget's disease of bone. *Laryngoscope*. 2004 Apr;114(4):598-606.

40. Hartikka H, Kuurila K, Körkkö J, Kaitila I, Grénman R, Pynnönen S, Hyland JC, Ala-Kokko L. Lack of correlation between the type of COL1A1 or COL1A2 mutation and hearing loss in osteogenesis imperfecta patients. *Hum Mutat*. 2004 Aug;24(2):147-54.

41. Higashi K, Matsuki C. Hearing impairment in Engelmann disease. *Am J Otol*. 1996 Jan;17(1): 26-9.

42. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*. 1997 May 30;89(5):755-64.

43. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB, Owen MJ. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*. 1997 May 30;89(5):765-71.

44. Erlebacher A, Derynck R. Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. *J Cell Biol*. 1996 Jan;132(1-2):195-210.

45. Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat Genet*. 1997 Jul;16(3):307-10.

46. Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S, Lindhout D, Cole WG, Henn W, Knoll JH, Owen MJ, Mertelsmann R, Zabel BU, Olsen BR. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell*. 1997 May 30;89(5):773-9.

47. Janssens K, Gershoni-Baruch R, Guañabens N, Migone N, Ralston S, Bonduelle M, Lissens W, Van Maldergem L, Vanhoenacker F, Verbruggen L, Van Hul W. Mutations in the gene encoding the latency-associated peptide of TGF-beta 1 cause Camurati-Engelmann disease. *Nat Genet*. 2000 Nov;26(3):273-5.

48. Janssens K, ten Dijke P, Ralston SH, Bergmann C, Van Hul W. Transforming growth factor-beta 1 mutations in Camurati-Engelmann disease lead to increased signaling by altering either activation or secretion of the mutant

protein. *J Biol Chem*. 2003 Feb 28;278(9):7718-24.

49. Sørensen MS. Temporal bone dynamics, the hard way. Formation, growth, modeling, repair and quantum type bone remodeling in the otic capsule. *Acta Otolaryngol Suppl*. 1994;512:1-22.

50. Sørensen MS, Jørgensen MB, Bretlau P. Distribution of bone remodeling units in the otic capsule of the rabbit. A semiquantitative morphometric study. *Acta Otolaryngol*. 1992;112(3):462-9.

51. Zehnder AF, Kristiansen AG, Adams JC, Merchant SN, McKenna MJ. Osteoprotegerin in the inner ear may inhibit bone remodeling in the otic capsule. *Laryngoscope*. 2005 Jan;115(1):172-7.

52. Zehnder AF, Kristiansen AG, Adams JC, Kujawa SG, Merchant SN, McKenna MJ. Osteoprotegrin knockout mice demonstrate abnormal remodeling of the otic capsule and progressive hearing loss. *Laryngoscope*. 2006 Feb;116(2):201-6.

53. Kanzaki S, Ito M, Takada Y, Ogawa K, Matsuo K. Resorption of auditory ossicles and hearing loss in mice lacking osteoprotegerin. *Bone*. 2006 Aug;39(2):414-9.

54. Kanzaki S, Takada Y, Ogawa K, Matsuo K. Bisphosphonate therapy ameliorates hearing loss in mice lacking osteoprotegerin. *J Bone Miner Res*. 2009 Jan;24(1):43-9.

55. Roschger P, Paschalis EP, Fratzl P, Klaushofer K. Bone mineralization density distribution in health and disease. *Bone*. 2008 Mar;42(3):456-66.

56. Fox SW, Lovibond AC. Current insights into the role of transforming growth factor-beta in bone resorption. *Mol Cell Endocrinol*. 2005 Nov 24;243(1-2):19-26.

57. Thys M, Schrauwen I, Vanderstraeten K, Janssens K, Dieltjens N, Van Den Bogaert K, Fransen E, Chen W, Ealy M, Claustres M, Cremers CR, Dhooge I, Declau F, Claes J, Van de Heyning P, Vincent R, Somers T, Offeciers E, Smith RJ, Van Camp G. The coding polymorphism T263I in TGF-beta1 is associated with otosclerosis in two independent populations. *Hum Mol Genet*. 2007 Sep 1;16(17):2021-30.