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## Mechanotransduction and Osteogenesis

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### 1. Introduction

It is well known that mechanical stimulation induces osteogenesis. Consciously or not, everyday we use mechanical loading to incite osteogenesis: normal daily activities like walking, or physical activities like gymnastics. It is easy to comprehend how biochemical factors (for example, hormones) activate biological reactions (for example, bone formation). In opposite, it is not simple to understand how mechanical forces cause biological reactions. The aim of this chapter is to describe the current knowledge about how bone cells react to mechanical stimuli, and what bone cells have to sense mechanical forces.

According to Wolff's Law, mechanical loads determine changes in bone structure (Duncan & Turner). In 1964, Frost proposed the **mechanostat model**, which is an upgrade of Wolff's Law. Within this model, relative deformations of bone are sensed by bone cells, which, in turn, produce or resorb bone tissue. The relative deformation, or strain, represents the ratio between the lengthening or shortening of a body and its original length. Strain is dimensionless and can be expressed as decimal fraction, percentage or  $\mu$ strain. For example, the strain of an 1 mm long body that – under action of an external load – lengthens or shortens 0.001 mm, is equal to 0.001 or 0.1% or 1000  $\mu$ strain. Deformations below 50-100  $\mu$ strain are in the **disuse** range, and result in bone resorption. Deformations between the ranges of 50-100 to 1000-1500  $\mu$ strain are in the **physiological** range. Physiological deformation can produce microfractures that are repaired; however, bone mass does not alter although the activation of osteogenesis. Deformations between the ranges of 1000-1500 to 3000  $\mu$ strain are in the **overuse** range, and produce microfractures, which are also repaired. Interestingly, in this case bone mass increases. There is no knowledge on how bone cells distinguish physiological and overuse deformations. Deformations above 3000  $\mu$ strain are in the **pathological overuse** range, and produce a number of microfractures that exceed bone repair capacity. As a result, microfractures accumulate, coalesce and weaken bone, ending in stress fractures (or fatigue fractures). Macroscopic fractures occur when relative deformation is over 25000  $\mu$ strain (Fig. 1) (Burr et al., 1998; Carter & Hayes, 1977; Frost, 2000, 2003; O'Brien et al., 2005). In conclusion, the role of mechanostat is to avoid mechanical deformations above 3000  $\mu$ strain, which may cause bone fracture.

Further, in addition to Frost's mechanostat model, it was realized that mechanostat can sense other physical parameters not only relative deformation: frequency, number of cycles resting periods, relative deformation distribution and local gradients of relative deformation (Torcasio et al., 2008). Relative deformation, frequency, number of cycles and resting periods

are the unique variables that can be controlled in mechanical assays. Moreover, there is no unit that encloses all these controllable variables. All of this impairs the analysis of bone cells response to mechanical stimulation, and may explain the difference in some values presented in this chapter.

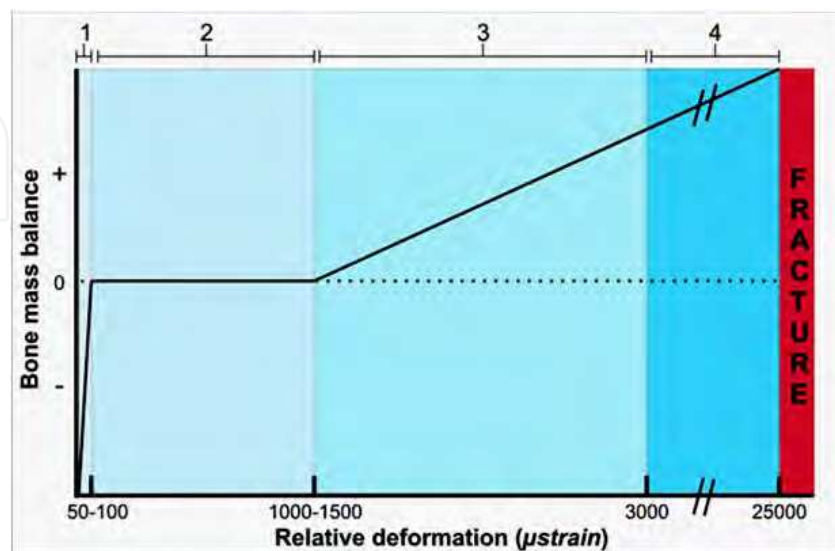


Fig. 1. Bone mass balance as a function of relative deformation. Within area 1, bone resorption rate is greater than bone formation rate, resulting in a negative balance (disuse range). Within area 2, bone resorption rate is equal bone formation rate, resulting in a neutral balance (physiological rate). Within area 3, bone resorption rate is lower than bone formation rate, resulting in a positive balance (overuse range). Within area 4, microfractures accumulate, resulting in decrease of bone resistance (pathological overuse range) that can lead to macroscopic bone fracture (area in red).

### 1.1 Frequency

The same strain applied on bone at 1-30 Hz frequencies increases osteogenesis. The opposite is not always true: different strains applied on bone at a fixed frequency may increase osteogenesis or not. Whether the strain will increase bone formation or not depends on the frequency of the stimulation. When the frequency is within the range of 5-10 Hz, bone exhibits the greatest osteogenesis rate. Above the range of 5-10 Hz, the osteogenesis rate decreases; probably, because mechanical loads at frequencies above 5-10 Hz exceed the capacity of bone cells to return to their previous shape like if bone tissue had become more rigid. If bone cells do not return to their not deformed shape, they will not undergo deformation; thus, they will not respond to mechanical deformation.

Curiously, vibratory mechanical loads induce bone formation at higher frequencies (17-90 Hz) and lower strain (5 μstrain). We hypothesize vibratory mechanical loads may stimulate a wider bone area, creating more local gradients of deformation; and hence, increasing mechanical stimulation on bone. Since various local gradients of deformation are created, more bone cells are incited to produce bone. In other words, there are more sites of osteogenesis; therefore, mechanical loads with lower strain at higher frequencies applied to bone will incite significant bone formation (Castillo et al., 2006; Fritton et al., 2000; Hsieh & Turner, 2001; Jacobs et al., 1998; Rubin et al., 2001; Torcasio et al., 2008; Warden & Turner, 2004; You et al., 2001).

### 1.2 Number of cycles

The product of the frequency and the duration of the stimulus is the number of cycles. As the number of cycles increases so does osteogenesis until a certain plateau (Burr et al., 2002; Umemura et al., 1997) (Fig. 2).

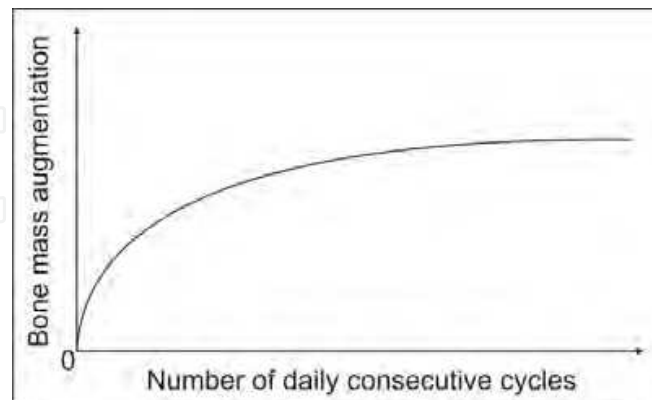


Fig. 2. Bone mass augmentation as a function of the number of consecutive cycles per day. After a certain number of consecutive cycles, which depends on the study design, bone mass augmentation ceases.

### 1.3 Resting periods

Continuous mechanical stimulation does not increase bone formation. Mechanical stimulation must have a pause otherwise bone cells osteogenic response will cease, because bone cells need a resting period to reorganize their cytoskeleton, recover ion concentration balance and dephosphorylate proteins to recover their mechanosensitivity. The resting period varies between studies from 8 to 48 hours. Since bone cells need a resting period to respond to mechanical deformation “at full strength”, the first stimulus may be the most important to determine how much bone formation a certain treatment can induce. One study supports this idea: the authors compared bone formation rate in the ulnae of rats subjected to 3 different protocols of mechanical stimulation. The first group was stimulated with progressively decreasing strains; the second group was stimulated with progressively increasing strains; and the third group was stimulated with constant strains. Bone formation rate was greater in the first group, followed by the third group (Burr et al., 2002; Pavalko et al., 1998; Robling et al., 2001, 2006; Schriefer et al., 2005; Tang et al., 2006).

## 2. Amplification of mechanical stimulation

Our bones in the skeleton can be subjected to 400 to 3000  $\mu\text{strain}$  during usual locomotion; however, it rarely exceeds 1000  $\mu\text{strain}$ . Nevertheless, *in vitro* bone cells response to mechanical stimulation is observed only with  $\sim 10000$ - $100000$   $\mu\text{strain}$  range, which is 10 to 100 fold greater than the usual strain bone is subjected. Interestingly, if bone was subjected to the same strain range bone cells need to be activated ( $\sim 10000$ - $100000$   $\mu\text{strain}$ ), bone would undergo fracture. You et al (2001) proposed a mathematical model to explain the different intensities needed to induce osteogenesis in the skeleton and in bone cells, pointing the actin cytoskeleton and the bone canaliculi system as strain amplifiers at the cellular level (Duncan & Turner, 1995; Fritton et al., 2000; Robling et al., 2006; Rubin & Lanyon, 1984; You et al., 2001).

## 2.1 Histological anatomy of bone

Schematically, long bones can be compared to a cylinder that contains several smaller cylinders (the Haversian canals), which intercommunicate via Volkmann's canals. Lamellae are the Haversian canals walls; disposed radially from each Haversian canal; composed of bone ECM (Table 1), which is basically hydroxyapatite (main inorganic component) and type I collagen (main organic component). Osteocytes are located within the lacunae, where they become imprisoned by the ECM synthesized during osteoblasts cellular differentiation. Osteocytes possess cytoplasmic processes (or dendrites) which are a prolongation of the cytoplasm. The cytoplasmic processes lacunae are called canaliculi (Fig 3).

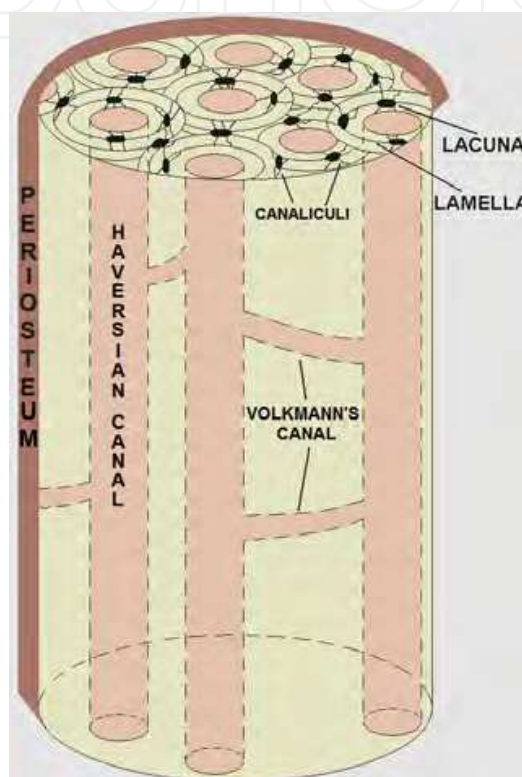


Fig. 3. Scheme of the histological anatomy of bone.

Between the canalicular wall and the cytoplasmic process is the pericellular space, whose diameter varies from 14 to 100 nm depending on the person age, osteocyte age, histological type of bone, and other features. Pericellular space diameter variation from 20 to 100 nm alters less than 40% the amplification of the mechanical stimulus within the cytoplasmic process. Within the pericellular space, there is a fluid with albumin and a PEM composed of proteoglycans and transverse fibrils. The osteocyte cytoplasmic process is anchored and centered in its canaliculus by these transverse fibrils. Within this system, You L et al., calculated that strains in the physiological range produce shear stresses from 0.5 to 3.0 Pa on cytoplasmic processes, but relative deformation from 8300 to 19700  $\mu strain$  at 1-20 Hz frequencies, which induce a cellular response. The larger the diameter of the space between glycosaminoglycans side chains, the lower is strain amplification. The calculation was based on the relationship between the albumin diameter ( $\sim 7\text{nm}$ ) and the space between the glycosaminoglycans side chains along a proteoglycan monomer. Within osteocytic cytoplasmic processes there are actin filaments arranged on the same axis of the process

Abbreviation	Meaning	Abbreviation	Meaning	Abbreviation	Meaning
AC	Adenylate Cyclase	GLAST	Glutamate/Aspartate Transporter	NOS	Nitric Oxide Synthase
cAMP	Cyclic Adenosine Monophosphate	cGMP	Cyclic Guanosine Monophosphate	OPG	Osteoprotegerin
AP	Activator Protein	GSK3b	Glycogen Synthase Kinase-3b	PDGF	Platelet-Derived Growth Factor
ATP	Adenosine Triphosphate	IGF	Insulin Growth-Like Factor	PEM	Pericellular Matrix
C/EBPβ	CCAAT-enhancer-binding protein	IGF-1R	IGF-1 Receptor	PI3K	Phosphatidylinositol 3-Kinase
cox	Cyclooxygenase	IP <sub>3</sub>	Inositol Triphosphate	PIP2	Phosphatidylinositol 4,5-Bisphosphate
CREB	cAMP Response Element Binding	IRS	Insulin Receptor Substrate	PKA and C	Protein Kinase A and C
Cx	Connexin	LIPUS	Low-Intensity Pulsed Ultrasound	PLA and PLC	Phospholipase A and C
DAG	Diacylglycerol	Lrp	Low-Density Lipoprotein Receptor-Related Protein	PTH	Parathyroid Hormone
EAAT	Excitatory Amino-acid Transporter	MAPK	Mitogen-Activated Protein Kinase	PYK2	Proline-Rich Tyrosine Kinase-2
ECM	Extracellular Matrix	mGluR	Metabotropic Glutamate Receptor	RANK(L)	Receptor Activator of NF-κB (Ligand)
ERα	Estrogen Receptor α	NF-κB	Nuclear Factor-κB	SH2	Src-homology-2
ERK	Extracellular Signal-Regulated Kinase	NMDA	N-Methyl-D-Aspartate	VEGF	Vascular Endothelial Growth Factor
FAK	Focal Adhesion Kinase	NO	Nitric Oxide		

Table 1. Abbreviations



membrane. Fimbrin is a rigid cross-linking protein that keeps actin filaments separated from each other ~25 nm of distance. The larger that distance, the higher the strain amplification at cytoplasmic processes level (Buckwalter et al., 1996a, 1996b; Owan & Triffitt, 1976; Sauren et al., 1992; Tanaka-Kamioka et al., 1998; You et al., 2001) (Fig 4).

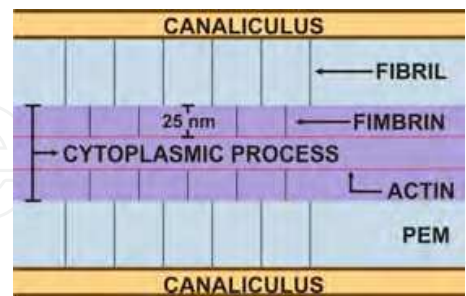


Fig. 4. *Actin cytoskeleton and fimbrins interaction.* Fimbrins keep actin filaments separated from each other ~25 nm of distance, which is optimum for mechanical stimulation amplification at the cytoplasmic process.

2.2 Drag force and shear stress

Bone deformation produces areas subjected to compression and tension stresses, creating pressure gradients within Haversian canals; and hence, forcing the fluid in canaliculi to flow from sites of compression stress (higher pressure) to sites of tension stress (lower pressure). This produces a fluid flow within the cytoplasmic processes pericellular space. The fluid flow imposes a drag force onto the PEM. Drag force is the resultant of the forces that oppose the relative motion of an object through a fluid. Considering 7 nm of diameter between the glycosaminoglycans side chains, You L et al (2001) calculated that,

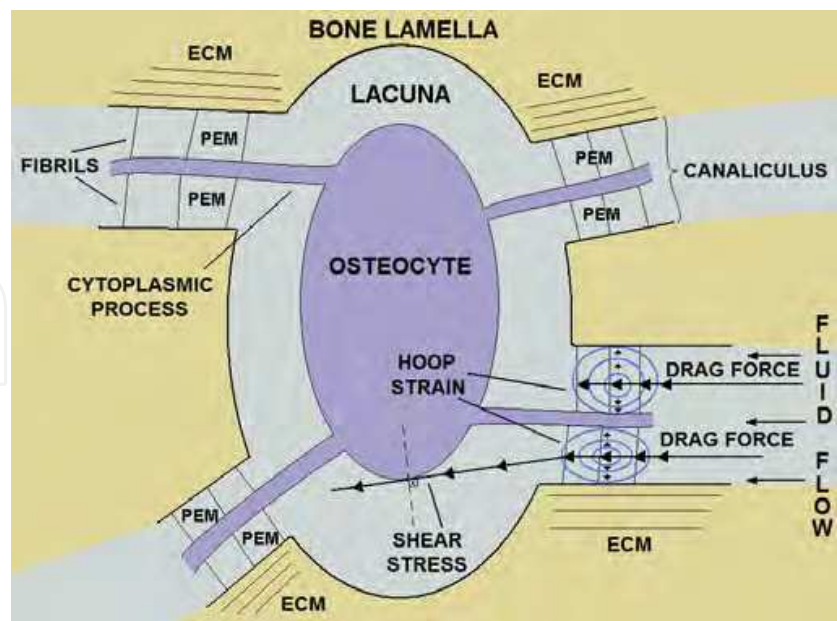


Fig. 5. *Mechanical stimulation amplification.* Mechanical deformation of bone tissue produces fluid flow, which, in turn, produces drag force on the cytoplasmic processes and the canaliculi walls. Besides, fluid flow produces a tangential force on the osteocyte cytoplasmic membrane, resulting in shear stress.

independently of the magnitude and frequency of the mechanical load, the drag force is 19.6 times larger than the shear force per unit length of cell process. Because of the histological anatomy of bone, mechanical deformations within the physiological range produce drag force that produces hoop strains on the membrane-cytoskeleton system of cytoplasmic processes, which are 20 to 100 times higher (or more) than the deformation experienced on the whole bone producing strains from 3000 to 50000  $\mu$ strain on the cytoplasmic process membrane. The larger the magnitude, or the frequency, of a mechanical loading, the higher is the strain amplification. Hoop strains are the normal tension forces on a body with circular symmetry. They produce compression and tension forces at the microscopic level (Fig. 5). According to the model proposed by You L et al, shear stress is not important for strain amplification *in vivo*. However, it does not mean that shear stress is disposable for the cellular response to mechanical load (Boutahar et al., 2004; Duncan & Turner, 1995; Hughes-Fulford, 2004; Kapur et al., 2003; Liedbert et al., 2006; Norvell et al., 2004; Scott et al., 2008; You et al., 2001).

### 3. The main mechanosensor cell

Osteoblasts, osteocytes and osteoclasts are the main bone cells. For decades, osteoblasts and osteoclasts were considered the protagonists of bone remodeling. For this reason, there are more reports with osteoclasts and osteoblasts than osteocytes, which comprise 90-95% of all bone cells. After realizing that osteocytes also respond to mechanical stimulation, authors inquire which is the main mechanosensor bone cell: osteoblasts or osteocytes (Bonewald, 2006; Buckwalter et al., 1996a).

Although there is no definitive proof, osteocytes are considered the cells that orchestrate bone remodeling through biochemical mediators that regulate the activity of osteoblasts and osteoclasts. Supporting this idea, it was demonstrated that osteocytes subjected to fluid flow stimulate osteoblasts to produce bone tissue; and that osteocytes produce prostaglandins faster than osteoblasts after mechanical stimulation. Prostaglandins mediate osteoclasts and osteoblasts activity. Moreover, it is more reasonable that osteocytes are the main mechanosensor bone cells since they comprise 90-95% of all bone cells and participate in the canicular system for strain amplification proposed by You L et al whereas osteoblasts are located at the periosteum, where strain does not undergo amplification (Bonewald, 2006; Chen et al., 1999; Cherian et al., 2005; Duncan & Turner, 1995; Goldspink, 1999; Gupta & Grande-Allen, 2006; Hsu et al., 2007; Klein-Nulend et al., 1995; Li et al., 2004; Plotkin et al., 2005; Taylor et al., 2007).

### 4. Piezoelectricity or streaming potential?

Fukada E and Yasuda I (1957) observed that bone is a viscoelastic material which possesses piezoelectric activity, producing negative electric charge in compression sites, and positive electric charge in tension sites. Furthermore, bone undergoes deformation when subjected to electric potentials. Interested in the study of piezoelectricity, Anderson JC and Eriksson C (1970, 1986) investigated the cause of those authors' observation.

They knew piezoelectricity does not occur in symmetric crystalline materials, and is as greater as asymmetric is its crystalline structure. Since hydroxyapatite is a symmetric crystalline material, bone piezoelectric property cannot be attributed to hydroxyapatite. Unlike the dried collagen, wet collagen, which is the *in vivo* collagen form, is not piezoelectric because water molecules interact with the collagen structure which becomes symmetric. On the other hand,



one third of the ECM collagen length interacts with hydroxyapatite, which blocks collagen interaction with some water molecules and impairs wet collagen structural changes, keeping the wet collagen asymmetric. With these data, one could hypothesize collagen is responsible for the electric potential detected after mechanical stimulation. Nevertheless, *in vivo* bone ECM collagen is embedded with a fluid containing high conductivity ions. As a result, the mechanically-induced fluid flow produces an electric potential, named streaming potential. Therefore, the electric potential observed after mechanical loading is the sum of the piezoelectric and the streaming potentials. Streaming potential depends on the type of the ion absorbed on a molecule surface (for example, collagen), inducing the formation of a diffuse layer composed of ions of the opposite charge. The fluid flow gives rise to a transport network of an ion type, determining a potential gradient (streaming potential), whose magnitude depends on the type of the molecule and the solution pH. Piezoelectric potential, in turn, depends only on the cellular mechanical deformation.

Anderson JC and Eriksson C observed that the molecule of collagen embedded within a fluid with pH 4.7 induces absorption of the same quantity of negative and positive charged ions, resulting in an isoelectric streaming potential; therefore, an electrode will detect only the piezoelectric potential of the molecule of collagen. In that experiment, the authors could not separate the bone ECM collagen from the hydroxyapatite; hence, they could not determine the streaming potential with pH 4.7 to 5.0. However, it was observed the lowest streaming potential within that pH range, suggesting the mechanically-induced electric potential in bone depends mainly on the streaming potential (Butcher et al., 2008; Qin et al., 2002) (Fig. 6).

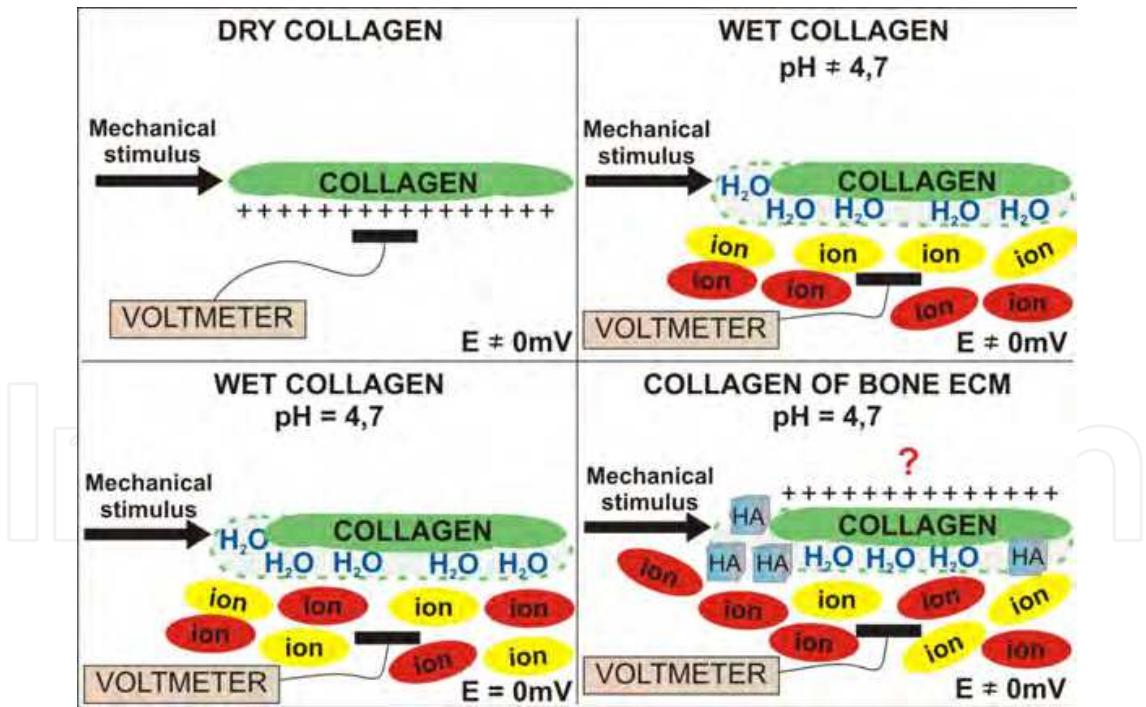


Fig. 6. Piezoelectricity x Streaming potential. Dried collagen is asymmetric and has piezoelectric activity. Hydrated collagen is symmetric and does not have piezoelectric activity. Streaming potential occurs when the fluid pH is different of 4.7; and does not occur when the fluid pH is 4.7. Bone ECM collagen is asymmetric although hydrated because it interacts with hydroxyapatite (HA). Therefore, collagen may exhibit piezoelectric activity and, principally, streaming potential.

Protein	Start of the event	Peak of event	Event duration
Akt	Increased activation: 5 minutes	15 minutes	At least 2 hours
ATP	Increased efflux: 1 minute	No data	No data
$\beta$ -catenin	Increased activation: 1 hour	No data	3 hours
Actin cytoskeleton	Reorganization: 1 hour	No data	No data
Collagen I	Increased synthesis: 3 days	3 days	No data
cox-2	Increased synthesis: 30-60 minutes	3-6 hours	At least 9 hours
Cx43	Increased synthesis: 2 hours	2 hours	24 hours
ERK	Increased activation: 0-15 minutes	15-240 minutes	2-4 hours
Sclerostin	Decreased synthesis: 24 hours	No data	No data
FAK	Increased activation: 0-5 minutes	0-30 minutes	1-4 hours
Alkaline phosphatase	Increased synthesis: 24-48 hours	No data	No data
GLAST (mRNA)	Decreased transcription: 6 hours	No data	No data
Cx43 hemichannels	Increased activation: immediately	Immediately	8-24 hours
$\alpha$ 2, $\alpha$ 5, $\beta$ 1 and $\beta$ 3 integrins subunits	Increased synthesis: 20 minutes	3-6 hours	No data
NF- $\kappa$ B	Increased activation: 30 minutes	30-60 minutes	No data
NO	Increased synthesis: 5-15 minutes (2 works); 12 hours (1 work)	5-15 minutes	24 hours
Osteopontin	Increased transcription: 1-3 days	No data	No data
Osteocalcin	Increased synthesis: 3 days	3 days	No data
p38	Increased activation: 15 minutes	30 minutes	90 minutes
PGE <sub>2</sub>	Increased synthesis: 1-6 hours (4 works); or 0-10 minutes (2 works)	18-24 hours	At least 24 hours
PGF <sub>2<math>\alpha</math></sub>	Increased synthesis: 5 minutes	5 minutes	10 minutes
PGI <sub>2</sub>	Increased synthesis: 30 minutes	No data	At least 1 hour
PI3K	Increased activation: 5 minutes	15 minutes	1 hour
PYK2	Increased activation: 30 minutes	4 hours	No data
PYK2-FAK	Coupling: 30 minutes	30 minutes	No data
Src	Increased activation: immediately	Immediately	4 hours
Src-FAK	Coupling: immediately	Immediately	4 hours
VEGF	Increased synthesis: 6 hours	12 hours	No data

Table 2. Chronology of the mechanotransduction events

PROTEIN	EFFECTS RELATED TO MECHANOTRANSDUCTION
AP-1	Activates CREB; increases type I collagen, alkaline phosphatase, iNOS, osteopontin and osteocalcin synthesis; and determines 48% of cox-2 transcription.
ATP	Activates P2Y <sub>2</sub> and P2X <sub>7</sub> ; increases P2Y <sub>2</sub> synthesis; and determines 80% of PGE <sub>2</sub> synthesis.
C/EBP	Determines 59% of cox-2 transcription.
Calcium	Increases PYK2, ERK and p38 activation; determines 83% of PGE <sub>2</sub> synthesis via G protein/AC pathway; determines 50% of ATP synthesis; *activates PLA <sub>2</sub> ; *increases IGF-I and TGF- $\beta$ synthesis; *complexes with calmodulin; and *releases ATP from vesicles.
calcium-calmodulin	Activates CREB, C/EBP $\beta$ , AC* and iNOS*.
$\beta$ -catenin	Activates Lef/TCF; and increases cox-2, Cx43 and c-jun synthesis.
cox-2	Increases Runx-2 synthesis; determines 100% of alkaline phosphatase synthesis, cellular proliferation and bone formation; and synthesizes PGE <sub>2</sub> and PGI <sub>2</sub> .
CREB	Determines 66% of cox-2 synthesis; and *increases c-fos synthesis.
Cx43	Determines 60% of PGE <sub>2</sub> efflux.
EP2/4	Activates AC.
ERK	Activates Lef/TCF, NF- $\kappa$ B and AP-1; Increases c-fos, c-jun and NO synthesis; determines 80% of osteocalcin synthesis, 80-100% of osteopontin synthesis, 100% of alkaline phosphatase and cox-2 synthesis, and 100% of cellular proliferation; and inhibits apoptosis.
Sclerostin	*Inhibits Wnt/ $\beta$ -catenin pathway.
FAK	Activates FAK-Src/Grb2/Sos/Ras/Raf/MEK/ERK-1/2 and FAK/PI3Kp85/Akt/NF- $\kappa$ B pathways; increases cox-2 synthesis; and *induces cellular migration.
c-fos	Complexes with c-jun, forming AP-1 heterodimer.
Alkaline phosphatase	Induces hydroxyapatite synthesis and ECM mineralization; and inhibits nucleation removers.
Frizzled proteins	*Activate G protein/PLC pathway; and compete with RANK*.
cGMP	*Closes sodium channels; and *induces vasodilatation.
GSK3b	*Degrades $\beta$ -catenin.
IGF-1	*Activates Ras/MEK/ERK/Tcf-Lef and PI3K/Akt pathways; *activates IRS-1/2; *induces ECM synthesis, osteoblasts and osteoblasts precursors differentiation, and chondrocytes proliferation; and, in adipocytes, increases FAK autophosphorylation by 30%* and *PYK2 phosphorylation.
IRS-1/2	*Activates the pathways of Grb2 and PI3K.
Lrp5	Increases the cellular response to mechanical stimulation, and *frizzled proteins synthesis.
mGluR	*Activates G protein/PLC pathway.
NF- $\kappa$ B	Increases cox-2, iNOS and *RANKL synthesis.

PROTEIN	EFFECTS RELATED TO MECHANOTRANSDUCTION
NO	Determines 100% of: cellular proliferation, alkaline phosphatase synthesis, and $\beta 1$ integrin subunit and ERK activation; *decreases bone resorption induced by PTH and vitamin D3; *induces: osteoblast precursors differentiation, 94% of OPG synthesis, osteocalcin synthesis, and NF- $\kappa$ B activation; *inhibits: osteoclastic activity, NF- $\kappa$ B activation, and 70-82% of RANKL synthesis; and *synthesizes cGMP.
iNOS	Increases PGE <sub>2</sub> synthesis; determines 90% of VEGF synthesis; and *synthesizes NO.
OPG	*Decreases osteoclasts recruitment; competes with RANK.
Osteopontin	*Constitutes bone ECM; *sets osteoclasts at resorption sites through $\alpha v \beta 3$ integrin binding; and *inhibits hydroxyapatite formation.
Osteocalcin	Captures calcium from the ECM; *inhibits ECM mineralization and hydroxyapatite formation.
P2X <sub>7</sub>	Increases: osteoblasts activity, 31% of the sensitivity to mechanical stimulation, and 50% of ERK activation; determines 61-73% of the osteogenesis rate; inhibits osteoclast activity; allows PGE <sub>2</sub> efflux and calcium influx.
P2Y <sub>2</sub>	Increases intracellular calcium concentration via G protein/PLC pathway; and allows calcium efflux.
p38	Determines 80% of osteopontin synthesis; and *phosphorylates C/EBP and CREB.
PDGF- $\beta$	*Induces: chondrocytes proliferation, osteoblast precursors differentiation and bone resorption.
PGE <sub>2</sub>	*Activates EP2/4 and protein G/AC pathway; induces osteogenesis; induces osteoclast precursors and osteoblast precursors proliferation and differentiation; induces calcium influx; inhibits collagen and RANKL synthesis*, GSK3b activity*, osteoclast activity and osteoblast proliferation; recruits osteoblasts to bone surface; and enhances gap junctions intercellular communication.
PGI <sub>2</sub>	*Activates protein G/AC pathway.
PI3K/ Akt	Determines 100% of cox-2 synthesis; inhibits apoptosis; *activates $\beta$ -catenin; and *inactivates GSK3b.
PKA	*Activates CREB and C/EBP $\beta$ ; *induces osteoblast differentiation and IGF-1 synthesis; and *phosphorylates calcium and potassium channels, prolonging their action.
PKC	*Activates MEK/ERK pathway; and *activates CREB and C/EBP $\beta$ .
PLA <sub>2</sub>	*Activates cox-1/2.
G protein	*Activates PLC/DAG and IP3/PKC pathway and AC/AMPC/ PKA pathway; *activates voltage-dependent L-type calcium channels, Ras and PI3K; and *determines 83% of PGE <sub>2</sub> synthesis.
PTH	*Activates PI3K/ Akt/ Bad, G protein/ AC and PKC/ERK pathways; *increases c-fos synthesis 2.5 fold, and IL-6 synthesis 11 fold; *decreases 90% of osteocalcin synthesis; and *induces RANK, RANKL, Runx2 and PGE <sub>2</sub> synthesis; and *induces bone resorption.

PROTEIN	EFFECTS RELATED TO MECHANOTRANSDUCTION
PYK2	Increases ERK-1/2 activation; and transphosphorylates FAK at Tyr-397, 576 and 925.
RANK	*Induces osteoclastogenesis and *inhibits osteoclast apoptosis; *RANKL activates RANK.
Runx2	*Decreases RANKL synthesis; and *induces alkaline phosphatase, ECM and OPG synthesis; *induces bone mineralization, and osteoblast precursors and osteoblasts differentiation.
Tcf/Lef	*Activates Runx2.
Vitamin D3	*Decreases OPG synthesis and bone resorption; and *induces RANK and RANKL synthesis.
VEGF	Increases alkaline phosphatase synthesis; and induces cellular and vascular proliferation*.
Wnt	*Activates $\beta$ -catenin, nuclear ERK and nuclear Akt; *increases osteopontin synthesis; and *inhibits glutamine synthetase (enzyme that degrades glutamate) and GSK3b.
*Data obtained from study not related to mechanotransduction.	

Table 3. Role of proteins

PROTEIN	PROPAGATION SPEED	EFFICIENCY
Cx43 gap junctions	0,5 $\mu\text{m/s}$	5-15 cells/field in 15-20 seconds (maintained for 3 hours, when the analysis ended)
P2Y <sub>2</sub>	10 $\mu\text{m/s}$	30-50 cells/field in 15-20 seconds

Table 4. Intercellular calcium transportation efficiency

1. Existence of NMDA channels in osteoblasts and osteoclasts.
2. Existence of mGluR4/8 in osteoblasts.
3. Mechanical stimulation decreases GLAST-1 expression.
4. NMDA channels absence decreases osteogenesis (without affecting osteoblasts life expectancy).
5. Calcium influx, which is critical to glutamate release from vesicles, increases markedly in response to mechanical loads.

Table 5. Features that support the memory system of bone existence

5. Mechanotransduction

In theory, mechanical deformation that reaches bone cells cytoplasmic membrane is transmitted to the nucleus through a complex network connecting the cytoplasmic membrane to the nucleus: the ECM/PEM-integrin-cytoskeleton-nucleus system. This system is supposed to activate various biochemical reactions that result in apoptosis inhibition, cellular proliferation, cell differentiation etc. This cascade of events, starting with mechanical deformation of bone cells, and ending with a cellular response (osteogenesis or bone resorption) is named mechanotransduction, which, we believe, is the upgrade of the mechanostat model (Tables 2 and 3).



### 5.1 The ECM/PEM-integrins-cytoskeleton-nucleus system

This system is thought to function as a lever system with various pivot points. The interactions between the molecules that participate in the ECM/PEM-integrins-cytoskeleton-nucleus system work as a pivot point of the lever system. The physicochemical characteristics of each pivot point can be altered accordingly to the molecules interacting within the system; therefore, leading to different responses to mechanical loads (Fig. 7) (Buckwalter et al., 1996a, 1996b; Duncan & Turner, 1995).

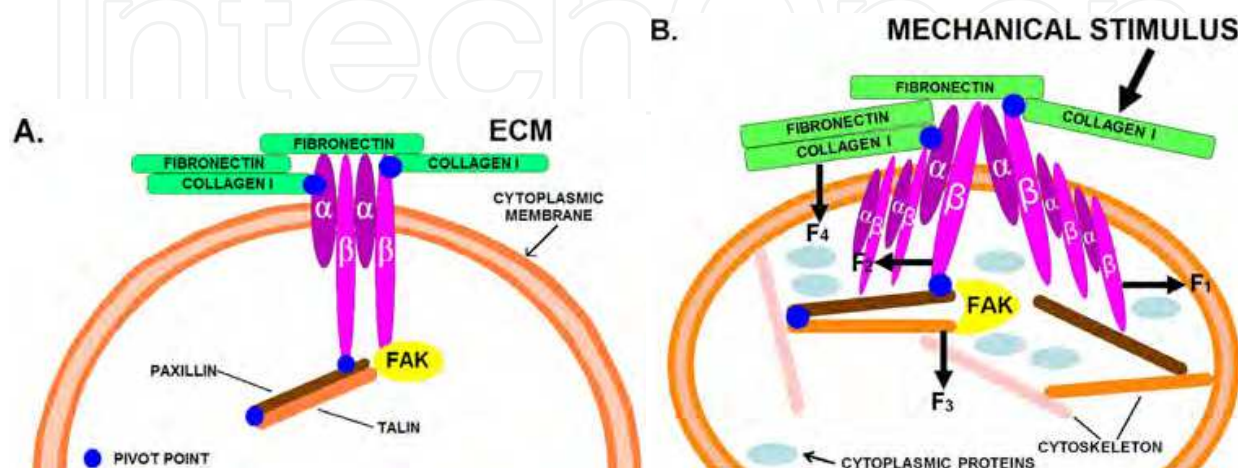


Fig. 7. Model for the lever system of bone cells. (A) Not stimulated bone cell. (B) The load is spread within the lever system via pivot points formed by interactions of the ECM/PEM-integrins-cytoskeleton-nucleus system, resulting in forces (schematically, F1, F2, F3 and F4) that modify integrins and FAK conformational structure. At the same time, integrins cluster, the cytoskeleton reorganizes, and cytoplasmic proteins are attracted to the focal adhesions, where the first mechanically-induced biochemical reactions occur.

#### 5.1.1 Integrins

Proteins interact with each component of the ECM/PEM-integrins-cytoskeleton-nucleus system. Within the constituents of the system, knowledge about integrins is greater. Integrins are considered the most important system component for mechanotransduction. Integrin refers to its role in integrating the intracellular environment (via cytoskeletal interactions) to the extracellular environment (ECM and PEM). Integrins are heterodimeric transmembrane glycoproteins of cellular adhesion. In humans, there are 24 types of integrins that result of the combination of the 18 types of  $\alpha$  subunits and 8 types of  $\beta$  subunits. Different combinations of  $\alpha$  and  $\beta$  subunits determine different interactions with ECM, cytoskeleton and cytoplasm components, as well as the affinity to a ligand. *In vitro*, osteoblasts express  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$  and  $\beta 5$  subunits; *in vivo*, they express  $\alpha 3$ ,  $\alpha 5$ ,  $\alpha v$ ,  $\beta 1$  and  $\beta 3$  subunits. Of these subunits,  $\alpha 2$ ,  $\alpha 5$ ,  $\beta 1$  and  $\beta 3$  subunits are responsive to mechanical load. Moreover,  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  integrins are also responsive to mechanical loads (Bennett et al., 2001; Gronthos et al., 1997; Sinha & Tuan, 1996).

#### 5.1.2 Focal adhesions

It is accepted that mechanical loading mobilizes the ECM/PEM-integrins-cytoskeleton-nucleus system, resulting in integrins structural changes that create high affinity sites for protein binding (for example, other integrins and cytoskeleton components). Mechanical

deformation induces integrin clustering, which enhances integrin affinity to other molecules, and increases integrins expression. Integrins clusters anchor the components of the cytoskeleton; induce cytoskeleton reorganization; and give rise to the focal adhesion (or focal contact), which is a dynamic and specialized structure originated from the response to mechanical stimulation, and vanishes in the absence of mechanical stimulation. Focal adhesion is located near the cytoplasmic membrane and the ECM, and recruits various molecules involved in the mechanotransduction: tyrosine kinases, ion channels, PLC, MAPK etc (Fig. 7) (Boutahar et al., 2004; van der Flier & Sonnenberg, 2001; Lee et al., 2000; Pommerenke et al., 2002; Scott et al., 2008; Tang et al., 2006; Yang et al., 2005).

## 5.2 Ion channels

Mechanosensitive ion channels activation by fluid flow alters membrane potential, which can be positive or negative. Positive membrane potential results from membrane depolarization – usually when sodium channels activation predominates –, leading to bone resorption. Negative membrane potential results from membrane hyperpolarization – usually when calcium-dependent potassium channels activation predominates –, leading to osteogenesis. Fluid flow speed, streaming potentials, and the magnitude and frequency of mechanical stimulation are variables that regulate ion channels activity. This feature allows distinct responses to mechanical loads because (1) multiple channels can be activated in different combinations, (2) ion channels can be activated for different intervals, (3) and ion channels can be fully or partially activated. At the moment, however, there is no tool to help predicting ion channels activity based on that variables (Butcher et al., 2008; Genetos et al., 2005; Qin et al., 2002; Riddle & Donahue, 2009; Salter et al., 1997, 2000; Scott et al., 2008; Yokota & Tanaka, 2005). Besides, there is limited information about ion channels on the subject due to lack of studies.

### 5.2.1 Mechanism of activation

Reports indicate that ion channels are not activated directly by strain. Instead, it seems they are activated through the ECM/PEM-integrins-cytoskeleton-nucleus system. As examples, it was found that  $\beta 1$  and  $\alpha v$  subunits of integrins,  $\alpha v \beta 5$  integrin and lack of the actin cytoskeleton impairs calcium-dependent potassium channels activation; and lack of  $\beta 1$  subunits of integrins and lack of the actin cytoskeleton impairs sodium channels activation (Desbois-Mouthon et al., 2001; Salter et al., 1997).

### 5.2.2 Calcium channels

Increased intracellular calcium concentration due to strain is crucial to enhance osteogenesis rate. Calcium intake depends on the activation of stretch-activated calcium channels, voltage-sensitive L-type calcium channels and endoplasmic reticulum  $IP_3$  receptors. Endoplasmic reticulum  $IP_3$  receptors are discussed to be the major responsible in determining intracellular calcium concentration. Therefore, it is speculated that an activator for the G protein/PLC/PIP2/DAG and  $IP_3$  pathway (for example, mGLUR, Wnt5, frizzled proteins), integrins, or G protein is activated by strain, resulting in  $IP_3$  synthesis, what leads to calcium release from endoplasmic reticulum and increases intracellular calcium concentration. Calcium forms complexes that activate AC, which, in turn, can phosphorylate voltage-sensitive L-type calcium channels and potassium channels through the G protein/AC/cAMP/PKA pathway. This event keeps these channels active for a longer time

and perpetuates bone cells response to mechanical loading through a positive feedback mechanism (Champe & Harvey, 2000; Chen et al., 2004; Duncan & Turner, 1995; Genetos et al., 2005; Godin et al., 2007; Lent, 2001; Li et al., 2003; Liedbert et al., 2006; Ma et al., 1999; Reich et al., 1997; Yamamoto et al., 2007) (Fig. 8).

### 5.2.3 Potassium channels

As seen in endothelial cells, in bone cells potassium channels can be activated directly by mechanical stimulation; or may also be activated by calcium influx through calcium-dependent potassium channels. Potassium channels activation leads to potassium efflux, resulting in membrane hyperpolarization (Duncan & Turner, 1995; Liedbert et al., 2006) (Fig. 8).

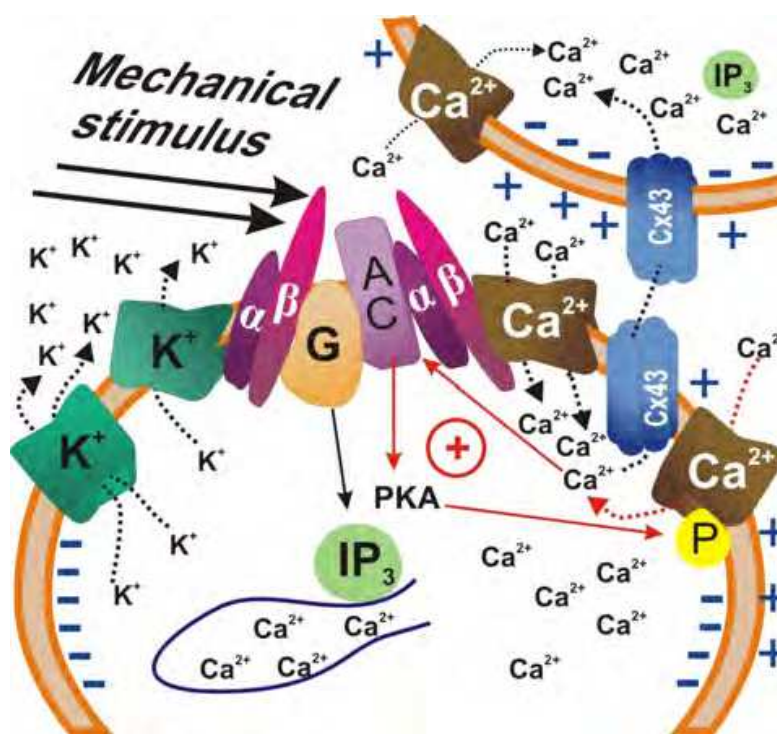


Fig. 8. *Ion channels activation.* Strain deforms the integrins, which may transmit the deformation to calcium ( $\text{Ca}^{2+}$ ) and potassium ( $\text{K}^+$ ) channels, producing calcium influx and potassium efflux, resulting in a negative action potential. At the same time, G protein (G) is activated by strain, and induces  $\text{IP}_3$  synthesis.  $\text{IP}_3$  is the main responsible for calcium influx. Calcium signaling pathways activate AC, resulting in PKA synthesis. PKA phosphorylates calcium and potassium channels (not showed) so that they remain active for longer, resulting in a positive feedback (arrows in red, and plus sign) that prolongs the cellular response to mechanical stimulation. Mechanical stimulation also activates Cx43 gap junctions, through which calcium,  $\text{IP}_3$  and other molecules transit and transmit mechanical stimulation to neighboring cells, which can also deflagrate a negative action potential.

### 5.2.4 Sodium channels

We believe sodium channels are inhibited by strain at the beginning of the mechanical stimulation, probably by cGMP or another inhibitor; and activated posteriorly so that the intracellular homeostasis is restored (Duncan & Turner, 1995; Liedbert et al., 2006).



### 5.3 FAK pathways

Within all molecules that interact with integrins in focal adhesions, FAK is considered to be critical to mechanotransduction. FAK is an adaptor protein capable of interacting with various proteins to form different protein complexes. This feature may enable FAK to enhance the cellular response to mechanical loading, and may result in different responses to strain. It is not known whether this tyrosine kinase is always bound to integrins, or is recruited to the integrins clusters during focal adhesions formation, or both situations.

Following integrins deformation, FAK may be deformed and its molecular structure mechanically-modified, resulting in autophosphorylation of FAK's tyrosine-397 (Tyr-397) residue. This event activates FAK by creating high affinity binding sites for SH2-domain-containing proteins like Src and p85 subunit of PI3K. In sequence, FAK conjugates with Src or PI3K, activating their pathways. ERK-1/2 and Akt are effector proteins of these pathways. FAK, ERK-1/2 and Akt promote cellular migration, proliferation and differentiation, and apoptosis inhibition. It is speculated that these actions are enhanced when FAK, ERK-1/2 or Akt migrates to the nucleus. Investigations suggest that FAK, ERK-1/2 and Akt migration to the nucleus is regulated by the cytoplasmic concentration of these proteins and the concentration of their phosphorylated forms (Chen et al., 1999; Cornillon et al., 2003; Desbois-Mouthon et al., 2001; Duncan & Turner, 1995; Giancotti & Rouslahti, 1999;

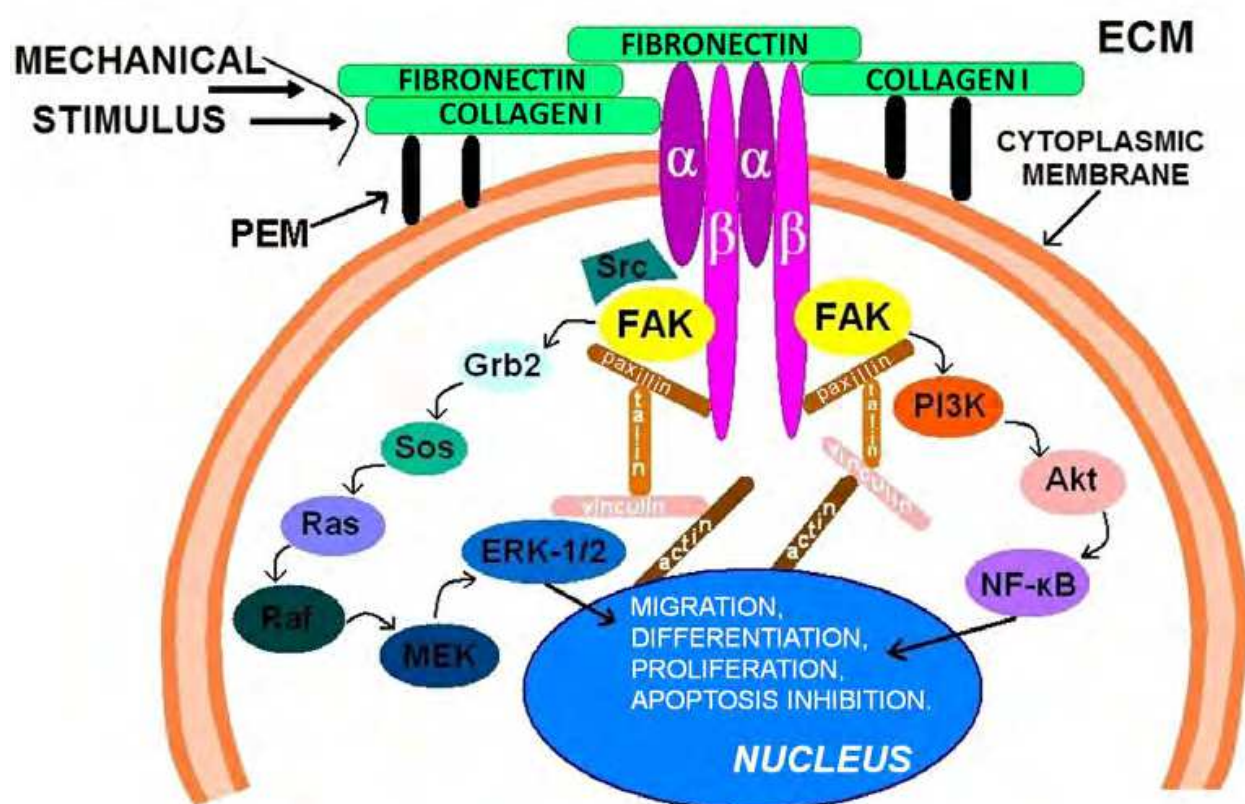


Fig. 9. *FAK signaling pathways*. FAK interacts with integrins and cytoskeleton components (actin, paxillin, talin and vinculin) – which also interact with the nucleus – and signal cellular mechanical deformation.

Hughes-Fulford, 2004; Kawamura et al., 2007; Liedbert et al., 2006; Mitra et al., 2005; Ogasawara et al., 2001; Raucci et al., 2008; Schlaepfer et al., 1998; Tang et al., 2006) (Fig. 7 and Fig. 9).

### 5.3.1 Ion channels

Curiously, mechanical loads induce FAK ligation to calcium-dependent potassium channels without FAK autophosphorylation at Tyr-397. However, the effect of this event to the cellular response to strain is not known yet. For example, it is not known whether the potassium channel activity is altered by that ligation (Rezzonico et al., 2003).

### 5.3.2 PYK2

This protein is highly homologous to FAK. Through an unknown mechanism, strain induces a calcium-dependent phosphorylation of PYK2 at Tyr-402, activating this protein. *In vitro*, PYK2 couples with FAK via SH2 and can transphosphorylate FAK at Tyr-397, Tyr-576 or Tyr-925. In opposite, FAK does not transphosphorylate PYK2. This indicates that PYK2 can enhance FAK tyrosine kinase activity because FAK phosphorylation at Tyr-397 gives rise to high affinity binding sites for SH2-containing-domain proteins. Supporting this hypothesis, it was found that PYK2 augments ERK-2 phosphorylation at Tyr-187 depending on PLC and calcium channels activity. Besides, PYK2 couples with Src like FAK, but this ligation does not depend on mechanical stimulation (Boutahar et al., 2004; Li et al., 1999; Liu et al., 2008).

### 5.3.3 p38

The activity of this MAPK increases with mechanical loads through an unknown mechanism. Within the pathways that result in p38 activation, FAK-Src/Grb2/Sos/Ras/PAK/MKK3/6 pathway may be deflagrated by strain because FAK is a mechanosensitive protein involved in this pathway. We found no study aiming to solve this issue (J. You et al., 2001).

## 5.4 Wnt pathways

Wnt is a family of 19 glycoproteins responsible for 88-99% of the mechanically-induced osteogenesis. Wnt1 class activates the canonical Wnt signaling pathway, in which complexes between Wnt1/3a, LRP5/6 and frizzled proteins are formed. Such complexes phosphorylate GSK3b, causing its inactivation; and hence, inhibit  $\beta$ -catenin degradation. Beta-catenin translocates to the nucleus where it accumulates, leading to Tcf/Lef transcription factor activation. Wnt also inhibits apoptosis via nuclear ERK and Akt activation. Wnt5a class participates in the non-canonical Wnt signaling pathway, and binds to frizzled proteins, leading to PLC activation via G proteins (Fig. 10).

There is exiguous information about Wnt pathways' relation to mechanotransduction. At the moment, it is known that (1) strain increases  $\beta$ -catenin activation and translocation to the nucleus, probably via PI3K activation, or decreasing sclerostin expression, which is an inhibitor of the canonical Wnt signaling pathway; (2) strain increases Wnt1, Wnt3a, Wnt5a and Lrp5 receptor expression; (3) and Lrp5 receptor hyperexpression increases the cellular response to mechanical loading, and decreases the strain needed to stimulate bone cells response to mechanical stimulation (Bonewals & Johnson, 2008; Johnson, 2004; Lau et al., 2006; Olkku & Mahonen, 2008; Robling et al., 2008; Turner, 2006; Yavropoulou & Yovos, 2007).



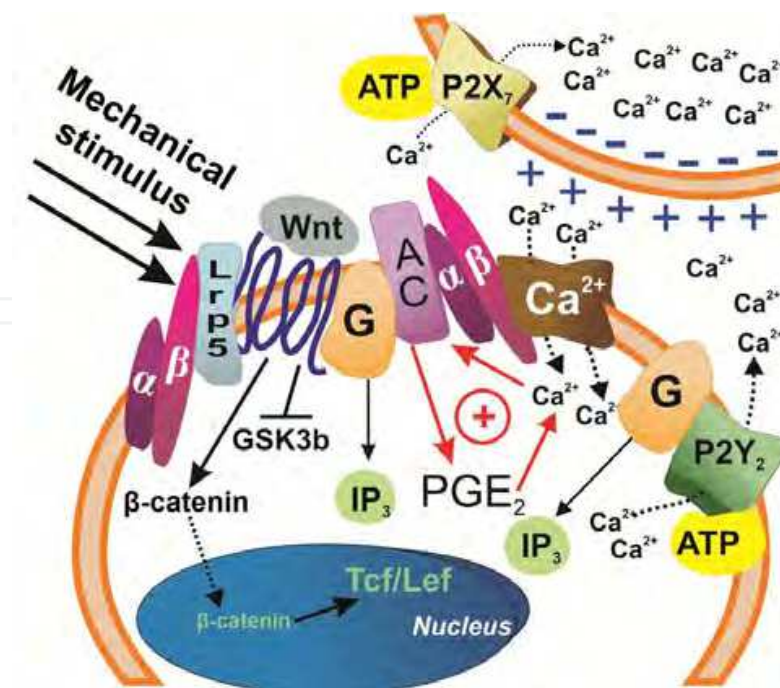


Fig. 10. Not well known models of mechanical stimulation signaling. Wnt complexes with the Lrp5 receptor and frizzled proteins (in blue), inhibiting GSK3b and activating Tcf/Lef (via  $\beta$ -catenin). The ATP produced after mechanical stimulation binds to its receptors (P2X<sub>7</sub> and P2Y<sub>2</sub>), inside the cell where ATP was produced; or in the neighboring cells, when secreted by the stimulated cell (not showed). P2X<sub>7</sub> activation in a neighboring cell, alike Cx43 gap junctions, provokes calcium influx, which may result in a negative action potential that transmits strain biochemically. P2Y<sub>2</sub> and frizzled proteins stimulate IP<sub>3</sub> synthesis via G protein, increasing calcium concentration (not showed). PGE<sub>2</sub> produced in response to mechanical stimulation induces calcium influx, which activates AC that stimulates PGE<sub>2</sub> synthesis via a positive feedback mechanism (red arrows and plus sign). PGE<sub>2</sub> migrates to other cells through gap junctions or P2X<sub>7</sub>.

## 5.5 Effector pathways

The signaling pathways activated by strain are part of bone mechanostat. Some products of these pathways are important to osteogenesis and are described below.

### 5.5.1 c-fos

Mechanical loads increase c-fos expression, which is a transcription factor that binds to c-jun, another transcription factor, forming the AP-1 heterodimer that induces osteogenesis (Judex et al., 2005; Mullender et al., 2004; Nomura & Takano-Yamamoto, 2000; Sikavitsas et al., 2001).

### 5.5.2 cox-1/2

Within cox isoforms, cox-2 (inducible cox) responds to mechanical stimulation with an increase in the amount of cox-2 mRNA. This protein participates in some mechanotransduction signaling pathways, and acts in the cytoplasm and nucleus of bone cells (Choudhary et al., 2008; Kapur et al., 2003; Ogasawara et al., 2001; Rouzer & Marnett, 2005; Tang et al., 2006).

### 5.5.3 Prostaglandins

The prostaglandins PGI<sub>2</sub>, PGF<sub>2α</sub> and PGE<sub>2</sub> are implicated in mechanically-induced osteogenesis. PGE<sub>2</sub> is responsible for 50-90% of the mechanically-induced osteogenesis; therefore, is pointed to be the most important prostaglandin in mechanotransduction. Besides, PGE<sub>2</sub> may be one of the regulators of the mechanostat because (1) its synthesis can increase via positive feedback (Fig. 10); (2) PGE<sub>2</sub> is transmitted to other cells through gap junctions, indicating that PGE<sub>2</sub> is involved in the intercellular transmission of mechanical stimulation; (3) and PGE<sub>2</sub> stimulates both osteogenesis and bone resorption. The underlying mechanism through which PGE<sub>2</sub> stimulates osteogenesis and bone resorption may be its intracellular concentration; however, this is a hypothesis. It is also possible that calcium concentration determines, indirectly, the cellular response to mechanical stimulation (osteogenesis or bone resorption) because this ion is responsible for 83% of PGE<sub>2</sub> synthesis. Additionally, the anabolic effects of PGE<sub>2</sub> are mediated by EP2 prostaglandin receptor and, mainly, by EP4 prostaglandin receptor, both located at the nuclear membrane (Cherian et al., 2005; Fortier et al., 2001; Genetos et al., 2005; Ke et al., 2003; Keila et al., 2001; Klein-Nulend et al., 1997; Li et al., 2005; Machwate et al., 2001; Mullender et al., 2004; Nomura & Takano-Yamamoto, 2000; Reich et al., 1997; Watanuki et al., 2002; Xu et al., 2007).

### 5.5.4 NO

Through an unknown mechanism, NO stimulates both osteogenesis and bone resorption. It participates in some osteogenesis pathways, and can decrease 50% of PTH-induced bone resorption and 68% of vitamin D-induced bone resorption (Fan et al., 2004; Kapur et al., 2003; Wang et al., 2004).

### 5.5.5 PDGF-β

It is described that PDGF-β stimulates chondrocytes proliferation, osteogenic cells differentiation into osteoblasts and bone resorption. The gene for PDGF-β has a shear stress response element (SSRE) region, which is present in the genes of other mechanosensitive proteins: cox-2, osteopontin, iNOS. For this reason, some authors believe PDGF-β has a role in the effector response to mechanical stimulation, although there is no report of PDGF-β involvement with mechanically-induced osteogenesis (Luo & Wang, 2004; Nomura & Takano-Yamamoto, 2000; Sikavitsas et al., 2001).

## 5.6 Biochemical transmission of mechanical stimulation

Each bone cell is subjected to different mechanical deformation intensities during one mechanical stimulation. But to produce significant bone mass, a great amount of bone cells may work together in response to mechanical stimulation. Besides, osteoblast precursor cells must be activated and migrate to the osteogenesis site; and osteoclasts must be inactive at the osteogenesis site. In order to these events occur bone cells are supposed to have means of intercellular communication.

### 5.6.1 Ion channels

Mechanical deformation activates ion channels (most importantly, calcium and potassium channels), generating a negative membrane potential. As the example of neuronal synapses, the negative membrane potential may be transmitted through the neighboring cells, which, in turn, activate biochemically their mechanotransduction pathways.

### 5.6.2 Cadherins

These single-chain transmembrane glycoproteins are expressed in the junction adherens of bone cells and link the cytoskeleton of adjacent cells through interactions between the extracellular segments of each cadherin. Like integrins, cadherins expression increases in response to strain. Both integrins and cadherins seem to have similar function: the first transmit the mechanical stimulation inside the deformed cell, and the latter transmit the mechanical stimulation to the adjacent cell (Pavalko et al., 2003).

### 5.6.3 Gap junctions

These specialized intercellular connections are pointed as the major contributor to intercellular transmission. Following mechanical stimulation, gap junctions allow the exchange of PGE<sub>2</sub> and other molecules smaller than 1 kDa (for example, calcium, IP<sub>3</sub> and cAMP) between adjacent cells (for example, osteocytes and periosteal cells). Each gap junction is composed of 2 hemichannels (each hemichannel belongs to each of the two adjacent cells). Each hemichannel allows communication between the cell and its ECM, and is composed of 6 connexins. Within the various types of connexins, Cx43 is the main constituent of the hemichannel, and is involved in the transmission of mechanical stimuli. In addition, mechanical loads increase Cx43 expression and phosphorylation, enhancing gap junctions intercellular communication; 60-100% of PGE<sub>2</sub> transmission to adjacent cells depends on Cx43; and calcium transmission between adjacent cells can occur via gap junctions (Cherian et al., 2005; Genetos et al., 2005; Miyauchi et al., 2006; Siller-Jackson et al., 2008; Stains & Civitelli, 2005; Yang et al., 2005) (Fig. 8).

### 5.6.4 ATP receptors

ATP binds to its transmembrane receptors P2X<sub>7</sub> and P2Y<sub>2</sub>. The first receptor is a non-selective ion channel that complexes with various proteins like  $\beta$ 2 subunits of integrins and  $\alpha$ -actinin, suggesting the involvement of this receptor in mechanotransduction. P2X<sub>7</sub> receptor activation is responsible for about 61-73% of the mechanically-induced bone formation rate; increases bone tissue mechanosensitivity by 31%; and is responsible for 50% of the mechanically-induced ERK-1/2 activation. P2Y<sub>2</sub> receptor seems to activate G protein/PLC/PIP<sub>2</sub>/IP<sub>3</sub> pathway, which increases calcium intake; and promotes calcium efflux after mechanical loads, more efficiently than gap junctions (Table 4). On the other hand, P2Y<sub>2</sub> is not important for ERK-1/2 activation, and there are no reports showing P2Y<sub>2</sub> activation directly by strain (Cherian et al., 2005; Genetos et al., 2005; Jorgensen et al., 1997; Ke et al., 2003; Li et al., 2005; Liu et al., 2008; Reich et al., 1995) (Fig. 10).

## 5.7 Memory system of bone

Some reports suggest that bone cells possess a memory system that prolongs the cellular response to mechanical stimulation. For example, it was documented *in vitro* that mechanically-induced calcium influx was greater when bone cells were subjected to a second load, 30 minutes after the first load. The underlying mechanism of this memory system is unknown. The positive feedbacks of PGE<sub>2</sub> production and calcium influx (described above) may be part of the underlying mechanism because they are positive feedbacks. In memory cells of the central nervous system, the action potential can be prolonged by glutamate activation of voltage-dependent calcium channels (NMDA-type

channels). This is called long-term potentiation. In those cells, it is described that following voltage-dependent calcium channels opening, calcium enters the neuron and binds to glutamate-containing vesicles releasing glutamate to the extracellular environment where glutamate binds to NMDA-type channels and mGluR. The activation of potassium channels and some classes of mGluR inhibit voltage-dependent calcium channels activation, blocking glutamate release. Glutamate actions end when GLAST (also called EAAT) takes glutamate from the extracellular environment and returns it back to the intracellular vesicles.

Based on the long-term potentiation mechanism, we believe there are five conditions in bone that makes possible the existence of a memory system in this tissue (Table 5). In addition, GLAST stops working when the extracellular concentration of sodium is low and the extracellular concentration of potassium is high. This situation is consistent with the available data: when extracellular potassium concentration is high, the cytoplasmic membrane hyperpolarizes (the calcium-dependent potassium channels open and the sodium channels close), leading to osteogenesis. Unfortunately, there are no reports about the NMDA channels and metabotropic receptors activation in bone cells subjected to mechanical loads (Godin et al., 2007; Hinoi et al., 2003; Lent, 2001; Mason, 2004; Nomura & Takano-Yamamoto, 2000; Spencer et al., 2007; Taylor, 2002).

## **5.8 Hormones and mechanotransduction**

Some hormones and growth factors like PTH and IGF-1 have synergistic effect with mechanical loads to induce osteogenesis. The underlying mechanism that produces the synergistic effect is unknown. On the other hand, it is known that such hormones and growth factors, and mechanical loads activate ERK, which is hyperactivated when double phosphorylated, resulting in ERK migration to the nucleus where ERK's action is potentialized. Therefore, we hypothesize that the stimuli from mechanical loads and biological factors hyperphosphorylate ERK, leading to a greater osteogenesis rate (Ebisuya et al., 2005; Yee et al., 2008).

### **5.8.1 PTH**

Increases 1.53-6 fold bone formation rate when associated with mechanical loads. The synergistic effect of PTH and mechanical loading depends 68-74% on voltage-dependent L-type calcium channels activation, probably because the synergistic effect of PTH may occur via PYK2-FAK/ERK, which is a calcium-dependent signaling pathway involved in mechanotransduction. Curiously in the presence of PTH, NO production augmentation is abolished even in the presence of mechanical stimulation (the study evaluated NO concentration until 30 minutes after mechanical loading) (Bakker et al., 2003; Chen et al., 2004; Choudhary et al., 2008; Fan et al., 2004; Ma et al., 1999; Ogasawara et al., 2001; Yamamoto et al., 2007).

### **5.8.2 IGF-1**

When associated with mechanical loading, cellular proliferation increases 1.8-3.8 fold, IGF-1R phosphorylation increases 7.5 fold, and ERK activation increases 2-9.5 fold. IGF-1 activity depends totally on the presence of integrins, and Akt activation rate is not altered by mechanical stimulation in the presence of IGF-1 (Desbois-Mouthon et al., 2001; Kapur et al., 2005; Nomura & Takano-Yamamoto, 2000; Sakata et al., 2004; Sekimoto et al., 2005; Sikavitsas et al., 2001).



### 5.8.3 Estrogen

It was not found any report about the synergistic effect of estrogen and mechanical loading. On the other hand, it is documented that ER- $\alpha$  absence decreases 70% of the mechanically-induced bone formation rate, and inhibits 100% of the mechanically-induced cellular proliferation (Lee et al., 2003).

### 5.9 Mechanotransduction *in vivo*

The majority of the reports about mechanically-induced osteogenesis are based on *in vitro* experimental models, probably because it is difficult to obtain bone cells and their proteins after *in vivo* experimental procedures. The beneficial effect of LIPUS stimulation (mechanical stimulation) has already been documented in lesioned bones, which exhibited early consolidation<sup>70</sup>. We developed an *in vivo* experimental model to assess the effect of the mechanical stimulation on bone proteins of intact tibia and fibula of rats. LIPUS was used to subject the animals to a daily 20-minute treatment for 7, 14 or 21 days. At the end of the treatment, we evaluated the expression and activation of FAK, ERK-1/2 and IRS-1 by immunoblotting assays. It was found that FAK, ERK-1/2 and IRS-1 expression increased in a non-cumulative manner indicating that the mechanostat blocks LIPUS osteogenic effects after a period of continuous stimulation. Increased FAK and ERK-1/2 activation was detected 15 hours after the last LIPUS stimulation at seven days of treatment, supporting the theory of the memory system of bone.

Additionally, LIPUS increased IRS-1 expression and activation (data not published) after one week of treatment. Since IRS-1 is involved in growth factors signaling pathways, those results suggest mechanical stimulation also acts in signaling pathways activated by growth factors. This hypothesis may serve as an explanation for the synergistic effect of some hormones with mechanical loading. There are few investigations on this issue *in vivo* and *in vitro*.

The sham LIPUS stimulation (device turned off) increased FAK activation after one week of treatment, indicating the muscle contraction of the stressed animals also possesses osteogenic effects, possibly because of the vibratory load on bone, which is a mechanical load of high frequency and low magnitude; or because of a paracrine stimulus from muscle to bone cells (Gusmão et al., 2007, 2010; Naruse et al., 2003; Warden et al., 2001).

## 6. Future directions

We consider that bone is not merely a structure for locomotion. It is a complex organ that also responds to mechanical stimulation. The underlying mechanism of mechanically-induced osteogenesis is not fully understood, and many questions need to be answered. Therefore, further studies shall investigate how bone cells distinguish physiological from overuse deformations; the regulatory activity of osteocytes on other bone cells; the mechanically-induced activity of ion channels, intercellular communication of bone cells and the memory system of bone; the physicochemical properties of the ECM/PEM-integrins-cytoskeleton-nucleus system components; the events that trigger FAK, ERK and Akt mechanically-induced migration to the nucleus; the synergistic effect of growth factors and hormones with mechanical loads; and so on.

In addition, the current data about mechanically-induced osteogenesis are not linked. For example, a little is known about the activation of FAK pathways, integrins, and ion channels by mechanical loading; however, we do not know how they work together, how their



combined activity influences bone response to mechanical stimulation, and how that bone response to mechanical stimulation macroscopically affects the skeleton. Therefore, we believe that studies shall be carried out to understand the events triggered by mechanical loading as a whole, because bone functions as a whole as well as the human body.

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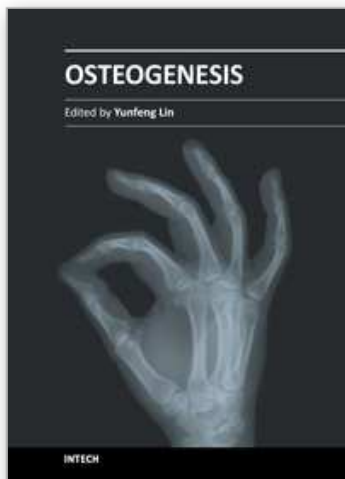
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This book provides an in-depth overview of current knowledge about Osteogenesis, including molecular mechanisms, transcriptional regulators, scaffolds, cell biology, mechanical stimuli, vascularization and osteogenesis related diseases. Hopefully, the publication of this book will help researchers in this field to decide where to focus their future efforts, and provide an overview for surgeons and clinicians who wish to be directed in the developments related to this fascinating subject.

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