Hormone Relaxin as Biomarker for Bone Health and Disease

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Abstract

Bone homeostasis is maintained by fine-tuning of the dynamic balance between bone resorption via osteoclasts and bone formation via osteoblasts. Bone metabolism-related biomarkers such as a soluble factor or type I collagen

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metabolism product specifically secreted by osteoblasts or osteoclasts are useful for evaluating the change in bone metabolism in a noninvasive manner in real time. Monitoring of bone metabolism-related biomarkers that are excreted in the urine or secreted into the bloodstream is quite useful for the diagnosis of various kinds of skeletal metabolism abnormalities. For example, an elevated level of a bone metabolism marker is a risk factor of bone fracture independent of bone density, as well as for bone density loss in the future. Relaxin (RLN) is a pleiotropic hormone of the insulin-like peptide hormone family, which is mainly secreted into the bloodstream from the ovary, uterus, and placenta during pregnancy. Therefore, RLN helps labor to progress by softening and widening the pubic symphysis and cervix, owing to its ability of remodeling the extracellular matrix by degrading collagen. The physiological roles of RLNs and relaxin family peptides through their receptors, relaxin family peptide receptors (RXFPs), in the reproductive system have been extensively studied. However, recent studies have shown that RLNs/RXFPs also play a key role in the cardiovascular system, renal function, organ protection, metabolism, cancer metastasis, and the central nervous system. The effectiveness of RLN for the treatment of acute heart failure is now assessed under phase III clinical trials. In addition to these broad physiological activities, its role in bone metabolism was also recently highlighted because of its ability to induce osteoclastogenesis, activate osteoclast function, and enhance osteoblast differentiation in vitro. In addition, the majority of men with *RXFP2* mutations presented with symptoms of osteoporosis, and *Rxfp2*-deficient mice showed a lower bone mass and reduced osteoclast surface compared to their wild-type littermates. This chapter provides an overview of the biological functions of RLN and its receptors (RXFPs), with particular focus on bone metabolism. In addition, the utility and possibility of RLNs/RXFPs as biomarkers for bone health and disease are discussed.

Keywords

Bone • Relaxin • Relaxin family peptide receptor • Osteoblast • Osteoclast • Bone remodeling • Collagen

List of Abbreviations

ALP	Alkaline phosphatase
BAP	Bone-specific alkaline phosphatase
BCE	Bone collagen equivalents
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BSP	Bone sialoprotein
BTM	Bone turnover marker
cAMP	Cyclic adenosine monophosphate
c-FMS	Colony-stimulating factor 1 receptor
CLIA	Chemiluminescence immunoassay
COL1α1	Collagen type I alpha 1
CREA	Urinary creatinine
CTX	Carboxy-terminal cross-linking telopeptide of type 1 collagen

DPD	Deoxypyridinoline
DXA	Dual-energy X-ray absorptiometry
ECLIA	Electrochemiluminescence immunoassay
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
ICTP	Carboxy-terminal cross-linking telopeptide of type 1 collagen generated by MMPs
IGF-1	Insulin-like growth factor-1
INSL	Insulin-like peptide
M-CSF	Macrophage colony-stimulating factor
MMPs	Matrix metalloproteinase
mRNA	Messenger RNA
NFATc1	Nuclear factor of activated T-cells cytoplasmic 1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
N-MID	Amino-terminal mid-fragment
NO	Nitric oxide
NTX	Amino-terminal cross-linking telopeptide of type 1 collagen
OC	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PBMC	Peripheral blood monocyte cells
PDL	Periodontal ligament
PICP	Carboxy-terminal propeptide of type 1 collagen
PINP	Amino-terminal propeptide of type 1 collagen
PTH	Parathyroid hormone
PYD	Pyridinoline
RANK	Receptor activator of NF-kB
RANKL	Receptor activator of NF-kappaB ligand
RIA	Radioimmunoassay
RLN (Rln)	Relaxin
RUNX2	Runt-related transcription factor 2
RXFP (Rxfp)	Relaxin family peptide receptor
sRANKL	Soluble RANKL
TGF-β	Transforming growth factor-β
TRAP	Tartrate-resistant acid phosphatase
TRAP5b	Tartrate-resistant acid phosphatase type 5b

Key Facts of Relaxin

- It is reported that relaxin is associated with increased knee joint laxity in pregnant women toward the end of the pregnancy period (Blecher and Richmond 1998).
- Phase III clinical trials have shown that recombinant human relaxin-2 (serelaxin) can be safely administered and has a promising pharmacological effect on patients with acute heart failure (Ponikowski et al. 2014; Teerlink et al. 2013).

- *Rxfp1* and *Rxfp2* mRNAs were expressed in developing mouse embryonic facial tissues, including Meckel's cartilage, the tongue, and tooth primordia (see Fig. 6) (Duarte et al. 2014a).
- RNL did not have any significant effects on human orthodontic tooth movement, and there was short-term relapse presumably because of systemic administration of this hormone, resulting in low concentrations in the local periodontal tissues. On the other hand, RLN reportedly prevents relapse after orthodontic tooth movement in rats (Hirate et al. 2012).
- RLN3 and RXFP3 control metabolism in humans via regulation of appetite, food intake, and body weight. A previous study revealed higher RLN3 levels in the serum of female patients with metabolic syndrome compared to those in the controls; thus, this hormone might be a candidate biomarker of metabolic disorder syndrome (Ghattas et al. 2013).

Definition of Words and Terms

Bone modeling	Bone modeling is the process of shaping bones during the developing stage and growing period. It changes the size and morphology of bones. In many instances, bone resorption and bone formation can take place independently.
Bone remodeling	Bone remodeling is the substitutive system of bone matrix, which involves removal of old bone matrix via osteoclast and new bone formation via osteoblasts. The bone remodeling process can be classified into five stages: (1) activation, (2) resorption, (3) reversal, (4) formation, and (5) termination. Osteoclasts migrate, become activated, and resorb on the surface of the bone that is covered with the bone-lining osteoblasts. Then, osteoblast precursor cells migrate to the resorption site, differentiate, and perform active bone matrix synthesis. The borderlines between old bone and new bone are called cement lines.
Osteoblast	Osteoblasts are derived from mesenchymal stem cells as a result of the action of multiple cytokines including BMPs, TGF-β, PHT, FGFs, IGFs, Wnts, and hedgehogs. Osteoblasts express specific markers depending on the cell differentiation stage. Runx 2 is a transcription factor, which is indispensable for osteoblast differentiation. Osteoblasts play an important role in extracellular matrix deposition, matrix mineralization, and osteoclast differentiation.
Osteoclast	Osteoclasts, a member of the monocyte/macrophage family, are the only cells that control mineralized bone resorption.

	The differentiation and the functions are strictly controlled by M-CSF, OPG, and RANK/RANKL signaling. Disorder in osteoclast function results in bone metabolic disorders, such as osteopetrosis, Paget's disease of bone, and osteoporosis.
Relaxin	Relaxin (RLN) is an insulin-like hormone that was first described as a factor that facilitates parturition by softening and lengthening the pubic symphysis and softening the cervix. This hormone is released in the bloodstream from the corpus luteum of the ovary, breast, placenta, and decidua in pregnant/ nonpregnant females and from prostate in males. RLN is known as a mediator of the hemodynamic changes during pregnancy. Recombinant human RLN2 is considered to have a promising pharmacological therapeutic effect on patients with acute heart failure.

Introduction

Bone is a supporting tissue and an organ of locomotion particular to vertebrates and also plays a role as a storage organ for several bioactive substances required for the living body, including differentiation or growth factors (e.g., TGF-β or IGF-1) (Tang et al. 2009; Xian et al. 2012) inside the bone matrix as well as minerals such as calcium or phosphorus. In addition, immune system cells and hematopoietic cells that control the biological defense mechanism are contained in the bone medullary cavity. Bone homeostasis is maintained by fine-tuning of the dynamic balance between bone resorption via osteoclasts and bone formation via osteoblasts. With regard to mammalian bone, bone formation is maintained by "modeling" and "remodeling" phenomena (Parfitt 1984). Bone modeling mainly occurs during periods of growth and is a mechanism of bone formation at the new site. On the other hand, bone remodeling is a reconstructive phenomenon involving the cylindrically structured basic multicellular unit (BMU) (Frost and Straatsma 1964; Matsuo and Irie 2008) called an "osteon" in the cortical bones or a "packet" in the cancellous bones. The strength and flexibility of the bone are maintained by bone remodeling; however, disruption of the balance of remodeling results in bone mass abnormality and the consequent development of various bone-related diseases. Bone remodeling is initiated by activation of the osteoclasts (Hattner et al. 1965; Martin and Rodan 2001; Parfitt 1984; Takahashi et al. 1964) and followed by the phases of resorption, reversal, and bone formation, which is characterized by collagenous matrix production and mineralization via osteoblasts (Raggatt and Partridge 2010), and then the termination phase. A series of processes from the activation to termination stage are repeated within approximately 3 months (Rosen et al. 2009). The

soluble molecules that are specifically secreted from osteoblasts or osteoclasts and the metabolic products of type I collagen are generally used as biomarkers of bone metabolism in clinical examinations (Table 1). Bone remodeling biomarkers in the blood or urine reflect the momentary sum of systemic bone resorption and formation progressing at the BMU. There are currently two major known groups of biochemical bone turnover markers, namely, bone formation markers and bone resorption markers. Bone formation is assessed with bone-specific alkaline phosphatase (BAP) (Ahmed and Gibbons 2015) and osteocalcin (OC), which are secreted by the osteoblasts at the early or late stage of differentiation, respectively. In addition, N- and C-terminal type I propeptides (PINP and PICP) (Pagani et al. 2005) reflect the activity of osteoblasts at the early differentiation stage. On the other hand, bone resorption is assessed by N- and C-terminal cross-linking telopeptides of type I collagen (NTX-I and CTX-I) (Pagani et al. 2005) generated by cathepsin K and C-terminal cross-linking telopeptides of type I collagen generated by metalloproteinases (MMPs; CTX-MMP or ICTP), which is a bone metastatic marker (Pagani et al. 2005). Moreover, the concentrations of deoxypyridinoline (DPD) in the urine and isoform 5b of tartrate-resistant acid phosphatase (TRAP5b), specifically secreted from osteoclasts into the bloodstream, are the bone resorption markers associated with increased fracture risk (Ivaska et al. 2010). The bone turnover markers (BTMs) provide a useful assessment of a clinical condition caused by abnormal bone metabolism such as osteoporosis and bone metastases. In addition, BTMs are a helpful index for the prediction of fragility fractures, response evaluations, and establishing an appropriate dose of anti-osteoporotic treatment (e.g., determining the metabolic effects and anti-fracture efficacy), which could improve compliance to treatment for osteoporosis.

Relaxin (RLN) is an insulin-like peptide hormone that is well known to facilitate parturition by inducing the softening and lengthening of the pubic symphysis and softening of the cervix (Hisaw 1926; Lu et al. 2005). RLN belongs to the relaxin family of peptides; among members of this family, RLN, insulin-like peptide 3 (INSL3), RLN3, and INSL5 interact with relaxin family peptide receptors (RXFPs) 1–4, respectively (Bathgate et al. 2005, 2006, 2013). Humans and apes possess the *RLN1–RLN3* and *RLN3–RLN6* genes, which encode H1–H3 relaxins and INSL3–INSL6 proteins, respectively (Bathgate et al. 2005). On the other hand, mice possess only *Rln2*, *Rln3*, *Insl3*, *Ins5*, and *Ins6* (Bathgate et al. 2005). Human RLN1 and RLN2, as well as RLN2 in other mammals, are commonly referred to as "relaxin" (Bathgate et al. 2005). RLN exerts a variety of effects in different types of cells. RLN blood levels are highest in the first trimester of pregnancy for the initiation of cardiovascular changes, although RLN is also produced in both males and females to exert broad physiological roles in paracrine- or autocrine-regulated mechanisms (Bathgate et al. 2006, 2013; Fig. 1). In particular, RLN inhibits fibroblast proliferation and differentiation (Samuel et al. 2004) but increases the production of matrix MMP1, MMP2, MMP9, and MMP13 (Ahmad et al. 2012;

Table 1 Biomarkers of bone turnover and reference intervals of each assay (Data from Jung and Lein (2014) with permission from the publisher)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
BAP	Serum plasma	Access ostase/ CLIA, automated	Beckman Coulter, Brea, CA, USA	Women, premenopausal, 3.2–18.8 µg/L Women, postmenopausal, 5.3–22.7 µg/L Men, 5.0–22.8 µg/L
		IDS-iSYS Ostase BAP/CLIA, automated Ostase BAP EIA/ELISA	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 6.0–22.7 µg/L Women, postmenopausal, 8.1–31.6 µg/L Men (>45 years), 7.5–26.4 µg/L as access ostase assay
		MicroVue BAP/ELISA	Quidel Corp., San Diego, CA, USA	Women, premenopausal, 11.6–29.6 U/L Women, postmenopausal, 14.2–42.7 U/L Men, 15.0–41.3 U/L
OC	Serum plasma	N-MID osteocalcin/ ECLIA, automated	Roche Diagnostics, Mannheim, Germany	Women, premenopausal, 11–43 µg/L Women, postmenopausal, 15–46 µg/L Men (>50 years),14–46 µg/L
		N-Mid Osteocalcin/ ELISAIDS-iSYSN-MID Osteocalcin/ CLIA, automated	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 12.8–55.0 µg/L Women, postmenopausal, 8.4–33.9 µg/L Men, 9.6–40.8 µg/L
		MicroVue Osteocalcin/ELISA	Quidel Corp., San Diego, CA, USA	Adults (>25 years), 3.7–10 µg/L
		Undercarboxylated Osteocalcin EIA Kit/ELISA	TaKaRa Bio Inc., Shiga, Japan	ca. 20% of total OC in serum; no further data
PICP	Serum plasma	MicroVue CICP/ ELISA	Quidel Corp., San Diego, CA, USA	Women, postmenopausal, 69–147 µg/L Men (>25 years), 76–163 µg/L
		Procollagen Type I C-Peptide (PIP)/ ELISA	TaKaRa Bio Inc., Shiga, Japan	Adults, 161–757 µg/L

(*continued*)

Table 1 (continued)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
PINP	Serum plasma	UniQ PINP/RIA	Orion Diagnostica Oy, Espoo, Finland	Women, 19–83 µg/L Men, 22–87 µg/L
		IDS-iSYS Intact PINP/CLIA, automated	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 19.3–76.3 µg/L Women, postmenopausal, 18.2–102.3 µg/L Men (>45 years), 19.1–77.0 µg/L
		Total P1NP/ECLIA, automated	Roche Diagnostics, Mannheim, Germany	Women, premenopausal, 13.8–60.9 µg/L Men, 13.9–85.5 µg/L

Bone resorption and osteoclastogenesis marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
DPD	Urine	MicroVue DPD/ELISA	Quidel Corp., San Diego, CA, USA	Women (25–44 years), 3.0–7.4 nmol/mmol CREA Men (25–55 years), 2.3–5.4 nmol/mmol CREA
Total DPD	Serum urine	MicroVue Total DPD/ELISA	Quidel Corp., San Diego, CA, USA	Women, (25–44 years), 2.18–4.68 nmol/L Men (25–55 years), 1.95–4.54 nmol/L 19–325 nmol/L
	Serum	MicroVue Serum PYD (only free)/ ELISA	Quidel Corp., San Diego, CA, USA	Adults, 1.09–2.79 nmol/L
	Urine	MicroVue PYD (free PYD + DPD)/ ELISA	Quidel Corp., San Diego, CA, USA	Women, premenopausal, 16.0–37.0 nmol/mmol CREA Men, 12.8–25.6 nmol/ mmol CREA
CTX	Serum plasma	ß-CrossLaps/ ECLIA, automated	Roche Diagnostics, Mannheim, Germany	Women, premenopausal, 31–568 ng/L Women, postmenopausal, 113–999 ng/L Men (30–50 years), 22–578 ng/L (>50–70 years), until to 692 ng/L (>70 years), until to 855 ng/L
		Serum CrossLaps/ ELISA	IBL, Toronto, Canada	Women, premenopausal, 112–738 ng/L Women, postmenopausal,

(*continued*)

Table 1 (continued)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
				142–1351 ng/L Men (30–50 years), 115–748 ng/L
		Serum CrossLaps (CTX-I)/ELISA	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 0.39–3.20 nmol/L Women, postmenopausal, 0.36–5.55 nmol/L
		IDS-iSYS CTX-I (CrossLaps)/ ECLIA, automated		Women, premenopausal, 50–670 ng/L Women, postmenopausal, 90–1050 ng/L Men (>45 years), 90–730 ng/L
	Urine	CrossLaps Urine/ ELISA	IBL, Toronto, Canada	Women, premenopausal, 67–544 µg/mmol CREA
		Urine BETA CrossLaps (CTX-I)/ ELISA	IDS Inc., Scottsdale, AZ, USA	Women, postmenopausal, 121–874 µg/mmol CREA Men (31–80 years), 54–559 µg/mmol CREA
		Alpha CrossLaps EIA/ELISA	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 0.10–0.99 µg/mmol CREA Women, postmenopausal, 0.17–2.26 µg/mmol CREA Men, 0.13–1.13 µg/mmol CREA
NTX	Serum plasma	Osteomark NTx Serum/ELISA	Alere Inc., Waltham, MA, USA	Women, premenopausal, 7.7–19.3 nmol BCE/L Men (31–80 years), 8.1–24.8 nmol BCE/L
	Urine	Osteomark NTx Urine/ELISA	Alere Inc., Waltham, MA, USA	Women, premenopausal, 14–74 nmol BCE/mmol CREA Men (31–87 years), 13–78 nmol BCE/mmol CREA
ICTP	Serum plasma	UniQ ICTP/ELISA and RIA	Orion Diagnostica Oy, Espoo, Finland	Women, 1.6–4.2 µg/L Men, 1.5–4.3 µg/L
TRAP5b	Serum plasma	MicroVue TRAP5B EIA/ELISA	Quidel Corp., San Diego, CA, USA	Women, premenopausal, 0.25–5.64 U/L Women, premenopausal, until to 9.12 U/L Men, 1.26–6.74 U/L

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Table 1 (continued)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
BSP	Serum plasma	Bone Sialoprotein (BSP)/ELISA	Immundiagnostik AG, Bensheim, Germany	Without data
OPN	EDTA-plasma	Quantikine ELISA Human Osteopontin	R&D Systems, Minneapolis, MN, USA	Adults, 46–144 µg/L
OPG	Serum plasma	Osteoprotegerin/ ELISA	Immundiagnostik AG, Bensheim, Germany	Adults, until 3.60 pmol/L
RANKL	Serum plasma	Total sRANKL/ ELISA	Immundiagnostik AG, Bensheim, Germany	Women, premenopausal, until 3.29 pmol/L Men until 1.66 pmol/L

Abbreviations: *BAP* bone-specific alkaline phosphatase, *BCE* bone collagen equivalents, *BSP* bone sialoprotein, *CLIA* chemiluminescence immunoassay, *CREA* urinary creatinine, *CTX* carboxy-terminal cross-linking telopeptide of type 1 collagen, *DPD* deoxypyridinoline, *ECLIA* electrochemiluminescence immunoassay, *ICTP* carboxy-terminal cross-linking telopeptide of type 1 collagen, generated by MMPs, *MMPs* matrix metalloproteinases, *NTX* amino-terminal cross-linking telopeptide of type 1 collagen, *OC* osteocalcin, *OPG* osteoprotegerin, *OPN* osteopontin, *PICP* carboxy-terminal propeptide of type 1 collagen, *PINP* amino-terminal propeptide of type 1 collagen, *PYD* pyridinoline, *RANKL* receptor activator of NF-kappaB ligand, *RIA* radioimmunoassay, *TRAP5b* tartrate-resistant acid phosphatase type 5b

Chow et al. 2012), resulting in a strong antifibrotic effect on various kinds of organs. RLN also influences bone metabolism by inducing osteoclastogenesis and the activation of mature osteoclasts expressing RXFP1 through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), receptor activator of NF-kB (RANK), tartrate-resistant acid phosphatase (TRAP), and cathepsins (Ferlin et al. 2010; Hombach-Klonisch et al. 2006). The physiological roles of RXFP, RLN, and INSL3 in bone metabolism have been elucidated in recent studies. Ferlin et al. reported that 64% of young men with *RXFP2* mutations had significantly reduced bone mass density without any apparent cause of osteoporosis and no aberration of testosterone levels and gonadal function (Ferlin et al. 2008). Interestingly, *Rxfp2*-deficient mice also presented a decreased bone mass, mineralizing surface, bone formation, and osteoclast surface in comparison with their wild-type littermates (Ferlin et al. 2008). The authors concluded that INSL3/RXFP2 signaling is essential for bone metabolism. In another study, Duarte et al. reported the expression of *Rxfp1* and *Rxfp2* during mouse craniofacial skeletal development and tooth development (Fig. 6) (Duarte et al. 2014a) and found that RLN enhanced osteoblastic differentiation and caused abnormal mineralization and extracellular matrix metabolism in vitro through *Rxfp2*, which was predominant over *Rxfp1* in MC3T3-E1 mouse calvarial osteoblasts (Duarte et al. 2014b). In addition, Moon et al. showed that in vivo administration of Rln enhanced bone morphogenetic protein (BMP)-2-induced bone formation and synergistically enhanced the BMP-2-induced osteoblast differentiation of mouse bone marrow stem cells and mouse embryonic C3H/10 T1/2
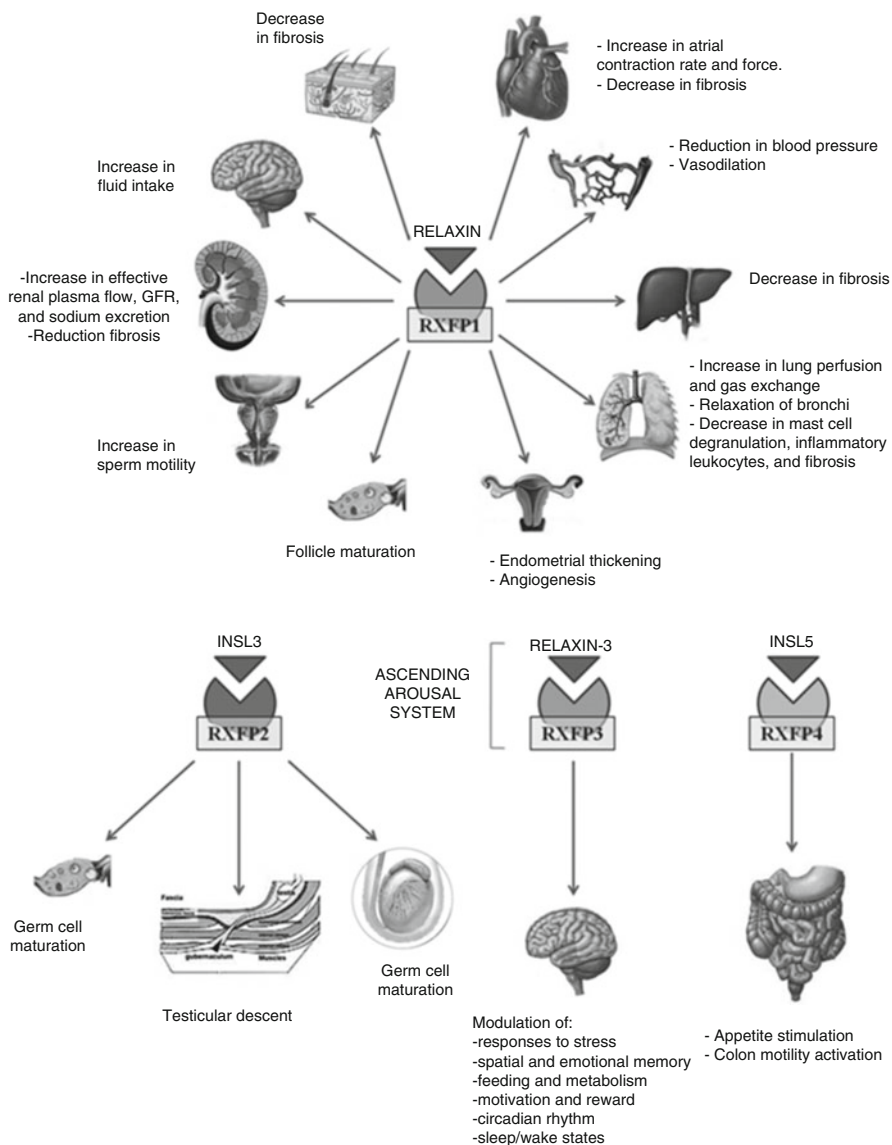
Fig. 1 **Pattern diagram of physiological role of RLN on multiple organs** Data from Cernaro et al. (Cernaro et al. 2014), with permission from the publisher.

fibroblasts through *Rxfp1* by augmenting and sustaining Smad and p38 phosphory-lation, which upregulated runt-related transcription factor 2 (Runx2) expression and activity (Moon et al. 2014). These results strongly suggest that RLNs might be useful for therapeutic applications in bone metabolic disorders and that RLNs and RXFPs

might be candidate biomarkers to reflect the condition of bone health and disease status.

Overview of Bone Turnover Biomarkers in the Serum/Plasma and Urine

Bone turnover biomarkers are generally classified into bone formation and bone resorption markers (Delmas et al. 2000). Analysis of the dynamics of bone turnover markers in the serum and/or urine is extremely useful as a diagnostic tool and for evaluation of the prognosis and treatment of patients with skeletal disorders. Table 1 shows the common bone markers that are tested in clinical settings (Jung and Lein 2014). The following sections provide a brief description of each marker listed in Table 1.

Biomarkers for Evaluating Bone Formation

An increased concentration of bone-specific alkaline phosphatase (BAP) in the serum primarily reflects accelerated osteoblast activity or secondarily indicates elevated bone resorption. There are two effective methods for measuring either the protein mass or enzyme activity of BAP (Gomez et al. 1995). Osteocalcin (OC) is secreted by mature osteoblasts in a vitamin K- and D3-dependent manner and is a calcium-binding protein, accounting for 25% of the non-collagenous proteins in the skeletal tissue. Proteolytic cleavage between amino acids 43 and 44 produces an intact molecule of 49 amino acids in the bloodstream (Garnero et al. 1994), and the mostly stable N-terminal N-MID fragment from amino acids 1–43 can be detected with an N-MID osteocalcin assay (see Table 1). Bone has a complex structure consisting of minerals based on hydroxyapatite and organic constituents based on type I collagen. Type I collagen is a triple helical-structured molecule containing two identical α1 (I) and α2 (I) chains. Type I procollagen is separated by proteases on the N- and C-terminal ends, accordingly referred to as the C-terminal or N-terminal propeptides of type I procollagen (PICP and PINP, respectively), which reflect the activity of osteoblasts at the early stage of differentiation.

Biomarkers for Evaluating Bone Resorption

The helix shape of bone collagen is stabilized by cross-links of lysine or hydroxylysine residues called pyridinoline (PYD) and deoxypyridinoline (DPD), which are released during bone resorption (Delmas et al. 2000). DPD is almost solely found in the bone and is thus a specific indicator of bone resorption. Methods have

been developed for the determination of free as well as total cross-links in the urine and serum. Type I collagen releases carboxy- as well as amino-terminally cross-linked telopeptides (CTX and NTX, respectively) into the bloodstream during bone resorption (Herrmann and Seibel 2008). CTX and NTX can be measured in both the serum and urine. Carboxy-terminal cross-linking telopeptide of type I collagen (ICTP) is a specific telopeptide that is separated from collagen by metalloproteinases. ICTP is a trivalent cross-linked telopeptide of 8.5 kDa, with two phenylalanine-rich domains between the two α1 collagen chains.

Osteoclasts specifically contain tartrate-resistant acid phosphatase type 5b (TRAP5b) as a bone-specific isoenzyme and release it into the bloodstream during bone resorption. The TRAP5b concentration in the serum reflects the number and activity of osteoclasts involved in the resorption process (Chao et al. 2010). TRAP5b has been specifically proposed for the diagnosis and monitoring of the treatment of metastatic bone disease, including breast, prostate, lung, and multiple myeloma (Chao et al. 2010). Bone sialoprotein (BSP) and osteopontin (OPN) are components of the non-collagenous bone matrix and are both expressed in not only osteoclasts and osteoclast-like cells but also in osteoblasts. BSP and OPN have been shown to be essential factors in the bone metastasis of osteotropic cancers from the breast, prostate, and lung (Kruger et al. 2014).

Although various types of cells express receptor activator of nuclear factor kappa B ligand (RANKL), chondrocytes, osteoblasts, and osteocytes are the main RANKL-expressing cells in the bone microenvironment under normal physiological conditions (Nakashima et al. 2012). The expression of RANKL is induced by bone resorption factors such as activated vitamin D_3, PTH, and inflammatory cytokines (Nakashima et al. 2012). RANK, the receptor of RANKL, is expressed on osteoclast precursor cells, and RANK/RANKL signaling activates the transcription factor nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) and controls osteoclastogenesis (Nakashima et al. 2012). Osteoprotegerin (OPG) is a soluble decoy receptor for RANK/RANKL signaling by binding to RANKL (Nakashima et al. 2012), indicating that RANK/RANKL signaling and its control mechanism mediated by OPG play indispensable roles in the bone resorption level by osteoclasts. The free RANKL and OPG-soluble RANKL (sRANKL) complex can be detected in the blood, and the so-called total sRANKL level can be differentially measured as an effective bone resorption biomarker.

Overview of Relaxin Family Peptides and Receptors

Relaxin is well known for its physiological role in the growth and differentiation of the reproductive tract during gestation (Bathgate et al. 2005, 2006, 2013). Relaxin plays roles in reproduction, the cardiovascular system, organ protection, metabolism, cancer metastasis, bone metabolism (including osteoclastogenesis and osteoblast differentiation), and as a neuropeptide in the brain (Fig. 1). Relaxin is a peptide

hormone that belongs to the relaxin-like hormone family, which is part of the insulin/insulin-like growth factor/relaxin-like hormone superfamily (Lu et al. 2005). The relaxin-like hormone family comprises relaxins 1, 2, and 3 (RLN1–RLN3) and insulin-like factors 3, 4, 5, and 6 (INSL3–INSL6). A group of G-protein-coupled receptors known as relaxin family peptide receptors 1–4 (RXFP1–RXFP4) shows tissue- and species-specific affinities to relaxin-like hormones. Relaxin, insulin-like peptide 3 (INSL3), RLN3, and INSL5 interact with RXFP1–RXFP4, respectively (Bathgate et al. 2005, 2006, 2013). Humans and apes possess the *RLN1–RLN3* and *INSL3–INSL6* genes, which encode H1–H3 relaxins and INSL3–INSL6 proteins, respectively (Bathgate et al. 2005). On the other hand, mice possess only *Rln2*, *Rln3*, *Insl3*, *Insl5*, and *Insl6* (Bathgate et al. 2005). Human RLN1 and RLN2, as well as RLN2 in other mammals, are commonly referred to as "relaxin" (Bathgate et al. 2005). RXFPs are G-protein-coupled receptors with seven transmembrane domains anchored to the cell membrane. RXFP1 and RXFP2 have large extracellular domains with characteristic leucine-rich repeats (LRRs) that allow them to bind to RLN1 and RLN2. Accordingly, RXFP1 and RXFP2 were previously referred to as LRR-containing G-protein-coupled receptors 7 and 8 (LGR7 and LGR8), respectively. Because RXFP3 and RXFP4 do not possess a large extracellular domain, they cannot bind to relaxin but they can bind to RLN3 and INSL5. Relaxin inhibits TGF-β-mediated collagen synthesis and increases the expression of MMPs in lung and kidney fibroblasts (Unemori et al. 1996). It also inhibits TGF-β-mediated cardiac fibroblast proliferation and differentiation, as well as collagen synthesis by increasing the expression of MMPs (Mookerjee et al. 2005). RLN3 is most concentrated in the brain, which suggests that it may have neurological effects. RLN3 is involved in stress responses and in the regulation of food intake. INSL4 is most abundant in the maternal decidua and can inhibit fetal growth by increasing cell apoptosis and reducing cell viability. INSL5 is abundant in the colon and is a possible marker of colorectal and neuroendocrine tumors (Bathgate et al. 2006). The activation of RXFP1 increases the accumulation of cyclic adenosine monophosphate (cAMP) and the rapid phosphorylation of mitogen-activated protein kinases 1 and 2 (ERK1/ERK2), resulting in activation of the nitric oxide (NO) signaling pathway. In the connective tissue, the activation of NO inactivates pSMAD2 and transforming growth factor-beta (TGF-β) to produce matrix metalloproteinase (MMP) 1, 2, 9, and 13, resulting in the degradation of collagen in the extracellular matrix. In osteoblast progenitor cells, RXFP2/INSL3 signaling induces ALP activity, extracellular matrix mineralization, and the activation of mitogen-activated kinase (MEK) and ERK1/ERK2. This results in increased production of type I collagen, osteonectin, OPN, TGF-β, macrophage colony-stimulating factor (M-CSF), and the PTH receptor, leading to osteoblast differentiation and osteoclastogenesis (Ferlin et al. 2011). In contrast to RXFP1 and RXFP2, RXFP3 and RXFP4 inhibit the activation of cAMP. RXFP3 activation induces the phosphorylation of MEK and ERK1/ERK2 and the activation of nuclear factor kappa B, subunit 1. RXFP4 increases intracellular calcium ion concentrations, although the mechanism of this effect remains unclear.

The Roles of Relaxin in Bone Development, Metabolism, and Disease

The Effects of Relaxin and Its Receptor on Craniofacial Skeletal Development and Osteoblast Activity

The craniofacial skeletal tissues are of mesenchymal origin, derived from the cranial neural crest and/or mesoderm. The boundary between these two types of mesenchyme is reported to lie between the parietal and frontal bones in the cranial vault and between the basisphenoid and basioccipital bones in the cranial base (Chai and Maxson 2006; Morriss-Kay 2001). The mechanism of skeletal development is divided into two main categories: intramembranous ossification and endochondral ossification. Calvarial bones, with the exceptions of a part of the great wing of the sphenoid bone, and facial bones, with the exception of the inferior nasal concha, ethmoid bone, and hyoid bone, are developed by the intramembranous ossification, which is characterized by direct ossification by the osteoblasts contained in mesenchymal cellular aggregates. The membranous bones are connected to each other by dense fibrous connections called sutures. Despite the wide-ranging effects of RLNs and RXFPs, their specific expression pattern and their effects on craniofacial skeletal development have scarcely been examined. A large number of signaling molecules have been described as modulators of craniofacial skeletal morphogenesis. Bone morphogenetic protein (BMP) 2 and 4 induce intramembranous ossification and guide the morphological changes of the craniofacial bones (Farhadieh et al. 2004). Runx2 is a signaling molecule exclusive to osteoblasts (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997) that is found in the cells of the osteoblastic lineage in embryonic mesenchymal condensations (Ducy et al. 1997) and mediates the commitment of mesenchymal cells into osteoblasts. The embryonic formation of craniofacial bones, especially the calvarial vault and mandibular bones, depends on the specific temporospatial expression of Runx2 (Maeno et al. 2011). After an osteoblast progenitor is committed to differentiate into an osteoblast, its maturation is characterized by BAP and collagen type 1 α1 (COL1α1) secretion into the extracellular matrix. BAP, a bone formation biomarker, is expressed in the osteoid and mineralized bone matrix of craniofacial bones and is induced by BMPs (Kim et al. 2004). COL1 is the main component of the bone matrix where minerals are deposited. The expression of *Col1α1*, the gene encoding Col1, is induced by both BMPs and Runx2 (Ortuno et al. 2013). OPN is expressed in well-differentiated osteoblasts and in more mature osteogenic tissues (Strauss et al. 1990). Along with OPN, mature osteoblasts express OCN, another bone formation marker, and bone sialoprotein (BSP). OCN is the protein responsible for the binding of calcium ions to the osteoid, and OPN induces the formation of mineral crystals. Fine-tuning of the tempo-spatial expression and function of OCN and OPN is indispensable for the proper development and growth of the craniofacial bones. Relaxin synergistically enhances BMP-2-induced osteoblast differentiation and bone formation through its receptor

Rxfp1 by augmenting and sustaining Smad and p38 phosphorylation, which in turn enhances Runx2 expression and activity (Moon et al. 2014). In addition, it has been shown that *Rxfp1* and *Rxfp2* mRNAs are expressed in the developing calvarial frontal bones (Duarte et al. 2014b; Fig. 2) and facial bones (Duarte et al. 2014b; Fig. 3). Relaxin was shown to enhance osteoblastic differentiation, mediated by enhanced *Runx2* expression and upregulation of BAP activity, which was
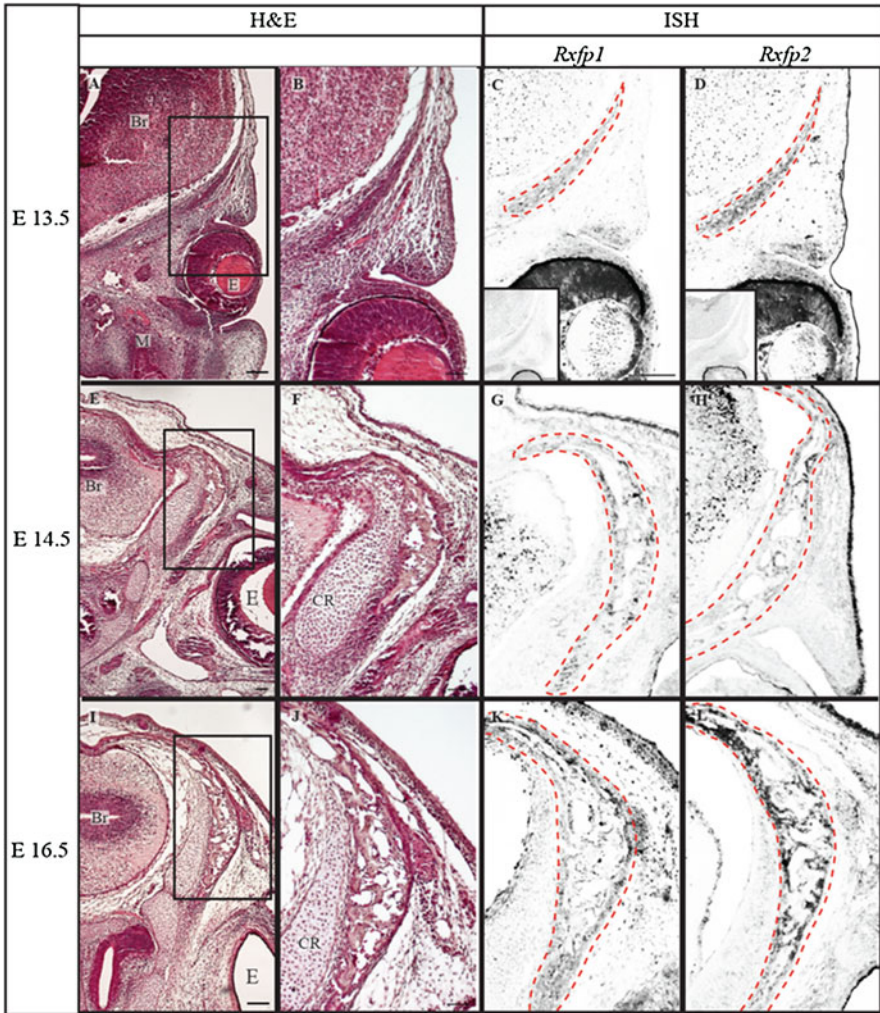

Fig. 2 **Expression of Relaxin/insulin-like family peptide receptors (Rxfp) 1 and 2 in developing mouse calvarial frontal bones**. Both Rxfp1 and 2 mRNA in the osteoblasts of the developing murine calvarial frontal bone from E13.5 to E16.5 by in situ hybridization (C, D, G, H, K, and L). Br, brain; CR, cartilaginous precursor of the cranial base; E, eye; and M, molar. Data from Duarte et al (Duarte et al. 2014a) with permission from the publisher.
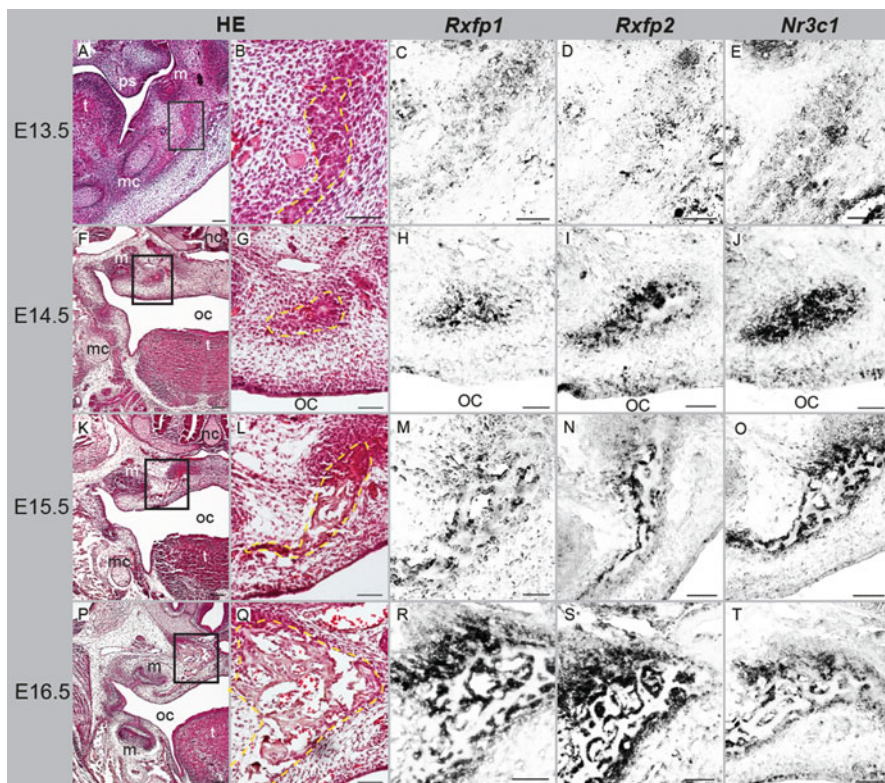
Fig. 3 **Expression patterns of Rxfp1 and Rxfp2 during the development of the mandibular bone (E13.5) and maxillary bone (E14.5–E18.5) determined by in situ hybridization**. Expression pattern of Rxfp1, Rxfp2 was observed in the mesenchymal condensations of the mandibular bone at E13.5 (C, D), and in the ossifying maxilla from E14.5 to E16.5 (H, I, M, N, R, and S). m, molar; mc, Meckel's cartilage; nc, nasal cartilage; oc, oral cavity; ps, palatal shelf; t, tongue. Data from Duarte et al (Duarte et al. 2014a) with permission from the publisher.

accompanied by ERK1/ERK2 phosphorylation through *Rxfp2* expression (predominant over *Rxfp1*) in MC3T3-E1 mouse calvarial osteoblasts, and thereby enhanced in vitro mineralization (Duarte et al. 2014b; Figs. 4 and 5). Moreover, relaxin has an effect on osteoblast mineralization, which is presumably derived from its capacity to increase the activities of MMP2 and MMP13 in osteoblasts (Duarte et al. 2014b; Fig. 5). These findings suggest a novel role for relaxin in craniofacial skeletal development and metabolism through Rxfp.

The Effects of Relaxin on Osteoclast Activation

The mature osteoclast, which arises from hematopoietic precursors, exhibits multinucleated giant cells and plays an indispensable role in bone resorption in the
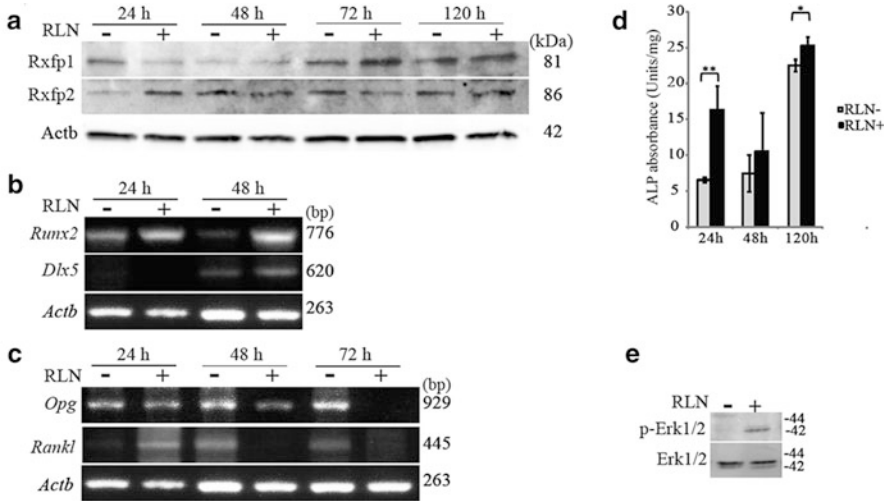
Fig. 4 RLN enhanced alkaline phosphatase (ALP) activity and Erk1/2 phosphorylation of mouse calvarial osteoblast cell line MC3T3-E1 cells. (A) RLN (20 ng/mL) enhanced Rxfp2 expression in MC3T3-E1 mouse calvarial osteoblasts, but inhibited expression of Rxfp1 after 24 h determined by Western blot analysis. (B and C) RLN enhanced expression of Runx2 after 48 h and Bmp2 after 72 and 120 h in MC3T3-E1 cells cultured in differentiation medium. On the other hand, RLN inhibited Opg and Rankl expression after 48 and 120 h culture period. (D) ALP activity and (E) ERK 1/2 phosphorylation were significantly increased by RLN (20 ng/mL) after 24 h culture in differentiation medium. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001). Data from Duarte et al (Duarte et al. 2014b) with permission from the publisher.

skeletal tissues. The differentiation and function of osteoclasts are strictly controlled by osteoblastic lineage cells, including osteoblasts, osteocytes, and stromal marrow cells. These cells express important cytokines such as M-CSF and RANKL. On the other hand, osteoclast progenitor cells express the transmembrane receptor tyrosine kinase c-FMS, which binds to M-CSF and RANK. The majority of the calcium-regulating hormones and cytokines that promote bone resorption induce the expression of RANKL in osteoblastic lineage cells. Osteoclasts secrete the soluble receptor OPG, which inhibits the RANK-RANKL interaction as a decoy receptor for RANKL. RANKL and M-CSF activate various intercellular signaling molecules to ultimately promote the expression of NFATc1, a master transcriptional factor for inducing osteoclast differentiation. Interestingly, human peripheral blood monocyte cells (PBMCs), the precursors of osteoclasts, were found to express *RXFP1* mRNA and respond to RLN2 by increasing the levels of tumor necrosis factor-α and interleukin-1β secretion (Kristiansson et al. 2005). Furthermore, primary cultured mature human osteoclasts derived from human PBMCs were shown to express *RXFP1* mRNA (Facciolli et al. 2009). Relaxin regulates the recruitment of leukocytes to the sites of inflammation and plays a role in the substrate adhesion and migration of mononuclear leukocytes (Figueiredo et al. 2009). RLN also influences bone metabolism by inducing osteoclastogenesis and the activation of mature
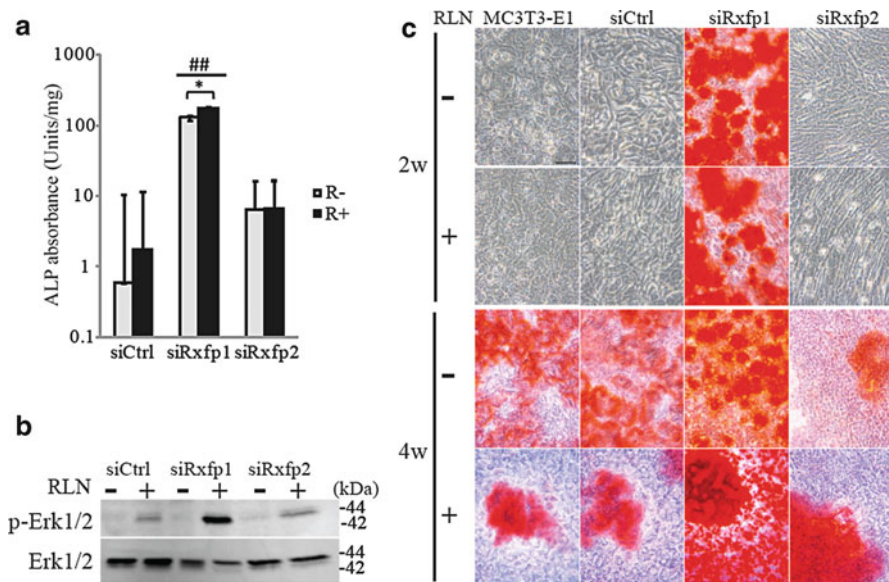
Fig. 5 RLN/Rxfp2 signaling enhanced ALP activity through Erk1/2 phosphorylation and enhanced mineralization of MC3T3-E1 cells. (A) ALP activity in siRxfp1 and siRxfp1/2, which means partially down regulation of Rxfp1/2 in cells, was significantly higher than in control and siRxfp2. RLN significantly increased ALP activity in siRxfp1 cells after 24 h culture. (B) Erk1/2 phosphorylation was observed in control, siRxfp1, and siRxfp2 samples after 24 h RLN (20 ng/mL) treatment; however, it was markedly higher in Rxfp1 cells. Phosphorylation of ERK1/2 in siRxfp1/2 cells was observed independent of RLN administration. (C) Significantly enhanced matrix mineralization was observed in siRxfp1 and siRxfp1/2 cells after 2 weeks culture with or without RLN administration. Data from Duarte et al (Duarte et al. 2014b) with permission from the publisher.

osteoclasts expressing RXFP1 through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), RANK, TRAP, and cathepsins (Ferlin et al. 2010; Hombach-Klonisch et al. 2006). Administration of RLN combined with estrogen increased OPG and decreased the RANKL/OPG protein ratio more than administration of estrogen alone in an adjuvant-induced arthritis rat model of rheumatoid arthritis (Ho et al. 2011). These observations indicate a new pharmacological role of relaxin in controlling bone resorption.

The relationship between INSL3/RXFP2 signaling and osteoporosis has been a topic of research interest. A comprehensive study involving clinical, biochemical, and hormonal analyses, including bone densitometry analysis through DXA, showed that 64% of young men with an *RXFP2* gene mutation resulting in a T222P amino acid substitution presented significantly reduced bone mineral density; their testosterone levels and gonadal function were normal, and no other apparent cause of osteoporosis was evident (Ferlin et al. 2008). Human and mouse osteoblasts express *RXFP2/ Rxfp2*, and administration of INSL3 to these osteoblasts results in cAMP production to affect cell proliferation in a dose- and time-dependent manner. In support of the human phenotype, bone histomorphometric and computed tomography analyses of
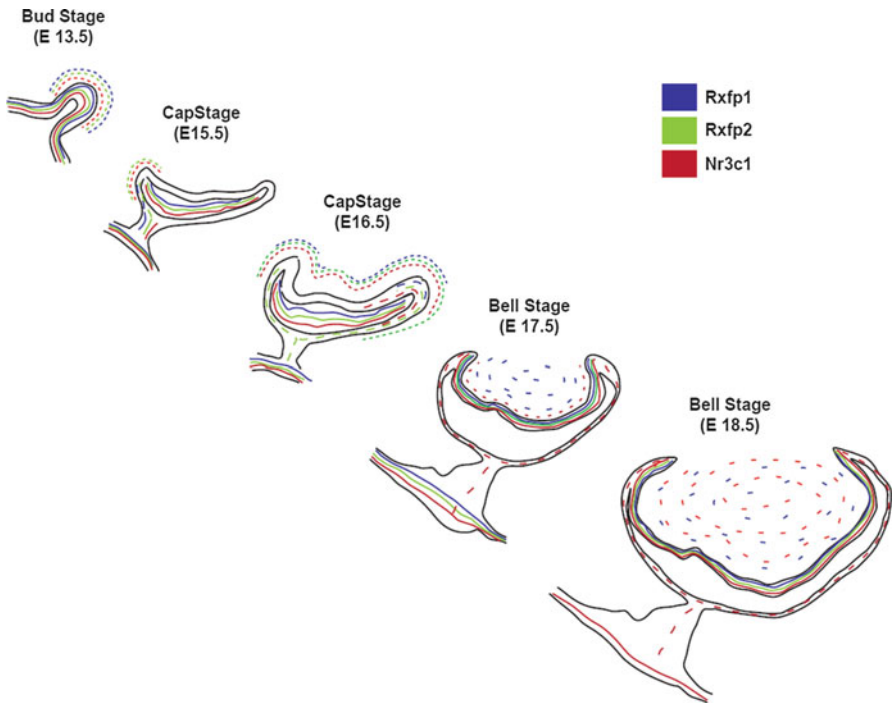
Fig. 6 Diagram of the mRNA expression patterns of relaxin family peptide receptors 1 and 2 (Rxfp1 and Rxfp2) and the glucocorticoid receptor (Nr3c1) during upper molar development. Data from Duarte et al (Duarte et al. 2014a) with permission from the publisher.

Rxfp2-deficient mice revealed a decreased bone mass, mineralizing surface, bone formation, and osteoclast surface compared with their wild-type littermates. These results suggest a significant role of INSL3/RXFP2 signaling in bone metabolism and link *RXFP2* gene mutations with human osteoporosis (Ferlin et al. 2008).

Given the physiological function of relaxin family peptides and RXFP2 on bone development, remodeling, metastasis, and metabolic disorders, these molecules are promising candidates as biomarkers to accurately reflect bone health and multiple bone-related disorders.

Potential Applications of Relaxin to the Prognosis of Other Diseases or Conditions

Relaxin and Orthodontic Treatment

A necessary and adequate force is required for orthodontic tooth movement, which is accompanied by remodeling of the soft tissue, including the periodontal ligament (PDL) and gingival tissue, and the alveolar bone supporting the teeth. Many

molecules, including local and systemic factors, have been suggested to be involved in the remodeling process of the alveolar bone and PDL during and after tooth movement, which influence on the rate of tooth movement, stability, and relapse after the treatment. The orthodontic compressive force induces alveolar bone resorption by osteoclasts, which is accompanied by the reorganization of periodontal soft tissues. Simultaneously, bone formation from active osteoblasts occurs at the alveolar bone surface of the tension side. Given the broad physiological activity of RLN, several experiments have been performed to test its clinical feasibility in orthodontic treatment (Hirate et al. 2012; Mcgorray et al. 2012; Yang et al. 2011). There are conflicting views regarding the action of relaxin on periodontal tissues. Relaxin was shown to reduce the level of PDL organization and mechanical strength, and to increase tooth mobility at early time points, but did not accelerate orthodontic tooth movement in rats (Madan et al. 2007). Relaxin receptors are localized in PDL fibroblasts (Stewart et al. 2005) and were found to decrease the expression of the collagen type I gene and increase the expression of MMP1 in stretched human PDL cells (Takano et al. 2009). The orthodontic force enhanced the expression of *Rln1* mRNA and the synthesis of Rln1 protein in the granulosa cells of the rat ovary (Yang et al. 2011). Accordingly, it is tempting to speculate that the RLN1 expressed in the ovary during orthodontic tooth movement might affect osteoclastogenesis on the pressure side and collagen turnover on the tension side. This speculation can be supported by other reports showing that relaxin may accelerate orthodontic tooth movement (Liu et al. 2005). The velocity of orthodontic tooth movement has been shown to be influenced by the hormones released during pregnancy (Hellsing and Hammarstrom 1991), and the levels of relaxin mRNA are altered in the rat ovary during pregnancy (Crish et al. 1986). However, it is not yet certain whether the increased expression of *Rln1* may directly affect tooth movement. Relaxin has been reported to be present in the cranial suture and PDL (Nicozisis et al. 2000). It has also been suggested that the effect of relaxin on PDL remodeling might reduce the rate of relapse after orthodontic treatment (Masella and Meister 2006). However, a randomized clinical trial was performed on humans given weekly injections of 50 µg of RLN or a placebo control for 8 weeks. Tooth movement was measured weekly by impressions. There was no significant difference between the RLN and placebo control groups regarding the acceleration and relapse rates, presumably quite low dose of RLN at the periodontal area (Mcgorray et al. 2012). The concentration of RLN in the bloodstream might reflect the efficacy of tooth movement, the adequacy of the orthodontic force, or the likelihood of relapse after orthodontic treatment. Nevertheless, the precise mechanism and effects of relaxin and RXFPs on orthodontic tooth movement or relapse should be elucidated with detailed investigations.

Summary Points

- Bone homeostasis is maintained by fine-tuning of the dynamic balance of "remodeling," which refers to the removal of old bone and subsequent formation of new bone by osteoclasts and osteoblasts, respectively, at the basic multicellular unit.

- Currently, the biomarkers used to assess bone metabolism are categorized into two groups: bone resorption markers and bone formation markers. The soluble molecules that are specifically secreted from osteoblasts or osteoclasts and the metabolic products of type 1 collagen are generally used as biomarkers of bone metabolism.
- RLN is an insulin-like peptide hormone that facilitates parturition by inducing the softening and lengthening of the pubic symphysis and softening of the cervix.
- RLN inhibits fibroblast proliferation and differentiation, but increases the production of matrix MMPs, resulting in a strong antifibrotic effect.
- RLN also influences bone metabolism by inducing osteoclastogenesis and activates mature osteoclasts through RXFP1.
- Significant bone loss, which can lead to osteoporosis, was observed in a large number of men with mutated *RXFP2* and in *Rxfp2*-deficient mice.
- In vivo administration of Rln enhanced BMP-2-induced bone formation through Rxfp1 by augmenting and sustaining Smad and p38 phosphorylation induced by BMP-2. This activated intracellular signaling upregulated Runx expression and activity, which enhanced osteoblast differentiation.
- These results suggest that RLNs can be useful for therapeutic applications in bone metabolic disorders and that RLN and RXFPs might be candidate biomarkers that reflect bone health and disease status.

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