Localization of the glucocorticoid receptor mRNA in cartilage and bone cells of the rat. An *in situ* hybridization study

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The *in vivo* localization of glucocorticoid receptor (GR) mRNA expression was studied in the cartilage and bone cells of the femur of young adult rats to compare its distribution with that of the GR protein, which had previously been shown histochemically in the same areas. To achieve this, we used a synthetic oligodeoxynucleotide as a probe, in line with the published human GR (hGR) cDNA sequence. The probe was coupled to fluorescein (FL), applying a rapid Fast-Tag TM FL nucleic acid labeling method. Negative controls were achieved by using sense sequences of the hGR oligoprobe, similarly coupled by using the Fast-Tag TM FL labeling kit. Dewaxed sections were treated for *in situ* hybridization (ISH) histochemistry with the antisense and sense oligoprobes. The ISH reaction product was more intense in the cytoplasm of proliferative and maturative chondrocytes of the growth plate cartilage than in that shown in the hypertrophic ones. In the metaphyseal secondary ossification zone, osteoblasts (OBs) and osteocytes (OCs) were variably labeled, whereas osteoclasts (OCLs) were always intensely stained. The labeling was also visible in some bone marrow cells, in articular chondrocytes, in the cells of tendon-bone junctions, and in the perichondrium and periosteal cells. Our results confirm a cellular co-location of GR protein and mRNA. In agreement with GR immunolocalization, the variability of labeling appeared to be related to the cell cycle, the stage of differentiation and cell-type differences.

Key words: glucocorticoid receptor, bone cells, osteoclast, osteoporosis, *in situ* hybridization.

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Glucocorticoid hormones (GCs) modulate a wide spectrum of cellular functions and physiological processes in animal tissues, including immune reactions, stress response, glycogen metabolism, and mineral homeostasis. Like other classes of steroid hormones, GCs act either by complexing with their specific intracytoplasmic glucocorticoid receptor (GR), which in its turn translocates to the nucleus and binds to specific sites of glucocorticoid response elements (GREs) in the chromatin, or by direct protein-protein interactions with other transcriptional factors (Gustafsson *et al.*1987, De Lange P *et al.* 1988, Distelhorst 1989, Bamberger *et al.*1996, De Lange Y *et al.* 1997). The presence of functional GRs is a prerequisite for a cellular response to hormones, which depends on cell cycle, differentiation stage, levels of GR expression, and cell and tissue specificity to the hormone (Gustafsson *et al.* 1987).

Recent re-examinations of the way GCs act have shown that steroids can also act directly on membranes through physicochemical interactions with them (Pilgrim 1999, Buttgereit *et al.* 2000), independently of steroid receptors in the cytoplasm, or by a more dynamic still incompletely known exchange process between chromatin and the nucleoplasmic compartment (McNally *et al.* 2000). This could open up new perspectives on our knowledge of the complex network of cellular interactions triggered by steroid stimulation.

As far as the effects of GCs on mineral homeostasis are concerned, several studies have shown their involvement in the mechanism of long bone elongation and bone remodeling *in vivo* (Lo Cascio *et al.* 1990, Quarles 1992, Chappard *et al.* 1996, Miyakoshi *et al.* 1997) and *in vitro* (Canalis 1982, Defranco *et al.* 1992, Delany *et al.* 1994, Doherty *et al.* 1995, Connaway *et al.* 1996). In particular, a prolonged high- dose treatment with GCs can lead to osteoporosis, as a result of a decrease in bone formation and/or increase in bone resorption. The

mechanisms through which GCs affect bone remodeling have not yet been defined, and the changes they induce in bone tissue are controversial, probably depending on the different experimental models used (Chavassieux *et al.* 1993, O' Connell *et al.* 1993, Saito *et al.* 1995).

GR protein expression has been shown *in vitro* in human osteoblasts (Liesegang *et al.* 1994, Dempster *et al.* 1997), canine (Kan *et al.* 1984), chick (Lee *et al.* 1978) and human (Di Battista *et al.* 1991) chondrocytes, and rat osteoclasts (Chen *et al.* 1977, Yoshioka et al 1980, Conaway *et al.* 1996,), and, *in vivo*, in chondrogenic regions of embryonic palate (Abbott *et al.* 1994), and in cartilage and bone cells of normal (Silvestrini *et al.* 1999) and GC-treated rats (Silvestrini et al 2000). Moreover, GR mRNA has been shown in several different cell types: *in vitro*, in HeLa cells (Burnstein et al 1991, Silva *et al.* 1994), human lymphocytes and rat pancreatic acinar cells (Rosewicz *et al.* 1988); in vivo in the brain (Tsujimoto *et al.* 1986), and pancreas (Lewis *et al.* 1988) and the pituitary gland (Ozawa *et al.* 1999, Matthews *et al.* 1995). Very few data concerning GR mRNA detection in cartilaginous and bone cells (Abbott *et al.* 1994, Condon et. al 1998) are available. GR protein and mRNA expression were reported to be co-located in normal conditions (Sweezey *et al.* 1998, Ozawa *et al.* 1999) and down-regulated after GC treatment (Burnsteinet al. 1991, Silva *et al.* 1994, Sweezey *et al.* 1998); discrepancies in their respective detection have only rarely been described (Antakly *et al.* 1989, Matthews *et al.* 1995), suggesting that various modalities of response to GC may exist in different cells and tissues.

The aim of this study was to localize GR mRNA expression in cartilage and bone cells in normal rats, and to compare its distribution with that of the GR protein distribution previously shown immuno-histochemically (Silvestrini *et al.* 1999), in order to identify which cells are responsive to GCs. To achieve this, a synthetic oligodeoxynucleotide, labeled by a Fast-Tag TM Fluorescein (FL) nucleic acid labeling method (Daniel *et al.* 1998), was used as probe.

Materials and Methods

Specimens

Six Sprague-Dawley male rats weighing 275-300 g were anesthetized with sodium pentothal, and killed with 4% paraformaldehyde dissolved in 0.1M phosphate buffer, at pH 7.3. The distal third of left femurs was removed, split longitudinally in half, decalcified in EDTA at pH 7.0 (Warshawsky and Moore 1967) for about 10 days, washed in phosphate buffer, and processed for paraffin embedding.

Oligonucleotide probe and Fast-Tag TM nucleic acid labeling method

In line with the published human glucocorticoid receptor (hGR) cDNA sequence (Hollenberg *et al.* 1985), we used a 40 base single-stranded synthetic oligonucleotide as probe (5′-TCTCT GGAAC ACTGG TCGAC CTATT GAGGT TTGCA ATGCT-3′), supplied by Oncogene Research Products, Cambridge, MA. This antisense sequence derives from sequences corresponding to the N-terminus of hGR. This GR oligo-probe was labeled by using the Fast TagTM fluorescein (FL) sample kit (Vector Lab Inc., Burlingame, CA, USA) (Daniel *et al.* 1998). The oligo-probe was coupled to the FastTagTM Reagent by heat activation (95°C for 10 min).

The GR sense probe, used for negative control, was synthesized by Life Technologies, Paisley, Scotland and similarly coupled to the Fast Tag TM FL Reagent.

Dot-blot assay

The labeling efficiency of the Fast TagTM FL system was estimated by comparing the detection sensitivity of the labeled hGR sense and antisense oligo-probes to a standardized sample of FastTagTM FL -l Hind III Dna, in a five side-by-side dot blot 10-fold dilution series, as shown in the kit. Detection and visualization of the immunoreaction on the dotted nylon membranes were performed by incubation with 1:5000 alkaline phosphatase anti-fluorescein antibody and by using BCIP/NBT alkaline phosphatase substrate (Vector Lab., Burlingame, CA, USA) (*data not shown*).

In situ hybridization (ISH) histochemistry

Sections from each specimen were mounted on slides coated with APES (3-aminopropyltriethoxysilan) (SIGMA, Steinheim, Germany) to avoid their detachment. After dewaxing, sections were pre-treated with proteinase K (SIGMA) 15mg/mL diluted in PBS for 30 min at 37°C, and washed with deionized water in DEPC (diethyl

pyrocarbonate) (SIGMA) to remove any contaminating nucleases, post-fixed in 4% buffered paraformaldehyde for 10 min, washed in PBS and in DEPC water. To avoid nonspecific bindings of the probe to positively charged free amino acid groups, the slides were washed with 1M triethanolamine (TEA) at pH 8.0 and with fresh TEA/acetic anhydride solution (0.25% acetic anhydride diluted in TEA) for 10 min, after which they were washed in DEPC water and in a 2× saline-sodium citrate solution (SSC); they were then dehydrated in DEPC graded ethanol from 50% to 100% and air dried.

Sections were incubated in the ISH buffer with 5ng/mL antisense FL-labeled probe for hGR overnight at 50°C in a humid chamber with 75% formamide. The ISH buffer was composed of 50% deionized formamide diluted in 4× SSC solution, 1× Denharts solution containing 0.2% Ficoll, polyvinylpyrrolidone in BSA (Sigma-Aldrich, St. Louis, MO, USA), 0.5 mg/mL of sheared salmon sperm DNA, 0.25 mg/mL of yeast tRNA, 10% dextran sulfate dissolved by heating and filtered with 0.45 mm syringe filter unit. Post-hybridization was performed with 2× SSC for 1h at 37°C, DNA-free RNAse (Boehringer, Germany), 40 mg/mL for 30 min at 37°C, 2× SSC for 5 min, 50% formamide in 2× SSC at 50°C for 30 min, two immersions in 2× SSC and 1× SSC of 30 min each at room temperature and DEPC water. After incubation with 10% goat serum plus 5% BSA and 0.1% Triton for 15 min the slides were incubated in 1: 200 goat alkaline phosphatase anti-fluorescein antibody in PBS with 1% BSA. After washing with PBS, the immuno-reaction was revealed by using the BCIP/NBT alkaline phosphatase substrate detection kit (Vector Laboratories, Inc., Burlingame, CA, USA) with the addition of levamisole to inhibit endogenous alkaline phosphatase activity. Slides were counterstained with Mayer's hemallum and mounted with an aqueous mounting medium.

Negative controls were achieved by using 5ng/mL sense sequences of probe previously coupled with FastTagTMFL as reported above; DNA-free RNAse treatment at 37°C from 2 to 4 hrs was performed before incubation with the probe. Incubation was also carried out in the hybridization buffer without the probe. An internal positive control resulted from the labeling of several bone marrow positive cells.

To test the efficiency of the ISH detection system, an FL-oligo-DNA probe for the detection of parathyroid hormone (PTH) mRNA sequences (Novocastra Lab., Newcastle upon Tyne, UK) was tested on human parathyroid sections treated side-by-side with ISH rat tibiae sections (data not shown).

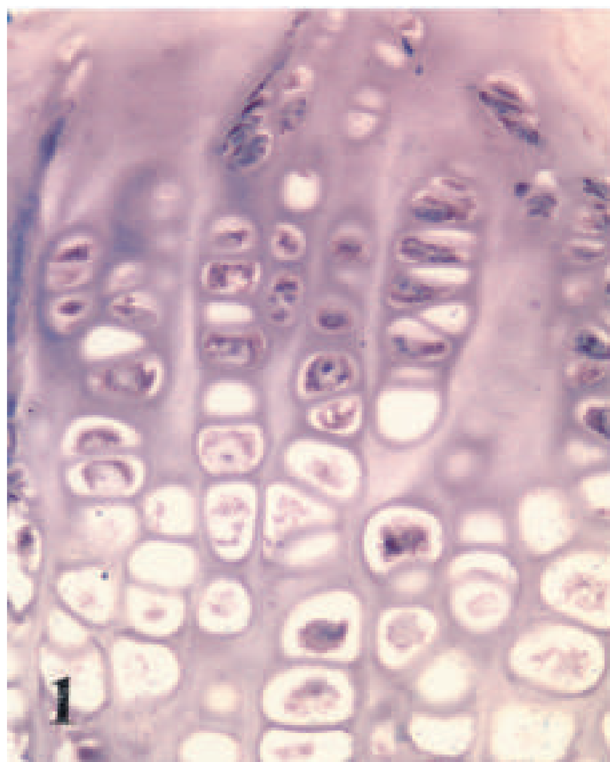

Figure 1. Growth plate cartilage: The reaction product of in situ hybridization (ISH) is visible in the cytoplasm of chondrocytes. The labeling of proliferative and maturative chondrocytes (above) is more intense than that of the hypertrophic ones (below). Matrix staining is aspecific. Nuclear Mayer's hemallum counterstaining. ×250.

Results

The ISH reaction product consisted of a granular dark blue-brownish violet precipitate in the cytoplasm.

Cartilage

In the epiphyseal growth plate, almost all the proliferative and maturative chondrocytes were labeled. Chondrocytes at the maturative stage were often more deeply stained than those at the proliferative stage, and both were more deeply labeled than hypertrophic chondrocytes (Figure 1). Perichondrium cells were highly positive. Along the chondro-osseous junction multinucleated cells corresponding to chondroclasts were deeply stained.

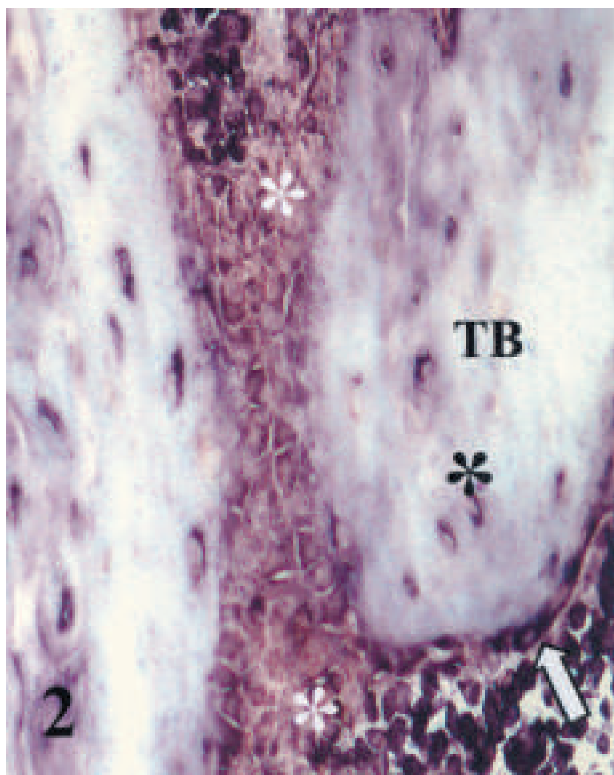

Figure 2. Metaphyseal secondary ossification area: osteogenic layer with labeled osteoblasts (white asterisks) is visible between two sections of trabecular bone (TB). A deeply stained osteoclast is shown on the left (white arrow). Osteocytes (black asterisk) are variably labeled. Nuclear Mayer's hemallum counterstaining. ×250.

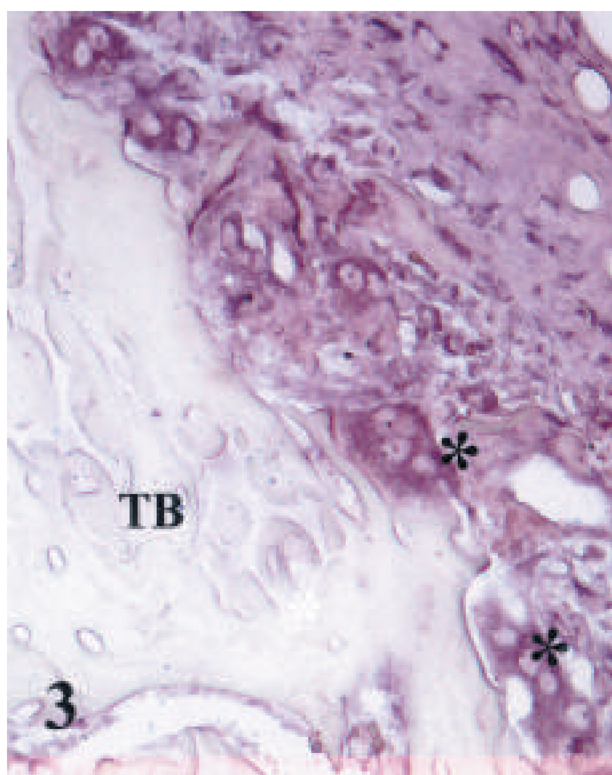

Figure 3. Periostium area: several osteoclasts (black asterisks) along a bone trabecula (TB) show staining of cytoplasm, whereas nuclei are unlabeled. The cells of the osteogenic layer are also positive. Nuclear Mayer's hemallum counterstaining. ×250.

The epiphyseal cartilage on the articular side showed staining both of proliferative and maturative/hypertrophic cells, a labeling pattern similar to that of the growth plate cartilage.

Part of the fibrocartilagineous cells at the bone-tendon interface, or enthesis (see Benjamin and Ralphs, 2001), were also deeply labeled. Labeling was chiefly found in chondrocytes contained in wide lacunae which were either scattered through the matrix or lined up along bundles of collagen fibers.

Bone

In the metaphyseal secondary ossification zone, osteoblasts (OBs) showed a variable degree of labeling, some were lightly labeled or hardly at all, whereas others showed a strong degree of labeling (Figure 2); by contrast, the OBs located in the cortical bone showed a more constant positive labeling. Lining cells were generally deeply stained. Osteoclasts (OCLs) were easily detectable because of the intense, diffuse labeling of their cytoplasm and the negativity of nuclei (Figure 2, 3). Osteocytes (OCs) were variably labeled (Figure 2). Cells of the osteogenic layer of the perichondrium, as well as OBs and OCLs, were intensely stained.

Nonspecific labeling of cartilage and bone matrices was sometimes visible. In this case, pre-treatment with 250 mg/mL RNAse A at 37°C for 30 min before proteinase K digestion removed the background, which was probably due to nucleic acid diffusion during the fixation procedure, without any visible reduction of ISH immuno-reaction.

Dot Blot assay: the limit of detection sensitivity of the two FastTagTM FL labeled (antisense and sense) probes being compared with the FL labeled standard -l Hind III DNA, was 0.2 pg/mL (*data not shown*). No differences in the efficiency of the ISH detection system was evident between the two GR FastTagTM FL or PTH FL-oligo-probes tested for ISH.

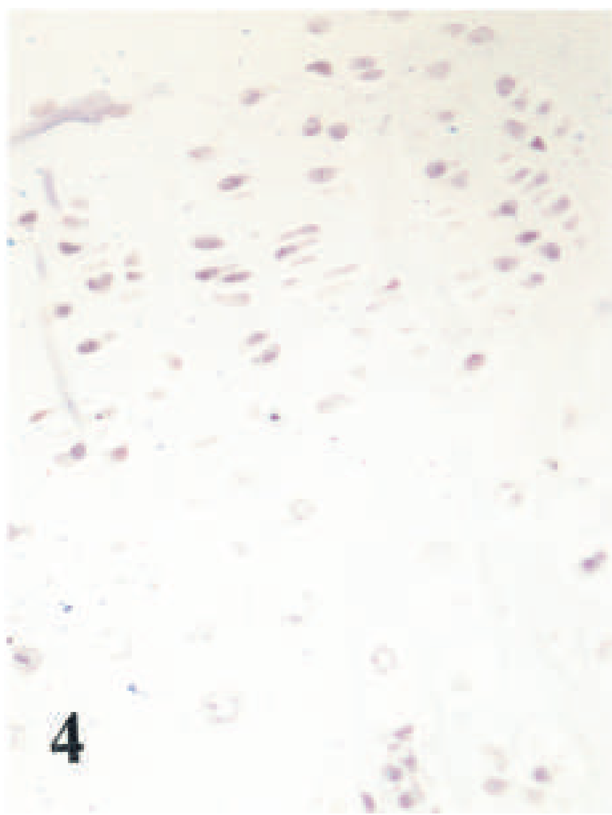

Figure 4. Growth plate cartilage: control section. Proliferative, maturative and hypertrophic chondrocytes are unlabeled. Nuclear Mayer's hemallum counterstaining. ×250.

Controls

no evidence of cell labeling was visible in the section treated with the sense oligo- probe coupled with FL FastTag™ (Figure 4).

Discussion

For the first time, the present study shows the *in vivo* distribution of GR mRNA in proliferative and maturative/hypertrophic chondrocytes of the growth plate, and in osteoblasts, lining cells, osteocytes and osteoclasts of the secondary metaphyseal ossification zone of the rat and periosteum. The GR mRNA expression generally correlates with previously immunodetected GR peptide in the same rat cells (Silvestrini *et al.* 1999), although small numerical differences were noted between cells identified using the two methods. The proliferative chondrocytes did, in fact, show a greater degree of labeling than that observed by immuno-histochemistry, probably because of a difference in the pattern of gene transcription and that of peptide expression or, more simply, because of a higher sensitivity of the ISH method. In this connection, the FastTagTM FL labeling used in this study, is a rapid, versatile method of coupling haptens, i. e., fluorochromes of affinity ligands to any nucleic acid (single or double-stranded DNA, RNA or oligonucleotides), by attaching a universal, photo or heat-activable moiety to which any sulfhydryl-reactive compound can be linked, so eliminating the need for multiple labeling kits (Daniel *et al.* 1998).

The labeling was also visible in some bone marrow cells, in chondrocytes of the epiphyseal cartilage on the articular side, in cells of tendon-bone interface, perichondrium and periosteal cells. In agreement with GR immunolocalization (Silvestrini *et al.* 1999), labeling showed variable intensity in different cell types, or even in the same cell type. In particular, besides cells that are invariably positive and deeply labeled, such as OCLs, labeling ranged from a slight or negative degree to a high degree, as in the case of OBs and OCs.

The co-localization of GR mRNA and protein appeared to be related to a different cell cycle or stage of differentiation, as suggested by the differences in degree of labeling between maturative and proliferative chondrocytes. In this connection, an ISH and immunohistochemical study carried out in the embryonic mouse secondary palate showed a strict correlation between the specific regional and temporal expression of GR mRNA and protein (Abbott *et al.* 1994). Moreover, ISH confirms the high presence of GR expression in periosteum and perichondrium cells, in agreement with analogous data obtained from embryonic tissues (Kitraki *et al.*1997), which showed high mRNA GR expression in cell populations in the earlier stages of their differentiation, which further supports a morphogenetic role for GCs.

In general, variations in the GR content or synthesis reflect variations in the degree of sensitivity to the hormone. This was demonstrated *in vitro* by a different response of OBs and OCLs to physiological or pharmacological hormone treatment (Wong *et al.* 1979), which suggested a higher resistance of OCLs to GCs. Moreover, our finding that GR mRNA and protein (Silvestrini *et al.* 1999) are invariably co-expressed in OCLs, and the fact that they did not show any detectable GR down-regulation to high-dose GC administration either by immunohistochemistry (Silvestrini *et al.* 2000) or by ISH meth-

ods (unpublished results), both support the general consensus view that there is no direct OCL involvement in GC-induced osteoporosis in the rat. In addition, the GR-mediated molecular interrelations which regulate the GC-induced response of OBs, OCs and OCLs are still unknown.

In this connection, the presence of GR mRNA and protein in the chondrocytes of bone-tendon interface, and the fact that apoptotic, i.e., TUNEL-positive, cells were detectable in the same area (Silvestrini et al., 1998), suggest a major sensitivity of these cells to hormonal or traumatic stimuli. This could be of clinical relevance in relationship to the development of enthesitis (Benjamin and McGonagle, 2001).

Recently, molecular GC effects have been found in some kinds of cell. They are based on the previously described (McConkey and Orrenius 1996) and now revisited (Buttgereit *et al.* 2000, Patschan *et al.* 2001) presence of GR membrane-bound receptors (Sanden *et al.* 2000, Patschan *et al.* 2001) and/or on physicochemical interactions with cellular membranes. These non-genomic mechanisms partly clarify the rapidity of hormonal action in the cell with respect to the classically genomic ones (Bamberger *et al.* 1996) and appear to occur in osteoporosis or apoptosis induced by GC-high-dose chronic administration (Patschan et al 2001).

OCs showed variable labeling; in this connection they appeared highly sensitive to high-dose GC-administration of GR protein content (Silvestrini *et al.* 2000), in agreement with their important role as the numerically best represented cells in bone, involved in transmitting mechanical, physicochemical or hormonal stimuli from or to other cells (Palazzini *et al.* 1998).

In conclusion, we have shown a co-localization of both GR protein and GRmRNA expression in cartilage and bone cells. The variability of labeling appeared related to the cell cycle, the stage of differentiation and cell-type differences. Recently, a GC-mediated decrease in circulating osteoprotegerin (OPG) — a novel cytokine inhibitor of differentiation and activation of OCLs — has been shown in a short-term GC administration (Sasaki *et al.* 2001). This opens up new perspectives on the possible existence of GR-mediated mechanisms of OPG regulation in bone, both in normal and in pathological conditions.

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