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Altered hypertrophic chondrocyte kinetics in GDF-5 deficient murine tibial growth plates

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Abstract

The growth/differentiation factors (GDFs) are a subgroup of the bone morphogenetic proteins best known for their role in joint formation and chondrogenesis. Mice deficient in one of these signaling proteins, GDF-5, exhibit numerous skeletal abnormalities, including shortened limb bones. The primary aim of this study was determine whether GDF-5 deficiency would alter the growth rate in growth plates from the long bones in mice and, if so, how this is achieved. Stereologic and cell kinetic parameters in proximal tibial growth plates from 5-week-old female GDF-5 −/− mice and control littermates were examined. GDF-5 deficiency resulted in a statistically significant reduction in growth rate (−14%, $p = 0.03$). The effect of genotype on growth rate was associated with an altered hypertrophic phase duration, with hypertrophic cells from GDF-5 deficient mice exhibiting a significantly longer phase duration compared to control littermates (+25%, $p = 0.006$). These data suggest that one way in which GDF-5 might modulate the rate of endochondral bone growth could be by affecting the duration of the hypertrophic phase in growth plate chondrocytes.

Keywords: Growth plate; GDF-5; Endochondral ossification; Growth/differentiation factors; Bone morphogenetic proteins; Chondrocyte

Introduction

One family of molecules known to play an import role in endochondral ossification is that of the bone morphogenetic proteins (BMPs): a highly conserved class of secreted signaling molecules belonging to the TGF-β superfamily. These proteins were originally identified based on their ability to induce ectopic cartilage and bone formation when implanted subcutaneously in rodents [15,21] and are now known to play a wide variety of roles in the skeletal system, including the promotion of cell proliferation, differentiation, and apoptosis, as well as influencing chondrogenesis, osteogenesis, and angiogenesis [7].

Growth/differentiation factor-5 (GDF-5) is a member of the GDF subfamily of bone morphogenetic proteins (BMPs). In 1994, Kingsley and coworkers discovered that the *brachypodism* (*bp*) phenotype in mice is caused by a mutation in the gene for GDF-5, thus identifying a unique animal model for studying the role of GDF-5 in vertebrates [16]. The *bp* mouse exhibits a range of skeletal abnormalities, including a reduction in the length of the long bones of the limbs, abnormal joint development in the limb and sternum, and a reduction in the number of bones in several of the digits [6,9,16]. It has been hypothesized that these abnormalities are due, at least in part, to a defect in the early stages of chondrogenesis and cell recruitment, as GDF-5 is capable of modulating chondrogenesis by increasing cell adhesion as well as chondrocyte proliferation [5]. GDF-5 also appears capable of promoting chondrocyte maturation and hypertrophy, but its role in these processes has not been fully characterized [3,10,17]. Based on the skeletal phenotype of the GDF-5 deficient *bp* mouse and the related human disorders of Hunter-Thompson and Grebe-type achromesomelic chondrodysplasias which are caused by mutations in the gene for CDMP-1/GDF-5 [14,18,19], it is likely that GDF-5 plays a role in endochondral ossification that may be elucidated by studying the growth plates of post-natal animals.

The objective of this study was to determine whether GDF-5 deficiency would alter the growth rate in growth

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plates from the long bones of *bp* mice. Second, we aimed to determine the basis for any observed growth rate differences (i.e. altered phase durations and/or structural parameters such as zone height or cell size). These studies were carried out by examining growth plate stereologic parameters and cell kinetics (i.e. dynamic parameters related to chondrocyte development and maturation) in the proximal tibial growth plates from 5-week-old female GDF-5 −/− mice and their age-matched, phenotypically normal control littermates. We found that GDF-5 deficiency resulted in a significant reduction of growth rate, which was associated with a significantly longer hypertrophic phase duration in GDF-5 deficient mice. These data suggest that one way in which GDF-5 may modulate the rate of endochondral bone growth could be by affecting the duration of the hypertrophic phase in growth plate chondrocytes.

Materials and methods

Animal model

Two groups of ten, 5-week-old healthy female mice were used to assess growth plate stereologic parameters and chondrocyte kinetics in the proximal tibia. This site was chosen as it provides the largest growth plate available for analysis in the mouse, thereby allowing fewer total animals to be used to obtain statistically significant results using systematic random sampling methods. Experimental animals consisted of GDF-5 −/− *brachypod* mice obtained from Jackson Laboratories (Bar Harbor, ME). The *bp* mutation arises from a frameshift mutation in the gene for GDF-5, resulting in a translational stop codon prior to the mature signaling portion of the molecule, this producing a functional null mutation in −/− mice [16]. Mutant animals are easily identified at birth due to the small size of their fore and hind paws. Control animals consisted of phenotypically normal littermates (note that genotyping was not performed to distinguish between +/− and +/+ animals). Animals were housed under 12 h light/dark conditions. At 5 weeks of age, all animals were injected intraperitoneally with 10 mg/kg of calceine in sterile 0.9% saline solution (Sigma Aldrich, St. Louis, MO) at 9:00 AM and euthanized exactly four days later at 9:00 AM by CO_2 inhalation. The calceine label was used to determine the growth rate for each individual growth plate in microns per day as described below. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Detailed methods for obtaining all stereologic and cell kinetic measurements were based on the work of Bailón-Plaza et al. [1] and are described below as adapted for the present study.

Sample preparation

Left and right tibiae were immediately dissected free from soft tissue and further cleaned under a dissecting microscope in fixative solution (2% glutaraldehyde in 0.1 M Cacodylate Buffer, pH 7.4, 330 mOsm). The proximal ends of the bones were cut with a sharp razor blade below the metaphysis. Specimens from the left limb were then cut in two along a mid-saggital plane, and specimens from the right limb were cut in two along a mid-frontal plane. One block from each side was placed in fixative solution containing 0.7% (w/v) ruthenium hexammine trichloride (RHT; Alfa Aesar, Ward Hill, MA) for 3–5 h on a rocker at room temperature to preserve the morphology of hypertophic chondrocytes [8]. The other block was placed in 40% ethanol and was not used further for the purposes of this study.

Samples were washed 3×5 min with 0.1 M Cacodylate Buffer, pH 7.4, 330 mOsm and then dehydrated through graded ethanol solutions. Infiltration in Epon plastic (Sigma Aldrich, St. Louis, MO) was performed with 4 changes of 15 min each in propylene oxide (PPO) followed by 24 h of PPO:Epon at 3:1, 24 h at 1:1, 72 h at 1:3, and 24 h in 100% Epon. Specimens were then allowed to polymerize for 7 days at 60 °C. One-micron thick vertical sections were cut using a cutting plane of 0° or 1.5° rotation about the vertical axis (randomly assigned to left or right blocks) using an autocut ultramicrotome (Leica, Vienna, Austria). All sections included bone from both the epiphysis and the metaphysis of each block. Alternating serial sections were either mounted on microscope slides, stained with toluidine blue and coverslipped for stereologic measurements, or mounted on slides without staining for visualization of calceine labels. A total of twenty alternating serial sections were processed in this way per side (left or right) for each mouse. All subsequent analyses were performed by a single blinded investigator (RTC).

Zone heights

Twelve stained sections per growth plate were viewed at 20× magnification and images captured using a Sony DKC 5000 color digital video camera (Sony Electronics, New York). Images were aligned so that the direction of growth was vertical on the computer screen prior to image capture. Using Adobe PhotoShop 5.0 (Adobe Systems, Palo Alto, CA), the heights of the growth plate and each growth plate zone were obtained by delineating the top of the growth plate, the junctions between the resting and proliferative zones, and between the proliferative and hypertrophic zones based on morphological characteristics of the chondrocytes, as well as the chondrosseous junction based on changes in matrix staining. The vertical height of each zone was then measured at four equidistant locations per section ($n = 48$ per growth plate). Values for each individual mouse were obtained by averaging all 96 measurements from the left and right sides.

Cell area fraction and mean cell volume

Using point counting methods on seventeen high magnification sections per growth plate (34 sections per mouse at approximately 800×), cell area fraction was estimated in the proliferative and hypertrophic zones (area occupied by cells in the region of interest as a percentage of the total area within the region of interest). Mean cell volume (MCV) was estimated in the proliferative and hypertrophic zones using point-sampled intercepts measured on cells lying on grid intersection points [4]. For each growth plate, seventeen sections were analyzed for MCV estimates to obtain a minimum of 300 intercepts per mouse which were then averaged together. A different grid angle was used on each field of view (3°R, 7°L, 15°R, 22°L, 34°R, 46°L, 64°R, 82°L, 5°R, 9°L, 16°R, 28°L, 40°R, 52°L, 76°R, 94°L, 1°R).

Cell numbers

Cell populations in the proliferative and hypertrophic zones were estimated by counting chondrocyte numbers in a reference volume of a cylinder of 1 mm diameter with height equal to the height of the growth plate zone being analyzed [1,22]. The number of cells per cylinder in a given zone was then calculated as

$$\text{cells/cylinder} = [0.25\pi \times (\text{cell area fraction}) \times (\text{zone height})]/\text{MCV} \quad (1)$$

where area fraction, zone height, and mean cell volume were obtained as previously described.

Growth rate

The calceine label was visualized using appropriate objectives on a Nikon Eclipse E600 epifluorescence microscope (Nikon Instruments, Melville, NY). The center of the green label and the chondrosseous junction were outlined on six sections per growth plate (12 per mouse) and the vertical distance between the two curves was measured at four equidistant locations per image. Measurements (μm) were divided by four (days) and then averaged together to obtain a single value for the growth rate of each growth plate for each mouse.

Cells lost per day and phase durations

Wilsman et al. have shown that chondrocyte activity in the growth plate is in steady-state over a 24 h period [22]. Thus, the number of proliferative cells produced each day via differentiation from the resting zone is the same as the number of cells that differentiate into hypertrophic chondrocytes as well as the number of cells lost each day at the chondrosseous junction [1,22]. Consequently, the number of cells lost per day can be estimated as the number of cells lost per day in a reference volume cylinder of 1mm diameter and height equal to the amount of growth that occurs in a 24 h period:

$$\text{cells lost/day} = [0.25\pi \times (\text{cell area fraction})_{\text{hyp}} \times (\text{growth rate})]/\text{MCV}_{\text{hyp}} \quad (2)$$

The durations of the proliferative and hypertrophic cell phases can then be calculated as

$$\text{phase duration} = (\text{cells/cylinder})/(\text{cells lost/h}) \quad (3)$$

Statistical analysis

The following parameters were analyzed using a one-factor ANOVA with genotype as the independent factor: growth rate; growth plate height and height of the resting, proliferative and hypertrophic zones; mean cell volume of the proliferative and hypertrophic zones; number of cells per cylinder in the proliferative and hypertrophic zones; proliferative and hypertrophic phase durations; and number of cells lost per day. All analyses were performed using the statistical software Statview (Abacus Concepts, Berkeley, CA) with a cutoff level for statistical significance chosen as $p < 0.05$.

Results

Histologically, there were no discernable distinctions in the morphology of GDF-5 deficient versus control growth plates (Fig. 1). Mutant proximal tibial growth plates exhibited a statistically significant 14% reduction in growth rate (Fig. 2; $p = 0.03$) as well as a 25% increase in hypertrophic cell phase duration ($p = 0.006$; Table 1). Based on reported *bp* bone lengths in the literature [9,11,17], we estimated that tibial growth rates should be approximately 70 μm/day in 5-week-old GDF-5 –/– mice and 85 μm/day in control littermates. These estimates agree well with the values obtained in the present study: 68 ± 11 μm/day in mutants vs. 79 ± 11 μm/day in controls. The number of hypertrophic cells per cylinder was 12% greater in mutants, but the effect of genotype on this variable was not quite large enough to reach statistical significance ($p = 0.08$). No differences in cell size or zone heights were detected between genotypes.

Discussion

The objective of this study was to determine whether GDF-5 deficiency significantly affects the rate of endo-

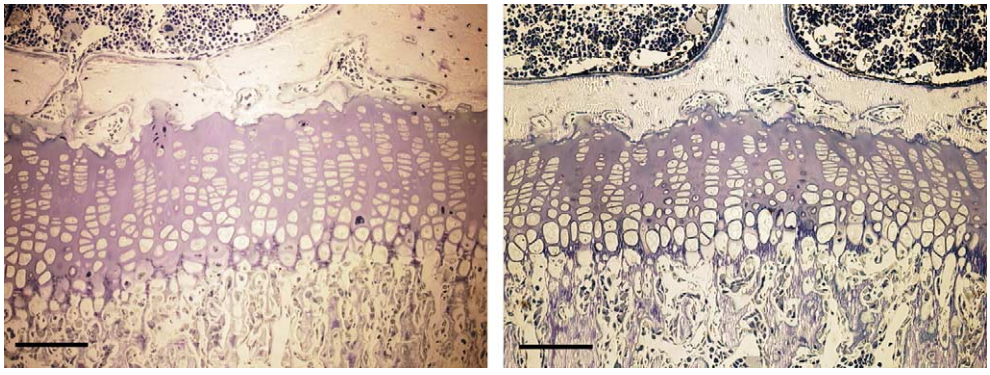

Fig. 1. Control (left) and GDF-5 deficient (right) proximal tibial growth plates stained with toluidine blue. Bar represents approximately 100 μm.

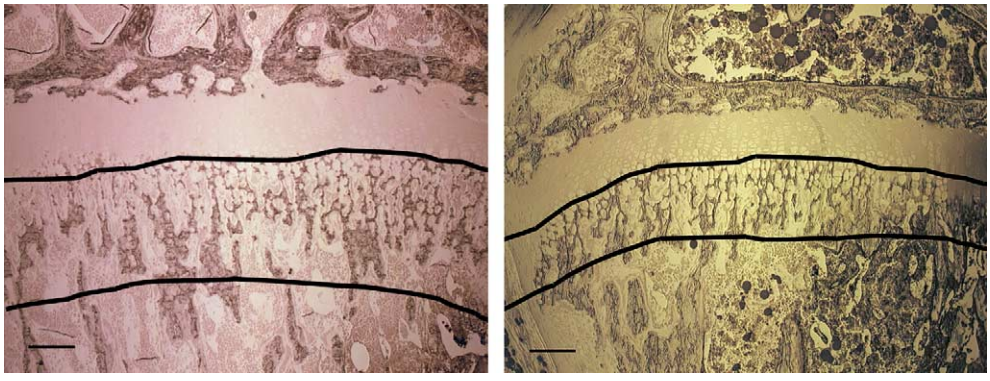

Fig. 2. Measurement technique used to determine growth rates in control (left) and GDF-5 deficient (right) proximal tibial growth plates. Upper curve delineates chondrosseous junction and lower curve indicates boundary of calceine label with region in between representing the total growth that has occurred in four days time. (Note that the calceine label itself is not shown on these figures.) Bar indicates approximately 100 μm.

Table 1
Proximal tibial growth plate stereologic parameters from 5-week-old female GDF-5 (−/−) and control mice

Parameter	Mutant $n = 10$	Control $n = 10$	Difference (%)
Growth rate (μm/day)	68.1 ± 11.2	79.4 ± 10.6	−14[a]
Growth plate height (μm)	203.3 ± 9.9	203.2 ± 11.5	<1
Resting zone height (μm)	31.9 ± 4.0	32.2 ± 3.7	<1
Proliferative zone height (μm)	92.1 ± 6.1	95.9 ± 7.1	−4
Proliferative zone mean cell volume (μm^3)	713 ± 88.2	658 ± 100.4	8
Hypertrophic zone height (μm)	79.4 ± 6.3	75.1 ± 6.8	6
Hypertrophic zone mean cell volume (μm^3)	3040 ± 434	3090 ± 478	−1
Cells lost/day	7530 ± 1631	8320 ± 1256	−9
Proliferative zone cells/cylinder (mm^{-3})	31,800 ± 4909	32,900 ± 4851	−3
Proliferative zone phase duration (h)	106.4 ± 23.6	96.7 ± 16.6	10
Hypertrophic zone cells/cylinder (mm^{-3})	8760 ± 1318	7830 ± 877	12
Hypertrophic zone phase duration (h)	28.7 ± 4.8	23.0 ± 3.3	25[a]

[a] $p < 0.05$.

chondral bone growth, and, if so, how this is achieved. We accomplished this by analyzing stereologic and cell kinetic parameters in proximal tibial growth plates from 5-week-old female GDF-5 −/− mice and their phenotypically normal control littermates. GDF-5 deficiency resulted in a significant reduction in growth rate (−14%; $p = 0.03$) that was associated with an altered hypertrophic phase duration, with hypertrophic cells from GDF-5 deficient mice exhibiting a significantly longer phase duration compared to control littermates (+25%, $p = 0.006$). These data suggest that one way in which GDF-5 can modulate the rate of endochondral bone growth may be by affecting the duration of the hypertrophic phase in growth plate chondrocytes.

Several pieces of evidence exist to support a possible link between GDF-5 and chondrocyte hypertrophy. Administration of exogenous GDF-5 protein has been shown to stimulate cartilage growth and differentiation, as evidenced by a 'halo' of more mature chondrocytes that were larger and produced more matrix in the immediate vicinity of GDF-5 coated beads implanted into chick digit cartilage [10,16]. In addition, Chang et al. were able to localize CDMP-1 (GDF-5) expression to hypertrophic chondrocytes in developing human long bones, although CDMP-1 was predominantly detected at the stage of precartilaginous mesenchymal condensation [2]. Further, strong expression of the closely related family member CDMP-2 (GDF-6) was found in hypertrophic chondrocytes, with protein found in the surrounding cartilaginous matrix. More recently, Coleman and Tuan have shown that overexpression of GDF-5 via retroviral infection of embryonic chick limb bud cells results in enhanced chondocyte hypertrophy and maturation [3]. In addition, Tsumaki et al. [20] demonstrated that transgenic mice with targetted expression of recombinant CDMP-1 exhibited a larger hypertrophic zone and smaller proliferative zone, presumably due to an accelerated rate of chondrocyte maturation from proliferation to hypertrophy. An increased number of chondrocyte progenitor cells was also observed by these investigators. In conjunction with the present study, the data from these other studies strongly support a possible role for GDF-5 (and/or related GDF family members) in the later stages of chondrocyte maturation and hypertrophy.

It is also possible, however, that the effect of GDF-5 on chondrocyte hypertrophic phase duration is an indirect one. For example, it has been shown that GDF-5 is capable of inducing angiogenesis in both chick chorioallantoic membrane and rat cornea assays [23]. The lengthening of the hypertrophic phase observed in the present study could be due to an indirect effect of GDF-5 action on the process of angiogenesis at the chondrosseous junction, although one would then expect to see a significant reduction in chondrocyte removal in GDF-5 deficient growth plates which was not found in the present study. More detailed molecular studies regarding the process of vascular invasion and removal of terminal chondrocytes will be required to determine if the observed effect of GDF-5 deficiency on hypertrophic phase duration can be explained by the angiogenic capabilities of this particular molecule.

An alternative explanation for the observed effects of GDF-5 deficiency on hypertrophic growth plate chondrocytes is possible overcompensation by related GDF family members (GDF-6 and/or GDF-7) in the absence of GDF-5. CDMP-2 (GDF-6) is strongly expressed in hypertrophic chondrocytes [2] and it is known to bind to BMPR-IA, the predominant type I receptor found in more mature regions of the growth plate. Although GDF-5 preferentially binds to BMPR-IB, it is capable of binding to IA in the presence of different type II receptors [13]. In addition, GDF-5 is highly expressed in the synovial joints of the limbs. Whether or not the action of this molecule at the joint surface can affect cellular activity some distance away within the growth plate remains to be determined. Immunolocalization and in situ hybridization detection of various GDF

proteins and mRNA as well as BMP receptor localization in mutant growth plates would help to clarify these possibilities, but were beyond the scope of the present study.

To date, only one other study has examined the growth plate of *brachypod* mice in any detail. Nakamura et al. [12] used tritiated thymidine autoradiography to show that the rate of chondrocyte proliferation was reduced in the distal femur and proximal tibia of 22-day-old mutant mice ($n = 3$ per group, gender unspecified). Although these authors report that the height of the hypertrophic zone "seemed to be shorter" in the mutants, no measurements of zone height were performed, and all other light and electron microscopy observations were described as normal. Given the extremely small sample sizes used in their study, the lack of gender specification and differences in methodology, it is difficult to compare our own results with those reported by these investigators. In addition, the *brp* mouse reported on by Nakamura et al. was discovered in Japan in 1974 and has not yet, to our knowledge, been confirmed to be the result of a mutation in GDF-5.

In conclusion, based on stereologic analyses of proximal tibial growth plates in 5-week-old female mice, we found that GDF-5 deficiency resulted in a significant reduction in growth rate. The effect of genotype on growth rate was associated with an altered hypertrophic phase duration, with hypertrophic cells from GDF-5 deficient mice exhibiting a longer phase duration compared to control littermates. These data suggest that one way in which GDF-5 can modulate the rate of endochondral ossification may be by affecting the duration of the hypertrophic phase in growth plate chondrocytes. The possible interaction of GDF-5 with related GDF and BMP family members will need to be examined in order to understand how various combinations of BMP signals regulate growth plate cellular activity.

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References

[1] Bailón-Plaza A, Lee AO, Veson EC, Farnum CE, van der Meulen MCH. BMP-5 deficiency alters chondrocyte activity in the mouse proximal tibial growth plate. Bone 1999;24:211–6.
[2] Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJ, et al. Cartilage-derived morphogenetic proteins. J Biol Chem 1994;269:28227–34.
[3] Coleman CM, Tuan RS. Growth/differentiation factor 5 enhances chondrocyte maturation and hypertrophy in vitro. Trans ORS 2003; 49th annual meeting: 0471.
[4] Cruz-Orive LM, Hunziker EB. Stereology for anisotropic cells: application to growth cartilage. J Microsc 1986;143:47–80.
[5] Francis-West PH, Abdelfattah A, Chen P, Allen C, Parish J, Ladher R, et al. Mechanisms of GDF-5 action during skeletal development. Development 1999;126:1305–15.
[6] Grünenberg H, Lee AJ. The anatomy and development of brachypodism in the mouse. J Embryol Exp Morphol 1973;30:119–41.
[7] Hogan BLM. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 1996;10:1580–94.
[8] Hunziker EB, Herrman W, Schenk RK. Improved cartilage fixation by ruthenium hexamine trichloride (RHT): a prerequisite for morphometry in growth cartilage. J Ultrastruct Res 1982;81:1–12.
[9] Landauer W. Brachypodism: a recessive mutation of house-mice. J Hered 1952;44:293–8.
[10] Merino R, Macias D, Gañan Y, Economides AN, Wang X, Wu Q, et al. Expression and function of GDF-5 during digit skeletogenesis in the embryonic chick leg bud. Dev Biol 1999;26:33–4.
[11] Mikic BM, Battaglia TC, Taylor EA, Clark RT. The effect of GDF-5 deficiency on femoral composition and mechanical behavior in mice. Bone 2002;30(5):733–7.
[12] Nakamura K, Hirosawa K, Hama K, Oda S-I. Defects in the growth plate of brachypodism mice—light and electron microscopic findings and cell proliferation by the method of tritiated thymidine autoradiography. J Jpn Orthop Assoc 1984;58:835–45.
[13] Nishito H, Ichijo H, Kimura M, Matsumoto T, Makishima F, Yamaguchi A, et al. Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. J Biol Chem 1996;271:21345–52.
[14] Polinkovsky A, Robin NH, Thomas TJ, Irons M, Lynn A, Goodman FR, et al. Mutations in CDMP-1 cause autosomal dominant brachydactyly type C. Nat Genet 1997;17:18–9.
[15] Sampath TK, Reddi AH. Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci USA 1981;78(12):7599–603.
[16] Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, Lee S-J. Limb alterations in brachypodism mice due to mutations in a new member of the TGF-beta superfamily. Nature 1994;368:639–43.
[17] Storm EE, Kingsley DM. Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. Development 1996;122:3969–79.
[18] Thomas JT, Kilpatrsick MW, Lin K, Erlcher L, Lembessis P, Costa T, et al. Disruption of human limb morphogenesis by a dominant negative mutations in CDMP1. Nat Genet 1997;17:58–64.
[19] Thomas JT, Lin K, Nandedkar M, Camargo M, Cervanka J, Luyten FP. A human chondrdysplasia due to a mutation in a TGF-beta superfamily member. Nat Genet 1996;12:315–7.
[20] Tsumaki N, Tanaka K, Arikawa-Hirasawa E, Nakase T, Kimura T, Thomas JT, et al. Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. J Cell Biol 1999;144:161–73.
[21] Urist M. Bone: formation by autoinduction. Science 1965;150:893–9.
[22] Wilsman NJ, Farnum CE, Leiferman EM, Fry M, Barreto C. Differential growth by growth plates as a function of multiple parameters of chondrocyte kinetics. J Orthop Res 1996;14:927–36.
[23] Yamashita H, Shimizu A, Nishitoh H, Ichijo H, Hanyu A, Morita I, et al. Growth/differentiation factor-5 induces angiogenesis in vivo. Exp Cell Res 1997;235:218–26.