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Intraspecies disparity in growth rate is associated with differences in expression of local growth plate regulators

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Tryfonidou MA, Hazewinkel HA, Riemers FM, Brinkhof B, Penning LC, Karperien M. Intraspecies disparity in growth rate is associated with differences in expression of local growth plate regulators. *Am J Physiol Endocrinol Metab* 299: E1044–E1052, 2010. First published September 21, 2010; doi:10.1152/ajpendo.00170.2010.—Disparities in longitudinal growth within a species can be partly explained by endocrinological differences. We hypothesized that regulatory networks acting locally in the growth plate may also be important. We tested this hypothesis by evaluating the IGF/IGFBP expression, the vitamin D pathway, and the PTHrP-Indian hedgehog (IHH) feedback loop in rib growth plates from 10- and 21-wk-old small- (Miniature Poodles, MP) and large-breed dogs (Great Danes, GD) using immunohistochemistry and quantitative (q)PCR. The rib growth plates of GD were 1.7 times thicker compared with those of MP, with larger proliferative (in absolute terms) and larger hypertrophic (in absolute and relative terms) zones. IGF/IGFBP gene expression profiling of the growth plates revealed decreased gene expression of *igfbp2*, *-4*, and *-6* and an unaltered expression of *igf-I* and *igf-II* and their respective receptors in GD vs. MP. Immunohistochemistry and qPCR findings showed that the vitamin D pathway was more active in GD than in MP. Staining for 1α - and 24-hydroxylase was more abundant and intense in GD and the gene expressions of *1\alpha*-hydroxylase and the vitamin D receptor-driven 24-hydroxylase were six- and eightfold higher in GD vs. MP, respectively. Consistent with the immunohistochemistry findings, the expression of mRNA for components of the parathyroid hormone-related peptide (PTHrP)-IHH loop was different in GD compared with MP, with there being a relative threefold downregulation of *Pthrp* and a tenfold upregulation of *Ihh* in GD vs. MP. These differences suggest that the effects of IHH in the regulation of chondrocyte proliferation and hypertrophy, both independently of PTHrP, can become more dominant during rapid growth rates. In conclusion, our data suggest that, in addition to modest endocrine differences, more pronounced changes in the expression of locally acting regulatory networks, such as the IGF system, vitamin D pathway, and PTHrP-IHH feedback loop are important contributors to within-species disparities in growth rates.

canine; growth plate; vitamin D; Indian hedgehog; parathyroid hormone-related peptide; insulin-like growth factor

THE REGULATION OF PREPUBERTAL longitudinal bone growth is complex and orchestrated by a large network of endocrine regulators, including GH, IGF-I, thyroxine (T_4), and vitamin D (14, 26). Although the GH-IGF-I axis is considered the most important endocrine modulator of longitudinal bone growth and body size (26), differences in body size, and thus in bone growth rate, are not solely due to endocrine differences in the

GH-IGF-I axis. For example, GH production, secretion, and/or action are apparently normal in children with idiopathic short stature, and these children achieve only a modest increase in height after long-term GH treatment (10). Longitudinal bone growth is also thought to be influenced by autocrine/paracrine signaling pathways acting at the level of the epiphyseal growth plate, regulating chondrocyte proliferation and differentiation. For example, both the vitamin D pathway and the PTHrP-Indian hedgehog (IHH) feedback loop have been shown to regulate growth plate activity in an intracrine manner (1, 39). $1,25(\text{OH})_2\text{D}_3$ acting through the vitamin D receptor (VDR) regulates intestinal absorption of calcium and phosphate as well as bone remodeling and is thought to be involved in the terminal differentiation of chondrocytes in endochondral ossification (23, 24). The growth-restraining feedback loop of parathyroid hormone-related peptide (PTHrP) and IHH plays a pivotal role during pre- and postnatal endochondral bone formation by determining the pace and synchrony of chondrocyte transition from proliferation into hypertrophic differentiation (17, 40). Moreover, IHH is a potent inducer of chondrocyte proliferation and hypertrophy independent of PTHrP (16, 21). Genome-wide association studies have shown that the VDR polymorphisms are associated with idiopathic short stature in humans (5) and that VDR gene polymorphisms (19, 35) and the IHH signaling pathway (18, 42) are in part responsible for the variation in final adult height.

The disparity in growth rate and, hence, in mature bone length is greater in dogs than in any other species. Although a single IGF-I allele was recently reported to be a major determinant of small size in dogs (34), other factors are also involved. For example, compared with small-breed dogs (e.g., Miniature Poodles, MP), Great Danes (GD) go through a period of juvenile gigantism, characterized by raised plasma GH levels that decline gradually during maturity (25, 38). During this period of growth, the antebrachium increases in length by $90.1 \pm 10.1\%$ in GD compared with $71.6 \pm 2.9\%$ in MP (38). Treatment of juvenile MP with GH does not reconstitute a GD growth pattern, even though this treatment results in fourfold higher plasma GH levels and in two- to threefold higher plasma IGF-I levels in MP than in GD (36, 37). We have previously shown a modest difference in plasma $1,25(\text{OH})_2\text{D}_3$ levels between these breeds, whereas there were no differences in renal *1\alpha*-hydroxylase (*1\alpha*-OHase) mRNA, plasma calcium, and PTH levels between the two breeds (38). Together, these observations indicate that, besides endocrine mechanisms, other local regulators play a major role in determining chondrocyte proliferation and differentiation in the epiphyseal growth plate.

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We hypothesized that differences in the expression and activity of regulatory networks acting locally in the epiphyseal growth plate are important mediators of within-species disparities in growth. Dogs represent an excellent model for studying this hypothesis, because there is large disparity in growth rates among different dog breeds (27). We studied the morphology and morphometry of the growth plate and the quantitative pattern of gene expression of components of the IGF axis, the vitamin D pathway, and the PTHrP-IHH feedback loop in growth plate samples from growing GD and MP raised under identical conditions. To take into account possible differences in skeletal maturity (33) with immunohistochemistry, both 10- and 21-wk-old dogs were used.

MATERIALS AND METHODS

Animals and diets. The *in vivo* experiments have been described elsewhere (38); all procedures were approved by the Utrecht University Ethics Committee for Animal Care and Use. Briefly, seven GD (3 male and 4 female) and seven MP (3 male and 4 female) were raised until the age of 21 wk on a balanced diet (9.5 g calcium and 7.5 g phosphate per 100 g diet dry matter and 11.4 μ g vitamin D₃ per kg diet). Growth curves and plasma levels of the relevant endocrine regulators during growth for both in GD and MP have been reported before and are summarized in Supplementary Fig. S1 (supplementary materials are found in the online version of this paper).

Tissue sampling and processing. At 10 wk of age, rib biopsies were obtained from the 9th left rib by partial rib resection under general anesthesia. After euthanasia, when the dogs were 21 wk age, biopsies were taken from the 8th and 9th right ribs. A single growth plate is responsible for rib length, and the 9th rib is the largest rib in the canine species and is thus a model of a rapidly growing growth plate. The rib growth plate and adjacent metaphysis share the morphology and morphometry of other rapidly growing growth plates, such as the proximal humerus, distal radius and ulna, and proximal tibia (11). In humans, the rib growth plate is an indicator of growth in skeletally immature patients, and its closure signifies growth cessation (12). The growth plate of the 8th rib with 1 mm of adjacent metaphysis was dissected from the surrounding tissues and immediately frozen in liquid nitrogen and stored at -70°C until further use. The 9th rib biopsy sample was cut longitudinally into slices containing costal and growth plate cartilage and part of the adjacent metaphysis. The slices were fixed in 4% buffered formalin and decalcified in 10% EDTA. Paired GD and MP samples were embedded in paraffin.

Morphometry of the 9th rib growth plate at 10 and 21 wk of age. A semiautomated image analyzing system using KS 400 software (Carl Zeiss Vision) was used to measure the mean width of the growth plate and the proliferative and hypertrophic zones in midsagittal sections of the growth plate. The proliferative zone comprised the region containing columnar chondrocytes of constant size. The hypertrophic zone was defined by collagen X expression. The number of cells within the proliferative zone was determined in triplicate per growth plate by defining the area comprising three columns of chondrocytes, to overcome the possible lack of continuous proliferative chondrocyte columns. The number of cells within the hypertrophic zone was determined at 10 locations in each growth plate because the hypertrophic chondrocytes are arranged in continuous columns. The perimeter of late hypertrophic chondrocytes, i.e., the last two rows of chondrocytes adjacent to the metaphyseal bone, was determined for the complete area of the growth plate.

Quantitative determination of the expression of target genes at 21 wk of age. The genes *igf-I*, *igf-I receptor (igf-I R)*, *igf-I binding proteins (igfbp) 1 through 6*, and *igf-II*, and *igf-II receptor (igf-II R)* were used to study the local IGF system. *Vdr*, *1 α -OHase*, and the VDR-driven *24-OHase* were used to study the vitamin D pathway. The genes *Pthrp* and its receptor *Pthrl* and *Ihh* and its receptors

Smoothed (Smo) and the IHH-driven *Patched (Ptc)* were used to study the PTHrP-IHH feedback loop.

Frozen growth plate tissue from the 8th rib was ground in pre-frozen cups of a micro-dismembrator (Micro-Dismembrator U, B. Braun Biotech International) using two cycles of 2,200 rpm for 45 s. Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Science). After on-column DNase-I treatment (Qiagen), cDNA synthesis was performed (iScript cDNA Synthesis Kit, Bio-Rad) according to standard operation protocols.

Primer design and testing. The primer sets were based on those available on public databases (www.ensembl.org or www.ncbi.nlm.nih.gov) and designed with Oligo explorer 1.1.0 software (www.genelink.com/tools/gl-downloads.asp; Supplementary Table S2). No minus-RT amplicons were observed.

Quantitative PCR. Quantitative PCR was performed using a Bio-Rad My-IQ PCR-machine and IQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Standard curves were prepared by plotting the log of the starting amount vs. the threshold cycle, using serial three- or fourfold dilutions of template. The amplification efficiency, E (%) = $[10^{(1/s)} - 1] \times 100$ (s = slope), of each standard curve was determined and was $>90\%$ and $<110\%$. For each experimental sample, the expression of three housekeeping genes (*Hprt*, *Gapdh*, and *Rps19*) was determined to control the quality/quantity of the template and to normalize expression (4). Relative quantification was calculated by means of the efficiency-corrected $\Delta\Delta C_T$ (ΔC_T) method (29): The expression ratio was calculated using the formula: ratio = $(E_{\text{target}})^{\Delta C_T(\text{target})} / (E_{\text{ref}})^{\Delta C_T(\text{ref})}$.

Quantitative determination of the expression of target genes of the vitamin D pathway and PTHrP-IHH feedback loop at 10 and 21 wk of age. The expression pattern of the VDR, *1 α -OHase*, and *24-OHase* was used to study the vitamin D pathway. The expression pattern of PTHrP, PTHrP1, and IHH was used to study the PTHrP-IHH feedback loop. All staining protocols were adjusted to the staining intensity and background in the growth plate cartilage (Supplementary Table S1). Five-micrometer-thick paraffin sections were cut, mounted on Super-Frost+ slides (Fischer Scientific), and deparaffinized through xylene (2×5 min) and graded ethanol (96, 80, 70, 60, and 30%; 5 min each), followed by two rinses with PBS. Midsagittal sections of paired samples of GD and MP were included in the same Pap-pen circle and were thus incubated under identical conditions. Thereafter, antigen retrieval was performed as described in Supplementary Table S1. After endogenous peroxidase inhibition for 5 min, sections were preincubated with blocking buffer for 30 min at room temperature (RT). The sections were incubated with primary antibody overnight at 4°C . For further processing, depending on the primary antibody, the EnVision-HRP detection system (Dako), the goat ImmunoCruz system (sc-2053, Santa Cruz Biotechnology), or specific secondary antibody was applied for 30 min at RT followed by incubation with streptavidin conjugated with horseradish peroxidase for 30 min at RT. All antibodies were visualized with the liquid DAB+ substrate chromogen system (Dako). In control experiments, the first antibody was omitted, and, depending on the antibody, either substitution of the primary antibody with its respective serum and/or competition of the first antibody with corresponding peptides available for IHH and PTH-PTHrP receptor (PTHrP1; sc-1196 P and sc-12777 P, respectively) was performed.

Statistical analysis. Statistical analyses were performed using SPSS for Windows 16.0 (SPSS). Results are presented as means \pm SE. Differences in the proliferative and the hypertrophic zones were analyzed by comparing the ratio of the mean width of the proliferative zone with the mean width of the growth plate and the ratio of the mean width of the hypertrophic zone with the mean width of the growth plate. Quality control of the real-time quantitative PCR data was done by including a serial dilution of template to control the amplification efficiency, and by correlating the C_T and the logarithm-transformed

concentration of the template (LOQ) through the simple linear regression model. A significant simple linear correlation between C_T and the LOQ values was found for all genes studied. The slope of the regression analysis was not significantly different from -1 . Differences within one group with respect to age were analyzed with a paired Student's *t*-test. The distribution-free nonparametric Mann-Whitney test was used to study differences in morphometry and in gene expression between the GD and MP. The expression of all genes investigated was not independent. Statistical modeling was used to investigate which genes were predictive of the growth plate morphology using principal component analysis with the ΔC_T for the genes representing separately the IGF/BPs (i.e., *igfbp 1–6*), the vitamin D pathway (i.e., *Vdr*, *1 α -OHase*, and *24-OHase*), the PTHrP component (i.e., *Pthrp* and *Pthrl*), and the IHH component of the PTHrP-IHH feedback loop (i.e., *Ihh*, *Smo*, and *Ptc*). An overall Kaiser-Meyer-Olkin (KMO) value of >0.500 , as a measure of sampling adequacy, was considered sufficient to continue with the factor analysis, and Bartlett's test of sphericity was used to assess the factorability of the data. A covariance matrix was chosen because the variance of ΔC_T was expected to explain differences in growth plate morphology and morphometry. Thereafter, stepwise backward regression analysis of the single variables and the derived components was performed with the growth plate width and the ratio of the mean width of the proliferative or hypertrophic zone to the mean width of the growth plate as dependent variables. Q-Q plots of the regression standardized residuals were generated.

RESULTS

Growth plate phenotype of growing large- and small-breed dogs. At 10 and 21 wk of age, the growth plate of the 9th rib was 1.7 times thicker in GD than in MP. In both groups, the width of the growth plate decreased significantly with age. Collagen X immunohistochemistry revealed considerable differences in the hypertrophic zone between GD and MP. Although the proliferative and hypertrophic zones were larger in absolute terms in GD of both ages, the relative ratio of the proliferative zone to the total growth plate width was significantly lower, and the relative ratio of the hypertrophic zone to the total growth plate width was significantly higher in GD than in MP. The number of proliferative chondrocytes per 100 μm^2 was similar in the two groups regardless of age, but the numbers of hypertrophic chondrocytes per column were 1.4 and 2.5 times higher in GD than in MP at 10 and 21 wk of age, respectively. In addition, the perimeter of late hypertrophic chondrocytes from GD was ~ 1.5 times higher than that of chondrocytes from MP at both ages. The absolute number of hypertrophic chondrocytes decreased with age only in the MP, whereas the perimeter of late hypertrophic chondrocytes decreased significantly with age in both groups (Fig. 1 and Table 1).

Pattern of gene expression of the IGF/IGFBP system. The level of expression of the genes *igf-I*, *igf-II*, and their respective receptors did not differ between 21-wk-old GD and MP within the rib growth plate, whereas from the group of IGF/BPs, only expressions of *igfbp-2*, *-4*, and *-6* were significantly decreased in GD compared with MP (Fig. 2).

Pattern of expression of components of the vitamin D pathway. Negative controls demonstrated the specificity of immunohistochemical staining (Supplementary Fig. S2 and Fig. 3). The patterns of expression of the vitamin D pathway components were similar at 10 and 21 wk of age; the immunohistochemistry data for the 10-wk samples are given in Supplementary Fig. S3.

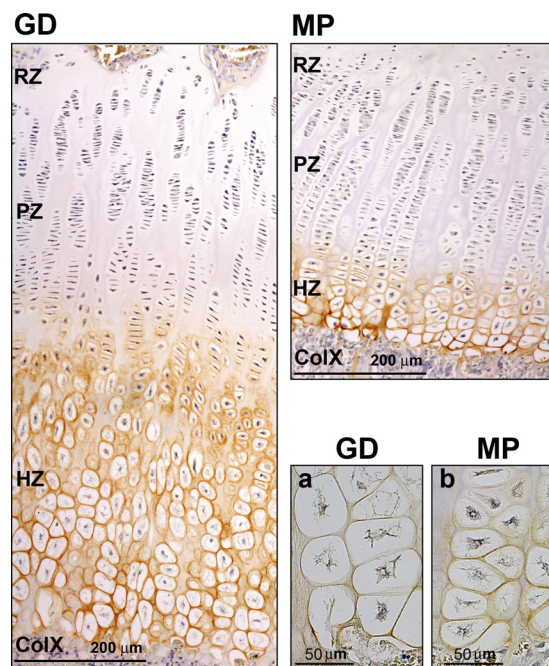


Fig. 1. Immunohistochemical staining for collagen X in the 9th rib growth plate of a large-breed dog (GD; Great Dane) and a small-breed dog (MP; Miniature Poodle) raised under the same dietary and housing conditions. Dogs were 21 wk old. Growth plate zones are indicated as follows: resting zone (RZ), proliferative zone (PZ), and hypertrophic zone (HZ) based on the expression pattern of collagen X. Details of the late HZ are depicted in *a* and *b*.

Intense nuclear staining of VDR in the resting, proliferative, and early hypertrophic chondrocytes was seen in GD, but only faint staining in MP. Cytoplasmic 1α -OHase staining was intense in resting cells, strong in proliferative and early hypertrophic chondrocytes, and less intense in late hypertrophic chondrocytes in GD, whereas cytoplasmic 1α -OHase staining was faint in proliferative and in some early and late hypertrophic chondrocytes in MP. A lower dilution of the primary VDR and 1α -OHase antibody (up to 1:25 for the VDR and 1:50 for 1α -OHase) did not reveal additional staining in the MP group (data not shown). 24-OHase staining was moderate in the cytoplasm of resting, proliferative, and early and late hypertrophic chondrocytes, and only at 21 wk of age was intense cytoplasmic staining seen in the middle part of the hypertrophic zone in GD. Despite use of a lower dilution (up to 1:10) of the 24-OHase antibody, only faint staining was seen in late hypertrophic chondrocytes in MP (data not shown). There were no evident differences between GD and MP in staining intensity with any antibody in the adjacent metaphysis. Osteoblasts were strongly positive for VDR, 1α -OHase, and 24-OHase and osteoclasts revealed nuclear VDR, cytoplasmic 1α -OHase and 24-OHase staining (Supplementary Fig. S6).

Quantitative PCR at 21 wk of age showed that *Vdr* mRNA expression was similar in GD and MP, even though VDR staining was more abundant in GD (Fig. 4), suggesting that posttranscriptional regulation of the *Vdr* occurred and may be indicative for higher VDR signaling in GD. Consistent with the increased VDR signaling at the level of the growth plate in GD, the levels of expression of mRNA for *1\alpha*-OHase and the VDR-driven *24-OHase* were six- and eightfold higher in GD than in MP, respectively.

Table 1. *Histomorphometric parameters of the 9th rib growth plate of growing GD and MP at 10 and 21 wk of age raised under the same housing and dietary conditions*

	GD		MP	
	10 wk	21 wk	10 wk	21 wk
Growth plate width, mm	1.13 ± 0.03 ^a	0.99 ± 0.10 ^{a,b}	0.69 ± 0.05	0.56 ± 0.01 ^c
Relative proliferative zone width, %	47.6 ± 2.7 ^a	50.4 ± 1.3 ^a	62.7 ± 1.6	69.6 ± 0.9 ^c
<i>n</i> proliferative chondrocytes/100 μm ²	4.7 ± 0.7	3.3 ± 0.25	3.8 ± 0.5	2.7 ± 0.15
Relative hypertrophic zone width, %	52.4 ± 2.7 ^a	49.6 ± 1.3 ^a	37.3 ± 1.6	30.4 ± 1.0 ^b
<i>n</i> hypertrophic chondrocytes/column	18 ± 1	18 ± 2 ^a	13 ± 1	7 ± 1 ^b
Surface of late hypertrophic chondrocytes, μm ²	1,378 ± 88 ^a	992 ± 155 ^{a,b}	984 ± 63	653 ± 114 ^b

Data are presented as means ± SE; *n* = 7 per breed. GD, Great Dane; MP, Miniature Poodle. Relative proliferative or hypertrophic zone width is expressed in relation to the mean of the growth plate width. ^a*P* < 0.001 GD vs. MP; ^b*P* < 0.001, ^c*P* < 0.05, significantly different between 10 and 21 wk of age, within one breed.

Expression of components of the PTHrP-IHH growth-restraining feedback loop. Appropriate negative controls demonstrated the specificity of immunohistochemical staining (Supplementary Fig. S4 and Fig. 5). The patterns of expression of the PTHrP-IHH pathway components were similar at 10 and 21 wk of age; the data for 10-wk samples are given in Supplementary Fig. S5.

Cytoplasmic staining for PTHrP was present throughout all zones of the growth plate in MP but was faint in GD and present only in the resting, proliferative, and early hypertrophic chondrocytes and not in late hypertrophic chondrocytes. In contrast, whereas cytoplasmic PTHR1 staining was only faintly present in proliferative chondrocytes in MP, it was abundantly present in resting, proliferative, and early hypertrophic chondrocytes in GD. Cytoplasmic IHH staining was weak in the resting chondrocytes, very faint to undetectable in proliferative chondrocytes, and intense in early hypertrophic chondrocytes, but less intense in late hypertrophic cells in MP. In contrast, IHH staining was more intense and more abundant in the proliferative and early hypertrophic zone in GD than in MP. In the adjacent metaphysis, there were no differences in the expression pattern between GD and MP. Osteoblasts were positive for PTHrP, PTHR1, and IHH, whereas osteoclasts were positive only for PTHrP (Supplementary Fig. S6).

Quantitative PCR of the samples taken at 21 wk of age showed that the relative expression of the *Pthrp* gene was significantly lower (3.3-fold) in GD than in MP, whereas the expressions of the genes for *Pthr1*, *Ihh*, *Smo*, and IHH-driven *Ptc* were significantly higher (3- to 10-fold) in GD than in MP (Fig. 6).

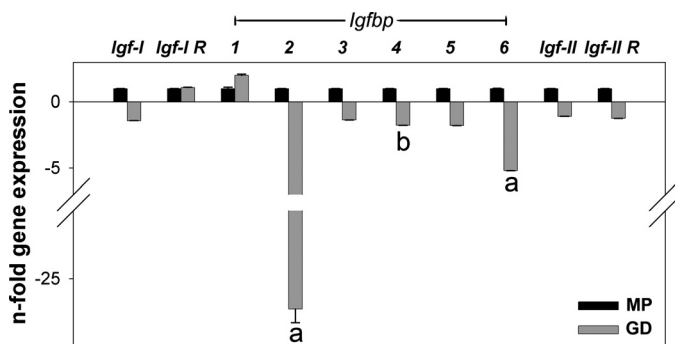


Fig. 2. Relative expression of mRNAs for components of the IGF system of the rib growth plate of GD and MP at 21 wk of age. mRNA expression of MP was set at 1; ^a*P* < 0.01; ^b*P* < 0.05.

Regression analysis of ΔC_T relative gene expression levels and growth plate width. Linear regression analysis of the ΔC_T of all genes studied revealed colinearity for *igfbp* 2, 4, and 6 with variance inflation factors ranging from 10 to 42. For *Ihh*, *Pthr1*, *1 α -OHase*, and *Smo*, variance inflation factors ranged from 12 to 16. Principal component analysis based on a covariance matrix resulted in one principal component for the IGFBP group and the vitamin D pathway and two for the PTHrP-IHH feedback loop (Table 2). Component loadings were calculated with the aid of regression. Subsequent regression analysis with growth plate width as independent variable revealed that *igf-II* and the group of *igfbp* 1–6 were the predictors of growth plate width ($R^2 = 0.716$, $P = 0.001$). Regression analysis with the ratio of the mean width of the proliferative zone to the mean width of the growth plate as independent variable revealed that the “PTHrP component” was the only predictor ($R^2 = 0.701$, $P < 0.0001$). The same predictor accounted also for the variance of the mean width of the hypertrophic zone to the mean width of the growth plate as independent variable.

DISCUSSION

Clinical data suggest that the physiological differences in longitudinal growth within a species can be only partly explained by endocrinological differences. We hypothesized that regulatory networks acting locally in the epiphyseal growth plate may also have a role in this growth disparity and evaluated the pattern of expression of the IGF system, the vitamin D pathway, and the PTHrP-IHH feedback loop in two dog breeds. We used dogs because the growth plate closes as it does in humans (27) and because this species shows the greatest physiological disparity in growth rate, resulting in an over 50-fold difference in mature body weight across dog breeds. To minimize the role of environmental factors on growth regulation, we raised GD and MP under controlled conditions. Despite the higher GH and IGF-I plasma levels in GD than in MP, there are only modest differences in the plasma levels of 1,25(OH)₂D₃ and no differences in plasma PTH levels or in the intestinal absorption of calcium and phosphate (mg/kg body wt) between GD and MP during this period of high mineral requirements and rapid growth (37, 38). Therefore, we postulated that local regulatory pathways within the epiphyseal growth plate would contribute significantly to the disparity in growth rates.

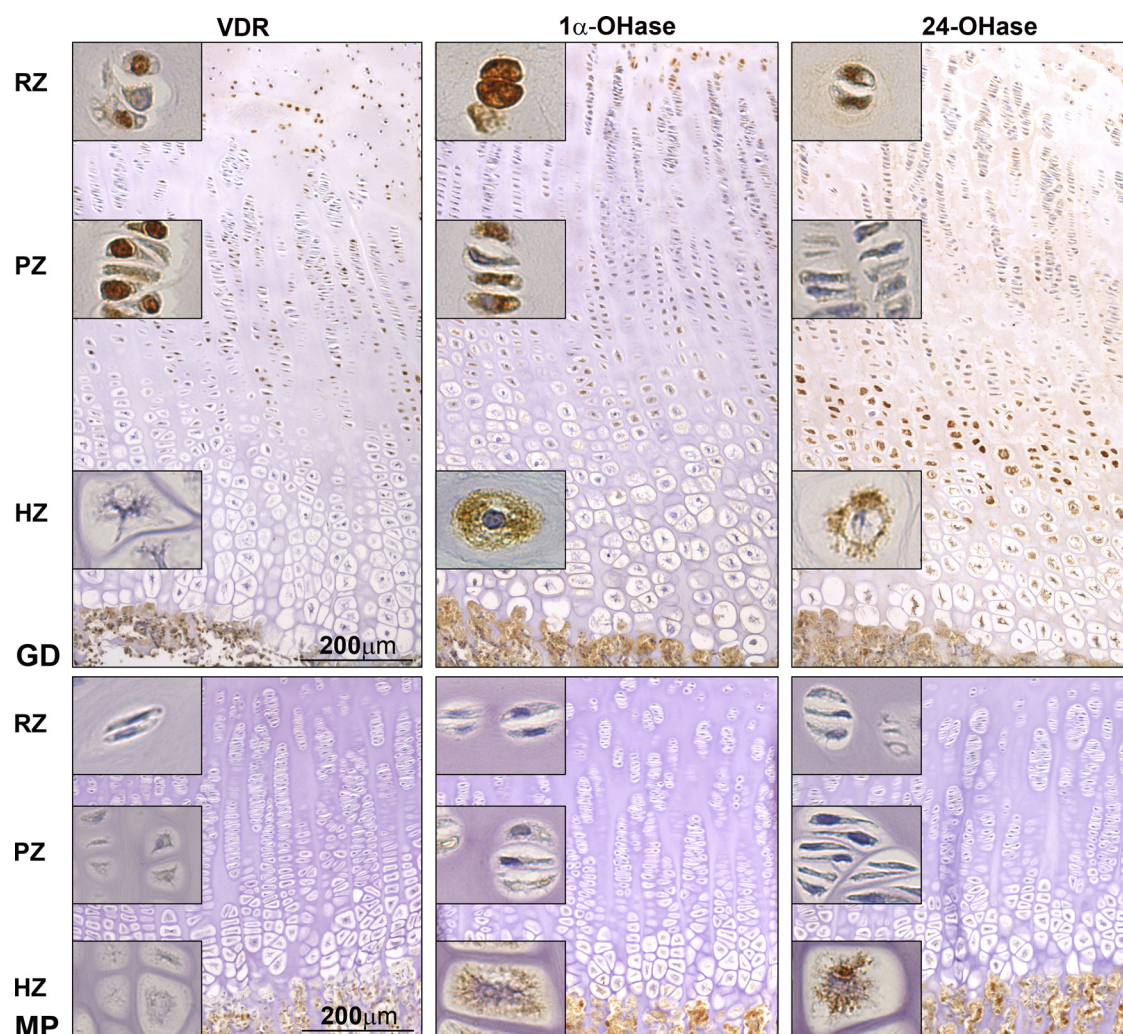


Fig. 3. Rib growth plate of GD and MP at 21 wk of age. Immunohistochemistry reveals the spatial and differential patterns of expression of components of the parathyroid hormone-related peptide-Indian hedgehog (PTHrP-IHH) pathway during rapid (GD) and slow (MP) growth rate within one species. Positive staining of osteoblasts in adjacent metaphyses in both panels is used as an internal control. Insets are higher magnifications.

As expected, dogs of the same age but with different growth rates had a strikingly different growth plate morphology and morphometry during this period of rapid growth. Chondrocyte proliferation/matrix production and chondrocyte hypertrophy

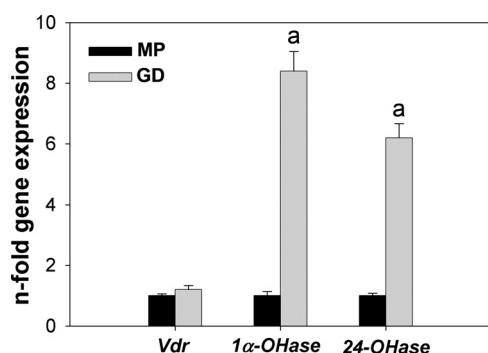


Fig. 4. Relative expression of mRNAs for components of the vitamin D pathway of the rib growth plate of GD and MP at 21 wk of age. vitamin D receptor (*Vdr*; mean C_T 29 ± 0.5), 1α -hydroxylase (*1α-OHase*; mean C_T 30.4 ± 0.7 vs. 33.6 ± 0.8 in GD vs. MP), and 24 -hydroxylase (*24-OHase*; mean C_T 31.2 ± 0.8 vs. 34.3 ± 0.8 in GD vs. MP) are upregulated, confirming the immunohistochemistry data. mRNA expression of MP is set at 1; ^a $P < 0.01$.

play equal roles in longitudinal skeletal growth (6, 7, 43). Although the growth plate was wider with a larger proliferative zone in absolute terms in GD than in MP, the number of proliferative chondrocytes per $100 \mu m^2$ did not differ, which indirectly suggests that the difference in size of the proliferative zone is not solely due to the production of more matrix in GD. The larger (in both absolute and relative terms) hypertrophic zone in GD than in MP can be attributed to an increase in both size and number of hypertrophic chondrocytes. The mechanisms underlying the difference in size of hypertrophic chondrocytes in GD and MP are poorly understood and are thought to involve a combination of systemic and intrinsic factors (20, 26, 32). The distinct histomorphometric differences between GD and MP suggest that different rates of chondrocyte replication, hypertrophic differentiation, and/or apoptosis contribute to the faster growth rate of GD compared with MP.

Differential gene expression profile of the local IGF system. The higher rate of longitudinal bone growth in the GD vs. the MP is at least facilitated by significant downregulation of the *igfbp2*, *-4*, and *-6* supported by quantitative PCR findings and statistical modeling. IGF-I stimulates growth plate chondrocyte

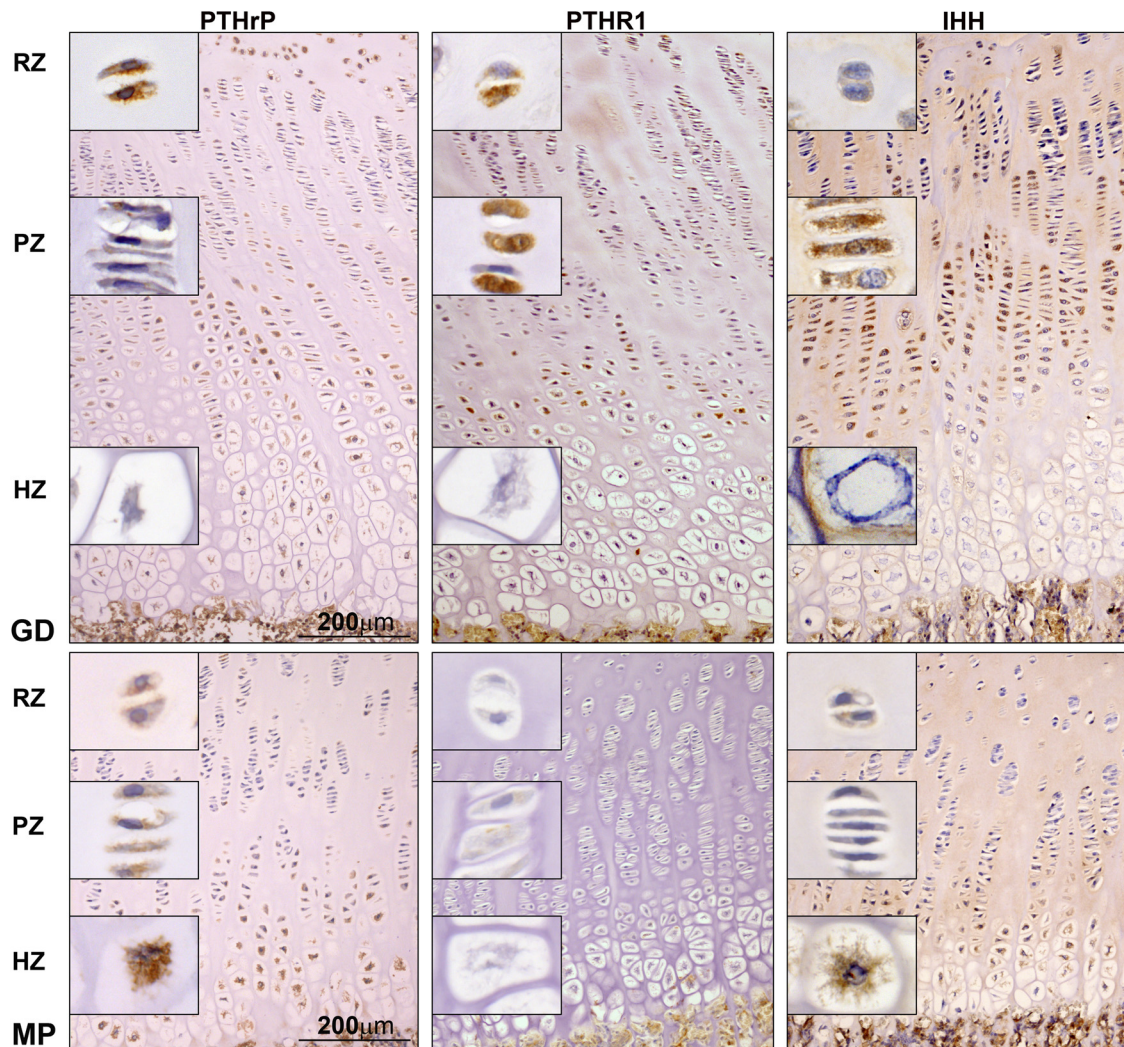


Fig. 5. Rib growth plate of GD and MP at 21 wk of age. Immunohistochemistry reveals the spatial and differential pattern of expression of components of the PTHrP-IHH pathway during rapid (GD) and slow (MP) growth rate within one species. Positive staining of osteoblasts in adjacent metaphyses in both panels is used as an internal control. Insets are higher magnifications.

proliferation and differentiation by both endocrine and para/autocrine mechanisms, and its actions are further regulated by the high-affinity IGFBP 1–6 expressed on growth plate level (28). This down-regulation of *igfbp2*, *-4*, and *-6* in GD may facilitate higher levels of free IGF-I, leading to a stimulation of growth (8, 15, 30). The regulation of IGFBPs by hormones and growth factors is very complex, and discussion of this is beyond the scope of the canine model as studied here.

The distinct morphological differences between GD and MP are accompanied by obvious differences in the growth plate level expression of mRNA and proteins of components of the vitamin D pathway and the PTHrP-IHH feedback loop. It is unlikely that the differences in immunohistochemical staining between GD and MP can be explained by interbreed differences in antibody reactivity, since all antibodies revealed the same expression pattern in the metaphysis of GD and MP.

Differential expression of components of the vitamin D pathway within the epiphyseal growth plate. The vitamin D pathway would seem to be more active in GD, as reflected by the intense and more extensive pattern of staining for all components of the vitamin D pathway and by the higher level

of expression of mRNA for *1 α -OHase* and the target gene *24-OHase* in GD compared with MP. Nuclear VDR and cytoplasmic *1 α -OHase* and *24-OHase* were expressed in the resting, proliferative, and early hypertrophic zones of the growth plate in GD, as also observed in the rat growth plate (13). Although dihydroxylated metabolites have been reported to be produced by chondrocytes (31), this is the first demonstration of specific cytoplasmic staining for *24-OHase* in the mammalian growth plate. Remarkably, such a difference does not exist in the kidney, suggesting that the regulation of the intracrine vitamin D pathway is organ specific (38). In line with this observation, there are only modest endocrine differences in the vitamin D pathway despite marked differences in the vitamin D pathway in the epiphyseal growth plate of GD compared with MP. Upregulation of the vitamin D pathway in the growth plate reflects its important intracrine role in the regulation of chondrocyte terminal differentiation and growth plate mineralization during the demanding period of rapid growth (3). In studies of chondrocyte-specific VDR knockout mice (23), as well as in *1 α -OHase* knockout and overexpressing mice (24), *1,25(OH) $_2$ D $_3$* is suggested to stimulate chondro-

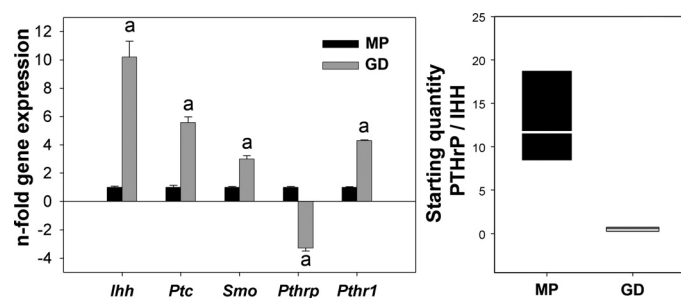


Fig. 6. Relative expression of the mRNAs for components of the PTHrP-IHH pathway (left) in the rib growth plate of GD and MP confirms the qualitative immunohistochemical data depicted in Fig. 4. Mean C_T values \pm SD for GD vs. MP are for *Pthrp* 28.3 ± 1.1 vs. 27.0 ± 0.7 ; *Pthr1* 25.9 ± 0.7 vs. 28.5 ± 0.7 ; *Ihh* 21.7 ± 0.6 vs. 25.7 ± 1.1 ; *Ptc* 30.1 ± 0.8 vs. 33.2 ± 1.5 ; *Smo* 30.1 ± 0.8 vs. 32.2 ± 0.7 , respectively. mRNA expression of MP is set at 1; $^aP < 0.01$. There is a distinct difference in the “set point” of the IHH-PTHrP feedback loop between GD and MP. The latter is illustrated in a box plot of the ratio of starting quantities of *Pthrp/Ihh* (right).

cytes to produce RANKL to induce osteoclast formation and activation. We have also found that *RANKL* mRNA levels are significantly upregulated (6-fold) in GD compared with MP (data not shown), and that GD have higher numbers of osteoclasts in the approximate metaphysis and more extensive bone remodeling, as determined in ^{45}Ca balance studies (38).

Biological significance of the differences in the PTHrP-IHH feedback loop at the epiphyseal growth plate. The localization of PTHrP, PTHR1, and IHH in the different zones of the growth plate of the two breeds in general resembles that described in prepubertal humans (16) and postnatal small laboratory animals (39, 41). The difference in staining for PTHrP, PTHR1, and IHH expression between GD and MP was supported by quantitative differences in gene expression. PTHrP staining was weaker and *Pthrp* mRNA and protein were less widely expressed in GD than in MP, which had higher *Pthr1* mRNA and protein expression. Moreover, the IHH pathway was upregulated and more widely present in GD than in MP, as demonstrated by the intense and more abundant

staining of IHH in the epiphyseal chondrocytes and the upregulated expression of mRNA for *Ihh* and its receptor *Smo*. This upregulation coincided with increased Hedgehog activity in GD as evidenced by the strong upregulation of the IHH target gene *Ptc*, which is commonly used as an indication of active Hedgehog signaling (41).

The clear differences in the PTHrP-IHH pathway between GD and MP probably contribute to the difference in growth plate morphology and growth rate between the two breeds. Despite lower and less extensive PTHrP expression in GD, the absolute width of the proliferative zone is much larger in GD than in MP. This contradicts the notion that the concentration of PTHrP determines the switch from a proliferative to a hypertrophic chondrocyte, with low levels accelerating and high levels decelerating the transition. The low levels of PTHrP might be in part compensated by the higher levels of PTHR1 expression in GD, which may have increased the sensitivity of the chondrocytes to PTHrP and in this way slowed the proliferative hypertrophic transition. Remarkably, *Ihh* levels are much higher in GD than in MP but they do not result in upregulation of *Pthrp* expression as one might expect in the feedback loop. This counterintuitive finding suggests that the growth-restraining loop of PTHrP-IHH attains a different equilibrium in rapidly growing GD compared with slower growing MP (Fig. 6). Studies of multiple endocrine systems, among which the PTH (2) and T_4 (9), in health and disease, suggest that feedback loops seek a new equilibrium according to the demands made on the system by disease, stimuli, etc., resulting in a new “set point”. We postulate that in rapid growing dogs the feedback loop is changed in such a way that PTHrP is relatively downregulated and IHH levels are upregulated. As a result of adjustment, the effects of IHH on the regulation of chondrocyte proliferation and hypertrophy, both of which are independent of PTHrP (21), become more dominant. Such a model can explain, at least in part, the increased absolute width of the proliferative and hypertrophic zones seen in GD. A set point change in rapid-growing GD in favor of a more dominant role of IHH in the feedback loop is

Table 2. Principal component analysis of ΔC_T values of genes with the aid of covariance matrix categorized in groups based on known biological relationships

	KMO	Bartlett's Test	Eigen Values	Total Variance Explained	Rescaled Loading Components
<i>Igfbp 1-6</i>	0.518	χ^2 56.435 df 15 $P < 0.001$	10.773	77.04%	0.055 <i>igfbp 1</i> 0.995 <i>igfbp 2</i> 0.268 <i>igfbp 3</i> 0.893 <i>igfbp 4</i> 0.717 <i>igfbp 5</i> 0.831 <i>igfbp 6</i>
<i>Vdr</i> , <i>1α-OHase</i> , and <i>24-OHase</i>	0.555	χ^2 10.268 df 3 $P = 0.016$	6.154	82.43%	0.904 <i>1α-OHase</i> 0.948 <i>24-OHase</i> 0.365 <i>Vdr</i>
<i>Pthrp</i> and <i>Pthr1</i>	0.500	χ^2 11.525 df 1 $P = 0.001$	2.561	89.83%	-0.940 <i>Pthrp</i> 0.955 <i>Pthr1</i>
<i>Ihh</i> , <i>Smo</i> , and <i>Ptc</i>	0.526	χ^2 13.305 df 3 $P = 0.004$	9.599	67.35%	0.474 <i>Ihh</i> 0.965 <i>Ptc</i> 0.588 <i>Smo</i>

KMO, Kaiser-Meyer-Okin measure of sampling adequacy. The IGFBP group contains *igfbp 1-6*. The vitamin D pathway is represented by the vitamin D receptor (*Vdr*), and the enzymes of production and catabolism of $1,25(\text{OH})_2\text{D}_3$ (*1 α -* and *24-hydroxylase*, respectively). The parathyroid hormone-related peptide-Indian hedgehog (PTHrP-IHH) feedback loop is split into 2 major groups, i.e., the PTHrP part, represented by *Pthrp* and its receptor *Pthr1*, and the IHH part, represented by *Ihh* and its receptors *smoothed* (*Smo*) and IHH-driven *Patched* (*Ptc*).

further underscored by regression analysis, which identified *Pthrp* and its receptor *Pthr1* as one of the major predictors of growth plate width in addition to *igf-I*, *igf-II*, and their receptors. On basis of genome-wide association studies regarding adult height (19, 44), the “more prominent” role of IHH in chondrocyte proliferation and hypertrophy independent of PTHrP will lead to changes in growth plate histomorphometry and growth rates. In the same way, the altered expression of the PTHrP-IHH feedback loop may facilitate the extended period of growth as seen in large- vs. small-breed dogs as well the larger circumference of the rib growth plate in GD compared with MP. Additional mechanistic studies on the regulation of the PTHrP-IHH feedback loop in the sense of a set point are warranted.

In conclusion, our data clearly indicate that, next to endocrine differences, prominent differences in expression of the *igfbp*'s and key components of the vitamin D pathway and PTHrP-IHH feedback loop at the growth plate are important contributors to disparities in growth rates. Although our experimental studies do not provide clues how the pronounced differences are established, they do support the notion that differences in transcription regulation of key components in organogenesis, rather than mutations in these components, may play an important role in species diversification (22).

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DISCLOSURES

No conflicts of interest are reported by the authors.

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