Intermittent PTH (1-34) injection rescues the retarded skeletal development and postnatal lethality of mice mimicking human achondroplasia and thanatophoric dysplasia

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Achondroplasia (ACH) and thanatophoric dysplasia (TD) are caused by gain-of-function mutations of fibroblast growth factor receptor 3 (FGFR3) and they are the most common forms of dwarfism and lethal dwarfism, respectively. Currently, there are few effective treatments for ACH. For the neonatal lethality of TD patients, no practical effective therapies are available. We here showed that systemic intermittent PTH (1-34) injection can rescue the lethal phenotype of TD type II (TDII) mice and significantly alleviate the retarded skeleton development of ACH mice. PTH-treated ACH mice had longer naso-anal length than ACH control mice, and the bone lengths of humeri and tibiae were rescued to be comparable with those of wild-type control mice. Our study also found that the premature fusion of cranial synchondroses in ACH mice was partially corrected after the PTH (1-34) treatment, suggesting that the PTH treatment may rescue the progressive narrowing of neurocentral synchondroses that cannot be readily corrected by surgery. In addition, we found that the PTH treatment can improve the osteopenia and bone structure of ACH mice. The increased expression of PTHrP and down-regulated FGFR3 level may be responsible for the positive effects of PTH on bone phenotype of ACH and TDII mice.

INTRODUCTION

Fibroblast growth factor receptor 3 (FGFR3) has been proved to be a negative regulator of endochondral bone growth (1). Mutations in the coding sequence of the FGFR3 gene can cause autosomal dominant human skeletal disorders, including achondroplasia (ACH) (2), hypochondroplasia (HCH) (3), thanatophoric dysplasia (TD) (3), and some other related disorders (4). ACH is the most common non-lethal form of dwarfism (5), which has the characteristic phenotypes of rhizomelic short limbs, macrocephaly, and lumbarlordosis. TD is a severe and early postnatal lethal skeletal dysplasia, and can be clinically divided into two types, TD type I and II (TDI and TDII) (6,7).

Recently, the roles of activated FGFR3 signals in bone development of ACH have been studied in vivo using transgenic (8) and knock-in (9,10) mouse models. Several studies suggested that the skeletal phenotypes in those mouse models mimicking human ACH and TD resulted from disturbed proliferation and differentiation of growth plate chondrocytes that lead to impaired endochondral bone growth (8,11). In order to study the mechanisms for the pathogenesis of ACH and TDII, we have generated several mouse models including mice carrying Fgrf3 G369C and K644E mutations, which

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cause ACH and TDII in humans, respectively (9,12). As expected, these mouse models display skeletal phenotypes similar to the corresponding human conditions, including short stature and dome-shaped skull. Specifically, mice carrying the Fgfr3 K644E mutation also exhibited underdeveloped thoracic cage, which leads to lethality within several hours after birth as a result of restricted respiratory function (12). Studies with these models have generated valuable knowledge regarding the mechanisms underlying the FGFR3-related skeletal dysplasias. FGFR3 is currently thought to inhibit chondrocyte proliferation, differentiation and matrix synthesis through its downstream pathways including MAPK, PLCγ, Stats and cell-cycle inhibitors such as p21 (10,13–15). FGFR3 is also found to promote chondrocyte apoptosis (15,16). Additionally, FGF/FGFR3 signaling has been found to have crosstalk with other signaling pathways critically involved in skeleton development.

Although no effective clinical therapy is available for TD patients, current treatments for ACH patients are mainly growth hormone treatment and distraction osteogenesis (17–19), which have limitations in effectiveness. Although the long-term effect of growth hormone therapy on ACH is not significant (20), distraction osteogenesis can extend the limbs of ACH patients only to some extent, and patients usually suffer from marked physical and economical burdens. Thus, there is urgent need to find new treatments for ACH/TD patients. Indeed, our increased understanding about the molecular mechanisms of ACH/TD has provided us with more biological measures to treat these diseases. Theoretically, down-regulating the activity of FGFR3 and/or its downstream signaling pathways may lead to a cure or alleviation of ACH/TD.

We have previously shown that ACH mice have decreased expression of PTHR1 in their growth plates, and PTHrP treatment can partially rescue the retarded growth of the cultured bone rudiments from ACH mice (21). Other studies have also shown that PTH treatment can improve the growth of cultured chondrocytes of femurs from mice with transgenic expression of activated FGFR3 in the growth plates (16,22). However, the actual in vivo effect of PTH signaling on the skeletal development of mice mimicking human ACH/TD and its action mechanism are still not fully clarified.

The purpose of this study was to elucidate whether the in vivo systemic administration of PTH can rescue the underdeveloped skeleton of ACH and TDII mice. We found that intermittent subcutaneous injection of PTH (1-34) can rescue the retarded skeletal development and prevent early postnatal lethality of ACH/TD mice, indicating that PTH can potentially be used in clinic to treat ACH and/or TD patients.

**RESULTS**

**PTH (1-34) rescues the lethal phenotype of mice mimicking human TDII**

We generated mice heterozygous for the Fgfr3 K644E mutation (Fgfr3+/K644E) (referred to as TDII) by crossing Fgfr3+/K644E mice with EIIa-Cre transgenic mice. All Fgfr3+/K644E pups without PTH treatment (n = 64) died within a few hours after birth due to the restricted respiration caused by retarded thoracic cage development, whereas all wild-type (WT) littermates (n = 79) developed normally. In contrast, TDII mice (n = 52) born by pregnant mice injected with PTH (1-34) survived for at least 5 days after birth, and 12 TDII (23.1%) mice survived for >90 days (Table 1).

Before death, TDII mice without PTH treatment invariably exhibited serious cyanosis and limited viability (Fig. 1B), whereas PTH (1-34)-treated TDII mice (Fig. 1C) showed ruddy skin and were viable, similar to that in WT pups. At postnatal day 1 (P1), TDII mice (Fig. 1F), compared with their WT littermate, showed decreased ossification in the spine and epiphyses, significantly curved axial skeleton, as well as notable round head (arrow in Fig. 1E). PTH-treated TDII mice (Fig. 1F) had significantly improved skeletal development, and they had larger total length and body size than those in untreated TDII mice, although they still exhibited round head (arrows). TDII mice showed remarkable bowing of tibia (Fig. 1K) and humerus (Fig. 1N) (arrows). The shortened ossified zone in TDII tibia was clearly exhibited (bars). Hindlimbs and forelimbs were better developed in PTH-treated TDII mice as evidenced by the longer ossified zone and milder curvature. We examined the histology of the P1 mouse long bones. The height of the whole epiphyseal growth plate was similar between TDII and WT mice (Fig. 1S–U). TDII mice had abnormal differentiation of growth plates (Fig. 1T). Small and round-shaped resting chondrocyte-like cells were randomly located in the proliferation and hypertrophic zones (arrow). TDII neonates died from respiratory failure early after birth (23). We examined the morphology of the postnatal lungs. WT mice had well-developed large alveoli. TDII pups displayed impaired lung development at P1 with fewer and poorly formed alveoli (Fig. 1W) compared with the WT controls (Fig. 1V), and PTH (1-34)-treated TDII mice (Fig. 1X) had more alveoli in their lungs compared with the untreated TDII mice.

At P5, TDII mice that survived after the PTH treatment had notable round head (Fig. 2A), disorganized hypertrophic zone (Fig. 2E and G) and less trabecular bone in lumbar vertebrae (Fig. 2C) compared with those in WT littermates (Fig. 2B, D and F). Although all those PTH-treated TDII mice survived early postnatal lethality, at the age of 2 months, they still exhibited small stature, short and round skull similar to

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All these mice received PTH (1-34) treatment (100 μg/kg body weight per day) during gestation from E13.5 to newborn via subcutaneous injection. M, male, F, female.
those of ACH mice (Fig. 2H). Soft X-ray analysis of PTH-treated TDII mice showed impaired growth of bones formed via endochondral ossification, including tibiae and vertebrae (Fig. 2I). PTH-treated TDII mice had shorter tibiae and caudal vertebrae, as well as decreased bone mass (Fig. 2I) compared with those of age-matched WT mice. Histological analysis revealed that PTH-treated TDII mice still had less trabecular bone in tibia than that in WT mice (Fig. 2L and M). All these data indicated that PTH (1-34) rescued the lethal phenotype of TDII mice and improved their skeletal phenotype.

**PTH (1-34) rescues the retarded bone development in ACH mice**

In order to study the effects of the PTH (1-34) treatment on ACH mouse growth and development, we measured the body weight and naso-anal length of ACH mice and their littersmates. Compared with vehicle-treated counterparts, ACH mice treated with PTH (1-34) showed no remarkable differences in body weights and lengths at P7. With age, PTH (1-34)-treated ACH mice had shorter tibiae and caudal vertebrae, as well as decreased bone mass (Fig. 2I) compared with those of age-matched WT mice. Histological analysis revealed that PTH-treated TDII mice still had less trabecular bone in tibia than that in WT mice (Fig. 2L and M). All these data indicated that PTH (1-34) rescued the lethal phenotype of TDII mice and improved their skeletal phenotype.

**Figure 1.** PTH (1-34) rescues the lethal phenotype of mice mimicking human TDII. (A–C) Morphology of the mutant (TDII) mice treated with PTH (1-34) and the littermate controls (WT) at P1. TDII mice showed notable cyanosis. (D–R) Alizarin red S and Alcian blue staining of the skeletons in WT mice, TDII mice and TDII mice treated with PTH at P1. (S–U) Histology (H&E staining) of the growth plates of proximal tibiae in WT mice and TDII mice treated with PTH at P1. The growth plates of TDII mice were disorganized: resting chondrocyte-like cells were located in the proliferative and hypertrophic zones (arrow in T). Dashed lines separate the resting, proliferative and hypertrophic zones (magnification × 40). (V–X) Morphology of the lungs (H&E staining, magnification × 200).

**PTH (1-34) partially rescues the premature fusion of cranial synchondroses and alleviates the retarded long bone growth and osteopenia in ACH mice**

Since premature fusion of synchondroses in the cranial base is a remarkable phenotype of ACH mice (10), we examined the synchondroses in each group. PTH appeared to partially rescue the premature fusion of the cranial synchondroses in ACH mice (Fig. 3A–H). At P7, premature fusion of sphenooccipital synchondroses was found obvious in ACH control mice (Fig. 3C, arrow), and it was partially rescued by the PTH treatment (Fig. 3D, arrow). Sphenooccipital synchondroses were completely closed and the anterior intraoccipital synchondroses (arrows) of ACH were encased in bone at P14 (Fig. 3G), whereas in PTH-treated ACH mice, premature fusion of intraoccipital synchondroses was partially rescued (Fig. 3H).

The number of BrdU-positive chondrocytes in the proliferating zone of P7 growth plates was found to be remarkably enhanced by the PTH treatment in both ACH and WT mice (Fig. 3I–L). The PTH (1-34)-treated ACH mice showed a significant increase in the thickness of hypertrophic zones compared with those in untreated ACH mice (Fig. 3M–P and W). At P8, the secondary ossification center began to be observed in WT mice and PTH-treated WT mice (Fig. 3Q.
and S), whereas it was not formed in untreated ACH control littermates. In the ACH control group, the location of the future secondary ossification center consisted of hypertrophic chondrocytes and resting chondrocyte-like cells, whereas in PTH-treated ACH mice, the secondary ossification center can be observed. At P14, the secondary ossification centers were well formed in each mouse group (data not shown), and the thickness of the growth plate proliferative zone was increased by the PTH treatment in ACH mice (Supplementary Material, Fig. S2A). At 2 months, the lengths of humeri and that of femurs of ACH mice were shorter than those of WT littermates, whereas in PTH-treated ACH group, the humeri and femurs were statistically longer when compared with ACH control (Fig. 3V). These results suggest that intermittent PTH (1-34) administration partially rescued the retarded
Table 2. Body weights of WT and ACH mice treated with or without PTH (1-34) (g) (n = 6–8)

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<td>PTH-treated WT</td>
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<tr>
<td>PTH-treated ACH</td>
<td>4.6 ± 0.1</td>
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aVersus control WT, P < 0.05.
bVersus control ACH, P < 0.05.

Table 3. Naso-anal lengths of WT and ACH mice treated with or without PTH (1-34) (cm) (n = 6–8)

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<tr>
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<tr>
<td>PTH-treated WT</td>
<td>2.7 ± 0.1</td>
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<tr>
<td>ACH control</td>
<td>2.3 ± 0.1</td>
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<tr>
<td>PTH-treated ACH</td>
<td>2.3 ± 0.1</td>
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aVersus control WT, P < 0.05.
bVersus control ACH, P < 0.05.

Since we observed previously that ACH mice have osteopenia (24), we further examined the bone phenotype at the adult stage. In both genotypes, bone mass of femurs was increased in 2-month-old mice after the PTH (1-34) treatment (Fig. 4A). Quantification of BMD by dual-energy X-ray absorptiometry (DEXA) revealed that the PTH treatment increased the total femoral BMD of ACH mice significantly by 16.1% (Fig. 4B). Micro-computed tomography (micro-CT) analysis of the distal metaphyses of femurs was used to assess the structural parameters, including bone volume/tissue volume (%), BV/TV, trabecular number (Tb.N), trabecular thickness (Tb.Th) and separation (Tb.Sp) in 2-month-old mice. The ACH control group displayed an increase of Tb.Sp, and decrease of BV/TV, Tb.N and Tb.Th, which indicated osteopenia as we previously reported (24).

In ACH mice, the PTH treatment increased BV/TV, Tb.N and Tb.Th markedly by 74.0, 29.4 and 23.1%, respectively, and decreased the Tb.Sp by 24.8%, indicating improvement of osteopenia in ACH mice after the PTH treatment. In WT littermates, PTH administration increased the Tb.N by 26.1% and decreased the Tb.Sp by 11.6% (Fig. 4C–G). The trabecular bone in the proximal tibia from 2-month-old ACH mice was shorter and sparser than that of WT mice, and the PTH treatment increased the trabecular number (Supplementary Material, Fig. S2B). These results are consistent with the results obtained by micro-CT examination. Mildly enhanced TRAP staining in the tibia was observed in ACH mice compared with WT mice, and no notable changes were observed in 2-month-old mice after the PTH treatment at the development stage (Supplementary Material, Fig. S2C). These observations indicate that the decreased bone mass and compromised architecture resulting from activated mutation in FGFR3 in adult mice can be ameliorated by the PTH treatment at the development stage.

**PTH treatment rescues the retarded growth of cultured tibiae from embryonic ACH mice**

PTH may have effects on cells other than skeletal cells, which may be responsible for the in vivo effects of the PTH (1-34) treatment on skeletons of ACH and TDII mice observed above. To clarify this issue, we then utilized an in vitro bone culture system to examine the direct effects of PTH (1-34) on bone growth. We found that the total lengths of cultured tibiae from both ACH and WT mice were gradually increased during the culture period (Fig. 5A). ACH tibiae cultured from both ACH and WT mice were gradually increased following the PTH treatment. In ACH tibiae, the PTH treatment increased the trabecular number (Supplementary Material, Fig. S2B). These results are consistent with the results obtained by micro-CT examination. Mildly enhanced TRAP staining in the tibia was observed in ACH mice compared with WT mice, and no notable changes were observed in 2-month-old mice after the PTH treatment at the development stage (Supplementary Material, Fig. S2C). These observations indicate that the decreased bone mass and compromised architecture resulting from activated mutation in FGFR3 in adult mice can be ameliorated by the PTH treatment at the development stage.

**PTH (1-34) stimulates the proliferation and differentiation of cultured chondrocytes**

To further explore the mechanism for PTH rescue, the effect of PTH on the proliferation and differentiation of
chondrocytes was evaluated in cultured primary chondrocytes. Although proliferation was decreased in chondrocytes derived from ACH mice compared with that of WT control ($P < 0.01$), intermittent PTH (1-34) treatments could promote the proliferation of chondrocytes from both WT ($P < 0.01$ versus WT control) and ACH mice ($P < 0.01$ versus ACH control) despite the fact that PTH-treated chondrocytes from ACH mice still had a lower proliferation compared with WT

**Figure 3.** PTH (1-34) increases the lengths of tibiae and femurs and alleviates the disorganized growth plates in ACH mice. (A–H) Alizarin red S and Alcian blue staining of the skeletons of mice in each group at P7 and P14. PTH treatment improved the premature fusion of cranial synchondroses in ACH mice. (I–L) Labeling of BrdU in the growth plates of each group, with PTH-treated mice showing more BrdU-positive cells in growth plates (magnification ×100). (M–P) Histological analysis of the growth plates of ACH mice in comparison with WT mice. The hypertrophic zone is denoted with bars (Safranin-O and fast green staining; magnification ×100). (Q–U) Comparison of secondary ossification centers of the proximal tibia from 8-day-old mice. The asterisk shows secondary ossification (H&E staining; magnification ×200). (V) Bone lengths of 2-month-old mice ($n = 6$). (W) Quantification of widths of hypertrophic zones in growth plates ($n = 6–8$). Graphs show mean values ± SD (two-way ANOVA, **$P < 0.01$; ***$P < 0.001$).
control ($P < 0.01$) (Fig. 6A). The changes in the differentiation of chondrocytes were evaluated by measuring the mRNA expression of genes related to chondrogenic differentiation using real-time PCR. The results showed that the expression levels of Col 2a1, Col 10a1 and PTHrP mRNA of chondrocytes from ACH mice were all increased after the PTH treatment (Fig. 6B). In addition, Alcian blue staining of chondrocytes showed that ACH chondrocytes secreted less cartilaginous matrix compared with WT control, whereas PTH (1-34) increased the secretion of extracellular matrix (ECM) in ACH chondrocytes (Fig. 6C), which may be involved in the rescuing effect of PTH on the retarded bone growth in ACH mice. To gain insights into the mechanism of PTH (1-34)’s effect on FGFR3-related skeletal dysplasia, the expression of FGFR3 in primary mouse chondrocytes was examined after exposure to PTH. The result showed that the levels of FGFR3 were higher in ACH chondrocytes than those in WT controls, and were down-regulated following $10^{-8}$ M PTH (1-34) treatment for 12 h in both WT and ACH cells (Fig. 6D).

**Figure 4.** PTH (1-34) ameliorates osteopenia in ACH mice. (A) Faxitron X-ray analysis of total femurs at 2 months. (B) BMD of femurs from ACH mice was significantly increased by the PTH treatment ($n = 8$). (C–G) Quantitative micro-CT analyses of distal femoral metaphysis at 2 months. Three-dimensional images show reduced trabecular bone in ACH mice and increased trabecular bone with the PTH treatment group (C). Quantification of the structural parameters showed that BV/TV, Tb.N and Tb.Th were significantly improved by the PTH treatment in ACH mice (D–G). Graphs show mean values ± SD (two-way ANOVA, $^*P < 0.05$; $^*^*P < 0.01$; $^*^*^*P < 0.001$).
study the effect of the PTH (1-34) treatment on the chondrogenic differentiation of mesenchyme cells from limb buds. Condensations of mesenchymal cells released from the limb buds of E11.5 WT and ACH mice plated at high density first appeared at day 2. The size and density of the cultured cell mass were increased up to day 6. The small, shallow condensations observed on day 2 were differentiated into nodules of rounded chondrocytes surrounded by flattened undifferentiated cells. At day 6 (5 days after the addition of PTH (1-34)), although there were no remarkable differences in the number of nodules between PTH-treated and -untreated ACH cells (Fig. 6E), the areas of nodules in the presence of PTH (1-34) were larger. The nodules in the PTH-treated group contained more cells and were much deeper and better formed than those in the non-treated group (Fig. 6F).

**Figure 5.** PTH (1-34) rescues FGFR3-mediated inhibition of growth in cultured murine tibiae. (A) Representative photographs showing the effects of the PTH treatment on the growth of cultured tibiae (cultured for 7 days) from ACH mice or WT mice (WT). (B) Quantification of increased percentage in total lengths of bones from WT and ACH mice cultured for 7 days in the absence or presence of PTH (1-34). (C) Changes in bone lengths of the calcified bone (CB) or cartilage (C) after 7-day cultures. ACH specimens had lower growth velocity compared with WT ones. PTH treatment promoted the growth of cartilage and had an inhibition of growth in calcified bone in both WT and ACH specimens (∆, increased length). Graphs show mean values ± SD (two-way ANOVA, n = 6–8, **P < 0.05; ***P < 0.001).

**DISCUSSION**

ACH and TD are the most common forms of dwarfism and lethal dwarfism, respectively, for which at present there are...
few effective treatments. ACH patients can be treated with growth hormone and the surgical limb-lengthening technique. While the recombinant hGH treatment can increase the short-term growth velocity in children with ACH/HCH, the increase cannot be readily achieved during the long-term treatment (20,26). Surgical limb lengthening may produce cosmetic and psychological benefits to some extent, but the extremely invasive nature, expense, risks and complications may make patients not to choose this surgery (18,27). Moreover, the stenosis of the foramen magnum and spinal canals cannot be readily corrected by surgery and may finally cause serious neurological complications, including hydrocephalus and sudden death in infancy as well as headaches in older ACH patients (28–30). As for the neonatal lethality of TD patients, no practical, effective therapies are available in clinics presently. TD patients usually already have development abnormality during gestation, which may lead to death before birth (31). The growth-promoting treatment and surgery are difficult to be carried out during pregnancy. So far, there are few reports about the treatment of postnatal TD patients, either (32).

Figure 6. PTH (1-34) stimulates proliferation and differentiation of cultured chondrocytes and also promotes limb bud mesenchymal cell differentiation to chondrocytes. (A) MTT proliferation assay showed decreased proliferation of ACH chondrocytes, and the PTH (1-34) treatment increased the proliferation in both WT and ACH chondrocytes on day 4. (B) mRNA expression of COL2A1, COL10A1 and PTHrP was evaluated by qRT-PCR in cultured chondrocytes from WT and ACH mice on day 4. Cyclophilin A was the internal standard and data are expressed as fold changes in relation to WT control (n = 5–6). (C) Alcian blue staining showed decreased cartilaginous matrix on day 4 in chondrocytes from ACH compared with that of WT cells, and the PTH (1-34) treatment increased ECM secretion in cultured chondrocytes from both WT and ACH mice. (D) There were no significant differences in the number of nodules between PTH-treated and -untreated cells on day 2, day 4 and day 6. (D) Effects of 10⁻⁸ M PTH (1-34) treatment for 12 h on the expression levels of FGFR3 as assessed by western blotting in primary chondrocytes isolated from WT and ACH mice. (F) The nodules formed at day 6 after 5-day culture in the presence of PTH (1-34) were larger in size and had more cells than those in the non-treated ACH group. Alcian blue staining was much darker in the PTH-treated group. Dashed circles show areas of nodules (magnification: left panels ×100, middle panels ×400, right panels ×400). Expressions of chondrocyte differentiation markers. Graphs show mean values ± SD (two-way ANOVA, **P < 0.01; ***P < 0.001).
Researchers have been working to find other approaches to treat or alleviate the skeletal phenotypes of FGFR3-related skeletal dysplasia. Current therapeutic strategies have focused on reducing signals activated by FGFR3 (33). Aviezer et al. (34) reported an inhibitor selective for FGFR3 reconstituted normal growth in cultured limb bones from ACH mice, but had little effect on live mice. Rauchenberger et al. (35) found an antibody against FGFR3 which can effectively block ligand-induced FGFR3 activation; however, there have been no subsequent reports about its in vivo effect on bone development. In addition, BMP signaling was found to rescue the reduced regions of proliferating and hypertrophic chondrocytes in growth plates of cultured limbs from ACH mice (36), but whether BMP can increase the bone length of ACH mice has not been reported. Furthermore, since overexpression of Snail1 in the developing bone has been shown to lead to Ach-like phenotype in mice by acting as a downstream signaling molecule of FGFR3 in chondrocytes that regulates both Stats and MAPK pathways (37), it is suggested that Snail1 may be a new encouraging non-surgical therapeutic target (31). Similarly, CNP has been found to prevent the shortening of achondroplastic bones by correcting the decreased ECM synthesis in the growth plates through its inhibition on the MAPK pathway of FGF signaling; however, CNP had no effects on the Stat1 pathway of FGF signaling that mediates the increased proliferation and the delayed differentiation of achondroplastic chondrocytes (38–40). Targeted overexpression of CNP in cartilage or systemic administration of CNP was shown to partially rescue the impaired skeletal growth of Ach mice (39). Since all these molecules have not been used clinically or have not been examined systematically, further work is needed to study their dosage, clinical safety and compliance before their eventual clinical application.

We previously revealed that the expression of PTHR1 in growth plates of ACH mice was decreased (21), and Li et al. (41) found that FGFR3 signaling down-regulated the expression of PTHR1 gene through the Jak/Stat pathway in chondrogenic cells (ATDC5). We and Ueda et al. (21,22) further found that PTHrP or PTH (1-34) can partially reverse the retarded long bone growth of the cultured bone rudiments from knock-in and transgenic ACH mice. As recombinant human PTH (1-34) (teriparatide) is the FDA-approved drug to treat osteoporosis in humans by promoting bone formation, these results indicate that the clinically available drug, PTH (1-34), may potentially be used to treat Ach or TD patients. However, so far there are no data about the in vivo effect of PTH on Ach/TD patients or mice. In the current study, the in vivo effects and action mechanisms of the PTH (1-34) treatment on skeletal development and survival have been explored in the ACH and TDII mice and cells isolated from these mice. We found that systemically administered PTH (1-34) can rescue the lethal phenotype of TDII mice. All TDII offspring born from pregnant mice injected with PTH (1-34) survived for 5 days or longer after birth, whereas all untreated TDII mice died several hours after treatment.
birth, which is consistent with our previous report (12). Since the lungs of PTH (1-34)-treated TDII mice were improved to have grossly normal alveolarization and the PTH treatment significantly promoted skeletal growth of TDII mice, it is likely that the prevention of the early postnatal lethality of TDII mice by PTH (1-34) is related to the improved lung alveolarization secondary to the ameliorated development of thoracic cavity.

In ACH mice, our study found that the PTH (1-34) treatment had significant positive effects. PTH-treated ACH mice had longer noso-anal lengths than ACH control mice, and the bone lengths of humeri and tibiae were rescued to be comparable with those of WT control mice. Interestingly, our study also found that the premature fusion of cranial synchondroses in ACH mice was delayed after the PTH (1-34) treatment, suggesting that the PTH treatment may alleviate the progressive narrowing of neurocentral synchondroses that cannot be readily corrected by surgery, and thus reduce the complications in the nervous system caused by the limited volume of the skull. In addition, as we observed previously that ACH mice have osteopenia (24), in the current study we further found that the PTH treatment also improved the bone mass and structure, which indicates that the PTH treatment at the development stage not only promotes the skeletal growth during the development stage, but also improves the bone quality at the adult stage. The mechanism for improved bone quality is not clear presently, although it may be secondary to the improved development of the skeleton.

The cellular mechanism for the rescue of ACH and TD mice by PTH is not clear. Impaired chondrocyte proliferation and differentiation, as well as increased chondrocyte apoptosis, have been attributed to the retarded growth of long bones in ACH/TD patients and mice (21,42). PTH/PTHrP/PTHR1 signaling can promote the proliferation of chondrocytes and inhibit the differentiation of chondrocytes through its downstream signaling molecules (25,43). Whereas PTHrP signaling plays a dominant role in regulating the pool of proliferating cells during limb development, FGFR3 signaling has a more prominent role in cartilage maturation (44). In the current study, we revealed that PTH (1-34)-treated ACH mice had an increased chondrocyte proliferative index and a thicker hypertrophic zone. These data suggest that the PTH treatment leads to increased chondrocyte proliferation and differentiation, which was further supported by results from studies on cultured primary chondrocytes. However, although the increased chondrocyte proliferation was expected as PTH/PTHrP/PTHR1 signaling can stimulate chondrocyte proliferation, the enhanced chondrocyte differentiation following the PTH treatment was unexpected as PTHrP/PTHR1 signaling is known to block chondrocyte differentiation (25). Since chondrocyte proliferation is tightly followed by differentiation, we speculate that the enhanced proliferation in PTH-treated ACH mice may override the inhibition of PTH on chondrocyte differentiation. Alternatively, the different effects of the continuous and intermittent PTH treatments may be responsible for the results. In fact, the inhibitory effects of PTH signaling on chondrocyte differentiation have been mainly concluded from patients or mice with gain-of-function or loss-of-function mutations of PTHR1 or PTHrP (45–49), in which the functional status of PTH pathway lasts for the life time, which differs with the intermittent action of PTH that was injected to mice in our current study. In bone metabolism, the effects of intermittent and continuous activation of PTH signaling are known to be different. Although intermittent PTH treatments have anabolic effects on bone remodeling by promoting osteoblast proliferation, differentiation and mineralization, continuous usage has opposite effects through its relatively stronger stimulation on osteoclasts (50,51). However, whether the enhanced chondrocyte differentiation observed in the current study following intermittent PTH treatments is related to the manner of PTH (1-34) administration needs to be further studied. Overall, we found that intermittent PTH (1-34) administration can promote chondrocyte proliferation and differentiation and rescue the bone growth retardation of ACH mice and postnatal lethality of TDII mice.

The molecular mechanisms for the effects of the PTH treatment on FGFR3-related skeletal dysplasia are not fully clarified currently. PTH signaling is transmitted through the G-protein-coupled receptor, which has classical downstream signaling pathways including PKA, PKC and MAPK/ERK (25,52). Whether these pathways are involved in the effects of PTH on the ACH/TD skeleton is not known at this moment. CNP has been found to rescue the skeletal phenotype of ACH mice by inhibiting the ERK pathway, a major downstream pathway of FGFR3 partially responsible for the retarded skeletal development of ACH (53). Since PTH can activate ERK (54), the PTH-activated ERK pathway may not likely be involved in the effects of PTH on the ACH skeleton. We found that the PTH treatment can decrease the expression of FGFR3 and the phosphorylation of FGFR3, which may be an important reason for the rescue effects of PTH on the ACH skeleton. The molecular mechanisms for the down-regulation of FGFR3 expression by PTH remain to be further explored. p21 is a cyclin-dependent kinase inhibitor and can cause cell-cycle arrest in the G1 phase (55). Besides the increased expression of p21 in mice mimicking human ACH (15), growth plate chondrocytes from ACH children and TDII fetuses were found to have increased expression of p21 (56,57). Since we found that the PTH treatment suppressed the expression of p21 in ATDC5 cells, we speculate that the decreased FGFR3 activity and p21 expression resulting from the PTH treatment are related to the rescued effect of PTH (1-34) on the retarded bone growth of TD and ACH mice. In addition, we also found that the PTH treatment led to increased expression of PTHrP in cultured chondrocytes. Since PTHrP promotes chondrocyte proliferation (25), our results suggest the increased PTHrP expression may also be responsible for the positive effect of PTH on bone development. Furthermore, we utilized siRNA approach to knockdown the PTHrP expression and found that Si PTHrP blunted the promoting effect of PTH (1-34) on the proliferation of ATDC5 cells. Thus, the increased expression of PTHrP level may also be involved in the promoting effects of PTH (1-34) on the retarded bone growth in ACH and TDII mice.

Although we revealed the beneficial effects of in vivo-administered PTH on the repressed skeletal development of ACH/TD mice, the effects are still limited, as the PTH-treated TDII mice still displayed short and round skull,
and the structure of long bone growth plates, although improved, was still disorganized with a morphology similar to that found in ACH mice. More research is needed to optimize the outcome of the PTH treatment.

Our preliminary data showed that additional postnatal PTH (1-34) treatment can lead to longer survival of TDII mice, but the mutants still have skeleton dysplasia. To further improve treatment effects, future studies are needed to investigate the timing and dosage of PTH administration, as well as the combination treatment of PTH with other measures such as growth hormone, BMP, CNP and/or surgical approach.

In addition, more research into long-term safety of the PTH treatment is needed. PTH was found to have the potential to cause osteosarcoma in rats administered daily up to 2 years (58), and human data showed that 2 patients in more than 430,000 osteoporotic persons who have received the PTH (1-34) treatment developed osteosarcoma (59). In the current study, the tibial and femoral metaphyses were checked attentively, where the primary osteosarcoma commonly occurs (58), and were found to have no malignancy in PTH (1-34)-treated mice. Additionally, since somatic gain-of-function mutations of FGFR3 have been found in cervical cancer, bladder cancer and multiple myeloma (60,61), we have also examined this issue carefully in PTH (1-34)-treated ACH mice, and found no obvious differences in the histology of important internal organs (heart, lung, liver, kidney and small intestine) between TDII and WT mice born from pregnant mice treated with PTH (1-34) (data not shown).

In summary, in our present study, we demonstrated that the PTH (1-34) treatment can rescue the retarded skeletal development and early postnatal lethality and improve the bone quality of ACH/TD mice possibly through its down-regulation of FGFR3 activity and up-regulation of PTHrP expression. Our study suggests that PTH can potentially be used in clinic to treat ACH and/or TD patients.

**MATERIALS AND METHODS**

**Animals**

*Fgfr3*+/G369C and *Fgfr3*+/K644Eneo mice were generated by us before (9,12), and *Ella-Cre* transgenic mice were kindly provided by Dr H. Westphal (62). To generate mice heterozygous for the mutation (*Fgfr3*+/K644E) (TDII), the neo gene was excised from *Fgfr3*+/K644Eneo construct, using Cre-loxP recombination by crossing with *Ella-Cre* transgenic mice. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Daping Hospital.

**Administration of PTH to mice**

Recombinant human PTH (1-34) was purchased from the Anaspec Institute (Fremont, Canada) and dissolved in vehicle (sterile water for injection). For *Fgfr3*+/G369C mice (ACH mice) and their WT littermates, PTH was administered subcutaneously at the dose of 100 μg/kg body weight per day for 4 weeks after birth. PTH was also administered subcutaneously at the dose of 100 μg/kg body weight in pregnant *Fgfr3*+/K644Eneo mice crossed with *Ella-Cre* mice at E13.5. Untreated animals were injected with the same volume of vehicle alone as control.

**Skeletal analysis and histology**

Briefly, whole-skeletons were harvested, stored in 70% ethanol and were subjected to high-resolution X-ray examination using Faxitron MX20, and the lengths of the bones were measured on the soft X-ray film. BMD was measured by DEXA (PIXimus Mouse 11 densitometer, GE Medical System, Madison, WI, USA). The whole-skeleton staining with Alizarin red S and Alcian blue was performed as described (63). For histological analysis, the right tibiae were fixed in 4% paraformaldehyde in 0.01 M PBS (pH 7.4), decalcified in 15% EDTA (pH 7.4) and embedded in paraffin as described (24). Six-micrometer-thick sections were sliced and stained with Safranin-O, Fast Green, hematoxylin and eosin (H&E). Analyses of the resting zone, proliferation zone and hypertrophic zone were conducted based on zonal definition as described by Reinholt et al. (64).

**BrdU labeling assay**

Mice were injected 100 μg/g body weight BrdU intraperitoneally (Sigma-Aldrich) 2 h before sacrifice. Sections of knee joints were incubated overnight with anti-BrdU antibody (Sigma-Aldrich), followed by a secondary antibody and DAB color development, and counterstaining with hematoxylin.

**Mesenchymal cell micro-mass cultures**

Cells released from E11.5 limb buds were plated in 20 spots containing 1 × 10⁶ cells/ml on 60 mm dishes and left for 1 h at 37°C before adding 4 ml of Dulbecco’s modified Eagle’s medium and F-12 (1:1) medium (Hyclone) containing 10% FBS and incubated overnight. Cultures were then rinsed once with serum-free medium before adding medium supplemented with 2% FBS with or without 10⁻⁸ M PTH (1-34) and incubating for 4 days with medium changes every second day. PTH (1-34) was added for 6 h, after which the medium was changed and the culture was maintained for another 42 h. Thus, every 48 h acts as a cycle to mimic the intermittent administration of PTH (1-34) (50). At the indicated times, dishes were rinsed three times with PBS (pH 7.4) before fixing for 1 h in 4% paraformaldehyde in PBS. Fixed cultures were rinsed three times for 5 min each time with PBS and stained with Alcian blue.
Primary chondrocyte cultures

Primary chondrocytes were isolated from cartilage of knee joints of P3 mice, using enzyme digestion (66). Cells were plated at a density of \(2 \times 10^6\) cells/ml, cultured in DMEM/F-12 (1:1) medium (Hyclone) supplemented with 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin and 10\% FBS until reaching subconfluence. PTH (1-34) \((10^{-8}\text{M})\) was added for 6 h in every 48 h cycle as described above.

Cell proliferation assay

Cell proliferation was detected using an in vitro colorimetric assay (Sigma-Aldrich). Chondrocytes were washed with PBS and incubated in 200 \(\mu\)l of medium containing 20 \(\mu\)l of MTT solution (5 mg/ml dissolved in 2% NaHCO3) for 4 h at 37\°C. Subsequently, MTT solution was removed and 150 \(\mu\)l of dimethyl sulfoxide (DMSO) was added. After being mixed for 10 min at room temperature, the purple formazan salt product resulting from the reduction of MTT was solubilized by DMSO and quantified spectrophotometrically at 490 nm.

In vitro culture of embryonic tibia bones

Culture of the embryonic tibia bone was conducted according to the protocol described before (22,67). Tibiae were harvested from E15.5 mice and were cultured in 48-well plates for 7 days with BGJb medium (Fitton-Jackson Modified) supplemented with 0.05 mg/ml ascorbic acid, 0.5 mg/ml L-glutamine, 10 mg/ml streptomycin, 10 U/ml penicillin, 1 mM \(\beta\)-glycerophosphate and 0.2\% BSA. The culture medium was changed every other day. The total lengths and longitudinal lengths of the cartilage and calcified bone were measured using Image-Pro Plus 7.0 (22). Changes in lengths were expressed as percentage increase relative to the value before the treatment [percentage increase = \((\text{length at day } 7 - \text{length at day } 0)/\text{length at day } 0\)].

Transfection with siRNA targeting PTHrP

siRNAs targeting PTHrP mRNA (Si PTHrP) and negative Si Control were designed and synthesized by Ribio Corporation (Guangzhou, China) and transiently transfected using the Lipofectamine\textsuperscript{TM} 2000 (Invitrogen), according to the manufacturer’s instructions. Forty-eight hours later, PTHrP mRNA levels were determined by real-time PCR after 48 h and the levels of PTHrP protein were analyzed by western blotting after (see what follows) 72 h.

Real-time PCR

Total RNA of cells was isolated using the Trizol reagent according to the manufacturer’s instructions (Invitrogen), which was used to assess effects of treatments on mRNA expressions of genes of interest. All reactions were performed in an Mx3000P PCR machine (Stratagene) using the Two-Step QuantiTect SYBR Green RT-PCR Kit (Takara), and the reaction conditions were optimized for each of the genes by changing the annealing temperature. Each run was replicated three times. Expression levels for each gene of interest were normalized to their corresponding values of endogenous control gene Cyclophilin A. PCR data are expressed as fold change in relation to WT control. The primer sequences are as follows: Collagen II (Col2a1): 5′-CTGGTGGAAGCAG CAAGAGCAA-3′ and 5′-CAGTGCACTATGAGGAAGGAAGGAAGGAAG-3′; Collagen X (col10a1): 5′-GCGACATTGACCCCAAAGAT-3′ and 5′-CATGA TGTCGAAGTGGAG-3′; PTHrP: 5′-CAGTCAGCAGTGAAGG-3′ and 5′-GGGTGTGTTGTGGGAGG-3′; Cyclophilin A: 5′-CGAGCTTA GCACCTGGAGA-3′ and 5′-TGGCGTGTAAGTCAACCAC-3.

Western blotting

Mouse primary chondrocytes and ATDC5 were lysed in an ice-cold buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS and a cocktail of protease inhibitors (Roche). Protein samples were resolved on a 10% SDS–PAGE gel and transferred onto a PVDF membrane (Millipore). Then antibodies against FGFR3, PTHrP and p21 (Santa Cruz, USA) were used to probe the blots followed by ECL signal detection.

Statistical analysis

Data were evaluated statistically in SPSS (Version 10.0 for Windows). Results are shown as mean value \(\pm\) SD. Statistics were analysed by Student’s t-test or two-way analysis of variance (ANOVA) with Turkey’s post hoc test. Significance was accepted at \(P\)-values < 0.05.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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