A novel loss-of-function mutation in Npr2 clarifies primary role in female reproduction and reveals a potential therapy for acromesomelic dysplasia, Maroteaux type

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We discovered a new spontaneous mutant allele of Npr2 named peewee (pwe) that exhibits severe disproportionate dwarfism and female infertility. The pwe phenotype is caused by a four base-pair deletion in exon 3 that generates a premature stop codon at codon 313 (L313X). The Npr2pwe/pwe mouse is a model for the human skeletal dysplasia acromesomelic dysplasia, Maroteaux type (AMDM). We conducted a thorough analysis of the female reproductive tract and report that the primary cause of Npr2pwe/pwe female infertility is premature oocyte meiotic resumption, while the pituitary and uterus appear to be normal. Npr2 is expressed in chondrocytes and osteoblasts. We determined that the loss of Npr2 causes a reduction in the hypertrophic and proliferative zones of the growth plate, but mineralization of skeletal elements is normal. Mutant tibiae have increased levels of the activated form of ERK1/2, consistent with the idea that natriuretic peptide receptor type 2 (NPR2) signaling inhibits the activation of the MEK/ERK mitogen activated protein kinase pathway. Treatment of fetal tibiae explants with mitogen activated protein kinase 1 and 2 inhibitors U0126 and PD325901 rescues the Npr2pwe/pwe growth defect, providing a promising foundation for skeletal dysplasia therapeutics.

INTRODUCTION

Disruptions in skeletal development and growth are classified as skeletal dysplasias and are an important contributor to severe short stature, occurring in approximately 1 out of every 5000 births (1). The effects on individuals with skeletal dysplasia can range from relatively minor to lethality (1–3) and multiple organ systems can be affected (1). The advent and improved accessibility to high-throughput sequencing technologies has brought forth a wealth of information regarding the genetic causes of skeletal dysplasias (1,4). According to the most recent nosology, there are 456 forms of skeletal dysplasia, of which 316 have been associated with mutations in one or more of 226 genes (4). This means that approximately 70% of the skeletal dysplasias have known genetic causes (4). The genetic causes of the remaining forms are currently unknown (1,3,4), and even those forms that have a known genetic cause may not have a clear molecular mechanism (1). Animal models of skeletal dysplasia have been useful in understanding the mechanisms by which these genes regulate proper skeletal development and growth and contribute to the pathology of skeletal dysplasia (1,5–7).

Some disorders of the skeleton also affect the reproductive organs (8–11). Infertility is estimated to affect 15% of the global population and 25% of these cases are idiopathic (12). There has been a surge of interest in the mechanisms of female infertility and sub-fertility with respect to reproductive aging, as many women in developed countries are delaying...
their age of first conception past their early 20s, when female fertility is at its zenith (13). Mouse models of female infertility have offered much in the way of understanding the genetic regulation of female fertility (14,15).

We have discovered a novel recessive, mutant allele of Npr2, the gene that encodes natriuretic peptide receptor type 2 (NPR2), which we have named peewee (pwe). The mutant mice have growth insufficiency, skeletal dysplasia and female infertility. Mutations in NPR2 cause a rare form of recessive skeletal dysplasia in humans, acromesomelic dysplasia, Maroteaux type (AMDM) (16), which affects the middle and distal portions of limbs and the shape of vertebrae (16,17). Npr2 encodes a membrane-bound guanylyl cyclase that generates the secondary messenger cyclic guanosine monophosphate (cGMP) upon binding its ligand, C-type natriuretic peptide (CNP) (18).

We report a comprehensive analysis of the role of Npr2 in the regulation of female fertility, and evidence that pharmacological inhibition of mitogen activated protein kinase 1 and 2 (MEK1/2) is sufficient to rescue the peewee growth defect in tibial explants. These findings offer promising clues as to signaling pathways that could be therapeutic targets in the treatment of AMDM and other forms of skeletal dysplasia.

RESULTS

The peewee growth defect

The peewee (pwe) mouse arose on the NAW/WI background, was outcrossed to obtain hybrid vigor and was maintained as a stock by breeding heterozygotes. Only 12% of the progeny were mutant, which is lower than expected for autosomal recessive inheritance. Outcrossing to Mus castaneus supported homozygote viability. Mutants constituted 27% of the progeny of this F1 × F1 intercross (28/103), consistent with the expected Mendelian ratio for an autosomal recessive allele.

Pwe homozygous mutants exhibit growth delay and disproportionate dwarfism that is evident at 2 weeks of age (Fig. 1A). Body weight and crown-rump length of mutants are 54 and 77%, respectively, of their unaffected littermates (Table 1). Pwe mice have a characteristic cranial dysmorphology that includes a domed skull and short snout (Fig. 1A). Malocclusion is frequent, but incompletely penetrant. All bones formed through endochondral ossification are significantly reduced in length (Table 1 and Fig. 2A, A–L).

The proximal skeletal elements of the appendicular skeleton (femur and humerus) are the most severely affected in the pwe mouse (Table 1 and Fig. 2A, C and E). All skeletal elements formed through endochondral ossification are ≤75% the length of unaffected skeletal elements (Table 1 and Fig. 2A, A–L). There is no reduction in the width of the skull, femur (thinner dimension) or ribs (Table 1). A fully penetrant aspect of the phenotype is a notch on the dorsal surface of the atlas (Fig. 2A and K).

Sections through 5-week-old male tibial growth plates suggest that the skeletal growth defect in pwe mice stems from a disruption in the regulation of chondrocyte differentiation, and potentially proliferation, due to the reduction in the height of the hypertrophic and proliferative zones of the growth plate (Fig. 2B). However, all zones of the growth plate are present, and their organization is maintained, meaning that the disturbance in skeletal growth may be due to a disruption in the number or rate in which the chondrocytes progress through the zones of the growth plate (Fig. 2B).

Molecular identification of the peewee mutation

Analysis of DNA samples from 15 affected animals from a [(pwe/+) × CAST/Ei] F1 × F1 intercross with a single nucleotide polymorphism (SNP)-mapping panel (19) placed the locus in a region on proximal mouse chromosome 4. Analysis of additional animals with more markers confirmed this location and narrowed the interval. The genotype of affected mice placed the mutation distal to the microsatellite marker...
Npr2 (data not shown). This deletion results in a frameshift that generates mutants revealed a four base-pair deletion in exon 3 of frame (L313X, Fig. 3B). Genotyping this mutation revealed a premature stop codon at codon 313, which encodes a NPR2 within the ligand-binding domain (18) (Fig. 3C).

We tested fertility by mating wild-type mice with Npr2 AMDM, which is caused by loss-of-function mutations of associated with this region of human chromosome 9 identified on human chromosome 9p13.3. A search for skeletal dysplasias which contains more than 80 genes and corresponds to Over a 3-month period, all unaffected females (data not shown).

D4Mit93 (Fig. 3A), and analysis of unaffected animals narrowed the critical interval to a 3 Mb region between D4Mit212 and D4Mit109 (Fig. 3A, one animal per marker), which contains more than 80 genes and corresponds to human chromosome 9p13.3. A search for skeletal dysplasias associated with this region of human chromosome 9 identified AMDM, which is caused by loss-of-function mutations of Npr2 in humans (16,17). Sequencing of Npr2 in known pwe mutants revealed a four base-pair deletion in exon 3 of Npr2 (Fig. 3B). This deletion results in a frameshift that generates a premature stop codon at codon 313, which encodes a leucine residue in the wild-type and reference open reading frame (L313X, Fig. 3B). Genotyping this mutation revealed that one apparently affected animal was actually a runted heterozygote, explaining the erroneous exclusion of Npr2 as a potential candidate gene early in our analysis (Fig. 3A, asterisk).

Npr2 encodes a membrane-bound guanylyl cyclase that is present at the plasma membrane as a dimer (18). Mutations in human AMDM patients occur throughout the length of the encoded NPR2 protein (16,20) (Fig. 3C). The premature stop codon at codon 313 in Npr2pwe/pwe mice truncates NPR2 within the ligand-binding domain (18) (Fig. 3C). Without an intact ligand-binding domain or any of the domains C-terminal to the premature stop codon, any translated protein from the pwe allele is predicted to be non-functional, generating a null allele (Fig. 3C). The growth insufficiency of pwe mutants is indistinguishable from that reported for other mouse null alleles of Npr2 (21–24).

### Table 1. Disproportionate dwarfism in pwee mutants

<table>
<thead>
<tr>
<th></th>
<th>Unaffected</th>
<th>pwee</th>
<th>Ratio*</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>25 ± 1</td>
<td>16 ± 1</td>
<td>0.54</td>
<td>0.001</td>
</tr>
<tr>
<td>Length of body and skeletal elements (mm)*</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Crown to rump</td>
<td>71.05 ± 0.96</td>
<td>54.43 ± 0.94</td>
<td>0.77</td>
<td>0.0003</td>
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<tr>
<td>Radius</td>
<td>10.40 ± 0.19</td>
<td>5.28 ± 0.13</td>
<td>0.51</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ulna</td>
<td>12.83 ± 0.25</td>
<td>7.16 ± 0.18</td>
<td>0.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Femur</td>
<td>15.12 ± 0.19</td>
<td>8.49 ± 0.21</td>
<td>0.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Humerus</td>
<td>11.52 ± 0.17</td>
<td>5.73 ± 0.17</td>
<td>0.64</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Tibia</td>
<td>15.84 ± 1.01</td>
<td>10.28 ± 0.29</td>
<td>0.65</td>
<td>0.001</td>
</tr>
<tr>
<td>Ilium</td>
<td>17.09 ± 0.39</td>
<td>12.59 ± 0.13</td>
<td>0.74</td>
<td>&lt;0.0001</td>
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<tr>
<td>Scapula</td>
<td>10.93 ± 0.15</td>
<td>8.22 ± 0.15</td>
<td>0.75</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Skull</td>
<td>23.38 ± 0.31</td>
<td>19.89 ± 0.41</td>
<td>0.85</td>
<td>0.0003</td>
</tr>
<tr>
<td>Width of skeletal elements (mm)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L6 vertebrae</td>
<td>3.51 ± 0.09</td>
<td>2.48 ± 0.08</td>
<td>0.71</td>
<td>0.001</td>
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<tr>
<td>Femur (a)</td>
<td>2.08 ± 0.04</td>
<td>1.83 ± 0.03</td>
<td>0.88</td>
<td>0.0005</td>
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<tr>
<td>Femur (b)</td>
<td>1.47 ± 0.04</td>
<td>1.36 ± 0.04</td>
<td>0.93</td>
<td>NS</td>
</tr>
<tr>
<td>Skull</td>
<td>11.52 ± 0.22</td>
<td>11.28 ± 0.35</td>
<td>0.98</td>
<td>NS</td>
</tr>
<tr>
<td>Rib</td>
<td>0.78 ± 0.05</td>
<td>0.83 ± 0.03</td>
<td>1.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Ratio of pwee to unaffected.

**Individual skeletal elements are listed from most affected to least affected.

**Femurs were measured in the thicker (a) and thinner (b) dimensions.

Npr2pwe/pwe female infertility

We tested fertility by mating wild-type mice with Npr2pwe/pwe mutants. Males were sub-fertile, but had no obvious abnormalities in the penian bone or testis histology (data not shown). Over a 3-month period, all unaffected females (n = 3) bore litters, while none of the Npr2pwe/pwe females (n = 3) bore litters (data not shown). Npr2 is expressed at all levels of the female reproductive tract in the rodent (18,25–28). Thus, Npr2pwe/pwe female infertility could be due to defects in any or all of these organs. We undertook a systematic approach to uncover critical sites of Npr2 action in the female reproductive tract.

We tested whether Npr2pwe/pwe females experienced puberty normally by observing the date of vaginal opening as an indicator of the onset of puberty in mice (29–31). The control animals experienced vaginal opening on average at postnatal day 21 (P21) as expected (Fig. 1B, range P18–P28, n = 11), while Npr2pwe/pwe females experienced vaginal opening from P33–P84 (Fig. 1B, n = 6). The extensive delay in puberty in individual Npr2pwe/pwe females appears to correlate with their body weight (Supplementary Material, Fig. S1A). Body weight is a known predictor of the onset of puberty in mice (30,31) and humans (32).

To determine whether post-pubertal Npr2pwe/pwe females were capable of cycling through estrus, vaginal smears were taken for 20 consecutive days and estrous stage was assigned by vaginal cytology (29). Control (n = 7) and Npr2pwe/pwe (n = 4) females cycled normally through all stages of the estrous cycle during this 20-day period (Fig. 4A). The amount of time spent in each of the stage of the cycle did not differ between mutants and controls (Fig. 4A). Thus, while Npr2pwe females have a delay in the onset of puberty, they are capable of cycling through estrus normally, suggesting that the hypothalamus and pituitary are functioning properly to regulate the estrous cycle (29,33). The diestrus uterine histology of Npr2pwe/pwe females is normal at 12 weeks of age, indicating that the uterine tissues, including the endometrium, myometrium and stromal glands, are intact (Fig. 4C).

To assess ovarian follicle development in the Npr2pwe/pwe female, we stimulated prepubertal females with pregnant mare’s serum gonadotropin (PMSG), which stimulates follicle growth to the preovulatory stage. Oocytes are arrested around birth at prophase I, and this is morphologically distinguished by the presence of a germinal vesicle (13,15,34–36). The germinal vesicle is normally maintained until the preovulatory follicle receives the surge of luteinizing hormone (LH) that promotes meiotic resumption and ovulation (13,15,34–36). At this point, the germinal vesicle will break down, the meiotic spindle is formed and the first meiotic division is completed with the extrusion of the first polar body (13,15,34–36). Preovulatory follicles in the ovaries of PMSG-primed control females contained oocytes (either released from ovarian follicles or in sections of whole ovaries) with intact germinal vesicles (Fig. 5A). However, Npr2pwe/pwe oocytes had progressed prematurely through meiosis; virtually all oocytes examined underwent premature germinal vesicle breakdown (GVBD) (Fig. 5A). Some had formed meiotic spindles, or extruded one or even two polar bodies (Fig. 5A). cGMP, which is generated by NPR2, is known to inhibit progression through meiosis (37,38). The level of cGMP in Npr2pwe/pwe antral follicles was reduced to undetectable levels (Fig. 5A). Thus, Npr2 is essential for cGMP production and maintenance of meiotic arrest in the oocyte.

To determine whether Npr2pwe/pwe females were capable of releasing fertilizable oocytes into the oviducts, we superovulated prepubertal Npr2pwe/pwe females as described in the Methods section and mated them with a proven wild-type.
male. Npr2\textsuperscript{pwe/pwe} females ovulated fewer oocytes than controls (Fig. 5B), and they underwent oocyte fragmentation, a precursor to oocyte cell death (39). None of the Npr2\textsuperscript{pwe/pwe} oocytes progressed to the two-cell embryo stage.

We tested the natural ability of Npr2\textsuperscript{pwe/pwe} females to ovulate by conducting natural mating experiments with mature, post-pubertal animals. Npr2\textsuperscript{pwe/pwe} females released similar numbers of oocytes when compared with controls (Npr2\textsuperscript{pwe/pwe}, n = 6 and control, n = 6), data not shown). We measured the levels of follicle-stimulating hormone (FSH) and estradiol. Levels of both the hormones at diestrus were normal (Table 2). Levels of LH and FSH from individual male and female pituitaries determined by radioimmunoassay were also normal (data not shown). Sections through Npr2\textsuperscript{pwe/pwe} ovaries at 8 and 12 weeks revealed the presence of corpora lutea (Fig. 4B), consistent with ovulation (33,40). Together, these data indicate that the hypothalamus and pituitary of Npr2\textsuperscript{pwe/pwe} females are functioning well enough to support ovulation and the ovary responds with appropriate steroid hormone production (33,40).

**Npr2 and bone tissue mineral density**

Npr2 is expressed in osteoblasts as well as chondrocytes (41), and data suggest that CNP-NPR2 signaling may augment osteoblast differentiation in combination with other factors (42). It is hypothesized that many disruptions in the growth plate also affect the normal function of osteoblasts or osteoclasts (7). We assessed whether bone was defective in mineralization or integrity by microscopic-computed tomography (microCT) scans of 12-week-old male femurs and L4 vertebrae (control, n = 6; mutant, n = 7). No major deficits in the
Elevated levels of phosphorylated ERK1/2 in Npr2<sup>pwe/pwe</sup> tibiae

The fibroblast growth factor (FGF) signaling axis is a potent activator of the MEK/ERK mitogen activated protein kinase (MAPK) pathway in the growth plate (43,44) (Fig. 6A). Gain-of-function mutations in the gene that encodes FGF receptor 3 (FGFR3) cause achondroplasia in humans (1,6,7,44,45). In cultured chondrocytes, activation of MEK/ERK leads to a reduction in the production of extracellular matrix proteins, differentiation and possibly proliferation (43,44). The cGMP generated by NPR2 upon binding CNP activates cGMP-dependent protein kinase II (PKGII), which goes on to inhibit the activation of RAF-1, the activator of MEK1/2 (43). Thus, NPR2 signaling halts the MEK/ERK MAPK cascade at the level of RAF-1 (Fig. 6A) through its activation of PKGII (43) in vitro.

We hypothesized that if Npr2<sup>pwe/pwe</sup> mice lack the critical receptor required for MEK/ERK inhibition, they should have increased levels of phosphorylated ERK1/2. Western blot analysis was performed using postnatal day three (P3) tibial lysates. Higher levels of phosphorylated ERK1/2 were observed in lysates prepared from mutant tibiae relative to wild-type (Fig. 6B). Thus, signaling through NPR2 inhibits the activation of the MEK/ERK MAPK pathway in intact bone.

**Therapy for the Npr2<sup>pwe/pwe</sup> growth defect**

We reasoned that treatment of Npr2<sup>pwe/pwe</sup> mice with a pharmacological inhibitor of the MEK/ERK MAPK pathway might improve bone growth. We cultured fetal tibiae harvested at embryonic day 16.5 for six days as explants in the presence of vehicle (DMSO) or the MEK1/2 inhibitors U0126 and PD325901 (Fig. 7A) (21,46–50). PD325901 is a potent MEK1/2 inhibitor (50), and has been used to study mouse cancers (46,47). After the culture period, the tibiae were photographed and measured (Fig. 7). Mutant tibiae were shorter than wild-type after treatment with vehicle, and both inhibitors induced growth of wild-type and mutant tibiae. PD235901 induced more growth than U0126. Two-way ANOVA and Bonferroni post-hoc analysis confirmed that the effects of both treatments were highly significant (Fig. 7B, P < 0.0001, n = 16 of both genotypes for U0126, n = 7 of both genotypes for PD325901). Drug-induced growth of the normal tibiae suggests that CNP signaling is rate limiting in normal mice. Western blots revealed reduced phosphorylated ERK1/2 in U0126-treated animals, demonstrating that the drug effectively inhibited MEK/ERK activation in explants from normal and mutant mice (Fig. 7C).

The histology of sections from cultured tibiae suggested that the primary effect of U0126 on wild-type and mutant tibiae was to expand the size of the hypertrophic zone (Fig. 7A). In situ hybridization was performed to assess expression of Col10a1 (51) (collagen 10, alpha subunit), a marker of hypertrophic chondrocytes (Fig. 7A). The cells in this expanded zone of the growth plate are marked with Col10a1 transcripts, confirming that these are hypertrophic chondrocytes.

**DISCUSSION**

We have identified the genetic defect in peewee, a new spontaneous mutant allele of Npr2, with growth insufficiency and female infertility. The phenotype is caused by a 4-bp deletion in exon 3 of Npr2 that generates a premature stop codon at
This leads to truncation of the receptor within the ligand-binding domain and a predicted null allele due to the loss of all domains C-terminal to the mutation. Other null alleles of Npr2 with similar skeletal and growth insufficiencies have been reported, but previous characterization focused on other aspects of Npr2 function including blood pressure regulation and gastrointestinal tract function (21,24). Here we report a comprehensive analysis of female infertility and bone growth in response to pharmacotherapy.

Since Npr2 is expressed in all the major organs of the female reproductive tract (18,25–28), we addressed the possibility that multiple organs could contribute to peewee female infertility. We show for the first time that Npr2-deficient females exhibit a delay in puberty, and this delay correlates with the body size (30,31). Once Npr2<sup>pwe/pwe</sup> females reach puberty, they progress through all stages of the estrous cycle and are capable of ovulating in response to exogenous and natural stimulation. Our results differ from previous reports that the Npr2 knock-out (KO) allele caused loss of cycling, uterine atrophy, reduced endometrial and myometrial layers, and absent stromal glands (21). The differences are likely due to genetic background effects. We characterized Npr2<sup>pwe/pwe</sup> on a mixed background that supports viability, but the mutation has reduced viability on other backgrounds, similar to reports for other Npr2 alleles. Susceptible background strains may offer less compensation for Npr2 deficiency in a variety of organs that express Npr2, including the uterus and pituitary gland. Genetic compensation could involve variable cross talk between other natriuretic peptide receptors and ligands.

Npr2<sup>pwe/pwe</sup> females have normal serum levels of FSH and estradiol at diestrus and naturally ovulate, indicating that...
hypothalamic stimulation of the pituitary by pulsatile GnRH secretion and pituitary secretion of FSH and LH (33) are occurring. This is surprising, given that Npr2 is expressed at all points of the female HPG axis (18,25–28), and natriuretic peptide signaling through NPR2 was proposed to play a role in gonadotropin production (27). Importantly, this work and the work of others (52) clearly demonstrate that Npr2 signaling is not required for HPG axis function in the female in the context of this genetic background.

The primary cause of female infertility in Npr2pwe/pwe females is premature oocyte meiotic resumption. We show that in Npr2pwe/pwe mice, impaired cGMP production in ovarian follicles is associated with premature oocyte maturation and impaired embryonic development. Our findings support the requirement for NPR2 to maintain the production of cGMP in ovarian follicles to ensure oocyte meiotic arrest (25). Although Npr2pwe/pwe females are able to ovulate in response to exogenous or endogenous gonadotropin stimulation, the oocytes undergo fragmentation (39). This has been reported in Npr2pwe/pwe mutants, mice with reduced levels of CNP (Nppclbab/lbab), and in the G-protein coupled receptor 3 mutant mouse (Gpr3−/−) (52). Gpr3−/− oocytes have reduced levels of cAMP, leading to premature meiotic resumption and oocyte fragmentation (39). Taken together, the primary site of Npr2pwe/pwe female infertility is the oocyte. None of the descriptions of people affected with AMDM discuss female fertility (16,17,20,53,54). Our studies suggest that if AMDM female patients are infertile due to a failure to maintain oocyte meiotic arrest, they may be able to bear children using donor eggs, provided that uterine and placental function are normal.

Figure 5. Npr2pwe/pwe female infertility is caused by a failure in meiotic arrest, leading to the release of oocytes that are not viable. (A) Oocytes from unaffected (+/−) and Npr2pwe/pwe mice were released from prepubertal PMSG-primed antral follicles by needle puncture and examined by light microscopy with DIC optics (left). Almost all oocytes had a single pronucleus while most oocytes from Npr2pwe/pwe follicles exhibited GVBD and premature formation of polar bodies. The percentage of oocytes that underwent GVBD is shown at the far right (top). Data are the mean ± SEM of three separate experiments. Histological sections from fixed ovary tissues of the same mice (center panels) revealed premature meiotic spindle formation in mutants. Extracts from prepubertal PMSG-primed antral follicles had measurable levels of cGMP in controls, but undetectable levels in mutants (far right, bottom). Data are the mean ± SEM of three separate experiments. (B) Superovulated prepubertal wild-type (+/+) mice released normal eggs, whereas nearly all mutant oocytes underwent fragmentation. When wild-type and Npr2pwe/pwe females were superovulated and mated with wild-type males, Npr2pwe/pwe females (black bars) released fewer oocytes compared with the wild-type (white bars). Most of the mutant oocytes were not fertilized and mutant zygotes failed to develop to two-cell embryos. Data are expressed as mean ± SEM (control, n = 6; mutant, n = 4).
nascent element (1, 5, 6). The trabecular and cortical tissue mineral density of femurs and L4 vertebral elements are normal in Np2pwe/pwe mice, even though the receptor is expressed in osteoblasts (41). It is clear that the main role of the CNP-NPR2 signaling axis in skeletal elements is to regulate their longitudinal growth through action in the growth plate. Without Npr2, chondrocyte hypertrophy is compromised, so the bones of mutant animals will never reach a normal adult length, although the bone tissue laid down is normal in its composition.

Both the proliferative and hypertrophic zones of the growth plates in Np2pwe/pwe animals appear reduced in size, consistent with the Npr2 KO mouse (21) and other spontaneous mutant alleles of Npr2 (22, 23). The loss of Npr2 does not affect the overall organization of the growth plate, but probably affects either the overall number of chondrocytes recruited to undergo hypertrophic differentiation, the rate in which they commit to this fate, and/or the rate in which they proliferate. We observed elevated activation of ERK1/2 in whole mutant tibial lysates, which demonstrates that NPR2 is required for ERK1/2 inhibition in vivo, as predicted by in vitro cell culture studies (43).

Treatment for skeletal dysplasia involves multiple lengthening surgeries for the long bones (1, 45). These surgeries are expensive, time consuming, fraught with many possible complications, and ultimately result in only a modest increase in bone length (1, 45). In general, the acceptance of short stature is advocated in the USA by patients, their families, and advocacy groups for individuals with severe short stature. There are currently no effective pharmacological therapies for individuals with skeletal dysplasia (1). Growth hormone treatment has been attempted in achondroplasia patients without much success (1, 45). C-type natriuretic peptide (CNP), the ligand of NPR2, is a very promising new therapeutic candidate that has been shown to rescue growth defects in mouse models of achondroplasia both genetically and pharmacologically (7, 49, 55, 56). While this therapeutic approach could offer hope for achondroplasia patients and their families, this form of therapy would be ineffective in AMDP patients that lack the receptor for CNP (16).

There are no reports of growth promoting pharmacotherapy for Npr2 mutants. Here, we report successful elongation of Np2pwe/pwe-mutant tibiae and inhibition of MEK/ERK MAPK signaling within these tibial explants through utilization of the MEK1/2 inhibitors U0126 and PD325901. We also show that MEK1/2 inhibitor treatment extends the length of the hypertrophic zone, consistent with the proposed role of ERK1/2 inhibition in the promotion of hypertrophic differentiation (7, 43, 49, 57). PD325901 treatment led to a more dramatic increase in the tibial length compared with U0126. This could be due to the fact that PD325901 is known to be a more potent MEK1/2 inhibitor, and has been successfully administered to the whole animal in cancer studies (47, 58). PD325901 has also been administered orally in clinical trials (59, 60). Thus, PD325901 as well as other structurally related agents that are currently undergoing clinical evaluation in cancer are promising therapeutic candidates for growth defects associated with excess MEK1/2 signaling, including patients with AMDP, achondroplasia or other appropriate skeletal dysplasias. The key to moving skeletal dysplasia therapeutics forward will be to deliver this class of agents specifically to the growth plate, and individuals with expertise in the realm of pharmacology will be better suited to address this issue.

We have identified a novel null allele of Npr2 and characterized the effect on long bone growth and female fertility. In addition, we have established a proof of principle for pharmacotherapy to correct poor bone growth. The next challenge is to develop efficacious delivery methods and treatment regimes in intact animals, minimizing effects on other tissues.

### MATERIALS AND METHODS

#### Generation of peewee mice and mapping of the mutation

The peewee mutation arose spontaneously on the NAW/WI strain in Glen Wolfe’s laboratory at the University of Kansas (Lawrence, KS). The mice were then transferred to Andrzej Bartke’s laboratory at Southern Illinois University (SIU, Carbondale, IL). The line was maintained at SIU by outcrossing for hybrid vigor. Upon transfer to the University of Michigan, in vitro fertilization was carried out with oocytes from C57BL/6J females (The Jackson Laboratory, Bar Harbor, ME) and sperm from a single homozygous peewee male from a mixed background including contributions from the NAW/WI, C57BL/6 and C3H/HeJ strains. The fertilized eggs were transferred to surrogate mothers yielding pwe heterozygotes that were a mixture of all these backgrounds. Heterozygotes were bred to C57BL/6 J X C3H/HeJ F1 mice (The Jackson Laboratory, Bar Harbor, ME), and the heterozygous progeny of this cross were intercrossed to generate mutant animals for analysis. These pwe heterozygotes were also crossed to Mus castaneus and F1 progeny were intercrossed to map the mutation by genotyping SNPs (19) and microsatellite markers.

All mice were housed in a specific pathogen free facility with 12 h light, 12 h dark cycle in ventilated cages with unlimited access to tap water and Purina 5020 chow. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals, and all experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guidelines of the Care and Use of Experimental Animals.

#### Skeletal preparation and histology

Skeletal preparations of whole, skinned adult mice were prepared by fixation in 95% ethanol overnight, stained with Alcian Blue, treated with 2% KOH, stained with Alizarin

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**Table 2. Normal hormone levels in peewee females**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Unaffected</th>
<th>peewee</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH* (ng/ml)</td>
<td>16.9 ± 10.8</td>
<td>17.8 ± 8.6</td>
</tr>
<tr>
<td>Estradiol* (pg/ml)</td>
<td>16.4 ± 4.7</td>
<td>15.1 ± 3.8</td>
</tr>
</tbody>
</table>

*Multiplex testing on serum samples collected at diestrus (control, n = 8; peewee, n = 8).

**ELISA on serum samples collected at diestrus (control, n = 8; peewee, n = 8).**
Red and stored in glycerol. Bones from affected and un-
affected animals were measured with calipers for determin-
ation of disproportionate dwarfism. Five-week-old male
tibiae were fixed overnight in 4% formaldehyde in phosphate
buffered saline (PBS), decalcified as described previously
(61), dehydrated through an ethanol series and embedded
in paraffin. Tibiae were sectioned and stained with hemotox-
ylin and eosin.

Analysis of the onset of puberty and estrous cyclicity
Females were observed for date of vaginal opening daily from
P18–P28, and estrous cycles were monitored by vaginal cy-
tology every day for a 20-day period between 8 and 12
weeks of age as described (29).

Hormone measurements
Females were sacrificed at 12 weeks of age at diestrus. Serum
was prepared from blood samples after clotting for 90 min at
room temperature, running a wooden toothpick along the
sides of the collection tube and spinning the samples at 2000
g for 15 min. The serum supernatant was removed and
stored at \( -20^\circ C \) until analysis. Analysis was performed by
the University of Virginia Ligand Core.

Analysis of oocytes, ovaries and uterus
Prepubertal female mice were stimulated with 5 IU PMSG to
stimulate follicle growth to the preovulatory follicle stage
and were euthanized 46 h later (62). Oocytes were released
from antral follicles from one ovary by needle puncture. The
other ovary was fixed overnight in Bouin’s fixative (Ricca
Chemicals), dehydrated in an ethanol series, followed by
\( n \)-butanol and embedded in paraffin. Sections were stained with
Mayer’s hemotoxylin and eosin. Uteri were dissected from
12-week-old females at diestrus, fixed for 1 h in 4% paraformal-
dehyde, dehydrated through an ethanol series and embedded in
paraffin. Sections were stained with hemotoxylin and eosin.

cGMP quantification
Intact antral follicles were dissected from PMSG-primed
ovaries of mutant and control females as previously described
(63). The follicles were processed for cGMP quantification
according to the manufacturer’s instructions (Cyclic GMP
EIA Assay, Cayman Chemical # 581021).

Superovulation
Prepubertal females were stimulated with 5 IU PMSG fol-
lowed 46 h later by 5 IU human chorionic gonadotropin
(hCG) to induce the superovulation of oocytes (62) and
placed with an experienced wild-type male overnight.
morning after hCG administration, females were observed for a copulation plug, and fertilized eggs were collected, counted and cultured in M16 media at 37°C in 5% CO2 for 4 days to observe normal early embryonic development (62).

Natural mating experiments

Twelve-week-old mutant and control females were placed with an experienced hybrid male (C57BL/6J X C3H/HeJ F1, The Jackson Laboratory, Bar Harbor, ME) for a week and observed daily for evidence of a copulation plug. The day of the plug, females were euthanized and their oviducts were flushed with a blunted needle filled with a 6% bovine serum albumin (BSA)/1× PBS solution. Oocytes were counted under a dissection microscope.

Micro-computed tomography

Specimens were dissected free of soft tissue and analyzed by micro-computed tomography (μCT, eXplore Locus SP, GE Healthcare Pre-Clinical Imaging, London, ON, Canada). Specimens were immersed in water and scanned 4 (femora) or 12 (vertebrae) at a time using the Parker method (180° plus a 20° fan angle) rotated at 80 kVp and 80 μA and filtered using both an acrylic beam flattener and a 0.02 in. aluminum filter. Images were reconstructed at an isotropic voxel size of 18 μm and calibrated for densitometry. A region of interest 20 and 25% of the mid-diaphysis was isolated from the femora for trabecular and cortical analyses, respectively. A region of interest including centrum of the vertebrae and excluding the spinal processes was segmented out for trabecular analysis. Manufacturer software (Micro-View v2.2, GE Healthcare Pre-Clinical Imaging) was used to calculate the trabecular bone volume fraction, bone surface-to-volume ratio, thickness (Tb.Th), number (N) and spacing (Tb.Sp) along with the bone mineral content, tissue mineral density, and periosteal and endosteal perimeters after applying a uniform threshold of 1200 Hounsfield Units (HU) for trabecular analyses and 2000 HU for cortical analyses.

Figure 7. MEK1/2 inhibitors rescue the Npr2pwe/pwe growth defect in explants. (A) Treatment with the inhibitor U0126 increases the length of mutant and wild-type tibiae by increasing the hypertrophic zone. Both wild-type and mutant whole tibiae are obviously longer than their DMSO (vehicle)-treated contralateral controls. Sections stained with nuclear fast red and alcin blue show the expansion of the hypertrophic zone (vertical arrows) upon treatment. In situ hybridization reveals Col10a1 transcripts in the hypertrophic zone of all samples. (B) Quantification of the tibial length in U0126 (n = 16 for both genotypes) and PD325901 (901) (n = 7 for both genotypes) treated mutants (+/-) relative to wild-type (+/+) . Treatment of both genotypes with either inhibitor leads to a highly significant increase in length (P < 0.0001). The PD325901-treated cohort is significantly longer than those treated with U0126 (P < 0.01). Letters indicate groups that are significantly different from one another (a,b,c), while the superscript numbers indicate the two different treatment groups (1: U0126, 2: PD325901). (C) Western blot analysis of phosphorylated ERK1/2 conducted on U0126-treated tibiae of both genotypes harvested at E16.5 and cultured in the presence of a vehicle (DMSO) or U0126 for 6 days indicates that U0126 inhibits activation of ERK1/2 without altering the levels of total ERK1/2 protein. Each lane contains lysates pooled from four individual tibiae of each genotype and treatment. Blot images are representative exposures of membranes incubated first with p-ERK1/2 primary antibody, stripped and incubated with total ERK1/2 primary antibody.
Tibial cultures and analysis

Tibial cultures were performed as previously described (21). U0126 was purchased from Promega (#V1121), and PD325901 was purchased from LC Laboratories (#P-9688). Both of these inhibitors were used at a final concentration of 10 μM. On the sixth day of culture, tibiae were photographed with a 2.5× objective lens under a dissection microscope and used for histological analysis or frozen at −80°C for protein analysis. Enlarged photographs were printed and measured with a ruler in centimeters to determine their lengths in arbitrary units. For histological analysis, cultured tibiae were fixed overnight in 4% formaldehyde in PBS, dehydrated through an ethanol series and embedded in paraffin. Nuclear Fast Red/Alcian Blue staining was performed as previously described (64). In situ hybridizations were performed as previously described (65). The Col10a1 probe (51) was a generous gift from Ernestina Schipani (Indiana University).

Western blotting

Total protein from cultured tibiae and P3 tibiae was extracted in a T-PER® buffer (Thermo/Pierce #78510) and Halt® protease and Phosphatase Inhibitor Cocktail (Thermo/Pierce #78840). Total protein was quantified using a BCA protein assay kit (Thermo/Pierce #23225). Proteins and ladder (BIO-RAD #161-0375) were run on 4–20% Mini-Protean TGXTM Gels from BIO-RAD (#456-1093S (10 well)) and transferred to an Amershams HybondTM-P membrane (GE Healthcare). Membranes were washed with a standard Tris-buffered saline solution using Tween-20 (TBS-Tween). ERK antibodies were purchased from Cell Signaling Technology (phospho-ERK1/2: #4370, total ERK1/2: #4695) and diluted as per the manufacturer’s instructions. Anti-beta actin was obtained from Abcam and used at a 1:25 000 dilution. Membranes were blocked and incubated with an antibody in a 1% BSA/TBS-Tween solution. A goat-anti-rabbit antibody was purchased from Thermo Scientific (#31462) and was diluted to 1:25 000. Membranes were washed with a standard Tris-buffered saline solution using Tween-20 (TBS-Tween). Goat-anti-rabbit conjugated to horseradish peroxidase second-ary antibody was purchased from Thermo Scientific (#34080) and stripped with RestoreTM western blot stripping buffer (Thermo/Pierce #34080) and stripped with RestoreTM western blot stripping buffer (Thermo/Pierce #21059).

AUTHORS’ CONTRIBUTIONS

K.A.G.: intellectual force in identification of the mutation, concept and execution of pharmacotherapy; histology of the skeleton, uterus and ovary; puberty and estrus assessments; wrote the manuscript with editing by S.A.C. M.L.B.: concept and execution of pharmacotherapy; histology of the skeleton, uterus and ovary; puberty and estrus assessments; V. Neel Professorship Funds (S.A.C.), Regents’ Fellowship for Cellular and Molecular Biology and Rackham Graduate School, (K.A.G.).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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