Increased maternofetal calcium flux in parathyroid hormone-related protein-null mice

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The role of parathyroid hormone-related protein (PTHrP) in fetal calcium homeostasis and placental calcium transport was examined in mice homozygous for the deletion of the PTHrP gene (*PTHrP*$^{-/-}$ null; NL) compared to *PTHrP*$^{+/+}$ (wild-type; WT) and *PTHrP*$^{+/-}$ (heterozygous; HZ) littermates. Fetal blood ionized calcium was significantly reduced in NL fetuses compared to WT and HZ groups at 18 days of pregnancy (dp) with abolition of the fetomaternal calcium gradient. *In situ* placental perfusion of the umbilical circulation at 18 dp was used to measure unidirectional clearance of ^{45}Ca across the placenta in maternofetal ($^{Ca}K_{mf}$) and fetoplacental ($^{Ca}K_{fp}$) directions; $^{Ca}K_{fp}$ was $< 5\%$ of $^{Ca}K_{mf}$ for all genotypes. At 18 dp, $^{Ca}K_{mf}$ across perfused placenta and intact placenta ($^{Ca}K_{mf(intact)}$) were similar and concordant with net calcium accretion rates *in vivo*. $^{Ca}K_{mf}$ was significantly raised in NL fetuses compared to WT and HZ littermates. Calcium accretion was significantly elevated in NL fetuses by 19 dp. Placental calbindin-D_{9K} expression in NL fetuses was marginally enhanced ($P < 0.07$) but expression of TRPV6/ECaC2 and plasma membrane Ca^{2+}-ATPase (PMCA) isoforms 1 and 4 were unaltered. We conclude that PTHrP is an important regulator of fetal calcium homeostasis with its predominant effect being on unidirectional maternofetal transfer, probably mediated by modifying placental calbindin-D_{9K} expression. *In situ* perfusion of mouse placenta is a robust methodology for allowing detailed dissection of placental transfer mechanisms in genetically modified mice.

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Provision of calcium to the developing fetus is essential for bone mineralization, which, if compromised, may increase the risk of developing osteoporosis later in life (Tobias & Cooper, 2004). Maximal fetal accretion of calcium occurs over the last third of pregnancy, and in the rat, as in other species, the rise in fetal calcium accretion is exponential over this period (Comar, 1956). Net calcium flux to the fetus ($^{Ca}J_{net}$) is the difference between unidirectional maternofetal ($^{Ca}J_{mf}$) and fetomaternal ($^{Ca}J_{fm}$) calcium fluxes (Sibley & Boyd, 1988). The rise in $^{Ca}J_{net}$ over gestation in the rat is a function of increased $^{Ca}J_{mf}$ (Glazier *et al.* 1992). Considerable data from a variety of species suggest that $^{Ca}J_{mf}$ is an active process, maintaining hypercalcaemia of the fetus relative to its mother, a feature of all species studied (Sibley & Boyd, 1988; Atkinson *et al.* 2006). $^{Ca}J_{fm}$ is thought to be a diffusional process (Husain *et al.* 2004).

In common with other calcium transporting epithelia, calcium transport across the placenta could plausibly involve three main steps (Belkacemi *et al.* 2005; Atkinson *et al.* 2006): firstly, diffusion into the trophoblast from maternal plasma down an electrochemical gradient through epithelial Ca^{2+} channels of the transient receptor potential (TRP) gene family; secondly, transfer across the trophoblast cytoplasm bound to the calcium binding protein calbindin-D_{9K} (Glazier *et al.* 1992); and, thirdly, active extrusion into the fetal compartment via plasma membrane Ca^{2+}-ATPase (PMCA) localized to the fetal facing basal plasma membrane (Fisher *et al.* 1987; Borke *et al.* 1989). Over the last third of gestation, gene and protein expression for calbindin-D_{9K} increases markedly in both mouse and rat placenta (Mathieu *et al.* 1989; Glazier *et al.* 1992; Hamilton *et al.* 2000; An *et al.* 2004) whereas rat placental Ca^{2+}-ATPase expression shows only a 2- to 3-fold increase (Glazier *et al.* 1992). This increase in rat placental calbindin-D_{9K} expression is associated with a concomitant, stoichiometric increase in unidirectional maternofetal ^{45}Ca clearance over this period leading to the

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speculation that calbindin-D_{9K} may be rate limiting for placental calcium transport in this species (Glazier *et al.* 1992).

One of the fetal factors implicated in the regulation of placental calcium transport is parathyroid hormone-related protein (PTHrP), which is produced by the placenta as well as by other fetal tissues (Tobias & Cooper, 2004). Although PTHrP was initially discovered as the humoral factor responsible for hypercalcaemia of malignancy, it is now known to have broad expression in a variety of fetal and adult tissues (reviewed in Philbrick *et al.* 1996). The versatility of PTHrP in performing multiple, yet distinct, biological effects through endocrine, autocrine, paracrine and intracrine activities arises from alternative splicing of the PTHrP gene, giving rise to three initial translation products which then undergo post-translational processing to generate N-terminal, mid-region and C-terminal mature peptide fragments.

Consistent with this concept, calcium transfer into blood used to perfuse the fetal circulation of the *in situ* placenta in thyroparathyroidectomised fetal lambs was increased by the addition of partially purified hPTHrP or recombinant PTHrP(1–84), PTHrP(1–108) and PTHrP(1–141) but not by synthetic PTHrP(1–34) (Abbas *et al.* 1989). This stimulation was rapid (within 1 h) and was also conferred by hPTHrP(67–86 amide) and hPTHrP(75–86 amide) fragments (Care *et al.* 1990) suggesting that this activity resides in the PTHrP(75–86) peptide sequence and that the N-terminus is not involved. By contrast, infusion of hPTHrP(1–34) or hPTHrP(75–86 amide) into the fetoplacental circulation of intact rat fetuses had no effect on the placental transport of calcium (Shaw *et al.* 1991).

The availability of fetal mice homozygous for deletion of the *PTHrP* gene ($PTHrP^{-/-}$ null; NL) (Karaplis *et al.* 1994) has allowed closer examination of the effect of fetal PTHrP on placental calcium transport. NL fetuses exhibit abnormalities of endochondral bone development with accelerated endochondral ossification, yet, despite broad PTHrP tissue expression in $PTHrP^{+/+}$ wild-type (WT) conceptuses, no gross morphological abnormalities were apparent in other tissues from NL fetuses (Karaplis *et al.* 1994). In NL fetuses, fetal blood ionized Ca^{2+} concentration is significantly reduced resulting in the abolition of the fetomaternal Ca^{2+} gradient (Kovacs *et al.* 1996; Tucci *et al.* 1996). The ratio of maternofetal transfer of ^{45}Ca normalized to that of ^{51}Cr-EDTA (used as a marker of diffusional permeability) was significantly reduced in NL fetuses when compared to WT and $PTHrP^{+/-}$ heterozygous (HZ) littermates (Kovacs *et al.* 1996), suggesting dependence of $^{Ca}J_{mf}$ on fetal PTHrP. Reversal of this effect by fetal injection 90 min before of hPTHrP(1–86) and hPTHrP(67–86), but not of hPTHrP(1–34), supports the concept that the mid-molecule region of PTHrP is functionally important in eliciting these acute effects. Curiously, in the light of these observations, others report a higher fetal calcium content in NL fetuses compared to WT littermates (Tucci *et al.* 1996), suggesting that $^{Ca}J_{net}$ is increased in the absence of fetal PTHrP. The explanation for these apparently divergent observations could be methodological, but may also reflect differences in the relative contribution of $^{Ca}J_{mf}$ and/or $^{Ca}J_{fm}$ to $^{Ca}J_{net}$. A more detailed examination of the role of fetal PTHrP on transplacental calcium fluxes is required.

To do this, we have developed a method in which the fetoplacental circulation is artificially perfused *in situ* in the mouse (Bond *et al.* 2006*a*) allowing separate measurement of J_{mf} and of fetoplacental calcium flux (J_{fp}) as an estimate of J_{fm}. This technique provides a powerful tool to explore the molecular mechanisms involved in the regulation of unidirectional placental solute fluxes.

Methods

Chemicals

All chemicals were purchased from either Sigma-Aldrich Co. Ltd (Poole, UK) or VWR International (Lutterworth, UK) unless otherwise stated.

Animals

Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986. PTHrP knockout mice generated by the targeted disruption in embryonic stem cells of exon IV, the major exon encoding mature murine PTHrP protein (Karaplis *et al.* 1994), were a kind gift from Professor H. Kronenberg (Harvard Medical School, USA).

Females (8–12 weeks old) and males (6 weeks to 8 months old) heterozygous for the deleted PTHrP gene were mated and the first day of gestation determined by the discovery of a copulation plug (term, 19–20 days). All animals were provided with nesting material and housed in cages maintained under a constant 12 h light–dark cycle at 21–23°C with free access to food (Beekay Rat and Mouse Diet, Bantin & Kingman, Hull, UK) and tap water. At the end of the experiment, all animals were killed by Schedule 1 procedure in accordance with the UK Animals (Scientific Procedures) Act of 1986.

Fetuses were genotyped using genomic DNA extracted from fetal tail tips (DNeasy, Qiagen, Crawley, UK) with primers specific to gene sequences for PTHrP (sense, 5′-GCTACTGCATGACAAGGGCAAGTCC-3′; antisense, 5′-GAGCCCTGCTGAACACAGTGAACAG-3′) and neomycin resistance (sense, 5′-GGAGAGGCTATTCGGCTATGAC-3′; antisense, 5′-CGCATTGCATCAGCCATGATGG-3′).

Placental calcium transfer studies

Measurement of unidirectional solute transfer across either the intact or perfused placenta is often expressed as solute clearance (Meschia *et al.* 1967), a formulation without any underlying mechanistic assumptions (Sibley, 1994). In the present investigation, unidirectional maternofetal clearance was measured both across perfused ($^{Ca}K_{mf}$) and intact ($^{Ca}K_{mf(intact)}$) placenta. Unidirectional fetoplacental clearance ($^{Ca}K_{fp}$) was measured using perfused placenta. Net flux ($^{Ca}J_{net}$) was calculated from calcium accretion rate *in vivo*. Unidirectional flux can be calculated as the algebraic product of unidirectional clearance and respective arterial concentration (Sibley, 1994).

Unidirectional maternofetal clearance of ^{45}Ca ($^{Ca}K_{mf}$) across the perfused placenta

$^{Ca}K_{mf}$ across the perfused placenta of mice at 18 days of pregnancy (dp) was measured as previously described (Bond *et al.* 2006*a*). The dam was anaesthetized with an intraperitoneal injection of 300 μl fentanyl/fluanisone (Hypnorm: Janssen Pharmaceutica, Belgium) and midazolam (Phoenix Pharma Ltd, Gloucester, UK) mixture (1 part fentanyl (0.315 mg ml^{-1}) and fluanisone (10 mg ml^{-1}), 1 part midazolam (5 mg ml^{-1}), and 2 parts water). Additional doses of anaesthetic were given intraperitoneally (2–5 μl (g dam weight)$^{-1}$) during the course of the experiment as required.

$^{Ca}K_{mf}$ was calculated (Sibley & Boyd, 1988) as:

$$^{Ca}K_{mf} = \frac{[v]\ Q}{[A]\ W}(\mu l\,min^{-1}(g\,placenta)^{-1}) \quad (1)$$

where [v] is fetal side placental venous effluent radioisotope concentration (dpm μl^{-1}), Q is the perfusion flow rate (μl min^{-1}), W is the placental wet weight (g) and $[A]$ is the maternal plasma radioisotope concentration (dpm μl^{-1}) taken at the mid-point of the perfusate collection period by curvilinear interpolation from a pooled maternal ^{45}Ca disappearance curve (Bond *et al.* 2006*a*).

Unidirectional fetoplacental clearance of ^{45}Ca ($^{Ca}K_{fp}$) using perfused placenta

In these experiments, the dam was similarly prepared on 18 dp as detailed previously for the measurement of $^{Ca}K_{mf}$ (Bond *et al.* 2006*a*), but the maternal tail vein was not cannulated. The arteriovenous difference of ^{45}Ca in Krebs Ringer solution (containing 10 nCi $^{45}CaCl_2$ ml^{-1}) used to perfuse the fetoplacental circulation allowed calculation of $^{Ca}K_{fp}$, as given in eqn (2):

$$^{Ca}K_{fp} = \frac{[a] - [v]\ Q}{[a]\ W}(\mu l\,min^{-1}(g\,placenta)^{-1}) \quad (2)$$

where [a] is the radioisotope concentration of arterial inflowing perfusate (dpm μl^{-1}).

Inclusion criteria for perfusion experiments

Data were analysed from those experiments where (a) perfusate effluent recovery was 95% of perfusate inflow, (b) $^{45}CaCl_2$ stock injectate radioactivity (dpm μl^{-1}) fell within 95% of the mean value for all experiments, and (c) the radioisotope injection volume was within 10% of the 50 μl injectate volume. Inclusion criteria (b) and (c) were introduced to ensure that the radioactivity injected into each mouse was comparable thereby minimizing variability in the maternal radioisotope disappearance curve (Bond *et al.* 2006*a*).

Unidirectional maternofetal clearance of ^{45}Ca across the intact placenta ($^{Ca}K_{mf(intact)}$)

$^{Ca}K_{mf(intact)}$ across the intact, unperfused placenta was measured on 18 dp using an adaptation of the method of Flexner & Pohl (1941) as previously described (Bond *et al.* 2006*a*).

$^{Ca}K_{mf(intact)}$ was calculated as:

$$^{Ca}K_{mf(intact)} = \frac{N_x}{W\int_0^x C_m(t)dt}(\mu l\,min^{-1}(g\,placenta)^{-1}) \quad (3)$$

where, N_x is total radiolabel accumulation (dpm) by the fetus (corrected for the fetal tail tip retained for genotyping) at x min after injection of radiolabel into the maternal vein and $\int_0^x C_m(t)dt$ is the time integral of radioisotope concentration in maternal plasma (dpm $\cdot$ min μl^{-1}) from 0 to x min (taken from the maternal plasma ^{45}Ca disappearance curve; Bond *et al.* 2006*a*).

Maternal plasma ^{45}Ca disappearance curve following injection into the maternal circulation

A maternal plasma ^{45}Ca disappearance curve was constructed from 78 dams at 18 dp and fitted to a one-phase exponential decay model ($r^2 > 0.8$) as previously described (Bond *et al.* 2006*a*).

Net transplacental flux of calcium ($^{Ca}J_{net}$)

Calcium content of fetal ash was measured at 17, 18 and 19 dp by atomic absorption spectrophotometry (Solaar

S-Series, Thermo Elemental, Unicam Ltd, Cambridge, MA, USA) as previously described (Husain *et al.* 1994). Tail tips were taken for genotyping as described above.

$^{Ca}J_{net}$ over 24 h (1440 min) between 17 to 18 or 18 to 19 dp was calculated as:

$$\frac{\text{Mean}\Delta\text{fetal calcium content}}{1440\ W}(\text{nmol min}^{-1}(\text{g placenta})^{-1}) \quad (4)$$

where W is mean placental wet weight (g) at either 18 or 19 dp, respectively.

Maternal whole blood ionized Ca^{2+} concentration

Terminal blood samples, taken at 18 dp from the carotid artery following decapitation, were analysed for ionized Ca^{2+} concentration (Bayer 865 Analyser, Siemens, Berkshire, UK).

Placental PTHrP expression

In order to assess the potential for endogenous placental production of PTHrP by maternal cells within decidua (Karperien *et al.* 1996), PTHrP gene and protein expression were examined in fetuses from within the same litter. Paired fetal genomic DNA and placental cDNA from each genotype was amplified using exon IV-specific primers (sense, 5′-GTTTCTTCCTCCACCATCTG-3′; antisense, 5′-ATCTGCCCTCATCGTCTG-3′) and placental PTHrP protein expression was examined by immunohistochemistry (see below).

Western blotting

Individual placentas harvested at 18 dp were homogenized in buffer containing 300 mM mannitol, 12 mM Hepes (pH 7.6) and 1% protease inhibitor cocktail (Sigma-Aldrich) for 30 s. The homogenate was retained or centrifuged at 2500 *g* for 5 min at 4°C (Sorvall Discovery 100SE, Kendro Laboratory Products, Bishop's Stortford, UK). Aliquots of this post-nuclear supernatant (PNS) were retained and the remaining PNS centrifuged at 100 000 *g* for 30 min at 4°C to obtain the membrane fraction. Both fractions were analysed for protein concentration (Bio-Rad Protein Assay) and stored at −80°C for further analysis.

Protein–SDS complexes were prepared with (Settle *et al.* 2004) or without (Bond *et al.* 2005) heat denaturation and SDS-PAGE performed followed by electrotransfer to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The antisera used were rabbit polyclonal anti-human TRPV6 (ECaC2, 1 : 200; Santa Cruz Biotechnology, Insight Biotechnology, Wembley, UK), rabbit polyclonal anti-rat calbindin-D_{9K} (1 : 1000; SWANT, Bellizona, Switzerland), rabbit polyclonal anti-human plasma membrane calcium ATPase isoforms 1 (PMCA1) and 4 (PMCA4, 1 : 1000; SWANT), goat polyclonal anti-human PTHrP (1 : 200; Santa Cruz Biotechnology) and rabbit polyclonal anti-human β-actin (1 : 1000; Abcam, Cambridge, UK). Negative controls were prepared by omission of primary antibody or pre-absorption with excess blocking peptide. Immunoreactive species were detected with horseradish peroxidase-conjugated secondary antibodies (1 : 2000, Dako, Ely, UK) using an enhanced chemiluminescence detection system (GE Healthcare). Immunoreactive signal density was measured by densitometry (Bio-Rad Molecular Analyst) and all signals fell within the linear range of detection.

PTHrP immunohistochemistry

PTHrP immunohistochemistry was performed on sections (5 μm) of paraffin-embedded placentas using standard techniques (Champion *et al.* 2004) after antigen retrieval by immersing slides in 0.01 M sodium citrate buffer pH 6 and microwaving on full power for 10 min. Non-specific binding was blocked with 10% rabbit serum. Goat polyclonal PTHrP antibody (2 μg ml^{-1}; Santa Cruz) or non-immune goat IgG as negative control were applied overnight at 4°C followed by incubation with biotinylated rabbit anti-goat IgG (1 : 100 Dako Ltd, UK) as secondary antibody. Sections were observed and photographed using a Leitz Dialux 22 microscope (Leica Microsystems, UK) and QICam 1394 digital camera (QImaging, UK).

Statistical analysis

All data are presented as mean ± S.E.M. where n is the number of fetuses/placentas. ANOVA with Bonferroni multiple comparison post hoc test was used to test the differences between experimental groups. Non-parametric comparisons were performed using Mann–Whitney and Kruskal–Wallis tests. $P < 0.05$ was taken as the significance level unless otherwise stated.

Abbreviations

PTHrP, parathyroid hormone-related protein; WT, wild-type; HZ, heterozygous; NL, null; TRPV, transient receptor potential vanilloid subfamily of calcium channels; ECaC, epithelial calcium channel; PMCA, plasma membrane Ca^{2+}-ATPase; dp, day of pregnancy; PNS, postnuclear supernatant; IPYS, intraplacental yolk sac; $^{Ca}K_{mf}$, unidirectional maternofetal clearance of calcium; $^{Ca}K_{fp}$, unidirectional fetoplacental clearance of calcium; $^{Ca}K_{mf(intact)}$, unidirectional maternofetal clearance of calcium across intact (unperfused)

Table 1. Fetal and placental weights, fetal : placental weight ratio and fetal plasma ionized Ca^{2+} concentration in WT (*PTHrP*$^{+/+}$), HZ (*PTHrP*$^{+/-}$) and NL (*PTHrP*$^{-/-}$) fetuses at 18 days of gestation

	WT	HZ	NL
Fetal weight (g)	0.90 ± 0.01 (74)	0.89 ± 0.01 (131)	0.84 ± 0.02[b] (45)
Placental weight (g)	0.084 ± 0.001 (74)	0.087 ± 0.001 (131)	0.090 ± 0.002[a] (45)
Fetal : placental weight ratio	10.9 ± 0.2 (74)	10.3 ± 0.1 (131)	9.5 ± 0.2[c,d] (45)
Fetal plasma ionized Ca^{2+} (mM)	1.45 ± 0.02 (15)	1.45 ± 0.02 (25)	1.25 ± 0.07[b,e] (6)

Data presented as mean ± S.E.M. (*n*). [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ *versus* WT; [d]$P < 0.05$, [e]$P < 0.001$ *versus* HZ (ANOVA followed by Bonferroni's multiple comparison test).

Table 2. Unidirectional maternofetal ($^{Ca}K_{mf}$) and fetoplacental ($^{Ca}K_{mf}$) clearance of ^{45}Ca across the perfused and intact placenta ($^{Ca}K_{mf(intact)}$) of WT (*PTHrP*$^{+/+}$), HZ (*PTHrP*$^{+/-}$) and NL (*PTHrP*$^{-/-}$) fetuses at 18 days of gestation

	WT	HZ	NL
$^{Ca}K_{mf}$ (μl min^{-1} (g placenta)$^{-1}$)	86.4 ± 20.7* (3)	94.9 ± 10.1** (13)	172.0 ± 19.4 (7)
$^{Ca}K_{fp}$ (μl min^{-1} (g placenta)$^{-1}$)	1.2 ± 0.3[a] (5)	3.4 ± 1.1[b] (4)	1.6 ± 0.9[b] (5)
$^{Ca}K_{mf(intact)}$ (μl min^{-1} (g placenta)$^{-1}$)	128.5 ± 10.5 (42)	123.0 ± 7.6 (67)	158.1 ± 14.9† (28)

Data presented as mean ± S.E.M. (*n*). *$P < 0.05$, **$P < 0.01$ *versus* NL (ANOVA followed by Bonferroni's multiple comparison test). [a]$P < 0.05$, [b]$P < 0.005$ *versus* $^{Ca}K_{mf}$ (Mann–Whitney test). †$P < 0.07$ for difference between groups (ANOVA).

placenta; $^{Ca}J_{net}$, net flux of calcium to the fetus; $^{Ca}J_{mf}$, unidirectional maternofetal calcium flux; $^{Ca}J_{fm}$, unidirectional fetomaternal calcium flux.

Results

Fetal and placental weight and fetal ionized Ca^{2+} concentration

Placental and fetal weights, fetal : placental weight ratio and fetal blood ionized Ca^{2+} concentration ($[Ca^{2+}]$) for WT, HZ and NL fetuses at 18 dp are given in Table 1. Fetal weight was lower, and placental weight higher in NL fetuses compared to WT, with no difference with respect to other group comparisons. In the NL group, this was reflected in a significantly reduced fetal : placental weight ratio relative to HZ and WT groups.

$[Ca^{2+}]$ in NL fetuses was significantly lower than WT and HZ fetuses. There was a marked fetomaternal Ca^{2+} gradient apparent in both WT and HZ fetuses with $[Ca^{2+}]$ significantly higher ($P < 0.001$) than maternal $[Ca^{2+}]$ (1.16 ± 0.02 mM, $n = 69$). However, in NL fetuses the Ca^{2+} gradient was abolished as fetal $[Ca^{2+}]$ was not significantly different to maternal $[Ca^{2+}]$.

Unidirectional clearances of ^{45}Ca across perfused and intact placenta

Table 2 gives unidirectional maternofetal clearance of calcium ($^{Ca}K_{mf}$) across the perfused placenta (taken as the mean value of clearance over 25–45 min, the period over which $^{Ca}K_{mf}$ was shown to be in steady state for all three genotypes; ANOVA, data not shown). $^{Ca}K_{mf}$ was significantly higher across the perfused placentas of NL fetuses compared to their WT and HZ counterparts. Mirroring this, $^{Ca}K_{mf}$ across the intact placenta ($^{Ca}K_{mf(intact)}$) of NL fetuses was 23–29% higher compared to WT and HZ groups (Table 2), the difference between groups being marginally significant ($P < 0.07$). For all genotypes $^{Ca}K_{mf}$ and $^{Ca}K_{mf(intact)}$ were not significantly different. There was no significant difference between groups in unidirectional fetoplacental clearance of calcium ($^{Ca}K_{fp}$) (taken as the mean value of clearance over 10–45 min, over which period values were at steady state for all genotypes; ANOVA, data not shown). $^{Ca}K_{fp}$ was < 5% of $^{Ca}K_{mf}$ in all groups.

Fetal calcium accretion and $^{Ca}J_{net}$

Table 3 shows fetal calcium content for all genotypes at 17, 18 and 19 dp normalized to ash weight. Ash weight, unlike wet weight (Table 1), was not significantly different between genotypes at each gestational age, in agreement with previous observations (Kovacs *et al.* 2001). Fetal calcium content was significantly elevated in NL fetuses at day 19 of pregnancy compared to WT and HZ fetuses, but no difference was observed between genotypes at the earlier gestational ages. The anticipated increase in fetal calcium accretion with advancing gestation was observed in all genotype groups between days 17–19 ($P < 0.0005$, ANOVA with Bonferroni's multiple comparison test). The calculated mean value for $^{Ca}J_{net}$ at day 18 is within the 95% confidence limits for $^{Ca}J_{mf}$ measured at the same gestation

Table 3. Fetal calcium content normalized to ash weight in WT (*PTHrP*$^{+/+}$), HZ (*PTHrP*$^{+/-}$) and NL (*PTHrP*$^{-/-}$) fetuses at 17, 18 and 19 days of gestation and estimates of $^{Ca}J_{mf}$ and $^{Ca}J_{net}$

	WT	HZ	NL
Day 17 (mmol (g ash)$^{-1}$)	2.23 ± 0.09 (6)	2.53 ± 0.04 (28)	2.46 ± 0.13 (9)
Day 18 (mmol (g ash)$^{-1}$)	3.15 ± 0.13 (30)	3.17 ± 0.09 (64)	3.56 ± 0.19 (17)
Day 19 (mmol (g ash)$^{-1}$)	3.45 ± 0.07** (29)	3.63 ± 0.08* (57)	4.12 ± 0.27 (18)
$^{Ca}J_{mf}$† (nmol min^{-1} (g placenta)$^{-1}$)	149 ± 12	143 ± 9	183 ± 17
$^{Ca}J_{net}$‡ (nmol min^{-1} (g placenta)$^{-1}$)			
Day 18	165	160	172
Day 19	234	236	315

Data presented as mean ± S.E.M. (*n*). $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* NL at day 19 (ANOVA followed by Bonferroni's multiple comparison test). † Values for $^{Ca}J_{mf}$ at day 18 calculated as:

$$^{Ca}J_{mf} = {}^{Ca}K_{mf(intact)}(\text{from Table 2}) \times (\text{maternal } [Ca^{2+}] \textit{ of } 1.16\,\text{mM}))$$

‡ Values for $^{Ca}J_{net}$ at day 18 and 19 calculated from mean calcium accretion over 24 h from days 17 to 18 and 18 to19, respectively, normalized to mean placental weight on day 18 or 19, respectively.

(Table 3). The rise in $^{Ca}J_{net}$ between day 18 and day 19 in NL fetuses was approximately double that for WT and HZ fetuses.

PTHrP gene expression

Placental cDNA from all three genotypes amplified PTHrP gene product (confirmed by sequencing) although product abundance appeared to be relatively lower in NL fetuses (Fig. 1*A*). However, no amplification product was visible in genomic DNA of NL fetuses (Fig. 1*B*).

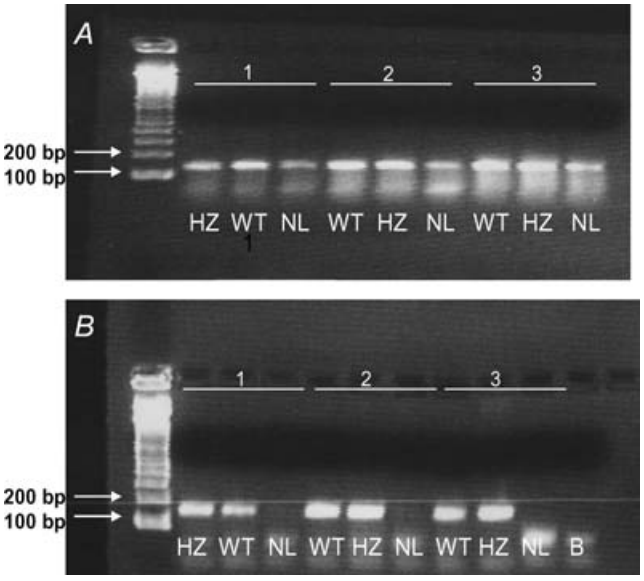

Figure 1. PCR of paired placental cDNA (*A*) and fetal genomic DNA (*B*) from WT (*PTHrP*$^{+/+}$), HZ (*PTHrP*$^{+/-}$) and NL (*PTHrP*$^{-/-}$) fetuses of 3 individual litters using exon IV-specific primers, the major exon encoding murine PTHrP

Lane B, H_2O replacement of DNA. 100 base pair (bp) ladder is shown on left. Amplification product of the predicted size (138 bp) was visible in placental cDNA from all three genotypes, whilst genomic DNA demonstrated the predicted pattern of amplification with no product observable in NL fetuses.

PTHrP immunohistochemistry

Figure 2 provides confirmation of the immunoreactive specificity of the anti-human PTHrP antibody for murine PTHrP and confirms negligible PTHrP protein expression in the placentas of NL fetuses. In placentas from WT and HZ fetuses, PTHrP immunoreactivity was intensely localized to the intraplacental yolk sac (IPYS) as well as being broadly distributed throughout the labyrinth trophoblast.

Expression of calcium transport proteins

Probing placental protein for calcium transport proteins that may mediate calcium epithelial influx (TRPV6/ECaC2; Fig. 3*A*), cytosolic translocation (calbindin-D_{9K}; Fig. 3*B*) and efflux (PMCA1 and PMCA4; Fig. 3*C* and *D*) revealed immunoreactive signals of appropriate size for all proteins. Signal intensity was not significantly different across genotype for TRPV6, PMCA1 and PMCA4 (Fig. 3*E*). However, in 7 of 9 litters examined calbindin-D_{9K} expression was higher in the placentas of NL fetuses compared to their WT and HZ littermates, as visible in Fig. 3*B*, with a mean increase in calbindin-D_{9K} expression of ∼37% (Fig. 3*E*; $P < 0.07$, Kruskal–Wallis test).

Discussion

In situ placental perfusion in the mouse (Bond *et al.* 2006*a*) has great potential as a methodology for measuring unidirectional clearances and fluxes in a species in which genetically modified strains are widely available. A striking finding of this study (Table 3) is the concordance

between transport measurements for an actively transported solute, calcium, obtained in three ways: across fetal-side perfused placenta; across unperfused *in situ* placenta in anaesthetized animals; and *in vivo*. These observations therefore provide considerable confirmatory confidence in this methodology.

The values of ${}^{Ca}K_{mf}$ per unit placental weight obtained here and our demonstration that calcium flux across the mouse placenta is highly asymmetric, with ${}^{Ca}J_{mf}$ prevailing and approaching ${}^{Ca}J_{net}$, accord with previous observations in the rat (Štulc & Štulcová, 1986; Mughal *et al.* 1989; Robinson *et al.* 1989). Also, the very low ratio of ${}^{Ca}K_{fm}$ to ${}^{Ca}K_{mf}$ is consistent with that found in the rat (Štulc & Štulcová, 1986) and, to a lesser degree, in women (Štulc *et al.* 1994).

The phenotype of PTHrP-null mutants, a domed skull, short snout and mandible with protruding tongue and shortened limbs (Karaplis *et al.* 1994), were clearly discernible at 18 dp. In such mutants, PTHrP allele expression was confirmed to be absent using primers to exon IV, the major exon encoding murine PTHrP. The reduction of ∼6% in the weight of PTHrP-null fetuses is consistent with previous observations (Kovacs *et al.* 2001) although others report a similar body weight at birth (Karaplis *et al.* 1994). The efficiency of placental nutrient transfer may be attenuated in NL fetuses as evidenced by the reduced fetal : placental weight ratio.

In agreement with previous observations (Kovacs *et al.* 1996; Tucci *et al.* 1996), PTHrP-null mutants had a significantly lower fetal blood ionized Ca^{2+} concentration resulting in abolition of the fetomaternal Ca^{2+} concentration gradient. In contrast, fetal blood ionized Ca^{2+} concentration in WT and HZ fetuses was comparable and significantly higher than maternal Ca^{2+}

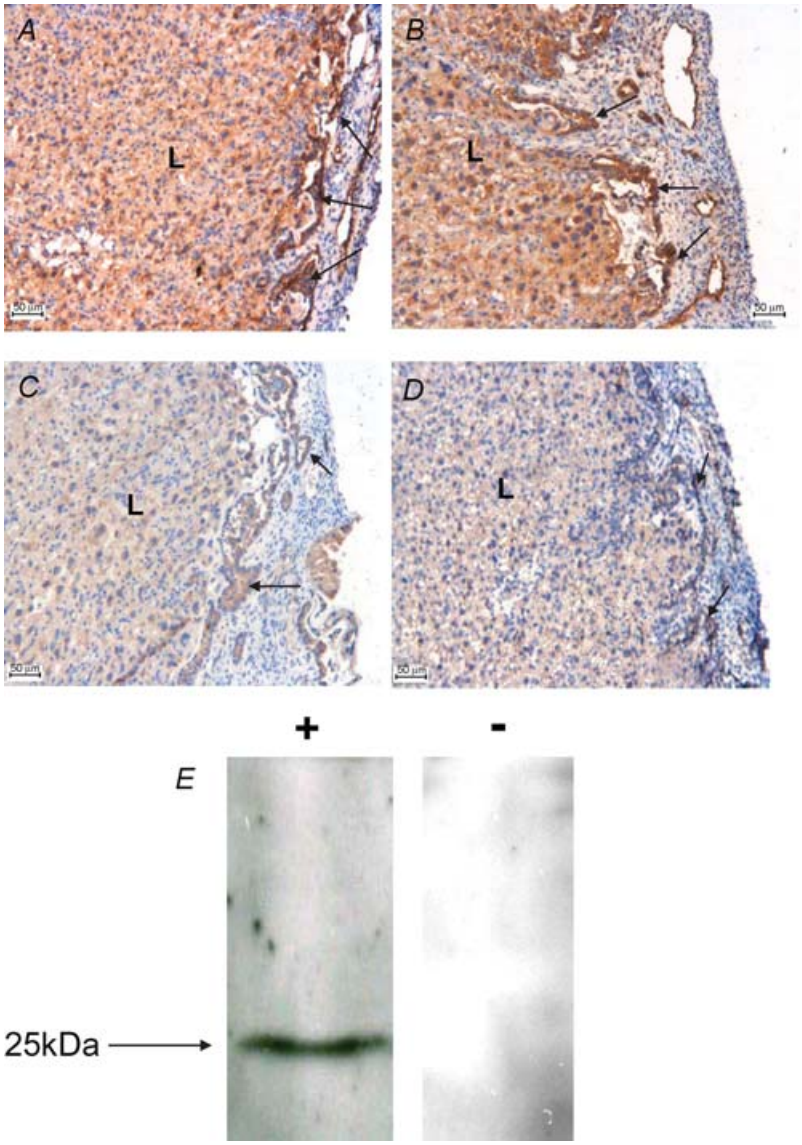

Figure 2. Immunohistochemistry of PTHrP in the placenta of mouse fetuses which were WT (*PTHrP*$^{+/+}$) (*A*), HZ (*PTHrP*$^{+/-}$) (*B*) or NL (*PTHrP*$^{-/-}$) (*C*) for PTHrP allele
Sections were incubated with goat anti-human PTHrP antibody (2 μg ml^{-1}; *A–C*) or goat IgG (2 μg ml^{-1}, *D*) as negative control. Intense staining was observed within the IPYS (arrows) and labyrinthine zone (L) of placentas of WT and HZ fetuses. In contrast in the NL fetus, placental staining was negligible and comparable to negative control, confirming a lack of placental PTHrP protein expression in the null mutant. All scale bars in *A–D* are 50 μm. *E*, Western blot of WT mouse placental lysate (50 μg protein lane^{-1}) probed with either: +, affinity purified goat anti-human PTHrP antibody (1 : 200; 1 μg ml^{-1}) alone giving a single immunoreactive signal of predicted size; or −, in the presence of 5× excess (1 : 40) blocking peptide which abolished signal.

concentration, also demonstrated previously (Kovacs *et al.* 1996; Tucci *et al.* 1996).

Previous studies have demonstrated a broad distribution of PTHrP mRNA and immunoreactivity in mouse placenta, being particularly abundant in the IPYS which lines the sinus of Duval connecting the yolk sac and the uterine lumen, but also detected in the labyrinth trophoblast (Kovacs *et al.* 2001, 2002), as observed here. As expected, PTHrP immunoreactivity was undetectable in the placental labyrinth of NL fetuses consistent with previous observations (Kovacs *et al.* 2001, 2002). We infer that PTHrP gene transcription in the placenta of NL fetuses (Fig. 1) arises from maternal cellular components of decidua (Karperien *et al.* 1996) although decidual PTHrP immunoreactivity in placenta from NL fetuses was undetectable (data not shown), suggesting paracrine effects of maternally derived PTHrP on placental function can be discounted.

Our starting hypothesis was that ${}^{Ca}J_{mf}$ would be down-regulated in placentas of NL fetuses based on previous evidence from PTHrP knockout mice (Kovacs *et al.* 1996) and studies examining the acute effects of PTHrP administration (outlined in the introduction). However, in contrast, our observations are all consistent in showing that calcium transport across the placenta of the NL fetus is, in fact, up-regulated. This assertion is based on the following evidence: (a) ${}^{Ca}K_{mf}$ was significantly elevated across the perfused placenta of the NL fetus compared to WT and HZ littermates, and at a significance level of $P < 0.07$ across the intact placenta (Table 2); (b) in the NL group there was a significant increase in fetal calcium accretion by day 19 of gestation consistent with an increased ${}^{Ca}J_{net}$ (Table 3); (c) the very low and similar values of ${}^{Ca}K_{fp}$ in all genotypes (Table 2) implies different fetomaternal fluxes beween genotypes cannot account for (b); and (d) placental expression of calbindin-D_{9K}, a molecular marker of placental calcium flux (Glazier *et al.* 1992) that predicts fetal calcium content (Verhaeghe *et al.* 1999), is probably raised ($P < 0.07$) in NL fetuses compared to WT and HZ littermates (Fig. 3).

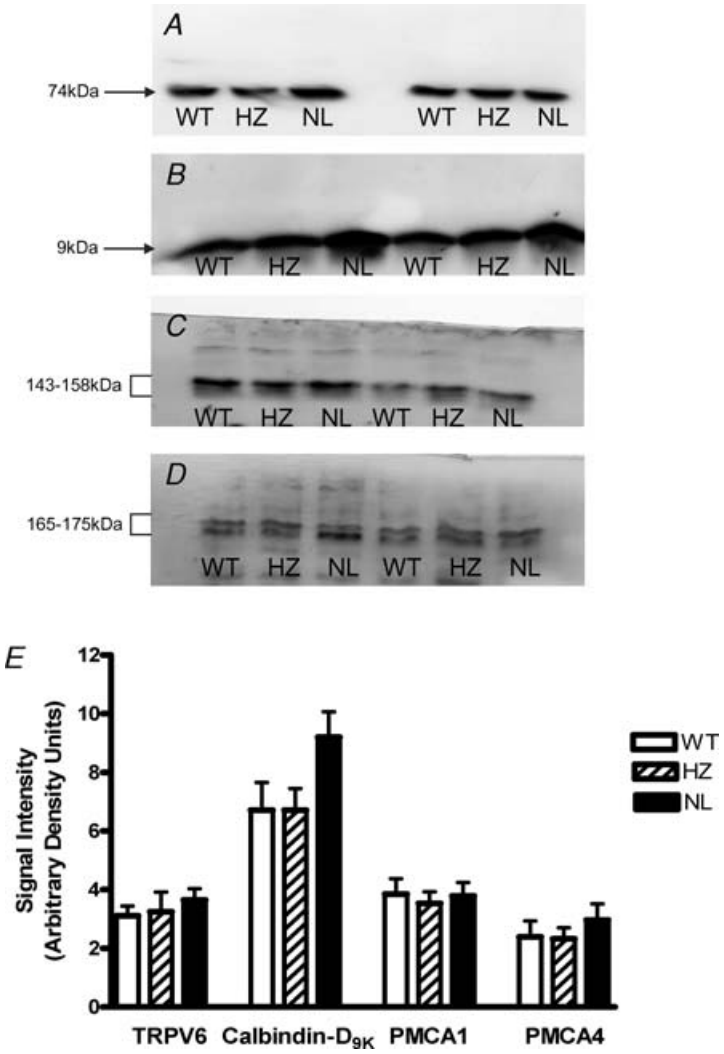

Figure 3. Western blots showing calcium transport protein expression in the placentas of WT (*PTHrP*$^{+/+}$), HZ (*PTHrP*$^{+/-}$) or NL (*PTHrP*$^{-/-}$) fetuses, matched from two litters

A, TRPV6 (ECaC2; 20 μg membrane protein lane^{-1}, 30 s exposure); *B*, calbindin-D_{9K} (20 μg PNS lane^{-1}, 15 s exposure); *C*, PMCA1 (30 μg membrane protein lane^{-1}, 5 min exposure); and *D*, PMCA4 (60 μg membrane protein lane^{-1}, 5 min exposure). For all blots the immunoreactive species detected accord with the predicted molecular mass of the target protein, with signal abolished by omission of primary antibody (not shown). *E*, densitometric analysis (mean + S.E.M.) of immunoreactive signal intensity for TRPV6 (ECaC2; $n = 6$), calbindin-D_{9K} ($n = 9$), PMCA1 ($n = 6$) and PMCA4 ($n = 6$) in placentas of WT, HZ and NL fetuses. Mean calbindin-D_{9K} expression in NL fetuses was ~37% higher than in WT and HZ littermates ($P < 0.07$, Kuskal–Wallis). All signals fell within the linear range of detection. For PMCA1 and PMCA4 signals comprising the triplet and doublet species, respectively, were analysed collectively. Equal protein loading was confirmed by densitometric analysis of replicate blots probed for β-actin (data not shown).

Our previous flux measurements in the mouse implicate active calcium transport mechanisms (Bond *et al.* 2006*a*), compatible with our demonstration and that of others (Kovacs *et al.* 2002) that calcium transport proteins mediating influx (TRPV6; ECaC2), transcytosolic movement (calbindin-D_{9K}) and efflux (PMCA) are co-expressed in mouse placenta. Of these, expression of calbindin-D_{9K} appears, as noted previously (Kovacs *et al.* 2002), to be specifically altered by a lack of fetal PTHrP.

The up-regulation in calbindin-D_{9K} expression parallels the increase in $^{Ca}K_{mf}$, as in the rat where placental calbindin-D_{9K} expression and $^{Ca}K_{mf}$ change in concert (Glazier *et al.* 1992; Husain *et al.* 1994). The cellular location of this altered calbindin-D_{9K} expression remains uncertain, as a variety of cell types other than the labyrinth within the murine placenta express calbindin-D_{9K} with a predominant localization to the IPYS (Mathieu *et al.* 1989; Ogura *et al.* 1998; Verhaeghe *et al.* 1999; Kovacs *et al.* 2002, 2005; Rummens *et al.* 2003). This distribution of calbindin-D_{9K} to the IPYS, along with a host of other calcitropic hormones and receptors (Kovacs *et al.* 2002, 2005), has led to the proposal that the IPYS, a unique structure restricted to rodent placentas and formed by invagination of the primitive yolk sac into the chorioallantoic placenta (Ogura *et al.* 1998), provides a potential pathway for maternofetal calcium transfer in addition to exchange across the labyrinth trophoblast (Kovacs *et al.* 2002). However, the measurements of $^{Ca}K_{mf}$ made here do not allow relative contribution of these pathways to be addressed. Mutant mice lacking an IPYS (Ogura *et al.* 1998) may be useful in examining this issue further.

The collective evidence presented here supports the notion that maternofetal calcium transport by the placenta of NL fetuses is increased compared to WT and HZ littermates. This contrasts with the conclusions of Kovacs *et al.* (1996, 2001, 2002). They found diminished placental calcium transport across the intact placenta, normal fetal calcium content and reduced placental calbindin-D_{9K} expression. However, there were several methodological differences between the two studies which might contribute to this disparity including: (a) the route, volume and amount of ^{45}Ca tracer (intracardiac, 100 μl, 50 μCi ^{45}Ca); (b) gestational age (17 dp); (c) anaesthetic (isoflurane inhalation); and (d) incorporation of ^{51}Cr-EDTA (50 μCi) as a paracellular marker with data expressed as ^{45}Ca/^{51}Cr ratio. A change in the ^{45}Ca/^{51}Cr ratio as an index of calcium transport by Kovacs *et al.* (1996) allows for the possibility that either the numerator or denominator is altered. Related to this, we have previously shown that the maternofetal clearance of ^{14}C-mannitol (a hydrophilic tracer with a similar diffusion coefficient in water to calcium) is significantly lower in the NL relative to its HZ and WT counterparts (Bond *et al.* 2006*b*), making interpretation following normalization to a paracellular marker under these circumstances a potential major source of error.

The interrelationships between fetal calcium homeostasis, placental calcium transport and fetal skeletal mineralization are complex, reliant upon the co-ordinated action of PTHrP and PTH (Tobias & Cooper, 2004; Miao *et al.* 2002). Fetal mice in which PTHrP or the PTHR1 gene is ablated or which lack parathyroid glands and so are unable to produce PTH (Hoxa3), all exhibit fetal hypocalcaemia, but show disparate patterns with regard to placental calcium transport, fetal PTH/PTHrP plasma concentration or fetal calcium accretion (Kovacs *et al.* 2001). This serves to emphasize that fetal calcaemia reflects the composite dynamics of calcium flux across both the placenta and fetal bone, calcium excretion by fetal kidney and its movement to and from amniotic fluid. The latter pathway is unlikely to confound the observations presented here as amniotic fluid calcium concentration is unaltered in NL fetuses (Kovacs *et al.* 2001).

The critical role of PTHrP in fetal skeletal morphogenesis is well established, acting in a paracrine manner to stimulate the proliferation of chondrocytes and delay their maturation (Karaplis *et al.* 1994; Miao *et al.* 2002; Tobias & Cooper, 2004). Derangement of this process by the targeted disruption of the PTHrP gene leads to lethal dyschondroplasia, premature differentiation of chondrocytes and advanced mineralization of the endochondral skeleton. This skeletal dysmorphogenesis is apparent as early as 14.5 days of gestation (Karaplis *et al.* 1994), a time at which PTHrP/PTH receptor expression is up-regulated in proliferating chondrocytes (MacLean & Kronenberg, 2005). Our demonstration that a lack of fetal PTHrP is associated with enhanced skeletal calcium deposition is compatible with the excessive skeletal mineralization and premature calcification/ossification found in NL fetuses (Karaplis *et al.* 1994) and agrees well with the observations of Tucci *et al.* (1996). In contrast, Kovacs *et al.* (2001) demonstrated comparable calcium content between genotypes, perhaps surprising in view of the excessive skeletal mineralization of NL fetuses. This excessive skeletal mineralization of the PTHrP-null mutant raises the possibility of an elevated fetal calcium demand, which we reason stimulates maternofetal calcium transport by a mechanism independent of fetal PTHrP, probably involving increased expression of calbindin-D_{9K}.

The regulatory stimuli involved in evoking this calbindin-D_{9K} response remain unclear. Fetal hypocalcaemia seems an unlikely candidate based on the lack of correspondence between placental calbindin-D_{9K} expression which is reduced or unchanged in the face of fetal hypocalcaemia (Kovacs *et al.* 2001, 2002). Other studies showing that expression of calbindin-D_{9K} in rodent placenta is not regulated by the biologically active form of vitamin D, 1,25$(OH)_2D_3$ (Glazier *et al.* 1995; Rummens *et al.* 2003; Kovacs *et al.* 2005) argue against its direct

involvement. A significant elevation in fetal serum PTH in both PTHrP- and PTHR1-null fetuses (Kovacs *et al.* 2001) is not associated with a consistent trend in calbindin-D_{9K} expression (Kovacs *et al.* 2002) suggesting a lack of direct regulation by PTH. Additionally PTH (1–84) had no effect on $^{Ca}K_{mf}$ across the placenta of intact rat fetuses (Robinson *et al.* 1989). Multiple regulatory factors may be involved in the trend to an altered placental calbindin-D_{9K} expression observed here.

In summary, this study provides clear evidence of increased maternofetal calcium transport across the placenta of PTHrP-null fetuses with increased fetal calcium accretion despite pronounced fetal hypocalcaemia and abolition of the fetomaternal calcium gradient. The asymmetric nature of this response is indicated by the lack of effect on $^{Ca}K_{fp}$. *In situ* placental perfusion of the mouse placenta provides a valuable tool to examine the regulation of unidirectional placental calcium flux, providing mechanistic insights regarding the role of fetal PTHrP.

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