BONE DEVELOPMENT AND MINERAL HOMEOSTASIS IN THE FETUS AND NEONATE: ROLES OF THE CALCIOTROPIC AND PHOSPHOTROPIC HORMONES

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Kovacs CS. Bone Development and Mineral Homeostasis in the Fetus and Neonate: Roles of the Calciotropic and Phosphotropic Hormones. Physiol Rev 94: 1143–1218, 2014; doi:10.1152/physrev.00014.2014.—Mineral and bone metabolism are regulated differently in utero compared with the adult. The fetal kidneys, intestines, and skeleton are not dominant sources of mineral supply for the fetus. Instead, the placenta meets the fetal need for mineral by actively transporting calcium, phosphorus, and magnesium from the maternal circulation. These minerals are maintained in the fetal circulation at higher concentrations than in the mother and normal adult, and such high levels appear necessary for the developing skeleton to accrete a normal amount of mineral by term. Parathyroid hormone (PTH) and calcitriol circulate at low concentrations in the fetal circulation. Fetal bone development and the regulation of serum minerals are critically dependent on PTH and PTH-related protein, but not vitamin D/calcitriol, fibroblast growth factor-23, calcitonin, or the sex steroids. After birth, the serum calcium falls and phosphorus rises before gradually reaching adult values over the subsequent 24–48 h. The intestines are the main source of mineral for the neonate, while the kidneys reabsorb mineral, and bone turnover contributes mineral to the circulation. This switch in the regulation of mineral homeostasis is triggered by loss of the placenta and a postnatal fall in serum calcium, and is followed in sequence by a rise in PTH and then an increase in calcitriol. Intestinal calcium absorption is initially a passive process facilitated by lactose, but later becomes active and calcitriol-dependent. However, calcitriol’s role can be bypassed by increasing the calcium content of the diet, or by parenteral administration of calcium.

I. INTRODUCTION

Normal calcium and bone homeostasis in the adult can be almost fully explained by the interactions of several regulatory hormones, including parathyroid hormone (PTH), calcitriol, fibroblast growth factor-23 (FGF23), calcitonin, and the sex steroids (estradiol and testosterone). Loss of any one of these hormones can have significant consequences for the adult. Hypoparathyroidism can cause fatalities due to hypocalcemia; it also causes hyperphosphatemia (leading to ectopic calcifications), hypercalciuria (leading to nephrolithiasis and nephrocalcinosis), low bone turnover, and possible neurological sequelae from basal ganglia calcifications. With vitamin D deficiency, the serum calcium may be normal or reduced (but not as low as in hypoparathyroidism), serum phosphorus is low, and undermineralization of the skeleton leads to rickets or osteomalacia. Loss of FGF23 causes hyperphosphatemia, extraskeletal calcifications, and early mortality. Loss of calcitonin increases bone resorption, an effect that may become most apparent during lactation. Deficiency of either of the sex steroids increases bone resorption and will lead to osteoporosis. The roles that these hormones play in regulating osteoblasts or osteoclasts have led to some of them (or their synthetic analogs) being used to treat osteoporosis and other skeletal disorders.

This review will make clear that mineral and bone metabolism are regulated differently in utero such that many of these hormones do not appear to have the crucial roles that they have in the adult.
The fetus has several developmental goals, which include the need to actively pump minerals across the placenta against concentration and electrochemical gradients, to maintain higher extracellular concentrations of minerals compared with the maternal circulation, and to adequately mineralize the skeleton before birth.

During embryonic development the early pattern for the endochondral skeleton is laid down, but it is during the later stages of fetal development that rapid bone formation and mineralization create a significant demand for mineral. The adequacy of the mineral supply is a limiting factor for how much mineral can be accreted by the skeleton before birth, and the availability of mineral also influences the function and activity of osteoblasts and osteoclasts. A human fetus typically accumulates ~30 g of calcium by term, with 80% of that mineral content obtained in the third trimester (121, 224, 683, 733, 734). The kidneys, intestines, and skeleton (through bone turnover) are not dominant sources of mineral supply for the normal fetus as they are for the adult (FIGURE 1). Instead, the placenta meets the fetal need for mineral by actively transporting calcium, phosphorus, and magnesium from the maternal circulation. To obtain 30 g of calcium by term, the placenta must deliver 120–150 mg·kg⁻¹·day⁻¹ of calcium during the third trimester, or more than 300 mg/day between weeks 35 and 38 (635, 734, 735, 761). The placenta appears capable of providing mineral even when faced with reduced concentrations of those same minerals in the maternal circulation. The fetus maintains higher mineral concentrations than in the mother or normal adult, and such high levels appear necessary for the skeleton to form and mineralize normally. As this review will demonstrate, fetal bone and mineral metabolism are critically dependent on PTH and PTH-related protein (PTHrP), whereas vitamin D/calcitriol, FGF23, calcitonin, and the sex steroids may not be required.

The intestines become the main source of mineral after birth, while the kidneys begin to reabsorb mineral, and normal bone turnover contributes additional mineral to the circulation. This switch in the regulation of mineral homeostasis is triggered by a postnatal fall in serum calcium, and followed in sequence by a rise in PTH and then an increase in calcitriol. FGF23 then begins to play an important role in regulating serum phosphorus, renal phosphorus excretion, and calcitriol synthesis and catabolism. Intestinal calcium absorption is initially a passive process facilitated by lactose, but later becomes active and calcitriol-dependent. However, calcitriol’s role can be bypassed by increasing the calcium content of the diet, or by administering parenteral calcium, thereby preventing or correcting the skeletal changes of rickets or osteomalacia, independent of calcitriol or vitamin D sufficiency.

There are only limited human data that directly examine regulation of mineral and bone homeostasis during fetal development. Much of those data consist of cord blood samples taken from normal and abnormal preterm and term fetuses and pathological examination of embryos and fetuses that died due to congenital abnormalities or obstetrical accidents. The main exception is with vitamin D and calcitriol, for which there have been randomized interventional trials, cohort studies, and associational studies, each examining possible effects of vitamin D deficiency on fetal and neonatal bone and mineral metabolism.

In contrast to the limited human data, mineral and bone homeostasis have been extensively examined in several animal species using surgical, genetic, and pharmacological approaches. Regulation of fetal mineral homeostasis was first studied by administering pharmacological doses of hormones or antibodies to the mother or fetus, by making the mother severely vitamin D deficient before pregnancy, or by removing the thyroid or parathyroids from the mother, fetus, or both. But most calcitropic and phospho-
tropic hormones cannot be adequately studied by these approaches because they are produced by more than one tissue. Global and conditional gene deletion models have enabled us to study fetuses that lack PTHrP, PTH, calcitriol, the vitamin D receptor, calcitonin, or FGF23 (see Table 1 for a glossary of mouse models to be discussed). It is through consideration of these animal data that it becomes possible to theorize how fetal mineral homeostasis is regulated in the human, and how this regulation changes after birth.

To avoid any confusion between animal and human data, within each subsection this review will sequentially discuss animal data followed by the extant human data. The animal data will be used to infer how mineral and bone homeostasis are regulated during fetal and neonatal development, and the sometimes limited human data will reveal whether there is agreement or disagreement with what the animal models suggest.

In this review, the term phosphorus is used for consistency and simplicity when referring to its presence in blood or bone. However, it is recognized that serum contains mainly inorganic phosphates (dihydrogen and monohydrogen phosphate), bone contains phosphorus largely in the form of hydroxyapatite, while the soft tissues and extracellular fluids contain organic phosphates in complex with carbohydrates, lipids, and proteins (37).

II. OVERVIEW OF FETAL MINERAL METABOLISM AND SKELETAL FORMATION

This section describes the normal circulating concentrations (Figure 2) and sources (Figure 1) of minerals and hormones during fetal development, and the participation

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<th>Table 1. Glossary of mouse models</th>
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<td>Casr null</td>
</tr>
<tr>
<td>Casr Chon, Pre, Ob, Oc, Para null</td>
</tr>
<tr>
<td>Ctcgrp null</td>
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<td>Ctr null</td>
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<td>Cyp27b1 null</td>
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Reference numbers are given in parentheses.
of parathyroids, kidneys, intestines, placenta, and bone in regulating fetal mineral metabolism. The roles of the individual calcitropic and phosphotropic hormones are discussed in sections IV–XI.

A. Serum Mineral Concentrations

1. Animal data

A consistent finding among mammalian fetuses is that the serum calcium concentration is significantly higher than the simultaneous maternal calcium. Typically the fetal serum calcium is 0.30–0.50 mM or higher than the maternal level in rhesus monkeys (189, 524), lambs (148, 201, 435, 505), calves (201, 715), rodents (201, 338, 340, 342, 346, 347, 623), pigs (98, 101, 353), and foals (201). The ionized calcium is also increased 0.25–0.50 mM above the maternal value in fetal rodents (148, 340, 422), confirming that true hypercalcemia is present relative to maternal and normal adult values. Although only ~50% of serum calcium is ionized in the adult rodent, within fetal rodents ~80% of calcium is free or ionized (148, 340). It is unknown how early in gestation the serum calcium becomes increased compared with the maternal value, but it has been demonstrated at all time points for which it has been technically possible to measure it. This includes the 35th day of gestation in lambs (93, 397), day 14 in rats (346, 347, 675), and day 15 in mice (330). Within rat fetuses, a small but progressive increase in total and ionized calcium occurs over the final 7 days, which may indicate that at an earlier time point there is no difference between embryonic/fetal and maternal calcium concentrations (346, 675).

This level of blood calcium, both total and ionized, exceeds the value that the calcium sensing receptor (CaSR) sets in the adult through its regulation of PTH synthesis and secretion, and which is represented by the simultaneous maternal serum calcium concentration (338). CaSR likely acts in response to the high serum calcium to suppress release of PTH (FIGURE 3). When one or both alleles of Casr bear an inactivating mutation, the fetal serum calcium and PTH increase above normal fetal values, confirming that CaSR can raise the fetal serum calcium by increasing PTH (338). That CaSR requires PTH to raise the fetal serum calcium was confirmed by the finding that if the PTH/PTHrP receptor (PTH1R) is also ablated in Pth1r/Casr double mutants, thereby blocking the actions of PTH, then inactivating mutations of Casr fail to increase the fetal serum calcium (338).

What sets the fetal blood calcium at its high target level in normal fetuses? One possibility is that novel calcium-sensing receptors are expressed during fetal development, which set the calcium concentration at specific values within the placental or fetal circulations. Alternatively, the active, forward flow of calcium across the placenta may be responsible for maintaining the high fetal blood calcium. If active

FIGURE 2. Schematic illustration of the longitudinal changes in calcium, phosphate, and calcitropic hormone levels that occur during the fetal and neonatal period in humans. Normal adult ranges are indicated by the shaded areas. The progression in PTHrP levels has been depicted by a dashed line to reflect that it is speculative. [From Kovacs and Kronenberg (339). Copyright 1997 The Endocrine Society; permission conveyed through the Copyright Clearance Center, Inc.]
placental calcium transfer is the explanation, then fetuses should maintain a relative gradient of serum calcium about the maternal level, and acute or chronic changes in maternal serum calcium should alter the fetal blood calcium.

However, the fetus does not maintain a set gradient of serum calcium relative to the maternal serum calcium; instead, it sets a fixed level of calcium independent of the maternal calcium value. This value can be defended and maintained despite significant challenges. The evidence includes that fetal rats were normocalcemic despite maternal hypocalcemia induced by a calcium-restricted diet (377), vitamin D deficiency (74, 241, 443), or thyroparathyroidectomy (67, 276). Fetal lambs also maintained normal serum calcium levels despite maternal hypocalcemia induced by parathyroidectomy (564). In fetal mice both Casr (257) and Vdr (372) deletion models have revealed that fetuses will maintain the ionized calcium level determined by their genotype, regardless of whether the mother is normocalcemic, hypercalcemic, or hypocalcemic (FIGURE 4) (338, 342). In these models, a lower maternal serum calcium means that the calcium gradient from mother to fetus is increased, whereas a higher maternal serum calcium means that the gradient is decreased. But in all of these models the fetal ionized calcium was unchanged, confirming that it is the blood level of calcium that is set and not a relative gradient compared with the mother. These findings are consistent with calcium-sensing receptors that determine the fetal blood calcium level, as opposed to the fetal blood calcium being determined by active placental calcium transport. Moreover, acute alterations in the maternal blood calcium of rodents and primates (such as by infusions of calcium, calcitriol, calcitonin, PTH, or EDTA) had minimal or no effect on the fetal blood calcium level (87, 214, 345, 478, 547). However, although a high fetal blood calcium level was maintained in fetal rats between the 12th and 17th days of gestation after maternal parathyroidectomy, it declined during the last several days of gestation, the interval when the skeleton is most rapidly accreting mineral (106, 208, 220).

What is the purpose of the fetus maintaining a higher blood concentration of calcium? The robust maintenance of this “fetal hypercalcemia” across numerous mammalian species suggests that it is physiologically important. But fetal hypocalcemia does not impair survival to the end of gestation, as shown by study of Pthrp null, Pth1r null, Hoxa3 null, Trpv6 null, Gp130 null, and Pthrp/Hoxa3 double mutant fetuses (336, 339, 341, 616, 659). Another possibility is that a high blood calcium level in utero may improve survival after birth. As discussed in section III, the postnatal onset of breathing, obligatory rise in pH, and loss of the placental calcium infusion contribute to a rapid fall in calcium within 12 h after birth. During the next 24 h, the serum calcium increases to the adult value. In rodents, the serum calcium drops more markedly by 40% after birth (201, 347). If the fetal blood calcium is lower than normal, conceivably the neonatal blood calcium will fall to an even lower value, thereby conferring an increased risk of hypocalcemic tetany and death. The early postnatal mortality of Pthrp null, Pth1r null, Pth null, Gp130 null, and Hoxa3 null fetuses is compatible with this possibility (297, 340, 341, 360, 616, 623). The high fetal calcium concentration also appears to be important for normal skeletal mineralization to be achieved, since hypocalcemia is associated with reduced ash weight and skeletal mineral content in Pth null, Hoxa3 null, Pth1r null, and Gcm2 null fetuses (see sects. VC and VIC) (336, 341, 623).

The serum ionized calcium concentration differs significantly among fetuses obtained from different commercially available strains of mice; for example, fetuses from the commonly used inbred C57BL/6 strain maintain a significantly lower ionized calcium compared with fetuses from outbred Black Swiss (340). Therefore, unknown genes also contribute to the regulation of fetal mineral homeostasis, or specifically the set calcium level maintained by normal fetuses.

Phosphorus plays key roles during endochondral bone development: it induces apoptosis of hypertrophic chondrocytes (158, 579) and is incorporated into osteoid before
Physiol Rev • VOL 94 • OCTOBER 2014 • www.prv.org

2. Human data

Human fetuses share the finding of an elevated serum calcium and ionized calcium, typically 0.30–0.50 mM above the maternal level (143, 148, 463, 523, 594, 595). This has been demonstrated at 15–20 wk of gestation by fetoscopy (453), and at delivery of preterm singleton and twin pregnancies (mean gestational age 33 wk) (152).

Serum phosphorus is typically ~0.5 mM higher in fetal than maternal blood (143, 523, 544, 594). Since calcium and phosphorus are each raised above adult normal values, fetal blood has a high calcium x phosphorus product (Ca x P) that may facilitate spontaneous calcium-phosphate crystals to form and cause soft tissue calcifications. In adults, a Ca x P value >5.6 mmol²/L² or 70 mg²/dl² is associated with calciphylaxis, coronary artery calcifications, and increased mortality (58, 115, 126, 724). However, this level may be needed to mineralize the fetal skeleton and may be nonhazardous in utero due to its relatively short duration.

Serum magnesium is also set independently of the mother’s concentration but is typically raised only 0.05 mM higher than the maternal value (60, 143, 523, 544, 594).

3. Summary of key points

Serum calcium, ionized calcium, and phosphorus concentrations are set at significantly higher concentration in fetuses than in the mother and can generally be maintained despite abnormal concentrations in the maternal circulation. Putative calcium-sensing receptors (other than the known CaSR) may set the fetal or placental calcium concentration in utero. Fetal ionized calcium level is set independently of the maternal calcium value. Data have been merged for simplicity and schematically represented in this illustration. In A, the ionized calcium of Vdr+/− and Vdr null mothers is represented by their respective horizontal lines ± SE. Despite normocalcemia in Vdr+/− mothers (who bore WT, Vdr+/−, and Vdr null fetuses) and hypocalcemia in Vdr null mothers (who bore Vdr+/− and Vdr null fetuses), the maternal ionized calcium did not affect the ionized calcium level of their respective fetuses. The fetuses maintained a set level independent of the maternal ionized calcium, and that value was no different in Vdr null fetuses compared with WT and Vdr+/− littermates. In B, the ionized calcium of WT and Casr+/− mothers is represented by the respective horizontal lines (± SE). Despite normocalcemia in WT mothers (who bore WT and Casr+/− fetuses) and hypercalcemia in Casr+/− mothers (who bore WT, Casr+/−, and Casr null fetuses), the maternal ionized calcium did not affect the ionized calcium level of their respective fetuses. The fetuses maintained a set level independent of the maternal ionized calcium, and that value was increased in Casr+/− and Casr null fetuses compared with their WT littermates. The background strain was Black Swiss for all mice represented in this figure. [Original data in A were published as two separate panels in Kovacs et al. (342). Original data in B were also published as two separate panels in Kovacs et al. (338) and adapted with permission of the American Society for Clinical Investigation, copyright 1998.]

![Figure 4](http://physrev.physiology.org/DownloadedFrom)
mnia” is not necessary for fetal viability, but may reduce the occurrence of neonatal hypocalcemia, and is required to mineralize the fetal skeleton.

B. Calciotropic and Phosphotropic Hormone Concentrations

1. PTH

A) ANIMAL DATA. PTH circulates at lower levels in the fetal circulation compared with the maternal value in rodents, lambs, and calves (118, 623, 675, 685, 715). These low values likely derive from the fetal sources because intact PTH does not cross the placenta of non-human primates, sheep, and rodents (196, 205, 341, 345, 478). The fetal parathyroids begin to express PTH by mid-gestation in rats and lambs (390, 602), and must be its dominant source because genetic ablation of the parathyroids (in Hoxa3 null fetal mice) results in undetectable serum PTH (336, 341). An additional source of PTH is the placenta, which in the mouse has been shown to express the Pth gene and may contribute a small amount of PTH to the circulation (623).

The normally low concentration of PTH in fetal blood remains important for maintaining the blood calcium concentration because fetal mice lacking PTH or the PTH/PTHrP receptor are each hypocalcemic compared with their WT littermates (340, 341, 623).

Why is PTH suppressed in fetal blood? As noted above, the high concentrations of total and ionized calcium in fetal blood likely activate CaSR on fetal parathyroids to suppress PTH synthesis and release (FIGURE 3). Several lines of evidence from fetal mice support this conclusion. Ablation of one or both alleles of Casr results in a progressive increase in PTH in fetal mice and an increase in the fetal blood calcium compared with WT fetal littermates (338). Conversely, in the absence of PTHrP (Pthrp null fetuses), the circulating PTH level rises but the fetal blood calcium is maintained at a level equal to the maternal value, rather than above it (336, 340). These data suggest that the fetal parathyroids are constrained by CaSR to maintain the blood calcium no higher than the adult level. Furthermore, when Casr is also ablated in Pthrp null fetuses to create Pthrp/Casr double-mutants, the fetal blood calcium rises well above the maternal level despite the absence of PTHrP (338).

The low circulating level of PTH does not mean that the fetal parathyroids are unable to produce greater amounts of PTH. PTH increases not only with ablation of one or both alleles of Casr (336, 340), but in response to EDTA-induced hypocalcemia in fetal lambs (631), calves (715), and rhesus monkeys (524). However, a second study in rhesus monkeys found no increase in fetal PTH in response to EDTA-induced hypocalcemia (189).

Active placental transport of calcium may contribute to the suppression of PTH by bringing calcium into the circulation through a route that does not require PTH. Furthermore, maternal hypercalcemia seems to enhance the forward flow of calcium to the fetus and further suppress the fetal PTH level, even if the fetal serum calcium does not change. For example, when wild-type (WT) and Casr+/− sister females are mated to the same Casr−/− males, the fetal PTH concentration is suppressed in offspring of hypercalcemic Casr+/− females compared with their genetic counterparts from normocalcemic WT females, even though the fetal blood calcium remains unaffected by the mother’s genotype or serum calcium (FIGURE 5) (338). Conversely, maternal hypocalcemia may impair the forward flow of calcium from mother to fetus, and force the fetal-placental unit to upregulate the mechanisms that maintain the fetal blood calcium and placental calcium transport. Consistent with this, maternal parathyroidectomy or calcitonin infusions in pregnant rats have been shown to cause a marked increase in fetal PTH, parathyroid gland hyperplasia, and bone resorption; the skeleton is undermineralized (104, 206, 541, 542, 625).

FIGURE 5. Maternal hypercalcemia suppresses fetal PTH while fetal expression of CaSR determines the relative fetal PTH level. Ablation of one or both copies of Casr results in a stepwise increase in PTH concentrations in the fetal circulation (A and B), confirming the ability of CaSR to regulate PTH release from the fetal parathyroids. However, fetuses obtained from hypercalcemic Casr+/− mothers (A) have lower PTH levels than in fetuses of the corresponding genotype obtained from normocalcemic WT mothers (B). Since maternal PTH cannot cross the placenta, the suppression of fetal PTH must be mediated by maternal serum calcium. The number of observations for each genotype is indicated in parentheses. [From Kovacs et al. (338). Copyright 1998 American Society for Clinical Investigation.]
B) HUMAN DATA. In human babies, PTH also circulates at low levels compared with the maternal circulation and normal adult values; very low (<0.5 pM) concentrations may be observed (20, 143, 168, 177, 235, 306, 501, 544, 571, 580, 598–600, 671, 736). This suppression of PTH has been observed as early as 19 wk of gestation in preterm babies (501, 571, 580, 599), although one study found suppressed PTH values in cord blood of infants aged 31–36 wk (152). The suppression of fetal PTH is considerable when it is realized that the maternal PTH value is also usually suppressed during pregnancy compared with normal adult values, at least in women from North America and Europe (328, 339). The parathyroids produce immunoreactive PTH beginning at 10 wk of gestation (367); whether the human placenta produces PTH has not been determined.

Maternal hypercalcemia can result in a normal or slightly increased cord blood calcium (131, 278, 610, 700) and suppression of the fetal parathyroids, which will later be realized as neonatal hypoparathyroidism, hypocalcemia, and tetany (see sect. XII, A, B, and G). These findings have been observed in babies born of mothers whose hypercalcemia during pregnancy was caused by primary hyperparathyroidism (79, 149, 304, 393, 609, 718), inactivating mutations of Casr (529), or hypercalcemia of malignancy (9, 131, 278, 454, 529, 673, 674, 700). Conversely, maternal hypocalcemia caused by hypoparathyroidism (11, 76, 389, 586, 652, 710) or pseudohypoparathyroidism (226, 710) has been associated with fetal parathyroid gland hyperplasia, normal cord blood calcium, increased PTH, and effects on the fetal skeleton that include increased resorption, demineralization, and fractures occurring in utero or during delivery (see sect. XII, C and D).

2. Calcitriol

A) ANIMAL DATA. Rodents and lambs have been most frequently used to study fetal mineral homeostasis, and in the data discussed thus far, they have yielded similar results. However, with respect to vitamin D physiology, there are some differences between fetal rodents and lambs. This may be in part due to the differences in placental structure and function that are discussed in section II F; in brief, rodents have hemochorial placentas (with similar structure and diffusional characteristics as human placentas), while lambs have epitheliochorial placentas (536).

Calcitriol typically circulates at <50% of the maternal value in fetal rodents (309, 342, 369, 709) and pigs (352, 353); in contrast, calcitriol significantly exceeds the maternal value in fetal lambs (4, 101, 155, 377, 505, 565). Radiolabeled calcitriol does not cross the rat placenta (475), which means that the low concentrations of calcitriol must be synthesized within the fetus or placenta. Placentas of lambs differ in that radiolabeled calcitriol crosses in both directions (155), and a high metabolic clearance rate of calcitriol within fetal lambs indicates that calcitriol produc-

tion is markedly upregulated compared with their ewes (566). Maternal nephrectomy in rats did not alter fetal calcitriol levels, confirming that these metabolites are independently synthesized in the fetal-placental unit (369). Fetal kidneys and placenta each express the 1α-hydroxylase (Cyp27b1), which converts 25-hydroxyvitamin D to calcitriol (234, 663, 729). Placental trophoblasts, yolk sac, and decidua also express 24-hydroxylase (Cyp24a1), which catabolizes 25-hydroxyvitamin D and calcitriol into inactive forms (30, 135, 728).

The fetal kidneys contribute a significant amount of calcitriol to the fetal circulation because fetal nephrectomy reduced the fetal calcitriol level by 60% in fetal lambs (564). This may mean that ~40% of calcitriol in the fetal circulation is produced by the placenta, at least in lambs. Although the data from lambs indicate that calcitriol can cross the placenta from the feta to maternal circulations (155), comparatively little calcitriol produced by the placenta may reach the maternal circulation in rodents. This conclusion is supported by the finding that maternal kidneys in pregnant mice have 35-fold higher expression of Cyp27b1 than in the placentas (310).

25-Hydroxyvitamin D readily crosses the rat placenta; consequently, the low level of calcitriol cannot be due to inadequate supply of its precursor (240). It is likely that renal 1α-hydroxylase expressed by fetal kidneys is suppressed by the high ionized calcium, high phosphorus, and low PTH concentrations typically observed in fetal blood. The 1α-hydroxylase is capable of responding to PTH in utero because Casr+/− and Casr null fetuses demonstrated a stepwise increase in both PTH and calcitriol levels (338), but the calcitriol levels of Casr null fetuses remained lower than in adult WT females (338, 342). Calcitriol levels did reach the adult normal range in Vdr null fetuses which lacked the vitamin D receptor (VDR) (192). As noted below, PTHrP circulates at high levels in fetal blood and through its mimicry of PTH action on the PTH/PTHrP receptor it might stimulate high fetal calcitriol concentrations. However, systematic studies of PTH1R binding and signaling induced by NH2-terminal PTH and PTHrP, comparison of their crystal structures, and clinical studies of the effect of NH2-terminal PTH and PTHrP infusions, suggest that PTHrP is much less potent than PTH at stimulating the 1α-hydroxylase (195, 269, 270, 740; reviewed in detail in Ref. 333).

FGF23 conceivably contributes to low calcitriol levels because it inhibits 1α-hydroxylase and stimulates the catabolic enzyme 24-hydroxylase. However, its effect during fetal development appears relatively insignificant because Fgf23 null fetuses had no change in serum calcitriol or the renal and placental expression of Cyp27b1 (395); however, Fgf23 null fetuses did have modest but significantly reduced renal expression of Cyp24a1 (395). Conversely, 11-fold increased FGF23 in Phex null male fetuses did cause a small
but statistically significant decrease in serum calcitriol, and this was accompanied by significantly increased expression of Cyp24a1 in placenta and fetal kidneys but no change in the expression of Cyp27b1 (395).

As has been demonstrated in the fetal rat, low serum calcitriol in WT fetuses may also be due to preferential catabolism of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D, instead of synthesis of calcitriol from 25-hydroxyvitamin D (369, 475). This results in fetal levels of 24-hydroxylated forms that are up to 40-fold higher than calcitriol in fetal rats and sheep (369, 475, 506). The high expression of Cyp24a1 in murine placentas is approximately equal to that of the maternal kidneys (310), confirming that the placenta has significant capacity to catabolize 25-hydroxyvitamin D and calcitriol into 24-hydroxylated forms.

B) HUMAN DATA. The human data are largely consistent with the rodent data mentioned above, and differ from data obtained from fetal lambs. Calcitriol circulates at low levels in fetal blood, typically <50% of the maternal value (190, 264, 599, 642, 736). Umbilical artery values are higher than those obtained from the umbilical vein (736), which suggests that it is the fetal kidneys and not the placenta that contribute much calcitriol to the fetal circulation. As in the animal models, 25-hydroxyvitamin D readily crosses the placenta (254) and achieves cord blood levels that are typically 75–100% of the maternal value at term with a fetal-maternal correlation coefficient of 0.8–0.9 (190, 264, 599, 713, 736). Calcitriol synthesis in the fetus is likely suppressed by the high serum calcium, high phosphorus, and low PTH concentrations typically observed in cord blood.

Human trophoblasts and maternal decidua express 1α-hydroxylase and convert 25-hydroxyvitamin D to calcitriol (156, 728, 758). Trophoblasts, yolk sac, and decidua also express 24-hydroxylase (30, 728). In one study of human placentas, CYP27B1 mRNA expression was higher than in adjacent decidua while CYP24A1 mRNA was lower (176), and there is evidence that CYP24A1 is methylated in human placenta (479). These findings have prompted other researchers to conclude that trophoblast synthesis of calcitriol is unopposed or “unfettered” by catabolism into 24-hydroxylated forms (383, 479). However, the available functional data suggest the opposite conclusion, that human placentas preferentially metabolize 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D instead of calcitriol (572), resulting in up to 40-fold higher concentrations of 24-hydroxylated forms compared with calcitriol in cord blood (152, 253). These findings are the same as in fetal rodents, as noted above.

Maternal calcitriol increases two- to threefold during pregnancy, and the placenta has often been assumed to be the source of this increase. However, the placenta contributes little or no calcitriol to the maternal circulation, as revealed by an anephric woman on dialysis whose serum calcitriol remained low before, during, and after a pregnancy (698). Instead, it is the maternal kidneys that upregulate the synthesis of calcitriol during pregnancy. This is also similar to the rodent data mentioned earlier.

3. PTHrP

A) ANIMAL DATA. Cytochemical assays have revealed that fetal blood contains high PTH-like bioactivity, which cannot be accounted for by the low immunoreactive levels of PTH (3, 20, 67). Following the discovery of PTHrP and the development of specific radioimmunoassays for it, studies in fetal pigs (7) and sheep (94, 396) determined that high immunoreactive levels of PTHrP correlated closely with, and likely accounted for, the high PTH-like bioactivity in fetal blood.

PTHrP has a full length of PTHrP1–139 in rodents compared with splice variants PTHrP1–139, PTHrP1–141, or PTHrP1–173 in humans. But PTHrP is a prohormone that is processed into several circulating peptides. In the human these include PTHrP1–36, PTHrP38–94, PTHrP38–101, PTHrP107–139, and possibly PTHrP141–173, each of which may have its own receptor and distinct role (333, 490, 517, 745). The structures of these peptides were originally determined through study of tumor cell lines transfected with the human PTHrP gene. Which of these PTHrP peptides circulate in fetal life of animals or humans has not been determined. Most available assays have been designed to detect fragments that contain PTHrP1–34 or PTHrP1–86. Notably, a PTHrP1–34 assay used in rodents or lambs will detect NH2-terminal PTHrP1–36 and full-length PTHrP1–139, whereas the PTHrP1–86 assay will only detect full-length PTHrP1–139, a form that may not be abundant due to rapid cleavage into PTHrP1–36.

Immunoreactive forms of PTHrP detected by a PTHrP1–86 assay did not cross the placentas of sheep and goats (537), but whether smaller fragments can cross the placenta has not been determined. Full-length PTHrP1–139 has about twice the molecular weight of PTH, and since PTH does not cross the placenta (196, 205, 341, 345, 478), full-length PTHrP is also unlikely to cross.

Numerous tissue sources may contribute to the circulating concentration of PTHrP, because in the embryo and fetus the gene is expressed by skeletal growth plates (299, 365), cardiac and vascular smooth muscle (89), trophoblasts (5, 336, 602), intraplacental yolk sac (336), amnion (68, 184), chorion (68), umbilical cord (185), and numerous other tissues and cell types. Venous umbilical PTHrP levels are higher than umbilical arterial levels in pigs, which suggests that the placenta may be an important source of circulating PTHrP in the fetus (7). As discussed in section II, the fetal parathyroids were originally proposed to be the source of PTHrP in fetal sheep, but the effect of fetal parathyroidectomy on the plasma PTHrP concentration was not reported.
Loss of fetal parathyroids did not alter the plasma PTHrP<sub>1–34</sub> concentration in Hoxa3 null and Gcm2 null fetuses (623). Pth1r null fetuses, which lack the PTH1R, have an 11-fold increase in PTHrP<sub>1–34</sub> and increased placental expression of PTHrP mRNA and protein (341). These findings are compatible with the placenta being the dominant source of PTHrP in the circulation of fetal rodents. Whether fetal parathyroid glands in rodents produce PTHrP remains unresolved and is discussed in section IIIC.

B) HUMAN DATA. As with animal fetuses, prior to the discovery of PTHrP it was recognized that human cord blood contained low immunoreactive intact PTH concentrations but high levels of PTH-like bioactivity as measured in cytochemical bioassays (20, 21, 571). The discrepancy is now explained by high circulating levels of PTHrP (600, 671), which mimics many of the actions of PTH on the PTH1R. The concentrations of PTH and PTHrP are often reported in unrelated units, but when expressed in equivalent units (pM), human cord blood PTHrP levels are 2–4 pM and up to 15-fold higher than the simultaneous levels of PTH (0.2–0.5 pM) (168, 306, 671).

A few studies have provided conflicting data by finding undetectable levels of PTHrP<sub>1–86</sub> in cord blood (592) or very low levels of PTHrP<sub>1–86</sub> that were no different among pregnant women, fetal samples obtained by cordocentesis, and cord blood samples (501). These results likely reflect several problems that are commonly found among clinical studies that included PTHrP measurements. First, PTHrP is subject to rapid cleavage and degradation in serum; consequently, blood samples are optimally collected as EDTA plasma with aprotinin (a protease inhibitor), kept chilled on ice, and promptly (within 15 min) centrifuged, separated, and frozen. Even when optimally collected as EDTA-aprotinin plasma and kept chilled on ice, PTHrP begins to degrade within 15 min of sample collection (273). Many studies did not approach this rigor of sample collection and preparation, and it is common to see use of stored sera that had been allowed to clot at room temperature for up to 60 min (678). All of these factors will result in failure to detect the full concentration of PTHrP that was present in the original blood sample, and may even result in undetectable PTHrP when it should be present. Second, the most commonly used assays in clinical studies have been those that purportedly measure PTHrP<sub>1–86</sub>. This form is not predicted to exist in humans (333), but the assay should detect full-length forms PTHrP<sub>1–139</sub>, PTHrP<sub>1–141</sub>, or PTHrP<sub>1–173</sub> if they are present in the circulation. However, it will not detect PTHrP<sub>1–36</sub>, which is conceivably the more abundant NH<sub>4</sub>-terminal form of PTHrP because it derives from all full-length forms. Conversely, a PTHrP<sub>1–34</sub> assay should detect each of PTHrP<sub>1–36</sub>, PTHrP<sub>1–139</sub>, PTHrP<sub>1–141</sub>, and PTHrP<sub>1–173</sub>. Standardization of sample requirements and PTHrP assays are sorely needed; in the meantime, it is preferable to use a PTHrP<sub>1–34</sub> assay and to use only plasma that has been rigorously collected and processed under the optimal conditions described here.

4. FGF23

A) ANIMAL DATA. FGF23, produced largely by osteocytes and osteoblasts, is a phosphorus-regulating hormone that controls the supply of phosphorus at the mineralizing surface of bone through actions on distal tissues (622). It downregulates the expression of sodium-phosphate cotransporters 2a and 2c (NaPi2a and NaPi2c) in proximal renal tubules (614). It also inhibits 1α-hydroxylase (Cyp27b1), increases expression of 24-hydroxylase (Cyp24a1), and inhibits intestinal expression of NaPi2b (614). In adults, increased effective circulating levels of FGF23 lead to hypophosphatemia and renal phosphate wasting, while loss of FGF23 causes hyperphosphatemia and impaired renal phosphate excretion (52).

FGF23 is predominantly expressed in rat fetal osteoblasts, in addition to thymus, liver, and kidney (754). Murine fetuses express FGF23 as early as embryonic day 12.5 in heart, liver, and somites; it later appears in bone (627). Using the sensitive method of real-time quantitative RT-PCR, comparative study of WT, Fgf23 null, and Phex null placental expression of NaPi2b (614). An independent study used the less sensitive method of conventional RT-PCR and did not find visible Fgf23 amplification on an agarose gel (484).

The concentration of intact FGF23 in fetal blood from WT fetuses was equal to the simultaneous maternal level in mice from the outbred Black Swiss strain for several mouse models (395), whereas the level was about half the maternal value in WT fetuses from the inbred C57BL/6 strain (194, 395). The maternal value in turn was approximately double the nonpregnant level as revealed through longitudinal studies in both strains of mice (194). The discrepancy in the fetal FGF23 blood levels between the Black Swiss and C57BL/6 mice, within the same laboratory and using the same intact FGF23 assay, indicates that the genetic background of the mice influences fetal FGF23 physiology. Phex null male fetuses and Phex<sup>+/−</sup> female fetuses had markedly increased intact FGF23 concentrations that equaled the simultaneous maternal concentration from their Phex<sup>+/+</sup> mothers (395), whereas in another study the FGF23 level in Phex<sup>+/−</sup> null male fetuses far exceeded the maternal concentration (484).

Although no formal studies have been done with radiolabeled FGF23, it evidently does not cross the murine placenta because Fgf23 null fetuses had undetectable FGF23 despite high levels present in the maternal circulation (395). Furthermore, the high FGF23 concentrations observed in Phex<sup>+/−</sup> mothers did not alter the circulating FGF23 level.
5. Calcitonin

A) ANIMAL DATA. Thyroid C-cells and calcitonin are detectable as early as the 30th day of an approximate 145-day gestation in fetal lambs (292), whereas in fetal mice and rats C-cells and calcitonin do not appear until day 15.5 or later out of a gestational period that lasts 19–22 days, respectively (22, 203, 284, 474, 645, 679). Near term, fetal calcitonin concentrations are maintained at higher concentrations than in the maternal circulation in rodents (201, 430) and lambs (201, 204). Calcitonin within the fetal circulation must derive from fetal sources because maternal calcitonin does not cross the rat or mouse placenta (212, 430). Rat and mouse placentas also express calcitonin mRNA and protein (284, 293, 337). The high circulating concentration of calcitonin may occur in response to the increased serum and ionized calcium that is normally present in the fetal circulation. Consistent with this, experimentally induced hypercalcemia in fetal pigs, lambs, calves, and nonhuman primates caused a further, marked increase in calcitonin, the magnitude of which correlated with the change in serum calcium (101, 380, 548). The relative contribution of thyroid and placenta to circulating calcitonin has not been determined. The fetal thyroid is a significant source because infusion of calcium chloride into the left thyroid artery of fetal lambs caused a 2- to 10-fold increase in calcitonin secretion (215).

B) HUMAN DATA. The thyroid C-cells differentiate around the 12th week of gestation (107), and calcitonin is detectable in those cells from as early as the 15th week of gestation (368). Serum calcitonin is increased to as much as twice the maternal level in both term and preterm babies (255, 343, 523, 583, 599, 736, 742). Trophoblasts of the placenta also express calcitonin and may, therefore, contribute to the amount in the fetal circulation (35).

6. Sex steroids

A) ANIMAL DATA. The dominant sex steroids, testosterone and estradiol, are considered as calcitropic hormones within this review because of their importance in regulating postnatal skeletal development and bone turnover. Testosterone in male fetal rats surges over the last several days of gestation before declining on the last day (720, 730). It exceeds the concentration in female fetuses (239, 272, 720, 730) and was higher than the maternal value in three studies (239, 720, 730), and equal to maternal in another (272). Testosterone was also higher in male compared with female fetal lambs (18). Conversely estradiol concentrations are similar between male and female rat fetuses (239), and exceed the maternal concentration (146, 239). In fetal lambs, testosterone and estradiol exceeded the maternal concentration at all time points (117, 650, 757).

B) HUMAN DATA. Human pituitary cells produce luteinizing hormone and follicle stimulating hormone from as early as 5 wk of gestation (619, 620), and the levels of both gonadotropins are higher in female fetuses throughout gestation (676). The gonadotropins and placenta-derived human chorionic gonadotropin drive the Leydig cells within the developing male testis to produce testosterone (29), whereas only negligible amounts of testosterone are produced in the ovaries or in the adrenals of both sexes (546). The ovaries, testes, and adrenals produce little or no estradiol until late in fetal life (91, 546). In male fetuses, testosterone exhibits higher concentrations between 11–17 wk of fetal age before subsequently declining to trough values (545, 739); at term, the concentration is modestly higher or no different than in female fetuses (301, 458, 509, 545, 682, 739). Estradiol levels are also similar between male and female fetuses (250, 509, 545, 682, 739). It is generally considered that in fetal humans estradiol and testosterone are present at low levels in cord blood near term compared with normal adult values (133, 676). Indeed, in most studies the fetal levels of estradiol and testosterone were much lower than simultaneous maternal concentrations at term (15, 179, 216, 458, 465, 509, 681, 682, 696). However, in a few studies either or both of testosterone and estradiol have been approximately equal to (250, 545) or significantly greater than (14, 357, 739) the maternal concentrations.

These discrepancies in relative fetal-to-maternal sex steroid levels are likely the result of whether the umbilical vein, artery, or mixed cord blood was sampled. By the third trimester, the placenta accounts for nearly all of the estrogens (estradiol, estrone, and estriol) in the maternal circulation (437), and it synthesizes them through aromatization of androgens that arise about equally from the fetal and maternal adrenals (382, 618). The concentrations of these estrogens progressively increase during pregnancy and reach peak values in the maternal circulation during the third trimester (382, 480, 697). If the umbilical vein or mixed...
cord blood are sampled, the levels will indicate high concentrations due to placental synthesis of sex steroids, whereas umbilical artery values reflect systemic levels within the fetus. Several studies have found that the umbilical artery estradiol or conjugated estrogen levels are significantly lower than the umbilical vein values (589, 617, 696). Such findings confirm that placental production of sex steroids confounds the cord blood measurements and that the umbilical artery values alone should be used to indicate systemic fetal concentrations. In the previously cited studies that reported elevated sex steroid levels in cord blood, it was mixed cord blood that had been sampled in two of them (14, 739), whereas the source was not specified in the third study (357).

7. Summary of key points

The human fetal circulation is characterized by low concentrations of PTH, calcitriol, and the sex steroids as well as high levels of PTHrP and calcitonin (FIGURE 2). Parathyroid CaSR likely suppresses PTH in response to the high fetal blood calcium. 25-Hydroxyvitamin D readily crosses the placenta whereas calcitriol does not. The low fetal calcitriol levels are likely due to suppression of the fetal renal 1α-hydroxylase (CYP27b1) by low PTH and high serum calcium and phosphorus, and increased activity of the catabolic enzyme 24-hydroxylase.

C. Fetal Parathyroids

1. Animal data

In rodents, the thymus and a single pair of parathyroids derive from the third pharyngeal pouch (56, 384). Parathyroids are friable such that fragments detach during embryonic migration, which likely accounts for parathyroid tissue within the thymus and adjacent tissues (232, 384, 385, 657, 705). Gcm2 is intensely expressed by the primordium derived from the third pharyngeal pouch in mice; therefore, deletion of Gcm2 should ablate the parathyroids and result in undetectable PTH, as occurs when Hoxa3 is deleted (232, 336, 341, 411). Consistent with this, the normal expression of PTH mRNA and protein in the common parathyroid/thymus primordium on embryonic day 11.5–12.5 is absent in Gcm2 null fetuses (385). However, a low level of PTH1 has been measured in the circulation of Gcm2 null fetuses even though the parathyroids (and parathyroid remnants in the thymus) have been eliminated (623). Pth is also expressed in normal placenta, and its expression there is upregulated in Gcm2 null fetuses compared with placentas from WT littermates (623), and so placental PTH may explain the low but detectable PTH blood levels in Gcm2 null fetuses. Adult Gcm2 null mice were originally reported to have normal circulating levels of PTH, a finding that is not easily explainable unless Gcm2-independent sources of PTH activate in the absence of parathyroids (238).

PTH mRNA and protein can also be detected by mid-gestation in the parathyroids of fetal rats and lambs (390, 602). It has been suggested that fetal parathyroids secrete both PTH and PTHrP. However, the evidence that fetal parathyroids express PTHrP mRNA and protein is inconsistent and inconclusive. PTHrP was first detected in parathyroids of fetal lambs by immunocytochemical methods with older polyclonal antibodies that did not cross react with PTH (5, 390, 396). These studies have not been repeated with newer and more specific monoclonal antibodies, nor has PTHrP mRNA expression been examined in parathyroids from fetal lambs. RT-PCR and in situ hybridization failed to detect PTHrP mRNA in fetal or adult rat parathyroids (601, 695), whereas PTHrP mRNA was detected by RT-PCR in a third study of adult rat parathyroids (274). The more sensitive technique of real time quantitative RT-PCR has not been used on fetal parathyroids from rats or lambs, while murine fetal parathyroids have not been directly examined at all for their ability to express PTHrP.

If the fetal parathyroids are a significant source of PTHrP in the fetal circulation, then fetal parathyroidectomy or genetic ablation of the parathyroids should reduce the circulating PTHrP level. However, the plasma PTHrP level was not measured in any of the studies in which fetal lambs and rats were parathyroidectomized. Instead, the parathyroid secretion of PTHrP was inferred from the finding that parathyroidectomy in fetal lambs caused a reduction in placental calcium transport that could be rescued by infusion of autologous blood or PTHrP, but not PTH (discussed in more detail in sects. IVB and VB). In contrast, genetic ablation of parathyroids in Hoxa3 null and Gcm2 null fetuses did not alter the plasma PTHrP level (341, 623), while an 11-fold upregulation of plasma PTHrP in PthIrt null mice was associated with upregulation of PTHrP mRNA and protein expression in the placenta but not in the region of the neck that contains the parathyroids (341). These findings in rats and mice suggest that the parathyroids are unlikely to be a significant source of PTHrP in the fetal circulation, whereas the data from fetal lambs remain consistent with parathyroid-localized expression of PTHrP.

Despite a low level of PTH in the fetal circulation, the evidence is consistent across all animal models that fetal parathyroids are required to maintain the normally high level of blood calcium. Loss of parathyroids from any cause results in fetal hypocalcemia, as demonstrated in fetal lambs (thyroparathyroidectomy followed by replacement of thyroxine) (1, 2), rats (thyroparathyroidectomy or decapitation) (520, 521, 554), and mice (loss of parathyroids through genetic ablation of Hoxa3 or Gcm2, or loss of PTH alone in Pth null fetuses) (336, 341, 623).

PTH and PTHrP both participate in the normal regulation of the fetal blood calcium, such that loss of either hormone results in fetal hypocalcemia, and loss of both leads to the
greatest decrease in blood calcium (336, 340, 341, 623). This is discussed in more detail in section VIA.

Fetal parathyroids are also required to regulate magnesium and phosphorus, since thyroparathyroidectomized fetal lambs, and Hoxa3 null, Gcm2 null, and Pth null fetal mice have hypomagnesemia and hyperphosphatemia (47, 341, 397, 398, 623). Loss of PTHrP itself causes hyperphosphatemia with a normal serum magnesium concentration (341, and unpublished data).

In sections IVB and VB, the evidence that the parathyroids regulate placental calcium transport is discussed.

2. Human data

In humans, parathyroids and thymus share a common developmental origin within the third pharyngeal pouch, while a second pair of parathyroids originate from the fourth pouch (56, 222, 518, 669). As in the animal models, the friable nature of developing parathyroids leads to thymic and other ectopic locations for fragments that detach during embryonic migration.

PTH is detectable by immunochemical methods within parathyroids beginning at 10 wk of gestation (367). Expression of PTHrP has not been sought in human fetal parathyroids, but PTHrP mRNA and protein have been detected in the oxyphil cells of adult normal, hyperplastic, and adenomatous parathyroids by a variety of methods (28, 137, 157, 277, 313, 314, 424, 552). Since adult parathyroids express PTHrP, it is conceivable that the fetal parathyroids do as well.

3. Summary of key points

Fetal parathyroids produce low amounts of PTH, but whether or not they produce PTHrP is unclear. The evidence is stronger for PTHrP production by parathyroids of fetal lambs than it is for fetal rodents or humans; species differences may explain these discrepancies.

D. Renal Mineral Reabsorption, Excretion, and the Amniotic Fluid

1. Animal data

In the adult, the kidneys play key roles in the regulation of mineral homeostasis: 1) by adjusting the relative reabsorption and excretion of minerals in response to PTH, calcitriol, calcitonin, FGF23, and CaSR; and 2) as the dominant site of expression of 1α-hydroxylase and synthesis of calcitriol.

The fetal kidneys may be of lesser importance because the placenta assumes control over mineral handling and synthesizes calcitriol. Another reason is that excreted mineral is not lost to the organism, because urine forms a large component of the amniotic fluid which is swallowed (autophagia), absorbed, and thereby recycled (563). Renal blood flow and glomerular filtration rate are also much lower during fetal development compared with after birth (237, 378, 605). Nevertheless, alterations in serum mineral concentrations and renal excretion of minerals have been observed in response to alterations in kidney function, or from manipulations in the factors that normally control renal tubular function.

In fetal lambs, bilateral nephrectomy led to hypocalcemia, hyperphosphatemia, increased PTH, and a 60% fall in the circulating level of calcitriol (455, 564). Additional fetal lambs underwent a control procedure that consisted of bilateral ureteral sectioning to permit fetal urine to drain into the fetal peritoneal cavity while retaining functional kidneys in situ. Ureteral sectioning had no effect on fetal calcium or calcitropic hormone levels (455). The investigators concluded that loss of renal 1α-hydroxylase and production of calcitriol resulted in altered fetal mineral homeostasis, rather than the loss of renal function to reabsorb and excrete minerals. However, other investigators performed bilateral nephrectomy in fetal lambs and observed an increase in serum phosphorus, no change in serum calcium, and a nonsignificant reduction in the excretion of calcium into the amniotic fluid and allantois (459). Bilateral nephrectomy in fetal rats did not alter the serum calcium or phosphate either; calcitriol and PTH were not measured in those experiments (105).

With respect to hormonal control of renal mineral handling, thyroparathyroidectomy in fetal lambs caused hypocalcemia, hyperphosphatemia, increased renal fractional excretion of calcium, and reduced renal phosphorus excretion (398). Pharmacological administration of NH2-terminal fragments of PTH or PTHrP increased serum calcium and reduced renal calcium excretion, but did not alter serum phosphorus or urine phosphorus excretion (142, 398). These results may indicate that thyroparathyroidectomy causes hypocalcemia in part through loss of the effects of parathyroid-derived PTH and PTHrP on the renal tubules. However, in contrast to fetal lambs, absence of parathyroids in Hoxa3 null and Gcm2 null fetal mice, loss of PTH1R in Pth1r null fetuses, and absence of PTH in Pth null fetuses, each caused hypocalemia, hyperphosphatemia, and reduced amniotic fluid calcium, which is a measure of renal calcium excretion (341, 623; and unpublished data). The low blood calcium in these murine fetuses must cause a reduced renal filtered load of calcium, which in turn may explain reduced excretion of calcium into the amniotic fluid. Conversely, fetal mice with high blood calcium have increased excretion of calcium into the amniotic fluid, which is likely the result of an increased renal filtered load of calcium (338).
Whether calcitriol itself plays any important role in fetal kidney function is uncertain. Data have already been cited about how circulating calcitriol levels are low in fetal rats but increased in fetal sheep. As already noted, fetal nephrectomy in lambs reduced the serum calcitriol level by 60% and was accompanied by a fall in serum calcium and an increase in serum phosphorus in one study (455), but serum calcium remained normal in another study of nephrectomized fetal lambs (459), and serum calcium and phosphorus were normal in nephrectomized fetal rats (105). The investigators in the first study assumed that loss of renal production of calcitriol caused the hypocalcemia and hyperphosphatemia and were able to show that pharmacological treatment with calcitriol reversed these abnormalities (455). However, in Vdr null fetal mice, ionized calcium and phosphorus, and amniotic fluid calcium and phosphorus, were not altered compared with WT littermates (342). It appears likely that renal production of calcitriol is relatively unimportant for fetal calcium homeostasis in most mammals, although it is possible that the situation is different for fetal sheep because of high circulating levels of calcitriol.

FGF23 increases renal phosphate excretion in the adult by downregulating expression of NaPi2a and NaPi2c in the renal tubules. Fetal kidneys show abundant expression of the FGF23 targets Klotho, NaPi2a and NaPi2c, Cyp27b1, Cyp24a1, and Fgfr1-Fgfr4 (395). But in Fgf23 null fetal mice, loss of FGF23 did not alter serum phosphorus or amniotic fluid phosphorus excretion, and there was no effect on expression of NaPi2 and NaPi2c in fetal kidneys (395). Conversely, in Phex null male fetuses, which have markedly increased circulating concentrations of FGF23, there was also no effect on serum phosphorus, amniotic fluid phosphorus excretion, and NaPi2 and NaPi2c expression in fetal kidneys (395). These results suggest that fetal kidneys do not share the key role in renal phosphate handling or responsiveness to FGF23 that the adult kidneys have.

2. Human data

There are no available mineral or calcitropic hormone measurements or skeletal data from anephric human fetuses, apart from noting that affected babies can have scoliosis, rib anomalies, and absent toes, radii, or thumbs (528). Those skeletal abnormalities may reflect the underlying genetic anomalies rather than an effect of absent kidney function. Bilateral renal agenesis presents with very low amniotic fluid volumes (oligohydramnios or anhydramnios) due to the lack of fetal urine production (147, 528), confirming that urine comprises much of the normal volume of amniotic fluid. In turn, the lack of amniotic fluid causes pulmonary hypoplasia, which has been confirmed by noting that a monoamniotic twin with bilateral renal agenesis was born with normal pulmonary function, presumably because the twin’s kidneys provided the normal volume of amniotic fluid (511). The absent kidney function and pulmonary hypoplasia contribute to the uniform fatality of this condition in the newborn period.

3. Summary of key points

The fetal kidneys are a likely source of the low level of calcitriol in the fetal circulation and are capable of excreting and reabsorbing minerals. Whether fetal kidneys contribute importantly to fetal mineral homeostasis is uncertain, especially since urine enters the amniotic fluid and can then be recycled through the process of autophagy.

E. Intestinal Mineral Absorption

1. Animal data

Adult intestines are the main route of mineral entry into the organism, with calcium being absorbed in part through passive processes, and actively through calcitriol-dependent mechanisms. Fetal intestines must play a minor role in mineral homeostasis because the only material available to be swallowed and absorbed is the amniotic fluid. No studies have examined the relative contribution of the fetal intestines to mineral homeostasis. However, data from newborns likely reflect the function of the intestines in utero. Intestinal calcium absorption in newborn rats is largely passive, nonsaturable, and not dependent on calcitriol (218, 219, 243). The vitamin D receptor is undetectable within enterocytes at days 7 and 14 after birth (242), which explains its lack of responsiveness to calcitriol at earlier stages (243).

2. Human data

Although the intestines are considered a minor player in the regulation of fetal mineral homeostasis, a significant volume of amniotic fluid (fetal urine) is normally swallowed and absorbed. When swallowing or absorption are not possible, such as in gastrointestinal atresias, an increased volume of amniotic fluid (polyhydramnios) is present at term (8, 141).

Intestinal calcium absorption has not been studied in human fetuses. However, a preterm baby at birth is the equivalent of a fetus of the same gestational age, and the functional capacity of a preterm neonate’s intestines may inform about the functional capacity of the fetal intestines. Studies in preterm neonates have shown that calcium absorption is proportional to intake and does not reach a maximum (i.e., is nonsaturable); these findings suggest that passive absorption of calcium is the dominant mechanism by which calcium is absorbed in preterm fetuses (44, 221, 581). Calcium absorption did not vary by vitamin D intake in preterm infants, consistent with passive absorption being the dominant mechanism of absorption rather than active, calcitriol-dependent mechanisms as seen in older infants and children.
The calcium requirement of the preterm neonate is very high at birth because the fetal skeleton is typically accreting ~300 mg or 120–150 mg/kg of calcium daily at that age (761). Current guidelines recommend a daily calcium intake of 120–200 mg/kg for preterm babies, which exceeds what the intestines should be capable of absorbing, so parenteral administration of calcium is needed (445). The postnatal changes in intestinal calcium absorption are discussed in more detail in section IIIE.

3. Summary of key points

The fetal intestines are likely a minor player in fetal mineral homeostasis because only amniotic fluid is available to be ingested, but their precise contribution has not been determined. Study of preterm babies have suggested that calcium absorption in the fetal intestines is likely passive and not regulated by calcitriol.

F. Placental Mineral Transport

This section reviews the mechanisms through which minerals are thought to be transported across the placenta, the methods used to study placental mineral transport, and the evidence that maternal factors influence the rate of transport. The effect of fetal sources of the calcitropic and phototropic hormones on placental calcium transport is discussed later within the relevant portions of sections IV–X.

1. Overview of mechanisms

A) ANIMAL DATA. Most of the calcium content of the newborn skeleton is obtained from the maternal circulation during the last third of gestation. In calves, 95% of the 673 g of calcium in the fetus is accreted during the last 3 mo of a 9-mo gestation, while in fetal rats 95% of the required 12.3 mg of calcium is accreted during the last 5 days of a 22-day gestation (121). Active placental calcium transport is necessary because diffusional flow is insufficient and the calcium exchange occurs against a substantial electrochemical gradient (95, 198). Additional studies have confirmed that it is an active process, dependent on Mg2+ and ATP (188). It has been estimated from study of perfused sheep placentas that active transport of calcium comprises two-thirds of the forward flow from mother to fetus (331).

Calcium transport is thought to occur via mechanisms and pathways that are similar to calcium transport across intestinal cells. Gated calcium channels [such as transient receptor potential cation channel, subfamily V, member 6 (TRPV6)] open within maternal-facing basement membranes to allow calcium entry into the relevant cells, calcium then shuttles to the opposite basement membrane bound to binding proteins, and then Ca2+-ATPase at the fetalfacing basement membranes actively pumps calcium into the fetal circulation (95, 659). The relevant calcium transporting cells are the trophoblasts and probably the intraplacental yolk sac cells within rodent placentas (63, 188, 337). The flow of calcium is not unidirectional, but the magnitude of backflux is difficult to measure; it may be <1% of the forward flow in sheep, rats, and mice (340, 507, 535, 660, 721), but 80% of the forward flow in primates (535).

The calcium binding protein calbindin-D9k transports calcium within renal and intestinal cells, and likely plays a similar role in trophoblasts and yolk sac cells (288). It is expressed as early as day 10 of gestation in rodent placentas (607), and its expression increases markedly over the last week of gestation in rats (81, 151, 227) and mice (150, 659). Other calcium binding proteins are also expressed within trophoblasts and intraplacental yolk sac cells and show increased expression over the same interval (251, 252, 692). Ca2+-ATPase expression doubles during the last 7 days of gestation (227, 693). TRPV6 expression increases 14-fold during the last 4 days of gestation in the mouse (659). All three of these calciotropic genes are expressed by trophoblasts, but within rodent placentas, they are most intensely expressed within the intraplacental yolk sac, a unique structure that also expresses other calciotropic genes at much higher intensity than in the surrounding trophoblasts (FIGURE 6) (659).

Varying levels of evidence support the role of calbindin-D9k, Ca2+-ATPase, and TRPV6 in placental calcium transport. Pthrp null and Trpv6 null fetuses exhibit reduced placental calcium transport, and this is accompanied by reduced expression of calbindin-D9k within the intraplacental yolk sac of each model (337, 659), but whether the reduced expression caused or resulted from a lower rate of placental calcium transfer is unknown. Deletion of calbindin-D9k does not alter serum calcium or intestinal calcium absorption in adult mice, but fetal minerals and placental calcium transport have not been measured (51, 351, 364). Ca2+-ATPase has been shown to be key because inhibition of its activity by dinitrophenol, ouabain, quercetin, or a neutralizing antibody leads to a reduction in the rate of calcium transport in perfused placentas from rats (63, 95). Pmca1 encodes the dominant placental Ca2+-ATPase, but ablation of it results in embryonic lethality (486, 530); consequently, placental calcium transport cannot be measured. Pmca2 or Pmca4 are isoforms that are also expressed within trophoblasts (456, 649), and the knockout mice each have mild phenotypes, but the fetal skeleton and placenta have not been examined (486, 530). The lack of an adult phenotype does not preclude a significant fetal phenotype, as seen by Trpv6 null adult mice that have no impairment of intestinal calcium absorption (51), whereas Trpv6 null fetuses have severe hypocalcemia, a 40% reduction in placental calcium transfer, and a 50% reduction in skeletal ash weight (659). Overall, the available data are consistent with important roles for Ca2+-ATPase and TRPV6 in regulating...
placental calcium transfer, while it remains unknown whether calbindin-D9k is essential.

Fewer studies have examined placental transport of other minerals. $^{32}$P transport across the rat placenta increases until term, paralleling the increase in placental calcium transport (315, 701, 702). Back-flux of $^{32}$P has been estimated to be ~7% of the forward (maternal to fetal) flow in lambs (660). The placenta expresses numerous phosphate-regulating genes that are present in the intestines and kidneys, including the sodium-phosphate channels NaPi2a, NaPi2b, NaPi2c; the FGF23 coreceptor Klotho, and all four FGF receptors (395, 483, 484). A low level of FGF23 expression has also been demonstrated (395).

$^{28}$Mg has been used to study active placental magnesium transport in lambs (47, 96), wherein the back-flow is ~30% of the forward flow (100). The factors that control magnesium transport are unknown.

B) HUMAN DATA. The human skeleton accretes more than 80% of its 30 g of calcium during the third trimester (121, 224, 683, 733, 734). Human placentas have been perfused in vitro, and this has led to estimates that active transport of calcium comprises about one-third of the forward flow from mother to fetus (655). Human trophoblasts express the same genes (TRPV6, calbindin-D9k, and Ca$_{2+}$-ATPase) and are expected to transport calcium in a manner similar to rodent placentas.

C) SUMMARY OF KEY POINTS. Active transport of calcium is required to meet the fetal requirement for mineral, and it is transported through mechanisms that are similar to those active within intestinal cells. The mechanisms governing phosphorus and magnesium absorption are not established.

2. Placental structure and expression of calcitropic and phosphotropic genes

A) ANIMAL DATA. The placentas of monkeys, lambs, pigs, goats, rats, and mice have each been studied as models of human placental structure and function, but there are significant differences among their macro- and microstructures that must be considered (174, 311, 536, 553, 575, 644).

Lambs, goats, and pigs have cotyledonal placentas, in which the structure is organized into 60–70 cotyledons that spread out over the entire uterine wall. Microscopically the placenta is epitheliochorial, in which the maternal and fetal blood spaces are separated by epithelial and mesenchymal barriers. Consequently, structural barriers between the maternal and fetal circulations are significantly reduced in hemochorial placentas.

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chorial compared with epitheliochorial placentas (575, 644). Rodent placentas are hemochorial with three layers of trophoblasts interposed between the maternal and fetal circulations (404, 536, 644). Maternal blood circulates in a labyrinthine series of small channels created by the trophoblasts (129). Rodent placentas also contain the intraplacental yolk sac, a bilayered membrane formed when part of the yolk sac becomes enfolded within the placenta; it is positioned directly between fetal and maternal blood vessels (FIGURE 6) (166, 167, 289). This structure shows more intense expression of many calcitropic genes compared with the surrounding trophoblasts, including calbindin-D$_{9k}$, Ca$^{2+}$-ATPase, PTHrP, PTH1R, TRPV6, vitamin D receptor, calcitonin, calcitonin receptor, and CaSR (35, 63, 81, 83, 84, 95, 150, 151, 293, 337, 338, 356, 423, 471, 608, 656, 659). Its location between the maternal and fetal vessels is optimal for it to be a site of maternal-fetal calcium and nutrient transport, independent of the trophoblast labyrinth (330, 337, 659). This route may enable rodents to meet proportionately larger calcium demands created by their large litters and short gestational interval.

It has not been possible to determine the extent to which the intraplacental yolk sac may contribute to placental calcium transport. Platelet-derived growth factor-α encodes the intraplacental yolk sac, but deletion of this gene leads to embryonic lethality (482). However, Pthrp null and Trpv6 null fetuses have reduced placental calcium transfer (340, 659), and this is accompanied by reduced expression of many calcitropic genes including calbindin-D$_{9k}$ and Ca$^{2+}$-ATPase within the intraplacental yolk sac of the Pthrp null, reduced expression of calbindin-D$_{9k}$ within the Trpv6 null, but normal expression of calbindin-D$_{9k}$ within the trophoblasts of the Pthrp null (337, 340, 659).

The mouse placenta expresses numerous FGF23 target genes including Klotho, NaPi2a, NaPi2b, NaPi2c, Cyp27b1, Cyp24a1, Fgfr1, Fgfr2, Fgfr3 and Fgfr4; there was also low-level expression of Fgf23 (395). Cellular localization of these genes has not been determined.

B) HUMAN DATA. Human placentas are discoid and hemochorial and are considered to have similar structure, function, and diffusional characteristics to placentas of mice and rats (174, 311, 536, 553). They are hemomonochorial, meaning that a single trophoblast cell layer separates maternal and fetal blood. Fingerlike projections (villi) protrude into a large intervillous maternal blood-filled space. There is no labyrinth as in the rodent placenta, nor is the intraplacental yolk sac present.

The trophoblasts express numerous calcitropic and phosphotrophic genes including calbindin-D$_{9k}$, Ca$^{2+}$-ATPase (isoforms 1 and 4), and TRPV6 (63, 95, 273), PTHrP (82, 162, 184), PTH1R (82, 183), CaSR (69), VDR (664), 1α-hydroxylase (156, 728, 758), calcitonin (35), calcitonin receptor (356, 471), and Klotho (483).

C) SUMMARY OF KEY POINTS. The epitheliochorial placentas of sheep are structurally and functionally different from the hemochorial placentas of humans and rodents, but there are also differences between human and rodent placentas. The rodent placenta is considered a close functional match to the human placenta. All in vivo data about placental function come from study of non-human placentas, whereas there are limited in vitro human data of placental gene expression and mineral transport.

3. Methods for studying placental mineral transport

Several methods have been used to assess placental mineral transport. There are significant differences among them that need to be reviewed to understand the inherent limitations in the data obtained from them.

A) IN SITU PLACENTAL PERFUSION IN RATS AND LAMBS. The most rigorous technique is in situ placental perfusion, which has been used in lambs, goats, guinea pigs, and rats (97, 433, 554, 654, 723). The procedure begins by removing the fetus and connecting the placenta to a semi-closed circuit via the umbilical vessels (FIGURE 7). Autologous fetal blood or a blood substitute is used to perfuse the placenta in situ (i.e., within the mother), but the flow rate and perfusion pressure are maintained at arbitrary set levels. $^{45}$Ca and $^{51}$Cr-EDTA are infused together into the maternal circulation ($^{32}$P or $^{28}$Mg are substituted for $^{45}$Ca to measure placental phosphorus or magnesium transport, respectively). Repeated blood samples are taken from the maternal circulation and umbilical vein to calculate radioisotope clearance rates. $^{51}$Cr-EDTA is not actively transported but diffuses across these placentas, so its appearance within the umbilical vein is considered to reflect diffusion of isotope. The rate of active calcium transfer is calculated from formulas that incorporate the speed at which the $^{45}$Ca concentration declines in the maternal circulation, and the rate at which the concentration of $^{45}$Ca increases relative to $^{51}$Cr-EDTA in the umbilical vein (554). Hormones, drugs, or antibodies can be infused into the umbilical artery (placental in-flow) to test their effects on fetal regulation of placental calcium transport, or into the maternal circulation to determine if there are maternal effects on the regulation of placental calcium transport (723). Significant limitations of this technique include that it is done in the absence of fetal control, the mother is deeply anesthetized with potential effects on physiological values. Indeed, when $^{32}$P accumulation in the placenta has been assessed before and up to 10 days after removal of the fetus and compared with an intact placenta and fetus within the same uterus, significant differences in phosphate concentration are presented at 1 h after the fetus is removed, are four to five times normal at 6 h, and become profound as the days pass (703). Significant advantages of this technique include that frequent sampling from the pla-

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central circuit and the mother enable similar experimental conditions to be maintained from one pregnant dam to the next. The same method has been used to study placental phosphorus and magnesium transport. Studies with magnesium have been very limited due to the short half-life of the isotope, hence the need to have it custom-synthesized locally and at high cost.

B) PLACENTAL TRANSPORT WITHIN INTACT MICE. The second method was developed for use in mice (FIGURE 8). An intracardiac injection of $^{45}$Ca and $^{51}$Cr-EDTA is administered to a pregnant mouse that remains anesthetized from isoflurane for $<30$ s. Between 5 and 30 min after the injection, a C-section is done to remove the fetuses, and the radioactivity within each fetus is quantified. $^{51}$Cr-EDTA controls for differences in diffusion, blood flow, or size of the individual placentas because it crosses the placenta by passive diffusion only. The relative rate of placental calcium transfer is calculated by normalizing the $^{45}$Ca activity to $^{51}$Cr-EDTA within each fetus; the $^{45}$Ca activity alone can also be compared (340). Additional adjustments can be made for placental weight, but these do not add significantly to the adjustment for $^{51}$Cr-EDTA. The utility of $^{51}$Cr-EDTA has been shown by a two- to threefold variation in its accumulation that can be present among WT fetuses of the same mother, while the $^{45}$Ca/$^{51}$Cr activity of the WT fetuses remains unchanged (unpublished data). This likely indicates that blood flow to any one placenta within the same murine uterus is constantly changing and will not be simultaneously equal among all placentas in a litter. Significant limitations of this procedure include that a relative and not an absolute rate of calcium transfer is obtained among the fetuses within each litter, and that after a blind intracardiac injection the amount of isotope reaching the maternal circulation may differ somewhat between experiments. However, each fetus within a litter is exposed to the same ratio of $^{45}$Ca to $^{51}$Cr-EDTA in the maternal blood. Significant strengths include that the dam is only briefly anesthetized, the fetuses remain intact, placental blood flow or perfusion pressure has not been changed, all placentas within a litter received the same concentrations of isotopes, and the 8–12 normal, heterozygous, and null fetuses within each litter serve as controls for each other, as well as enabling comparisons from one litter to the next. The fetuses can also be treated with hormones prior to the intracardiac injection of isotopes (FIGURE 8). The procedure has also been adapted to study placental phosphorus transport (395).

C) IN SITU PLACENTAL PERFUSION ADAPTED TO MICE. The third method has adapted the in situ placental perfusion technique for the small scale of murine placentas. Mouse placentas are $<1$ cm in diameter with tiny umbilical vessels, which represents substantial technical challenges for a perfusion experiment. One placenta is selected, and its fetus is removed. The placental and umbilical vasculature are first maximally vasodilated with nitroglycerin in order that the umbilical vessels can be successfully catheterized and hooked up to a semi-closed circuit (61, 62). $^{45}$Ca is infused alone, without $^{51}$Cr-EDTA or another isotope to control for diffusional differences or placental size. Numerous maternal or umbilical blood samples cannot be collected because
the recoverable blood volumes of adult and fetal mice are too small, so maternal-fetal clearance of $^{45}$Ca cannot be directly calculated. Instead, the rate of disappearance of $^{45}$Ca from the maternal circulation was determined by sampling a single time point from different mothers, and creating a disappearance curve from the aggregate results (61, 62). This curve allows the maternal-fetal $^{45}$Ca clearance to be estimated under the assumption that experimental conditions remain identical from mouse to mouse. Significant strengths of this procedure are that it provides a quantitative estimate of the rate of placental calcium transfer in mice, and that it may be possible to study the effects of different hormones or drugs. This technique shares the significant weaknesses mentioned earlier for the in situ perfusion technique in fetal lambs and rats. Additional significant concerns include that an artifactual result may occur from diluting the placental vessels with nitroglycerin, especially if placental vasculature, blood pressure, or responsiveness to vasodilators differ between WT and mutant placentas. Furthermore, the lack of repeated blood sampling and the use of a derived clearance curve mean that the amount of isotope in the maternal and placental circulations are assumed rather than directly measured. As noted above, blood flow can vary two- to threefold from one murine placenta to the next within the same uterus based on the $^{51}$Cr activity in each fetus after its administration to the mother, but this technique requires a single placenta and an arbitrary perfusion pressure and rate.

D) INDIRECT MEASURES. A fourth technique has involved measuring the calcium or phosphorus content of the fetal skeleton and inferring the rate of placental mineral transport from that. In one approach, the pregnant ewe receives a single infusion of $^{45}$Ca or $^{32}$P 7 days before slaughter followed by daily treatment with a calcitropic hormone, and at the end of the experiment, the $^{45}$Ca or $^{32}$P content of the fetal skeleton is measured (38, 165). In a second approach, the pregnant ewe is immobilized and catheterized, and calcitropic hormones or diluent are injected three times daily into fetal lambs for 12 days. At the end of the experiment, the ash weight and mineral content of the fetal skeleton are determined (40). At best, these two approaches are measures of net accretion of mineral by the fetal skeleton, and only very indirect measures of what the rate of placental calcium or phosphorus transport might have been during the experiment. The amount of mineral in the skeleton is a composite result from cycling within several compartments of the fetus (blood, amniotic fluid, soft tissues, and bone), the effect of the hormones to directly stimulate or inhibit bone formation and mineralization independent of placental mineral transport, and back-flux of mineral across the placenta into the maternal circulation (330).

Some investigators have interpreted a fall in fetal blood calcium to the maternal level or below (so-called “reversal of the maternal-fetal gradient”) to indicate that placental calcium transport has decreased. This is not a valid approach; as results will show in sections IV–X, the blood calcium level is regulated independently of the rate of placental calcium transport.

E) ISOLATED PLACENTAL MEMBRANES AND VESICLES. The fetal-facing basement membranes of human syncytiotrophoblasts have been isolated and induced to form vesicles in vitro, with the fetal side facing inward. These can then be used to study the effects of calcitropic hormones and inhibitors to induce signal transduction or stimulate $^{45}$Ca accumulation within the vesicle (649). In such studies, accumulation of $^{45}$Ca within the cultured vesicles reflects the transport of $^{45}$Ca across the basement membrane, so it is a surrogate for measurement of placental calcium transport in the intact animal.

Other investigators have created vesicles from either maternal-facing or fetal-facing basement membranes, but with each membrane oriented so that calcium will be transported outward into the media. The vesicle is loaded with $^{45}$Ca and then efflux is measured under the influence of PTHrP, PTH, competitive antagonists of PTH or PTHrP, and other hormones (178).
Alternatively, other investigators have used isolated maternal-facing brush-border membranes, and fetal-facing basement plasma membranes of syncytiotrophoblasts, to study the expression of calcitropic hormone and their receptors, and the effects of calcitropic hormones and inhibitors on signal transduction within these cells (354, 355). No estimate of placental calcium transport is provided with these approaches.

F) SUMMARY OF KEY POINTS. Several methods exist for estimating the rate of placental mineral transport, and each has advantages and disadvantages that must be considered in interpreting the data. The placental perfusion technique in rats and lambs provides the most quantitative data but occurs in the absence of fetal control, and placental function begins to deteriorate within an hour after removal of the fetus. The placental transport technique within intact mice enables relative transport among fetal genotypes within a litter to be accurately determined while each fetus remains undisturbed and in control of placental function.

4. Maternal regulation of placental mineral transport

A) ANIMAL DATA. The fetal-placental unit regulates placental mineral transport, but it remains unclear to what extent (if any) maternal hormones or other factors provide any direct regulation of placental mineral transport. Indirect maternal regulation may occur because the concentration of minerals within the maternal circulation represents the supply from which the placenta must extract the necessary minerals. For example, if the maternal blood calcium is reduced by diet or altered maternal hormone levels, it might be anticipated to reduce the rate of placental calcium transport.

However, several studies suggest that placental calcium transport is normal even in the presence of maternal hypocalcemia or specific hormone deficiencies. For example, calcium transport across perfused placentas of sheep remained normal despite significant maternal hypocalcemia induced by parathyroidectomy or a severely calcium-deficient diet (99, 723). Similarly, hypocalcemia in Vdr null and Pth null mothers did not impair placental calcium transfer or reduce the skeletal mineral content in their WT or mutant fetuses (342, 623). Loss of calcitonin in pregnant Ctcgrp null mice did not alter placental calcium transfer or the ash weight and calcium content of the fetal skeleton (430). Excess FGF23 and hypophosphatemia in Phex+/− females did not alter placental phosphorus transport to any of the fetuses nor the phosphorus content of the skeleton (395).

On the other hand, when pregnant ewes were made hypocalcemic by a very low calcium diet, their fetal lambs had slightly delayed ossification and ash weight, despite no change in fetal levels of calcium, phosphorus, PTH, calcitriol, and a bone resorption marker (377). Similarly, when pregnant rats were hypocalcemic due to thyroparathyroidectomy or calcitonin infusions, the fetal bones showed increased resorption when subsequently cultured in vitro (541, 542), the fetal parathyroids enlarged (206, 623), and fetal femur length and mineral ash content were reduced (104). These findings in lambs and rats are compatible with a reduction in placental calcium transport that is sufficient to delay primary mineralization of bone or induce secondary hyperparathyroidism in the fetuses, while enabling the fetuses to maintain normal concentrations of minerals within the circulation. Conversely, acute (295) and chronic (338) maternal hypercalcemia in rodents have been shown to suppress the fetal PTH level (FIGURE 5). This suggests that maternal hypercalcemia increases the flow of calcium across the placenta, thereby suppressing the fetal parathyroids. Furthermore, when the perfusate calcium concentration is increased on the fetal side of a perfused placenta, the rate of active transport is not decreased in the perfused guinea pig placenta (433). This means that hypercalcemia in the fetus does not suppress placental calcium transport, which implies that placental calcium transport is regulated independently of the fetal blood calcium. With placental calcium transport being nonsuppressible by fetal hypercalcemia, this may explain why maternal hypercalcemia can have adverse consequences on fetal parathyroid function (see sect. XII, A, B, and G).

The impact of maternal hypercalcemia and hypocalcemia have on fetal bone metabolism in intact fetuses contradicts the normal placental calcium transport results obtained in the study of isolated, perfused placentas of sheep and rats. The problem may be that the placental perfusion model is not sensitive enough to detect a small reduction in placental calcium transport, one that may be sufficient over the course of gestation to cause fetal secondary hyperparathyroidism and skeletal resorption. Alternatively, the absence of the fetus may lead to an artifactually normal result that does not reflect what occurs when the fetus remains in control of placental function.

When the mother is hypocalcemic, placental mechanisms may upregulate to extract the needed mineral from a lower maternal concentration. Consistent with this, when a maternal calcium infusion was administered to pregnant rats, it caused an acute and marked rise in the serum calcium of fetuses whose mothers were hypocalcemic from thyroparathyroidectomy, but caused no increase in serum calcium of fetuses from normal pregnant rats (106).

The effect of maternal hormone deficiencies on placental mineral transport has also been inferred by determining the net skeletal calcium content of vitamin D-deficient rats (228), thyroidecomitized, thyroxine-supplemented (“calcitonin-deficient”) sheep (38, 163), and sheep that received daily administration of prolactin or bromocriptine (39). In each study, the fetal skeleton had normal mineral content, implying that placental mineral transport had been normal in utero. The lack of an effect of maternal vitamin D or calcitonin deficiency has been confirmed by direct measure-
ment of placental calcium transport in Vdr null and Ctcgrp null mothers compared with their WT sisters, as discussed below in sections VIII B and IX B (342, 430).

A competitive but weak PTHrP antagonist, PTHrP<sup>7–34</sup>, was infused via osmotic mini-pumps into the circulation of pregnant rats between day 8 and 15 of gestation and was found to decrease fetal weight, inconsistently reduce placental weight, and to increase the number of apoptotic cells within the placenta (677). The investigators inferred that administration of PTHrP<sup>7–34</sup> causes fetal-placental growth restriction, but these findings have not been independently replicated. Since the Pthrp null fetal mouse does not show evidence of growth restriction or an alteration in placental size or weight (62, 340), and PTHrP<sup>7–34</sup> is unlikely to cross the placenta, these purported actions of PTHrP<sup>7–34</sup> must be exerted on the maternal side of the placenta.

B) HUMAN DATA. There are, of course, no direct measurements in human babies of placental mineral transport. However, there are indirect indications that placental calcium transport may be altered when maternal serum calcium is significantly increased or decreased. Maternal hypercalcemia due to primary hyperparathyroidism or familial hypocalciuric hypercalcemia causes suppression of the fetal parathyroid glands that can last for months after birth or be permanent (54, 79, 393, 417, 493, 529, 609, 674). Maternal hypercalcemia likely increases the flow of calcium across the placenta, thereby suppressing the fetal parathyroids. Conversely, maternal hypocalcemia caused by hypoparathyroidism or pseudohypoparathyroidism has been associated with intrauterine fetal hyperparathyroidism, skeletal demineralization, intrauterine fractures, and bowing of the long bones (226, 389, 652, 710). This is compatible with reduced placental calcium transfer that prompts secondary hyperparathyroidism to develop in the fetus.

C) SUMMARY OF KEY POINTS. There is no direct evidence that maternal hormones regulate placental mineral transport. Study of isolated perfused placentas of sheep suggest that maternal hypocalcemia does not reduce placenta calcium transport. However, the functional outcomes in animals and humans (hypoparathyroidism in fetuses from hypercalcemic mothers, and secondary hyperparathyroidism in fetuses from hypocalcemic mothers) indicate that significant disturbances in maternal serum mineral levels must alter placental mineral transport despite failure of the perfused placentas to demonstrate it.

G. Endochondral Bone Formation, Mineralization, and Remodeling
1. Animal data

The details of fetal skeletal formation are well beyond the scope of this review; instead, a brief overview is provided to supply context for the subsequent sections that describe specific skeletal actions of the calciotropic and phosphotrophic hormones.

Patterning of the early embryonic skeleton has been studied in most detail within the mouse. Numerous genes and signaling pathways are required, include Hox genes, Wnts, Hedgehogs, bone morphogenetic proteins, fibroblast growth factors, Notch/Delta, and others (751). Mesenchymal cells are first laid down where bones of the axial and appendicular skeletons will form. In a few places, such as the flat bones of the skull, these mesenchymal cells differentiate directly into osteoblasts that then form intramembranous bones. But most of the skeleton forms through the multistep process of endochondral bone formation, in which mesenchymal progenitors differentiate first into chondroblasts and chondrocytes. A relatively avascular cartilaginous template of each bone is formed, with proliferating chondrocytes at each end that lengthen the template away from the center. The proliferating and early differentiating cells respond to growth factors and hormonal signals such as PTHrP, which prevent chondrocytes from terminally differentiating. As the distance increases from the zone of proliferating chondrocytes, older chondrocytes no longer receive the signals that prevent them from undergoing terminal differentiation, so they become hypertrophic and then undergo apoptosis. Dead chondrocytes and matrix are removed by chondroclasts, new blood vessels form, and osteoblasts begin to lay down the primary spongosia of bone. Primary ossification centers are present in most endochondral bones of the mouse at term (an exception being small bones within the digits), while most secondary ossification centers develop after birth.

It is not until primary ossification centers have formed that the skeleton begins to accrete substantial mineral content; simultaneous with this, the placenta ramps up the expression of calciotropic genes and the rate of mineral delivery. As previously mentioned, the fetal rat skeleton accretes 95% of its mineral content during the last 5 days of a 22-day gestation (121).

While the net flow of mineral is into bone to support bone modeling, some turnover or remodeling of the newly formed bone occurs in utero, which allows calcium to reenter the circulation and maintain the serum calcium level. This concept is supported by the presence of osteoclasts and expression of osteoclast-specific genes within fetal bones (336, 359) and the presence of bone resorption markers in serum and amniotic fluid (338). As described in detail in sections IV A and VI A, Pthrp null and Pth1r null fetuses are hypocalcemic, which may be due in part to loss of normal skeletal responsiveness or bone turnover. In support of this, expression of a constitutively active PTH1R (Jansen receptor) within the endochondral skeleton increased the fetal serum calcium, including in double-mutants that also...
lacked Pit1 (330, 596). Furthermore, the fetal skeleton can undergo increased osteoclast-mediated resorption in response to such factors as maternal hypocalcemia (104, 377) or an inactivating mutation of CaSR (338), and present with an undermineralized skeleton or fractures at birth.

2. Human data

In human fetuses, a complete cartilaginous scaffold of the endochondral skeleton forms by the 8th week of gestation. Primary ossification centers form in vertebrae and long bones between the 8th to 12th wk, but most mineralization does not occur until the third trimester when 80% of the ash weight and mineral content is accreted (121, 224, 683, 733, 734). Secondary ossification centers form in the femurs by the 34th week. The rate of mineral accretion accelerates from ~60 mg/day at week 25 to >300 mg/day from the 35th through 38th weeks, before tapering off slightly in the last 2 wk (635, 734, 735, 761). Normal skeletal turnover likely contributes to maintaining the high level of calcium within the fetal circulation, and skeletal resorption will increase in response to severe maternal hypocalcemia caused by hypoparathyroidism.

3. Summary of key points

The skeleton is developing and mineralizing during fetal development, but still participates in the normal regulation of serum mineral levels, and will be adversely resorbed if the mineral supply from the placenta is insufficient.

III. OVERVIEW OF NEONATAL MINERAL HOMEOSTASIS

The alterations in circulating concentrations of minerals and hormones that occur during the neonatal period are depicted in Figure 2. This section describes the changes in function and roles that are invoked at birth in parathyroids, kidneys, intestines, and endochondral skeleton.

A. Serum Mineral Concentrations

1. Animal data

The regulation of bone and mineral metabolism changes significantly after birth. Severing the umbilical cord means that the placental mineral pump is lost, and the neonate must initiate mechanisms to absorb mineral from the intestines and reabsorb mineral in the kidney tubules. The onset of breathing induces a rise in pH from relatively acidic levels in utero, and this will contribute to a fall in the ionized calcium.

The serum calcium and ionized calcium fall 40% during the first 6–12 h after birth in rodents (201, 347). Total calcium then rises steadily to reach adult levels by 7–14 days after birth (201, 422). Ionized calcium displays a bimodal pattern, increasing to ~80% of the high fetal value during the subsequent 12–36 h, and then declining to normal adult levels over the subsequent 7 days (422). Albunin increases steadily such that the albunin-bound fraction will account for ~50% of the serum calcium, in contrast to only 20% of the total during fetal life (422). Neonatal lambs differ significantly by maintaining a high serum calcium, similar to the fetal value, until 3–4 mo of age (94, 201).

Phosphorus follows a similar time course in rodents, dropping significantly in the first 2–4 h after birth, increasing significantly by 6 h after birth, and declining again over the subsequent day (201). The early decline is consistent with the loss of the placental phosphorus infusion, while the subsequent rise corresponds to a transient hypoparathyroid state of the newborn. Conversely in lambs, and similar to what occurs with calcium, serum phosphorus increases over the first 24 h and stays elevated for at least a week (403).

2. Human data

After birth, the decline in serum and ionized calcium are typically 20–30% over the first 12–24 h (32, 143, 387, 594). In one study, the ionized calcium fell from the mean umbilical cord level of 1.45 mM to a mean of 1.20 mM by 24 h after birth (387). After that, the serum and ionized calcium follow a pattern similar to the rodent data, achieving normal adult values after several days. A single study reported that delivery by elective C-section resulted in a lower blood calcium and higher PTH levels at birth compared with babies delivered vaginally (31). Most studies have not distinguished mode of delivery among the neonatal blood values. The use of formula or breast milk influences the postnatal time course of calcium as well. The fall is greater in infants fed formula (32, 686) because the high phosphorus content of formula causes hyperphosphatemia and complexes calcium within the circulation.

Phosphorus rises over the first 24–48 h after delivery (32, 582, 594); after that, it declines toward adult values, consistent with resolution of transient hypoparathyroidism in the newborn.

3. Summary of key points

The early neonatal period in humans and rodents is characterized by a significant fall in ionized and total calcium and a rise in phosphorus over the first 12 h, which largely corrects over the succeeding 24 h.
B. Calciotropic and Phosphotropic Hormone Concentrations

1. Animal data

In newborn rats, PTH increases markedly by 6–12 h after birth (199, 675), with the peak level corresponding to the trough level in serum calcium (675). After that, PTH declines toward adult values over the subsequent 2 wk (199, 675).

Calcitriol does not increase until during the third week after birth (near weaning); during that time, 24-hydroxylase actively produces the inactive metabolite 24,25-dihydroxyvitamin D while little or no calcitriol is produced (201, 729). Similar findings were found in newborn lambs up to 11 days after birth with preferential catabolism of 25-hydroxyvitamin D and no production of calcitriol, not even in response to PTH administration (201, 326). These studies suggest that the neonatal kidney is initially resistant to PTH and sluggish in its ability to produce calcitriol.

PTHrP becomes undetectable in the adult circulation, but exactly when this occurs after birth has not been rigorously determined. The placenta and extraembryonic membranes are sources of PTHrP secretion that are lost at birth; if these are the main sources, then a prompt fall in PTHrP may be another trigger that causes the parathyroids to upregulate PTH synthesis and secretion. However, neonatal rat pups were found to have persistently high PTHrP1–34 concentrations, in the range of 20–40 pM (401). Neonatal lambs also have persistent PTHrP immunoreactivity in the parathyroid glands, and serum bioactivity attributable to PTHrP, over the first several months after birth (94, 396); however, circulating PTHrP measurements were not done to confirm this. Data cited earlier from fetal lambs indicated that the parathyroid gland may be a key source of PTHrP; the persistently elevated serum calcium and bioactivity attributable to PTHrP in neonates may be an indication of persistent secretion of PTHrP by the parathyroids.

Milk contains very high concentrations of PTHrP which, if absorbed into the neonatal circulation, could also explain persistently high circulating levels of PTHrP. Neonatal goats that consumed mother’s milk had significantly higher PTHrP compared with kids that received only formula, which may imply that PTHrP had been absorbed into the circulation (559). No study has examined a time course of PTHrP levels in neonatal blood after suckling, or used radiolabeled PTHrP in milk to determine if it reaches the neonatal bloodstream.

Scant data are available pertaining to serum FGF23 levels soon after birth. In WT mice, FGF23 was 10-fold higher than adult values at 12 h after birth (34).

Calcitonin increases during the newborn period in rats (209), calves (201), and foals (201, 210). In the neonatal lamb it increases and persists for at least 6 wk after birth before falling to adult levels (204, 215).

There is a surge in testosterone level of neonatal rats and mice within the first 2–3 h after birth, which rapidly declines to baseline by 6 h (124, 461). After this, testosterone is equivalent between male and female mice for the rest of the newborn period (498, 499, 730). Estradiol is also low in both sexes (498).

2. Human data

The postnatal time course of PTH was originally delineated using older COOH-terminal assays, and it was found that PTH remained low over the first 12–24 h, and did not reach peak levels until 48 h or later (32, 143, 177, 595, 598, 685). Subsequent studies used intact PTH assays but obtained measurements solely at birth and 24 h later, and demonstrated that PTH rose significantly (31, 386, 388, 416, 444, 473, 580). Those studies did not determine when the PTH value increased or whether the peak level was achieved at 24 h or later.

Calcitriol is low in cord blood and increases to adult values during the first 2 days after birth, likely in response to the rise in PTH (144, 642). A similar increase to peak levels by the fourth day after birth occurs in preterm infants supplemented with vitamin D (231, 582).

It is not known whether the neonatal parathyroids synthesize PTHrP, or how soon after birth the circulating PTHrP level becomes undetectable. A single study reported that the plasma PTHrP1–34 level was reduced 50% between birth and day 1 in healthy newborns, but it then increased back to the cord blood level by day 2 (72). As in the animal models, human milk is a very potent source of PTHrP that could support the circulating level if absorbed; by comparison, PTHrP concentrations are low to absent in infant formulas (306, 392, 488). The breast milk content of PTHrP doubled between 2 and 10 days after delivery, but serum PTHrP was very low in the neonates and did not change between those time points (678). The investigators concluded that PTHrP was not absorbed from milk; however, the blood collections were not timed to breastfeeding, and the authors did not consider that PTHrP has a short half-life in blood. Moreover, the assay requires plasma, but the authors measured PTHrP1–86 in stored sera, so the low PTHrP values may reflect the inevitable degradation of PTHrP in sera, and use of an assay that does not detect PTHrP1–34.

Intact FGF23 increased fourfold between birth and 4–5 days of age (483, 661), whereas COOH-terminal FGF23 maintained the high values of fetal life (661).
Calcitonin may increase up to 10-fold over cord blood levels during the first 48 h after birth (32, 53, 444, 595), before gradually declining over the subsequent days or weeks (32, 583).

Estradiol remains very low in male and female newborns and stays that way until puberty (91, 739). Testosterone remains very low in females, but in males it surges within the first 24 h (124), and again in the second week after birth until about 4 mo of age, after which it declines to very low levels and remains that way until puberty (133, 739).

3. Summary of key points
Calciotropic hormones levels rapidly progress from fetal values to levels that are similar to the adult (FIGURE 2). PTH, calcitriol, and intact FGF23 increase; PTHrP disappears from the circulation after an uncertain time course; and calcitonin may stay elevated for several weeks.

C. Neonatal Parathyroid Function

1. Animal data
The parathyroids are important for control of serum mineral concentrations in the neonate. Parathyroidectomy in the newborn rat pup caused a fall in serum calcium and an increase in serum phosphorus (199, 344). Notably the length, morphology, trabecular content, and calcium and phosphorus content of the neonatal skeleton were unaffected by parathyroidectomy in the neonate compared with sham controls, indicating that the development of the skeleton and accretion of mineral is not dependent on PTH during this time period (344). Whether the neonatal parathyroids produce PTHrP is uncertain, but suggestive evidence for it comes from neonatal lambs which maintain increased serum calcium and high PTH-like bioactivity, similar to what is present during fetal development.

2. Human data
The increase in PTH follows the early postnatal drop in the ionized calcium, and precedes (and is likely responsible for) the subsequent rise in ionized calcium and calcitriol, and decline in phosphorus. Consistent with the concept that the neonatal parathyroids recover from suppression experienced during fetal development, in the 48 h after birth the parathyroids are sluggish in their response to acute hypocalcemia, such as due to exchange transfusion with citrated blood (143, 595, 685, 686). This sluggishness resolves with increasing postnatal age (685, 686) but becomes prolonged after maternal hypercalcemia during pregnancy (see sect. XII, A and B).

3. Summary of key points
A transient phase of relative hypoparathyroidism or sluggish responsiveness by the parathyroids occurs during the first 24 h after birth and contributes to the development of postnatal hypocalcemia and hyperphosphatemia. The postnatal awakening of parathyroid function explains the subsequent increase in calcium and calcitriol, and decline in serum phosphorus.

D. Renal Mineral Reabsorption and Excretion

1. Animal data
The neonatal kidneys begin to actively reabsorb and excrete minerals. Over the first 24 h there is reduced cAMP responsiveness to PTH in vitro compared with the response obtained during fetal life (675). The rat kidney does not show expression of VDR or responsiveness to calcitriol until the third week after birth (633). TRPV6 and calbindin-D9k show a progressive increase in expression, reaching a maximum at 3 wk after birth (634). There is low level of expression of CaSR at birth that increases after the first postnatal day in mice (112).

2. Human data
Urine calcium excretion is low at birth despite the high level of serum calcium in cord blood and the suppressed PTH level. After the first 5–7 days, urine calcium excretion increases over the subsequent weeks despite a lower serum calcium and higher PTH concentration (298, 473). The change in renal calcium excretion is likely due to the developmental increase in renal blood flow and glomerular filtration rate that occurs over the same interval (171, 237, 298). Additional factors include loss of the effects of PTHrP to reabsorb calcium, increased expression of CaSR, and increased responsiveness to calcitriol.

Phosphorus excretion is also low at birth and increases with postnatal age (378, 605). This likely reflects not only the increasing level of PTH in the circulation, but (as in the rodent) a developmental increase in renal responsiveness to PTH that has been demonstrated in term and preterm infants (378, 406).

The rapid rise in calcitriol after birth likely arises from synthesis in the kidneys, which are stimulated by PTH.

3. Summary of key points
The neonatal kidneys undergo developmental changes that progressively increase their responsiveness to PTH, calcitriol, and CaSR. Sluggish kidney function over the first several days after birth likely contributes to the development of neonatal hypocalcemia.
E. Intestinal Mineral Absorption

1. Animal data

In newborn rodent pups, calcium absorption occurs mainly through passive, nonsaturable processes that are not dependent on calcitriol (218, 219, 243, 510–512). Milk contains a high content of lactose, which directly increases paracellular calcium absorption and net absorption of dietary calcium (86, 366, 500). As the days pass, intestinal cells begin to express VDR (242), increase their expression of calbindin-D9k (80, 634) and TRPV6 (634), and display evidence to express VDR (242), increase their expression of calbindin-D9k and TRPV6 have reached maximally upregulated expression compared with the day of birth (634).

2. Human data

As noted earlier, preterm human infants demonstrate calcium absorption that is proportional to intake, with a linear relationship between intake and absorption; this is consistent with passive absorption of calcium (44, 46, 221, 581). Absorption did not vary by vitamin D intake in preterm infants, which is also consistent with passive, non-vitamin D-dependent absorption (75). An interventional study found that preterm infants showed no calcemic response to short courses of supraphysiological doses of calcitriol (0.1–3.0 μg/kg), which suggests that the preterm intestines are refractory to calcitriol (708). For perspective, the adult requirement in hypoparathyroidism is typically a total daily dose of 0.25–1.0 μg. A second interventional study used 4.0 μg/kg of calcitriol administered intravenously for three consecutive days and found a significant increase in serum calcium (325). However, that effect was not the result of an alteration in intestinal calcium absorption but likely reflected an increase in bone resorption: oral intake was minimal, osteocalcin increased significantly, and urine calcium/creatinine ratio did not change (325).

A small study of preterm neonates compared human milk, human milk plus 1,200 IU vitamin D, and human milk plus 1,200 IU vitamin D and phosphorus, and found that only the group receiving phosphorus supplementation had a significant increase in calcium retention (603). This is also consistent with a lack of effect of vitamin D/calcitriol to increase calcium absorption and retention in the preterm intestines. The preterm infant is also limited by reduced intestinal absorption of vitamin D that increases with postnatal age (263). Another study supplemented the preterm neonate with 1,200 IU vitamin D or 0.5 μg calcitriol, both of which increased intestinal absorption of calcium but required up to 4 wk to achieve the full effect, with use of calcitriol being more effective than vitamin D (604).

Similar to the data from rodents, calcium absorption in neonates is enhanced by the lactose content of milk (317, 318). Intestinal absorption and retention of calcium increase with both postnatal age and gestational age, with additional variations superimposed by the composition of the milk (45, 221, 256, 485, 604, 613). These findings suggest a maturation effect toward an active, calcitriol-dependent, saturable mechanism of intestinal calcium absorption as seen in older children and adults. It may be inferred from these data that, similar to the rodent intestine, the human neonate’s intestines undergo progressive upregulation in expression of the vitamin D receptor. However, longitudinal changes in expression of VDR have not been studied in human neonatal intestines. Similarly, it is unclear when the neonatal intestines change from passive to active absorption, although the responsiveness to calcitriol at 4 wk of age in one clinical study may be an indication that active absorption has become more prominent by that age (604).

The preterm infant must supply a high rate of calcium to the skeleton as if it were in utero, but this exceeds what can be achieved with breast milk or standard infant formulas. In the first week after birth, most nutrition is provided parenterally because the intestines are not able to absorb a sufficient amount (445). As oral feeds are implemented, special formulas high in calcium are needed to meet the preterm neonate’s need for mineral. In the very low birth weight preterm neonate, a daily calcium intake of 120–200 mg/kg is usually recommended with much of that being given parenterally (445). As the postnatal days pass for the preterm baby, the skeletal demand for mineral declines from the high fetal rate and can eventually be met with breast milk or formula.

The efficiency of intestinal calcium absorption is influenced greatly by the nutrition that the neonate receives. Absorption of calcium is more efficient from breast milk than from infant formula regardless of vitamin D supplementation (223, 604, 732). More than 90% of the phosphate content of breast milk and formula is absorbed (604), but the higher phosphate content of formula can lead to double the serum phosphorus level compared with neonates that consume breast milk (32, 223, 604). High serum phosphate lowers serum calcium and is a cause of neonatal hypocalcemia (686). The differences between breast milk and formula have had clinical effects, with breast-fed infants having higher serum calcium levels and a lower risk of hypocalcemia compared with formula-fed infants (32, 223).

3. Summary of key points

A developmentally programmed maturation alters intestine calcium absorption from passive, nonsaturable mechanisms to an active, calcitriol-dependent process. This has been well established in the neonatal rodent, but there is sufficient evidence to accept that it probably occurs in human
neonates as well. This explains why preterm babies are dependent on passive absorption of mineral until the intestines are capable of responding to calcitriol.

F. Skeletal Metabolism

1. Animal data

The efficiency of intestinal mineral absorption has significant effects on the neonatal skeleton. Neonatal rodents are largely protected from abnormalities due to the effects of lactose to enhance absorption of calcium. Consequently, despite significant abnormalities such as hypoparathyroidism, vitamin D deficiency, or loss of VDR, the neonatal rodent maintains normal bone mineralization until the time of weaning. It is at that point in time, when the intestines are dependent on calcitriol-regulated active intestinal calcium absorption, that the skeleton begins to develop deficits in mineral content and altered development (see sects. VD and VIII).

Normal peak bone density and skeletal calcium content differ markedly among mouse strains (48), with the commonly used inbred C57BL/6 strain having a significantly lower bone mass than the outbred Black Swiss strains (192, 309, 310, 744). Serum and ionized calcium are also significantly lower in C57BL/6 compared with Black Swiss mice (340), but there are no obvious differences in calciotropic and phosphotropic hormone levels between the two strains. Unidentified genes and proteins that affect bone metabolism and development likely explain this interstrain variability.

2. Human data

The late term fetus accumulates skeletal calcium at a rate of over 300 mg/day or \( \sim 150 \text{ mg·kg}^{-1}\cdot\text{day}^{-1} \) (635, 734, 735, 761), whereas this drops to \( \sim 30–40 \text{ mg·kg}^{-1}\cdot\text{day}^{-1} \) in the normal neonate (683). The skeleton is highly dependent on the intestines to supply needed mineral; any deficiency in the diet or in the ability to absorb mineral will cause impaired bone mineralization and increased resorption of the skeleton. This is especially true for the preterm neonate for whom breast milk or standard formula would provide insufficient amount of calcium. However, the ability of the intestines to passively absorb mineral in proportion to intake enables special formulas that are high in calcium to provide the needed mineral, after an initial phase in which most of the nutrition is administered parenterally.

A consequence of inadequate intestinal delivery of calcium and phosphorus is rickets of prematurity. This is not due to vitamin D deficiency because it is not prevented by supplementation of the mother during pregnancy, nor is it responsive to treatment of the neonate with vitamin D or calcitriol (10, 92, 323, 432, 510). It is precipitated by loss of the placental calcium pump at a time when skeletal demand for calcium is very high, normal oral calcium intake is insufficient to meet the demand, and the intestines are developmentally immature, and likely lack expression of VDRs, to deliver calcium through active absorption (92, 510). Bone mineral content is initially appropriate for gestational age at birth, but it fails to increase appropriately if left untreated (235, 236, 324, 446, 640, 641, 690). Physical and radiological signs of rickets of prematurity may develop, including rachitic rosary, chest deformity, osteopenia, pathological (especially rib) fractures, metaphyseal stippling, and flaring and widening of the epiphyses of long bones (92, 513, 514). Special oral formulas or parenteral preparations containing high calcium and phosphorus content will correct the bone demineralization and allow the skeleton to develop normally (10, 92, 323, 432, 641, 643). Follow-up at 3–4 yr of age has shown normal bone mineral content in children who earlier had rickets or low bone mineral content of prematurity (266).

3. Summary of key points

The neonatal skeleton accrues mineral at a rapid rate and but will quickly become demineralized if intestinal mineral delivery is inadequate. Infants that are preterm, severely vitamin D deficient, lack calcitriol or the VDR, or are hypoparathyroid/aparathyroid, are especially prone to this complication.

IV. PARATHYROID HORMONE-RELATED PROTEIN

In the decades that followed the development of specific PTH assays, it was recognized that fetal blood contains high PTH-like bioactivity but low immunoreactive levels of PTH (3, 20, 21, 67, 571). PTHrP was cloned in 1987 (410, 646, 658), and subsequent work confirmed that it mimics many of the biological actions of PTH (333), circulates at high levels in fetal blood compared with PTH (168, 306, 600, 671), and accounts for the high PTH-like bioactivity in fetal blood (7, 94, 396). The relative difference in circulating levels of PTHrP and PTH may even be underestimated because the commonly used PTHrP\(^{1–86}\) assay does not detect biologically active PTHrP\(^{1–36}\). These data led many to hypothesize that PTHrP is the main calcium-and-bone-regulating hormone of fetal life, and that the suppressed levels of PTH indicate that it is relatively unimportant until after birth. Given this context, PTHrP’s role in fetal and neonatal mineral metabolism will be discussed first, followed by PTH in section V.

A. Regulation of Serum Minerals

Comparison of Pthrp null fetuses to their WT and Pthrp\(^{+/-}\) littermates, which share the same intrauterine environment,
has confirmed the importance of PTHrP in regulating aspects of fetal mineral and bone metabolism. PthrP null fetuses have a hypoparathyroid-like phenotype characterized by hypocalcemia (both total and ionized levels), and a serum phosphorus that is raised even further above the normally high fetal phosphorus level (62, 340, 341, 694). Serum magnesium remains normal (330). Notably the fetal ionized calcium falls to a level equal to the maternal ionized calcium (340, 694). Serum PTH increases threefold in PthrP null fetuses (336), likely through CaSR-mediated effects to increase serum calcium to the normal adult level but no higher. This conclusion is supported by additional observations in double-mutant fetuses. When one or both alleles of CaSR are inactivated in PthrP null fetuses (i.e., $PthrP^{+/+}$ fetuses and $PthrP/Casr$ double mutants), serum PTH and ionized calcium levels increase significantly (338). However, when the NH$_2$-terminal actions of PTH and PTHrP are blocked by deletion of PTH1R ($Pth1r$ null fetuses), simultaneous ablation of one or both alleles of CaSR does not alter the ionized calcium (i.e., $Pth1r$ null/Casr$^{+/+}$ fetuses and $Pth1r$/Casr double mutants) (338).

Section V that follows notes that hypocalcemia occurs in several models lacking PTH or parathyroids, but within each there is no compensatory increase in plasma PTHrP (341, 623). Therefore, PTHrP is unresponsive to falls in the ambient fetal blood calcium, which may imply that it is autonomously produced and regulated by, and in response to, local factors within the placenta such as the flow of calcium.

The source of PTHrP in the fetal circulation cannot be determined from the $PthrP$ null model because PTHrP is absent in all fetal tissues.

### B. Regulation of Placental Mineral Transport

Several lines of evidence indicate that PTHrP is an important regulator of the active transport of calcium and magnesium across the placenta from mother to fetus.

Fetal lambs were thyroparathyroidectomized and treated with thyroid hormone; several days later, the fetus was removed from the uterus and its placenta was studied using the in situ placental perfusion model (see details in section II3). The rate of increase in the concentration of $^{45}\text{Ca}^{51}\text{Cr}-\text{EDTA}$ in the umbilical vein portion of the circuit (placental outflow) was significantly reduced after prior fetal thyroparathyroidectomy, compared with the rate observed in placenta from separate ewes whose fetuses had been intact (97, 723). Furthermore, infusion of autologous blood from intact fetal lambs (sometimes the twin from the same ewe) restored the rate of increase in $^{45}\text{Ca}^{51}\text{Cr}-\text{EDTA}$ to normal values (97). These results showed that prior fetal thyroparathyroidectomy reduced the basal rate of placental calcium transport when measured several days later but, importantly, in the absence of the fetus which had been removed hours before. The investigators concluded that fetal parathyroids produce a factor that stimulates placental calcium transport, such that its absence causes the rate of transport to fall.

The same investigators went on to infuse specific peptide sequences of PTHrP or PTH into the umbilical artery, to determine their effects on placental calcium transport. Peptides that contained a mid-region portion of PTHrP ($\text{PTHrP}_{1-141}$, $\text{PTHrP}_{1-86}$, and $\text{PTHrP}_{67-86}$) increased the rate of change in the concentration of $^{45}\text{Ca}^{51}\text{Cr}-\text{EDTA}$ in the umbilical vein, whereas $\text{PTHrP}_{1-34}$ and $\text{PTH}_{1-34}$ had no effect (6, 96, 556). When $\text{PTHrP}_{1-34}$ was later determined to be the structure of mid-regional $\text{PTHrP}$ (490, 517), these investigators repeated the experiments and confirmed that $\text{PTHrP}_{1-34}$ also significantly increased the rate of placental calcium transport in perfused placentas from previously thyroparathyroidectomized fetal lambs (745). This approach has also been used to show that mid-molecular PTHrP stimulates placental magnesium transport, but not phosphate transport (43, 47, 96, 653).

The conclusion of these experiments was twofold: 1) that PTHrP stimulates placental calcium and magnesium transport and 2) that the relevant source of PTHrP is the fetal parathyroids. However, no measurements of plasma PTHrP were done in thyroparathyroidectomized lambs, so it remains unknown whether fetal parathyroidectomy reduced the circulating level of PTHrP.

The in situ placental perfusion model has also been used in rats, and at first glance the evidence obtained seems inconsistent with the data obtained from perfused placentas of lambs. Decapitation was done to crudely mimic parathyroidectomy, and (similar to the effects of thyroparathyroidectomy in fetal lambs) this resulted in a fall in placenta calcium transport (554). That deficit in placental calcium transport was increased slightly (but remained well below the normal value) after infusion of PTH$_{1-34}$ (554); no PTHrP peptides were tested in the decapitation model. However, in perfused placentas from intact fetuses, PTH$_{1-34}$, PTHrP$_{1-34}$, and PTHrP$_{67-86}$ each had no significant effect on placental calcium transport (554, 611). These data do not contradict the findings in ovine placentas because mid-molecular PTHrP was not tested in the decapitated fetal rat model, and the efficacy of PTH$_{1-34}$ in the decapitation model was very modest compared with that of mid-molecular PTHrP in placentas from thyroparathyroidectomized fetal sheep. Furthermore, the failure of mid-molecular PTHrP to stimulate placental calcium transport in placentas from intact rats may be the result of PTHrP-mediated effects already being maximally exerted. Data described in the next paragraph corroborate this conclusion.
The creation of Pthrp null fetuses (297) enabled a different approach to measure placental calcium transport within intact fetuses using the methodology described in section IIF3. $^{45}$Ca and $^{51}$Cr-EDTA were administered by intracardiac injection to pregnant mice, and the intact fetuses were removed 5, 15, or 30 min later. Compared with their WT and Pthrp$^{+/−}$ littermates, $^{45}$Ca/$^{51}$Cr-EDTA accumulation in Pthrp null fetuses was 25% lower after 5 min and 40% lower after 30 min (340). To confirm that this reduction was specific to loss of PTHrP, a laparotomy was done to administer an intra-abdominal injection of a specific peptide form of PTHrP, PTH, or diluent to several fetuses. Sixty minutes later, $^{45}$Ca and $^{51}$Cr-EDTA were administered to the mother by intracardiac injection, and the fetuses were removed 30 min after that. PTHrP$^{1−86}$ and PTHrP$^{67−86}$ increased the accumulation of $^{45}$Ca/$^{51}$Cr-EDTA to a value not different from WT littermates, whereas PTH$^{1−34}$ and PTHrP$^{1−34}$ had no significant effect (340). Importantly, injections of mid-molecular PTHrP had no effect in WT fetuses, which suggests that the WT fetus may already have maximally stimulated PTHrP responses on the placenta, such that pharmacological treatment had no additional effect. This is consistent with the data cited in the preceding paragraph, in which mid-molecular PTHrP failed to stimulate placental calcium transport in placentas from intact rats.

These results from intact Pthrp null fetuses, and the aforementioned data from in situ perfused placentas from thyroparathyroidectomized fetal lambs, both showed that mid-molecular forms of PTHrP stimulate placental calcium transport, whereas peptides of PTHrP that contain only the NH$_2$-terminal form, and PTH itself, have no significant effect. Analysis of Pthrp null placentas revealed reduced mRNA and protein levels of Ca$^{2+}$-ATPase and calbindin-D$_{9k}$ within the intraplacental yolk sac compared with placentas from WT littermates (337), which may indicate that PTHrP acts in part through the stimulation of Ca$^{2+}$-ATPase and calbindin-D$_{9k}$ expression. Alternatively, the reductions in Ca$^{2+}$-ATPase and calbindin-D$_{9k}$ expression could be a consequence of reduced placental calcium transport rather than its cause. That these reductions in Ca$^{2+}$-ATPase and calbindin-D$_{9k}$ expression may be physiologically significant is supported by Trpv6 null fetuses, which have markedly reduced placental calcium transport, and loss of Trpv6, Ca$^{2+}$-ATPase, and calbindin-D$_{9k}$ expression within the intraplacental yolk sac (659). Notably, the reduction of Ca$^{2+}$-ATPase and calbindin-D$_{9k}$ expression within the Pthrp null placenta was limited to the intraplacental yolk sac which normally expresses PTHrP (337). No difference in Ca$^{2+}$-ATPase and calbindin-D$_{9k}$ mRNA or protein expression was found within the adjacent trophoblasts or when the entire placenta was analyzed by Western blot (62, 337).

The results described so far are consistent in revealing a role for PTHrP to stimulate placental calcium transport in fetal lambs and mice. Contrary evidence comes from the in situ placental perfusion model, miniaturized for use in fetal mice (see details in sect. IIF3) (61, 62). A fetal mouse was removed, and nitroglycerin was used to dilate the placental vasculature before cannulating the umbilical vessels and performing the placental perfusion technique. $^{45}$Ca was administered alone, and a single sample was later obtained from the maternal circulation and the placental circuit. With the use of this technique, Pthrp null placentas had an increased rate of placental $^{45}$Ca transport compared with perfused WT placentas from separate mothers. The investigators concluded that PTHrP inhibits rather than stimulates placental calcium transfer.

There are several possible reasons why the results of these experiments were the opposite of what the prior studies found in lambs and mice had revealed. The likeliest is that the use of nitroglycerin to dilate the placental vasculature may have created an artifactual result when the vascular expression and actions of PTHrP are considered. PTHrP is expressed within vascular smooth muscle wherein it has actions to relax smooth muscle and thereby cause vasodilation and increased blood flow (517, 551). PTHrP and PTH share vasodilatory effects when injected into living animals (120, 127, 247, 464, 497), and overexpression of Pthrp or Pth1r within vascular smooth muscle cells of mice leads to lower blood pressure, increased perfusion pressure, and reduced vasoconstriction in response to challenge with angiotensin II (476). When PTHrP was deleted from vascular smooth muscle cells by using a vascular smooth muscle-driven promoter for Cre in Pthrp floxed mice, the mice showed reduced renal perfusion and glomerular filtration, consistent with renovascular constriction (533). Therefore, a similar increase in vascular constriction and reduced blood flow may be present in the Pthrp null placenta compared with the WT and Pthrp$^{+/−}$ placentas. Consequently, nitroglycerin may have differential vasodilatory effects in Pthrp null placentas which, combined with placental function being studied well after the fetus has been removed, may have artifactualy increased the apparent transport of calcium across the perfused placenta.
That PTHrP stimulates rather than inhibits placental calcium transport is supported by studies in other mouse models wherein the placental calcium transport technique for intact fetal mice was used. Pth1r null fetuses (360) have greater than 10-fold increases in circulating levels of both PTHrP and PTH (336, 341). They lack PTH1R and so cannot respond to NH2-terminal actions of PTH or PTHrP, but should be responsive to actions of mid-molecular PTHrP on its (as yet uncloned) receptor(s). The placental calcium transport rate was increased in Pth1r null fetuses to 150% of the value of their WT fetal littermates (340), in keeping with anticipated effects of high plasma PTHrP concentrations to increase the rate of placental calcium transport. Aparathyroid Hoxa3 null fetuses and Pth null fetuses each have normal plasma PTHrP concentrations and normal rates of placental calcium transport (336, 341, 623). Casr null mice have significantly reduced plasma PTHrP levels and reduced placental calcium transport (330, 338). The concordance between plasma PTHrP concentrations and placental calcium transport among these different mouse models (Pthrp null, Pth1r null, Hoxa3 null, Pth null, and Casr null fetuses) are consistent with the conclusion that PTHrP’s role is to stimulate placental calcium transport.

Previously the increased fetal blood mineral concentrations were considered to be proof of active transport of calcium across the placenta, and elimination of the maternal-fetal gradients in the relative concentrations of calcium or magnesium (such as by fetal thyroparathyroidectomy) were considered to be evidence that active transport had been eliminated (97, 100, 198). However, study of these murine gene deletion models have revealed that placental calcium transport is not a direct determinant of the fetal blood calcium level or skeletal mineral content. The placenta supplies the mineral which may then be incorporated into bone or soft tissues, remain in the circulation or extracellular fluids, be excreted into the urine and amniotic fluid, or be transported back across the placenta and into the maternal circulation. The blood levels of calcium and incorporation of calcium into bone are regulated separately from the placental calcium transport.

Although the Pthrp null model does not address whether fetal parathyroid glands produce PTHrP or even if parathyroid-derived PTHrP stimulates placental calcium transfer, when considered together with results from Hoxa3 null and Pth1r null fetuses (sects. V and VI), the studies in fetal mice are more consistent with a placental source of PTHrP. The studies in fetal sheep and mice both found that a mid-molecular form of PTHrP stimulates placental calcium transfer, but differ on what its source might be. These seemingly discrepant results between fetal lambs and mice might be due to species difference; there are no comparable human data.

An indirect approach to estimating placental calcium transport was carried out in fetal lambs that received thrice-daily injections of PTHrP1–34 or PTHrP1–86, with or without calcitonin, for a total of 12 days. Each form of PTHrP significantly increased the mineral content of the fetal skeleton compared with control (diluent), and the simultaneous use of calcitonin blocked the effect of PTHrP (40). The authors inferred that both forms of PTHrP stimulated placental calcium transport and that calcitonin countered PTHrP’s actions on the placenta. However, these results can be explained by the effects of both NH2-terminal forms of PTHrP to stimulate bone formation and mineralization, and for calcitonin to inhibit bone resorption (and, secondarily, bone formation), without implicating any direct effect of calcitonin on placental calcium transport.

Magnesium and phosphorus are each actively transported across the in situ perfused placentas of rats (612, 653) and lambs (47, 96, 397). Mid-molecular PTHrP stimulated magnesium transport across the in situ perfused ovine placentas (47, 96, 397) but not rodent placentas (611), and it did not affect placental phosphorus transfer (43, 397, 653). Placental transport of these minerals has not been examined in Pthrp null fetuses.

C. Regulation of Endochondral Bone Formation

PTHrP is produced by perichondrial cells and proliferating chondrocytes, and its receptor (PTH1R) is expressed further down the growth plate, within prehypertrophic chondrocytes, preosteoblasts, and osteoblasts. PTHrP acts in concert with Indian hedgehog and Wnt signaling to delay terminal differentiation and hypertrophy of chondrocytes (300, 714). PTHrP’s role is clearly illustrated by the Pthrp null skeleton, which has premature chondrocyte hypertrophy, apoptosis, and initiation of primary bone formation; the result is a chondrodysplasia with shortened limbs (FIGURE 9) (297, 300, 360). The normally cartilaginous portions of the ribs turn into bone, and bones that normally mineralize after birth become mineralized in utero (297). In contrast to the effects of loss of PTHrP, overexpression of PTHrP or PTH1R...
has the opposite effect of delaying chondrocyte hypertrophy, resulting in a cartilaginous skeleton at birth that slowly undergoes bone formation and mineralization over the succeeding weeks (596, 726).

Although PTHrP’s anabolic actions on preosteoblasts and osteoblasts have been lost, Pthrp null fetuses show normal to increased expression of osteoblast-specific genes and proteins, including collagen α1(I), collagenase-3, osteocalcin, and osteopontin (297, 359). This is likely because the three-fold compensatory increase in circulating PTH (336) stimulates osteoblasts and prevents the deficits in osteoblast function that loss of PTHrP should otherwise have caused. This conclusion is borne out by careful comparison of Pthrp null to Pth1r null fetuses, as described in section VI C. Even though increased PTH seemingly ameliorates the skeletal effects of PTHrP deletion prior to birth, mice that lack PTHrP expression only within preosteoblasts and osteoblasts have reduced bone formation and bone mass by 6 wk of age (438).
In striking contrast to the physiological findings of reduced placental calcium transport and hypocalcemia, the ash weight and mineral content of the Pthrp null skeleton has been variably reported to be normal or modestly increased (FIGURE 9) (62, 336, 340, 694). This seemingly contradictory finding is likely explained by the early, accelerated, and abnormal calcification that occurs within the Pthrp null skeleton. As described above, bones that normally do not mineralize until after birth become prematurely mineralized in utero; also, normally cartilaginous structures (such as the medial aspects of the ribs and the sternum) are transformed into bone (297). Consequently, the Pthrp null skeleton matures faster and accretes more mineral sooner, and in normal and abnormal locations, thereby leading to a seemingly normal or increased mineral content despite its slightly smaller size and reduced rate of placental calcium transport.

Complete loss of PTHrP causes death often within minutes of birth, although a few pups have lived for 5 days. The cause of death may be multifactorial, including severe hypocalcemia, with cardiac arrhythmias, a rigid rib cage that restricts ventilation, and stiff lungs due to abnormal development of alveolar type II cells and low surfactant (297, 340, 570).

D. Role in the Neonate

The abrupt fall in serum calcium experienced by most mammals after birth is consistent with loss of placental PTHrP, but there are insufficient data to confirm that PTHrP has been lost from the neonatal circulation. Conversely, neonatal lambs do not experience a fall in serum calcium after birth, but maintain the same high levels that were present during fetal life until 3–4 mo of age. When the calcium level finally declines in lambs, it corresponds to the age when increased PTH-like bioactivity attributable to PTHrP finally subsides (94). This result seemingly indicates that parathyroids of neonatal lambs produce PTHrP for some months after birth.

PTHrP continues to be produced in the growth plates of endochondral bone wherein it delays terminal differentiation of chondrocytes, and within osteoblasts wherein it directs bone formation and mineralization, and inhibits apoptosis. Its importance has been illustrated by several mouse models. Posnatally, Pthrp+/− mice develop modestly accelerated endochondral ossification and deficits in bone microarchitecture and mass (23). When Pth null mice are made haploinsufficient for Pthrp, osteoporogenitor cell recruitment decreases, osteoblast apoptosis increases, bone formation diminishes, and trabecular bone mass is reduced (442). Conditional deletion of Pthrp from osteoblasts leads to an osteoporotic skeleton by the age of maturity (438, 441).

The very high concentration of PTHrP in milk seemingly declares that it is important, but for what physiological purpose? Passive immunization of day-old neonatal mice with antibodies to NH2-terminal PTHrP did not affect serum calcium or the calcium content of their ashed skeletons at 48 h (348), but a longer duration may have been needed to definitively determine if the skeleton would become abnormal. Oral administration of PTHrP1–34 to neonatal mice had no effect, whereas subcutaneously administered PTHrP1–34 increased serum calcium significantly (348); from these data, investigators concluded that PTHrP is not absorbed into the circulation of the neonatal mouse. Deletion of the Pthrp gene from mammary tissue at the onset of lactation resulted in milk that lacks detectable PTHrP, but the growth rate of the pups was qualitatively described as no different than expected (706). Collectively, these results suggest that eliminating PTHrP from milk is of no consequence to the neonate.

However, the same investigators recently examined pups that nursed from mothers in which none, one, or two of the Pthrp alleles had been deleted from mammary tissue at the onset of lactation (409). Deletion of one Pthrp allele reduced the milk PTHrP content, whereas loss of both alleles eliminated PTHrP, but the calcium content of milk was unaltered (409). At day 12, there was an inverse dose-response relationship between the PTHrP content of milk and the ash calcium content of the pups (409), with loss of milk PTHrP leading to increased skeletal calcium. Does PTHrP reduce net intestinal absorption of calcium? That seems unlikely given the previously cited evidence that the calcium content of milk is more readily bioavailable than from formula, and results in a higher serum calcium in neonates. Does some functional portion of PTHrP get absorbed into the neonatal circulation and act on osteoblasts to reduce mineral accretion? Such a pathway could explain how milk consumption might lead to a higher serum calcium but a lower mineral content of the skeleton. It would give PTHrP the evolutionary role of protecting against neonatal hypocalcemia by preventing the skeleton from accreting too much of the mineral in the circulation.

E. Human Data

Possible effects of PTHrP on human trophoblast function or placental calcium transport have been investigated in vitro. Vesicles prepared from fetal-facing basement membranes of syncytiotrophoblasts (32–37 wk of gestation) showed a linear increase in ATP-dependent accumulation of 45Ca within the vesicles (649), which is consistent with a progressively increasing rate of active placental calcium transport in late gestation. Mid-molecular PTHrP38–94 stimulated ATP-dependent uptake of 45Ca calcium in a concentration-dependent fashion starting at the physiological dose of 5 pg/ml (648), while PTH, PTHrP1–34, PTHrP67–86, and calcitonin had only modest effects at pharmacological doses (50 or 250 pg/ml) (648). 45Ca uptake was inhibited by a protein kinase C inhibitor, while PTHrP38–94 increased inositol tri-
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sphosphate production and protein kinase C phosphorylation. PTHrP may also promote trophoblast survival since PTHrP$^{1–34}$ prevented cytrophoblast apoptosis while PTHrP$^{67–86}$ had no effect (128).

In contrast to those findings, other investigators created similar vesicles from maternal-facing and fetal-facing basement membranes of human syncytiotrophoblasts, but with the direction of calcium transport oriented outwards into the media. The vesicles were loaded with $^{45}$Ca and then efflux of the isotope was measured. PTHrP$^{1–34}$ was most potent to increase $^{45}$Ca efflux; PTH$^{1–34}$ required a fourfold higher dose; PTHrP$^{67–94}$ was without effect (178). The NH$_2$-terminal antagonist PTHrP$^{7–34}$ significantly reduced the effect of both peptides. These results suggest that NH$_2$-terminal PTHrP can stimulate transmembrane calcium transport while the mid-molecular form cannot.

As noted previously, cord blood NH$_2$-terminal PTHrP concentrations are 15-fold higher than PTH when expressed in equivalent pM units (330), although this may underestimate the true concentration of PTHrP depending on the assay and how the blood sample was collected. The plasma concentration of mid-molecular PTHrP$^{38–94}$ was increased almost twofold in cord blood of intrauterine growth restricted babies compared with healthy neonates at term (647). When vesicles of fetal-facing basement membranes of syncytiotrophoblasts were made from the placentas of these same growth-restricted babies, $^{45}$Ca uptake increased 50% into the vesicles from IUGR babies over those from healthy babies (647). These data positively correlate an increase in the concentration of mid-molecular PTHrP with an in vitro measure of placental calcium transport.

The effects of NH$_2$-terminal PTHrP fragments have also been studied on isolated maternal-facing and fetal-facing basement membranes from term human placentas. PTHrP$^{1–34}$ stimulated phosphoinositide turnover, phospholipase C, and protein kinase C in both membrane fractions (355, 362). It was equipotent to PTH$^{1–34}$ on the phospholipase C pathway but more potent than PTH at stimulating protein kinase C activity (362).

An equivalent to the murine Pthrp null state has not yet been identified in human fetuses, but is expected to cause intrauterine death. An autosomal dominant microdeletion in the human PTHrP gene (PTHLH) has been linked to brachydactyly type E, which is characterized by short stature and shortened metacarpals and metatarsals (316). This finding is consistent with a role for PTHrP in regulating the formation of the endochondral skeleton in humans.

Whether the very high concentrations of PTHrP in human milk have effects in the neonate to alter intestinal calcium absorption or bone metabolism has not been directly investigated. Studies during the first 6 to 12 mo after birth have shown variable results of higher, lower, or no difference in bone mineral content gains achieved by human milk versus formula-fed babies (90, 145, 636, 722). However, several studies have found consistent and significant positive correlations between the duration that an infant received human milk versus formula and the bone mass measured by DXA as a child, adolescent, or young adult (186, 290, 450). These correlations do not prove causation nor do they directly implicate the PTHrP content of human milk. However, the results are intriguing, especially given that the PTHrP content is one of the most striking differences in the composition of human milk versus formula.

F. Summary of Key Points

The bulk of the available data are consistent with PTHrP playing several key roles in fetal bone and mineral metabolism, including the regulation of serum calcium and phosphorus, stimulation of placental calcium and magnesium transport, control of endochondral bone development by delaying terminal differentiation of chondrocytes, and several effects that stimulate formation, recruitment, activity, and survival of osteoblasts. Although PTHrP is needed to maintain a normal serum calcium in the fetus, PTHrP does not increase in response to systemic hypocalcemia. Animal and human data support the conclusion that it is a mid-molecular form of PTHrP, likely PTHrP$^{38–94}$, which stimulates placental calcium transfer through actions on an as yet unidentified receptor. It remains unclear whether placenta, parathyroids, or both are the key source of PTHrP in the fetal circulation, nor is it certain when PTHrP disappears from the postnatal circulation. Through its local production in chondrocytes and osteoblasts, PTHrP continues to play key roles in regulating skeletal development in the neonate, infant, and child. Exactly what role the high content of PTHrP in milk has is unknown, but there are intriguing clues that it may modulate intestinal calcium absorption or mineral accretion by the skeleton.

V. PARATHYROID HORMONE

The low levels of PTH in late term fetuses, which rise significantly after birth, suggested that PTH may be unimportant for fetal bone and mineral homeostasis. However, this is now known to be incorrect.

A. Regulation of Serum Minerals

The most specific evidence that PTH regulates fetal blood calcium is that hypocalcemia occurs when Pth is ablated in fetal mice (623), and when PTH antisera is infused into fetal rats (197). Hypocalcemia also occurs after parathyroidectomy in fetal lambs and rats (1, 2, 97, 105, 520), and genetic ablation of parathyroids in mice (341); however, hypocalcemia due to loss of parathyroids may be caused in
part by loss of other parathyroid-derived factors such as PTHrP. Consistent with this possibility, in fetal mice loss of parathyroidectomy or decapitation in fetal rats causes the blood calcium to fall to a level equal to or above the maternal level (105, 519). This was interpreted by the investigators to imply persistence of active transport of calcium in the absence of maternal and fetal parathyroids. The experiments were done prior to the discovery of PTHrP and may indicate more persistent actions of placental PTHrP on serum calcium and placental calcium transport.

In section IV, it was noted that in Pthrp null fetuses, serum PTH increases and likely maintains the blood calcium at the normal adult level. But in several mouse models that are hypocalcemic due to PTH deficiency (Pth null, Gcm2 null, and aparathyroid Hoxa3 null fetuses), there is no compensatory increase in plasma PTHrP (341, 623). These findings confirm that PTH is responsive to falls in the ambient fetal blood calcium whereas PTHrP is not.

Although thyroparathyroidectomy or decapitation in fetal rats causes the blood calcium to fall to a level equal to or above the maternal level, when the mother was also thyroparathyroidectomized, the fetal serum calcium remained significantly higher than the maternal serum calcium (105, 519). This was interpreted by the investigators to imply persistence of active transport of calcium in the absence of maternal and fetal parathyroids. The experiments were done prior to the discovery of PTHrP and may indicate persistent actions of placental PTHrP on serum calcium and placental calcium transport.

The low level of PTH in the fetal circulation also regulates serum phosphorus and magnesium because hyperphosphatemia and hypomagnesemia occur in Pth null fetuses (623). Hyperphosphatemia and a modest reduction in serum magnesium also occur in thyroparathyroidectomized fetal lambs and rats, and aparathyroid fetal mice (105, 341, 397, 398, 520, 623). Notably calcitriol levels do not change in thyroparathyroidectomized fetal lambs (1, 97), indicating that fetal production of calcitriol is not dependent on PTH or parathyroids.

Therefore, PTH is a key regulator of fetal blood calcium, phosphorus, and magnesium, despite the low circulating levels that originally suggested otherwise. In fact, PTH may have a greater role in determining the serum calcium level than PTHrP, as revealed through study of double-mutants (see section VI).

**B. Regulation of Placental Mineral Transport**

The PTH1R is expressed by murine trophoblasts but is most intensely expressed within the intraplacental yolk sac (337). That intense expression implies that NH2-terminal PTH (≥N-terminal PTHrP) has some function within the placenta, but most in vivo studies have failed to find an effect of PTH on placental mineral transport. These included studies of in situ perfused placentas from thyroparathyroidectomized fetal sheep (6, 96, 556), and placental calcium transfer in intact Pthrp null and WT fetuses (340). However, Pthrp null fetuses have threefold higher PTH which may have maximized any effect that exogenously administered PTH could have had.

Exogenous PTH1–34 did modestly stimulate calcium transport in perfused placentas from rat fetuses that had been decapitated to crudely mimic parathyroidectomy (554), but it had no effect in perfused placentas from intact fetuses (554, 611). Placentas from thyroparathyroidectomized rat fetuses were not studied.

Comparative study of Pth null and Gcm2 null fetuses within a similar genetic background has provided evidence of a possible role for PTH in stimulating placental mineral transport (623). Placental45Ca transfer appeared normal at baseline in Pth null fetuses, but microarray and real-time quantitative RT-PCR analysis of Pth null placentas revealed significantly reduced expression in Trpv6, Sg100 (Calbindin-D1K), Vdr and other cation transporters (623). Pth null fetuses treated in utero with PTH1–84 responded with a 30% increased rate of 45Ca accumulation, confirming that exogenous PTH can stimulate placental calcium transfer in vivo when endogenous PTH is absent (623). Gcm2 null fetuses have a similar phenotype to Pth nulls with hypocalcemia and hyperphosphatemia, but differ by having a low but detectable concentration of circulating PTH compared with their WT littermates. They also differ from Pth nulls by having a significantly increased rate of placental calcium transfer, and increased placental expression of Trpv6, Sg100, and Vdr relative to WT littermates (623). These observations led to the discovery that there is a low level of expression of Pth mRNA within WT placentas, which is absent in Pth null placentas, and significantly increased in Gcm2 null placentas (623). Therefore, PTH may regulate placental calcium transfer through local expression of PTH and PTH1R, in addition to PTH having possible effects from within the systemic circulation.

PTH does not regulate phosphorus transfer in perfused placentas from fetal lambs (330), and it probably does not regulate magnesium transport either because parathyroid extracts and PTHrP1–34 failed to have effects (47, 96). Placental phosphorus and magnesium transfer have not been studied in aparathyroid or Pth null fetal mice.

Overall, PTH may act systemically and locally within placenta to regulate the transfer of calcium and other solutes. Its effects may be weaker or secondary to that of PTHrP, since Pthrp null fetuses have reduced placental calcium transfer.
transfer despite a threefold increase in endogenous PTH. If so, then loss of both PTH and PTHrP should cause a greater decline in placental calcium transport than loss of PTHrP alone, but this has not been investigated.

C. Regulation of Endochondral Bone Formation

Thyroparathyroidectomized fetal lambs displayed reduced mineralization of the fetal skeleton at term (2), but whether this is due to loss of PTH, PTHrP, or both could not be determined. Specifically, examination of the fourth lumbar vertebra revealed reduced ash weight, eroded surface, and mineralizing surface, but increased bone volume, trabecular thickness, osteoid thickness, and osteoid surface. This was described as a rachitic phenotype by the investigators, but shows the effect of loss of parathyroids and PTH to reduce skeletal mineralization, thereby resulting in the accumulation of unmineralized osteoid.

Study of the more recently developed murine models has shown that PTH is an important determinant of skeletal mineralization during fetal development, but its effects on endochondral bone development appear weaker and somewhat variable compared with loss of PTHrP (624).

Hoxa3 null fetuses lack parathyroid glands and PTH and (within the Black Swiss outbred background) have very low skeletal mineral content, but normal cartilaginous and osseous components of the endochondral skeleton (FIGURE 9) (336, 341). These include normal expression of chondrocyte and osteoblast-specific genes, lengths of the long bones, and cellular morphology and lengths of the tibial growth plate and trabecular compartment (336, 341). The skeleton is essentially normal except for a significant deficit in mineral content (FIGURE 9).

In contrast, Ptb null fetuses (within the inbred C57BL/6 background) were initially reported to have slightly shorter tibial metaphyses, shortened metacarpals and metatarsals, smaller vertebrae, reduced trabecular bone volumes, and fewer osteoclasts and osteoblasts (439). These structural changes largely affected osseous and not chondrocytic parameters, including reduced expression of osteoblast-specific genes.

Why should loss of PTH lead to a bone phenotype that loss of parathyroids does not share? To resolve this, Ptb nulls and PTH-deficient Gcm2 nulls were back-crossed into the outbred Black Swiss strain to match that of Hoxa3 nulls (623). In this strain Ptb nulls and Gcm2 nulls each had normal skeletal lengths and morphology, and a modestly reduced mineral content that was not as severe as in aparathyroid Hoxa3 nulls (336, 623). Therefore, loss of PTH caused an osteoblast-deficiency phenotype only in the C57BL/6 strain. Black Swiss fetuses and their mothers have 0.25 mM higher serum calcium, and adults have higher bone mass, compared with C57BL/6 mice (192, 309, 310, 340, 744). These and other differences between the background strains may explain why absence of PTH causes variable effects on bone.

Overall, PTH is a key regulator of skeletal mineralization in the fetus, as observed in lambs and mice. This may occur indirectly through PTH’s actions to support the fetal blood calcium; when serum calcium falls, substrate availability is reduced and mineral accretion is impaired. PTH may also directly regulate osteoblast development and function during fetal development, with its effects varying by genetic background.

D. Role in the Neonate

PTH becomes a major controller of mineral and bone homeostasis in the hours after birth. The parathyroids increase synthesis and secretion of PTH, which acts to raise the blood calcium, lower phosphorus, stimulate calcitriol synthesis, inhibit calcitriol catabolism, reabsorb calcium in the kidney tubules, and upregulate bone formation.

The importance of PTH is most clearly evident through the study of Ptb null mice, which are born in the expected Mendelian ratios at birth. They are hypocalcemic and hyperphosphatemic at birth, but it is around the time of weaning at 3 wk of age that sporadic sudden deaths begin to occur from presumed hypocalcemia-induced tetany and arrhythmias (310). The timing is consistent with the maturation of intestinal calcium absorption from a passive to an active, calcitriol-dependent process, and with Ptb nulls being unable to normally stimulate calcitriol synthesis. These deaths are prevented by using a calcium-phosphate-lactose-enriched “rescue diet” that increases passive absorption of mineral (310). A similar sporadic mortality occurs in Gcm2 null mice when maintained in outbred background strains, but mortality is 70–100% in the inbred C57BL/6 strain for reasons that appear unrelated to loss of PTH (384).

By 2 wk after birth, Ptb null mice in the C57BL/6 background are slightly smaller than their WT siblings due to shortening of the long bones. Histomorphometric assessment has shown that chondrocytic parameters are still largely normal, but abnormalities within the osseous component include a significant reduction in mineral apposition rate, trabecular bone volume, and mineralization (as assessed by von Kossa) (748).

The hypocalcemia in Ptb nulls contributes to their increased mortality and reduced skeletal mineralization independent of any effect of PTH to stimulate the formation of calcitriol. This has been tested in Ptb/Cyp27b1 double mutants that lack PTH and calcitriol, are hypocalcemic, hyperphosphatemic, and hypercalcuiic, have shortened skeletal lengths
and reduced trabecular bone volumes, and die before weaning at 3 wk of age (748). When PTH or PTHrP injections were administered daily starting at day 4 after birth to increase the serum calcium, survival improved such that the pups could be analyzed at 1 mo of age (749). The serum calcium increased significantly, hyperphosphatemia persisted, urine calcium excretion decreased, and there were significantly improved skeletal lengths, trabecular bone volumes, and mineral content (749). These results could not be mediated by calcitriol because the double-mutants lacked the ability to stimulate calcitriol synthesis in response to PTH or PTHrP treatment (749). In a separate report, Pth/Cyp27b1 double mutants were treated with exogenous calcitriol, and the results were quite similar with improved survival, increased serum calcium, lowered serum phosphorus, reduced urine calcium excretion, and improved skeletal lengths, trabecular bone volumes, and mineral content (747). These effects were not mediated by PTH since the double-mutants lacked it. Since similar outcomes occurred when the serum calcium of Pth/Cyp27b1 double mutants was raised by treatment with PTH, PTHrP, or calcitriol, these results may indicate the importance of serum calcium to support osteoblast function and skeletal mineralization, as well as to prevent deaths due to hypocalcemia. Calcium should exert its effects through the CaSR expressed by chondrocytes, osteoblasts, and osteocytes (see sect. VIIC).

E. Human Data

Human trophoblasts express PTH1R (82, 183), which makes it likely that PTH exerts biological effects within the placenta. PTH1–34 stimulated 45Ca accumulation into vesicles prepared from fetal-facing basement membranes of human syncytiotrophoblasts, but the minimum dose needed was 10-fold or more than that needed for mid-molecular PTHrP38–94 (648). In vesicles oriented to transport calcium outward, PTH1–34 stimulated 45Ca efflux but required a fourfold greater dose than PTHrP1–34 (178). These findings indicate that PTH has the ability to stimulate calcium transport in human trophoblasts when studied in vitro, but that it is less potent than PTHrP. NH2-terminal PTHrP may be the endogenous ligand for the PTH1R expressed within human trophoblasts, unless PTH is produced locally within the human placenta.

Congenital hypoparathyroidism can occur from genetic deletion of the parathyroids (DiGeorge and other 22q11.2 deletion syndromes, ablation of Gcm2, etc.) and from activating mutations of the calcium sensing receptor (70). The animal data predict that hypoparathyroid babies should have hypocalcemia, hyperphosphatemia, and impaired skeletal mineralization at birth. However, due to the sporadic nature of these conditions and lack of symptoms at birth, no cord blood or skeletal radiographs are available.

Symptomatic hypocalcemia is not inevitable after genetic loss of parathyroids: in a large series of 22q11.2 deletions, only 60% of affected individuals developed hypocalcemia at some point in their lives (578). Many presented as neonates, but in others, the presentation was in childhood or as late as 18 years of age (578). In another series of 12 cases with confirmed hypocalcemia, only 4 were symptomatic; 10 were diagnosed before 1 mo of age, and the remaining 2 presented at 3 mo and 12 yr of age (71). Serum phosphorus was inconsistently measured and not always elevated. In another series of 10 cases with confirmed hypoparathyroidism, the age at diagnosis ranged from 9 days to 13 yr (12). It seems likely that when hypocalcemia begins in utero as a result of failure of the parathyroids to form, the individual adapts to the low calcium and is less likely to become symptomatic at birth. This contrasts to the marked symptoms that occur after surgically induced hypoparathyroidism in children or adults.

Pseudohypoparathyroidism or PTH resistance also causes hypocalcemia, but it develops later in childhood and is usually preceded by hyperphosphatemia and elevated PTH (412, 470, 687). Consequently, pseudohypoparathyroid newborns should have normal cord blood calcium and are unlikely to become hypocalcemic as neonates.

Neonatal or infantile primary hyperparathyroidism has been described in older literature (57, 405, 562), but these cases likely represented neonatal severe primary hyperparathyroidism mediated by homozygous or compound heterozygous inactivating mutations of CaSR (see sect. VIIID). The earliest that conventional primary hyperparathyroidism caused by adenomas or 4-gland hyperplasia has been reported is 4 years of age, and these are usually in the setting of multiple endocrine neoplasia types 1 or 2, or familial primary hyperparathyroidism (321).

F. Summary of Key Points

Despite its low levels in the fetal circulation, PTH is an important determinant of serum calcium and phosphorus. It may play a modest role in stimulating placental calcium transport by reaching trophoblasts from the systemic circulation or through localized expression within the placenta. Alternatively, expression of PTH1R within trophoblasts may indicate a role for NH2-terminal PTHrP that is only fulfilled by PTH when secondary hyperparathyroidism occurs in utero. PTH contributes to endochondral bone development in the fetus mainly by supporting the mineralization of bone. This occurs by PTH maintaining the normal concentration or supply of minerals in the circulation, as well as through possible direct effects of PTH on osteoblasts that may be independent of the higher circulating concentrations of PTHrP. Postnatally, PTH increases after the first 12 h and assumes its well-known roles as a dominant regulator of bone and mineral status, through actions in osteoblasts, kidneys, and (indirectly by stimulating formation of calcitriol) the intestines.
VI. ROLE OF PTHrP AND PTH CONSIDERED IN COMBINATION

PTHrP and PTH overlap in their contribution to the regulation of fetal bone and mineral metabolism. This has been made most clear by comparative study of double mutants that lack either PTHrP and PTH, or PTHrP and the parathyroids, and by ablation of their common NH2-terminal receptor, PTH1R.

A. Regulation of Serum Minerals

It is clear that PTH and PTHrP each participates in the regulation of the fetal blood calcium, such that loss of either hormone causes fetal hypocalcemia (336, 340, 341, 623). Their individual effects appear additive as revealed by studies of a double-mutant colony in which PTHrP, parathyroids, or both were deleted, and compared with the findings in Pth1r null fetuses that lack the PTH1R (FIGURE 10). Compared with WT littersmates, Pthrp null fetuses had a modestly reduced blood calcium (equal to the maternal level), aparathyroid Hoxa3 null fetuses had a markedly reduced blood calcium (well below maternal values), and Pthrp/Hoxa3 double mutants had the lowest blood calcium (336). The ionized calcium level caused by simultaneous loss of parathyroids and PTHrP was equivalent to the ionized calcium level of Pth1r null fetuses in the same Black Swiss strain each had an ionized calcium that was equal to the maternal level, and not reduced well below it as in aparathyroid Hoxa3 null fetuses (623). The greater decline in ionized calcium within aparathyroid Hoxa3 null fetuses (336, 623) might imply that parathyroid-produced PTHrP has been lost; however, plasma PTHrP is unchanged among WT, Hoxa3+/−, and Hoxa3 null fetal littersmates (336, 341). It is possible that the parathyroids contribute to fetal mineral homeostasis directly or indirectly through the release of other factors. For example, the chromogranins have been estimated to represent 50% of the protein output of the parathyroids (305, 606). Chromogranin expression increases with hypercalcemia and decreases with hypercalcemia (160, 467), and chromogranins have been shown to inhibit PTH synthesis and release (26, 576); therefore, chromogranins could conceivably have distal effects to regulate the production of PTHrP by the placenta (248, 319). However, the possible role that non-PTH secretory products of the parathyroids may have on mineral homeostasis has not been studied in the fetus or adult. Pth/Hoxa3 double mutants should reveal whether loss of parathyroids adds to the phenotype caused by loss of PTH, but that model has not been studied. Similarly, although Pthrp/Pth double mutants have been examined for their effect on skeletal development (439), no measurements of serum calcium or phosphorus have been reported.

As noted earlier in section VA, Pth null and aparathyroid Gcm2 null fetuses in the same Black Swiss strain each had an ionized calcium that was equal to the maternal level, and not reduced well below it as in aparathyroid Hoxa3 null fetuses (623). Pth nulls have hyperplastic parathyroids that conceivably could produce PTHrP and compensate for the loss of PTH (439); however, no increase in expression of PTHrP was observed in the Pth null fetuses (623). Upregulation of placental PTH expression was found in aparathyroid Gcm2 null fetuses, and that may have blunted the fall in blood calcium caused by loss of parathyroids (623). The fact that the ionized calcium of Pthrp null fetuses in the same background is equal to the maternal level should not be taken as evidence that the magnitude of PTHrP’s effect is to raise the blood calcium above the maternal level. Pthrp null fetuses have a significant compensatory increase in PTH; without it, the serum calcium of Pthrp null fetuses would likely have been much lower and approaching the level observed in Pthrp/Hoxa3 null double mutants (336). Pth1r null fetuses have the lowest ionized calcium level, and the absence of PTH1R is accompanied by markedly increased circulating concentrations of PTH and PTHrP (336, 340, 341). Although the parathyroids of Pth1r null fetuses were not directly examined, there was no increase in PTHrP mRNA expression in the neck region that contains the parathyroids (341). However, there was increased expression of PTHrP mRNA (by Northern blot and in situ hybridization).
and protein (by immunohistochemistry) within placenta and liver of Pth1r null fetuses (340, 341). These findings provide additional evidence that the fetal parathyroids are unlikely to be a dominant source of PTHrP in the fetus, and that placenta and possibly liver may provide much of the PTHrP that is normally present in the fetal circulation.

B. Regulation of Placental Mineral Transport

Pth1r null fetuses exhibit a significantly increased relative rate of placental calcium transport compared with their WT littermates (340). Since PTHrP circulates at 11-fold higher levels in these fetuses compared with WT (341), and loss of the PTH1R blocks NH2-terminal actions of PTHrP, it seems likely that high circulating levels of mid-molecular PTHrP are acting on a mid-molecular receptor to stimulate placental calcium transport (336, 340). These results support earlier findings that mid-molecular PTHrP stimulated placental calcium transport in fetal lambs and mice (sect. IVB). The mid-molecular receptor for PTHrP has yet to be cloned.

C. Regulation of Endochondral Bone Formation

Pthrp/Hoxa3 double mutants have an overall appearance and skeletal phenotype that combines the Pthrp null chondrodysplasia with the undermineralized skeleton of the Hoxa3 null; they are also globally smaller in size (336). Combining these two genetic deletions confirms that loss of fetal parathyroids leads to undermineralization of the fetal skeleton without affecting the underlying cartilaginous and osseous structure. In contrast, loss of PTHrP alone causes marked acceleration in chondrocyte differentiation and initiation of primary ossification centres, and both accelerated and abnormal mineralization of bone (336).

Pthrp/Pth double mutants also show the Pthrp null chondrodysplasia combined with undermineralized skeleton of the Pth null; they too are globally smaller than WT siblings (439). However, the Pth null has mild defects in osteoblast parameters that appear only within the C57BL/6 genetic background and not the Black Swiss background (see sect. VC).

Pth1r nulls have a similar global phenotype as the Pthrp nulls, including accelerated endochondral ossification, dysplasia, and ossification of the cartilaginous portions of ribs; however, they are globally much smaller than their WT siblings (360). They also have a greater deficit in skeletal mineral content than that caused by loss of either ligand alone (336, 340). This may result from their much lower ionized calcium level, combined with loss of the effects of PTHrP or PTH to stimulate osteoblasts.

There are other differences in the skeletal phenotypes of Pth1r null and Pthrp null fetuses. Whereas Pthrp null fetuses have normal expression of osteoblast genes, and normal or increased mineralization (359), Pth1r null fetuses show reduced expression of collagenase-3, osteocalcin, and osteopontin and have significantly reduced mineralization (359). The explanation may be the increased circulating levels of PTH in Pthrp null fetuses. The chondrocytic portion of the growth plate is avascular, which will prevent systemic PTH from compensating for loss of PTHrP production and its local actions within chondrocytes. But systematically high PTH levels should easily reach the highly vascular bone compartment to maintain apparently normal expression of osteoblast genes. Such actions of PTH are lost in Pth1r nulls, resulting in downregulation of these osteoblast-specific genes. Furthermore, the Pth1r null skeleton is undermineralized despite a relative upregulation in placental calcium transfer (as described in sect. VIB). This likely indicates that, notwithstanding relatively increased delivery of calcium from the maternal circulation in Pth1r nulls compared with their WT littermates, calcium cannot be incorporated into bone without NH2-terminal actions of PTH or PTHrP, so it returns to the maternal circulation.

An activating mutation of the PTH1R creates the opposite phenotype of the Pth1r null fetuses, exaggerating what are likely the normal NH2-terminal actions of PTHrP to delay chondrocyte hypertrophy. The result is a largely cartilaginous at birth (596). Its appearance is similar to the effect of PTHrP overexpression within the developing skeleton (726).

D. Role in the Neonate

Pthrp/Pth double mutants and Pthrp/Hoxa3 double mutants die soon after birth (336, 341, 439). This is likely a consequence of the lower blood calcium that these double mutants must have, in addition to the early mortality conferred by ablation of Pthrp or Hoxa3 alone. In the original C57BL/6 background, Pth1r null fetal mice die at E11.5–12.5 due to widespread cardiomyocyte apoptosis, which implies important roles for PTH1R in early embryogenesis (532). When crossed into the outbred Black Swiss strain, they survive to the end of gestation before dying promptly in a manner similar to Pthrp nulls (340, 360). Consequently, there are no neonatal data on the effects of combined loss of PTH and PTHrP.

E. Human Data

As noted earlier, human trophoblasts express PTH1R (82, 183), which implies that PTH or PTHrP exert NH2-terminal biological effects within the placenta. Loss of PTH1R causes Blomstrand chondrodysplasia, which is characterized by a chondrodysplasia quite similar to what has been
found in Pth1r null fetal mice (296, 489). It is embryonically lethal; therefore, there are no cord blood measurements of calcium, phosphorus, etc.

F. Summary of Key Points

PTH and PTHrP have additive effects on the serum calcium level, and in turn affect mineralization of the fetal skeleton. Loss of PTH or PTHrP have equivalent effects on the serum calcium, whereas loss of parathyroids in Hoxa3 null fetuses or loss of PTH1R causes more significant hypocalcemia. PTHrP has a more profound effect on regulating endochondral bone development, primarily by regulating the fate of chondrocytes and the initiation of osteoblast activity. PTH has more modest effects on osseous portions of the skeleton, but these become much more apparent after birth. Combin- ing absence of PTHrP with deletion of PTH results in a skeleton that bears the Pthrp-null chondrodysplasia but is also undermineralized as in the Pth null or Hoxa3 null skeleton. Upregulation of PTH likely compensates for loss of NH2-terminal actions of PTHrP on bone within Pthrp null fetuses.

VII. CALCIUM-SENSING RECEPTOR

CaSR is considered here because it plays a pivotal role in regulating mineral and bone homeostasis in the adult, through its regulation of PTH synthesis and release, and renal tubular handling of calcium. Furthermore, calcium functions like a calciotropic hormone by binding to its receptor in different tissues, including parathyroids, bone, kidneys, and placenta. Calcium appears to have direct actions that influence the function of osteoblasts and chondrocytes.

A. Regulation of Serum Minerals

As discussed in section II, A and B, CaSR does not set the high level of calcium found in normal fetuses, but it suppresses PTH in response to it. Ablation of one (Casr+/−) or both (Casr null) alleles of CaSR results in an equal increase in the fetal blood calcium above the WT value (FIGURE 3), and a dose-dependent increase in circulating PTH (FIGURE 4) and calcitriol (338). The increase in calcium depends on PTH because simultaneous ablation of the PTH1R in Pth1r/Casr double mutants prevents Casr ablation from increasing the blood calcium above the low level present in Pth1r null fetuses (338). It is puzzling that ionized calcium of Casr null fetuses is not higher than in Casr+/− fetuses; the intrauterine environment may constrain Casr null fetuses from achieving the higher blood calcium level that they reach after birth. CaSR is likely responsible for the ionized calcium level of Pthrp null fetuses being maintained at the maternal level rather than falling below it, through its actions to stimulate PTH to achieve the adult set point of calcium (see also sects. IVA and VA) (338, 340).

It is evident from data discussed earlier that CaSR does not stimulate PTHrP to maintain the fetal blood calcium, because in Hoxa null fetuses, the ionized calcium is reduced well below the maternal concentration, PTH is undetectable, and the plasma PTHrP level does not increase in compensation (336, 341). However, CaSR may have effects to downregulate the expression of PTHrP, because circulating plasma PTHrP is significantly lower in Casr null fetal mice compared with their siblings (334). The increased ionized calcium in Casr nulls may be a factor, or CaSR may directly regulate PTHrP through their colocalized expression within trophoblasts and intraplacental yolk sac.

In the adult, ablation of CaSR decreases renal calcium clearance in a dose-dependent fashion (257), leading to hypercalcemia combined with hypocalciuria. However, Casr+/− and Casr null fetal mice each have increased amniotic fluid calcium with no difference between them (338). This striking difference from the adult phenotype is likely the result of two factors. First, fetal kidneys express very low levels of Casr mRNA until the first postnatal day (112); consequently, the renal phenotype potentially caused by Casr ablation is absent. Second, the renal filtered load of calcium should be increased equally in both genotypes due to their similar increments in ionized calcium. Therefore, in the absence of modulation by CaSR, the increased filtered load of calcium simply leads to increased renal excretion of calcium into the amniotic fluid.

CaSR has been selectively ablated from parathyroid glands (Casr Para null), but the effect that this may have on fetal mineral homeostasis has not been reported (109).

An activating mutation of Casr has been described in Nuf mice (271). No fetal data have been published, but it is anticipated that they should have a similar phenotype as Pth null fetuses, with reduced skeletal mineralization and low PTH.

B. Regulation of Placental Mineral Transport

As noted earlier, CaSR is expressed in murine trophoblasts and intraplacental yolk sac cells (337). Placental transport of 45Ca is significantly and dose-dependently reduced in Casr+/− and Casr null fetuses compared with their WT siblings (338). It is unknown why placental calcium transfer is reduced. If the CaSR senses calcium flow within the placenta, or regulates placental PTHrP synthesis, CaSR ablation could explain a decrease in placental calcium transport. The lower plasma PTHrP levels in Casr null fetuses could result from either postulated mechanism. Alternatively, the elevated serum calcium or secondary hyperparathyroidism...
that occur in Casr null fetuses might also lead to downregulation of placental calcium transfer.

Additional studies of Pthrp/Casr double mutant fetuses revealed that although ablation of Casr reduced placental calcium transport in fetuses that had two normal Pthrp alleles, ablation of one or both alleles of Casr had no effect on placental calcium transport when both Pthrp alleles were ablated (338). This result is compatible with downregulation of Pthrp explaining reduced placental calcium transport in both Pthrp null and Casr null fetuses.

There have been no studies of the Casr activating mutation from Nuf mice of placental calcium transport.

C. Regulation of Endochondral Bone Formation

CaSR is expressed by chondrocytes and osteoblasts, with its highest expression seen within the hypertrophic chondrocytes (109, 110). Chondrocyte-specific ablation of CaSR (Casr\textsubscript{Chon} null) caused embryonic lethality with severely delayed cartilage and bone development (109), but when the chondrocyte-specific deletion was induced between embryonic day 16 and 18 by use of a tamoxifen-driven promoter, the fetal skeleton was shortened and undermineralized due to delayed terminal differentiation of chondrocytes (109). These results indicate that CaSR promotes chondrocyte differentiation and that its absence delays differentiation, actions that oppose those of PTHrP within the growth plate. This is consistent with the known effect of high extracellular calcium concentrations to promote chondrocyte differentiation (108, 111, 257); ablating CaSR must block this effect.

Ablation of CaSR has also been carried out in preosteoblasts, immature osteoblasts, mature osteoblasts, and osteocytes (Casr\textsubscript{Pre}, Casr\textsubscript{OB} and Casr\textsubscript{OC} null), and in each the fetal skeleton is unremarkable, although skeletal abnormalities develop later (see below) (109, 169, 549).

Bone resorption is increased in global Casr-null fetuses, as suggested by the increased circulating PTH levels, increased excretion of the bone resorption marker deoxypyridinoline into amniotic fluid, and a significant reduction in the ash weight and mineral content of the fetal skeleton (331, 338). Since Casr ablation reduces placental calcium transport and increases excretion of calcium into the amniotic fluid, the increased circulating calcium concentration must be maintained through resorption of mineral from the fetal skeleton, thus compromising bone strength and mineral content before birth. The endochondral skeleton has normal lengths and microscopic morphology at birth in Casr null mice apart from the reduced mineral content, and this contrasts with the significant abnormalities found in the chondrocyte-specific knockout of Casr. The explanation for this discrepancy is thought to be that, although exon 5 is deleted in the global Casr null, a splice variant lacking exon 5 is produced which is able to form heterodimers and provide calcium sensing in certain tissues of the global Casr null (187). The chondrocyte-specific deletion of Casr does not produce that splice variant.

Castr has also been deleted selectively from parathyroid cells (Casr\textsubscript{Para} null), but the fetal phenotype of this model has not been reported (109). It is unremarkable in size at birth but will likely be found to have hypercalcemia and a demineralized skeleton in utero.

D. Role in the Neonate

Postnatally, global Casr nulls develop severe hyperparathyroidism with grossly enlarged parathyroids, hypercalcemia, fluid depletion (calcium diuresis), and progressive growth failure (257). Most die before 3 wk of age, but surgical or genetic deletion of the parathyroids will enable them to survive (327). Casr nulls are the murine equivalent of neonatal severe primary hyperparathyroidism in humans. Casr\textsuperscript{null} neonates are hypercalcemic but have normal life spans and fertility, analogous to familial benign hypercalcemia (familial hypercalcemic hypercalciuria, FHH).

The skeleton is normal at birth when Casr is ablated from early osteoblasts (Casr\textsubscript{Pre} null), but the mice fail to thrive, develop multiple fractures, and die at 1 mo (109, 169). There are severe abnormalities including decreased osteoblasts, osteoblast apoptosis, osteoclasts, and increased osteoid, all of which contribute to a soft and disorganized bone structure (109, 169). In contrast, deletion of Casr from mature osteoblasts and osteocytes (Casr\textsubscript{Ob} null and Casr\textsubscript{OC} null) lead to viable mice that later develop osteoporosis with increased numbers of osteoclasts (109, 549).

Postnatally the parathyroid-specific knockout of CaSR (Casr\textsubscript{Para} null) develops severe hypercalcemia, markedly elevated PTH, growth retardation, multiple fractures, a severely demineralized skeleton, and marked delay in osteoblast differentiation (109). These findings suggest that much of the phenotype of the global Casr null is mediated by hyperparathyroidism, with little or no effect from Casr ablation in chondrocytes, osteoblasts, and kidney tubules.

The neonatal phenotype of Nuf mice has not been reported. At 4 wk of age (the earliest time point described) they have hypocalcemia, hyperphosphatemia, low PTH, and ectopic calcifications in multiple tissues; they are also prone to sudden death (271).

E. Human Data

No case reports have described blood calcium or PTH values in fetuses or newborns with inactivating mutations of
CASR. These births are sporadic, and serum calcium is not routinely measured in healthy newborns. The findings from Casr<sup>+/−</sup> fetal mice predict that human babies with FHH will be hypercalcemic in utero and that the cord blood calcium will be increased. Since hypercalcemia begins during fetal development, this likely explains why individuals with FHH are adapted to a high ionized calcium, and feel hypocalcemic if their blood calcium is reduced to “normal” values. This parallels the findings in disorders causing hypocalcemia during fetal development, in which affected neonates, children, and adults may remain asymptomatic for hypocalcemia (see sect. VE).

Neonatal severe primary hyperparathyroidism is usually caused by homozygous or compound heterozygous inactivating mutations of CASR (193, 394), and occasionally by heterozygous mutations through a presumed dominant-negative effect (33, 481). It is usually not identified at birth, but the findings from Casr null fetal mice predict that hypercalcemia will be present in utero and in cord blood. The neonate is at very high risk to develop severe and life-threatening hypercalcemia, dehydration, failure to thrive, neurological disturbances, nephrocalcinosis, and skeletal demineralization with fractures. The presentation can be delayed as much as a month after birth (557), in part because maternal hypercalcemia has a suppressive or constraining effect on parathyroid function in utero (see sect. XIIIB). Parathyroids are grossly enlarged and serum PTH is quite high. This is an almost invariably fatal condition unless parathyroidectomy is urgently performed (57), although some very rare cases have been reported to spontaneously resolve into milder hypercalcemia that did not require surgery (57, 405, 562, 719). Bisphosphonates and the calcimimetic drug Cinacalcet have been used to lower the serum calcium temporarily to enable the newborn to have surgery at an older age (719, 737).

Activating Casr mutations cause autosomal dominant hypocalcemia with hyperphosphatemia, and normal (inappropriately low) PTH; hypercalcemia is variably present (508, 526). The presentation is expected to be similar to disorders causing hypoparathyroidism, as described in section VE. Cord blood calcium was in the adult-normal range in one newborn, but below the expected fetal value, and recurrent apneic seizures began shortly after birth (670). In another affected neonate, the cord blood calcium and ionized calcium were both low, but the baby showed no hypocalcemic symptoms (217). The variable postnatal presentation is exemplified by the development of hypocalcemic seizures at day 7 of age in a baby carrying an activating mutation of CaSR (113). Subsequent investigations confirmed that his sister (age 3 yr), mother (age 31), and grandfather (age 63) all had the same activating mutation causing hypocalcemia, low PTH, hypercalcemia, nephrocalcinosis, and nephrolithiasis (113). The sister subsequently developed tetany but only during episodes of gastroenteritis. Furthermore, a survey of 25 affected patients found that only 2 had developed hypocalcemia within the first 30 days after birth (540). The overall mean age that hypocalcemia was diagnosed was 28 yr and only 50% had ever been symptomatic (540).

F. Summary of Key Points

Global inactivating mutations of the CaSR cause hypercalcemia in utero. If homozygous or compound heterozygous mutations are present, then skeletal demineralization may develop prior to birth. Postnatally, single inactivating mutations of CaSR cause benign life-long hypercalcemia (FHH), whereas two inactivating mutations (and occasionally a single mutated allele) cause life-threatening severe hypercalcemia and hyperparathyroidism. The mutations may occur de novo or be inherited, in which case the mother may also have FHH; the effect of maternal hypercalcemia on the fetus and neonate is discussed in section XIIB.

Activating mutations resemble hypoparathyroidism with hypocalcemia, hyperphosphatemia, and inappropriately normal or low PTH; these individuals are indistinguishable from those with genetic causes of hypoparathyroidism, unless they are tested to demonstrate their abundant PTH secretory reserve, or genetic testing reveals CASR mutations.

VIII. CALCITRIOL AND VITAMIN D

A. Regulation of Serum Minerals

Consistent evidence from multiple species and models confirms that vitamin D, calcitriol, and the vitamin D receptor are not required for the fetus to be able to maintain normal serum mineral concentrations. This includes severely vitamin D deficient rats (74, 228, 241, 443), 1α-hydroxylase-null pigs (352, 353), and Vdr<sup>Bos</sup> and Vdr<sup>Leu</sup> null fetuses (342, 376), in which serum calcium, ionized calcium, and phosphorus were all normal in utero (FIGURE 4). PTH was also normal in Vdr<sup>Bos</sup> and Vdr<sup>Leu</sup> null fetuses, indicating that absence of VDR did not induce compensatory secondary hyperparathyroidism, as occurs after birth (342, 376).

Four groups independently made Vdr null mice, of which only the Vdr<sup>Bos</sup> null truly lacks VDRs. The three other models (Vdr<sup>Leu</sup>, Vdr<sup>Mum</sup>, and Vdr<sup>Tok</sup>) each deleted the exon that encodes the first zinc finger of VDR, but a splice variant is secreted which has normal ligand binding ability but no DNA binding ability (175, 376, 574, 704, 755). Of these four models, studies have been performed in Vdr<sup>Bos</sup> null and Vdr<sup>Leu</sup> fetuses. Inconsistent data have been obtained from the Vdr<sup>Leu</sup> model that expresses an aberrant but functional
VDR (376, 574), and these data contrast somewhat with data obtained from $Vd^{\text{pos}}$ null fetuses that lack VDRs (342, 372).

In the first fetal study that used $Vd^{\text{leu}}$ mice, the investigators obtained $Vd^{\text{leu}}$ null mothers by mating $Vd^{\text{leu}}$ females to $Vd^{\text{leu}}$ null males, and then mated those $Vd^{\text{leu}}$ null mothers to WT males so that all fetuses were $Vd^{\text{leu}}$. The progeny of those studies were compared with unrelated WT females mated to WT males. The authors observed the fetoplasm calcium to be paradoxically increased in $Vd^{\text{leu}}$ fetuses compared with WT fetuses from unrelated mothers, but the serum calcium was normalized (reduced) in $Vd^{\text{leu}}$ fetuses whose mothers had been placed on the calcium-phosphate-lactose-enriched “rescue diet” (574). The ionized calcium (the physiologically relevant fraction) was not measured, nor were the “rescue diet” (574). The ionized calcium (the physiologically relevant fraction) was not measured, nor were the unrelated WT control mothers and their fetuses studied on the enriched diet. It is not possible to determine from that study whether the maternal genotype or the fetal genotype led to the apparent changes in serum calcium on the normal ground (342). In short, there was no evidence of transplacental passage of calcitriol in the $Vd^{\text{pos}}$ model, and earlier cited studies determined that calcitriol does not cross the rodent placenta (see sect. II B) (475). Therefore, it seems unlikely that maternal calcitriol caused increased mortality in $Vd^{\text{leu}}$ mice.

These data from $Vd^{\text{leu}}$ fetal mice need to be interpreted cautiously because of possible aberrant signaling in $Vd^{\text{leu}}$ fetuses, and because unrelated controls were used in the first study. The studies in $Vd^{\text{pos}}$ fetuses are not affected by aberrant VDRs and have compared WT to $Vd^{\text{pos}}$ null fetuses, and $Vd^{\text{pos}}$ to $Vd^{\text{leu}}$ null fetuses, within their respective litters.

Other investigators have found possible calcitriol-mediated effects on the fetal serum calcium level. Pharmacological doses of calcitriol given to pregnant guinea pigs increased the fetal serum calcium and phosphorus (164). However, the observed effect was likely indirect through a documented increase in maternal serum calcium and phosphorus; a direct effect on the fetus is unlikely because calcitriol does not cross the rodent placenta (475). Surprisingly, vitamin D deficiency in fetal guinea pigs lowered calcitriol but caused a paradoxical increased serum calcium (573). Their pregnant mothers had 25-hydroxyvitamin D levels of ~400 nM (573), which is in a toxic range for humans or rodents and, therefore, is not relevant for vitamin D physiology in those species. Furthermore, with a maternal 25-hydroxyvitamin D level of 400 nM compared with a level of ~20 nM on a vitamin D-deficient diet, the guinea pig studies should be considered as contrasting the effects of maternal hypervitaminosis D to vitamin D deficiency, as opposed demonstrating effects of vitamin D sufficiency vs. deficiency.
Infusion of an antiserum to calcitriol into fetal lambs for 72 h reportedly lowered the blood calcium level; however, no data, controls, or details about the antiserum were shown to confirm that brief statement (564). Direct injection of calcitriol into decapitated rats from thyroparathyroidectomized mothers caused an increase in serum calcium, a decrease in serum phosphorus, and an increase in ash weight; there was no effect in intact or decapitated fetuses of normal mothers (103). What those pharmacological effects of calcitriol in decapitated rat fetuses might mean for normal fetal physiology is not clear.

Bilateral nephrectomy in fetal sheep reduced serum calcitriol by 60% and also lowered the ionized and total calcium, and increased serum phosphorus and PTH (455). The abnormal serum calcium and phosphorus levels could be corrected by direct administration of calcitriol to the fetus (455). The investigators inferred that loss of renal production of calcitriol explained the results rather than uremia from loss of kidney function. However, the calcitriol-induced changes in serum calcium and phosphorus are pharmacological effects that were not tested in intact lambs. Furthermore, the control procedure involved leaving the fetal kidneys in situ while diverting the ureters so that the urine drained into the fetal peritoneal cavity instead of the amniotic fluid. Consequently, it is difficult to interpret these results and the suitability of the renal diversion procedure as a control for bilateral nephrectomy. In the control procedure, the pooling of urine in the peritoneum means that fluid, solute, and waste products are retained in a compartment that may not lead to the equivalent effects that uremia or absent renal function may have on the fetus.

The results in nephrectomized fetal lambs contrast with those from nephrectomized fetal rats, in which the serum calcitriol level fell, but there was no effect on the serum calcium (105). Species differences may explain why nephrectomy causes hypocalcemia in lambs but not rats.

In summary, vitamin D deficiency in fetal rats, loss of 1α-hydroxylase in fetal pigs, loss of VDR in VdrBos null fetal mice, and loss of VDR in a recent study from VdrLeu null fetal mice, have shown that the serum calcium, phosphorus, PTH, and renal excretion of calcium and phosphorus are unchanged despite the absence of vitamin D, calcitriol, or VDR. Data from VdrLeu+/− fetal mice are inconsistent between the two studies reported by the same group (376, 574), but it is only their most recent study that provided data from VdrLeu−/− null fetuses (376). Some of the problems with the VdrLeu+/− fetal data include the presence of a mutant VDR that may cause aberrant signaling, and use of unrelated control mice in the first study. The remaining animal data provide evidence that pharmacological doses of calcitriol can have effects in fetuses that may not be relevant to normal physiology, while the suitability of a control procedure for bilateral nephrectomy in fetal lambs clouds the interpretation of the data from that study.

B. Regulation of Placental Mineral Transport

Where placental calcium transport has been directly measured, it is clear that calcitriol is not required because the calcium transport rate was normal in severely vitamin D deficient rats (228), and increased in both VdrBos and VdrLeu null fetal mice (342, 376). The placentas of severely vitamin D deficient rats and both VdrBos and VdrLeu null fetuses displayed normal expression of calbindinD-9k and Ca2+-ATPase (228, 342, 413, 574, 709), while PTHrP and TRPV6 were upregulated in both VdrBos and VdrLeu null placentas (342, 376). These findings indicate that the mechanisms that regulate transplacental movement of calcium do not require calcitriol. Furthermore, the findings in VdrBos and VdrLeu null fetuses may indicate that the normal role of calcitriol is to inhibit placental calcium transport, such that the rate increases when VDR has been ablated.

In contrast, Care (95) nephrectomized five fetal lambs and judged that the rate of placental calcium transport fell across their in situ perfused placentas compared with the placentas from intact lambs. A pharmacological dose of calcitriol was reported to increase the rate of calcium transfer in three placentas from the nephrectomized lambs (95). Surprisingly, the data were published only in a review article and without a statistical analysis, but this author’s assessment of the raw data has revealed that there was no statistically significant difference among the three groups (P = 0.28), nor in the comparison of the placental calcium transfer rates before and after treatment (P = 0.14). The effect of calcitriol was not assessed in the placentas of intact lambs. Therefore, the conclusion that nephrectomy reduced placental calcium transport and that it was rescued by calcitriol is not justified by the available data.

An indirect approach to measuring placental mineral transport was carried out in pregnant ewes. A single infusion of 45Ca or 32P was followed by daily treatment with pharmacological doses of 1α-hydroxycholecalciferol or diluent. At the end of a week-long treatment period, the amount of 45Ca and 32P was higher in fetal tissues from ewes that had received the calcitriol treatments (165). The same investigators tested pharmacological doses of calcitriol or diluent administered to pregnant guinea pigs for 10 days, after which it was found that the mineral content of the fetuses was higher in the offspring of calcitriol-treated animals than in those from diluent-treated animals (164). A dose of 45Ca was also given to the pregnant guinea pigs, and the amount of radioactivity in fetal blood samples was increased 30–90 min later in the fetuses whose mothers had previously received treatment with calcitriol. In both reports, the investigators inferred that placental calcium and phosphorus...
transport had been increased, but the approaches were too indirect to support that conclusion. Both treatments caused a significant increase in maternal serum calcium and phosphorus, and in turn that would have increased the availability of mineral to be transferred to the fetuses without invoking any direct actions of 1α-hydroxycholecalciferol or calcitriol on trophoblasts. A simple calcium infusion has been shown to acutely increase the flow of calcium from mother to fetus (106), so a role for calcitriol in stimulating placental calcium transport cannot be inferred when use of calcitriol raises the maternal blood calcium.

C. Regulation of Endochondral Bone Formation

Calcitriol is not required for the formation and mineralization of the endochondral skeleton during fetal development. Severely vitamin D-deficient rats (74, 241, 443) and both Vdr<sup>Bos</sup> and Vdr<sup>Leu</sup> null fetuses (342, 376) have normal skeletal lengths, morphology, ash weight, and mineral content at term (FIGURE 11). In the two remaining models, Vdr<sup>Mia</sup> and Vdr<sup>Tok</sup> null, the fetuses were also described as normal at birth and until the end of weaning, but no fetal data have been published (175, 755). Chondrocyte and osteoblast gene expression pertinent to endochondral bone development were also normal within the growth plates and trabecular compartment of Vdr<sup>Bos</sup> null fetal tibias (342).

Additional studies mated Vdr<sup>Bos</sup><sub>+/−</sub> and Vdr<sup>Bos</sup> null sisters to the same Vdr<sup>Bos</sup><sub>+/−</sub> males to determine whether the maternal Vdr null status influenced the fetal phenotype. WT, Vdr<sup>Bos</sup><sub>+/−</sub>, and Vdr<sup>Bos</sup> null fetuses born of Vdr<sup>Bos</sup><sub>+/−</sub> mothers were indistinguishable from each other, but had a slightly larger size, weight, and skeletal mineral content than their Vdr<sup>Bos</sup><sub>+/−</sub> and Vdr<sup>Bos</sup> null counterparts born of Vdr<sup>Bos</sup> null mothers (342). When adjusted for their smaller size or weight, the ash weight and mineral content of the Vdr<sup>Bos</sup> null mothers’ offspring were no different from that of Vdr<sup>Bos</sup><sub>+/−</sub> mothers (342). Vdr<sup>Bos</sup> null mothers were also placed on the calcium-phosphate-lactose-enriched “rescue diet” and compared with Vdr<sup>Bos</sup> null mothers that consumed the regular diet, and this had no effect on the size, weight, or mineral content of the offspring. Thus fetal loss of VDR in the Vdr<sup>Bos</sup> model had no effect on skeletal development or mineral content compared with respective littersmates, maternal loss of VDR resulted in a slightly smaller fetal size (but proportionately normal mineral content) that was independent of the fetal genotype, and the maternal diet had no influence on fetal size or skeletal ash weight and mineral content (342).

These results contrast with those obtained from the Vdr<sup>Leu</sup> null model (574), which, as described in section VIII A, in the first study mated Vdr<sup>Leu</sup> null mothers to WT males so that all fetuses were Vdr<sup>Leu</sup><sub>+/−</sub>, and compared them with fetuses from unrelated WT females mated to WT males. Fetal skeletal mineralization was reduced in Vdr<sup>Leu</sup><sub>+/−</sub> fetuses compared with unrelated WT fetuses, but improved when their mothers were treated during pregnancy with the “rescue diet” (574). This change was in the opposite direction of the fetal serum calcium, which was reduced when the Vdr<sup>Leu</sup> null mother was placed on the enriched diet. But in the second study, the authors mated Vdr<sup>Leu</sup> null mothers to Vdr<sup>Leu</sup><sub>+/−</sub> males so that the offspring were Vdr<sup>Leu</sup> null and Vdr<sup>Leu</sup><sub>+/−</sub>, and compared them to WT fetuses from related WT females mated to WT males. Fetal skeletal mineralization was normal in Vdr<sup>Leu</sup><sub>+/−</sub> fetuses but reduced in Vdr<sup>Leu</sup><sub>−/−</sub> fetuses from Vdr<sup>Leu</sup> null mothers, while the previous finding of reduced serum calcium was not seen. When the mothers were placed on the “rescue diet” during pregnancy, the skeletal mineralization was normal in Vdr<sup>Leu</sup><sub>+/−</sub> fetuses. As previously postulated in section VIII A, it is possible that the reduced mineralization of Vdr<sup>Leu</sup><sub>+/−</sub> compared with Vdr<sup>Leu</sup> null and WT fetuses, which was not seen in the Vdr<sup>Bos</sup> null model, is due to aberrant VDR signaling introduced by the alternately spliced receptor that is expressed by Vdr<sup>Leu</sup><sub>+/−</sub> mice. It seems a likely explanation since the Vdr null skeleton was normal in both the Vdr<sup>Bos</sup> and Vdr<sup>Leu</sup> null fetuses; it was only the Vdr<sup>Leu</sup><sub>+/−</sub> skeleton that differed between the two models (342, 376). This would be made clearer if Vdr<sup>Leu</sup><sub>+/−</sub> × Vdr<sup>Leu</sup><sub>−/−</sub> matings were done so that WT, Vdr<sup>Leu</sup><sub>−/−</sub>, and Vdr<sup>Leu</sup> null fetuses can be compared within the same litters. If abnormal VDR signaling explains the phenotype of Vdr<sup>Leu</sup><sub>+/−</sub> fetuses, then WT and Vdr<sup>Leu</sup> null fetuses will have normal skeletons and mineral content while Vdr<sup>Leu</sup><sub>−/−</sub> fetuses will continue to be abnormal. Furthermore, to test the investigators’ hypothesis that the Vdr<sup>Leu</sup><sub>+/−</sub> skeletal phenotype is caused by increased calcitriol in the circulation of Vdr<sup>Leu</sup> null mothers, Vdr<sup>Leu</sup><sub>−/−</sub> fetuses obtained from Vdr<sup>Leu</sup> null mothers should be compared with Vdr<sup>Leu</sup><sub>+/−</sub> fetuses obtained from WT mothers.

Fetal guinea pigs differ from the results described for fetal rats and mice, in that 8 wk of a vitamin D-deficient diet caused reduced fetal body weight and total body bone mineral content, and increased osteoid with expansion of the hypertrophic zone of chondrocytes (573). However, as noted earlier, the comparison may not be physiologically relevant because the maternal 25-hydroxyvitamin D was 400 nM on the vitamin D-replete diet and ~20 nM on the D-deficient diet, with fetuses having a 25-hydroxyvitamin D level of ~200 nM when their mothers were on the vitamin D-replete diet. It is a comparison of the skeletal effects of hypervitaminosis D to vitamin D deficiency, rather than vitamin D sufficiency to vitamin D deficiency. Furthermore, the vitamin D-replete and vitamin D-deficient diets were made by different manufacturers and differed in more than just vitamin D content, including higher vitamin C and lower phosphorus content in the vitamin D-deficient diet. Since the standard and control diet differed in more than
just the nutrient being studied, it is possible that dietary differences influenced the phenotypes of vitamin D “replete” and deficient guinea pigs.

Overall, while there are some differences among the models, vitamin D deficient rats and Vdr<sup>Bos</sup> and Vdr<sup>Leu</sup> null fetal mice show normal endochondral bone development and mineralization by term. This is very convincing evidence that calcitriol is not required during fetal development to regulate skeletal development and mineralization.

D. Role in the Neonate

Multiple models of disrupted vitamin D physiology have been studied during the neonatal period, including severely vitamin D-deficient rats (74, 241, 443), four different Vdr null mice (175, 372, 704, 755), and two different models of 1α-hydroxylase null (Cyp27b1 null) mice (138, 496). All of these appear normal at birth, although chemistries and ash weights have yet to be reported in any of the Cyp27b1 null models at birth. All models remain phenotypically normal through most of the succeeding 3 wk that the mother is lactating. As described earlier, near the time of weaning the intestines become less permeable to passive absorption of calcium, and more dependent on active, calcitriol-dependent intestinal calcium absorption. It is at this point in time that all of these models begin to develop hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and skeletal evidence of rickets (138, 241, 371, 372, 443, 496). However, a calcium-phosphate-lactose-enriched “rescue diet” bypasses the need for vitamin D/calcitriol or its receptor and allows each of these models to maintain normal serum chemistries, skeletal morphology, and skeletal mineral content (24, 64, 65, 140, 154, 175, 371, 372, 496, 638, 639, 704, 755).

These animal data clearly demonstrate that vitamin D, calcitriol, and its receptor are not required for normal fetal calcium homeostasis, skeletal development, and mineralization. It is at the time of weaning, when the intestines become the route of mineral delivery, that calcitriol becomes required. Furthermore, since arguably all of the skeletal man-

![Image of fetal skeletons stained with alizarin red and alcian blue](image1)

**FIGURE 11.** Skeletal morphology and mineral content in Vdr null fetuses. A and B show images of fetal skeletons (ED 18.5) stained with alizarin red (for mineral) and alcian blue (for cartilage). Skeletal morphology of the Vdr null was consistently normal, as shown by the normal crown-rump length, lengths of long bones, and mineralization pattern of the Vdr null (B) and its WT sibling (A). C and D are von Kossa preparations (counterstained with methyl green) of the upper halves of fetal tibias (ED 18.5), which include the growth plates and part of the tibial shafts. The overall morphology of the tibias, the lengths of the growth plates, and periosteal thickness were normal in Vdr null (D) compared with WT (C), and a normal amount of mineral (black) was present in the shafts of the tibias. E shows the calcium content (by atomic absorption spectroscopy) of Vdr null fetuses compared with WT (C), and a normal amount of mineral was present in the shafts of the tibias. F compares the magnesium content among the littermates. The calcium and magnesium content were normal in Vdr nulls. Ash weight was also normal (not shown). Scale bar indicates 100 μm for C and D. [Adapted from Kovacs et al. (342).]
ifestations of vitamin D-related rickets can be prevented by use of a high-calcium diet, these findings suggest that the main role of calcitriol with respect to the skeleton is not through direct actions on chondrocytes or osteoblasts, but indirect through upregulation of intestinal mineral delivery to provide mineral for chondrocytes and osteoblasts to use. This point has been confirmed by the observation that selective expression of Vdr in intestinal cells rescued the skeletal phenotype of Vdr null mice (375, 746), whereas after selective ablation of Vdr from intestinal cells a rachitic phenotype develops (375), and that phenotype can also be produced by dietary calcium restriction (375). Moreover, ablating Vdr or Cyp27b1 from cells within the skeleton does not cause rickets either. The skeleton is normal if Vdr is selectively ablated from chondrocytes, mature osteoblasts, or osteocytes (375, 421), and a high bone mass phenotype with reduced resorptive surfaces occurs when Vdr is ablated from osteoblasts (750). Ablating Cyp27b1 from chondrocytes does not reproduce the rachitic growth plate, whereas low phosphorus will cause the widened, irregular growth plate (466). The only evidence from mice supporting a possible direct role for calcitriol or VDR in bone cells is that Cyp27b1/Vdr null double mutants have a skeletal phenotype that is not completely prevented by the calcium-phosphate-lactose-enriched “rescue diet” (495).

E. Human Data

1. Observational studies and case reports

The most rigorous assessment of the fetal skeleton at birth involved babies that had died of obstetrical accidents: seven from mothers with clinically diagnosed osteomalacia and vitamin D deficiency and eight from otherwise healthy mothers (427). This 1925 report has been cited as confirming the existence of prenatal rickets, despite the fact that the paper concluded otherwise. The investigators obtained plain radiographs of the fetal long bones, and reduced the femurs to ash to measure the mineral content by atomic absorption spectroscopy. One “osteomalacic parent” baby was born prematurely at 7.5 mo and had an ash weight that was less than half of the other 14 near-term or term fetuses, which is a normal finding given that more than 80% of the mineral content of the human skeleton is deposited during the third trimester (224, 683, 734). But that single fetus’s low ash weight led to the investigators’ impression that “osteomalacic parent” babies had softer bone and lower ash weight compared with normal babies. No statistical analysis was done, but the raw data were presented in a table that permits such an analysis by modern methods. The calcium content (mean ± SE) was 374.2 ± 3.0 per thousand in “osteomalacic parent” babies versus 371.8 ± 13.9 per thousand in normal babies. Similarly, the phosphorus content was 189.0 ± 1.1 per thousand in “osteomalacic parent” babies versus 189.6 ± 1.6 per thousand in normal babies. The radiographs revealed no signs of rickets, and centers of ossification were normal (427). The authors speculated about “a curious cupping” at the ulnar ends in two babies born to osteomalacic mothers, but that is now known to be a normal variant (225). Maxwell et al. (427) concluded “there is no evidence of prenatal rickets” despite his initial enthusiasm that he would find such evidence. Rickets could not be present with normal skeletal mineral content because unmineralized osteoid is pathognomonic for the condition.

Epidemiological data show that hypocalcemia and rickets due to vitamin D deficiency are not usually diagnosed until weeks to months after birth; the peak incidence is between 6 and 18 mo, even in regions where vitamin D deficiency is endemic (17, 49, 332, 510). Clinically recognized cases within the first few weeks after birth are rare and the subject of individual case reports. Vitamin D deficiency is endemic in India, so clinical suspicion for its presence is high. Over 16 years Teotia et al. (666) examined 165 babies born of women with severe osteomalacia. Of these, only six babies showed abnormalities: three had postnatal hypocalcemia and three had postnatal rickets without hypocalcemia (666). In another paper summarizing their experience, Teotia et al. (667) wrote that congenital rickets is “uncommon and develops only when maternal mineral and vitamin D stores have been completely exhausted.”

This clinical experience of the natural history of rickets concurs with the results from the animal models in that the fetal skeleton should be normal at birth with rickets developing later. As explained earlier, intestinal calcium absorption is largely passive at birth but becomes calcitriol dependent as the baby matures (330, 332, 335); this is why vitamin D-dependent rickets usually develops after calcitriol becomes required to maintain the supply of calcium for the developing skeleton. Vitamin D-deficiency rickets also needs to be distinguished from rickets of prematurity, which is the result of the preterm skeleton having a much higher demand for calcium than what the preterm intestines can absorb from a normal intake of milk or formula (see sect. IIIE).

A few rare, isolated case reports from the past 100 years have demonstrated that physical, radiographic, or histological changes consistent with rickets may be detected at birth (50, 279, 400, 426, 429, 448, 577, 741), although only two of these reports have biochemical confirmation of low 25-hydroxyvitamin D levels (279, 448), while one measured serum vitamin D which is normally low in cord blood (400). This is an important distinction because skeletal evidence of rickets is not synonymous with vitamin D deficiency. Instead, rickets means an undermineralized skeleton that is primarily caused by deficiency of calcium or phosphorus, of which vitamin D deficiency or genetic disturbances of vitamin D physiology are but a few of the causes (539). The differential diagnosis of the rachitic-appearing fetal and
neonatal skeleton encompasses at least 50 different conditions, including fetal hypoparathyroidism, skeletal dysplasias, metaphyseal chondrodysplasias, hypophosphatasias, type V osteogenesis imperfecta, and scurvy. Consequently, it is possible that some of these cases of rickets were not due to vitamin D deficiency. For example, in a 1932 case report, the cord blood calcium was normal (2.65 mM) in the presence of an apparently undermineralized skeleton. In other cases, the babies hemorrhaged from multiple orifices before dying, which suggests other severe disturbances such as scurvy or vitamin K deficiency.

In other reports that described rickets as being present “at birth,” the diagnosis was actually made within the first or second week after birth, so the skeletal status at birth remains unknown. Neonatal rickets is a more appropriate term for such cases, as opposed to “congenital rickets,” because the latter implies that the condition was present at birth. Since the neonate normally adds 30–40 mg/kg of calcium per day to the skeleton while maintaining appropriate blood calcium and phosphorus levels, any impairment in intestinal absorption of mineral can quickly lead to progressive demineralization of the skeleton, and hypocalcemia. This is what happens in rickets of prematurity, for example, which is not caused by vitamin D deficiency but by the skeletal demand for calcium being far higher in the preterm baby than in the normal neonate. In one illustrative case of possible neonatal vitamin D-deficiency rickets, the clinicians anticipated from the maternal history that rickets might be present in the infant. However, convincing radiographic findings were not present at day 2 but had developed by day 16, confirming that radiographic evidence can develop rapidly after birth.

In many cases of neonatal rickets attributed by the authors to vitamin D deficiency, the cause was clearly not isolated vitamin D deficiency. Instead, the mothers had such problems as severe anorexia, malnutrition, malabsorption from celiac disease or pancreatic insufficiency, lactose intolerance, extreme avoidance of calcium and dairy food sources, use of drugs that interfere with vitamin D metabolism, or very low intakes of both calcium and vitamin D. This fits with Teotia’s impression that rickets will be present at birth only in extreme cases of complete nutritional exhaustion, or as Maxwell described in 1935, “women who have, in the matter of diet, been living near the starvation-line.” Vitamin D deficiency itself is normally not sufficient to adversely affect the development or mineralization of the fetal skeleton. But combined with other significant maternal nutritional deficiencies, an abnormal skeleton may be present at birth.

Additional case reports and case series have used questionable criteria to make a diagnosis of rickets, whether or not it is due to vitamin D deficiency. Craniotabes (the subjective determination that the skull is softer than normal) is a common criterion used alone to make the diagnosis of rickets in many of these reports, but public health data from several countries have shown craniotabes to be present in 30–50% of healthy infants, and to have no correlation with maternal or neonatal 25-hydroxyvitamin D levels or skeletal evidence of rickets. Consequently, craniotabes is not considered a reliable indicator of rickets or of vitamin D deficiency; its presentation may be predicted more by ethnicity and gestational age than vitamin D sufficiency. As mentioned earlier, distal ulnar cupping is a normal variant that should not be used to diagnose rickets. Although a large or slowly closing anterior fontanelle can be seen with rickets, these are nonspecific changes shared among more than 25 metabolic, genetic, and skeletal disorders. Use of widened fontanelle alone to indicate vitamin D deficiency led to misdiagnosis and maltreatment of a newborn with hypophosphatasia. Among the questionable reports that claimed vitamin D-deficient rickets to be present at birth are those that relied on the normal variant of ulnar cupping or craniotabes. One report noted craniotabes at birth but the skeletal radiographs were normal, but by 2 wk of age the baby had developed hypocalcemia, hyperphosphatemia, elevated alkaline phosphatase; the hyperphosphatemia indicates hypoparathyroidism and not vitamin D deficiency. The biochemical abnormalities normalized in the months following initiation of vitamin D treatment, but this likely reflected improvement in the hypoparathyroidism rather than being proof of vitamin D deficiency.

A single-authored report from Japan is particularly interesting for the claim that 60 of 255 healthy newborn infants had radiographic evidence of rickets at the wrist. The serum calcium, ionized calcium, phosphorus, and alkaline phosphatase were normal in cord blood and in the newborns over the first 8 days after birth, with no differences between the babies with “rickets” versus those without. The normal chemistries (especially calcium and alkaline phosphatase) are an indication that if the radiographic findings (ulnar cupping, etc.) represented true abnormalities, they were unlikely to be the result of vitamin D deficiency. Similarly, a single observer reported that rachitic rosaries and ulnar cupping were present in 75 of 858 consecutive newborn infants in Kuwait, who also had normal serum calcium, phosphorus, and alkaline phosphatase. Would other observers have agreed with that single observer’s subjective impression that rachitic rosary was present? 25-Hydroxyvitamin D was measured only in the 255 healthy newborn infants, and to have no correlation with maternal or neonatal 25-hydroxyvitamin D levels or skeletal evidence of rickets. Consequently, craniotabes is not considered a reliable indicator of rickets or of vitamin D deficiency; its presentation may be predicted more by ethnicity and gestational age than vitamin D sufficiency. As mentioned earlier, distal ulnar cupping is a normal variant that should not be used to diagnose rickets. Although a large or slowly closing anterior fontanelle can be seen with rickets, these are nonspecific changes shared among more than 25 metabolic, genetic, and skeletal disorders. Use of widened fontanelle alone to indicate vitamin D deficiency led to misdiagnosis and maltreatment of a newborn with hypophosphatasia (449). Among the questionable reports that claimed vitamin D-deficient rickets to be present at birth are those that relied on the normal variant of ulnar cupping or craniotabes (491, 534, 577, 752) or craniotabes (191, 752). One report noted craniotabes at birth but the skeletal radiographs were normal, but by 2 wk of age the baby had developed hypocalcemia, hyperphosphatemia, elevated alkaline phosphatase; the hyperphosphatemia indicates hypoparathyroidism and not vitamin D deficiency (191). The biochemical abnormalities normalized in the months following initiation of vitamin D treatment, but this likely reflected improvement in the hypoparathyroidism rather than being proof of vitamin D deficiency.
for the apparent findings. Another report from Japan suggested that the presence of craniotabes in 22% of consecutive newborns meant rickets, but since larger surveys have already shown that craniotabes is present in up to 30–50% of healthy newborns and to have no association with vitamin D sufficiency, that conclusion is not supportable (46).

A curious report from China examined 124 babies that had died either before or sometime after birth, with no data provided about the cause of death, the mothers’ health or obstetrical history, or the percentage that these babies represented of the catchment population (381). Sixteen fetuses and 28 neonates were said to have histological evidence of rickets (381). However, the two main criteria used by the investigator included an island (apophysis) of chondrocytes in the diaphysis, and an irregular growth plate (381). Islands or protuberances of cartilage into bone and an irregular growth plate are not standard or defining criteria for rickets and can be seen in a variety of other congenital bone disorders, including achondroplasias, osteopetrosis, collagen disorders, and other skeletal dysplasias (16, 88, 268, 503, 550, 680). The defining histological criterion for rickets is increased osteoid (unmineralized bone), but the report did not describe this. Nor were any data provided to support the hypothesis that the abnormalities were due to vitamin D deficiency and not any of the other conditions on the differential. Consequently, vitamin D-deficiency rickets cannot be confirmed to be present with any confidence from those data.

Overall, out of 100 years of published literature, these reports represent a very uncommon occurrence. For those cases that may have been truly due to vitamin D deficiency, it should be clear that these are exceptional cases and not reflective of vitamin D deficiency during pregnancy that is commonly seen today. Severe hypocalcemia does not usually occur in vitamin D deficiency because secondary hyperparathyroidism minimizes the fall in serum calcium. But the descriptions of the mothers in the case reports above underscore how extreme their conditions were, with serum calcium levels often half normal, signs of globally poor nutrition, carpopedal spasms, positive Chvostek’s signs, hypotonia, severe osteomalacia, severe back pain, waddling gait, etc. The confounding presence of other nutritional deficiencies makes it difficult to be certain the degree to which vitamin D deficiency contributed to the fetal or neonatal phenotype. But it seems likely that vitamin D deficiency alone did not explain these rare presentations.

Additional cohort studies have reported that bone mineral content in newborns did not differ by measures of vitamin D sufficiency in the mother or baby (122, 725). Congdon et al. (122) studied infants born to 45 untreated Asian women, 19 Asian women who had received 1,000 IU vitamin D during the third trimester, and 12 unsupplemented Caucasian women. Mean cord blood concentrations of 25-hydroxyvitamin D were 5.9, 15.2, and 33.4 nM, respectively (122). There were no significant differences in serum calcium, phosphorus, and alkaline phosphatase, while bone mineral content of the forearm, measured within 5 days of birth, did not differ among groups (122). Weiler et al. (725) measured bone mineral content of the lumbar spine, femur, and whole body by DXA within 15 days after birth in 50 healthy term infants. Mean cord blood 25-hydroxyvitamin D was 73 and 36 nM in the sufficient and insufficient Caucasians, 44 nM in Asians, and 27 nM in First Nations infants. Differences in bone mineral content were seen that were attributable to ethnicity but not vitamin D sufficiency (725).

Viljakainen et al. (712) divided a cohort of 87 children into two groups above and below the median cord blood 25-hydroxyvitamin D, and measured DXA soon after birth and at 14 mo. There were no differences in anthropometric parameters or tibial bone mineral density at either time point. Tibial bone mineral content and cross-sectional area of the tibia were slightly lower at baseline in the cohort with lower 25-hydroxyvitamin D; the difference in bone mineral content was gone at 14 mo while a difference in cross-sectional area persisted. Notably bone mineral content is affected by bone size or area, whereas bone mineral density corrects for this. The investigators noted disparities in sex and size of the newborns above and below the median 25-hydroxyvitamin D level, and those disparities may account for the transient difference in bone mineral content noted at birth.

If vitamin D deficiency alone could readily cause prenatal rickets, then even more severe abnormalities would be expected to occur in fetuses and neonates bearing inherited mutations of vitamin D physiology, including the inability to synthesize calcitriol (1α-hydroxylase deficiency; pseudovitamin D deficiency rickets; vitamin D dependent rickets type I; VDDR-I) or the expression of non-functional vitamin D receptors (hereditary vitamin D-resistant rickets; vitamin D dependent rickets type II; VDDR-II). But these babies appear normal at birth (apart from alopecia in VDDR-II) and have normal calcium levels (66, 180, 312, 408, 538, 621, 662, 665). In both conditions the child is fated to develop hypocalcemia, hypophosphatemia, and rickets. VDDR-I presents within the first 3–12 mo after birth; VDDR-II can also present in infancy but has often been diagnosed during the second year or even later (36, 66, 230, 258, 312, 538, 621, 662, 665, 727). Mortality can also occur in infancy or childhood due to the severity of the hypocalcemia (36, 374). Pseudofractures and fractures can occur in both conditions, but bowing of the long bones and metaphyseal widening are much more common (36, 66, 180, 230, 258, 312, 408, 538, 621, 662, 665, 727).

Available clinical data from pediatric and older age VDDR type II children are also consistent with the animal data in...
that calcitriol’s role to regulate intestinal calcium absorption can be bypassed. Repeated intravenous or intracaval calcium infusions, or high oral dose calcium, have been shown to normalize calcium, phosphorus, PTH, alkaline phosphatase; rickets was prevented or healed in these children (36, 259, 312, 727). In one case series, the use of parenteral calcium caused growth velocity to increase, bone pain to decrease, and biochemical abnormalities to normalize within a year; it also enabled some affected children to walk for the first time ever (259). Rickets healed more rapidly in younger patients and when parenteral calcium was used instead of high dose oral calcium. For VDDR type I, the high-calcium approach is usually not needed because physiological doses of calcitriol or 1α-hydroxyvitamin D should normalize intestinal calcium absorption (662).

Once weight bearing begins, the natural history of untreated vitamin D-deficiency rickets includes progressive deformities of the long bones (enlargement of the wrist and bowing of the distal radius and ulna, and lateral bowing of the femur and tibia), distorted growth plates, and short stature (513, 514). These changes are the result of the softer, malleable structure of rachitic bone which bends in response to weight-bearing activity, and the resulting varus and valgus deformities can be very dramatic. Notably, fractures of the long bones are not a dominant feature of rickets, but when they do occur they are single and not multiple. A study of 736 Nigerian children aged 18 mo or older found that previous fracture was present in 9% with active rickets, 7% with early or nearly healed rickets, and 9% of normal children (668). The other clinical features that predicted rickets were age <5, height-for-age Z-score <2 standard deviations, leg pain during walking, wrist enlargement, and costochondral enlargement (668).

Oddly, despite the evidence that overt rickets is not associated with an increased risk of fracture, vitamin D insufficiency has been proposed to cause marked skeletal fragility in infants such that it would mimic the multiple fractures seen in physically abused infants and others with osteogenesis imperfecta (303). Moreover, it has been suggested that for a 6-mo window of time the infant is transiently susceptible to fragility from vitamin D insufficiency in what has been termed “temporary brittle bone disease” (504). Since severe vitamin D deficiency causes slowly developing deformities of the limbs without more fractures than in normal children (668), how could the more modest condition of vitamin D insufficiency be a cause of overt skeletal fragility with multiple atraumatic fractures? This seems a theory that lacks credible evidence to support it; consequently, it has been thoroughly discredited by others (181, 182, 286, 287, 414, 629, 637, 651). The Society for Pediatric Radiology and the European Society of Paediatric Radiology jointly wrote that the diagnosis fails to meet minimum standards of reliable scientific information that can be presented in the courts of law “because it lacks appropriate grounding in scientific methods and procedures...it is not generally accepted within the field of radiology, but is instead based on the unsupported speculation and subjective beliefs of a very small number of medical professionals” (436).

Overall, the human genetic disorders, the population surveillance data on the incidence of rickets, 100 years of case reports of extreme cases, and much of the clinical observational studies pertaining to vitamin D deficiency, are consistent with the animal models in that hypocalcemia and rickets are not likely to be present at birth. Instead, hypocalcemia and rickets will develop postnatally in children with severe vitamin D deficiency or genetic disorders causing loss of calcitriol or VDR, with the peak age of presentation occurring not at birth but between 6 and 18 mo of age. Furthermore, calcitriol’s role in stimulating intestinal calcium absorption can be bypassed by enriching the diet or administering calcium parenterally. Left untreated, the long bones are malleable, resulting in limb deformities, short stature, and eventually osteoarthritic changes from the deformed joints.

2. Interventional studies

One of the earliest randomized trials of vitamin D supplementation still stands as the most definitive because it involved women who were severely vitamin D deficient by today’s standards (77). Brooke et al. (77) studied 126 Asian women living in England, 59 who were treated with vitamin D and 67 controls. The mean 25-hydroxyvitamin D level was 20 nM at baseline and the treatment group achieved a level of 168 nM at term; this marked difference was ideal for determining if correction of vitamin D deficiency resulted in any maternal or fetal benefits. The treatment group nominally received 1,000 IU vitamin D per day but must have received 10,000 IU per day or more to have reached that increment in 25-hydroxyvitamin D. The mean cord blood 25-hydroxyvitamin D level was 10 nM in control babies and 138 nM in babies born of the treated mothers. Cord blood calcium and phosphorus showed no statistically significant differences between the control and treated babies. Treatment also had no effect on birth weight, crown-heel length, forearm length, triceps skinfold thickness, and head circumference, but a small decrease in femur/tuber diameter was observed (77). There was also a higher alkaline phosphatase level in control babies, but fractionation confirmed that this was entirely accounted for by the placental fraction. The babies were examined radiologically, and there were no signs of rickets in either group (77).

Interestingly, at days 3 and 6 after birth, the control group had a significantly lower blood calcium than in the vitamin D-replete babies (2.18 ± 0.04 vs. 2.30 ± 0.04 mM, P < 0.05) (77). Five infants in the control group had symptomatic hypocalcemia (irritability and serum calcium <1.8 mM), whereas none of vitamin D-replete babies became hypocalcemic (77). These results mirror the findings in the
previously discussed animal studies, in which serum minerals will be normal at birth but fall postnatally when severe vitamin D deficiency is present. In one of two follow-up analyses of the same data (which have been mistaken as separate trials by other reviewers), a slightly greater increment in weight and length over the subsequent 12 mo was seen in the babies of vitamin D-supplemented mothers (78, 425).

Cockburn et al. (116) randomized 164 women to receive either 400 IU vitamin D beginning at the 12th week of pregnancy or nothing. Cord blood 25-hydroxyvitamin D was 28 versus 20 nM in the treated versus controls, and there was no significant difference in cord blood calcium or phosphorus. However, by the sixth day, calcium was significantly higher while phosphorus was significant lower in the infants whose mothers had received vitamin D compared with controls. A lower phosphorus is not understandable in the context of a higher 25-hydroxyvitamin D level.

In a smaller study of 77 women from France, Mallet et al. (407) administered 1,000 IU vitamin D2 daily during the third trimester, or a single dose of 200,000 IU vitamin D2 at the start of the third trimester, or no supplementation (407). The two treatment arms raised cord blood 25-hydroxyvitamin D to 15.7 and 18.2 nM compared with 5.3 nM in controls. There were no significant differences in maternal, cord blood, and day 2 or 6 neonatal calcium levels, hypocalcemic tetany, and skeletal or anthropometric parameters of the babies.

A randomized study by Delvin of 40 women in France compared 1,000 IU vitamin D3 supplementation daily to placebo starting at 6 mo of pregnancy, and raised the cord blood 25-hydroxyvitamin D from 17.5 to 45 nM (153). There were no effects on skeletal parameters or on the cord blood calcium, phosphorus, or PTH. However, at 5 days of age, the treated group had a significantly higher serum and ionized calcium (153).

Marya performed two studies in vitamin D-deficient Asian women in India (419, 420). In the first study there were three treatment arms: 800,000 IU vitamin D2 was given in the 7th and 8th months of pregnancy to 20 women, 1,200 IU vitamin D2 per day were given to 25 women during the third trimester, and 75 women received nothing. There was a small increase in uncorrected cord calcium in babies of high-dose mothers compared with babies of untreated mothers (2.68 vs. 2.52 mM in untreated), but no change in serum phosphorus or other benefit (420). The second study administered 600,000 IU vitamin D2 in the 7th and 8th months of pregnancy to 100 women and gave nothing to 100 others. A significant increase in uncorrected cord blood calcium was observed in the treatment group (2.77 vs. 2.57 mM) with no difference in cord blood phosphorus (419). A slightly greater birth weight, crown-heel length, and head circumference were also found in the babies from treated mothers (419). Cord blood 25-hydroxyvitamin D was not measured in either study. The lack of disclosure of methodological details in the two studies, especially whether and how the subjects were truly randomized, leaves the results somewhat questionable.

Yu et al. (756) conducted a randomized unblinded trial in London of 180 women, with three arms beginning at week 27 of pregnancy: 800 IU vitamin D2 per day, a single dose of 200,000 IU of vitamin D3, or no treatment (756). Mean cord 25-hydroxyvitamin D was significantly higher in the two treatment groups compared with the controls (26, 25, and 17 nM, respectively). However, there were no significant differences in gestational age at delivery, birth weight, or incidence of low birth weight.

Roth et al. (567) completed a trial in Bangladesh (NCT01126528) in which 160 women were randomized to 35,000 IU/wk vitamin D3 or placebo beginning at 26–29 wk until delivery. Achieved cord blood levels of 25-hydroxyvitamin D were 103 versus 30 nM, respectively. There was no effect on length, weight, head circumference, and femur length at birth (568). Cord blood calcium levels were done but have not been reported.

A similar study by Hashemipour et al. (246) in Iran randomized 160 women to receive 50,000 IU vitamin D per week or 400 IU daily beginning at 26–28 wk for 8 wk (246). Cord blood 25-hydroxyvitamin D was 69 versus 27 nM, and cord blood calcium (not corrected for albumin) was also significantly higher in the vitamin D-treated babies at 2.48 versus 2.28 mM. There was no difference in the incidence of hypocalcemia between the groups. Small but statistically significant increases in newborn weight, length, and head circumference were seen in the vitamin D-treated babies.

Grant et al. (233) completed a study in New Zealand (ACTRN1261000483055) that randomized 260 women to placebo, 1,000 IU vitamin D daily, or 2,000 IU vitamin D daily. Cord blood 25-hydroxyvitamin D levels were 33, 60, and 65 nM, respectively. There was no difference in serum calcium among the three groups at 2.58, 2.62, and 2.60 mM, respectively (233). Postnatally the infants continued in their treatment arms by receiving placebo, 400 IU, or 800 IU of vitamin D daily. 25-Hydroxyvitamin D rose faster in the high-dose group, but by 6 mo of age the values were no different among the three groups of infants (78, 85, and 84 nM, respectively), and there was no difference in serum calcium at any postnatal time point (233).

Kalra et al. (294) completed a trial in India in which 97 women were randomized to one dose of 60,000 IU vitamin D in the second trimester or 120,000 IU vitamin D in each of the second and third trimesters; 43 other women who
received standard care served as controls. Achieved cord blood 25-hydroxyvitamin D median values were 28.2, 24.1, and 18.5 nM, respectively, and there was no difference in cord blood calcium across the groups (294). Small increments in length, head circumference, and weight, and a slight decrease in anterior fontanelle length, were seen in the both vitamin D groups compared with the nonrandomized controls (294).

Hollis et al. (262) recently completed two larger trials. The first (NCT00292591) randomized women at 12–16 wk of gestation to receive 400, 2,000, or 4,000 IU vitamin D3 per day; 350 completed the study out of the planned 516 enrollment (262). The primary outcome of the first study was a combination of the 25-hydroxyvitamin D levels and bone mineral density of both mother and infant; however, the BMD values have not been published. Achieved cord blood mineral density of both mother and infant; however, the BMD values have not been published. Achieved cord blood 25-hydroxyvitamin D was 46, 57, and 66 nM, respectively. The second study (NCT00412087) randomized women at 12–16 wk of gestation to receive 2,000 or 4,000 IU vitamin D3 per day; 160 completed this study out of the planned 559 (717). Achieved cord blood 25-hydroxyvitamin D was 55 and 68 nM, respectively. The cord blood calcium values for both studies, and the bone density results for the first study, have been described verbally by the authors in public presentations as showing no significant differences (716). In the first study, 4,000 IU vitamin D had no effect on gestational age at delivery, birth weight, mode of delivery, need for level II or III neonatal care, cord blood calcium, preterm birth, preterm labor, preeclampsia, infection, or BMD (261, 262, 716). Post-hoc analyses of both studies have suggested that some combinations of nonskeletal obstetric or neonatal outcomes may show a trend to improvement with higher achieved 25-hydroxyvitamin D level, but only after arbitrary exclusion of certain races from the analysis (261, 262, 265, 716).

Whether vitamin D supplementation during pregnancy has any effect on neonatal bone mass may be determined by the Maternal Vitamin D Osteoporosis Study (MAVIDOS), in which more than 1,000 women have been randomized to receive 1,000 IU vitamin D or placebo daily from 14 wk of gestation until term (245). The primary outcome is the whole body bone mineral content of the neonates as assessed by DXA.

Overall, these trials have not shown any compelling evidence that higher dose vitamin D supplementation achieving any benefit related to calcium and bone metabolism prior to birth, although there is likely a decreased risk of hypocalcemia after birth when the starting 25-hydroxyvitamin D level is very low, as in the Brooke study. When the basal level of 25-hydroxyvitamin D is higher than that, there may be no additional benefit from supplementation.

3. Associational studies

A number of associational studies have recently drawn attention for their inferences that higher intakes of vitamin D may be needed during pregnancy to ensure optimal bone health in the fetus, infant, and child. These studies have examined associations between estimated maternal intake of vitamin D or single measurements of maternal 25-hydroxyvitamin D during pregnancy, and various skeletal outcomes in the offspring. In agreement with data from observational studies and randomized trials discussed above, there were no significant associations found with weight, skeletal lengths, or bone mineral density at birth (281, 285, 363, 399, 460, 713).

A study by Morley et al. (460) concluded that maternal 25-hydroxyvitamin D below 28 nM predicted a slightly shorter knee-heel length in newborns as assessed by a handheld device. However, left unstated in the abstract was that the association was not statistically significant after correcting for gestational age of the babies.

Seemingly opposing results were obtained in studies by Mahon et al. and Viljakainen et al. in which increased metaphyseal area of the femur or tibia was associated with lower (399) or higher (713) maternal 25-hydroxyvitamin D during pregnancy. Curiously, for each study the investigators concluded that an adverse effect of low 25-hydroxyvitamin D had been found. Mahon et al. (399) considered greater cross-sectional area of the femoral metaphysis to be evidence of prenatal rickets in 242 mother-baby pairs assessed by high-resolution ultrasound, while Viljakainen et al. (713) considered greater cross-sectional area of the tibial metaphysis to be an indication of stronger bone in 125 mother-baby pairs assessed by pQCT. More recently Ioannou, together with Mahon and colleagues, adapted their ultrasound technique to calculate femoral volumes in 357 mother-baby pairs (281). Maternal 25-hydroxyvitamin below 75 nM was associated with slightly smaller femur length, width, and volume, an association that is in the opposite direction from the original report by Mahon and co-workers (281). Although maternal 25-hydroxyvitamin D level predicted fetal femoral volume in a univariate analysis, there was no association after adjusting for maternal height and skinfold thickness (280). The seemingly contradictory results in the studies by Mahon, Viljakainen, and Ioannou could indicate that vitamin D has complex, site-specific effects on skeletal development, or these could be chance findings with no true causal link between maternal 25-hydroxyvitamin D during pregnancy and the areas or volumes of fetal long bones.

A study by Javaid et al. (285) has drawn much attention. In 198 mother-child pairs there were no significant associations between maternal serum 25-hydroxyvitamin D during pregnancy and birth weight, length, placental weight, abdominal circumference, head circumference, or cord blood
calcium (285). There were also no associations of maternal 25-hydroxyvitamin D with skeletal and anthropometric parameters in the infants at age 9 mo. But at 9 years of age, children whose mothers had had a 25(OH)D below 27.5 nM during pregnancy had a slightly but significantly lower bone mineral content by DXA than children of mothers whose 25-hydroxyvitamin D had been ≥50 nM. These data confirm the theory that vitamin D exposure during fetal development programs bone mass attained later in childhood (123). However, a recent study by Lawlor et al. (363) analyzed ~20 times the number of mother-child pairs than Javaid (3,960 vs. 198) and found no significant association between maternal 25-hydroxyvitamin D during pregnancy and offspring bone mass at age 9 years, as assessed by DXA. Lawlor et al. (363) had almost eight times the number of women with 25-hydroxyvitamin D below 27.5 nM (220 vs. 28 mothers) and should have had greater power to detect an association. But the contradictions continue, because Lawlor’s colleagues originally reported from the same database that estimated maternal UVB exposure in the third trimester of pregnancy in 6,995 mother-offspring pairs positively predicted offspring bone mass at age 9 years as assessed by DXA (593). The difference between the two studies by Lawlor and colleagues may be that estimated UVB exposure is a less accurate variable than the measurement of 25-hydroxyvitamin D. Overall, it is challenging to conclude anything at present from these contradictory studies, which could be reporting chance associations rather than indications of causal links.

These associational studies are confounded by factors that contribute to low maternal 25-hydroxyvitamin D, including maternal obesity, lower socioeconomic status, poorer nutrition, and lack of exercise, prenatal care, vitamin supplementation, etc. Consequently, a lower value of 25-hydroxyvitamin D may simply predict a less healthy pregnant woman, rather than revealing cause-and-effect results of low maternal 25-hydroxyvitamin D on fetal bone health. Furthermore, the mother’s 25-hydroxyvitamin D value in pregnancy may predict factors that will remain unchanged and be shared or held in common with the child (lower socioeconomic status, poorer nutrition, obesity, etc.), in turn influencing childhood growth and bone mass at age 9 years without invoking a causative role for 25-hydroxyvitamin D exposure prior to birth.

Some of the associational studies have much larger sample sizes than in the clinical trials of vitamin D supplementation, and therefore may be detecting real differences that the trials could not. Alternatively, the associational studies may simply be reporting “noise” caused by residual confounding, which in turn could explain the contradictory findings. A basic principle of associational studies is that causation cannot be proven from the results, but hypotheses may be raised that warrant further testing.

F. Summary of Key Points

Overall, the available animal and human data indicate that cord blood calcium, phosphorus, and skeletal morphology and mineral content should be normal at birth despite vitamin D deficiency, VDDR-I, or VDDR-II. In rare instances where the mother is more profoundly unwell due to vitamin D deficiency combined with global malnutrition or other factors, the baby may develop skeletal signs of rickets that are detectable at birth. Consistently, it is after birth that hypocalcemia and rickets will develop if severe vitamin D deficiency persists, or VDDR-I and VDDR-II are not recognized and treated. The peak incidence of vitamin D deficiency rickets is at 6–18 mo after birth. Larger-scale clinical trials of vitamin D supplementation during pregnancy that systematically measure skeletal mineral content and morphology in newborns, and larger and carefully executed associational studies, are needed to be certain whether vitamin D deficiency in utero has any effects on skeletal mineralization at term or later in childhood. Several systematic reviews agree with the conclusion that current data are insufficient to know if vitamin D supplementation during pregnancy confers any skeletal or nonskeletal benefits on fetuses (244, 560, 561).

IX. CALCITONIN

Ctcgrp encodes both calcitonin and calcitonin gene-related peptide-α, and ablation of it results in Ctcgrp null fetuses appearing in the expected Mendelian ratios. In contrast, ablation of the calcitonin receptor (Ctr) causes embryonic lethality at mid-gestation for unknown reasons (134, 361). Ctcgrp null mice live normal lifespans and are fertile, but show a small but significant reduction in the number of viable fetuses the day before expected birth compared with Ctcgrp+/− mothers (430). Calcitonin is expressed in the endometrium during the time of implantation (349), and attenuation of its expression blocks implantation of the early embryo (760). Taken together, these findings may indicate that calcitonin plays critical roles during implantation and during development until mid-gestation. That Ctcgrp null fetuses do not experience embryonic lethality may indicate that maternal calcitonin compensates for its absence in the early embryo before the placental barrier is in place, whereas such rescue would not be possible in Ctr nulls due to lack of the receptor. Alternatively, Ctr may also mediate signaling by unknown ligands that are critical during embryonic development.

A. Regulation of Serum Minerals

There is some evidence for calcitonin having a role in regulating fetal mineral concentrations. Infusion of calcitonin antiserum into fetal rats at day 21.5 of gestation caused a 0.16 mM increase in blood calcium 1 h later (200), while
pharmacological doses of calcitonin injected into fetal rats or lambs caused hypocalcemia and hypophosphatemia (41, 211). However, when calcitonin deficiency was created by fetal thyroidectomy followed by thyroxine replacement, the blood calcium in fetal lambs was unaltered (97).

The potential role of calcitonin has been investigated in Ctcgrp null fetal mice. Serum calcium and phosphorus were normal, but serum magnesium was significantly reduced compared with the littermates (430). This finding also occurred in Ctcgrp null fetuses from both Ctcgrp+/− and Ctcgrp null mothers (430) and indicates that loss of either calcitonin or calcitonin gene-related peptide-α affects magnesium homeostasis.

Calcium, phosphorus, and magnesium content of amniotic fluid were normal in Ctcgrp null fetuses. PTH showed a nonsignificant increase among Ctcgrp+/− and Ctcgrp null fetuses compared with WT.

B. Regulation of Placental Mineral Transport

A reduction in placental mineral transport has been inferred from intact lambs in which pharmacological doses of calcitonin blunted the effect of PTHrP1–34 and PTHrP1–36 to increase the mineral content of the skeleton at term (40). However, that indirect measurement could have resulted from reduced incorporation of calcium into bone, as opposed to a specific reduction in placental calcium transport. In contrast, fetal thyroidectomy with subsequent thyroxine replacement did not alter placental calcium transport in sheep (298).

Placental transport of calcium was unaltered in Ctcgrp null fetuses from both Ctcgrp+/− and Ctcgrp null mothers (430). Magnesium transport has not been measured.

C. Regulation of Endochondral Bone Formation

Endochondral bone development appeared normal in Ctcgrp null fetuses as assessed by examination of whole mount fetuses, tibial histomorphometry, and gene expression within the developing endochondral skeleton (430). The ash weight and calcium content were also normal, but there was a modest but statistically significant reduction in skeletal magnesium content of Ctcgrp null fetuses which paralleled the reduction in serum magnesium (430).

D. Role in the Neonate

No disturbances in mineral metabolism have been reported in Ctcgrp null neonates.

E. Human Data

Genetic calcitonin deficiency has not been described in humans. A few early studies suggested that the normal high calcitonin levels in the neonate could contribute to hypocalcemia in preterm infants (558, 583, 595). However, subsequent studies found that gestational age at birth was a more significant determinant of neonatal calcitonin levels than the postnatal fall in serum calcium or the development of hypocalcemia (444, 558, 707). Therefore, the available human data suggest that calcitonin may have little role in mineral homeostasis of the neonate.

F. Summary of Key Points

Calcitonin may play critical roles during implantation and early embryonic development, which could explain the embryonic lethality caused by loss of CTR. Loss of calcitonin and calcitonin gene-related peptide-α caused a selective reduction in serum magnesium and skeletal magnesium content, but no other disturbance in bone and mineral metabolism. Whether loss of calcitonin or calcitonin gene-related peptide-α exerts this effect on magnesium is unknown.

X. FIBROBLAST GROWTH FACTOR-23

Fibroblast growth factor-23 (FGF23) plays a key role in regulating phosphorus metabolism in postnatal mice and humans. FGF23 forms a complex with membrane-bound or soluble forms of its co-receptor Klotho and one of four FGF receptors (usually FGFR1c), and downregulates expression of sodium-phosphate cotransporters 2a and 2c (NaPi2a and NaPi2c) within the proximal renal tubules (350, 614, 699, 731). It also inhibits the renal 1α-hydroxylase (Cyp27b1), increases expression of 24-hydroxylase (Cyp24a1), inhibits PTH, and inhibits intestinal expression of NaPi2b (161, 267, 415, 614). Loss of FGF23 leads to impaired renal phosphorus excretion, hyperphosphatemia, increased calcitriol, increased intestinal phosphorus absorption, skeletal abnormalities, extraskeletal calcifications, and early mortality (161, 267, 415). Conversely, excess FGF23 causes enhanced renal phosphorus excretion, hypophosphatemia, low calcitriol, reduced intestinal phosphorus absorption, and rickets or osteomalacia (19, 161, 267).

A. Regulation of Serum Minerals

Intact FGF23 is undetectable in Fgf23 null fetal sera, but serum phosphorus and calcium, amniotic fluid phosphorus and calcium, calcitriol, and PTH are all normal (395). Conversely, Phex null male fetuses (also known as Hyp fetuses) have 7.8-fold increased intact FGF23 in one study (395) and 1,000-fold increased in another (484). Despite elevated FGF23, Phex null males have normal serum phosphorus and calcium, amniotic fluid phosphorus and calcium, and
PTH (395, 484). Serum calcitriol was halved in Phex null males compared with WT fetuses (395, 484).

Fetal kidneys showed abundant expression of Klotho, NaPi2a and NaPi2c, Cyp27b1, Cyp24a1, and Fgfr1-Fgfr4 (395). But in Fgf23 null fetal kidneys, the only change in expression was downregulation of Cyp24a1 (395). Conversely, in Phex null male fetal kidneys there was significantly increased expression of Cyp24a1 (395, 484) and decreased expression of Klotho and Fgfr2 (395), whereas all other FGF23 targets showed no changes (395, 484).

These results show that neither loss nor excess of FGF23 disturbs serum phosphorus or renal excretion of phosphorus into amniotic fluid, but excess FGF23 did lower calcium into amniotic fluid, but excess FGF23 did lower calcitriol, likely through increased Cyp24a1-mediated catabolism. The relative lack of responsiveness to deficiency or excess of FGF23 was not due to failure of expression of FGF23 target genes.

### B. Regulation of Placental Mineral Transport

Murine placentas showed abundant expression of Klotho, NaPi2a, NaPi2b, NaPi2c (NaPi2b expression was greater than NaPi2a and NaPi2c), Cyp27b1, Cyp24a1, and Fgfr1-Fgfr4; there was also low level expression of Fgf23 (395). But in Fgf23 null fetal placentas the only significant change was loss of Fgf23 (395). Conversely, in Phex null male placentas, there was significantly increased expression of Cyp24a1 (395, 484) and a threefold increase in Fgf23 (P = 0.054) (395), whereas all other FGF23 targets showed unchanged expression (395, 484).

Placental transport of $^{32}$P was assessed using the procedure for intact fetuses. Fgf23 null and Phex null male fetuses showed a normal rate of placental $^{32}$P transport at 5 min, confirming that neither absence nor excess of FGF23 affected placental phosphorus transport (395).

Therefore, FGF23 does not regulate placental phosphorus transport, despite expression of the relevant FGF23 target genes within the placenta.

### C. Regulation of Endochondral Bone Formation

In Fgf23 null and Phex null male fetuses, endochondral development appears normal the day prior to birth. Tibias showed no alteration in the length or cellular morphology of the cartilaginous or osseous compartments, and a normal distribution of mineral (395). Skeletal ash weight and ash mineral content (calcium, magnesium, phosphorus) were also unchanged in Fgf23 null and Phex null male fetuses compared with their WT siblings (395).

### D. Role in the Neonate

Scant data are available pertaining to the role of FGF23 in phosphorus metabolism of the neonate. In WT mice, FGF23 spikes to 10-fold higher than adult values within 12 h after birth, concurrent with a transient increase in serum phosphorus (34). But this increase appears nonessential because Fgf23 null mice have normal serum calcium and phosphorus at delivery (395) and 1 day after birth (615). Hyperphosphatemia and increased calcitriol are evident in Fgf23 nulls at 10 days after birth, and hypercalcemia by 2 wk (615). Reduced renal phosphorus excretion is present at 3 wk after birth in Fgf23 nulls (the earliest time point studied) (626). Fgf23 nulls are born with normal length and weight but are smaller by 2 wk of age (615). By 3 wk of age, the skeleton is undermineralized and progressive limb deformities develop, but whole body mineral content is increased due to excessive mineralization of soft tissues, including heart, lungs, and kidneys (468, 615, 626, 627). Mortality is 100% by 10–12 wk (468, 615, 626, 627).

Phex null mice are similarly indistinguishable from their WT siblings at birth, and as adults they are commonly identified by low serum phosphorus rather than genotyped. Although serum phosphorus is normal at birth (395), Phex null pups surprisingly develop modestly increased serum phosphorus compared with WT from 2 h until 10 h of age (34). Hypophosphatemia begins at 24 h and is accompanied by increased FGF23, low calcitriol, and high PTH (34). By 72 h, the tibias display slight shortening, normal bone volumes, but fewer osteoblasts and osteoclasts within the trabecular compartment (34).

The neonatal findings reveal that absence or excess FGF23 exert powerful effects on mineral metabolism in the neonate, in striking contrast to a lack of effect in the fetus.

### E. Human Data

Intact FGF23 rises from the low levels in cord blood to reach adult values by 5 days after birth (661). Genetic loss of FGF23 action causes familial hyperphosphatemic tumor calcinosis, which commonly presents during the first two decades of life with calcific tumor masses, hyperphosphatemia, elevated calcitriol, and normal or elevated serum calcium (19, 161, 267, 494, 531). It has presented as early as 18 days after birth with a calcific mass and hyperphosphatemia (527, 628) and as late as age 65 (494). Specific causes include missense mutations in FGF23, inactivating mutations in GALNT3 which normally O-glycosylates FGF23 to prevent its premature cleavage and inactivation, and inactivating mutations in KLOTHO which make FGF23 unable to activate its receptor (19, 161, 267). Hereditary disorders of excessive FGF23 action include X-linked hypophosphatemic rickets, autosomal dominant hy-
pophosphatemic rickets, autosomal recessive hypophosphatemic rickets, and McCune-Albright syndrome (267). All display hypophosphatemia, relatively high FGF23, inappropriately normal or low calcitriol, normal to low PTH, normal calcium, myopathy, and rickets or osteomalacia (19, 161, 267). X-linked hypophosphatemic rickets is the seminal example of excess FGF23 action. It can be diagnosed during the first year when suspected by family history and early onset of hypophosphatemia (452), but in the absence of family history it more often presents at 2 or 3 years of age with bowing of the long bones or growth failure, or at later ages with short stature (102, 172, 588). In four cases in which affected parents prompted an early diagnosis, serum phosphorus was low in all four between 2 and 6 wk of age, tubular reabsorption of phosphorus was low at 9 days of age in one but normal in the others until 6 mo, and radiographic findings of rickets did not develop until 3–6 mo (452).

F. Summary of Key Points

FGF23 does not contribute importantly to fetal regulation of serum phosphorus, placental phosphorus transport, or skeletal development and mineralization. Its effects may be curtailed by the limited role that the fetal kidneys play in mineral homeostasis. FGF23 begins to have significant effects within days after birth such that deficiency or excess of FGF23 alter serum phosphorus and calcitriol, although clinical manifestations may not appear until years later.

XI. SEX STEROIDS

A. Animal Data

Mice lacking estrogen receptor alpha or beta, or the aromatase, have normal skeletal lengths at birth and do not develop altered skeletal metabolism until several weeks after birth (125, 391, 462, 711, 738). At 3 wk of age (the first postnatal measurement) in male estrogen receptor knockout mice, there was no difference in total weight, crown-rump length, femur length, and bone density at multiple skeletal sites (711). Therefore, it is likely that no difference is present at the end of fetal development and that mineral metabolism is undisturbed through at least 3 wk of age. However, no fetal studies have been done.

Estradiol exerts its effects on osteoblasts in part by downregulating the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) and upregulating the expression of its decoy receptor osteoprotegerin (OPG). The RANKL-OPG-RANK system plays a critical role in regulating the formation, recruitment, and function of osteoclasts within the adult. However, it may be relatively unimportant from the perspective of the fetal skeleton and regulation of mineral homeostasis, and in turn, this may explain why loss of the estrogen receptor has no obvious effect during fetal development. Deletion of RANKL results in osteopetrotic changes in mice by 2 days after birth, whereas growth and serum calcium, phosphorus, and alkaline phosphatase remain normal until weaning (322). Deletion of RANK similarly leads to a normal appearance at birth. However, the mice are stunted and osteopetrotic by 3 wk of age, tooth eruption fails to occur, and they also develop hypocalcemia, hypophosphatemia, and secondary hyperparathyroidism (370). An opposing phenotype of severe osteoporosis with arterial calcifications occurs in mice lacking OPG (85, 447). Although grossly normal until a month of age, slight trabecular and cortical thinning are evident in femurs and vertebral bodies within 7 days after birth, and fractures of spine and long bones can occur before weaning (85). No studies have been done in fetuses.

B. Human Data

The only human data come from case reports, such as a man with an inactivating mutation of the estrogen receptor who had normal birth weight, length, and early development (630). He later presented with a severe form of osteoporosis.

C. Summary of Key Points

Overall, although estradiol and testosterone play important roles in regulating calcium and bone metabolism in the adult, in part through regulating RANKL and OPG, the available evidence is compatible with these hormones (and the RANKL-OPG system) being unimportant for mineral and bone metabolism in the fetus and neonate. Detailed study of bone and mineral metabolism in these models during fetal development is needed.

XII. FETAL AND NEONATAL RESPONSES TO MATERNAL MINERAL DISTURBANCES

A. Primary Hyperparathyroidism in the Mother

1. Animal data

Maternal hypercalcemia adversely affects fetal parathyroid function. An acute infusion of calcium raised the serum calcium of pregnant ewes from 2.25 to 3.75 mM, and this in turn caused an increase in the fetal serum calcium from 3.00 to 3.5 mM and a >50% fall in the fetal PTH level (295). In contrast, exogenous PTH administration to the mother failed to raise the fetal serum calcium level (295), consistent with the failure of PTH to be transported across the placenta. These results suggest that maternal hypercalcemia
will increase the fetal blood calcium and, in turn, suppress the fetal parathyroids. Furthermore, hypercalcemia within the fetal circulation did not suppress active transport of calcium across the perfused guinea pig placenta (433). The lack of a compensatory decrease in placental calcium transport means that the effect of maternal hypercalcemia can be magnified to cause fetal hypercalcemia and suppression of fetal parathyroid function.

2. Human data

Maternal hypercalcemia likely increases the flow of calcium across the placenta, and in turn it may lead to a higher than normal cord blood calcium (610). Importantly, maternal hypercalcemia suppresses the fetal parathyroids and can cause significant fetal morbidity and mortality in up to 80% of cases (597). These outcomes include stillbirth, miscarriage, and neonatal tetany. Comparison of older to more recent case series suggest that these outcomes have improved (609); however, a recent sobering report found that fetal death occurred during the second or third trimester in 30 of 62 medically managed cases, representing a 3.5-fold increased risk of fetal mortality compared with surgically treated cases (477).

After experiencing higher than normal calcium levels in utero, the neonatal calcium level may decline more slowly after birth (610). Failure of the neonate’s parathyroids to upregulate promptly after birth may precipitate hypocalcemia and tetany. In several case series 50% of neonates had tetany or other complications of maternal hypercalcemia, while 25–30% of them died (149, 304, 393, 718). Hyperparathyroidism normally resolves by 3–5 mo after birth (609), but permanent hypoparathyroidism has also occurred (79, 393, 609). Some cases have had delayed onset or recognition until several weeks or months after birth (282, 469, 688).

A common clinical impression is that mild maternal hypercalcemia during pregnancy may be unlikely to cause fetal or neonatal problems. However, neonatal hypocalcemia and tetany have occurred after seemingly mild cases of primary hyperparathyroidism (522). A twin pregnancy complicated by maternal hypercalcemia provides a seminal example of the variability: one neonate remained normocalcemic while the other had hypocalcemic seizures (431).

B. Familial Hypocalciuric Hypercalcemia in the Mother

1. Animal data

Study of Casr+/- mice, the murine equivalent of FHH, has shown that fetal PTH is suppressed compared with the PTH values obtained in fetuses from WT mothers (338).

2. Human data

Despite the seemingly benign nature of hypercalcemia in adults with FHH, maternal hypercalcemia during pregnancy has caused suppression of the fetal parathyroids, and subsequent neonatal hypocalcemia and tetany (529, 673, 674). This includes the heterozygous neonate who may still develop symptomatic hypocalcemia and tetany, before eventually becoming hypercalcemic when the parathyroids gain full function and achieve the higher calcium level set by the mutated CaSR (672).

C. Hypoparathyroidism and Activating Mutations of the Calcium-Sensing Receptor in the Mother

1. Animal data

Maternal hypocalcemia compromises the supply of mineral available to the fetus. Thyroparathyroidectomy has been used in rats and ewes to mimic the effects of maternal hypocalcemia due to hypoparathyroidism during pregnancy. These fetal rats (67, 276) and lambs (564) had normal serum calcium levels despite severe maternal hypocalcemia, although the serum calcium declined during the last several days of gestation in other studies of parathyroidectomized rats (106, 208, 220). A normal serum calcium likely requires upregulation of placental calcium transfer to maintain it, and this is supported by the observation that an acute calcium infusion caused a marked rise in serum calcium of fetuses from thyroparathyroidectomized rats, but not in fetuses from thyroidectomized rats (106). The fetal skeleton may also be compromised by resorbing itself to maintain the normal high level of calcium in fetal blood, as seen by enlargement of the fetal parathyroids (206, 625), increased resorption in fetal rat bones that requires intact fetal parathyroids for it to occur (542), and reduced femur length and mineral ash content (104). But even with overt hypocalcemia throughout pregnancy, the neonate can have a perfectly normal skeleton (217).

2. Human data

When maternal hypocalcemia during pregnancy has been prolonged and severe, fetal hyperparathyroidism has developed, in turn causing a severely demineralized skeleton, and fractures in utero or during birth (11, 76, 389, 586, 652). Spontaneous abortion, stillbirth, and neonatal death have also occurred (25, 170, 302). The cord blood calcium is variably normal, low, or even increased, whereas the fetal parathyroids may be enlarged and hyperplastic. Although this is secondary hyperparathyroidism in the fetus, the function remains autonomous for a time, such that even if the fetus is normal at birth, hypercalcemia and skeletal demineralization can develop in the neonate before the parathyroid function subsides to nor-
The potential effect of maternal vitamin D deficiency during pregnancy has been addressed in detail in section VIII.

Pregnancies have been uneventful in women who lack functional vitamin D receptors (VDDR-II) (408). Depending on the nature of the mutation, there may be a partial response to calcitriol or cholecalciferol or complete loss of calcitriol-induced effects. The calcium content of the diet may need to be increased to maintain a near-normal ionized or albumin-corrected serum calcium.

If maternal hypercalcemia occurs from excess consumption of calcitriol, 1α-cholecalciferol, or vitamin D (hypervitaminosis D), then fetal parathyroid suppression and neonatal hypocalcemia and hypoparathyroidism can result. This effect is mediated by the high maternal calcium concentration, and not directly by the vitamin D metabolites. If women consume modestly high doses of vitamin D during pregnancy but remain normocalcemic, the neonate is unlikely to be adversely affected based on available clinical data. This was shown by a randomized clinical trial that administered 400, 2,000 or 4,000 IU of vitamin D daily to women during the second half of pregnancy (262). No maternal or fetal hypercalcemia was reported during the study. Maternal 25-hydroxyvitamin D at the end of pregnancy ranged from 79 nM in the low-dose group to 111 nM in the highest dose group, while fetal 25-hydroxyvitamin D values in the corresponding offspring were 46 and 66 nM (262). Intense placental activity of 24-hydroxylase causes preferential catabolism of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D (as described earlier in sect. II B2), and likely explains why the fetal rise in 25-hydroxyvitamin D was more modest than in the mothers.
F. Maternal Magnesium Infusions (Tocolytic Therapy)

Intravenous magnesium is used in pregnant women to inhibit uterine contractions of premature labor and to prevent seizures from eclampsia. The infusion causes fetal hypermagnesemia, suppressed PTH and parathyroid responsiveness, and variable effects on the total and ionized calcium of neonates (130, 159). Prolonged exposure has also caused defective ossification of bone and enamel in the teeth (358), and abnormal mineralization within the metaphyses of long bones (132, 402, 587). Severe hypermagnesemia (>7 mg/dl) is more likely to cause hypotonia, respiratory depression, and bone abnormalities (159, 379, 402, 591).

G. Hypercalcemia of Malignancy in the Mother

Maternal hypercalcemia in these cases is more extreme and likely to suppress fetal parathyroid function, thereby provoking neonatal hypocalcemia, tetany, and possibly permanent hypoparathyroidism. Hypercalcemia may be present in cord blood and the first few postnatal samples (131, 278, 700), but the calcium will fall and may reach severely hypocalcemic values with attendant risk of respiratory distress, tetany, and seizures (9, 454). One of four babies died where the outcome was reported (454).

H. Pseudohyperparathyroidism in the Mother

Excess maternal production of PTHrP causes pseudohyperparathyroidism, a less common cause of maternal hypercalcemia during pregnancy. It can occur as a result of excess mammary production of PTHrP and present with hypercalcemia during late pregnancy or in the puerperium (283, 307, 590); it has also occurred in nonpregnant women with large breasts (418). Placental PTHrP can also cause pseudohyperparathyroidism, as shown by a pregnant woman who presented with severe hypercalcemia (5.25 mM), undetectable PTH, and a serum PTHrP of 21 pM (173). Six hours after an urgent C-section, she was profoundly hypocalcemic with undetectable PTHrP and elevated PTH. Her breasts were of unremarkable size and she did not breast feed. The rapid change in her status after delivery is most consistent with the placenta being the source of PTHrP (173). In all cases of pseudohyperparathyroidism, hypercalcemia may be present in cord blood (590), and in turn maternal hypercalcemia increases the risk of neonatal hypocalcemia and hypoparathyroidism.

I. Maternal Diabetes Causing Fetal and Neonatal Hypoparathyroidism

Poorly controlled maternal diabetes during pregnancy can cause hypocalcemia, seizures, and tetany within the first

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**FIGURE 12.** Relative roles of PTH, PTHrP, and calcitriol during fetal and neonatal life. The placenta is the main source of mineral during fetal life. PTH and PTHrP are expressed within the placenta but may also act on it from systemic sources to stimulate calcium transfer. The intestines are a trivial source of mineral in the fetus but are the main source for the neonate. Intestinal calcium absorption is initially passive but later becomes active, saturable, and calcitriol-dependent in the infant. Within the endochondral skeleton, PTHrP is produced by proliferating chondrocytes and perichondrial cells (arrowheads) and delays terminal differentiation of prehypertrophic chondrocytes. PTHrP is also produced within preosteoblasts and osteoblasts and stimulates bone formation (semicircular arrows). During fetal life PTHrP and PTH act together to maintain high blood calcium and phosphorus levels to facilitate mineralization; loss of either PTHrP or PTH causes hypocalcemia and hyperphosphatemia. Calcitriol is not required to regulate blood calcium, endochondral bone formation, or skeletal mineralization in the fetus. [From Kovacs (329), with permission from Elsevier.]
24–72 h after birth. Hyperphosphatemia is commonly present, indicating that transient neonatal hypoparathyroidism is contributing to the hypocalcemia. The mechanism for this is unknown. One case series found high ionized and total serum calcium levels in cord blood of infants of diabetic mothers (684), which could account for suppression of the fetal parathyroids. But why maternal diabetes should cause fetal hypercalcemia is unclear, unless some aspect of the maternal diabetic state leads to increased maternal-fetal flow of calcium. Another possibility is that glucosuria caused by uncontrolled diabetes during pregnancy may cause renal wasting of magnesium and maternal hypomagnesemia, which in turn could cause fetal hypomagnesemia and parathyroid suppression. However, a small clinical trial found no effect of postnatal magnesium supplementation on the incidence of neonatal hypocalcemia in infants of diabetic mothers (434). Other factors that contribute to hypocalcemia in babies born of diabetic mothers are associated increased risks of preterm birth, lung immaturity, and asphyxia.

XIII. CONCLUSIONS

The fetal and neonatal skeletons require adequate mineral delivery to develop and mineralize normally (Figure 12). Placental mineral transfer requires PTHrP and possibly PTH, but not calcitriol, calcitonin, or FGF23. PTH and PTHrP work together to maintain serum calcium and phosphorus at high levels in utero for optimal bone mineralization, while calcitriol, calcitonin, and FGF23 are not required. PTH and PTHrP are required to regulate endochondral bone development during gestation, whereas calcitriol, calcitonin, FGF23, and the sex steroids are not. During the neonatal period, intestinal calcium absorption becomes dependent on vitamin D/calcitriol. This means that skeletal development and mineralization in turn become dependent on vitamin D/calcitriol as well. However, this is not an absolute requirement because the role of calcitriol can be bypassed by increasing the calcium content of the diet or by administering parenteral calcium.

ACKNOWLEDGMENTS

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GRANTS

This work was supported by Canadian Institutes of Health Research Grants 133413, 126469, and 84253 as well as by Research & Development Corporation of Newfoundland and Labrador Grant 5404.1143.102.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author.

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