Calcitropic gene expression suggests a role for the intraplacental yolk sac in maternal-fetal calcium exchange

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Kovacs, Christopher S., Linda L. Chafe, Mandy L. Woodland, Kirsten R. McDonald, Neva J. Fudge, and Peter J. Wookey. Calcitropic gene expression suggests a role for the intraplacental yolk sac in maternal-fetal calcium exchange. Am J Physiol Endocrinol Metab 282: E721–E732, 2002. First published November 20, 2001; 10.1152/ajpendo.00369.2001.—The expression of calcitropic genes and proteins was localized within murine placenta during late gestation (the time frame of active calcium transfer) with an analysis of several gene-deletion mouse models by immunohistochemistry and in situ hybridization. Parathyroid hormone-related protein (PThrP), the PTH/PTHrP receptor, calcium receptor, calbindin-D9k, Ca2+-ATPase, and vitamin D receptor were all highly expressed in a localized structure of the murine placenta, the intraplacental yolk sac, compared with trophoblasts. In the PThrP gene-deleted or Pthrp-null placenta in which placental calcium transfer is decreased, calbindin-D9k expression was downregulated in the intraplacental yolk sac but not in the trophoblasts. These observations indicated that the intraplacental yolk sac contains calcium transfer and calcium-sensing capability and that it is a probable route of maternal-fetal calcium exchange in the mouse.

Evidence that parathyroid hormone (PTH), calcitriol, and calcitonin might regulate placental calcium transfer. The mechanisms by which active calcium exchange occurs across the placenta are not well understood. Analogous to calcium transfer across the intestinal mucosa and kidney (25), it has been proposed that calcium diffuses into calcium-transporting cells through maternal-facing basement membranes, is carried across these cells by calcium-binding proteins (calbindin-D9k and other calbindins), and is actively extruded at the fetal-facing basement membranes by Ca2+-ATPase (8). The placental expression of calbindin-D9k increases 135-fold over the last 7 days of gestation in the rat (17), whereas the expression of the Ca2+-ATPase increases 2-fold over the same interval (17, 49). These observations are consistent with the hypothesis that calbindin-D9k and Ca2+-ATPase are required for maternal-fetal calcium transfer in late gestation.

The factors that regulate placental calcium transfer are also, at best, only partly elucidated. There is evidence from thyroparathyroidectomized sheep (9, 47), and from the Pthrp gene knockout model (35), that midmolecular forms of parathyroid hormone-related protein (PThrP) stimulate placental calcium transfer. The calcium-sensing receptor (CaSR) also appears to influence the rate of placental calcium transfer (33). Evidence that parathyroid hormone (PTH), calcitriol, and calcitonin might regulate placental calcium transfer is contradictory and less certain (see Ref. 36 and a review in Ref. 34). It is likely that some of the discrepancies are due to the markedly different placental structures in the mammals that have been studied, including humans and other primates, rodents, and sheep.

Calcitropic gene expression suggests a role for the intraplacental yolk sac in maternal-fetal calcium exchange.
Murine and human placentas are hemochorial (i.e., maternal blood freely bathes the fetal tissues within the placenta) and have similar function and permeability (31, 46). However, the structures of murine and human placentas do differ in other respects. The murine placenta is trichorial, whereas the human placenta is monochorial. There are three types of trophoblasts in the murine placenta, including the labyrinthine or syncytiotrophoblasts, the spongiotrophoblasts, and the giant trophoblasts; in contrast, the human placenta contains syncytiotrophoblasts and cytotrophoblasts. The labyrinthine trophoblasts make up the bulk of the murine placenta and are considered to be the dominant site of maternal-fetal exchange. The giant trophoblasts and spongiotrophoblasts invade the decidual tissue and also express numerous hormones.

An often overlooked structure contained in rodent placenta is the intraplacental yolk sac (IPYS), which, as the name implies, consists of part of the primitive yolk sac that later became incorporated into the placenta (Fig. 1, A and B) (12). The primitive yolk sac participates in nutrient exchange between the fetal and maternal circulations before the formation of the placenta (12, 26). Like the yolk sac from which it derives, the IPYS is a bilayered membrane, consisting of tall columnar cells on the visceral or endothelial side overlying fetal vessels, and smaller parietal or cuboidal cells on the epithelial side that overlie a thick basement membrane (Reichert’s membrane) and the maternal blood spaces (Fig. 1C). These two layers of the IPYS are separated by a potential space (sinus of Duval), which communicates with the yolk sac cavity and, thereby, the uterine lumen. Given its anatomic position between fetal vessels and maternal blood spaces at the fetal pole of the placenta, it is well situated for exchange of substances between mother and fetus (Fig. 1C). The IPYS is found exclusively in rodent placentas (rat, mouse, gerbil, and hamster) (26); a corresponding structure has not been described in human, other primates, or ruminant placentas.

Since the original description of the IPYS in rodent placenta by Duval (15), it has been reported that the IPYS contains abundant expression of calbindin-D9k, greater than that observed in the surrounding trophoblasts (5). Although the placental expression of calbindin-D9k increases 35-fold during the time of rapid calcium transfer in the rat (17), the increase is more marked in the IPYS than in the trophoblasts (41). The IPYS has also been reported to express Ca\(^{2+}\)-ATPase (3). These observations suggest that the IPYS may have some role in maternal-fetal calcium exchange, but this possibility has not been further explored. More recently, it has been observed that the IPYS does not behave simply as an incorporated remnant in the placenta; instead, it actively invaginates and expands in volume during the last 4–5 days of gestation in the mouse, the time frame of rapid calcium transfer (45). In the absence of the gene-encoding platelet-derived growth factor receptor-\(\alpha\), the IPYS does not form, and the embryo often dies in midgestation (45). Whether the lack of IPYS contributes to the embryonic lethality of that knockout has not been determined. Apart from these recent observations, the IPYS has been largely ignored and considered to be a nonfunctional remnant of the primitive yolk sac (12).

Although several calcitropic factors [such as PTHrP, PTH/PTHrP receptor, and vitamin D receptor (VDR)] have been reported to be expressed in the placenta, the specific localization of expression of many of these genes (particularly in the murine placenta) has not been reported. Technical limitations clouded interpretations; for example, radiolabeled calcitriol bound to placenta, suggesting the presence of VDRs (48). However, since the cloning of the VDR gene (Vdr), placental expression of VDR mRNA or protein has not been reported. Determination of the specific intraplacental localization of these calcitropic genes has particular relevance in the definition of the routes along which calcium might be transferred from mother to fetus, and which genes might be involved in that process.

The purpose of this study was twofold. First, we wanted to localize the expression of calcitropic genes within the murine placenta during late gestation (the time frame of active calcium transfer), utilizing placentas from specific knockout mice to rigorously confirm the specificity of the mRNA and proteins detected. Second, we hypothesized that upregulation or down-regulation of placental calcium transfer would result in alterations in calcitropic gene and protein expression within placental cells that are involved in maternal-fetal calcium transfer. To test that hypothesis, we systematically examined placentas of mice in which we have previously noted that placental calcium transfer is downregulated (Pthrp-null fetuses) and upregulated (PTH/PTHrP receptor (Pthr1)-null fetuses) (35).

We found that PTHR1P, PTH/PTHrP receptor, CaSR, calbindin-D9k, Ca\(^{2+}\)-ATPase, and VDR were particularly highly expressed in the IPYS of the murine placenta compared with the trophoblasts. In the Pthrp-null placenta, the expression of calbindin-D9k mRNA and protein was downregulated in the IPYS, but not in the trophoblasts; no such downregulation was observed in the Pthr1-null placentas. The IPYS may be an important route of calcium exchange between mother and fetus in rodents that utilizes a strategy from the process of eggshell calcification.

**METHODS**

**Knockout mice and genotyping.** Pthrp (28), Pthr1 (38), Casr (22), Vdr (39), and calcitonin (Ct) (23) gene knockout mice were obtained by targeted disruption of the murine genes in embryonic stem cells, as previously described. Heterozygous mice were mated overnight; the presence of a vaginal mucus plug on the morning after mating marked ED 0.5. Normal gestation in these mice is 19 days. All mice were given a standard chow diet and water ad libitum. All studies were performed with the prior approval of the Institutional Animal Care Committee of Memorial University of Newfoundland.

Genomic DNA was obtained from fetal tails, and genotyping was accomplished by PCR with primers that were specific...
to the Pthrp, Pthr1, Casr, and Vdr gene sequences (22, 35, 38, 39) and the Ct gene sequence in a single-tube, 36-cycle PCR reaction utilizing a PTC-200 Peltier Thermal Cycler (MJ Research, Cambridge, MA). The Ct genotyping was accomplished in a three-primer system [one in the retained portion of wild-type (wt) allele, one in a deleted portion of the wt allele, and one in the inserted neomycin sequence] that utilized the following specific sequences: CAG GAT CAA GAG TCA CCG CT; GGA GCC TGC GCT CCA GCG AA; and GGT GGA TGT GGA ATG TGT GC.

**Tissue collection.** Wt and null placentas obtained from ED 16.5–18.5 pregnancies were studied, because this time frame corresponds to the interval of rapid maternal-fetal transfer of calcium. Placentas were obtained after placental perfusion with paraformaldehyde to minimize the degradation in mRNA or protein levels that might occur during fixation and processing of the RNase and protease-rich placental tissues (36). After this, the placentas were removed and placed in 10% formalin for standard processing, embedding in paraffin, and sectioning.

**Riboprobes and antibodies.** cDNAs used included murine calbindin-D28k (42) and murine calbindin-D9k (51) (gifts of S. Christakos). Human Ca2+-ATPase (37) (gift of R. Kumar), murine α-fetoprotein (gift of Margaret Baron), rat PTH/PTHrP receptor (2) and rat PTHrP (29) (gifts of H. M. Kronenberg), murine CaSR (55) (gift of C. Ho-Pao), murine 57-kDa calcium-binding protein (57-kDa calbindin) (50) (gift of R. S. Tuan), murine VDR (27) (gift of T. Kawada), murine calcitonin (gift of G. J. Cote), murine placental lactogen (24) and murine prolactin (40) (gifts of D. Linzer), and murine nodule (56) (gift of M. Kuehn). Antibodies included rabbit anti-rat calcitonin receptor antibody (2) and rat PTHrP (29) (gifts of H. M. Kronenberg), murine anti-human erythrocyte Ca2+-ATPase (Pside, rabbit anti-rat PTHrP (30) (gift of J. Moseley and T. J. Martin), mouse anti-CaSR (ADD antibody) (18) (gift of K. V. Rogers (NPS Pharmaceuticals) and A. M. Spiegel and P. K. Goldsmith (Metabolic Diseases Branch, NIDDK/NIH) rabbit anti-human VDR (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human calcitonin (Dako, Carpenteria, CA), and rabbit anti-rat PTH/PTHrP receptor (Berkley Antibody/Covance Research Products, Berkeley, CA).

The rabbit anti-rat calcitonin receptor antibody was developed as follows, with techniques previously described (53). Antibodies were raised to a synthetic peptide corresponding to an epitope on the rat calcitonin receptor, located in the cytoplasm and after the seventh transmembrane domain and having the sequence KGLPIYICHQEPRNPPVSNN-NH2. Antisera were partially purified by chromatography by use of 1-ml columns packed with protein A-Sepharose (Pharmacia) and low-pressure chromatography, eluted at low pH, neutralized, dialyzed, and finally lyophilized under vacuum.

**Ribobrope labeling.** For in situ hybridization, the plasmids were linearized with appropriate restriction enzymes and labeled with 125 μCi of 35S-labeled UTP using an SP6/T7 Transcription Kit (Promega/Fisher Scientific, Burlington, ON, Canada) and the appropriate polymerase. Unincorporated nucleotides were removed with the NucTrap columns (Stratagene, La Jolla, CA) as per package instructions.

**In situ hybridization.** In situ hybridization was performed on 5-μm tissue sections, as described previously (32, 36). Slides were then dipped into NTB-2 liquid emulsion, dried, and exposed to X-ray film for 2-10 days. After washing the films in running water, they were developed, fixed, and then dipped into Kodak NTB-2 photographic emulsion. Slides were then mounted on glass slides, dehydrated, and cleared with xylene. Tissue sections were then mounted over standard coverslips with Permount (Fisher Scientific, Burlington, ON, Canada) and the appropriate coverslip. Films were then placed in desiccators and allowed to dry for several weeks before being mounted over glass slides with Eukitt (Tissue Banking). Slides were then dipped into NTB-2 liquid emulsion, dried, and exposed to X-ray film for 2-10 days. After washing the films in running water, they were developed, fixed, and then dipped into Kodak NTB-2 photographic emulsion. Slides were then mounted on glass slides, dehydrated, and cleared with xylene. Tissue sections were then mounted over standard coverslips with Permount (Fisher Scientific, Burlington, ON, Canada) and the appropriate coverslip. Films were then placed in desiccators and allowed to dry for several weeks before being mounted over glass slides with Eukitt (Tissue Banking).

**Results.** Histology of placental tissue sections processed for in situ hybridization of the murine calcitonin receptor revealed a differential distribution of calcitonin receptor mRNA in the IPYS. In the control sections, the calcitonin receptor was found to be highly expressed in the parietal yolk sac, whereas the columnar or visceral yolk sac layer was in apposition to this layer, separated by the yolk sac cavity. The yolk sac bilayer that is overlying the dome of the placenta forms an apposition to this layer, separated by the yolk sac cavity. The parietal layer and Reichert’s membrane overlie maternal and fetal blood spaces, as visualized in detail in C. In the IPYS, the parietal layer and Reichert’s membrane overlie maternal blood spaces and vessels, and the columnar layer overlies fetal blood vessels. Between these layers is the sinus of Duval, which communicates with the yolk sac cavity and the uterine lumen.

**Discussion.** The role of the calcitonin receptor in the regulation of calcium homeostasis in the placenta is not well understood. However, recent studies have suggested that the calcitonin receptor plays a role in the regulation of calcium transport across the placental barrier. The calcitonin receptor is expressed in the placenta, and its expression is increased during pregnancy. The calcitonin receptor is also expressed in the amniotic fluid, suggesting that it may play a role in the regulation of calcium transport across the placental barrier.

**Conclusions.** The results of this study suggest that the calcitonin receptor plays a role in the regulation of calcium transport across the placental barrier. The calcitonin receptor is expressed in the placenta, and its expression is increased during pregnancy. The calcitonin receptor is also expressed in the amniotic fluid, suggesting that it may play a role in the regulation of calcium transport across the placental barrier.

**Fig. 1.** Formation and detailed anatomy of the intraplacental yolk sac (IPYS). The bilayered primitive yolk sac cavity (A) functions as a primitive placenta early in gestation. Later in gestation (B), the yolk sac has been compressed such that it completely lines the uterine cavity (decidua) that is not in contact with the placenta, and it overlies the dome of the placenta. The parietal layer and Reichert’s membrane are in contact with the decidua and the dome of the placenta, whereas the columnar or visceral yolk sac layer is in apposition to this layer, separated by the yolk sac cavity. The yolk sac bilayer that is overlying the dome of the placenta forms finger-like projections into the placenta near the insertion of the fetal vessels (boxed area in B, the IPYS). The IPYS is positioned between maternal and fetal blood spaces, as visualized in detail in C. In the IPYS, the parietal layer and Reichert’s membrane overlie maternal blood spaces and vessels, and the columnar layer overlies fetal blood vessels. Between these layers is the sinus of Duval, which communicates with the yolk sac cavity and the uterine lumen.
stored in light-tight boxes, and kept at 4°C until developed (3 days to 6 wk). The emulsion was developed using standard developer and fixer, and the sections were then counterstained with hematoyxin-eosin.

**Immunohistochemistry.** Immunohistochemistry on 5-μm sections was performed using standard technique with secondary antibody, ABC reagent, and DAB-Tris-peroxidase kits obtained from Vector (Burlington). Sections were counterstained with Contrast Red (GIBCO BRL, Burlington) or 1% methyl green, washed, dehydrated, and mounted. Assessments of staining intensity were determined in a blinded fashion (no knowledge of the genotype). The reproducibility of the results was confirmed independently on at least three separate litters.

For immunohistochemistry of CaSR and PTH/PTHrP receptor, sections were treated to reduce cross-linking and unmask the epitopes. After the deparaffinization and rehydration steps, sections were incubated in 4% paraformaldehyde for 15 min, then in 10 μg/ml of proteinase K (GIBCO BRL) in PBS at 37°C for 15 min, and then in 4% paraformaldehyde again for 15 min to stop the reaction. After this, the standard immunohistochemical technique was resumed with administration of the blocking serum.

For each primary antibody, the appropriate concentration and incubation conditions were determined empirically. Concentrations ranged from 1:250 to 1:1,000, with optimal staining generally observed at 1:500. The specific details of PTHrP immunohistochemistry have been previously described (36). For remaining primary antibodies, incubations were at room temperature for 1–2 h, except for anti-VDR and anti-CaSR antibodies, which were applied at 4°C overnight. The CaSR and PTH/PTHrP receptor antibodies were diluted in 1% bovine serum albumin in PBS; the PTHrP antibody was diluted in PBS containing 5% newborn bovine serum (GIBCO BRL); all other primary antibodies were diluted in PBS containing 2% blocking serum.

**Controls.** There were several levels of controls for the immunohistochemistry and in situ hybridization. Pthrp-null, Pthrl-null, Casr-null, Vdr-null, and Ct-null placentas were used to confirm the specificity of the respective cDNAs and antibodies. All comparisons between wt and null placentas were made between placentas that had been obtained from within the same litter (i.e., siblings) and that had been fixed, embedded, sectioned, and treated together. For immunohistochemistry, adjacent control sections also had the primary antibody omitted. For in situ hybridization, sense and antisense riboprobes made from the same cDNA were applied on adjacent sections; also, different antisense probes (i.e., different cDNAs) were studied on adjacent sections in the same experiments. Because of the practicalities of space limitations, it is not possible to show all controls in the figures.

**RESULTS**

For consistency with the literature, the layers of the IPYS will be referred to as the columnar layer and the parietal layer. The yolk sac proper (i.e., the bulk of the yolk sac, which is outside the placenta) will be referred to as the extraplacental yolk sac and its layers as the visceral layer and the parietal layer. The columnar layer of the IPYS is contiguous with the visceral layer of the extraplacental yolk sac, and both parietal layers are also contiguous.

**Calbindin-D9k and calbindin-D28k.** Calbindin-D9k mRNA was intensely expressed in the columnar cells of the IPYS, with little or no signal detected in the parietal cells (Fig. 2, a and c). The signal was typically so intense that the columnar cells were blackened and obliterated by silver grains on the bright-field image, but silver grain deposition was not readily apparent in the remainder of the placenta (Fig. 2a). However, on the dark-field images, calbindin-D9k mRNA was clearly present at a much lower intensity in the labyrinthine trophoblasts than in the visceral layer of the extraplacental yolk sac (Fig. 2c). Immunohistochemistry demonstrated that calbindin-D9k was present in both columnar and parietal IPYS cells, although more intensely on the columnar side (Fig. 2g).

In contrast to the intense expression of calbindin-D9k, another vitamin D-dependent calcium-binding protein, calbindin-D28k, was not detectable by in situ hybridization of murine placenta (data not shown).

**Ca2+-ATPase.** Examination of Ca2+-ATPase mRNA and protein revealed an expression pattern similar to that of calbindin-D9k. By in situ hybridization, the mRNA signal was most intense in the columnar cells of the IPYS, resulting in a blackened bright-field image (Fig. 3a), whereas the less intense expression in the labyrinthine trophoblasts and visceral layer of the extraplacental yolk sac was apparent only on the corresponding dark-field image (Fig. 3b). Immunohistochemistry determined that both the columnar and parietal cells of the IPYS express Ca2+-ATPase, although the staining is more intense on the columnar side (concordant with the mRNA signal intensity) (Fig. 3c and d).

**PTH/PTHrP receptor.** PTH/PTHrP receptor mRNA was most intensely expressed in the parietal cells of the IPYS overlying Reichert’s membrane, with reduced mRNA signal intensity evident in the columnar cells of the IPYS (Fig. 3, e, f, and g). Labyrinthine trophoblasts and the visceral layer of the extraplacental yolk sac showed no detectable PTH/PTHrP receptor mRNA in bright- or dark-field images. Immunohistochemistry with anti-PTH/PTHrP receptor antibody showed a similar pattern of immunoreactivity confined to the parietal layer of the IPYS, in addition to adjacent epithelial cells of maternal blood vessels (not shown). In control experiments using Pthrl-null placentas, the PTH/PTHrP receptor mRNA and protein were absent (data not shown).

**PThrP.** PThrP mRNA was present throughout the placenta, including the IPYS, trophoblasts, and visceral layer of the extraplacental yolk sac, but the signal was most intense in the parietal cells of the IPYS (Fig. 3, h and i). The PThrP mRNA was absent in sections obtained from Pthrp-null placentas (Fig. 3j). When anti-PThrP antibody was used, the peptide was found to be diffusely expressed in all three trophoblast types, the IPYS, and the visceral layer of the extraplacental yolk sac. The highest intensity of PThrP immunoreactivity was observed in the spongiotrophoblasts and IPYS. Within the IPYS, the columnar cells had the highest intensity of staining, with less apparent staining in the parietal cells of the IPYS (Fig. 3k). With use of sections obtained from Pthrp-null placentas, the
specificity of the anti-PTHrP antibody was confirmed (Fig. 3f).

**CaSR.** CaSR mRNA was detectable by in situ hybridization only in the IPYS cells and not in the trophoblasts or the extraplacental yolk sac. Although more intense on the parietal side, CaSR mRNA could be detected on the columnar side of the IPYS as well (Fig. 3m and n). Placentas obtained from Casr-null mice could not be used as controls for in situ hybridization, because a truncated (mutant) CaSR mRNA is transcribed and is detected by Northern blot and in situ hybridization (unpublished data). By immunohistochemistry, with a monoclonal antibody directed against a region of the CaSR that has been deleted in the Casr-null mice, the CaSR was found to be expressed in both layers of the IPYS and in the surrounding trophoblasts of wt placenta (Fig. 3o) and absent in placenta obtained from Casr-null mice (Fig. 3p).

**VDR.** By use of riboprobes generated from a cDNA for the murine VDR, a low level of mRNA signal was
detected in wt placenta that was not strikingly different from the background signal observed in Vdr-null placentas (not shown). This finding indicated that VDR mRNA must be present at a low level, if present at all. With use of antibody to VDR, and when wt were compared with Vdr-null placentas, nuclear staining for VDR was detected in many columnar cells of the IPYS and in a few parietal cells (Fig. 3, q and r). No apparent VDR immunoreactivity could be detected in trophoblasts or the extraplacental yolk sac.

**Calcitonin.** Calcitonin mRNA was found to be diffusely expressed throughout the placenta, including the IPYS and the labyrinthine trophoblasts (Fig. 4, a and b). No apparent signal was detected in the extraplacental yolk sac. No calcitonin mRNA was detected in Ct-null placentas (Fig. 4, c and d), confirming the specificity of the signal detected in wt placenta. Calcitonin immunoreactivity was present at low levels diffusely in the labyrinthine trophoblasts and in both layers of the IPYS of wt placenta (Fig. 4e), with no immunoreactivity detected in Ct-null placenta (Fig. 4f).

**Calcitonin receptor.** Antibody to the calcitonin receptor revealed modest but diffuse staining in the IPYS and labyrinthine trophoblasts, with the most intense staining in the visceral and parietal layers of the extraplacental yolk sac (Fig. 4, g and h).

**57-kDa calbindin.** The expression of 57-kDa calbindin was most apparent in spongiotrophoblasts and giant trophoblasts at the periphery and base of the placenta and in the visceral layer of the extraplacental yolk sac (Fig. 4, i and j). There was comparatively less intense mRNA signal visualized in the labyrinthine trophoblasts, and no definite mRNA was detected in either layer of the IPYS.

**α-Fetoprotein, nodal, placental lactogen, and prolactin.** As a guide to the relative expression of calcitropic factors in the IPYS, we also examined the expression of other placental markers, including α-fetoprotein (a yolk sac marker, Fig. 4, k and l), nodal (a spongiotrophoblast marker, not shown), placental lactogen, and proliferin (markers of giant trophoblasts, not shown). None of these factors was expressed in the IPYS, as determined by in situ hybridization using specific riboprobes. α-Fetoprotein was intensely expressed in the visceral layer of the extraplacental yolk sac, but immediately upon entry into the placenta, the corresponding columnar cells of the IPYS did not express α-fetoprotein (Fig. 4, k and l). Nodal was expressed in spongiotrophoblasts, and placental lactogen and proliferin were expressed in giant trophoblasts, and none of these appeared to be present in the IPYS (data not shown).

**Pthrp-null and Pthr1-null placentas.** Previously, Pthrp-null fetuses had been shown to have reduced placental calcium transfer compared with their littermates, whereas Pthr1-null placentas had been shown to have increased placental calcium transfer (35). Placentas of wt and Pthrp-null siblings were examined to determine whether reduced placental calcium transfer was associated with any abnormality in structure or in gene or protein expression. Pthr1-null placentas were similarly examined to determine whether increased placental calcium transfer was accompanied by any changes in placental structure or in gene or protein expression. The absence of PTHrP mRNA and protein in Pthrp-null placentas has already been demonstrated (Fig. 3, j and l). The IPYS was far less often present in sections obtained from Pthrp-null placentas, but it was readily and abundantly present in wt and Pthr1-null placentas. In Pthrp-null sections that did have IPYS, calbindin-D9k mRNA signal intensity was sharply reduced in the Pthrp-null placentas, such that it was not apparent in the bright-field image (Fig. 2, b vs. a) and was apparent only in the dark-field image (Fig. 2, d vs. c). The expression of the corresponding protein was also reduced in intensity, as judged by immunohistochemistry by use of anti-calbindin-D9k antibody (Fig. 2, f and h vs. e and g). The reduction in calbindin-D9k was specific, because adjacent sections...
DISCUSSION

This study has comprehensively examined calcium-binding proteins, calcitropic hormones, and their receptors in the murine placenta during late gestation in the interval of rapid calcium exchange. We have used the semi-quantitative technique of mRNA in situ hybridization, aided by the use of specific antibodies for immunohistochemistry where available, and by the use of placentas obtained from specific gene knockout models. The null placentas have enabled us to more rigorously test the specificity of the mRNAs and proteins that were detected. We have determined that many of these factors are expressed more intensely in the IPYS than in the trophoblasts (summarized in Table 1), a finding that may indicate the importance of the IPYS in maternal-fetal calcium transfer. This led us to hypothesize that abnormalities in calcitropic gene expression would be present in placentas in which placental calcium transfer is downregulated and upregulated. We tested this hypothesis by examining placentas obtained from Pthrp-null fetuses (in which placental calcium transfer is downregulated) and Pthr1-null fetuses (in which placental calcium transfer is upregulated). We found that Pthrp-null placentas have less IPYS and reduced expression of calbindin-D9k mRNA and protein in the IPYS; these findings are not present in Pthr1-null placentas.

These results indicate that the columnar cells of the IPYS have the most intense expression (mRNA and protein) of Ca\(^{2+}\)-ATPase and calbindin D9k, two of the components thought to be necessary for maternal-fetal calcium transfer. In addition, the IPYS expresses other calcitropic hormones and receptors more intensely than in the surrounding trophoblasts, including PTHrP adjacent to the PTH/PTHrP receptor, CaSR, and VDR; the IPYS also expresses calcitonin and the calcitonin receptor. The localization of PTHrP to the

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**Fig. 4.** Expression patterns of calcitonin (a-f), calcitonin receptor (g and h), 57-kDa calbindin (i and j), and α-fetoprotein (k and l). Calcitonin mRNA is present throughout labyrinthine trophoblasts and both parietal and columnar layers of the IPYS in wt placenta (a, bright field; b, dark field). Its expression is very low in the visceral layer of extraplacental yolk sac. Corresponding bright-field (c) and dark-field (d) images from gene encoding calcitonin (Ct)-null placentas show no signal, confirming the specificity of the mRNA detected in the wt placenta. Calcitonin immunoreactivity is present in both layers of IPYS in wt (e) compared with Ct-null placenta (f). By immunohistochemistry, calcitonin receptor is present at low levels throughout the labyrinthine trophoblasts and the IPYS but is most intensely expressed in the parietal (outer rim of placenta) and visceral layers of extraplacental yolk sac (g, nonimmune control; h, anti-calcitonin receptor antibody). 57-kDa calbindin mRNA was most intensely expressed in the visceral layer of extraplacental yolk sac and in the spongiotrophoblasts and giant trophoblasts situated near the periphery and base of the placenta (i, bright field; j, dark field); its expression was much less intense in the labyrinthine trophoblasts and not apparent in the IPYS. α-Fetoprotein mRNA was detected only in the extraplacental yolk sac; immediately upon entry into the placenta, the IPYS no longer expresses α-fetoprotein (k, bright field; l, dark field). Arrows, columnar IPYS; arrowheads, parietal IPYS overlying Reichert’s membrane; Y, visceral layer of extraplacental yolk sac; T, labyrinthine trophoblasts; G/S, giant trophoblasts and spongiotrophoblasts. Calibration bars, 50 μm.
Table 1. Summary of the relative expression of calcitropic genes in murine placenta

<table>
<thead>
<tr>
<th>Gene</th>
<th>IPYS</th>
<th>LAB</th>
<th>SPONG</th>
<th>GIANT</th>
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<td>Calbindin-D9k</td>
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IPYS, intraplacental yolk sac; LAB, labyrinthine trophoblasts; SPONG, spongiophoblasts; GIANT, giant trophoblasts; YOLK, extraplacental yolk sac; PTH, parathyroid hormone; PTHrP, PTH-related protein; CaSR, calcium-sensing receptor; VDR, vitamin D receptor.

The observation that fewer sections of Pthrp-null placenta have IPYS suggests that the absence of PTHrP may impair formation or growth of the IPYS. Furthermore, because the IPYS appeared to be present in normal amounts in Pthrl-null sections, any action of PTHrP to regulate IPYS formation must be independent of the PTH/PTHrP receptor. Placental calcium transfer is regulated by the PTHrP midmolecule, independent of the PTH/PTHrP receptor (1, 10, 35, 54); the development and expansion of the IPYS may similarly require the PTHrP midmolecule. The reduced rate of placental calcium transfer in Pthrp-null fetuses may be partly due to the decreased volume of IPYS.

Apart from calbindin-D9k, the placental localization of calcium-binding proteins and calcitropic hormones has not been systematically studied in other species. However, it is evident that the localization of calbindin-D9k differs significantly among species, and this may indicate that calcium transfer occurs at different sites of the placenta within different species. In human and other primate placentas, calbindin-D9k is highly expressed in trophoblasts. As noted in this report and the published literature, in rodent placentas it is not the trophoblasts (which make up the bulk of the placenta) but the IPYS cells that express the highest levels (mRNA and protein) of calbindin-D9k during the time frame of rapid placental calcium transfer (4, 7, 13, 14).

In the epitheliocorial placentas of sheep, cows, and goats, calbindin-D9k is mainly concentrated not in the trophoblasts of the placentome (which has the greater surface area for maternal-fetal exchange) but in a smaller structure, the interplacentomal trophoblast cells (i.e., the flat intercytodelonary trophoblasts) (43, 44, 52). Thus the hemochorial placentas of rodents and the epitheliocorial placentas of ruminants have concentrated the expression of calbindin-D9k in small structures within the placenta, as opposed to the trophoblasts, which make up the bulk of the placenta.

In both rodents and ruminants compared with humans, the amount of calcium transferred to the fetus from the mother is proportionately much greater, and the time frame in which the transfer can be accomplished is much shorter. A human fetus typically accumulates 21 g of calcium by term, requiring an average daily transfer of 200 mg calcium from the mother in the third trimester (16). A fetal rat accretes ~12 mg of calcium in the last 5 days of gestation, which requires that the mother provide 24 mg of calcium per day to a litter of 10 fetuses (11). A fetal lamb accretes 75 g of calcium by term, which requires that the mother provide 6 g of calcium per day to her typically twin lambs in late pregnancy (19). Rodents and ruminants may have developed these specialized areas within the placenta in order for calcium transfer to be facilitated.

The question remains as to how the IPYS might be involved in maternal-fetal calcium transfer, i.e., by what route(s) can the IPYS effect calcium transfer? The answer to this question may be apparent by considering the anatomic localization of the IPYS and the process of eggshell calcification in birds and (especially) egg-laying mammals.
First, as noted in this report, the IPYS abundantly expresses many of the proteins and receptors that might be required for calcium transfer to occur. Second, the IPYS is positioned between thin-walled fetal vessels and maternal blood spaces at the fetal pole of the placenta. Calcium and other substances might transfer from maternal to fetal vessels directly across IPYS and the sinus of Duval. Third, the IPYS communicates with the yolk sac cavity and, through the parietal yolk sac layer that overlies the uterine decidua, it communicates with the uterine epithelium that is not in contact with the placenta itself (Fig. 1B). Calcium and other substances excreted or secreted by uterine epithelium may, therefore, be transported by the parietal yolk sac layer into the yolk sac cavity and thence to the IPYS. Exuberant calcium secretion by uterine epithelial cells is a fundamental step in the process of eggshell calcification in birds and egg-laying mammals. Figure 5 schematically depicts these two postulated routes by which calcium may reach the fetal circulation via the IPYS.

Thus the IPYS may have evolved to permit more rapid transfer of calcium (and possibly other nutrients or minerals) by utilizing all of the uterine lumen to secrete calcium into the yolk sac (and thence to the IPYS and fetal circulation), instead of limiting maternal-fetal exchange to the uterine-placental interface. As well, the IPYS may provide a direct short-circuit between fetal and maternal circulations across the sinus of Duval within the rodent placenta. Bruns et al. (6) have previously proposed that the process of eggshell calcification (calcium secretion by uterine epithelial cells) suggests that one function of the IPYS is to take calcium from the uterine epithelium and transfer it to the fetus. Therefore, it may be that rodents have adapted a strategy of egg-laying animals to maximally increase the surface area through which maternal-fetal calcium transfer can occur in late gestation. If this hypothesis is correct, fetuses that completely lack IPYS [platelet-derived growth factor receptor-α-null fetuses (45)] should have reduced placental calcium transfer and impaired skeletal mineralization near term.

In conclusion, our observation of intense expression of calcitropic genes, calbindin-D₉k, and Ca²⁺-ATPase (“calcium transfer machinery”) in the IPYS of murine placenta implies function, and we speculate that one role of the IPYS is to transfer calcium to the fetus. The concentrated expression of calcitropic factors within the IPYS may enable calcium transfer to be locally regulated (e.g., calcium sensing by the CaSR and stimulation of active maternal-fetal calcium transfer by PTHrP), and the growth and physical size of the IPYS may be controlled by some of these same factors (e.g., PTHrP and platelet-derived growth factor receptor-α). The reduction in placental calcium transfer rate in Pthrp-null fetuses may be a consequence of loss of the tropic effect of PTHrP on growth of the IPYS and the local effect of PTHrP to stimulate placental calcium transfer.

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Fig. 5. Schematic illustration of the IPYS showing a columnar and a parietal cell, the latter overlying Reichert’s membrane; the cells are separated by the sinus of Duval, which communicates with the yolk sac cavity and the uterine lumen. The cellular localization of some of the calcitropic factors studied in this paper are shown; others (e.g., calcitonin and its receptor) have been omitted for clarity. Calcium transfer is postulated to occur through a short (straight arrow) and a long (curved arrow) circuit. The short circuit is directly across the bilayer from maternal blood spaces, through parietal to columnar cells, and thence to fetal circulation (and backward flow may be possible). The long circuit is from (not depicted) the uterine decidua to the parietal cells of the extraplacental yolk sac, through the yolk sac cavity to the sinus of Duval, to the columnar cells of the IPYS, and thence to the fetal circulation. Diffusional and vesicular transfer of calcium ions is represented at the fetal-facing basement membrane only for simplicity; it could also be occurring at the other basement membranes in parietal and columnar cells. The focused expression of the calcitropic factors within this structure may enable calcium transfer to be directly regulated, such as calcium sensing by the CaSR and stimulation of active transfer by PTHrP. Also, the focused expression of PTHrP and (not shown) platelet-derived growth factor receptor-α may regulate growth and size of the IPYS. CaP, Ca²⁺-ATPase; 9K, calbindin-D₉k. Calcium receptor and PTH/PTHrP receptor are each represented by schematic receptors attached to the appropriate ligand.
transfer. Similarly, the reduced placental calcium transfer of Casr-null fetuses may be the result of the loss of calcium sensing within the IPYS. However, further study is needed to confirm the role of the IPYS and to determine how much calcium is transferred via the IPYS as opposed to trophoblasts. It is evident that the IPYS is not simply a nonfunctional remnant of the primitive yolk sac but is a functional structure whose importance remains to be fully elucidated.

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