Localization of parathyroid hormone-related protein in the preimplantation mouse embryo is associated with events of blastocyst hatching

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Abstract

Purpose To determine the pattern of expression of parathyroid hormone-related protein (PTHrP) and its receptor, parathyroid hormone receptor 1 (PTHr1), in mouse embryos in different stages of preimplantation development.

Methods Embryos were cultured from the pronuclear zygote stage and harvested as 2-cell, 4-cell and 8-cell embryos, morulae and blastocysts. RT-PCR was carried out on mRNAs of these and of trophoblast outgrowths for detection of PTHrP and PTHr1. Whole mounted embryos intact or stripped of zonae pellucidae were immunofluorescently stained for PTHrP and PTH receptor and observed with confocal microscopy.

Results PTHrP mRNA was present in the pronuclear zygote, not present in 2-cell, 4-cell and uncompact 8-cell embryos, present in the 8-cell compacting embryo, and not detected in 16-cell morulae or blastocysts. The mRNA was present in trophoblasts growing on fibronectin beds. mRNA for PTHr1 was detected in the pronuclear zygote, then undetected until the compacted 8-cell stage and thereafter. PTH receptor protein was observed in 2-cell embryos, morulae and in the inner cell mass and trophectoderm of blastocysts. PTHrP was observed dispersed in the cytoplasm of 2-cell, 4-cell and uncompact 8-cell embryos, and in distinct foci near the nuclei of morulae. In blastocysts, PTHrP appeared on the apical surface of only

trophoblast cells which had extruded from the zona pellucida. Fully hatched blastocysts expressed the protein on the apical side of all trophoblasts. When morulae were prematurely stripped of their zonae, PTHrP was observed on the embryos' outer surface. **Conclusions** PTHrP protein is expressed throughout early embryo development, and its receptor PTHr1 is expressed from the morula stage. Embryo hatching is associated with translocation of PTHrP to the apical plasma membrane of trophoblasts. PTHrP may thus have autocrine effects on the developing blastocyst.

Keywords Parathyroid hormone-related protein (PTHrP) · Parathyroid hormone receptor (PTH receptor, PTHr1) · Preimplantation embryo · Blastocyst hatching

Introduction

Parathyroid hormone-related protein (PTHrP) is a growth factor that was first isolated as a product of tumors associated with humoral hypercalcemia [1, 2]. Due to its high degree of structural homology with parathyroid hormone, PTHrP is able to interact with PTH receptor [3]. While PTH is a product of the parathyroid gland, PTHrP has been identified in a variety of tissues, including skin [4, 5], mammary tissue [6], pancreas [7], ovary [8], placenta [9, 10] and uterine myometrium and endometrium [4, 5, 11]. Earlier studies from our laboratory showed that PTHrP stimulates the outgrowth of trophoblast cells of mouse blastocysts cultured on fibronectin beds. Transforming growth factor- β caused a similar increase of outgrowth through up-regulation of endogenous PTHrP production by preimplantation or outgrowing embryos [11]. We therefore examined the pattern of expression of PTHrP and the PTH receptor (PTHr1) mRNAs as well as localization of the proteins at different stages of development in early mouse embryos.

Capsule Parathyroid hormone-related protein (PTHrP) is expressed by preimplantation embryos and its localization is markedly altered in trophoblast cells during blastocyst hatching.

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Materials and methods

Embryo collection and culture

Embryos were collected and cultured as previously described by Nowak et al. [11]. All embryos were produced by mating outbred CF1 females (Crl:CF1 BR, 6–8 weeks old; Charles River, Wilmington, MA) with hybrid BDF males (B6D2F1/Crl BR; Charles River). Mice were maintained on a 14L:10D cycle (lights-on at 0,500 h). Female mice were superovulated by injection of 5 IU eCG (Sigma, St. Louis, MO) i.p. at 1,330 h, followed by injection of 5 IU hCG (Sigma) 48 h later. Females were placed in cages with males and examined the following morning (Day 1) for the presence of a vaginal plug indicating mating had occurred.

For experiments for immunofluorescent staining, embryos were cultured from the pronuclear zygote stage. Female mice were killed between 1,330 h and 1,430 h on Day 1. Zygotes were flushed from each excised oviduct with 0.1 ml HEPES-buffered medium KSOM [12]. Zygotes from several mice were pooled and washed through one droplet of flushing medium containing 0.65 mg/ml hyaluronidase (Sigma) to remove cumulus cells; they were then washed through three further droplets of HEPES-buffered KSOM. Zygotes were cultured in dishes (Costar, Cambridge, MA) in medium KSOM [12, 13]. Four 50- μ l drops of medium were placed in each dish under 5 ml mineral oil (Sigma). The dishes were made up the day before embryos were harvested and were equilibrated overnight in an atmosphere of 6 % CO₂, 5 % O₂ and 89 % N₂ in a plastic culture chamber (Billups-Rothberg, Del Mar, CA) inside a 37 °C incubator. Zygotes, 5 at a time, were taken randomly from the collection pool and transferred to a culture dish, through two serial washes of culture drops, then to a third culture medium drop. Culture dishes were returned to the culture chamber at 37 °C, which was gas-equilibrated and then sealed.

For immunofluorescent staining of zona-stripped and zona-intact morulae and blastocysts, 2-cell embryos were harvested from excised oviducts on Day 2 and washed and cultured as described above.

RT-PCR

Mouse embryos were collected as pronuclear zygotes and cultured to the 2-cell, 4-cell, uncompact 8 cell, compacted 8 cell, morula, early blastocyst, expanded blastocyst, and hatched blastocyst stages, and also after undergoing outgrowth for 3 days in vitro on fibronectin beds [11]. The number collected at each stage ranged from 15 to 39. Embryos were collected, washed in PBS, pooled by developmental stage, then transferred to TRIZOL to be frozen and stored at -70 °C. RNA was isolated from embryos using TRIZOL extraction with the addition of glycogen to allow for better

precipitation of the very small amounts of RNA. The RNA samples were treated with DNase prior to reverse transcription to eliminate genomic contamination. Each RNA sample that was reverse-transcribed contained the equivalent of four embryos. PCR amplification was carried out for each set of primers on the mouse embryo cDNAs. The negative control consisted of a sample tube containing all reagents for PCR amplification, but with no cDNA added, only water. All PCR products were gel purified and their identities confirmed by dye-primer cycle sequencing.

Nested PCR was used for analysis of the PTHrP mRNA expression. The sequences of the first set of primers were: Primer A (upstream, bp 36–52 of PTHrP cDNA): 5' aggcgtgtcagt 3' and Primer B (downstream, bp 52–73 of cDNA): 5' ttccatatactattaca 3'. The sequences for the second set of primers (internal to Primers A and B) were: Primer C (upstream, bp 12–30 of cDNA): 5' tgcgtaacatcagctat t 3' and Primer D (downstream, bp 24–41 of cDNA): 5' acgtgtccttggagatc 3'. cDNA samples were first amplified for 30 cycles using Primers A and B. Three μ l of the product from the first round of amplification were then re-amplified for a further 30 cycles using Primers C and D. We also carried out nested PCR for analysis of expression of PTHR1 mRNA. The sequences of the first set of primers were: Primer A (upstream, bp 667–683 of PTHR1 cDNA): 5' gcaatggcagctgggag 3' and Primer B (downstream: bp 1,590–1,610 of cDNA): 5'ccattgcagaacag tatat 3'. The sequences for the second set of primers (internal to primers A and B) were: Primer C (upstream, bp 1,137–1,157 of cDNA): 5' cacagcctcatcttcatgggc 3' and Primer D (downstream, bp 1,308–1,323 of cDNA): 5'tgccaggatgggcacctg 3'. cDNA samples underwent two rounds of amplification, the first with primers A and B, the second with primers C and D. Primers used for β -actin to confirm integrity of RNA were Primer A (upstream, 5' ctcttccagccttctctctg 3' and Primer B (downstream, 5' gaagcatttgcgttgacgat 3') with a product size of 335 bp. All PCR reactions were repeated on four different lots of embryos.

Fluorescent staining and confocal microscopy

Embryos were washed three times in PBS + BSA 4 mg/ml (PBS/BSA), then fixed by transfer to a droplet of 0.1 M PIPES, 5 mM magnesium chloride, 2.5 mM EGTA, 3 % paraformaldehyde for 30 min at room temperature. Embryos were washed again twice with PBS/BSA then incubated 45 min at room temperature in 0.2 % Triton in PBS/BSA. Following this, embryos were incubated in PBS 3 % fetal calf serum, 0.1 % tween 20, 0.02 % sodium azide for 2 h at room temperature, then in PBS, 1 % fetal calf serum, 0.03 % tween 20, 50 mM glycine for 15 min at room temperature. Embryos were then washed three times in PBS 1 % fetal calf serum, 0.03 % tween. For specific staining for PTHrP, the embryos were transferred to a 50 μ l drop of PBS containing rabbit anti-PTHrP (Oncogene Research Products, Cambridge, MA) at a

concentration of 5 µg/ml for 1 h in a humidified incubator at 37 °C (control consists of PBS/BSA). Separately, for specific staining for PTH-R, embryos were transferred to a 50 µl drop of PBS containing rabbit anti-PTH-R (Sigma Aldrich, St. Louis, MO). At the end of this incubation, embryos were washed through three droplets of PBS 3 % fetal calf serum, 0.1 % tween, 0.02 % sodium azide and incubated in a fourth droplet of this solution for 30 min at room temperature. Embryos were then transferred to a 50 µl drop of PBS containing fluorescein-conjugated goat anti-rabbit IgG (affinity purified; cat. no. 100825, Boehringer-Mannheim, Indianapolis, IN) at a dilution of 1:100, for 1 h at 37°C. Following this, embryos were washed through two droplets of PBS 3 % fetal calf serum, 0.1 % tween, 0.02 % sodium azide (room temp), then through a series of droplets of PBS 3 % goat serum containing glycerol at successively increasing concentrations: 2.5 %, 5 %, 10 %, 20 % and 50 %. Embryos were mounted in tape wells on glass slides in 50 % glycerol, propidium iodide 10 µg/ml and 1,4-diazabicyclo [2, 2, 2]-octane and then sealed under coverslips.

Fixed and stained embryos were observed using a Zeiss Laser Scan 410 Inverted microscope with an argon laser set at an excitation wavelength of 488 nm. Fluorescent emission from propidium iodide was detected using a 590 nm long pass filter, emission from fluorescein using a 510–525 nm long pass filter. Optical sections across the *z* axis were recorded using Zeiss LSM 3.80 software and retrieved using Adobe Photoshop 3.0. A KPT Sharpen Intensity 2.0 filter was used on all images to reduce background fluorescence, and for printing purposes, an Unsharp Mask filter was used to increase image resolution.

Mechanical hatching

Embryos cultured from the 2-cell stage were taken for immunofluorescent staining for PTHrP as four groups: zona-intact morulae, zona-intact blastocysts, zona-stripped morulae and zona-stripped blastocysts. Embryos to be stripped of their zonae pellucidae were removed from culture by group to a drop of HEPES-buffered KSOM 0.5 % protease (Sigma P8811) previously warmed to 37 °C, and observed continuously until the zonae were visibly weakened, for no longer than 2 min. Embryos were then washed through two drops of HEPES-buffered KSOM and to a third drop where they were drawn and redrawn through a pipette until zonae were mechanically stripped away. Stripped embryos were transferred to a culture drop and returned to incubate at 37 °C for 30 min, then removed for fixing and staining.

Results

RT-PCR was undertaken to determine at what stages of development mouse embryos begin to express the mRNAs for

PTHrP and PTHR1. The results are shown in Fig. 1. Detection of a 335 bp amplification product for β -actin in all samples confirmed the integrity of each RNA sample. A 466 bp product corresponding to PTHrP mRNA was present in the one cell zygotes but was not detected in the early or late 2-cell, 4-cell or uncompact 8-cell stages. The mRNA then was expressed transiently in the compacting 8-cell embryos. Expression of PTHrP mRNA then disappeared until it was detected again in blastocysts undergoing outgrowth on fibronectin beds in vitro. This pattern of PTHrP mRNA expression was intriguing as its expression in the embryo appears to be correlated with two stages that involve important morphological changes, compaction and outgrowth. We also carried out RT-PCR for PTHR1 in the various stages of embryos. Our results showed that one cell zygotes expressed the 188 bp product corresponding to the PTHR1 mRNA. The source of this mRNA in the one cell zygotes was maternal mRNA which was degraded by the 2-cell stage. Once embryos reached the compacted 8-cell stage the mRNA for PTHR1 reappeared and was evident in all subsequent stages of development.

In Fig. 2 are shown images of whole mounted embryos immunofluorescently stained for PTHR1 (red is the fluorescent signal for propidium iodide-stained nuclei, green is fluorescent signal for PTHR1). Results shown are representative of all embryos (minimum ten) at each stage. Receptor protein for PTH was present in the 2-cell embryo, however we were unable to detect any PTHR1 protein in either 4-cell or 8-cell stage embryos (2B,C). In the morula the PTHR1 protein was once again apparent as discrete foci near the nuclei (2D), and in the blastocyst the receptor was evident in both the inner cell mass and in the trophectoderm (2E).

Fig. 1 RT-PCR for PTHrP, PTHR1 and β -actin mRNAs in mouse embryos at various stages of development. Samples analyzed were: 1C- fertilized zygotes; E2C- early 2-cell embryos; L2C- late 2 cell embryos; 3C- four cell embryos; UNC8- uncompact 8-cell embryos; C8- compacted 8-cell embryos; M- morulae; EB- early blastocysts; HB- hatched blastocysts; OUTB- blastocysts cultured on fibronectin beds for 72 h. Expected amplification product sizes were 466 bp for PTHrP, 166 bp for PTHR1, and 335 bp for β -actin. Each sample for amplification consisted of mRNA equivalent to 4 embryos and was carried out on four different lots of embryos

Fig. 2 Expression of PTHR1 protein in mouse embryos at various stages of development visualized by immunofluorescent staining. Images show PTHR1 protein expression as green staining in (a) 2-cell; (b) 4-cell; (c) 8-

cell; (d) morula; (e) blastocyst and (f) negative control. Red color represents the nuclear propidium iodide stain

Figure 3 shows PTHrP protein expression in cultured embryos at all stages, with immunostaining dispersed in the cytoplasm of the pronuclear zygote (3B) and of cells of the 2-cell embryo (3C). The 4-cell (3D) and uncompact 8-cell (3E) embryos displayed more concentrated areas of staining

within their cytoplasm. Discrete foci of intense staining for PTHrP could be observed near the nuclei of cells of the morula (3F). The final frame of Fig. 3g shows immunofluorescent staining for PTHrP in a hatching blastocyst, with intense immunoreactivity only in trophoblast cells free of the zona

Fig. 3 Expression of PTHrP protein in mouse embryos at various stages of development visualized by immunofluorescence staining. Images show PTHrP protein expression as green staining in (a) negative control;

(b) one cell zygotes; (c) 2-cell; (d) 4-cell; (e) 8-cell; (f) morula; (g) hatching blastocyst. Red color represents the nuclear propidium iodide stain

Fig. 4 Expression of PTHrP protein in hatching (**b, c**) and fully hatched blastocysts (**d**). Only trophoblast cells of the portion of the blastocyst extruded from the zona pellucida showed intense staining across the

pellucida. The negative control for specific immunostaining is shown in **3A**.

Hatching and fully hatched blastocysts immunostained for PTHrP are presented in Fig. 4. Only trophoblast cells of the portion of the blastocyst extruded from the zona pellucida showed intense staining across the apical surface of the cells. The hatched blastocyst completely free of the zona showed this same pattern of staining for PTHrP in all trophoblasts.

We next tested whether mechanical removal of the zona pellucida from embryos would cause the same distribution of PTHrP protein observed in response to spontaneous hatching. The results of this experiment are shown in Fig. 5. Blastocysts hatched spontaneously (**5C**) or mechanically (**5D**) demonstrated similar patterns of immunostaining, with intense staining on the apical surfaces of all trophoblast cells. In comparison with zona-intact morulae (**5A**), morulae prematurely

stripped of their zonae (**5B**) showed PTHrP protein expression across the surface of the cells constituting the outer surface of the embryo. The morulae enclosed within the zona showed immunostaining only in discrete foci around the nuclei.

Discussion

Embryo hatching is a necessary process in order for attachment of the blastocyst to the uterine epithelium to occur. While some of the events of hatching are beginning to be understood, there is still a substantial lack of knowledge about the mechanisms utilized by the embryo to initiate hatching and that allow it to escape from the zona pellucida. We investigated the expression of PTHrP and its receptor, PTHR1, during early embryo development and hatching. Our findings show

Fig. 5 PTHrP protein expression in zona-intact morulae (**a**); morula prematurely stripped of its zona (**b**); blastocysts hatched spontaneously (**c**) or blastocysts that had zona removed mechanically (**d**) and blastocyst in the process of hatching (**e**). Removal of the zona resulted in localization of PTHrP protein to the outer surface of cells

that PTHrP protein is expressed throughout early embryo development but that localization of PTHrP in the trophoctoderm is altered dramatically as the blastocyst escapes from the zona pellucida. The translocation of PTHrP protein from the cytoplasm to the apical plasma membrane also occurred in morulae that were stripped of their zona. The PTHR1 is expressed beginning at the morula stage and is present in both the inner cell mass and trophoctoderm cells of the blastocyst. Thus, PTHrP secreted by the developing embryo may have autocrine effects on the blastocyst as it progresses through implantation.

The results of our study differ somewhat from those reported recently by Guo et al. [14]. These investigators analyzed PTHrP mRNA expression at different stages of development in mouse embryos using qPCR. They found that PTHrP mRNA was expressed in mouse oocytes and all stages of embryo development while we detected expression only in one cell zygotes, compacted 8-cell embryos and blastocysts undergoing outgrowth. These differences could be due to differences in sensitivity of the methods used. Similar to our results Guo et al. also observed highest expression of PTHrP mRNA at the 8 cell stage and lowest expression in blastocysts. SiRNA knockdown of PTHrP in mouse embryos significantly decreased blastocyst formation in vitro and also reduced expression of the pluripotency-associated genes *Nanog* and *Pou5f1*. These results suggest that PTHrP plays an important role during preimplantation embryonic development.

We reported in a previous study that mouse blastocysts secrete PTHrP in response to transforming growth factor- β and that PTHrP stimulates blastocyst outgrowth in an in vitro culture system [11]. Subsequent studies by El-Hashash et al [15] determined that PTHrP stimulates expression and organization of actin stress fibers and suppresses E-cadherin expression in murine trophoblast giant cells. Other studies have demonstrated that PTHrP stimulates secretion of metalloproteinases MMP-2 and MMP-13 by fibroblasts and osteoblasts [16, 17] and dipeptidyl peptidase II by rat astrocytes [18]. These findings support a role for PTHrP during embryo implantation to facilitate migration and invasion of trophoblast cells.

Mammalian blastocyst hatching is a necessary event for successful implantation of the embryo yet it is still not well understood. Hatching is thought to be under cellular and molecular control but also probably involves responses to biomechanical signals [19]. One of the findings in regards to the ultrastructure of blastocysts at the time of hatching is the presence of actin-based trophoctodermal projections (TEPs) into the zona pellucida [19, 20]. These TEPs appear at the abembryonic pole of hatching blastocysts of guinea pigs and hamsters but at the embryonic pole in primates [20, 21]. Recent studies have shown that the TEPs may serve as cargo carriers for the various enzymes and proteases that the blastocyst requires for hatching from the zona [19, 22]. The cell

signaling pathways involved in regulating hatching are beginning to be unraveled. Studies by Lu et al. [23] showed that the small-conductance calcium-activated K^+ channels (SK3) regulate blastocyst hatching in mouse and human embryos by controlling the intracellular calcium concentrations. In fact, the levels of SK3 expression were significantly lower in human blastocyst that failed to hatch. SK3 protein was localized predominantly in the trophoctoderm cell membranes of expanded mouse blastocysts and siRNA knockdown of SK3 reduced intracellular calcium levels and inhibited hatching. The beta-catenin signaling pathway has also been shown to be an important regulator of hatching. Aberrant activation of beta-catenin with LiCl strongly inhibited hatching of mouse blastocysts as well as adhesion and outgrowth [24]. Other potential intracellular regulators include cyclooxygenases and nuclear factor κ B [19].

In conclusion, we have shown that PTHrP and PTHR1 are expressed by preimplantation mouse embryos and that localization of PTHrP protein is dramatically altered in trophoctoderm cells as the blastocyst undergoes hatching. A similar relocation of PTHrP protein occurs in morulae when the zona is removed mechanically. We do not know how this alteration in protein localization is regulated but our findings support studies by others that PTHrP is important for peri-implantation development. Additional studies focusing on the mechanisms regulating this protein movement within the trophoctoderm cells at the time of hatching are warranted.

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