The Roles of Parathyroid Hormone-Like Hormone during Mouse Preimplantation Embryonic Development

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Abstract
Parathyroid hormone-like hormone (PTHLH) was first identified as a parathyroid hormone (PTH)-like factor responsible for humoral hypercalcemia in malignancies in the 1980s. Previous studies demonstrated that PTHLH is expressed in multiple tissues and is an important regulator of cellular and organ growth, development, migration, differentiation, and survival. However, there is a lack of data on the expression and function of PTHLH during preimplantation embryonic development. In this study, we investigated the expression characteristics and functions of PTHLH during mouse preimplantation embryonic development. The results show that Pthlh is expressed in mouse oocytes and preimplantation embryos at all developmental stages, with the highest expression at the 32-cell stage oocytes and at the lowest expression at the blastocyst stage of preimplantation embryos. The siRNA-mediated depletion of Pthlh at the 32-cell stage embryos significantly decreased the blastocyst formation rate, while this effect could be corrected by culturing the Pthlh-depleted embryos in the medium containing PTHLH protein. Moreover, expression of the pluripotency-related genes Nanog and Pou5f1 was significantly reduced in Pthlh-depleted embryos at the morula stage. Additionally, histone acetylation patterns were altered by Pthlh depletion. These results suggest that PTHLH plays important roles during mouse preimplantation embryonic development.

Introduction
Parathyroid hormone-like hormone (PTHLH), also known as parathyroid hormone-related protein (PTHrP), was first identified as a parathyroid hormone (PTH)-like factor responsible for humoral hypercalcemia in malignancies in the 1980s [1]. Moniz et al. reported for the first time that PTHLH was expressed during normal human fetal development [2]. Unlike PTH, PTHLH expression was observed in many fetal tissues and adult tissues, even in the absence of hypercalcemia [3,4]. PTHLH has multiple physiological functions, including the regulation of morphogenesis, cell proliferation and differentiation, and transplacental calcium transport [5]. Pthlh null mice are characterized by chondrodysplasia, accelerated chondrocyte differentiation, endochondral ossification, and lethality minutes following birth [6]. Pthlh+/− mice demonstrate dysplastic long bone formation and early lethality, either in utero or within one to two days following birth [7].

Although many studies have reported that PTHLH is expressed in multiple tissues and plays multiple functions, few studies have investigated its expression characteristics and biological functions during preimplantation embryonic development. Van de Stolpe et al. reported that PTHLH is an endogenous inducer of parietal endoderm differentiation in the preimplantation mouse embryos, thus constituting the first identified example of an embryonic inducer in preimplantation mammalian development and the earliest hormone receptor system involved in embryogenesis defined to date [8]. They also showed for the first time that in the preimplantation mouse embryos, PTHLH was detected from the late morula stage onwards primarily in developing trophectoderm cells [8]. However, there was no functional study on the preimplantation embryonic development other than differentiation. Hereafter, Nowak et al. reported that PTHLH (1–34) and PTHLH (1–141) had no effect on the incidence of blastocyst formation in mouse preimplantation embryonic development [9]. Watson et al. reported that the supplementation of serum-free cSOFMaa oocyte maturation medium with PTHLH (1–141) resulted in a concentration-dependent increase in the development of bovine zygotes up to the blastocyst stage in vitro [10]. Both studies investigated the effects of exogenous PTHLH on preimplantation embryonic development, but the biological functions of endogenous PTHLH during mouse preimplantation embryonic development are yet unclear. Based on the extensive roles of PTHLH during the development and the expression of PTHLH in the preimplantation mouse embryos, we hypothesized that the endogenous PTHLH might play important roles during mouse preimplantation embryonic development by affecting the developmental capacity of mouse preimplantation embryos, the quality of blastocyst and the transcription of the preimplantation embryos.
In this study, for the first time, the expression characteristics of \textit{Pthlh} were investigated, and siRNA-mediated depletion of \textit{Pthlh} was used to investigate the involvement of endogenous PTHLH in mouse preimplantation embryonic development. The effects of down-regulation of this gene on developmental capacity, the expression of downstream or functionally related genes and histone acetylation dynamics were also examined.

**Results**

\textbf{Pthlh Expression during Mouse Preimplantation Embryonic Development}

The expression of \textit{Pthlh} in mouse oocytes and preimplantation embryos was examined using quantitative real-time PCR. The results show that \textit{Pthlh} is expressed in mouse oocytes and preimplantation embryos at all developmental stages, with the highest level observed at the MII stage of the oocytes. \textit{Pthlh} expression decreased dramatically following the 1-cell stage and reached its lowest level at the blastocyst stage (Fig. 1).

**Effects of \textit{Pthlh} Depletion on Mouse Preimplantation Embryonic Development**

The effects of \textit{Pthlh} depletion on early mouse preimplantation embryonic development were assessed by introducing \textit{Pthlh} siRNA into the oocytes at the MII stage and the preimplantation embryos at the 1-cell stage. To ensure the efficiency of the siRNA-mediated knockdown prior to further studies, the expression levels of \textit{Pthlh} were examined using quantitative real-time PCR and Western blot. The results showed that \textit{Pthlh} expression level was decreased by 72.1% in the \textit{Pthlh} siRNA-injected ICSI embryos compared with the control embryos at the 2-cell stage (Fig. 2A). The results also showed that the expression of \textit{Pthlh} was reduced by 84.7% in the \textit{Pthlh} siRNA-injected embryos compared with those from the control groups (100.0%) (P<0.01) at the 2-cell stage (Fig. 2B), and the protein level in the \textit{Pthlh} siRNA-injected group also decreased compared with the control groups (Fig. 2D). However, compared with the control groups, the \textit{Pthlh} expression level was increased in the \textit{Pthlh} siRNA-injected group (100.0% vs 163.2%) (P<0.01) (Fig. 2C).

To investigate the effects of \textit{Pthlh} depletion on blastocyst formation, 9 experiments were performed. The developmental rate and cell number at the blastocyst stage were examined. The \textit{Pthlh} siRNA-injected and the control embryos were cultured in vitro until the control embryos developed to the expanded blastocyst stage. Notably, 51.7% embryos in the control siRNA-injected ICSI group and 52.5% of the un.injected ICSI embryos developed to the blastocyst stage, while only 24.5% embryos in the \textit{Pthlh} siRNA-injected ICSI group developed to the blastocyst stage (P<0.01) (Table 1). Additionally, 83.1% of the control siRNA-injected embryos and 83.7% of the un.injected embryos developed to the blastocyst stage, whereas only 38.0% of the \textit{Pthlh} siRNA-injected embryos developed to the blastocyst stage (P<0.01), and 49.0% of the \textit{Pthlh} siRNA-injected embryos arrested at the morula stage (P<0.01) (Table 2, Table S1). The total cell number of the \textit{Pthlh}-depleted embryos at the blastocyst stage (59.9±2.7, n = 21) was not significantly different from that of the control siRNA-injected group (63.3±2.7, n = 19) and the un injected group (63.8±2.9, n = 20).

\textbf{Pthlh Depletion Down-regulates the Expression of Pou5f1 and Nanog}

To investigate the effects of \textit{Pthlh} depletion on the expression of the pluripotency-related genes \textit{Nanog} and \textit{Pou5f1} during mouse preimplantation embryonic development, quantitative real-time PCR and immunofluorescence staining were performed. Quantitative real-time PCR results showed that the expression levels of \textit{Pthlh}, \textit{Pou5f1} and \textit{Nanog} were reduced by 50.3%, 55.0% and 59.3%, respectively, in the \textit{Pthlh} siRNA-injected group compared with the control groups (100.0%) (P<0.01) at the morula stage (60 h post-siRNA injection) (Fig. 3). Decreased expression of OCT4 and NANOG was also demonstrated by immunofluorescence staining at the morula stage (Fig. 4, Fig. S1).

\textbf{Effects of \textit{Pthlh} Depletion on the Histone Acetylation of Mouse Preimplantation Embryos}

Immunofluorescence staining showed the following changes in histone acetylation of preimplantation embryos: the acetylation of lysine 9 on histone H3 (H3K9) and the acetylation of lysine 14 on histone H3 (H3K14) were both significantly decreased at the 2-cell, 4-cell, 8-cell and morula stages (Fig. 5, Fig. 6, Fig. S2, Fig. S3).

\textbf{Effects of PTHLH (1–141) on the Development of Embryos Injected with \textit{Pthlh} siRNA}

To investigate whether the exogenous target protein can improve the blastocyst development of the embryos injected with \textit{Pthlh} siRNA, the PTHLH (1–141) was synthesized \textit{in vitro}. After \textit{Pthlh} siRNA injection, the embryos were cultured in KSOMAA with or without 10 ng/ml PTHLH (1–141). 76.8% of embryos cultured in KSOMAA with 10 ng/ml PTHLH (1–141) reached the blastocyst stage (Table 3), showing that the protein PTHLH (1–141) could improve the blastocyst development of the embryos injected with \textit{Pthlh} siRNA.

**Discussion**

In the present study, we determine for the first time that \textit{Pthlh} is expressed in mouse oocytes and preimplantation embryos at all developmental stages and PTHLH plays important roles during mouse preimplantation embryonic development.

Before the functional studies of endogenous PTHLH during mouse preimplantation embryonic development were performed, the expression characteristics of \textit{Pthlh} were investigated by quantitative real-time PCR. We observed the expression of \textit{Pthlh} in mouse oocytes and preimplantation embryos at all developmental stages, with the highest level observed at the MII stage of the oocytes and the lowest level at the blastocyst stage of the

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**Figure 1. \textit{Pthlh} expression in mouse oocytes and preimplantation embryos.** Quantitative real-time PCR was applied to detect the expression of \textit{Pthlh} in GV oocytes, MII oocytes, and in embryos at the 1-cell (1-C), 2-cell (2-C), 4-cell (4-C), 8-cell (8-C), morula (MO) and blastocyst (BL) stages. Transcripts levels were normalized against \textit{Gapdh} expression. Data are presented as means ± SEM. doi:10.1371/journal.pone.0040528.g001
Given that the expression level of Pthlh decreased dramatically after the 1-cell stage, we examined the effects of Pthlh knockdown on preimplantation embryonic development by performing the siRNA-mediated Pthlh depletion at the MII stage oocytes and 1-cell stage embryos. Our data clearly indicated that the expression of Pthlh could be efficiently knocked down using siRNA mediated gene silencing in early preimplantation embryos. Further functional studies of PTHLH during mouse preimplantation embryonic development were performed based on the successful knockdown.

First, we investigated the effects of PTHLH on blastocyst formation. The formation of the blastocyst is an important landmark in the early developmental axis [11]. Blastocyst formation is often used as a criterion for developmental competence in in vitro embryo production systems. In the present study, we assessed blastocyst formation following the microinjection of Pthlh siRNA into mouse embryos at the 1-cell stage.

**Table 2. Embryonic development following the microinjection of Pthlh siRNA into mouse embryos at the 1-cell stage.**

| Treatment         | Total | 2-c (%) | 4-c (%) | 8-c (%) | Morula (%) | Blastocyst (%) |
|-------------------|-------|---------|---------|---------|------------|----------------|
| uninjected        | 210   | 3 (1.4) | 7 (3.4) | 4 (1.9) | 16 (7.6)   | 180 (85.7)     |
| Control siRNA     | 308   | 13 (4.2)| 12 (3.9)| 4 (1.3) | 23 (7.5)   | 256 (83.1)    |
| Pthlh siRNA       | 361   | 16 (4.4)| 17 (4.7)| 11 (3.1)| 177 (49.0)*| 140 (38.8)*    |

*p<0.01 compared with the control groups.

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**Table 1. Effect of Pthlh siRNA on development of mouse ICSI embryos.**

| Treatment     | No. of embryos (No. exp) | No. of blastocysts (%) |
|---------------|---------------------------|------------------------|
| uninjected    | 80 (3)                    | 42 (52.5)              |
| Control siRNA | 87 (3)                    | 45 (51.7)              |
| Pthlh siRNA   | 102 (3)                   | 25 (24.5)*             |

*p<0.01 compared with the control groups.

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study, Pthlh depletion was used to examine the role of PTHLH during mouse early embryonic development, especially blastocyst formation. The siRNA-mediated depletion of Pthlh at both MII oocytes and 1-cell embryos affected the blastocyst formation of mouse ICSI or fertilized embryos significantly. Nowak et al. reported that exogenous PTHLH (1–141) had no effect on the incidence of blastocyst formation in mouse preimplantation embryonic development [9]. However, we found that the PTHLH (1–141) had positive effect on the blastocyst formation rate of Pthlh-depleted embryos, which suggest that the lack of endogenous PTHLH can be corrected by exogenous PTHLH. The significantly decreased blastocyst formation rate of Pthlh-depleted embryos and the fact that PTHLH (1–141) could increase the blastocyst formation rate of the Pthlh-depleted embryos indicate that PTHLH is required for blastocyst formation.

Many transcription factors are required for reprogramming during mouse preimplantation embryonic development. The pluripotent state of the early embryo is established and maintained in vivo by a transcriptional network driven by a number of core regulatory genes, including Oct4/Pou5f1 and Nanog. Blastocyst

Figure 3. The effects of Pthlh siRNA injection on the expression of Pthlh (A), Nanog (B) and Pou5f1 (C) in mouse morula stage embryos. The uninjected embryos and the control siRNA-injected embryos were used as controls. Data are presented as means ± SEM. *p<0.01. doi:10.1371/journal.pone.0040528.g003
formation shows the segregation of the two cell lineages in mammalian preimplantation embryos, the inner cell mass (ICM) and trophectoderm (TE). As the important regulators of pluripotency in mammalian embryos, both Oct4 and Nanog play important roles in the formation of the ICM. OCT4 is the earliest expressed transcription factor known to be crucial in murine preimplantation embryonic development [12,13]. Nanog (-/-) embryos do not develop beyond implantation [14], and NANOG specifically demarcates the nascent epiblast [15], whereas ICM without Nanog cannot differentiate into primitive endoderm [14]. In the present study, the significantly decreased expression of Nanog and Pou5f1 in Pthlh-depleted embryos indicates that Pthlh depletion can affect the preimplantation embryonic development by decreasing the expression of the pluripotency-related genes. There is no evidence
Figure 5. The effects of Pthlh siRNA injection on the acetylation of lysine 9 on histone H3 (Ac-H3K9) in mouse preimplantation embryos.

A) The staining pattern of Ac-H3K9 in the uninjected (a–a' to e–e'), the control siRNA-injected (f–f' to j–j') and the Pthlh siRNA-injected (k–k' to o–o') embryos at the 2-cell stage (a–a', f–f' and k–k'); the 4-cell stage (b–b', g–g' and l–l'); the 8-cell stage (c–c', h–h' and m–m'); the morula stage (d–d', i–i' and n–n') and the blastocyst stage (e–e', j–j' and o–o'). Ac-H3K9, green; DNA, blue. Bar = 20 μm.

B) Quantification of Ac-H3K9/DNA signal intensity in the Pthlh siRNA-injected and the control embryos (n = 12). Data are presented as means ± SEM. *P < 0.05.

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Figure 6. The effects of Pthlh siRNA injection on the acetylation of H3K14 (Ac-H3K14) in mouse preimplantation embryos. A) The staining pattern of Ac-H3K14 in the uninjected (a-a' to e-e'), the control siRNA-injected (f-f' to j-j') and the Pthlh siRNA-injected (k-k' to o-o') embryos at the 2-cell stage (a-a', f-f' and k-k'); the 4-cell stage (b-b', g-g' and l-l'); the 8-cell stage (c-c', h-h' and m-m'); the morula stage (d-d', i-i' and n-n') and the blastocyst stage (e-e', j-j' and o-o'). Ac-H3K14, green; DNA, blue. Bar = 20 μm. B) Quantification of Ac-H3K14/DNA signal intensity in the Pthlh siRNA-injected embryos and the control embryos (n = 12). Data are presented as means ± SEM. *P < 0.05, **P < 0.01.
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showed that how the PTHLH affects the transcription factors (Oct4/Pou5f1 and Nanog) in any cell types. Previous studies showed that the PTHLH can play roles through a complex network of signaling pathways involving cAMP, PKC and ERK/MAPK pathways [16]. Therefore, we speculated that the PTHLH might affect the expression of the pluripotency-related genes indirectly through those pathways during preimplantation embryonic development, but the mechanism needs further investigation.

Apart from the transcriptional network, epigenetic modifications play critical roles in mammalian embryo development. The acetylation of lysine residues on the tails of histones H3 and H4 is a reversible process that plays critical roles in maintaining higher-order chromatin structure and in regulating various chromatin processes, including transcription, DNA repair and replication. In the present study, the dynamic changes of the acetylation of different lysine residues on core histones H3 (H3K9 and H3K14) in Pthlh-depleted mouse embryos demonstrated that PTHLH can affect histone acetylation modification during preimplantation embryonic development. Although there is no evidence showed that the PTHLH exerts direct effects on histone acetylation during preimplantation embryonic development, previous studies reported that the PTHLH exerts direct effects on histone deacetylase 4 (HDAC4) [17,18]. Since the activity of histone deacetylase can be affected by PTHLH, the histone acetylation can be affected as a result during preimplantation embryonic development.

In the present study, our data provide the first evidence of the functions of endogenous PTHLH during mouse preimplantation embryonic development. PTHLH influences blastocyst formation, Nanog and Pou5f1 expression and the histone acetylation in mouse preimplantation embryos, indicating that Pthlh plays very important roles in the development of mouse preimplantation embryos.

Materials and Methods

All chemicals and media were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned.

Ethics Statement

Mice care and handling were conducted in accordance with the Animal Research Committee guidelines of the Institute of Zoology, Chinese Academy of Sciences. The institute does not issue a number to any animal study, but there is an ethical committee to guide animal use. Each study requires the permit to use animals from the committee, and this study was approved by the Animal Research Committee of the Institute of Zoology, Chinese Academy of Sciences. The institute does not require any animal study permit or ethical committee approval.

Microinjection, ICSI and in vitro Culture

Microinjections were performed in M2 medium. MII stage oocytes and 1-cell stage embryos were microinjected with Pthlh siRNA (sc-39696, Santa Cruz, Fig. 2B, Fig. S4). MII stage oocytes were performed intracytoplasmic sperm injection (ICSI) 3 h post-siRNA microinjection, a single sperm head was microinjected into the MII oocyte assisted with a Piezo-drill micromanipulator. Oocytes and embryos injected with control siRNA (GenePharma) were used as sham control. All injected embryos and uninjected control embryos were cultured in KSOMaa (Millaire) following microinjection for 4 days in a 37°C, 5% CO₂ atmosphere.

RNA Isolation, Reverse-transcriptase and Quantitative Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated from 40 oocytes or preimplantation embryos of the injection groups and the control groups at the different developmental stages using the RNeasy Micro kit (74004, Qiagen) following the manufacturer’s manual. Oocytes and preimplantation embryos were transferred to lysis buffer and treated with the RNase-free DNase supplied in the kit. The mRNA was reverse-transcribed into cDNA using oligo (dT) primers and the PrimeScript™ 1st strand cDNA synthesis kit (D6110, TaKaRa) according to the manufacturer’s instructions.

To measure mRNA levels, real-time RT-PCR analyses were performed using the ABI Prism 7500 real-time PCR system (Applied Biosystems). SYBR Premix Ex Taq™ reagents (DRR041, TaKaRa) were used for monitoring amplification, and the results were evaluated with the 7500 software program (v 2.0.1, Applied Biosystems). Real-time PCR primers were designed using the PrimerExpress software (Applied Biosystems) purchased from Invitrogen. All primers are shown in Table 4. The reaction mixture had a final volume of 20 μl and contained 10 μl SYBR Premix Ex Taq™ (2×), 0.4 μl forward primer (10 μM), 0.4 μl

### Table 3. Effect of PTHLH (1–141) on development of mouse embryos injected with Pthlh siRNA.

| Treatment | No. of embryos (No. exp) | No. of blastocysts (%) |
|-----------|--------------------------|------------------------|
| KSOMaa    | 43 (3)                   | 17 (39.5)              |
| KSOMaa+10 ng/ml PTHLH (1–141) | 56 (3)                   | 43 (76.8)*             |

*p<0.01 compared with the control group.

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reverse primer (10 μM), 0.4 μl ROX Reference Dye (50x), 2.0 μl cDNA and 6.8 μl water. The PCR thermal cycling parameters were 95°C for 10 s and 40 cycles of 5 s at 95°C and 34 s at 60°C. Following the PCR reaction, the specificity of amplification for each gene was evaluated by monitoring the dissociation (melting) curve. Expression levels of different transcripts were normalized to the housekeeping gene Gapdh within the log-linear phase of the amplification curve using the ΔΔCT method.

Western Blot Analysis

350 mouse embryos were collected in SDS sample buffer and heated for 5 min at 100°C. The proteins were subjected to 12% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked for 2 h in Tris-buffered saline-Tween (TBST) containing 5% nonfat dry milk at room temperature. The blocked membranes then were incubated overnight at 4°C with affinity-purified goat polyclonal anti-PTHLH antibody (1:500; Santa Cruz) or mouse monoclonal anti-GAPDH antibody (1:1,000, Zhong Shan Jin Qiao) in TBST. After washing three times in TBST with each time for 10 minutes, the membrane was incubated for 1 hour at 37°C with peroxidase-conjugated rabbit anti-goat IgG (1:1,000, Zhong Shan Jin Qiao) or peroxidase-conjugated rabbit anti-mouse IgG (1:1,000, Zhong Shan Jin Qiao), respectively. Finally, the membrane was processed using the SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific).

Synthesis of Pthlh mRNA and PTHLH (1–141) Protein

The PTHLH (1–141) gene fragment was amplified by PCR with the following primers: F: GCGAATTCTATGCTGCCTGG-GAGGCCTGGTTCT (NM_008084) and R: GCCCTGAGTCGCTTCTTTTTCTC (NM_008084), respectively. The PCR thermal cycling parameters were 94°C for 1 min and 35 cycles of 10 s at 94°C, 10 s at 60°C, and 30 s at 72°C. Following the PCR reaction, the specificity of amplification for each gene was evaluated by monitoring the dissociation (melting) curve. Expression levels of different transcripts were normalized to the housekeeping gene Gapdh within the log-linear phase of the amplification curve using the ΔΔCT method.

Immunoﬂuorescence and Confocal Microscopy

All steps were performed at room temperature unless otherwise noted. Collected embryos were fixed with 4% paraformaldehyde for 30 min and permeabilized for 30 min with 0.2% Triton X-100 in PBS. Following three washes, all samples were incubated in a blocking solution (1% BSA and 0.05% Tween-20 in PBS) for at least 1 h. Embryos were then incubated with antibodies to H3K9 acetylation (1:250, Upstate), H3K14 acetylation (1:250, Upstate), NANO (1:500, Cosmo Bio) or OCT4 (1:500, Santa Cruz) overnight at 4°C. Following three washes, embryos were incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse second antibody (1:100; Zhong Shan Jin Qiao) or goat anti-rabbit second antibody (1:100; Zhong Shan Jin Qiao) for 1 h. Following three washes in washing buffer, DNA was stained with Hoechst 33342 (10 μg/ml), and all samples were mounted in antifade solution. Stained and slide-mounted embryos were observed on a Zeiss LSM 710 microscope (Carl Zeiss, Germany). The same instrument settings were used for samples of the same development stage. Nuclear intensities of integrated fluorescence were measured with the ImageJ software (image processing and analysis in Java, http://rsb.info.nih.gov/ij/) as previously described [23,24]. All individual nuclei in embryos at the 2-cell, 4-cell, 8-cell, 12 nuclei per morula and 20 nuclei per blastocyst stages were outlined manually. Following background subtraction, total fluorescence intensities of all individual nuclei in embryos at the 2-cell, 4-cell, 8-cell, 12 nuclei per morula and 20 nuclei per blastocyst stages were captured. For quantification, the ratios of the fluorescence intensity of OCT4, NANO, acetylated histone H3-lysine 9 (Ac-H3K9) or acetylated histone H3-lysine 14 (Ac-H3K14) and that of Hoechst 33342 DNA signal were compared. All collected images were assembled using the Adobe Photoshop software (Adobe Systems, San Jose, CA) without contrast or brightness adjustments. At least five embryos at every developmental stage were processed for each experiment and the experiments were replicated at least three times.

Cell Number Counting at the Blastocyst Stage

Cell numbers were determined as previously described for blastocysts cultured 90 h following microinjection [25]. Briefly, acidic Tyrode’s solution was used to remove the zona pellucida of blastocyst stage embryos. Zona-free embryos were then exposed to 10% (v/v) rabbit anti-mouse whole serum for 30 min at 37°C, and then incubated in a 10% (v/v) guinea pig complement with propidium iodide (PI) and Hoechst 33342 for 15 min at room

| Gene name | Accession no. | Primer sequence (5′-3′) | Annealing temperature (°C) | Product size (bp) |
|-----------|---------------|--------------------------|-----------------------------|-------------------|
| Pthlh     | NM_008970     | For-AATGCATTTGGATCAACACTTCT | 60                          | 75                |
|           |               | Rev-GCCTGGCAAAAGGGAAAA    |                             |                   |
| Pth1r     | NM_011999     | For-GCTCTCTGCAACCCACCAAT  | 60                          | 103               |
|           |               | Rev-GGGAACCTGCTAGATACTTG  |                             |                   |
| Nanog     | NM_028016     | For- CCGATTTCTTACACGTTCCA | 60                          | 123               |
|           |               | Rev-GGCCCTGAGAGACACAGC    |                             |                   |
| Pou5f1    | NM_013633     | For- TGTCCTGGATCACTGCTG   | 60                          | 82                |
|           |               | Rev-TGTCCTGGATCACTGCTG    |                             |                   |
| Gapdh     | NM_08084      | For-TGGGCAAGGAGATTTGTCGCC | 60                          | 156               |
|           |               | Rev-AAGATGGTGATGGGGTTCCCG |                             |                   |
temperature. Embryos were then immediately examined using an inverted NIKON fluorescence microscope (TE 200, Japan). Cells were counted directly under the microscope.

Statistical Analysis
Statistical analyses of real-time PCR data, total cell number of blastocyst stage embryos and fluorescence intensity values were evaluated by one-way analysis of variance (ANOVA) with the SPSS 13.0 software. A value of P<0.05 was considered statistically significant.

Supporting Information
Figure S1 The effects of Pthlh siRNA injection on OCT4 and NANOG expression in mouse morula stage embryos. Staining pattern of NANOG or OCT4 in the Pthlh siRNA-injected and the control embryos at the morula stage; OCT4/NANOG, green; DNA, blue. Bar = 20 μm.

Figure S2 The effects of Pthlh siRNA injection on the acetylation of lysine 9 on histone H3 (Ac-H3K9) in mouse preimplantation embryos. The staining pattern of Ac-H3K9 in the Pthlh siRNA-injected and the control siRNA-injected preimplantation embryos at the 2-cell stage; the 4-cell stage; the 8-cell stage; the morula stage and the blastocyst stage. Ac-H3K9, green; DNA, blue. Bar = 20 μm.

Figure S3 The effects of Pthlh siRNA injection on the acetylation of lysine 12 on histone H3 (Ac-H3K12) in mouse preimplantation embryos. The staining pattern of Ac-H3K14 in the Pthlh siRNA-injected and the control siRNA-injected preimplantation embryos at the 2-cell stage; the 4-cell stage; the 8-cell stage; the morula stage and the blastocyst stage. Ac-H3K14, green; DNA, blue. Bar = 20 μm.

Table S1 Effect of Pthlh siRNA on development of embryos from mice in nature oestrus.

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Author Contributions
Conceived and designed the experiments: ZH QYS. Performed the experiments: LG ZH. Analyzed the data: LG DQM XWL Contributed reagents/materials/analysis tools: YH. Wrote the paper: LG ZH.

References
1. Suva LJ, Winslow GA, Wettenhall RE, Hammonds RG, Moseley JM, et al. (1987) A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science 237: 493-496.  
2. Moniz C, Burton PB, Malik AN, Dixit M, Banga JP, et al. (1990) Parathyroid hormone-related peptide in normal human fetal development. J Mol Endocrinol 5: 239-266.  
3. Kartosozzani V, Moseley J, McKiehie B, Chou ST, Hardi DK, et al. (1997) Temporal expression of PTHrP during endochondral bone formation in mouse and intramembranous bone formation in an in vivo rabbit model. Bone 21: 383-392.  
4. Escande B, Lindner V, Massfelder T, Helwig JJ, Simeoni U (2001) Developmental aspects of parathyroid hormone-related protein biology. Semin Perinatol 25: 76-84.  
5. Wyszomierski JJ, Stewart AF (1998) The physiology of parathyroid hormone-related protein: an emerging role as a developmental factor. Annu Rev Physiol 60: 431-460.  
6. Karaplis AC, Lux A, Glowacki J, Bronson RT, Tylusiewicz VI, et al. (1994) Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev 8: 257-219.  
7. Schipani E, Kresse K, Juppner H (1995) A constitutively active mutant PTH/PTHrP receptor in an osteosarcoma cell line. J Biol Chem 270: 810-816.  
8. Mehta S, Foygel K, Choi B, Jun S, Leong DE, Lee A, et al. (2008) A novel and critical role for Oct4 as a regulator of the maternal-embryonic transition. PLoS One 3: e1190.

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11. Duranthon V, Watson AJ, Lonergan P (2008) Preimplantation embryo programming: transcription, epigenetics, and culture environment. Reproduction 135: 141-150.

12. Rosner MH, Vigano MA, Ozato K, Timmons PM, Poirier F, et al. (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature 345: 686-692.