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Original article

Possible effects of pituitary adenylate cyclase activating polypeptide (PACAP) on early embryo implantation marker HB-EGF in mouse

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ABSTRACT

Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated as a hypothalamic neuropeptide stimulating adenylate cyclase activity. Besides its neuroprotective effects, numerous data proved its role in reproductive processes. However, there are limited data on its role in preimplantation embryo development and implantation. Our aim was to analyse the mRNA expression of *Adcyap1* (coding region of PACAP) and *Hbegf* [coding region of HB-EGF (Heparin-binding EGF-like growth factor)] in embryos and pregnant uterus to investigate the possible correlation between them. Eight-week-old BDF1 mice were superovulated and subsequently mated overnight or left in their cage after hCG treatment. Day4 embryos were flushed from mated females. After morphological analysis, *Adcyap1* and *Hbegf* gene expression of embryos and uterine tissues was assessed with qPCR.

Our results showed significantly higher *Adcyap1* and *Hbegf* mRNA levels in females producing embryos compared to non-mated ones. Robust elevation of *Adcyap1* and slight elevation of *Hbegf* were detected in females with blastocyst embryos compared with non-blastocysts. We found low rate of *Hbegf* mRNA expression in uncompacted embryos, whereas morulae and blastocysts expressed high amounts of *Hbegf*. However, we did not find detectable *Adcyap1* mRNA in embryos. Strong correlation was found between uterine tissue and embryonic *Hbegf* levels, slight correlation between uterine *Adcyap1* and *Hbegf* levels. Uterine tissue *Adcyap1* and embryonic *Hbegf* showed no correlation. In summary, our present data show, for the first time, the correlation between PACAP and HB-EGF mRNA expression suggesting that PACAP might play a role during the peri-implantation period of early mouse embryo development.

1. Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated as a hypothalamic neuropeptide that stimulates adenylate cyclase activity, and thus, the release of several hormones in the pituitary [1]. PACAP plays diverse roles in the endocrine system, also influencing the gonadal axis in both males and females [2–5]. The peptide can be found in the ovary playing important roles in steroid synthesis [6], folliculogenesis and granulosa cell proliferation [7,8]. PACAP stimulates the production of estradiol and inhibin in the ovary and affects oviduct motility [9]. Moreover, PACAP accelerates meiotic maturation in rat and mouse oocytes [10,11]. The production of PACAP and its receptors is stimulated by gonadotropins in preovulatory follicles [12,13]. The study of *Zhao et al.* has shown that PACAP mRNA expression gradually increases in pregnancy in the corpus luteum suggesting its involvement in the maintenance of mid-term and late pregnancy [14]. Our recent study has shown positive effects of PACAP on preimplantation mouse embryo development [15]. A study by *Isaac and Sherwood (2008)* suggests that PACAP has an important role in the implantation of embryos [16]. PACAP and its receptors are also expressed in the placenta and increase during pregnancy both on the maternal and fetal sides [17]. Human studies show that maternal serum levels of PACAP increase markedly during the last trimester of pregnancy [18].

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family and performs a variety of functions influencing cell growth and differentiation in different cells [19]. It has drawn specific interest because of its expression pattern in the uterus and its paracrine and juxtacrine interactions with the embryonic ErbBs (primary receptors of the HB-EGF) during implantation in mice and humans [20]. In mice, HB-EGF is first expressed 6 − 7 hours before the attachment reaction in the uterus at the site of the blastocyst apposition and persists

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during decidualization [21], reaching its peak level on Days 5–8 after ovulation (Days 1–7 after the attachment reaction) [22]. HB-EGF also plays an essential role in feto-maternal communication both in mice and humans [23]. The time course of its expression is very similar to hCG, which gonadotropin is important in the early preimplantation human embryo development taking also part in the maternal recognition of pregnancy.

Although there are plenty of data on the role of PACAP in the mammalian reproduction, there is limited information about its connection with early embryo development and implantation. The aim of our study was to analyse the mRNA expression of PACAP and HB-EGF in both the embryos and the uterus and to analyze possible correlations between them.

2. Materials and methods

2.1. Animal housing and mating

Procedures with animals were performed following good veterinary practice established for animal welfare according to Hungarian national laws in force. The protocol of the animal experiment was approved by the Food Chain Safety and Animal Health Directorate of Pest County's Government Office (PE/EA/1101–7/2017). Eight-week-old BDF1 (National Institute of Oncology, Budapest, Hungary) mice were kept under a 12 h light/12 h dark schedule at a temperature of 21 °C with 30 % relative humidity in the air. Feed and drinking water were available *ad libitum*. Superovulated (7.5 IU eCG ip.; 7.5 IU hCG ip. 48 h after eCG (Alvetra und Werfft, Austria)) female mice were placed together overnight with mature males after the hCG treatment (mated group, n = 12), or left in their cage after the hCG treatment (non-mated group, n = 12).

2.2. Embryo and tissue collection

Embryos were obtained on Day4 from uterine horns. Both uterine horns were flushed with PBS + 20 % FBS. Embryo morphology was assessed with Olympus SZX7 (Olympus Corp., Shinjuku, Tokyo, Japan) stereomicroscope and embryos were divided into three groups based on the compaction: uncompacted, morula, and blastocyst.

Following the embryo collection, 5 × 5 mm sections of uterine horns were collected. Tissue samples were divided into two groups: non-blastocyst, if only uncompacted ones and morulae were found in the female; blastocyst if there was at least one blastocyst in the uterine horn.

2.3. RNA extraction

Total RNAs from single embryos and uterus tissues were extracted using the column-based Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. In-column DNase treatment was applied to remove residual DNA. Embryos were treated separately and tissue samples were mechanically shattered with handheld homogenizer.

2.4. cDNA preparation and quantitative PCR

Reverse transcription of the RNA samples was carried out using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) with oligoDT primers according to the manufacturer's instructions. The PCR analysis was performed with FastStart Essential DNA Green Master (Roche Diagnostics, Basel, Switzerland). The mRNA expression levels of *Adcyap1* and *Hbegf* were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Data from gene expression analysis were calculated as follows: $2^{\Delta Ct}$, where $\Delta Ct = Ct_{housekeeping\ gene} - Ct_{studied\ gene}$. Table 1. shows the base sequences of forward and reverse primers for *Adcyap1*, *Gapdh*, and *Hbegf*, respectively. PCR cycling conditions: initial denaturation and enzyme activation at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 30 s; annealing at 59 °C for 30 s (*Hbegf* and *Gapdh*) or 49.6 °C for 30 s (*Adcyap1*); extension at 72 °C for 30 s. The PCR analysis was performed using Roche LightCycler Nano real-time PCR system (Roche Diagnostics, Basel, Switzerland).

2.5. Statistical analysis

The data were analysed with R v3.0.0 software (R Development Core Team). Differences of *Adcyap1* and *Hbegf* tissue expressions in mated non-blastocyst vs. blastocyst females and in mated vs. non-mated females were analysed with Mann-Whitney *U* test General linear mixed model was used to analyse (1) the effect of elevated tissue *Adcyap1* expression on embryo development stages, and (2) the *Hbegf* expression of embryos at different stages. The possible correlation between embryonic and tissue *Hbegf* and *Adcyap1* levels were analysed with Pearson correlation test. Differences at a probability value (p) < 0.05 were considered significant. Values are given as mean ± S.D.

3. Results

3.1. Tissue mRNA expression of Adcyap1 and Hbegf in mated and non-mated females

Increased mRNA expression was found of both *Adcyap1* and *Hbegf* in the uterine tissues of mated females (0.0029 ± 0.0030 and 0.011 ± 0.010, respectively) compared to non-mated ones (0.00068 ± 0.00024 and 0.0009 ± 0.0007, respectively) (Fig. 1A and B).

3.2. Adcyap1 mRNA expression in uterine tissue samples and embryos

Relative uterine tissue mRNA expression levels were assessed at different developmental stages of embryos (Fig. 2). Our results show that higher rates of expression belong to female uteruses containing blastocysts compared to females with non-blastocysts (0.0053 ± 0.0021 vs 0.0004 ± 0.0008, respectively; $p = 0.018$). Regarding the uterine tissue concentrations, 0.001 increase in the relative *Adcyap1* mRNA level was associated with 3.92 times greater probability of blastocyst occurrence in females (95 % CI: 7.78–79.03).

Adcyap1 RNA extraction and cDNA synthesis were not successful in embryos in neither developmental stage. Extraction from pooled samples (10 or 30 embryos) resulted in no measurable RNA amounts either (data not shown).

3.3. Hbegf mRNA expression in uterine tissue samples and embryos

Regarding the uterine tissue samples, we found slightly higher rates of *Hbegf* mRNA expression in females with blastocysts compared to females with non-blastocysts (0.016 ± 0.009 vs 0.006 ± 0.008, respectively, $p = 0.026$) (Fig. 3A).

Embryonic expression of *Hbegf* was measured in each developmental stage. Significant differences were found among all groups (blastocyst VS morula – $p < 0.05$; blastocyst VS uncompacted – $p < 0.001$; morula VS uncompacted – $p < 0.001$) (Fig. 4). Strong correlation was found between uterine and embryonic *Hbegf* levels ($R^2 = 0.63$, $p < 0.0001$) (Fig. 5A).

3.4. Correlation between Adcyap1 and hbegf mRNA expression

The possible correlation between *Adcyap1* and *Hbegf* in the peri-implantation period was evaluated with correlation test. Endometrial *Adcyap1* and *Hbegf* expressions showed slight correlation ($R^2 = 0.31$, $p < 0.005$) (Fig. 5B). No correlation was found in the case of uterine tissue *Adcyap1* and embryonic *Hbegf* (Fig. 5C).

Table 1
Sequences of forward and reverse primers for *Adcyap1*, *Gapdh* and *Hbegf*.

Gene	Forward	Reverse	Reference
Adcyap1	CCGAAAACAAATGGCTGTCAAG	CTGTGCATTCTCTAGTGCTTCA	Xu et al., 2016 [24]
Gapdh	GCTACACTGAGGACCAGGTTGT	CTCCTGTTATTATGGGGGTCTG	Xu et al., 2016 [24]
Hbegf	CTGAAGGTTCCTATAGCTCAGGTCCT	GAGAGACCCATGCCTCAGGAAATAC	Designed with SnapGene software (GSL Biotech LLC)

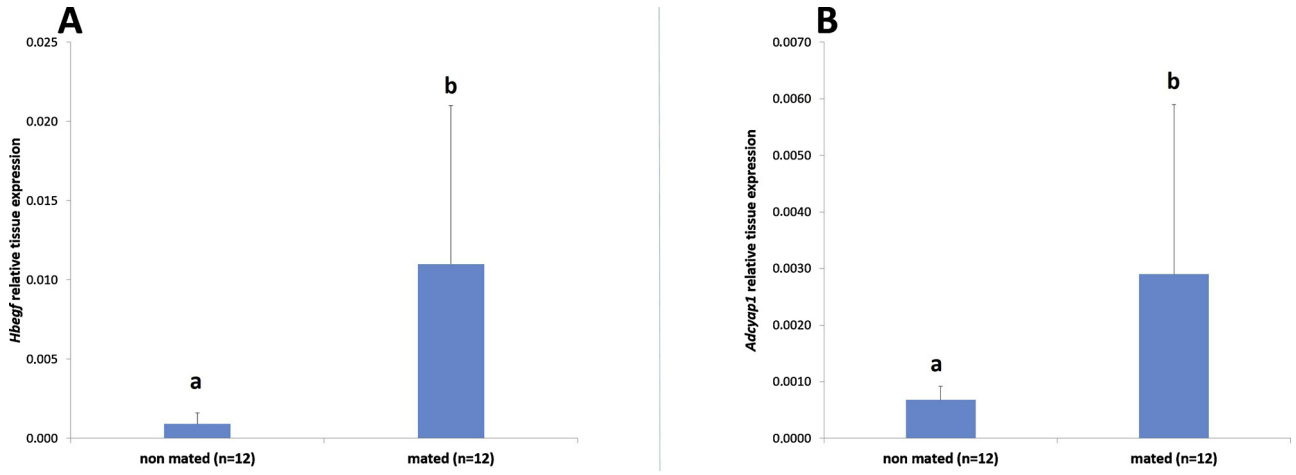

Fig. 1. Expression of *Hbegf* (A) and *Adcyap1* (B) in the uterine tissues of mated and non-mated females. (A: a,b)P < 0.05; (B: a,b)P < 0. 01. Data are presented as mean ± SD.

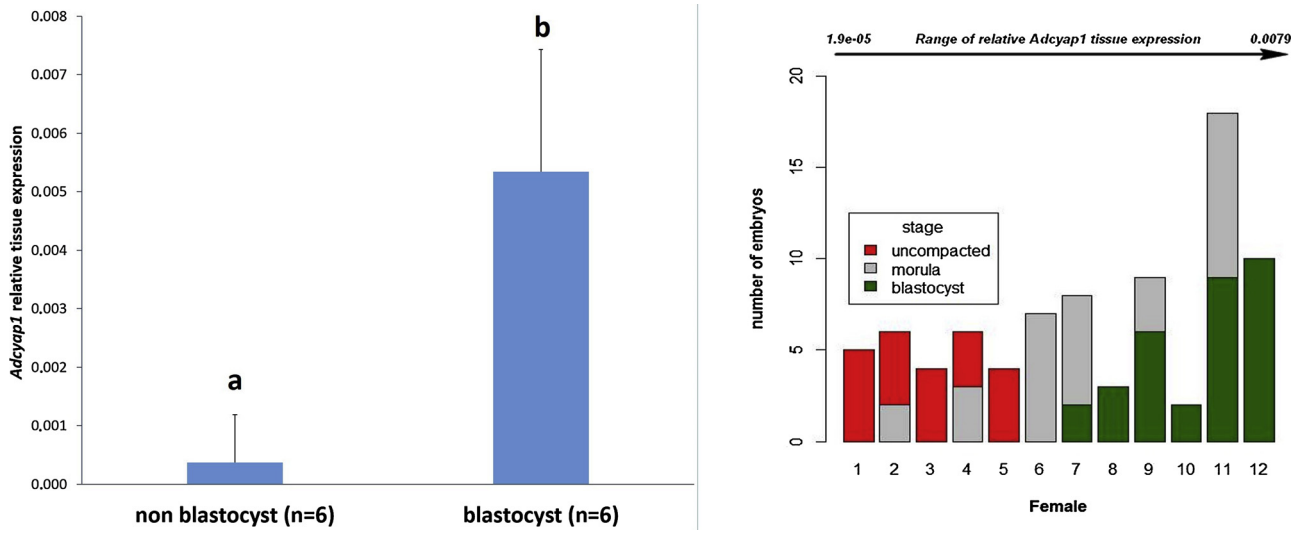

Fig. 2. (A) Relative *Adcyap1* uterine tissue expression levels belonging to females bearing blastocysts and non-blastocysts. (a,b)P < 0.05. Data are presented as mean ± SD. (B) Number and stages of embryos obtained from each female. X axis represents the *Adcyap1* expression rate of uterine tissue. Stages were divided into 3 categories (uncompacted, n = 20; morula, n = 30; blastocyst, n = 32) for visualization purposes.

4. Discussion

Our results showed higher *Adcyap1* and *Hbegf* mRNA levels in females producing embryos after mating compared to non-mated ones. Literature data suggest that the continuously elevated progesterone during early pregnancy increases the concentration of PACAP in vitro. In a study by *Oride et al. (2016),* rat placenta cells were treated with 10 μM progesterone in vitro [25]. This treatment resulted in elevated PACAP mRNA expression. Authors also found that PACAP treatment stimulated hCG production via increased hCG mRNA expression. Similarly to HB-EGF, hCG also reaches its highest levels during peri-implantation period [26]. Increased HB-EGF levels are the evidence of feto-maternal communication before and during implantation [23]. *Spencer et al. (2001)* studied PACAP mRNA levels in decidual endometrium of pseudopregnant and pregnant rats every 3 days between Days 3–15 of pregnancy [27]. Expression levels were zero on Day 3, whereas remarkable increase was detected on Day 6 both in the pseudopregnant and pregnant females. These data are in accordance with our findings in non pregnant (nonmated) mice where we detected low rates of mRNA expression on Day4.

To our knowledge, there is no data on the *Adcyap1* or *Adcyap1r1* (coding region of PAC1, the specific receptor of PACAP) mRNA expression in early, preimplantation embryos. The earliest investigated stage was Day8.5. In that study [28], embryos did not show measurable *Adcyap1r1* signals on Day8.5, but the expression was increased on Day9.5. The same research group examined Day11.5 mouse fetuses,

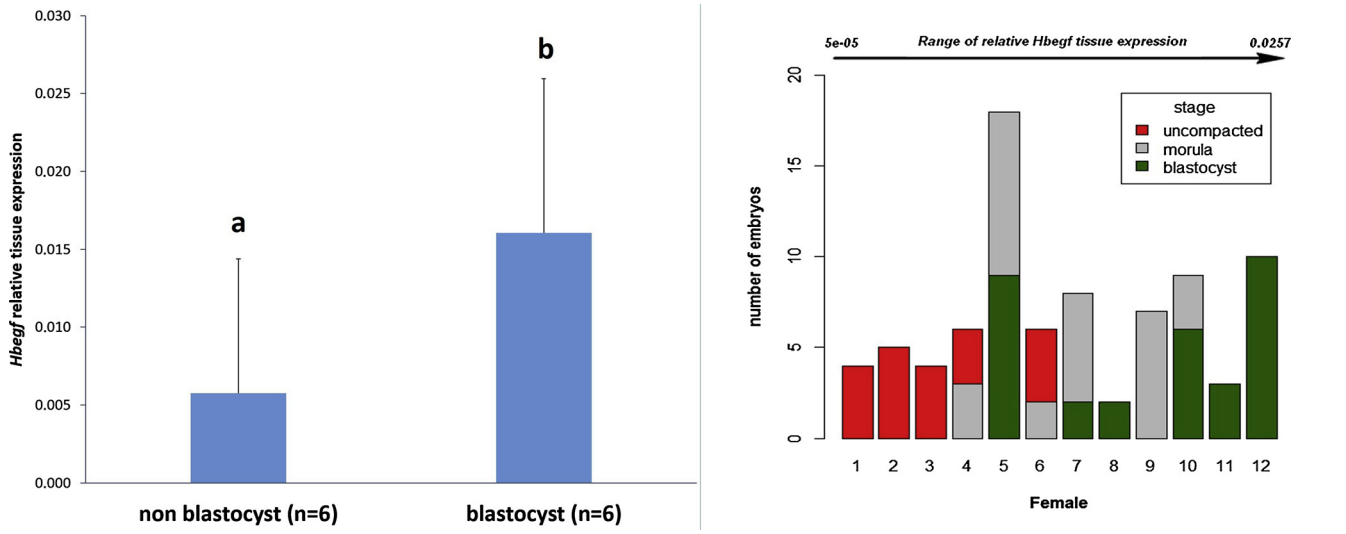

Fig. 3. (A) Relative *Hbegf* uterine tissue expression levels belonging to females bearing blastocysts and non-blastocysts (a,b)$P < 0.05$. Data are presented as mean ± SD (B) Number and stages of embryos obtained from each female. X axis represents the *Hbegf* expression rate of uterine tissue. Stages were divided into 3 categories (uncompacted, n = 20; morula, n = 30; blastocyst, n = 32) for visualization purposes.

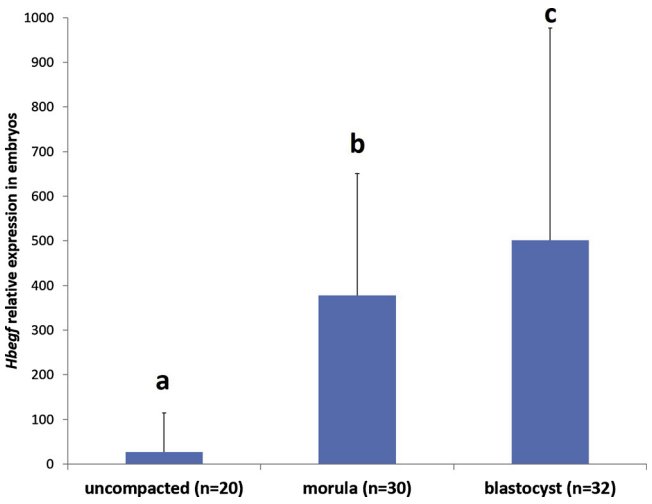

Fig. 4. *Hbegf* mRNA expression of embryos at different stages on the 4th day post coitum. (a,b)$P < 0.001$; (a,c)$P < 0.001$; (b,c)$P < 0.05$. Data are presented as mean ± SD.

which also had a measurable and relatively high quantity of *Adcyap1* mRNA [29]. In our study, no *Adcyap1* mRNA expression was detected in early embryos (Day4). Further studies are needed to investigate if it is due to the low amount of mRNA (under detection limit) or the lack of PACAP production in preimplantation embryos as the abovementioned studies suggest.

Our results show correlation between uterine *Adcyap1* expression levels and embryo development. Higher values of *Adcyap1* are associated with more developed embryos. The analysis showed that the elevation of *Adcyap1* expression by 0.001 gives 3.92-fold greater chance to a more advanced embryo. Furthermore, we found positive correlation of *Adcyap1* expression with the uterine *Hbegf* mRNA expression, suggesting the role of PACAP during early pregnancy and in the peri-implantation period. In contrast, tissue *Adcyap1* and embryonic *Hbegf* were not correlated which suggests that PACAP has no or limited effect on the HB-EGF production in the embryos during this period. However, this hypothesis needs further examinations. *Isaac and Sherwood (2008)* found a relationship between PACAP and implantation, assessing the implantation sites on Day6.5 [16]. They found decreased implantation rate in PACAP null mice. To our knowledge, our data provide the first evidence on the possible role of different PACAP expression levels on implantation.

The blastocyst, upon encountering the maternal interface, initiates a two-way communication several hours before the attachment reaction/

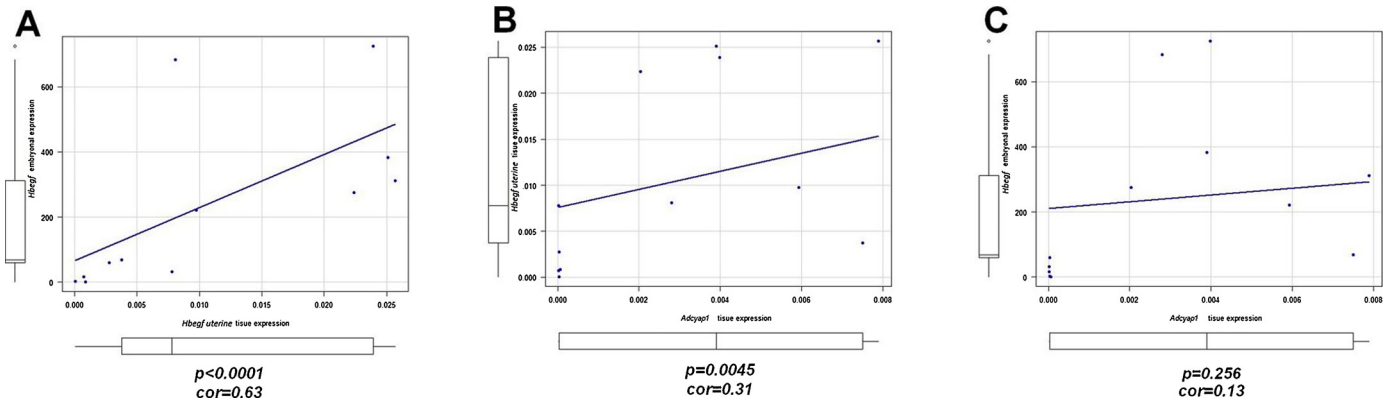

Fig. 5. Correlation between endometrial and embryonic *Hbegf* (A), between endometrial *Adcyap1* and *Hbegf* (B), and between endometrial *Adcyap1* and embryonic *Hbegf* expression levels. cor = Pearson's correlation coefficient.

process occurs. HB-EGF first appears in epithelial cells juxtaposed with blastocysts around 4 pm on Day 4 of pregnancy (100 h post hCG) [30]. HB-EGF induction is not triggered by the dormant blastocyst or embryo [31], consequently a low quality embryo results in low HB-EGF mRNA expression in the endometrium. In accordance with these findings, our results show lower expression of HB-EGF mRNA in the uterine tissue when non-blastocyst embryos are present in the uterus. As a study by *Wang et al. (2000)* shows, endometrium signals back to the embryo by HB-EGF to activate the program of trophoblast differentiation [32]. This process is required for adhesive functions during subsequent attachment and invasion, and takes place only when properly developed embryos (blastocysts) are present. Our findings on the correlation of tissue and embryonic *Hbegf* expression confirm this theory. Furthermore, we found low rate of *Hbegf* mRNA in uncompacted embryos, whereas morulae and in particular blastocysts expressed high amount of *Hbegf* mRNA. These data are in accordance with previous studies [33,34] where the positive effect of HB-EGF on preimplantation embryo development and hatching have been proved.

In summary, our present data show, for the first time, the correlation between PACAP and HB-EGF, a growth factor involved in implantation. These results suggest that PACAP might play a role during the peri-implantation period of early mouse embryo development.

Declaration of Competing Interest

The Authors declare that there is no conflict of interest.

Acknowledgments

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