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# Research Article
# Mitotic Autocrine and Paracrine Roles of Gastrin-releasing Peptide (GRP) in the Placental Tissues of Seven Months Pregnant Cows

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## Abstract

**Background and Objective:** The gastrin-releasing peptide (GRP) has been proposed as an autocrine and/or paracrine growth factor in the female reproductive tract based on the presence of GRP immunoreactivity in the reproductive tract of human, sheep and cow but unproven at the cellular level. Since, GRP mediates their important functions by binding to cell surface gastrin-releasing peptide receptor (GRPR), the present study was aimed to clear out the mitogenic activity of GRP in the placenta of the cow based on the immunolocalization of GRPR and Proliferating Cell Nuclear Antigen (PCNA) at the cellular level. **Materials and Methods:** Three placental tissues of 7 months pregnant cows were used for light microscopic immunohistochemical staining using the polymerized reporter enzyme staining system. **Results:** Immunohistochemical results showed that GRPR immunoreactivity was found in the cell membrane of mononucleated trophoblast cells, while PCNA immunoreactivity was detected in the nuclear part of mononucleated trophoblast cells. No GRPR and PCNA immunoreactivity was visualized in both mononucleated and multinucleated trophoblast giant cells. Gastrin-releasing peptide receptor and PCNA immunoreactive cells were also not detetcted in the maternal epithelium of 7 months placental tissues of the cows. **Conclusion:** The present results suggested that in the placental tissues of 7 months pregnant cows, GRP plays mitotic roles to promote proliferation and differentiation of mononucleated trophoblast cells via autocrine and paracrine loops as indicated by the presence of GRPR and PCNA immunoreactivities in the mononucleated trophoblast cells of the present study and the existance of GRP immunoreactivities in the trophoblast and both mononucleated trophoblast cells and multinucleated trophoblast giant cells of the former studies.

Key words: GRP, GRPR, PCNA, 7 months pregnancy, placental tissue, cow

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The gastrin-releasing peptide (GRP) was first isolated from porcine gastric and intestinal extracts as a mammalian homologue of amphibian bombesin[1]. As the name denotes, the peptide stimulates gastric secretion and regulates the releases of most gastrointestinal hormones[2]. Traditionally, GRP is localized almost exclusively in neurons and neuroendocrine cells and thought to act mainly as neurotransmitter[1,3]. In normal tissues, GRP can stimulate growth of bronchial epithelial cells, gastrointestinal epithelial cells and incisor development[4]. In a series of small cell and non-small cell lung cancer cells, GRP was suggested to have a growth promoting effect via an autocrine and/or paracrine loop[5]. Recently, GRP has been proposed as an autocrine and/or paracrine growth factor in the female reproductive tract on the base of the presence of GRP immunoreactivity in the reproductive tract of human[6], sheep[7-9] and cow[10,11]. Since, GRP mediates their important functions by binding to the cell surface gastrin-releasing peptide receptor (GRPR), the present study aims to clear out the mitogenic activity of GRP base on the localization of GRP, GRPR and Proliferating Cell Nuclear Antigen (PCNA) at the cellular level of the 7 months placental tissues of the cows.

In normal bovine placentome, chorionic villi consist of vascularized mesenchyme provide with a simple layer of trophoblast epithelial cells, which are arranged in an irregular manner. The trophoblast epithelium consist of typically mononucleated trophoblast epithelial cells and binucleated trophoblast giant cells, scattered at random throughout the trophoblast epithelial cells[12]. Trophoblast epithelial cells have a large, round to ovoid nucleus which is situated basally or centrally within the cell. Any trophoblast epithelial cells can give rise to a binucleated trophoblast giant cells via mitotic division[13]. Numerous studies have revealed evidence for binucleated giant cells migration and subsequent fusion with an uterine epithelial cell[14]. There is no data about the relations between the presence and function of GRPR in the tropoblastic cells and uterine epithelial cells of bovine placentome.

## MATERIALS AND METHODS

Three placenta of 7 months pregnant Friesian Holstein cows were used in the study. After fixation in Bouin's fluid for 24 h, the tissue samples were dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections were cut serially at 4 μm in thickness. For histological evaluation, sections were stained with hematoxylin and eosin (HE).

For light microscopic immunohistochemical staining, the ImmPRESS™ polymerized reporter enzyme staining system (Vector Laboratories Inc., Burlingame, CA, USA) was employed. Tissue sections were first deparaffinized in xylene, rehydrated in descending series of ethanol concentration, washed in distilled water and then processed by target retrieval solution (S1699; DakoCytomation, Inc., Carpinteria, CA, USA) for 15 min at 95°C for retrieve of each antigen. After washing in 0.01 M phosphate-buffered saline, subsequently sections were both incubated with 0.3% $H_2O_2$ in methanol for 10 min to block endogenous peroxidase activity. After treatment with normal goat serum for 30 min at room temperature, the sections were incubated with rabbit anti-human GRPR polyclonal antibody (1:500, GTX13339, GeneTex, Inc. San Antonio, USA) and rabbit anti-human PCNA polyclonal antibody (1:100, MBS9413373, MyBioSource, Inc.San Diego, CA, USA), overnight at 4°C as the primary antibodies and with ImmPRESS anti-rabbit IgG (ImmPRESS™ reagent, MP-7401, Vector Laboratories, Inc.) as the secondary antibody for 30 min at room temperature. Then the binding sites were visualized by 0.02% 3,3'–diaminobenzidine tetrahydrochloride (DAB) in 50 mM tris-HCL (pH 7.4) containing 0.006% $H_2O_2$. Some sections were counterstained with Mayer's hematoxylin. Stained sections were examined with Nikon conventional microscope and photomicrographs were taken with Nikon digital sight DS-5M camera (Nikon, Tokyo, Japan).

The negative control conducted to assess the specifity of antibody was replacement of the primary antibody with normal rabbit serum in serial sections.

## RESULTS

This immunohistochemical study showed that GRPR immunoreactivity was found in the mononucleated trophoblast cells (Fig. 1d), while PCNA immunoreactivity was detected in the nuclear part of mononucleated trophoblast cells (Fig. 1e) in the bovine placental tissues of same 7 months pregnant period, as indicated by arrows with white border. No GRPR (Fig. 1d) and PCNA (Fig. 1e) immunoreactivity was visualized in both mononucleated and binucleated trophoblast giant cells in the placental tissues of 7 months pregnant cows, as indicated by black stars. The GRPR (Fig. 1d) and PCNA (Fig. 1e) immunoreactive cells were also not detetcted in the epithelium of the maternal placenta used in the present study as indicated by white stars. Negative control for GRPR (Fig. 1b, c) and PCNA (Fig. 1f) immunohistochemical staining were performed with no primary antibodies on serial slides respective to positive slides. The outline of tropoblast cells was shown using HE staining method (Fig. 1a).

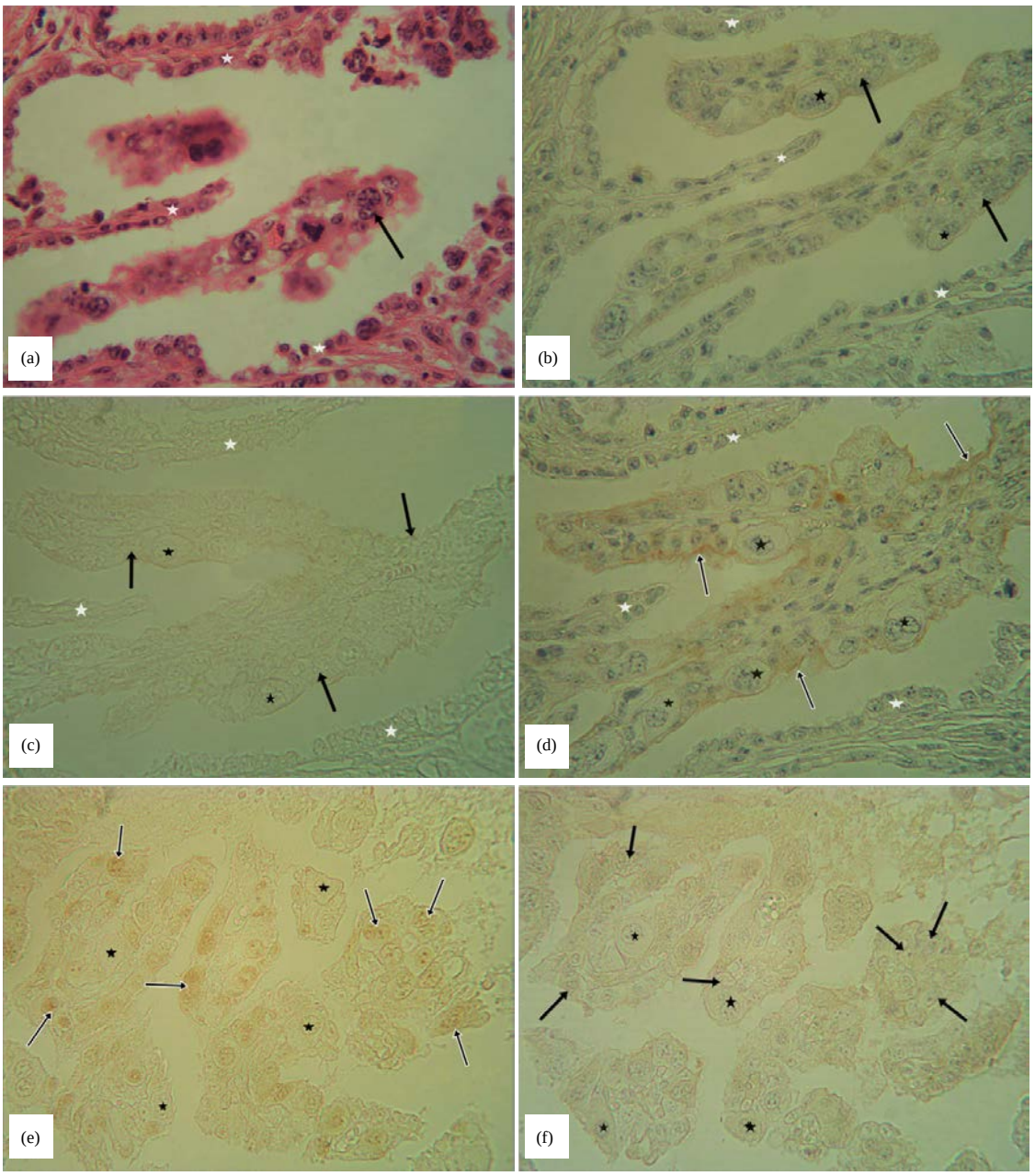


Fig. 1(a-f): Gastrin-releasing peptide receptor (GRPR) and proliferating cell nuclear antigen (PCNA) immunoreactive cells in the placental tissues of 7 months cows (HE and immunohistochemistry; 520x). The serial sections of 7 months placental tissues were used to visualized the outline of (a) Tropoblast cells using HE staining, (b) Negative control with no GRPR primary antibody followed by Mayer's haematoxillin conterstain, (c) Without counterstain, (d) Positive staining of GRPR immunoreactivy. The GRPR immunoreactivity was found predominantly in the cell membrane of (d) Trophoblast cells, while PCNA immunoreactivity was detected in the nuclear part of (e) Mononucleated trophoblast cells as indicated by arrows with white border. (d) No GRPR and (e) PCNA immunoreactivity was visualized in both mononucleated and multinucleated trophoblastic giant cells as indicated by black stars. (d) GRPR and (e) PCNA immunoreactive cells were also not detetcted in the maternal epithelium as indicated by white stars. No PCNA immunoreactivity was found in (f) Negative control serial section of E slide. Solid arrows in the negative control slides indicate the same cells respective to the immunoreactive positive cells

The results may significantly explain the mitotic activity (as indicated by the presence of PCNA immunoreactivity) of mononucleated trophoblast cells, which induced by GRP (as indicated by the presence of GRPR immunoreactivity). The autocrine loop of GRP confirmed on the based of the presence boths of GRPR and GRP in mononucleated trophoblastic cells, while paracrine loop of GRP among different cell types confirmed on the based of the presence of GRPR in the mononucleated trophoblastic cells and GRP in the mononucleated and multinucleated trophoblastic giant cells.

## DISCUSSION

In the present study, GRPR immunoreactivity was found in the mononucleated trophoblast cells. This result is consistent with our previous study on placental GRPR with fetal developmental stages (under the submission). On the othet hand, PCNA immunoreactivity was detected in the nuclear part of mononucleated trophoblast cells. The GRPR is a G protein-coupled receptor whose endogenous ligand is gastrin releasing peptide[15]. The presence of GRPR in the cell membrane of trophoblastic cells in this study agree with the statement of Wootten *et al.*[16] that G protein-coupled receptor located in the cell membrane. Proliferating cell nuclear antigen was originally identified as an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle[17] is in line with the result of this study that found PCNA in the nuclear part of mononucleated trophoblast cells. Otherwise, no GRPR and PCNA immunoreactivity was visualized in both mononucleated and multinucleated trophoblastic giant cells. Moreover, GRPR and PCNA immunoreactive cells were also not detetcted in the maternal epithelium of 7 months placental tissues of the cows used in the present study.

It has been reported that in the placental tissues of cow, GRP immunoreactivity was detected in trophoblast and both mononucleated and multinucleated trophoblastic giant cells[10]. Otherwise, GRPR and PCNA immunoreactivity were only found in the mononucleated trophoblast cells of the present study. Since, in the ruminant placenta, the mononucleated trophoblastic cells directly differentiate to both mononucleated and multinucleated trophoblastic giant cells[18], so GRP may play a mitotic role to promote proliferation and differentiation of mononucleated and multinucleated trophoblast giant cells from trophoblast cells. The presence of GRP immunoreactivity in all trophoblast type cells and the existance of GRPR and PCNA immunoreactivity only in mononucleate trophoblast cells indicated that GRP release from mononucleated trophoblast cells and then via autocrine loop coupled with its receptor in mononucleated trophoblast cells to initiate the proliferation and diferentiation to trophoblastic giant cells. In the other side, the GRP release from mononucleated and multinucleated trophoblastic giant cells was work in trophoblast cells via paracrine loop.

The GRP immunoreactivity was also detected in the cryptal epithelial hybrid cells of the placental tissues of the cow[10]. In the present study, GRPR and PCNA immunoreactivity were only found in the mononucleated trophobast cells. Since, trinucleate cells as cryptal epithelial hybrid cells are formed by binucleated giant cells which fused with the apex of a single uterine epithelial cell[19,20], so the role of GRP from this cells may take a similar pattern to trophoblastic giant cells.

Trophoblast binucleated giant cells are vital in maintenance of pregnancy, as they produce hormones, including the placental lactogen/prolactin-related protein family and pregnancy associated glycoprotein family[18,21]. In the placental tissues of cow, GRP immunoreactivity was detected in trophoblast and both mononucleated and multinucleated trophoblastic giant cells[10]. The present study, revealed the precences of GRPR and PCNA immunoreactivity in trophobast cells of 7 months placental tissues of cow in the same period, which mean that the functional site of GRP is trophoblast cells and may corelate with mitotic activity of trophoblastic cells. So, it may suggest that GRP did not have a direct role to the function of binucleated trophoblast giant cells as mention before[10], but directly influencing the proliferation and differentiation of mononucleated trophoblastic cells into the binucleated giant cells.

## CONCLUSION

The results suggested that in the placental tissues of 7 months pregnant cows, GRP may play mitotic roles to promote proliferation and differentiation of mononucleated trophoblast cells via autocrine and paracrine loops, as indicated by the presence of GRPR and PCNA immunoreactivities in the mononucleated trophoblast cells of the present study and the existance of GRP immunoreactivities in trophoblast and both mononucleated and multinucleated trophoblastic giant cells of the former studies.

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