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IMMUNOLOCALIZATION OF THE SMAD4 PROTEIN IN POSTNATAL PORCINE UTERUS

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ABSTRACT

Transforming growth factor beta (TGF-β) regulates a series of effects on biological events, including uterine morphogenesis. SMAD4, downstream signal protein of the TGF superfamily, is an essential factor for mediating the TGF-β superfamily signaling. In the current study, our objective was to investigate the localization and expression of SMAD4 in postnatal porcine uterus using immunohistochemistry to provide experimental clues illustrating the mechanism of TGF-β superfamily signaling during the process of uterine development. Our results revealed that SMAD4 was detected among all samples examined, and widely and differentially expressed in the uterine luminal and glandular epithelium, myometrium and stroma in different age groups. Our findings strongly suggested that TGF-β superfamily signaling may be involved in postnatal uterine development in pigs.

Key words: SMAD4, TGF-β superfamily, Uterus, Pig

INTRODUCTION

Histogenesis of the pig uterus begins during fetal life, but rapidly develops and is completed during the postnatal period. Transformation of the porcine uterine wall from histoarchitectural infancy to maturity occurs between birth and postnatal day 120. Morphogenetic events, including appearance and proliferation of uterine glands, development of endometrial folds, and growth of the myometrium, are considered to be regulated by locally dynamic uterine cell-cell and cell-extracellular matrix interactions (Bal and Getty, 1970; Bartol *et al.*, 1993; Erices and Schnurrbusch, 1979; Erices *et al.*, 1976; Gray *et al.*, 2001; Hadek and Getty, 1959). Recently, activin receptors and inhibitors, both of which are members of the transforming growth factor-β (TGF-β) superfamily, are hypothesized to be potential regulators of postnatal uterine morphogenesis (Carpenter *et al.*, 2003; Hayashi *et al.*, 2003).

The TGF-β superfamily is comprised of over 40 members, such as TGF-βs, activin, inhibin, Müllerian-inhibiting substance (MIS), growth and differentiation factors (GDFs) and the bone morphogenetic proteins (Cai *et al.*, 2012; Chen *et al.*, 2012; Miyazono *et al.*, 2010; Wen *et al.*, 2011). Members of the TGF-β superfamily are widely expressed in the mammalian uterus. BMP-6 and 7 are co-expressed in the endometrial epithelial and subjacent stromal cells in the non-pregnant uterus (Lyons *et al.*, 1989; Ozkaynak *et al.*, 1997; Shimasaki *et al.*, 2004). TGF-β2 and TGF-β3 are differentially present in the luminal and glandular epithelium, myometrium and the decidua at early stages of pregnancy (Das *et al.*, 1992). Activin receptors and inhibins were detected in the endometrial luminal and glandular epithelium and myometrium in the neonatal ovine uterus (Hayashi *et al.*, 2003). The presence of members of the TGF-β superfamily in the uterus indicated that TGF-β signaling plays instrumental roles in the regulation of uterine function and development. However, the precise details of how TGF-β signaling functions are still not very clear. In order to reveal the mechanisms of the TGF-β superfamily members and evaluate their effects on uterine function, SMAD4, a downstream signal protein of the TGF-β superfamily, was investigated in the current study.

A well-established TGF-β signaling is the SMAD-dependent pathway (Wen *et al.*, 2011). SMAD proteins, the major intracellular signal transducers for the TGF-β family receptors, can be divided into three types: receptor-regulated SMADs (R-SMADs: SMAD 1, 2, 3, 5 and 8), inhibitory SMADs (I-SMADs: SMAD 6 and 7), and a common-mediator SMAD (SMAD4) (Cai *et al.*, 2012; Massague *et al.*, 2005; Miyazono *et al.*, 2010; Pangas, 2012). Homodimers or heterodimers of the TGF-β family ligands bind to and activate a pair of receptors, and then phosphorylate the intracellular R-SMADs. The phosphorylation of R-SMADs combines with SMAD4, and this complex translocates to the nucleus to regulate expression of target genes through interaction with numerous other factors including transcription factors and transcriptional co-activators or co-repressors (Bruce and Sapkota, 2012; Miyazono *et al.*, 2010). All of this evidence suggests that SMAD4 is a central mediator of TGF-β signaling in the regulation of cellular responses. Although the distribution and expression patterns of SMAD4 mRNA and protein have been described in mice (Liu *et al.*, 2004), rat (Lin *et al.*,

2004) and human (Piestrzeniewicz-Ulanska *et al.*, 2003), little information is available about the SMAD4 distribution pattern in the postnatal porcine uterus.

In order to provide experimental clues illustrating the mechanism of TGF-β superfamily signaling during the process of uterine development, it is necessary to determine whether and where SMAD4 (co-SMAD) is expressed in postnatal porcine uterus. In our current study, the histological examination and immunolocalization of SMAD4 in postnatal porcine uterus was determined by hematoxylin and eosin (HE) staining and immunohistochemistry.

MATERIALS AND METHODS

Animals and sample preparation: Sixty (10/time point) Purebred Landrace pigs, at postnatal days 1, 7, 21, 35, 60 and adulthood, were included in the present study. The animals were housed on Jiangpu Farm of Nanjing Agricultural University, Nanjing, China under standard conditions of lighting and temperature and provided with commercial feed three times per day and tap water *ad libitum*. They were slaughtered on specific postnatal days and uteri were collected free of surrounding tissue. Subsequently, uteri were fixed in 4% paraformaldehyde at room temperature for 24 h, dehydrated in a graded series of ethanol (70, 85, 95 and 100%) and embedded in paraffin wax for histologic examination and immunohistochemistry. All procedures involving animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University, China.

Histological examination: For histological examination, uteri embedded in paraffin were cut at 6 um. Sections were then stained with hematoxylin and eosin (HE), and uterine morphological changes observed under a light microscope (Nikon Inc., NY, USA).

Immuno histochemistry: To examine the localization pattern of SMAD4 in postnatal porcine uterus, immunohistochemistry was carried out using the streptavidin-biotin peroxidase complex (SABC) method with polyclonal antibodies to SMAD4 (Boster Biological Technology, Ltd. Wuhan, China). Uterine segments were sectioned (6 μm in thickness) and mounted on slides. Sections were deparaffinized and rehydrated through a consecutive series of xylenes and ethanol, treated with 0.9% hydrogen peroxide, and diluted with phosphate-buffered saline (PBS, pH 7.4) for 1 h to extinguish endogenous peroxidase activity. Sections were then blocked with 5% bovine serum albumin (BSA) for 1 h to avoid non-specific staining. Subsequently, sections were incubated overnight at room temperature with primary antibody to SMAD4 (1:100), and incubated with secondary antibody, goat anti-rabbit IgG (Boster Biological Technology, Ltd. Wuhan, China) diluted in PBS for over 2 h in a humidified box at room temperature. Finally, immunoreactivity was visualized by addition of diaminobenzidine (DAB) substrate (Boster Biological Technology, Ltd. Wuhan, China). The reacted sections were counterstained with hematoxylin solution and mounted using neutral gum and a coverslip. Negative control sections were incubated with normal rabbit serum instead of primary antibody. In order to evaluate relative levels of immunostaining within the different uterine structural components and cell types, three independent observers blinded to the experimental procedures were asked to examine the sections using a method similar to our previously published description work: -, no staining detected; +, weak; ++, moderate; +++, strong staining (Ding *et al.*, 2012; Kim *et al.*, 2005; Shi *et al.*, 2004; Zhang *et al.*, 2011). Relative levels of immunostaining for SMAD4 were assessed and repeated at least four times.

RESULTS

Histological examination of uterine development in postnatal pigs: After H&E staining, developmental characteristics of porcine uteri were observed from postnatal day 1 to adulthood. At day 1, the porcine uterine wall was composed of shallow, luminal epithelial depressions supported by unorganized endometrial stroma, surrounded by myometrium, and endometrial glands are absent. These observations did not change substantially at day 7. At day 21, simple, coiled tubular glands were observed throughout the endometrial stroma to the adluminal border of the myometrium. At day 35, well-developed uterine lumens were observed. Endometrial gland was densely and extensively present throughout the endometrial stroma. Particularly, both inner circular and outer longitudinal myometrial muscle layers were prominently observed. At day 60, the endometrial glands continued to grow in a rapid manner. Mature uterine histoarchitecture was observed in adult pigs, suggesting that it was morphologically and functionally mature.

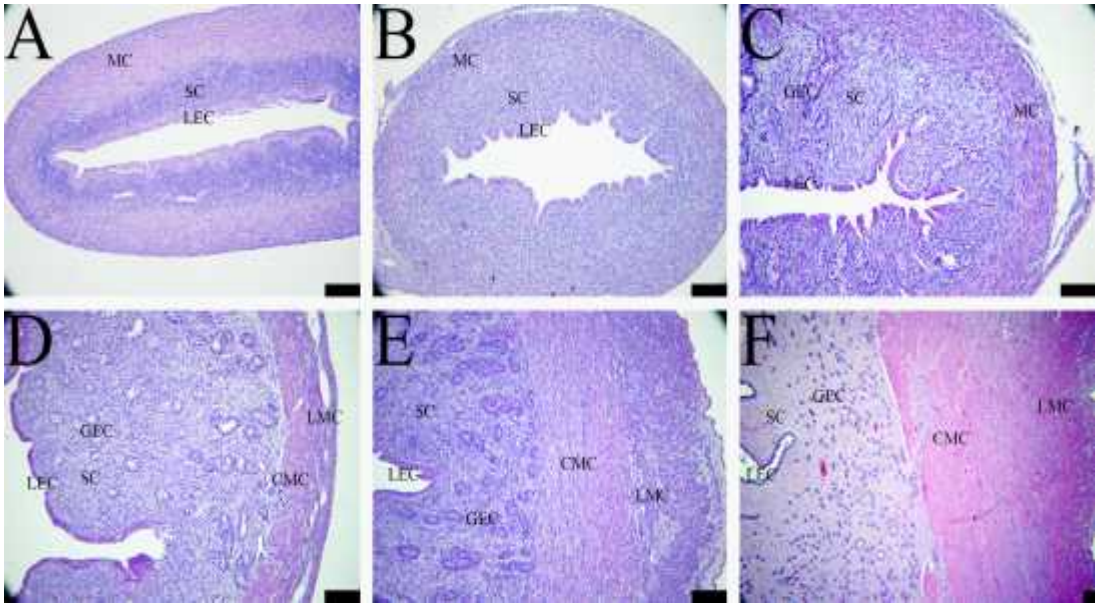

Fig. 1. Histologic examination depicting developmental characteristics of porcine uterus from day 1 to adulthood. At days 1 and 7 (A and B), the porcine uterine wall was composed of shallow, luminal epithelial depressions, endometrial stroma and myometrium, and endometrial glands are absent. At day 21 (C), simple, coiled tubular glands are apparent. At day 35 (D), endometrial gland are well developed and extensively present throughout the endometrial stroma. Particularly, both inner circular and outer longitudinal myometrial muscle layers are observed markedly. At day 60 (E), endometrial gland rapidly continue to grow. Mature uterine histoarchitecture is observed in adult pigs (F). LEC: luminal epithelial cells; GEC: glandular epithelial cells; SC: stromal cells; MC: myometrial cells; CMC: circular layer of myometrium; LMC: longitudinal layer of myometrium. Scale bars: 10 µm

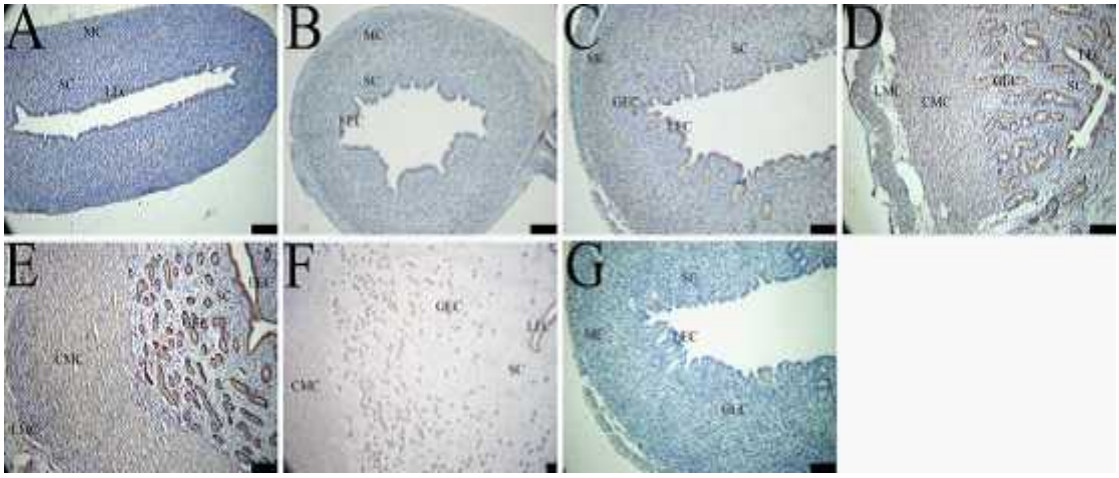

Fig. 2. Immunolocalization of SMAD4 in porcine uterus collected from day 1 to adulthood. A positive reaction was observed as brown staining and the counterstained background appears blue in color. At days 1 and 7, SMAD4 was weakly immunolocalized to the uterine luminal epithelium and myometrium (A and B). At day 21, SMAD4 was moderately immunolocalized to the uterine luminal and glandular epithelium and weakly immunolocalized to the myometrium (C). However, at day 35, SMAD4 was moderately and extensively immunolocalized to the uterine luminal and glandular epithelium and myometrium (D). In contrast, the most intense staining for SMAD4 was observed in the uterine luminal and glandular epithelium and myometrium at day 60 and adulthood (E and F). Furthermore, scattered individual stromal cells also stained positively for SMAD4 protein. No specific staining was observed in the negative control sections (G). LEC: luminal epithelial cells; GEC: glandular epithelial cells; SC: stromal cells; MC: myometrial cells; CMC: circular layer of myometrium; LMC: longitudinal layer of myometrium. Scale bars: 10 µm.

Immunolocalization of SMAD4 in postnatal porcine uterus: The immunolocalization of SMAD4 was performed in porcine uteri collected from postnatal day 1 to adulthood. The intensity of SMAD4 immunostaining was assessed using a method described above. Our results showed that SMAD4 was detected among all samples examined and markedly immunolocalized to the uterine luminal and glandular epithelium and myometrium (Table1 and Figs. 2, 3). At day 1, SMAD4 was weakly immunolocalized to the uterine luminal epithelium and myometrium (Table1 and Figs. 2A, 3A). A similar uterine localization pattern was observed for SMAD4 at day 7 (Table1 and Figs. 2B, 3B). At day 21, SMAD4 was moderately immunolocalized to the uterine luminal and glandular epithelium and weakly immunolocalized to the myometrium (Table1 and Figs. 2C, 3C). However, at day 35, SMAD4 was moderately and extensively immunolocalized to the uterine luminal and glandular epithelium and myometrium (Table1 and Figs. 2D, 3D). In contrast, the most intense staining for SMAD4 was observed in the uterine luminal and glandular epithelium and myometrium at day 60 (Table1 and Figs. 2E, 3E). The intensity of SMAD4 immunostaining did not change markedly toward adulthood (Table1 and Figs. 2F, 3F). Furthermore, scattered individual stromal cells also stained positively for SMAD4 protein. No specific immunostaining was observed in the negative control sections (Figs. 2G and 3G).

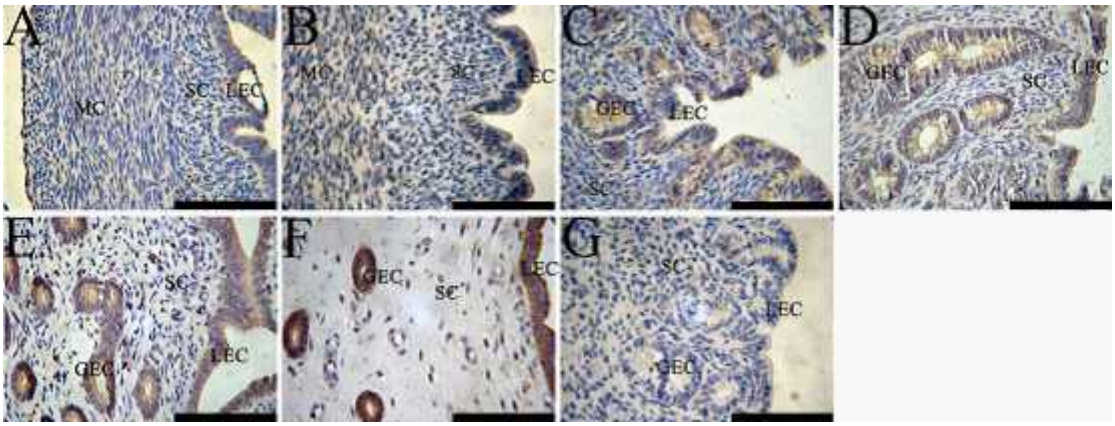

Fig. 3. Higher magnification views of SMAD4 immunoreactivity in porcine uterus collected from day 1 to adulthood. A positive reaction was observed as brown staining, and the counterstained background appears blue in color. Immunostaining for SMAD4 was weakly detected in the uterine luminal epithelium and myometrium at days 1 and 7 (A and B), whereas it was moderately detected in the uterine luminal and glandular epithelium at days 21 and 35 (C and D). In contrast, the most intense staining for SMAD4 was observed in the uterine luminal and glandular epithelium at day 60 and adulthood (E and F). Furthermore, scattered individual stromal cells also stained SMAD4 protein positive. No specific staining was observed in the negative control sections (G). LEC: luminal epithelial cells; GEC: glandular epithelial cells; SC: stromal cells; MC: myometrial cells; Scale bars: 10 µm.

Table 1. Relative levels of immunostaining for SMAD4 in porcine uterus collected from postnatal day1 to adulthood.

Uterine development	Intensity of SMAD4 immunostaining					
	PND1	PND7	PND21	PND35	PND60	Adulthood
Glandular epithelial cells	NA	NA	++	++	+++	+++
Luminal epithelial cells	+	+	++	++	+++	+++
Myometrial cells	+	+	+	++	+++	+++
Stromal cells	-	+	+	+	+	+

Staining intensity: -, no staining detected; +, weak; ++, moderate; +++, strong staining. NA: not available; PND: postnatal day.

DISCUSSION

SMAD4 is a downstream intracellular signal transducer of the TGF-β superfamily. The differential distribution and expression patterns of SMAD4 may reflect phosphorylation of its upstream signaling molecules, the TGF-β superfamily proteins and its receptors (Wen *et al*., 2011). In the current study, we first investigated the cellular localization of SMAD4 in the postnatal porcine uterus. Our findings suggested that TGF-β superfamily signaling plays an important role in the process of uterine development.

Uterine gland morphogenesis and growth is a postnatal event in pigs. Endometrial glands are absent at birth, but they rapidly develop during early postnatal period. In many cases, endometrial adenogenesis usually undergoes three developmental processes, including bud formation, tubulogenesis and the branching morphogenesis of the tubular, coiled glands penetrating the stroma, until the tips reach the inner circular layer of the myometrium. The overall processes of endometrial adenogenesis become mature by PND 120 (Bal and Getty, 1970; Dyck and Swierstra, 1983; Erices and Schnurrbusch, 1979; Gray *et al.*, 2001; Hadek and Getty, 1959; Spencer *et al.*, 1993). Although characteristics of early uterine gland morphogenesis in pigs are understood, there is a lack of knowledge regarding the mechanisms that regulate endometrial gland morphogenesis and inherent differentiated functions. Some studies have recently reported potential mechanisms of endometrial gland morphogenesis. One particularly important hypothesis suggested is that the activin-follistatin system, both of which belong to the TGF-β superfamily, may play regulatory roles in postnatal uterine development during the critical period of endometrial gland morphogenesis (Carpenter *et al.*, 2003; Hayashi *et al.*, 2003). Thus, SMAD4 protein, an essential factor mediating the TGF-β superfamily signaling pathway was investigated in the current study. Our results showed that SMAD4 was extensively present in the uterine luminal and glandular epithelium, myometrium and stroma in different age groups. Interestingly, at days 1 and 7, SMAD4 was weakly immunolocalized to the uterine luminal epithelium, myometrium and stroma; while SMAD4 was moderately or strongly immunolocalized to the uterine luminal and glandular epithelium, myometrium and stroma at other days. The differential distribution of SMAD4 demonstrates that this protein may be involved in endometrial gland morphogenesis, although conversely, it may also reflect changes in its upstream signaling molecules, *i.e.*, the TGF-β superfamily (Wen *et al.*, 2011; Zhang *et al.*, 2012). In addition, with further development of endometrial glands, expression of SMAD4 was gradually increased and reached its zenith at day 60 and adulthood, indicating that TGF-β superfamily signaling may be involved in endometrial gland morphogenesis in a time-dependent manner. Collectively, our findings strongly suggest that the TGF-β superfamily signaling molecules may play a pivotal role in the regulation of endometrial gland morphogenesis in a time-dependent manner in postnatal pigs.

Although there is no direct evidence proving a connection between the TGF-β superfamily and endometrial stromal cell migration, our results showed that SMAD4 was detected in the stromal cell, which gradually became scattered and disordered with development of the porcine uterine wall. This phenomenon indicates the possibility that TGF-ß superfamily signaling may be involved in endometrial stromal cell migration. However, the precise mechanism of action for these proteins remains to be explored.

In summary, our overall results demonstrated that SMAD4 was extensively expressed in various uterine structural components and cell types in pigs. Combining the immunolocalization of SMAD4 in the current study with previous results in mammals, we speculate that TGF-β superfamily signaling may be involved in postnatal uterine development in pigs. These findings will be helpful in elucidating regulatory mechanisms of uterine development, and are expected to provide a theoretical basis for regulation of reproduction in pigs.

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