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MATERNAL-FETAL MEDICINE

The investigation of hippo signaling pathway in mouse uterus during peri-implantation period

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Abstract

Purpose Events in the uterus during the peri-implantation period include embryo development, acquisition of uterine receptivity, implantation and decidualization. Hippo signaling pathway regulates cell proliferation, apoptosis and differentiation. We aimed to determine localization and expressions of pYAP (Phospho Yes-associated protein), YAP (Yes-associated protein), TEAD1 (TEA domain family member 1) and CTGF (Connective tissue growth factor), members of the Hippo signaling pathway, in the mouse uterus during the peri-implantation period.

Methods Pregnant mice were randomly separated into 5 groups: 1st, 4th, 5th, 6th, and 8th days of pregnancy groups. Non-pregnant female mice in estrous phase were included in the estrous group. Uteri and implantation sites were collected. Also, inter-implantation sites were collected from the 5th day of pregnancy group. pYAP, YAP, TEAD-1 and CTGF were detected by immunohistochemistry and Western blotting.

Results We observed that the expressions of YAP, TEAD-1 and CTGF were increased in the luminal and glandular epithelium on the 1st and 4th days of pregnancy when epithelial proliferation occurred. pYAP expression was high, and YAP and CTGF expressions were low in the luminal epithelium of the implantation sites on the 5th day of pregnancy, when epithelial differentiation occurred. pYAP expression was low, YAP and CTGF expressions were high at implantation sites on the 6th and 8th days of pregnancy, where decidua was formed.

Conclusion Our findings suggest that the Hippo signaling pathway might be involved in implantation and decidualization. Our findings will guide further studies and may help to elucidate underlying causes of implantation failure and pregnancy loss.

Keywords Implantation · Decidualization · pYAP · YAP · TEAD1 · CTGF

Introduction

Implantation is one of the crucial steps for a successful pregnancy and requires cross-talk between the blastocyst and maternal endometrium [1]. Ovarian hormone progesterone (P_4) and estrogen (E_2) support blastocyst and uterine receptivity. The uterine receptivity for mice consists of three phases. These are pre-receptive, receptive, and non-receptive (refractory) phases [2]. The uterus is pre-receptive on days 1–3 when mice with vaginal plug are admitted on the first day of pregnancy, then the uterus becomes receptive on days 4–5 of pregnancy. From the 5th day of pregnancy, the uterus becomes refractory for the embryo [2–4]. In mice, the attachment of blastocyst to the endometrium surface occurs at night on the 4th day of pregnancy. The avascular primer decidual zone (PDZ) is formed on the 5th day of pregnancy, where stroma cells surrounding the blastocyst. Around the PDZ, the secondary decidual zone (SDZ) is formed on day 6, and PDZ degenerates on the 8th day of pregnancy [5]. Embryo implantation is a complex process that comprises molecular and cellular interactions such as signaling molecules, morphogens, growth factors, homeotic proteins, and transcription factors. These interactions must occur in an optimal time frame [6]. Understanding these events' physiology is important for new treatments of implantation failure.

The Hippo signaling pathway was first discovered as a signaling pathway that regulates organ size in *Drosophila melanogaster* [7]. The Hippo signaling pathway and its function have also been conserved in mammals [8, 9]. The Hippo signaling pathway regulates cell number by controlling cell proliferation, death, and differentiation in both Drosophila

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and mammals [10]. YAP (Yes-associated protein) has important roles in early embryonic development. YAP has a nuclear localization in the trophectoderm while it represents cytoplasmic localization in the inner cell mass of mouse embryos at the pre-implantation period. The diverse distribution of YAP is important for lineage specification [10]. In mice, it was reported that the overexpression of YAP leads to a dramatic increase in liver or heart size [8, 11]. The most important components of the mammalian Hippo pathway are MST 1/2 (mammalian Ste20-like kinases 1/2), LATS 1/2 (large tumor suppressor 1/2), and their cofactor SAV1 (Salvador) and adaptor protein MOB1 (Mps1-binder-related). MST autophosphorylates itself, then phosphorylates Sav1 and constitutes an active complex. MST also can phosphorylate LATS kinases and co-activators MOB1. LATS1 or LATS2 then phosphorylate the downstream targets of the Hippo pathway, coactivator YAP and TAZ (PDZ-binding motif), enabling 14-3-3 proteins to bind and sequester YAP/TAZ in the cytoplasm. Phosphorylated YAP activates 14-3-3 proteins to provide protein degradation. The phosphorylation of YAP from S127 and TAZ from S89 residues constitutes the 14-3-3 binding sites. When the Hippo pathway is inactivated, YAP and TAZ translocate to the nucleus, where they treat as co-activators for several transcription factors. YAP and TAZ lack a DBD (DNA-binding domain) and therefore influence transcription by interacting with DNA-binding proteins [12]. YAP/TAZ interacts with the members of TEA (Transcriptional enhanced associate) domain family to activate cell proliferation genes [13]. There are four *Tead* genes (*Tead 1–4*) in mammals, and these genes have the same domain structure [14]. TEAD family members play a key role in normal cell growth. The dysregulation of these genes is associated with tumor formation [15]. TEAD transcription factors are the best characterized binding partners of YAP/TAZ [16]. Target genes of the YAP/TAZ-TEAD complex are genes such as *CYR61*, *CTGF, AREG, VIM* [13, 17–19]. CTGF was first described in 1991 as a 38 kDa protein of human vascular endothelial cells in vitro [20]. It has been shown that CTGF is highly expressed in many pathological conditions such as atherosclerosis [21], scleroderma [22, 23], desmoplasia [24], pulmonary fibrosis [25], and renal fibrosis [26]. The production of CTGF in response to mechanical tissue injuries suggests that this growth factor plays a role in tissue repair and regeneration. Among the biological effects of CTGF are cell proliferation, extracellular matrix production, and cell adhesion [20, 27–32]. CTGF has also been shown to play a role in apoptosis [33]. It is known that *Ccn2 / Ctgf* deletion in ovarian granulosa cells in mice leads to subfertility and abnormal follicle development [34].

Hippo signaling pathway had been shown to play critical roles in embryonic development. During pre-implantation period, two cell types, inner cell mass (ICM) and trophoectoderm (TE) are formed at the early blastocyst stage [35]. Hippo pathway is required for lineage formation of mouse pre-implantation embryos. Defects in differentiation into ICM and TE lineages occur as a result of disruption of the Hippo signaling pathway [36]. Hippo signaling pathway member YAP promotes maintenance of cytotrophoblast progenitors, via stimulating proliferation and expression of stemness-associated genes. YAP–TEAD4 complexes directly suppress genes promoting trophoblast cell fusion [37]. These results showed that Hippo pathway may be involved in the determination and diferentiation of the cell fate of pre-implantation embryos. After the embryo reaches the implantation site, the attachment of the blastocyst to the uterine epithelium initiates the decidualization process that includes the differentiation of stromal cells to decidual cells. This progesterone-induced cellular differentiation process is a pre-requisite for a successful pregnancy [38]. Chen et al. showed increased expression of YAP in decidual cells compared to human endometrial stromal cells and suggested that YAP plays a role in decidualization [39]. Hippo signaling pathway had been shown to play a critical role in endometriosis, the investigators showed increased expression of YAP and decreased expression of p-YAP in ectopic and eutopic endometrium compared with normal endometrium [40]. In another study, after eutopic endometrial stromal cells (ESC) were treated with rapamycin, mTOR (mammalian target of rapamycin) was suppressed and autophagy increased; however, YAP expression was downregulated. These results suggested a negative relationship between YAP and autophagy in eutopic ESCs [41]. It is important to note that disruptions in placental formation and its functions may lead to pregnancy disorders such as preterm birth and pre-eclampsia resulting in health complications [42]. YAP levels were shown to be downregulated in patients with pre-eclampsia [43]. In another study, TEAD4 was shown to maintain the stemness and promote self-renewal of trophoblast progenitors in post-implantation embryos to ensure placentation and progression of pregnancy [44]. However, there is no study investigating the localization and expression of Hippo pathway members in the peri-implantation period mouse uterus and implantation sites. In this study, we aimed to evaluate the localization and expressions of the Hippo signaling pathway members pYAP, YAP, TEAD1, and CTGF and whether they have roles during peri-implantation period mouse uterus and implantation sites.

Materials and methods

Six to eight-week-old female Balb/C mice ($n = 60$ for all groups) and twelve-week-old male Balb/C mice ($n = 30$) were obtained from Akdeniz University Animal Research Unit, Antalya, Turkey. Mice were left at 22–24 °C room

temperature with a 12-h light/dark cycle and with free access to mice chow and tap water ad libitum.

Two or three female mice were kept with male mice overnight for mating, and the female mice were checked for vaginal plug the next morning. Female mice observed to have vaginal plugs were admitted on the 1st day of pregnancy [45]. Pregnant mice were randomly separated into 5 groups: 1st day of pregnancy group, D1 ($n=10$), 4th day of pregnancy group, D4 ($n=10$), 5th day of pregnancy group, D5 ($n=10$), 6th day of pregnancy group, D6 ($n=10$) and 8th day of pregnancy group, D8 ($n=10$).

Non-pregnant female mice in the estrous phase were included in the estrous group ($n=10$). A vaginal smear was performed to determine the estrous phase of the non-pregnant female mice. The vaginal smear was stained with 0.1% cresyl violet (#105235, Merck, Darmstadt, Germany). When squamous, large, nucleated epithelial cells were seen in the smear, mice were admitted at the estrous phase. The 1st day of pregnancy was confirmed by recovering zygotes from the oviducts; the 4th day of pregnancy was confirmed by recovering blastocysts from the uterus. Implantation sites on the 5th and 6th days of pregnancy were visualized by an intravenous injection of 1% Chicago Blue dye solution (#C8679, Sigma Aldrich, St. Louis, MO, USA, prepared in 0.9% NaCl) before sacrification [46]. Mice were anesthetized (Ketamine-Xylazine Mix, 0.1 ml/10 g Body Weight, intraperitonally), implantation sites were collected, and euthanized by cervical dislocation. Also, inter-implantation sites were collected from the 5th day of pregnancy group (D5-I). Collected tissues were fixed in formalin for immunohistochemistry, stored in liquid nitrogen for Western blotting.

Tissue processing

Tissues were fixed in 10% formalin (#15512, Merck, Darmstadt, Germany) (100 ml 37% formaldehyde, 900 ml distilled water, pH ~ 7) at room temperature for 24 h. Formalin was cleaned with tap water, then tissues were dehydrated with immersion in 70%, 80%, 90% ethanol (#100986, Merck, Darmstadt, Germany) for 24 h each and 100% ethanol for 4 h. After dehydration, tissues were cleared in xylene (#16446, Sigma Aldrich, Italy) and embedded in paraffin wax (#107337, Merck, Darmstadt, Germany). Cleared tissues were kept in paraffin in an oven at 56ºC and then embedded in paraffin.

Immunohistochemistry

After 5 µm paraffin sections were taken, deparaffinization and rehydration were performed. Citrate buffer (pH 6.0) was used for antigen retrieval using microwave. Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H_2O_2 (#108597, Merck, Darmstadt, Germany) for 20 min. After washing with PBS, sections were incubated with a blocking solution (Ultra UV Block, #TA-125UB, LabVision Corporation, Fremont, CA, USA) to block non-specific binding. Excess serum was drained, and sections were incubated with rabbit monoclonal anti-pYAP (Phospho S127) (#ab76252; Abcam) at 1:100 dilution, rabbit monoclonal anti- YAP (#ab205270, Abcam) at 1:1000 dilution, rabbit monoclonal anti-TEAD1 (#ab133533; Abcam) at 1:100 and rabbit polyclonal anti-CTGF (#ab6992; Abcam) at 1:1000 + 4 ºC overnight. The primary antibodies were substituted with normal rabbit IgG (#sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the same dilutions as the specific antibodies. The next day, sections were incubated with biotinylated anti-rabbit secondary antibody (#BA-1000, Vector Laboratories, Burlingame, USA) at 1:500 dilution at room temperature for 45 min, followed by incubation with HRP-conjugated streptavidin complex (#TS125HR, Thermo Scientific, Fremont, CA, USA) for 20 min at room temperature. Sections were treated with Di Amino Benzidine (DAB) chromogen (#D4168, Sigma Aldrich, St. Louis, MO, USA) for 2 min and then washed in tap water. Mayer's Hematoxylin (#109249 Merck, Darmstadt, Germany) was utilized for counterstaining of the sections, and they were dehydrated and mounted with Entellan mounting medium (#107961; Merck, Darmstadt, German). Sections were examined and photographed by Nikon Eclipse E200 microscope.

H-SCORE evaluations

pYAP, YAP, TEAD1, and CTGF expressions were evaluated by H-SCORE analysis in the luminal epithelium, glandular epithelium, and stroma of the sections of experimental groups. [47]. Immunopositive cells were counted in 5 different areas under 200X original magnification under the Nikon Eclipse E200 microscope. Three sections were randomly selected from every group. The evaluations were recorded as percentages of positively stained cells in each of four intensity categories which were denoted as 0 (no staining), 1 + (weak but detectable above control), 2 + (distinct), 3 + (intense). The evaluation was conducted by two blinded observers, and an H-SCORE value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [H-SCORE = $\sum$·Pi (i + 1) for each tissue, where i is the intensity score, and Pi is the corresponding percentage of the cells].

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blotting

Protein extraction and immunoblot analysis were performed as described previously [48]. Proteins were subjected to SDS polyacrylamide gel electrophoresis. At the end of

electrophoresis the proteins were transferred onto PVDF (polyvinylidene difluoride) membrane (#1620177, Bio-Rad, Portland, ME, USA) overnight. The next day, the membranes were blocked for 1 h with 5% BSA (#A9647, Sigma Aldrich, St. Louis, MO, USA) prepared in Tris-buffered saline (TBS; pH 7.2–7.4). Membranes were incubated with 1:1000 dilution of rabbit monoclonal anti-pYAP (Phospho S127) (#ab76252; Abcam), 1:1000 dilution of rabbit monoclonal anti-YAP (#ab205270, Abcam), 1:1000 dilution of rabbit monoclonal anti-TEAD1 (#ab133533; Abcam) and 1:1000 dilution of rabbit polyclonal anti-CTGF (#ab6992, Abcam), prepared in 5% BSA, overnight at + 4 °C. After washing the membranes with TBS they were incubated with horseradish peroxidase (HRP) conjugated secondary antibody (#PI-1000, Vector Laboratories, Burlingame, USA) diluted in 5% BSA for 1 h at room temperature. The membranes were incubated for 5 min within the Super Signal Chemiluminesans (CL)-HRP substrate system (#34080, Thermo Fisher Scientific, Rockford, USA), and the signals on membranes were then transferred on to hyper film (#28-9068-37 Amersham Hyperfilm ECL, GE Healthcare, Buckinghamshire, UK) in a dark room. Membranes were also labeled by an identical protocol for binding of a 1:5000 dilution of a rabbit monoclonal anti-beta actin antibody (#4970, Cell Signaling Technology, Danvers, MA, USA) as an internal control to confirm the equal loading of the samples. Experiments were repeated three times. The quantities of pYAP (Phospho S127), YAP, TEAD1, and CTGF protein expressions were determined using NIH image analysis software (Image J Version 1.36b; National Institutes of Health, MD, USA).

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by post hoc Tukey's HSD test. Probability values of less than 0.05 were considered significant; values are presented as mean ± SEM (standard error of the mean).

Results

Immunohistochemistry results

pYAP immunohistochemistry

pYAP showed cytoplasmic and nuclear localization, and its expression intensity showed differences between the experimental groups.

In the luminal epithelium, pYAP showed a similar and weak expression in estrous, D1, and D4 groups. Expression of pYAP at implantation sites on the 5th day of pregnancy was very strong and increased compared to all groups ($p < 0.0001$). A decrease in pYAP expression was observed at implantation sites on the 6th day of pregnancy compared to all experimental groups ($p < 0.0001$) (Fig. 1).

In the glandular epithelium, pYAP expression increased at inter-implantation sites on the 5th day of pregnancy compared to the 1st day of pregnancy (p: 0.0395). A decrease in pYAP expression at implantation sites on the 6th day of pregnancy compared to all experimental groups was observed ($p < 0.0001$) (Fig. 1).

Expression of pYAP in the stroma was increased at implantation sites on the 5th day of pregnancy compared to the estrous phase uterus ($p < 0.0001$). The expression of pYAP was decreased at the inter-implantation sites compared to the implantation sites on the 5th day of pregnancy ($p < 0.0001$). pYAP expression on the 6th day of pregnancy was lower than all groups ($p < 0.0001$). pYAP expression was very weak both in the primary decidual zone (PDZ) and the secondary decidual zone (SDZ) at implantation sites on the 6th day of pregnancy. pYAP expression was weak in the SDZ on the 8th day of pregnancy. (Fig. 1).

YAP immunohistochemistry

YAP was nuclear-localized, and its expression intensity showed differences between the experimental groups.

In the luminal epithelium, an increase of YAP expression was observed in the uteri on the 1st day of pregnancy and inter-implantation sites on the 5th day of pregnancy compared to the estrous group uteri ($p < 0.0001$). Also, in the luminal epithelium of implantation sites on the 6th day of pregnancy, where blastocyst did not attach, YAP expression was higher than other experimental groups ($p < 0.001$) (Fig. 2).

The expression of YAP in the glandular epithelium was increased in the uteri on the 1st day of pregnancy compared to the estrous group uteri ($p < 0.0001$) and reached the highest level among all experimental groups. YAP expression in the uteri on the 4th day of pregnancy decreased compared to the uteri of the estrous ($p < 0.001$) and the 1st day of pregnancy groups ($p < 0.0001$). The expression of YAP at the implantation and inter-implantation sites on the 5th day of pregnancy increased compared to the uteri on the 4th day of pregnancy, but this increase was not statistically significant ($p > 0.05$). The expression of YAP at the implantation sites on the 6th day of pregnancy was the lowest among all experimental groups, and this decrease was statistically significant ($p < 0.0001$) except for the uteri on the 4th day of pregnancy ($p > 0.05$) (Fig. 2).

The expression of YAP in the stroma was similar in the uteri of the estrous group, the uteri on the 1st day of pregnancy, and inter-implantation sites on the 5th day of pregnancy. YAP expression increased as the pregnancy progressed, and it was observed to reach its highest level on the 5th and 6th days of pregnancy ($p < 0.0001$). The expression

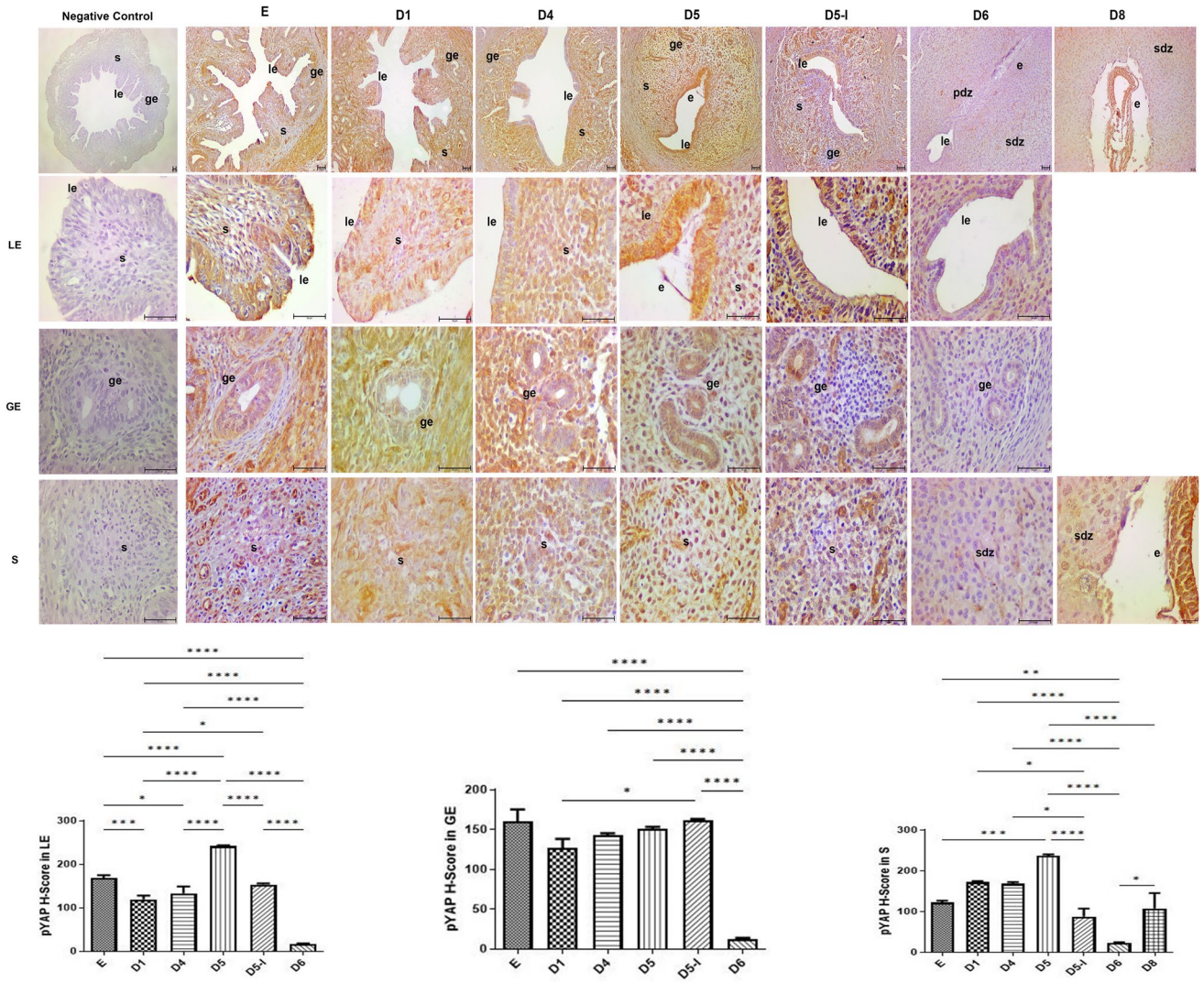


Fig. 1 pYAP immunohistochemistry in the luminal epithelium, the glandular epithelium, and the stroma of uterus, implantation, and inter-implantation sites of the experimental groups. For figures LE, GE, and S H-Score analysis; asterisks indicate significant difference (*$p<0.05$), (**,***$p<0.001$), (****$p<0.0001$). *le* Luminal epithelium, *ge* Glandular epithelium, *s* Stroma, *e* Embryo, *pdz* Primary decidual zone, *sdz* Secondary decidual zone. Scale bars: 50 µm

of YAP in the PDZ was observed to be negligible compared to the SDZ on the 6th day of pregnancy. YAP expression was strong in the SDZ of the 8th day of pregnancy (Fig. 2).

TEAD1 immunohistochemistry

TEAD1 was nuclear-localized and the intensity of TEAD1 expression showed differences between the experimental groups.

TEAD1 expression was decreased in the luminal epithelium of the uteri on the 1st and 4th days of pregnancy compared to the uteri of the estrous group, and this decrease was statistically significant on the 4th day of pregnancy ($p<0.05$). TEAD1 expression was increased from the 4th day of pregnancy and reached the highest level on the 6th day of pregnancy ($p<0.0001$) (Fig. 3).

TEAD1 expression in the glandular epithelium of all groups was generally close to each other. The lowest TEAD1 expression in the glandular epithelium was in the uteri on the 1st day of pregnancy. TEAD1 expression was increased in the uteri on the 4th day of pregnancy ($p<0.05$), implantation sites on the 5th and 6th days of pregnancy ($p<0.0001$), inter-implantation sites on the 5th day of pregnancy ($p<0.05$) compared to the uteri on the 1st day of pregnancy; the most significant increase was observed at the implantation sites on the 5th day of pregnancy (Fig. 3).

TEAD1 expression in the stroma was increased in the uteri on the 4th day of pregnancy, implantation sites on

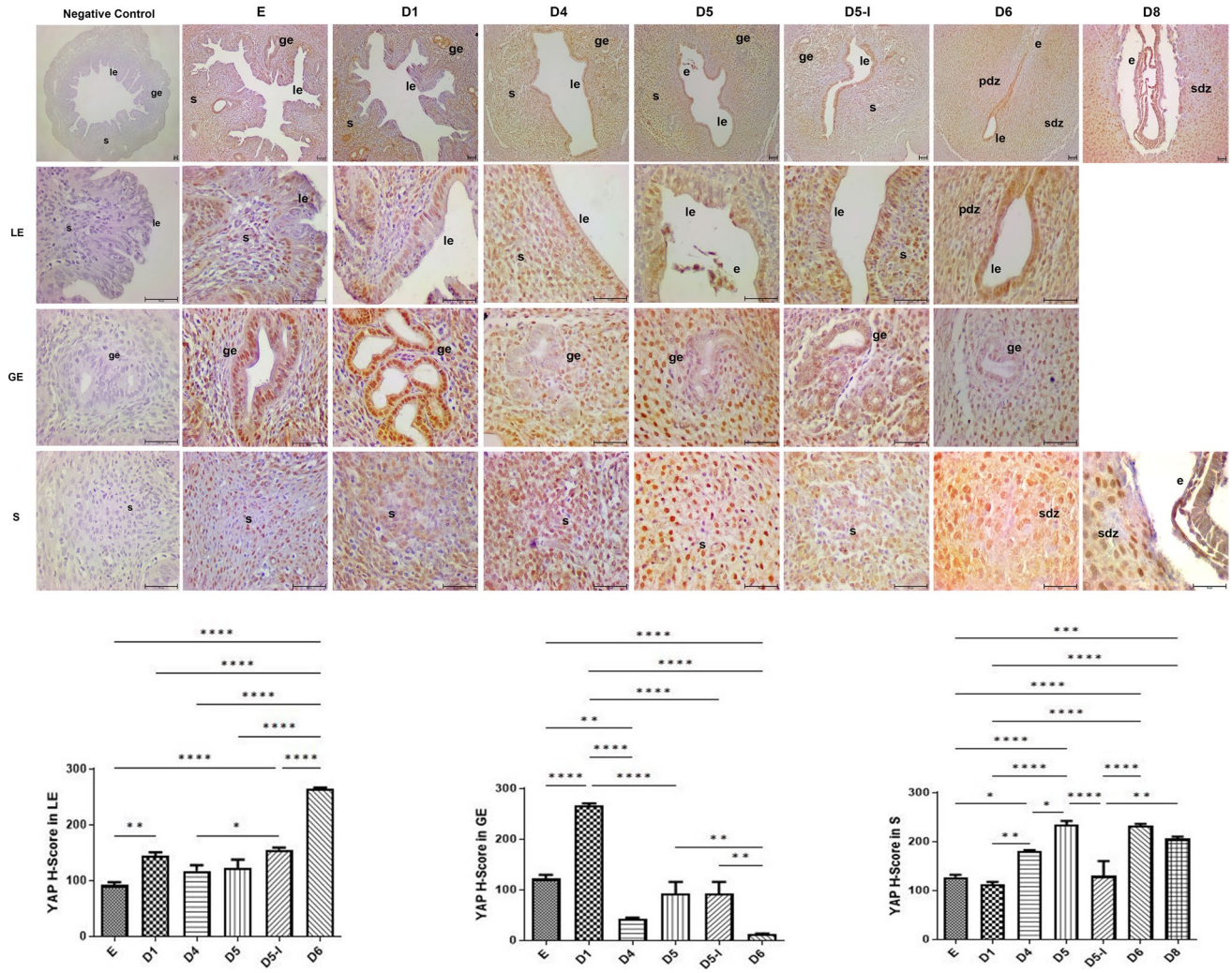


Fig. 2 YAP immunohistochemistry in the luminal epithelium, the glandular epithelium, and the stroma of uterus, implantation, and inter-implantation sites of the experimental groups. For figures LE, GE, and S H-Score analysis; asterisks indicate significant difference (*$p<0.05$), (**,***$p<0.001$), (****$p<0.0001$). *le* Luminal epithelium, *ge* Glandular epithelium, *s* Stroma, *e* Embryo, *pdz* Primary decidual zone, *sdz* Secondary decidual zone. Scale bars: 50 µm

the 5th and 6th days of pregnancy, and inter-implantation sites on the 5th day of pregnancy ($p<0.0001$) compared to the uteri on the estrous and the 1st day of pregnancy and, the highest increase was observed at the implantation site on the 6th day of pregnancy. A decrease of TEAD1 expression was observed at the implantation sites on the 8th day of pregnancy compared to all experimental groups ($p<0.0001$) except the uteri on the 1st day of pregnancy ($p>0.05$). TEAD1 expression was strong both in the primary (PDZ) and secondary decidual zones (SDZ) on the 6th day of pregnancy. TEAD1 expression was also strong in the secondary decidual zone (SDZ) on the 8th day of pregnancy. (Fig. 3).

CTGF immunohistochemistry

CTGF was both nuclear and mostly cytoplasmic localized and, CTGF expression intensity was different between the experimental groups.

In the luminal epithelium, CTGF expression was at the highest level in the estrous group. It was gradually decreased in the uteri on the 1st and 4th days of the pregnancy and implantation sites on the 5th, 6th, and 8th days of pregnancy and inter-implantation sites on the 5th day of pregnancy compared to the estrous group ($p<0.0001$). While there was no statistically significant difference for CTGF expression between the uteri on the 4th day of pregnancy,

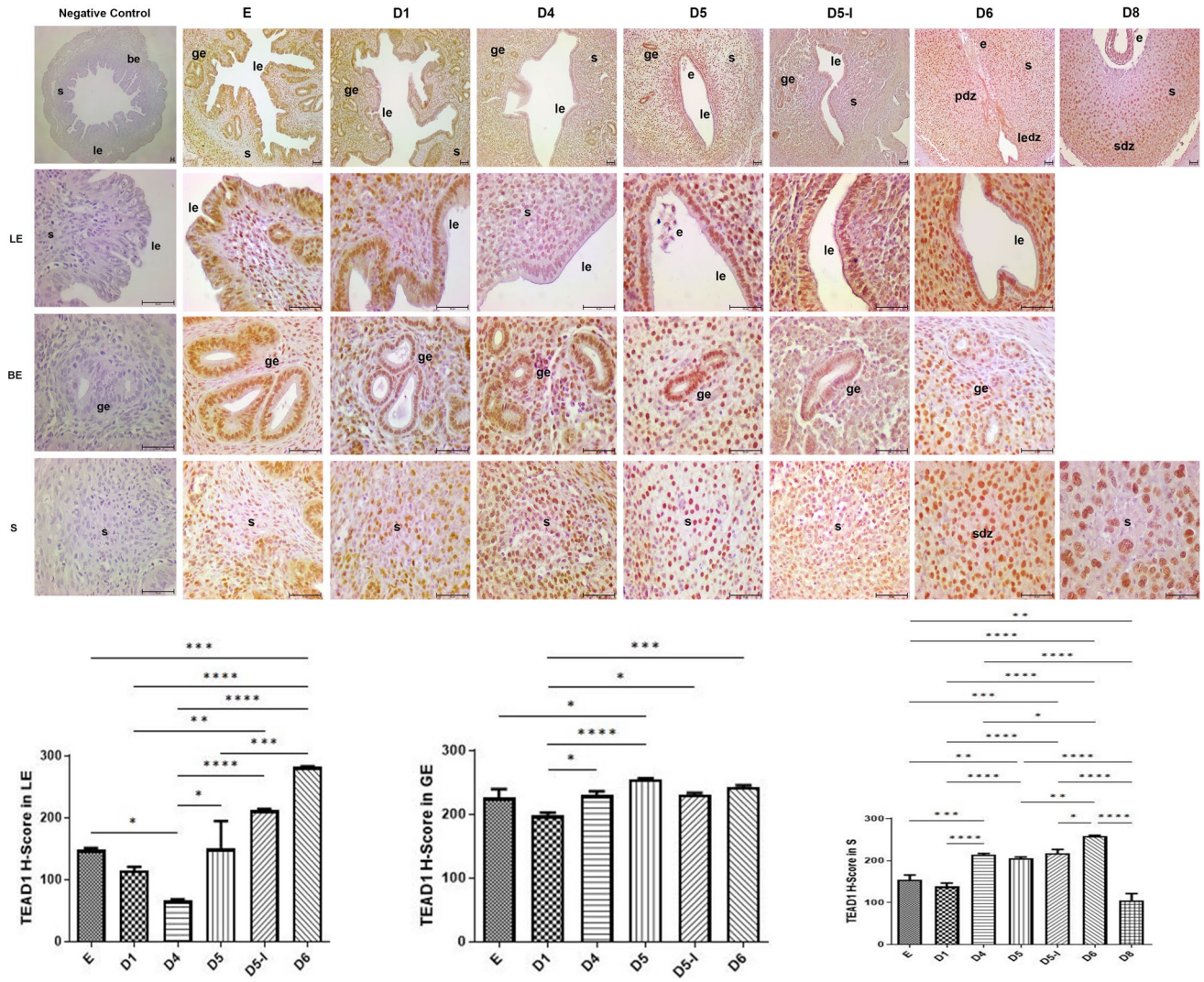


Fig. 3 TEAD1 immunohistochemistry in the luminal epithelium, the glandular epithelium, and the stroma of uterus, implantation, and inter-implantation sites of the experimental groups.. For figures LE, GE, and S H-Score analysis; asterisks indicate significant difference (*$p<0.05$), (**,***$p<0.001$), (****$p<0.0001$). *le* Luminal epithelium, *ge* Glandular epithelium, *s* Stroma, *e* Embryo, *pdz* Primary decidual zone, *sdz* Secondary decidual zone. Scale bars: 50 µm

the implantation sites and the inter-implantation sites on the 5th day of pregnancy ($p>0.05$), a decrease was observed at implantation sites on the 6th day of pregnancy compared to all experimental groups ($p<0.0001$) (Fig. 4).

In the glandular epithelium, CTGF expression was at the highest level in the estrous group uteri compared to all groups. CTGF expression in the uteri on the 1st and 4th days, at the implantation sites on the 5th and 6th days, and the inter-implantation sites on the 5th day of pregnancy were gradually decreased compared to the estrous group uteri ($p<0.0001$). A decrease was observed in the uteri on the 4th day of pregnancy, implantation sites on the 5th and 6th days of pregnancy and, inter-implantation sites on the 5th day of pregnancy compared to the uteri on the 1st day of pregnancy ($p<0.0001$). In addition, a decrease was observed at the inter-implantation sites on the 5th day of pregnancy ($p<0.05$) and implantation sites on the 6th day of pregnancy ($p<0.0001$) compared to the uteri on the 4th day of pregnancy (Fig. 4).

In the stroma, CTGF expression was at the lowest level in the estrous group uteri compared to the other groups. CTGF expression was dramatically increased in the later days of pregnancy, and the greatest increase was observed at the implantation sites on the 8th day of pregnancy ($p<0.0001$). While there was no CTGF expression in the primary decidual zone (PDZ), it was strong in the secondary decidual zone (SDZ) on the 6th day of pregnancy. CTGF expression was very strong in the secondary decidual zone on the 8th day of pregnancy (Fig. 4).

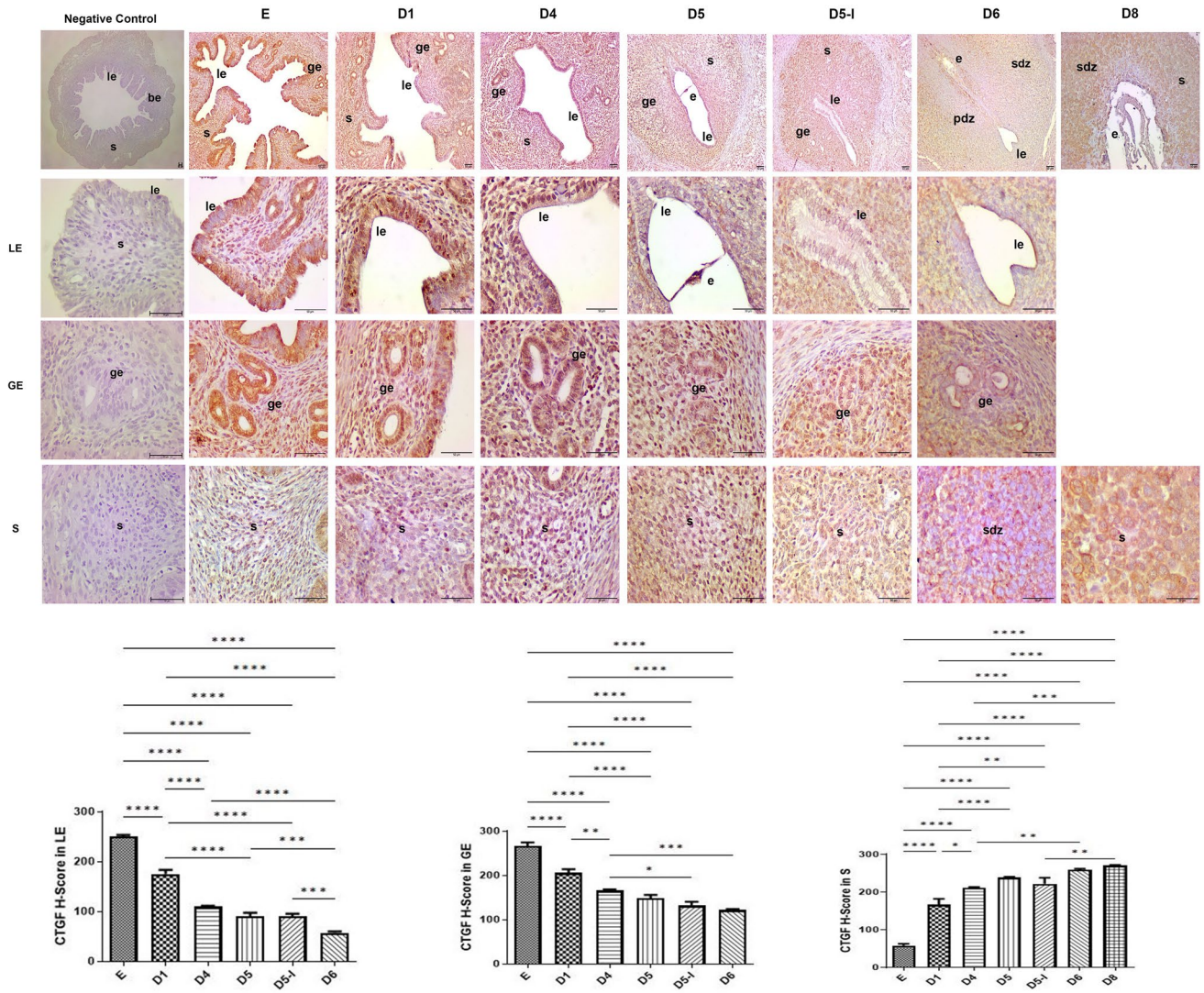


Fig. 4 CTGF immunohistochemistry in the luminal epithelium, the glandular epithelium, and the stroma of uterus, implantation, and inter-implantation sites of the experimental groups.. For figures LE, GE, and S H-Score analysis; asterisks indicate significant difference (*$p<0.05$), (**,***$p<0.001$), (****$p<0.0001$). *le* Luminal epithelium, *ge* Glandular epithelium, *s* Stroma, *e* Embryo, *pdz* Primary decidual zone, *sdz* Secondary decidual zone. Scale bars: 50 µm

Western blot results

According to the results of Western blot analysis for pYAP, no statistically significant difference was observed between the uteri of estrous and 1st day of pregnancy ($p=0.9973$), 4th day of pregnancy ($p>0.9999$), implantation sites of 5th day of pregnancy ($p>0.9999$), inter-implantation sites of 5th day of pregnancy ($p=0.9984$), and also 6th day of pregnancy ($p=0.757$). A decrease was observed at the implantation sites on the 6th day of pregnancy compared to the uteri on the 1st day and the inter-implantation sites on the 5th day of pregnancy ($p<0.05$). An decrease was observed at the implantation sites on the 8th day of pregnancy compared to all experimental groups ($p<0.0001$) (Fig. 5).

Western blot analysis for YAP showed that expression was statistically significantly higher in other experimental groups than the uteri of the estrous and the 1st day of pregnancy groups, and the highest YAP expression was observed at the implantation sites on the 5th, 6th, and 8th days of pregnancy among all experimental groups ($p<0.0001$) (Fig. 6).

TEAD1 Western blot analysis showed that expression levels in the uteri of the estrous and the 1st day of pregnancy groups were similar. The highest TEAD1 expression was observed in the uteri on the 4th day of pregnancy, and this was statistically significant compared to the uteri of the estrous and the 1st day of pregnancy groups ($p<0.0001$). TEAD1 expression was statistically significantly decreased

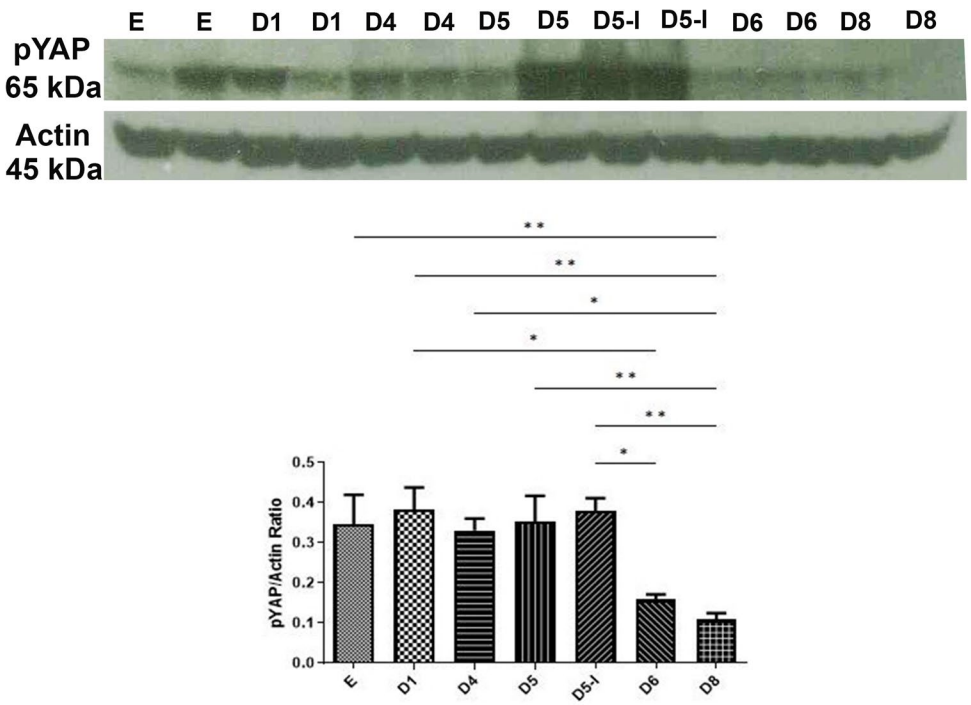


Fig. 5 pYAP Western Blot in the uterus and implantation sites of all experimental groups and analysis. Asterisks indicate significant difference (*$p < 0.05$), (**,***$p < 0.001$), (****$p < 0.0001$)

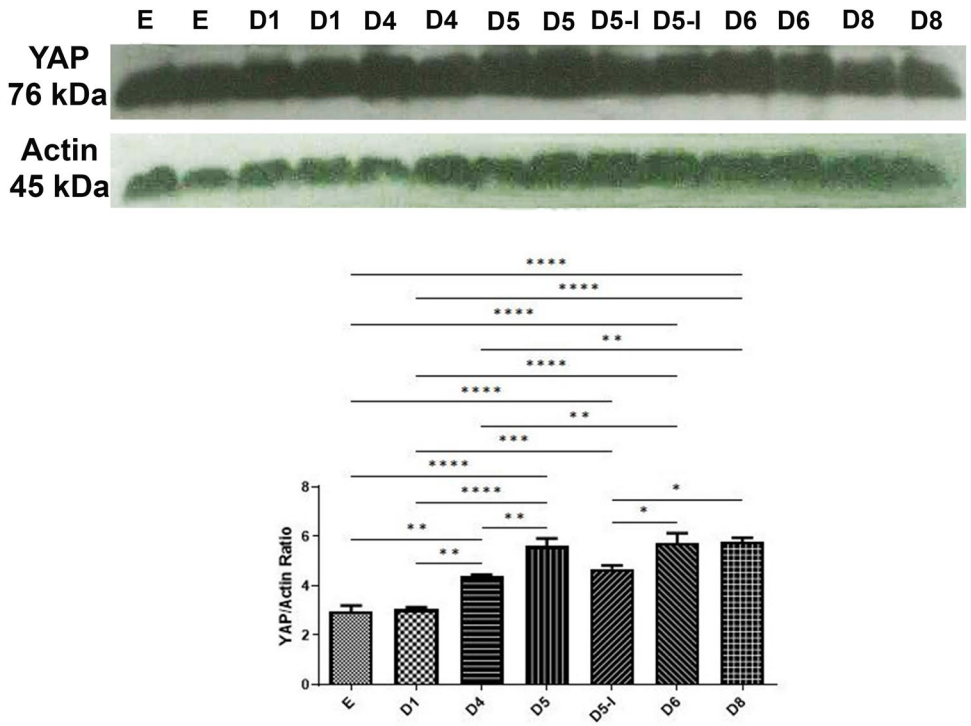


Fig. 6 YAP Western Blot in the uterus and implantation sites of all experimental groups and analysis. Asterisks indicate significant difference (*$p < 0.05$), (**,***$p < 0.001$), (****$p < 0.0001$)

at the implantation sites on the 5th ($p < 0.05$), 6th and 8th days of pregnancy ($p < 0.0001$) and, inter-implantation sites on the 5th day of pregnancy ($p < 0.001$), compared to the uteri on the 4th day of pregnancy. The lowest TEAD1 expression was observed at the implantation sites on the 8th day of pregnancy among all experimental groups (Fig. 7).

According to the results of Western blot analysis for CTGF, similar expression levels were observed in the uteri of the estrous and the 1st day of pregnancy groups. An increase was observed in the uteri on the 4th day of pregnancy ($p < 0.0001$), implantation and inter-implantation sites ($p < 0.05$) on the 5th day of pregnancy and, implantation

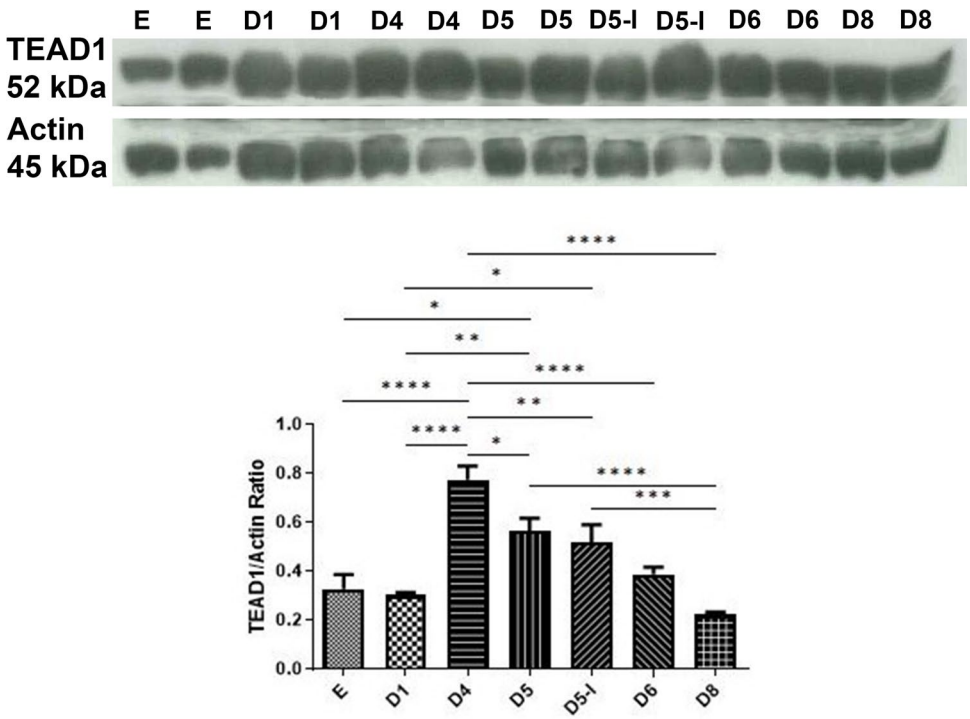


Fig. 7 TEAD1 Western Blot in the uterus and implantation sites of all experimental groups and analysis. Asterisks indicate significant difference (*$p < 0.05$), (**,***$p < 0.001$), (****$p < 0.0001$)

sites on the 6th and 8th days of pregnancy ($p < 0.0001$). An increase was observed in the uteri on the 4th day of pregnancy ($p < 0.0001$), implantation sites on the 6th and 8th days of pregnancy compared to the uteri on the 1st day of pregnancy ($p < 0.0001$). The highest CTGF expression was observed at the implantation sites on the 8th day of pregnancy among experimental groups (Fig. 8).

Discussion

In the implantation process, which can be defined as the nidation of the embryo in the uterus, mutual communication via many signaling pathways play a role between the maternal endometrium and the embryo [4, 49]. Implantation takes place when the uterus is receptive. The receptive

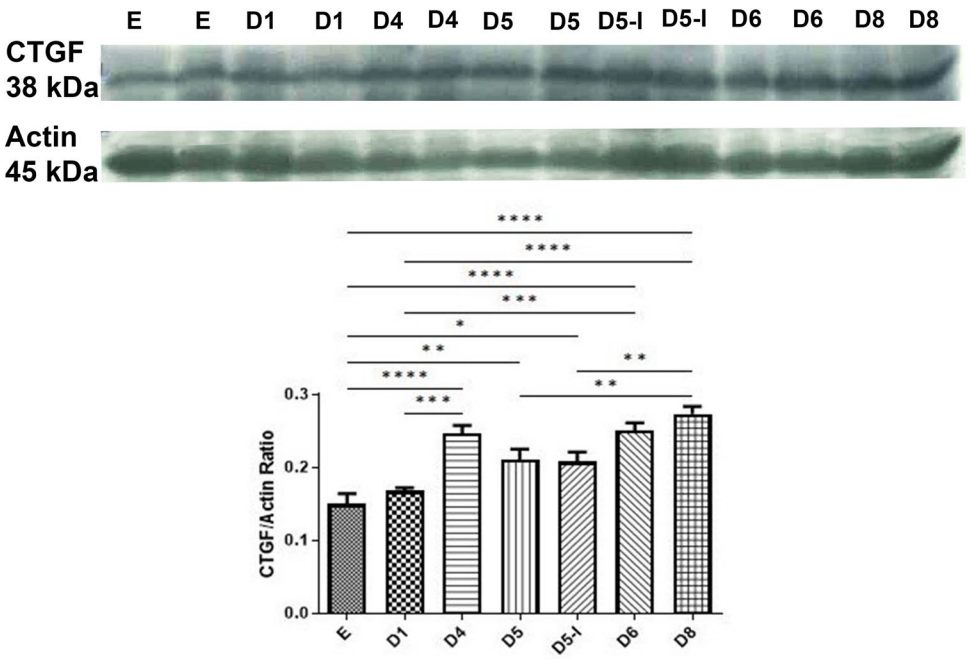


Fig. 8 CTGF Western Blot in the uterus and implantation sites of all experimental groups and analysis. Asterisks indicate significant difference (*$p < 0.05$), (**,***$p < 0.001$), (****$p < 0.0001$)

phase is also called the implantation window and, ovarian steroid hormones play important roles during this phase. For successful implantation, the embryo must develop synchronously to the blastocyst stage, and the uterus must be in the receptive stage.

There are studies in the literature investigating the expressions and/or roles of the Hippo signaling pathway in the uterus, endometrial stromal cells, placenta under different conditions, but there is no study whether this pathway plays a role in implantation and/or decidualization processes in mice. In our study, we investigated the localization and expressions of pYAP, YAP, TEAD1, and CTGF, which are members of the Hippo signaling pathway, in the luminal epithelium, glandular epithelium, and stroma of uterus/implantation sites during the mouse peri-implantation period.

According to our study, on the 1st day of pregnancy YAP, TEAD1, and CTGF were expressed in the luminal and glandular epithelium. Our results may show that luminal and glandular epithelium proliferates under the influence of estradiol, but there is no proliferation yet in the stroma. In a study, STK4 (Serine/threonine protein kinase 4), a component of the Hippo signaling pathway, was shown to be regulated by estrogen in the uterus. STK4 was highly and rapidly expressed in the luminal and glandular epithelium, depending on the estrogen level. These results suggested a new potential for STK4 to regulate uterine dynamics during the estrus cycle [50]. In another study performed by Wood et al., PCNA expression was found to be at the highest level in the luminal epithelium and stroma of the estrous phase mouse uteri [51]. Our findings about stroma are not compatible with the findings of Wood et al. however, PCNA is not an absolute marker of proliferation, and there are publications in the literature stating that stroma proliferation starts on the 4th day of pregnancy [49, 52]. On the 4th day of pregnancy, we observed that the expressions of YAP, TEAD1 and CTGF in the stroma are increased but decreased in the luminal and glandular epithelium. These results led us to think that proliferation takes place under the influence of increasing progesterone in stroma cells, and proliferation ceases in epithelial cells.

According to our results, pYAP expression was at the highest level in the luminal epithelium of the implantation sites on the 5th day of pregnancy. It is known that luminal epithelium does not proliferate since it is differentiated on the 5th day of pregnancy in mice [52]. From this point, our findings are compatible with the literature. We observed that both YAP and pYAP expressions were high in the stroma of the implantation sites on the 5th day of pregnancy. Stroma cells around the blastocyst undergoing differentiation that will form the primary decidual zone (PDZ) expressed pYAP. Also, stroma cells that will take part in the secondary decidual zone (SDZ) expressed YAP for proliferation. Supporting our hypothesis, we observed that CTGF, which is induced by the YAP-TEAD1 complex, was less expressed in stroma cells around the blastocyst than in other stroma sites. Our findings were supported by the findings of Yuan et al. In that study, Yuan et al. performed Ki67 immunohistochemistry at the implantation sites of *Scrib*$^{f/f}$ mice on the 5th day of pregnancy (they used *Scrib*$^{f/f}$ mice as a control group) and showed that stroma cells around the embryo did not express Ki67, since they stopped proliferation and begin to differentiate to form the PDZ [53].

Our results showed that although YAP and TEAD1 expression were high, CTGF expression was low in the luminal epithelium of the implantation sites on the 6th day of pregnancy. There is expected to be no proliferation in the luminal epithelium because these cells are removed by entosis and apoptosis during this day of pregnancy [54]. Similar to our work, Ota et al. showed that the distribution of *Yap* and *Tead1* did not always correlate with proliferation in mouse embryos [55]. Compatible with the literature, we think that the high YAP-TEAD1 expression observed in the luminal epithelium of the implantation sites on the 6th day of pregnancy is not associated with proliferation.

According to YAP, TEAD1, and CTGF Western blot and stroma H-SCORE results at the implantation sites on the 6th and 8th days of pregnancy, although YAP and CTGF expressions were high, TEAD1 expression was relatively low on these days. These findings made us think that YAP could induce CTGF expression by complexing with another TEAD (TEAD 2, 3, or 4). Similarly, Zhao et al. showed that YAP induces CTGF expression by forming a complex with TEAD4 in HEK293, HEK293-T, NIH-3T3, and ACHN cells [18].

We found that YAP and TEAD1 were not expressed in a correlated way in the glandular epithelium of all experimental groups. For example, while TEAD1 expression was high, YAP expression was low on the 4th, 5th, and 6th days of pregnancy. However, we observed that CTGF was less expressed in the glandular epithelium as the pregnancy progressed, where the expression of the YAP-TEAD1 complex was induced. It is known that on the 4th day of pregnancy, proliferation shifts from epithelial cells to stroma cells [52]. In this respect, our finding is compatible with the literature. The absence of a correlated expression between YAP and TEAD1 made us think that TEAD1 could provide the expression of different genes by forming a complex with co-activators other than YAP. Malt et al. showed that TEAD1 plays a role in apoptosis by forming a complex with Livin in HeLa, MCF7, and BUA cell lines [56]. Besides, Osman et al. studied vascular smooth muscle cells of *Tead1* heterozygous mice and showed that TEAD1 formed a complex with MCAT (muscle CAT element) and provided transcriptional induction of SLC1A5 (Solute Carrier Family 1 Member 5), enabling the proliferation of vascular smooth muscle cells [57].

In mice, the uterus becomes receptive only on the 4th day of pregnancy, and on the 5th day in the afternoon, it becomes non-receptive [3]. The key events that occur following implantation are the proliferation and differentiation of stromal cells into decidua cells to support embryo development. In mice, the stroma cells around the blastocyst stop proliferation and differentiate into the primary decidual zone (PDZ) [58, 59]. PDZ begins to form in the afternoon on the 5th day of pregnancy and is fully formed on the 6th day of pregnancy [59]. As the secondary decidua zone (SDZ) begins to form, the PDZ disappears. SDZ is the layer of stromal cells that proliferate and differentiate around the PDZ and peaks on the 8th day of pregnancy.

According to our results, there was no pYAP expression both in PDZ and SDZ at the implantation sites on the 6th day of pregnancy. While YAP was not expressed in PDZ, it was expressed in SDZ. TEAD1 was expressed both in PDZ and SDZ. CTGF was not expressed in PDZ but expressed in SDZ. These findings made us think that on the 6th day of pregnancy, PDZ regresses, and SDZ continues to proliferate, so the YAP-TEAD1 complex stimulates CTGF expression and provides proliferation in SDZ. TEAD1 expression in PDZ without the expression of YAP made us think again that TEAD1 might have other functions by forming a complex with other co-activators. TEAD1 may play a role in apoptosis by forming a complex with Livin [56, 57].

In one study, CTGF expression was determined in uteri of mice on different days of pseudo-pregnancy (vaginal plaque accepted as 0.5 day of pseudo-pregnancy) and mice treated with E_2 and/or P_4 after ovariectomy [60]. In this study, CTGF was most prominently expressed in epithelial cells on 0.5–2.5. days of pseudo-pregnancy, and it was decreased in the epithelium and increased in the stroma and endothelium on 3.5–4.5 days of pseudo-pregnancy. The researchers concluded that CTGF expression is independent of the blastocyst, depends on maternal factors, and is regulated by steroid hormones. It is known that in pseudopregnant mice (females mated with vasectomized males), the steroid hormonal milieu within the uterus is similarly maintained [61]. These findings are consistent with the results we obtained. We also observed CTGF was prominently expressed in the luminal and glandular epithelium on the 1st day of pregnancy. We found CTGF expression in the stroma was increased from the 1st day to the 8th day of pregnancy.

CTGF has roles in the regulation of endothelial cell function and angiogenesis. Vascular defects were seen during embryogenesis in CTGF knock-out mice [62]. It is known that estrogen and progesterone have different effects on angiogenesis in vivo. While estrogen increases vascular permeability in the uterus and inhibits angiogenesis, progesterone induces angiogenesis with little effect on vascular permeability [63]. In our study, we observed the increase of CTGF expression in the stroma from the estrous phase to the 8th day of pregnancy. This result made us think that CTGF may play a role in extracellular matrix remodeling and angiogenesis processes in the stroma during pregnancy. CTGF expression was lower in the uteri of the estrous and the first day of pregnancy groups, where estrogen is more effective, and angiogenesis has not occurred yet. On the 4th day of pregnancy, when angiogenesis increases with the effect of progesterone, CTGF expression was also increased.

Endometrium cells undergo proliferation, differentiation, and extracellular matrix remodeling under the influence of molecules regulated by the steroid hormones. In addition to the healthy development of the embryo, synchronous uterus receptivity, cross-talk between embryo and uterus, differentiation of stroma cells to decidua cells, angiogenesis, and placental development are very critical for a successful pregnancy. In our study, we examined the expressions of pYAP, YAP, TEAD1, and CTGF, members of the Hippo signaling pathway, in the uterus/implantation sites of mice during the peri-implantation period. The Hippo signaling pathway shows dynamic change during the peri-implantation process. The potential role of Hippo signaling pathway during the peri-implantation period in the mouse uterus is shown in Fig. 9. In conclusion, it is possible to say that the Hippo signaling pathway has roles in implantation and decidualization processes.

To the best of our knowledge this is the first study revealing the role of the Hippo signaling pathway during the peri-implantation period mouse uterus and implantation sites. We think that these findings are important to elucidate the mechanisms such as implantation failure and recurrent pregnancy losses encountered in the clinic.

Conclusion

In our study, the localization and expressions of the members of the Hippo signaling pathway, pYAP, YAP, TEAD1, and CTGF, were investigated by immunohistochemistry and Western blot methods in the uterus/implantation sites of mice in the estrous phase and peri-implantation period. It is known that embryo implants on the 5th day of the pregnancy and on this pregnancy day we observed that pYAP was at the highest level in the luminal epithelium of uterus. YAP was at the highest level in the stroma of implantation sites on the 6th and 8th days of pregnancy when decidualization occurs. TEAD1, a transcription factor that enables the expression of CTGF by forming a complex with YAP, was not always expressed in correlation with YAP and CTGF. CTGF gradually decreased in the luminal and glandular epithelium from the estrous phase to the 6th day of pregnancy. In the stroma of the experimental groups, CTGF gradually increased from the estrous phase to the 8th day of pregnancy. The findings of our study suggested that the Hippo signaling pathway

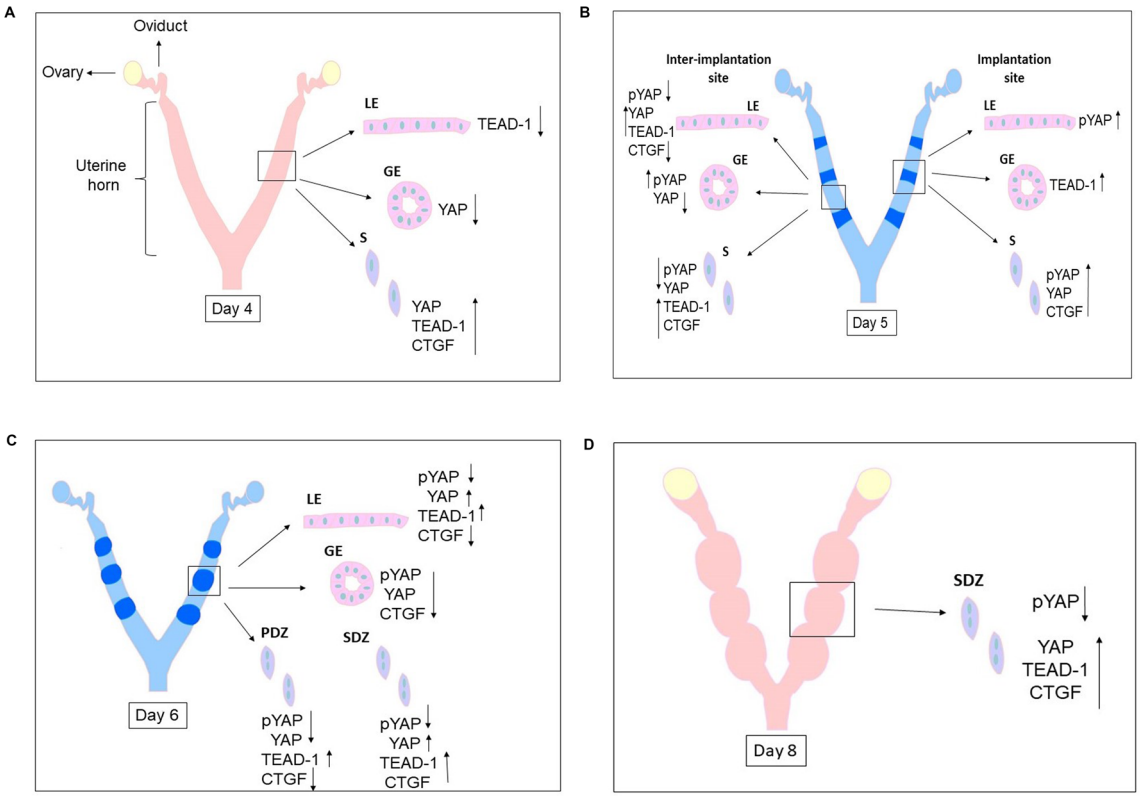


Fig. 9 Schematic illustration of the potential role of Hippo signaling pathway during peri-implantation period mouse uterus **A** Day 4 of pregnancy, **B** Day 5 of pregnancy, **C** Day 6 of pregnancy, **D** Day 8 of pregnancy, *LE* Luminal epithelium, *GE* Glandular epithelium, *S*: Stroma

during early pregnancy may take a role in implantation and decidualization processes. We think that our results may shed light on the mechanisms of recurrent pregnancy losses and implantation disorders seen in the clinic.

Author contributions EG: conceptualization, investigation, formal analysis, writing—original draft, visualization. CNB: formal analysis. IU: review & editing, NA: conceptualization, writing—review & editing, funding acquisition.

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Data availability Available upon request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was performed in line with the principles of the Declaration of Helsinki. The experimental protocol was approved by the Animal Care and Use Committee of Akdeniz University Faculty of Medicine (Number of the Ethical Approval 735/2018.02.001).

Consent to participate Not applicable.

Consent for publication Not applicable.

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