

IMMUNOLOCALIZATION OF HOXA11 AND HLX PROTEINS IN COW PLACENTA DURING PREGNANCY

Uğur Topaloğlu^{1*}, Muzaffer Aydın Ketani¹, Mehmet Erdem Akbalık¹, Hakan Sağsöz¹, Berna Güney Saruhan¹, Bayram Bayram²

¹Department of Histology and Embryology, Faculty of Veterinary Medicine Dicle University, Diyarbakır 21280, ²Laboratory and Veterinary Health, Idil Vocational College Sırnak University, Şırnak 73200, Turkey

*Corresponding author, E-mail: ugur.topaloglu@dicle.edu.tr

Abstract: In addition to its many functions, the placenta is characterized by the intensity and characteristics of its hormonal functions. It has been reported that the development and vascularization of the placenta with normal fetal growth is regulated by many factors such as growth factors, transcriptional factors, and homeobox proteins. This study was conducted to determine the distribution and possible physiological roles of HOXA11 and HLX in the uterus and placenta of pregnant cows. In this study, 27 pregnant Holstein breed cow uteri and placentas obtained from private slaughterhouses were used as tissue samples. In order to determine the pregnancy period of the obtained uterus, the ages of the fetuses were calculated according to the age calculation formula. The uteri were classified under three different groups as the first (69-89 days), second (99-178 days), and third (190-269 days) periods of pregnancy so that there are 9 animals in each group. Tissue samples were then subjected to immunohistochemistry to demonstrate the presence of HOXA11 and HLX proteins. It was observed that HOXA11 and HLX immunoreactions occurred prominently in the luminal and glandular epithelial cells of the uterus during pregnancy, and the maternal epithelial cells of the placenta and UTC and TGC/BNCs. In addition, it was determined that HOXA11 immunoreaction also formed in some vascular endothelial cells. Immunohistochemical findings in this study suggested that Hoxa11 and HLX may affect the proliferation and differentiation of cells in the uterus and placenta during pregnancy in cows, and that these proteins may contribute to normal uterine physiology, placentation formation, and continuity of pregnancy in cows.

Key words: Hox proteins; HOXA11; HLX; placenta

Introduction

Prenatal growth and development affect lifetime well-being and general health, status, which is directly proportional to the placental transport of nutrients and oxygen. As the placenta is subjected to significant morphological and histological changes during pregnancy to provide for fetal growth, gaining insight into the developmental changes which may affect the growth and development of fetus/embryo is crucial (1). Besides its many functions, the placenta is characterized by its hormonal functions. Hormones secreted by

trophoblasts and syncytiotrophoblast cells along the placental villi have important roles in the birth mechanism as well as in the formation and maintenance of pregnancy and the growth and development of the fetus (2).

The formation of the placenta in Eutherian mammals requires interaction between the chorioallantoic membrane and the maternal uterine tissue. Different degrees of invasiveness are observed among different types of placenta. The human hemochorial placenta has invasive trophoblasts that come into direct contact with maternal blood. On the other hand, in the epitheliochorial placenta observed in ruminants, the uterine epithelium and maternal blood vessels remain intact throughout pregnancy. On the contrary, the cow placenta is a more complex

placental type in which different sites may have different levels of fetal-maternal interaction (3). The cotyledonary placenta in cattle is classified as synepitheliochorial based upon the presence of fetal-maternal syncytium and the number of cell layers between maternal and fetal circulations (4). Synepitheliochorial placenta is the most critical region of fetal-maternal communication for the continuation of pregnancy and embryonic development in ruminants (5). Normal fetal growth and development are regulated by maternal, fetal, placental, and environmental factors in accordance with the genetically predetermined growth potential. However, it was reported that the development and vascularization of the placenta in fetal growth are governed by many elements such as growth factors, transcriptional factors, and homeobox proteins (6).

Homeobox proteins have many subunits such as HOX/Hox, DLX/Dlx, MSX/Msx, HMX/Hmx, and PBC in the genomes of vertebrates such as humans and mice (7), HOX proteins are essential regulators of the fate and identity of the cell in the embryonic process, and their roles in embryonic stem cell differentiation and morphogenesis are important. Hox proteins have been reported to have many important roles according to their order and location on the chromosome, and their activation feature (6, 8). These roles appear in implantation, cell and tissue differentiation of developing embryos, the angiogenesis and neovasculogenesis of adult processes of the embryo, and the development of the vertebrate nervous and reproductive systems. However, it has been pointed out that they play important roles in adults, such as controlling the extraembryonic development of the placenta, sexual cycle regulating and the physiology of the uterus during pregnancy (6, 8, 9).

In mammals, along the paramesonephric ducts (Müllerian ducts), it has been shown that HOXA11, which is related to the abdominal-B (Abd-B), plays a role in the composition of the female genital anatomy and is expressed in the cervix (10). HOXA11, which is very important for the development and function of the female reproductive system, and has been reported to be necessary for the successful implantation of the blastocyst in mammals (11, 12). HOXA11 is responsible for significant changes in the reproductive biology of mammals (especially changes in the cellular functions of the female reproductive organs such as the uterus, cervix, vagina, and placenta). However, it has been reported

that HOXA11 has plays critical roles in the proliferation and differentiation of endometrium cells in response to varying estrogen and progesterone concentrations, in the acceptance of the offspring by the uterus, and in the maintenance of pregnancy. Mutation defects occur in the organs of the female genital system, especially the uterus, and as a result infertility occurs (12, 13, 14, 15).

Similar to the *Drosophila*'s homeobox gene H2.0, Hlx is a distinct homeobox protein whose expression and protein sequence are highly conserved among mammals, birds, and fish. Hlx, which was initially detected in hematopoietic cells of myelomonocytic and B lymphocyte lineages in mice, is 86.5% identical to its homolog HLX (HB24/HLX1) in humans, and its homeodomain areas are completely preserved (16, 17). It has been pointed out that the HLX/hlx protein generally has important roles such as visceral organogenesis and enteric nervous system development, and is detected at the highest level in the normal liver, gall bladder, and mesenchyme of the developing mouse embryo. In recent studies, it has been reported that it was isolated in human and mouse placenta and is a potential regulator of placental development (16, 17, 18, 19, 20). It has also been reported that HLX has an active role in the proliferation, differentiation, and migration of extra villous trophoblast (EVT) cells in the human placenta (19, 21). Thus, it was reported that HLX is important for normal placental formation and, its deficiency causes fetal growth retardation and pregnancy impairment together with abnormal trophoblasts (22).

The present study was carried out to reveal the expressions of HOXA11 and HLX proteins, subunits of homeobox proteins, in cow uterus and placenta, which are critical for reproductive fertility and maintenance of animal husbandry, and also to identify their possible physiological roles in these tissues.

Material and methods

Obtaining the Tissue Samples

In the study, 27 pregnant Holstein breed cow uteri and placentas obtained from cows slaughtered in Diyarbakir and Hatay, Turkey were used as tissue samples (23). In order to determine the pregnancy period of the obtained uteri, the ages of the fetuses

were calculated. For this purpose, Crown-Rump Length (CRL) was measured. Then, the age of the fetus was calculated using the equation „ $y: 54.6 \text{ cm} + 2.46 (x) \text{ cm}$ “, which was obtained as a result of the linear relationship between CRL and fetal age. In the calculation applied, y is a constant representing the fetal age, and x denotes CRL (24). According to this age calculation formula, the uteri were classified under three different groups as: the first (69-89 days), second (99-178 days), and third (190-269 days) periods of pregnancy.

Routine Histological Tissue Tracking and Staining

We obtained tissue samples in the placentomal and interplacentomal region of the pregnant cornu uteri in three different groups were subjected to fixation in 10% neutral formalin solution for 24 hours. The tissues were then washed with running tap water for 24 hours. Following the washing process, the tissues were treated with through graded alcohols, methyl benzoate, and benzol series. Subsequently, the tissues were kept in an incubator at 58 °C in paraffin dissolved in benzol for 30 minutes in order to acclimate the tissues to paraffin. Then, the tissues were kept in clean, melted paraffin in an oven at 58 °C for 4 hours. After these procedures, the tissues were enclosed in liquid paraffin and transformed into blocks. Serial sections of 5- μm thickness were taken from the prepared paraffin blocks in a rotary microtome (Leica RM-2125, Germany). The sections were put on Poly-L-Lysine (PLL) coated slides and subjected to immunohistochemistry staining.

Immunohistochemical Staining

Indirect streptavidin-biotin complex method of immunohistochemistry was performed on the serial sections taken on Poly-L-Lysine (PLL) slides to determine the presence of HOXA11 and HLX proteins (23). Serial sections were taken in distilled water after passing through xylol for deparaffinization and through alcohol series for dehydration. Then, the sections were kept in 3% H_2O_2 prepared with methanol for 20 minutes to prevent endogenous peroxidase activity in the tissues. In the next step, the preparations were washed three times in 0.01M phosphate buffer saline (PBS) for 5 minutes each. Following washing, citrate buffer (0.01M, pH 6) was prepared for antigen retrieval. The sections were

boiled at 95 °C for 20 minutes and then left for cooling. After cooling, the sections were washed in 0.01M PBS. Afterwards, they were incubated with protein blocking solution (Ultra V Block, Thermo Fisher Scientific, Lab Vision Corporation) at room temperature for 15 minutes in order to prevent non-specific staining in the tissues. Afterwards, normal sections were incubated with HOXA11 (Rabbit polyclonal, catalog no: PA5-57341, Invitrogen) and HLX (Rabbit polyclonal, catalog no: PA5-44857, Invitrogen) primary antibodies diluted to 1/100, and negative control preparations were incubated with PBS overnight at 4 °C in order to determine the accuracy of staining. After incubation, the preparations were washed three times in 0.01M PBS for 5 minutes. Subsequently, they were subjected to biotinylated secondary antibody (Histostain Plus Bulk Kit, Zymed) for 20 min at room temperature and binding to the primary antibody was obtained. After this treatment, the preparations were washed three times again in 0.01M PBS for 5 minutes. Subsequently, the sections were reacted in streptavidin peroxidase solution (Histostain Plus Bulk Kit, Zymed) for 20 minutes. They were then washed three times for 5 minutes in 0.01M PBS. After these processes, the preparations were incubated with 3'3'-diaminobenzidine hydrochloride (DAB) for 5-15 minutes to observe the antigen-antibody reaction. After the immunoreaction is visualized, the preparations were subjected to Mayer's hematoxylin for 2-3 minutes for background staining. Immediately after this, they were washed in running water. Then, the preparations washed in distilled water were passed through the alcohol and xylol series and closed with Entellan. The images of the preparations were taken with a Nikon Eclipse E400 (Nikon, Tokyo, Japan) research microscope attached to a Nikon Coolpix 4500 digital camera.

Semi-quantitative Evaluation

Immunohistochemical staining was assessed semi-quantitatively based on the intensity score. Intensity score was determined according to the positive staining intensity in the cells. Accordingly, the scoring process in the immunohistochemical staining results was carried out following this rubric: (0: Negative) no staining observed in cells at 40 and 100X magnification, (1: Weak) staining observed in cells only at 40 and 100X magnification, (2: Medium) staining easily observed in cells at 20X magnifications, (3: Strong) staining observed in cells at 4X and 10X magnifications (25).

Table 1: In cow placenta and uterus in the first period of pregnancy (69-89 days, n:9), in the second period (99-178 days, n:9), in the third period (190-269 days, n:9) distribution of HOXA11 and HLX proteins according to immunostaining intensity score (IS)

		Endometrium			Placenta maternalis		Placenta fetalis			
		Luminal Epithelium	Glandular Epithelium	Stroma	Smooth Muscle	Maternal Epithelium	Maternal Stroma	UTC	TGC/BNC	Fötal Stroma
		IS	IS	IS	IS	IS	IS	IS	IS	IS
HOXA11	I. Period	2,11±0,6 ^a	2,00±0,7 ^a	1,67±0,7 ^b	0,67±0,7 ^a	0,67±0,5 ^a	0,22±0,44 ^a	1,44±0,73 ^b	0,89±0,6 ^{ab}	0,33±0,5 ^a
	II. Period	1,89±0,6 ^a	1,78±0,67 ^a	0,33±0,5 ^a	0,44±0,53 ^a	1,67±0,5 ^b	0,56±0,53 ^{ab}	2,11±0,6 ^b	1,33±0,5 ^b	0,67±0,71 ^a
	III. Period	2,00±0,5 ^a	1,44±0,53 ^a	0,56±0,73 ^a	0,67±0,5 ^a	1,11±0,6 ^{ab}	1,11±0,6 ^b	0,67±0,5 ^a	0,44±0,53 ^a	0,78±0,67 ^a
HLX	I. Period	1,67±0,5 ^a	1,56±0,53 ^a	0,89±0,33 ^a	0,78±0,44 ^a	1,33±0,5 ^a	0,78±0,44 ^a	1,22±0,44 ^a	1,22±0,44 ^a	0,33±0,5 ^a
	II. Period	1,78±0,44 ^a	1,78±0,44 ^a	0,67±0,50 ^a	0,33±0,50 ^a	1,44±0,53 ^a	0,78±0,44 ^a	1,33±0,5 ^a	1,44±0,53 ^a	0,67±0,5 ^a
	III. Period	1,56±0,53 ^a	1,33±0,50 ^a	0,78±0,44 ^a	0,56±0,53 ^a	1,11±0,6 ^a	0,33±0,5 ^a	0,89±0,33 ^a	1,00±0,00 ^a	0,22±0,44 ^a

Different superscripts in the same row show the statistical difference (^a-^b*p<0,05) in expression levels depending on the periods, and common superscripts show the similarity between the periods

The sections were examined independently by three expert histologists (U.T, M.A.K, M.E.A) and calculations were made by taking the mean of the scores reported by the observers. Positive immunoreaction parts of HOXA11 and HLX in the placentomal and interplacentomal regions were scanned at 40x, 100x, 200x, and 400x magnifications, and thereby, high expression areas were detected. Four sections randomly selected for each section were evaluated. The mean of the obtained scores was taken as a single score. The followings were assessed as placentomal and interplacentomal parts: maternal epithelial and stromal cells, fetal trophoblast and stromal cells, luminal and glandular epithelial cells of the uterus, and stromal and smooth muscle cells. We did not assess blood vessels in detail; however, the general appearance of the vessels was observed. In this study, 1090 observations were recorded for each tissue and 2320 observations were recorded for each uteri.

Statistical analysis

The data obtained as a result of immunohistochemical density scoring were evaluated statistically with SPSS Statistics for Windows, Version 25.0 (Armonk, NY, USA). The normality test was applied to the scores before the statistical analysis. In the normality test, the Shapiro-Wilk test was used depending on the number of samples in each parameter. The datasets for which normality tests were shown to be p<0.05 were analyzed using Kruskal-Wallis test (a non-parametric test). Tamhane's T2 test was used for multiple comparisons between groups. The data obtained as a result are shown as

mean ± standard deviation (SD). Any p value less than 0.05 was considered statistically significant (Table 1).

Results

HOXA11

In the fetal part of the placenta, it was found that HOXA11 immunoreaction in the chorion epithelium was localized in the nucleus and cytoplasm of some uninucleate trophoblast cells (UTC) and binucleated trophoblast giant cells (TGC/BNC) in the first and second periods, and only in the cytoplasm of the third period cells. Furthermore, HOXA11 was observed to induce a weak immunoreaction in fetal mesenchyme along with some fetal stromal cells (Figure 1A, B, C). It was observed that the moderate intensity immunoreaction observed in UTCs in the first and second periods significantly reduced in the third period cells (p<0.05). However, it was confirmed that the intensity of immunoreactions in TGC/BNCs decreased significantly in the third period compared to the second period (p<0.05), and took a value between of the first and that of the second period. It was observed that HOXA11 immunoreaction was very weak to weak in the epithelial and stromal cells of the maternal placenta. However, it was determined that the reaction intensity in maternal epithelial cells in the second period was slightly stronger compared to the other periods (Figure 1D, E, F). It was found that the observed immunoreaction increased significantly in the second period for maternal epithelium and in the third period for maternal stromal cells (p<0.05).

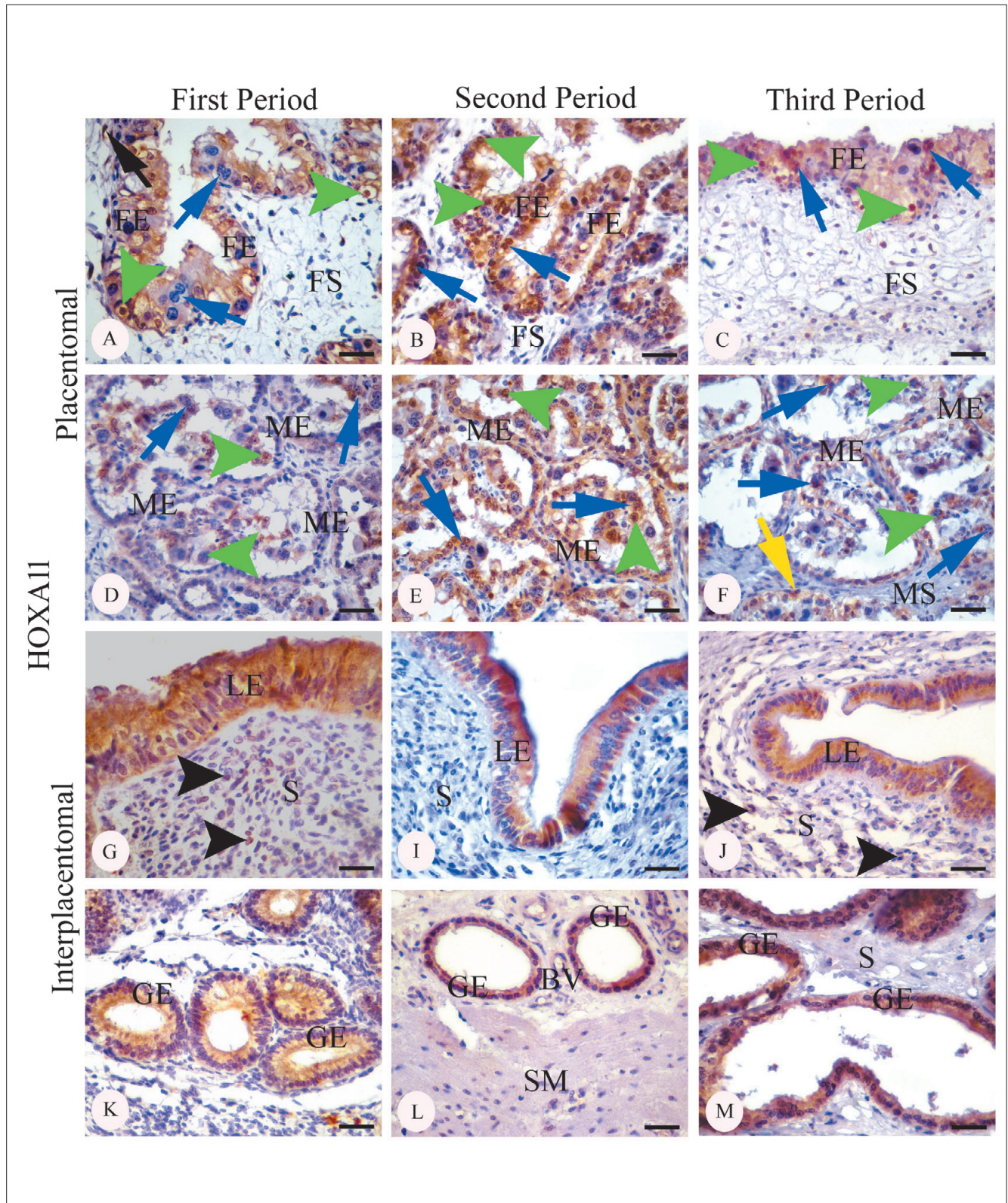


Figure 1: Immunolocalisation of HOXA11 in bovine placental and interplacental part, in the first period of pregnancy (79 days; A, D, G, K), second period (106 days; B, E, I, L), third period (249 days; C, F, J, M). FE: Fetal Epithelial, FS: Fetal Stroma, Green Arrowhead: Uninucleated trophoblast cell, Blue arrow: Binucleated trophoblast cell, Black arrow: Fetal stroma cells, ME: Maternal Epithelium, MS: Maternal Stroma, Yellow arrow: Maternal stroma cell, LE: Luminal Epithelium, S: Stroma, Black arrowhead: Stroma cell, GE: Gland Epithelium, SM: Smooth Muscle layer, BV: Blood Vessel. Scale Bar: 25 μ m

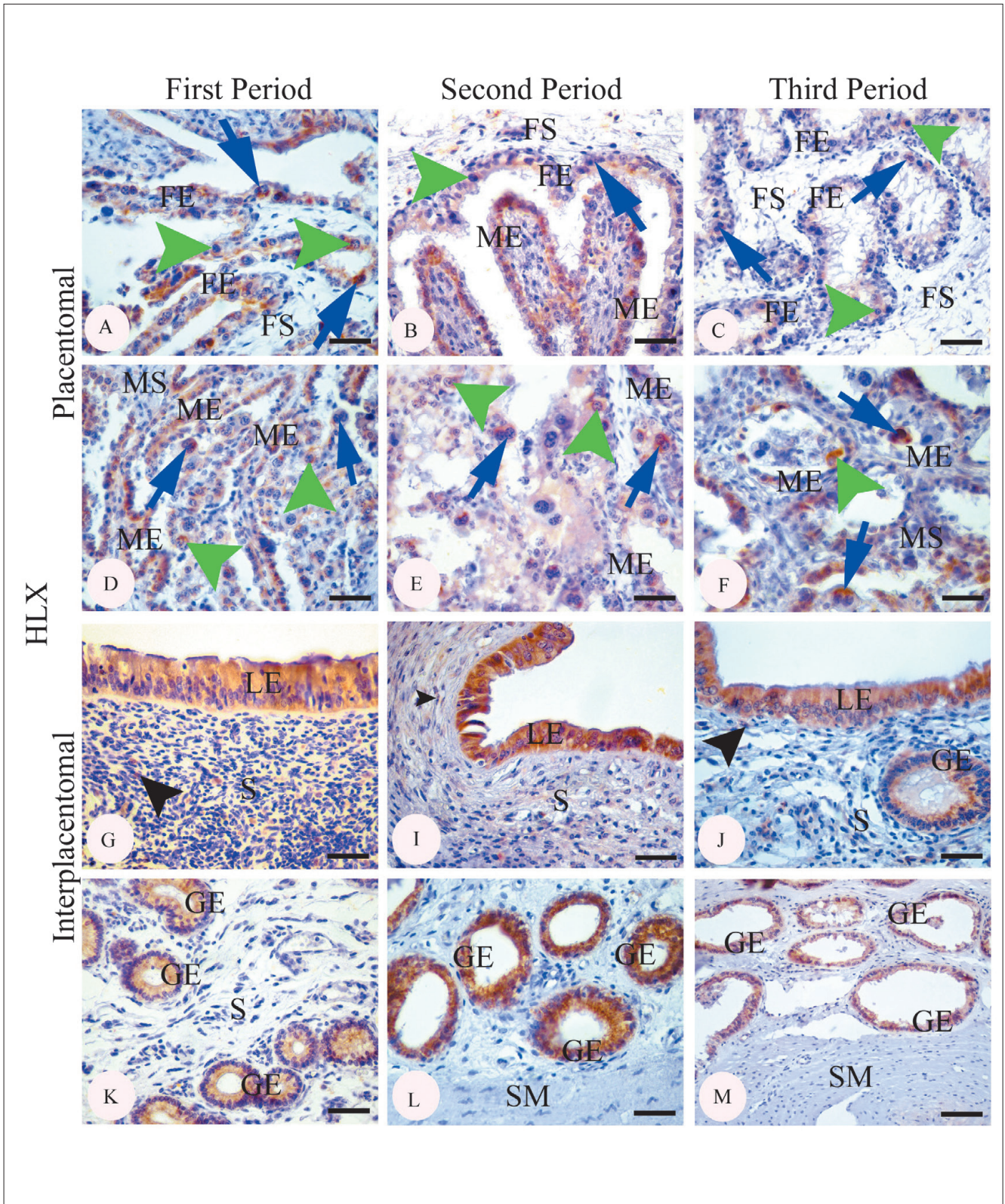


Figure 2: Immunolocalisation of HLX in bovine placental and interplacental part, in the first period of pregnancy (75 days; A, D, G, K), second period (123 days; B, E, I, L), third period (202 days; C, F, J, M). FE: Fetal Epithelial, FS: Fetal Stroma, Green Arrowhead: Uninucleated trophoblast cell, Blue arrow: Binucleated trophoblast cell, ME: Maternal Epithelium, MS: Maternal Stroma, LE: Luminal Epithelium, S: Stroma, Black arrowhead: Stroma cell, GE: Gland Epithelium, SM: Smooth Muscle layer cell. Scale Bar: (A, B, C, D, E, F, G, I, J, L): 25 μ m, (K, M) 50 μ m

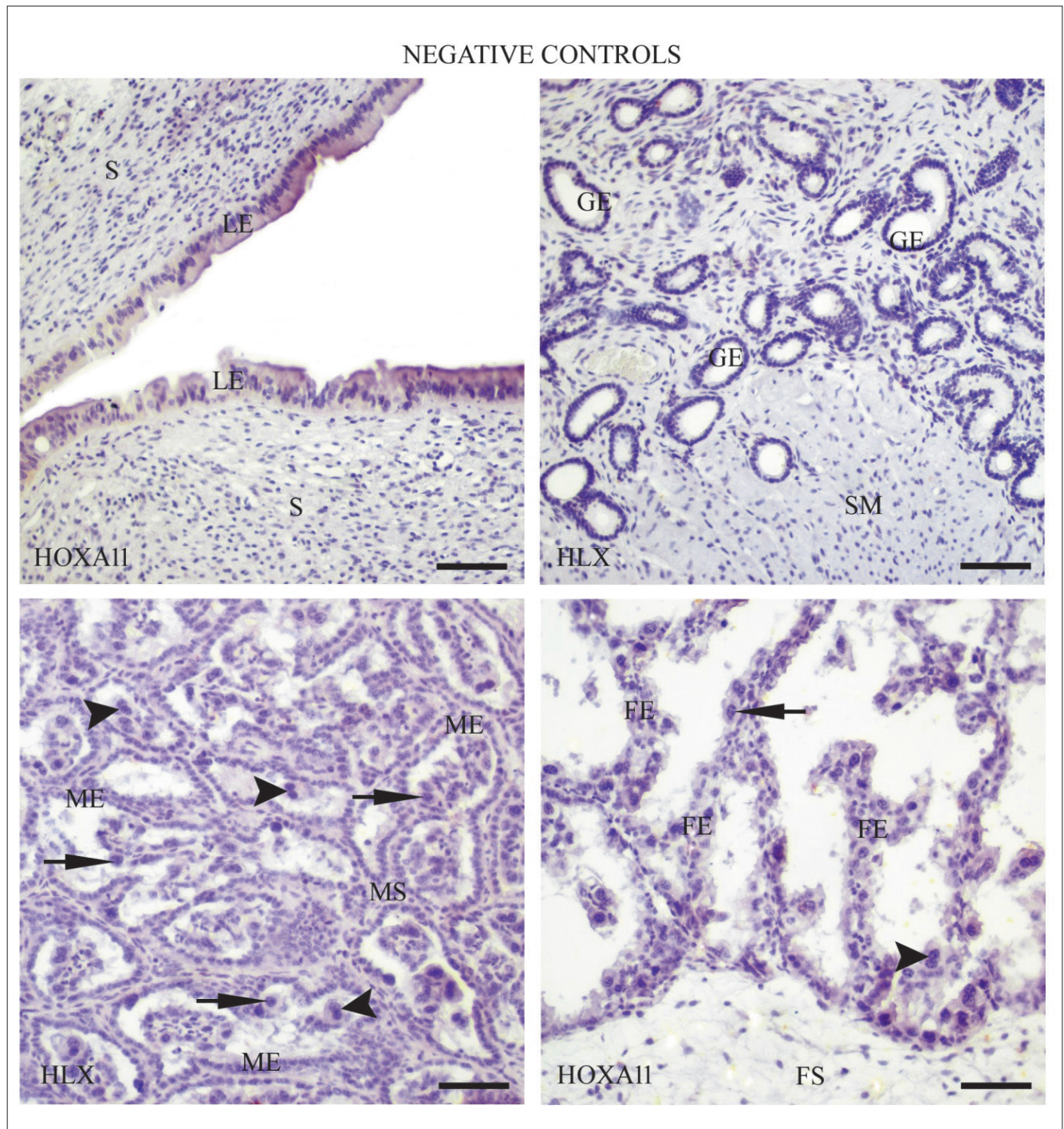


Figure 3: Negative control immunoreactivity for HOXA11 and HLX proteins. LE: Luminal Epithelium, S: Stroma, GE: Gland Epithelium, SM: Smooth Muscle layer, FE: Fetal Epithelial, FS: Fetal Stroma, ME: Maternal Epithelium, MS: Maternal Stroma, Arrow: Uninucleated trophoblast cell, Arrowhead: Binucleated trophoblast cell. Scale Bar: 50 μ m

It was observed that a moderate positive immunoreaction occurred in the luminal and glandular epithelial cells of the endometrium in all three periods of pregnancy. It was found that especially the immunoreaction was localized in the apical cytoplasm of the luminal and gland epithelial

cells (Figure 1G, I, J, K, L, M). It was observed that immunoreactivity was statistically similar in endometrial epithelium, gland, and muscle layer during the entire pregnancy ($p > 0.05$). Moreover, it was determined that HOXA11 produced a positive immunoreaction in some stromal cells

of the uterus in the first and third periods, as well as in some vascular endothelial cells in the second period (Figure 1G, J, L). It was observed that immunoreaction in stromal cells decreased significantly in the second period compared to the first period ($p < 0.05$).

HLX

It was observed that the immunoreaction in UTC and TGC/BNC in the chorion epithelium belonging to the fetal part of the placenta was found to be in weak density in the cytoplasm in all three periods of pregnancy. On the other hand, a very weak immunoreaction was observed in the fetal stroma and mesenchyme. The HLX immunoreaction observed in the maternal part of the placenta was determined to be weak and weak-to-middle for the epithelium in all three periods of pregnancy, and it was found to be in very weak intensity for the stroma (Figure 2A, B, C, D, E, F). As a result of the statistical evaluation of the scores obtained from the placental parts, it was observed that there was no significant difference in the immunoreaction intensity of HLX in each period of pregnancy ($p > 0.05$).

HLX immunoreaction observed positively in pregnant cow uteri was found to occur in the cytoplasm of luminal and glandular epithelial cells from weak-to-medium density in all three periods of pregnancy. Furthermore, it was found that a very weak immunoreaction occurred in some stromal and smooth muscle cells of the uterus (Figure 2G, I, J, K, L, M). The statistical evaluation of the scores showed that the HLX immunoreaction was at similar levels in the uterus in all periods of pregnancy ($p > 0.05$).

It was observed that no immunoreaction occurred in negative control sections where PBS was applied instead of primary antibody, which verified the accuracy of the immunoreactivity in the interplacentomal and placentomal regions (Figure 3).

Discussion

Homeobox proteins encode transcription factors controlling embryonic development, and regulate the proliferation and differentiation of cells throughout the reproductive cycle. It has also been stated that they play critical roles in the

growth and development of the endometrium together with estrogen and progesterone hormones that regulate endometrial physiology (26).

Homeobox proteins have been reported as one of the many transcription factors that regulate and control the proliferation and differentiation of placental cells. Furthermore, it has also been reported that they can contribute to the normal and pathological development of the human placenta, as in embryogenesis and organogenesis (27). Moreover, studies have indicated that developmental, morphological, and angiogenesis defects occur in the placenta caused by the absence of homeobox protein (28).

It has been reported that HOXA11, which is necessary for female fertility, is expressed in the cervix together with the lower segment of the uterus during the development of the female genital tract (29, 30). Studies have reported that HOXA11/Hoxa11 protein is expressed in adult human and mouse endometrium, and this expression reaches the highest level in the secretory phase of the sexual cycle in humans in relation with estrogen and progesterone. It was reported that the hox protein in mice (Hoxa11) and humans (HOXA11) plays a critical role in embryo implantation and it is expressed in the endometrial glands and stroma during the estrus cycle (31). In an experimental study in wild-type mice, it was reported that the Hoxa11 gene was localized in stromal cells in the endometrium; however, it was shown to be absent in the glandular epithelium. It was pointed out that infertility occurs as a result of uterine anomalies caused by the mutation of this gene (15). In another study, it was reported that HOXA11/Hoxa11 expression, which was observed at high levels during the secretory phase of the normal endometrium in humans and in the normal endometrium of mice, did not occur in uterus with endometriosis. In addition, the impairment of HOXA11 expression was reported to promote abnormal epithelial cell differentiation leading to epithelial ovarian neoplasia and decreased glandular and stromal tissue development in mice (26). In another study conducted in humans, it was stated that HOXA11 was strongly localized in the stromal, glandular, and luminal epithelial cell nuclei of the endometrium (32). In a study conducted on pigs, it was reported that HOXA11 was expressed in the uterus and it was shown that this expression, which was high at the beginning of pregnancy, decreased towards the end of pregnancy (33).

In the presented study, it was observed that HOXA11 was expressed in the cow endometrium as well as in that of humans, mice, and pigs. Hsieh-li et al. (1995) reported that, unlike other studies, no reaction occurred in the glandular epithelium of the wild-type mouse endometrium. However, parallel to the findings of Xu et al. (2014) in humans, in our study, it was found that a similar level of expression occurred in all three periods of pregnancy in luminal, glandular, and some stromal cells. However, in our study, a significant decrease was found in the expression of stromal cells in the second and third periods of pregnancy ($p < 0.05$). These findings suggest that HOXA11 can contribute to endometrial functions during pregnancy in cows, to the proliferation and differentiation of epithelial and glandular cells, and to pregnancy physiology. Moreover, it was suggested that the reduced immunoreaction in stromal cells in the second period of gestation may be due to the stagnancy in the differentiation of stromal cells. Furthermore, the observation of HOXA11 immunoreactivity in some vascular endothelium suggested that this protein may play a role in the proliferation of some endothelial cells.

Although it is known that homeobox proteins are localized in placental cells, information about their roles is sparse. In a study performed in human placenta, it was observed that HOXA11 reacted positively in the cells of the placental villi, cytotrophoblast, intermediate trophoblast, and syncytiotrophoblast cells in the last period of pregnancy. This demonstrated that the HOXA11 gene expression has important roles in the formation and development of the human placenta, as well as in the regulation of differentiation and proliferation of the trophoblast cells. Furthermore, the fact that this protein is expressed in placental cells with choriocarcinoma shows that it plays a role in the development and pathogenesis of tumor diseases (34). In another study, it was observed that HOXA11 induces a strong immunoreaction in cytotrophoblast nuclei. However, it was reported that this immunoreaction is reduced in syncytiotrophoblasts (35).

Studies on the expression of HOXA11 in the placenta are scarce and its function has not been fully revealed. In parallel with the studies in the human placenta, in our study, HOXA11 was found to be expressed in cow placenta. It was found that the expression increased significantly in the stroma of the maternal placenta in the third period

($p < 0.05$), and decreased significantly in the UTC and TGC/BNC in the fetal placenta in the same period ($p < 0.05$). Thus, it was thought that HOXA11 could perform autocrine and/or paracrine functions in stromal cells in cow placenta as well as in human placenta. Furthermore, placental development and continuity may have critical roles in the proliferation and differentiation of trophoblasts. In addition, it is thought that the immunoreaction increasing in the second period of the maternal placenta occurs due to the metabolic activities of the differentiated epithelial cells.

H2.0-like homeobox (HLX, HB24) is one of the molecules that can control tissue growth caused by epithelial-mesenchymal cell interactions during human endometrial morphogenesis (36). In a study conducted in human endometrium, it was reported that the HB24 (HLX) protein was detected in the glandular and luminal epithelial cells and in the stromal cells of the endometrium (37). The effects of the Hlx gene, the homologue of HLX in rats, on endometrial or placental development are unknown. However, the Hlx gene has been reported to be concentrated in the lung, heart, skeletal muscle, and hematopoietic tissues and cells in adult mice; moderately intensely expressed in the liver, uterus, and ovaries; and it is expressed at a little intensity in the brain, kidney, and testis (36, 38). As in humans and mice, it was observed in our study that HLX induces a positive immunoreaction in the cow uterus. It was determined that immunoreaction occurred in the cytoplasm of the cells from weak-to-medium density in luminal and glandular epithelial cells in all three periods of pregnancy. It was also observed that a very weak reaction occurred in some stromal and smooth muscle cells. Thus, it was thought that HLX may have functional roles in the normal physiology of the cow uterus, in the preparation of the uterus for pregnancy, and in maintaining the pregnancy, as in humans.

Evidence has been presented that HLX (previously HLX1 and HB24), a member of the Homeobox gene family, may play an important role in normal placentation and development, especially trophoblast proliferation and differentiation (19, 22, 39). It was reported that HLX, which has an important regulatory role for the human placenta, is expressed in the villi and extravillous cytotrophoblast cells as well as in endothelial cells and fetal microcapillaries (22). In another study, it was stated that HLX expression seen in trophoblasts and stromal

cells in human placenta in early gestation (8-12 weeks) is dense in the nucleus and weak in the cytoplasm. In addition, expression was observed in villus cytotrophoblasts, endovascular, and multinuclear (giant cells) cytotrophoblasts, but not in syncytiotrophoblasts or syncytiums (39, 40, 41). In studies performed in human placentas with fetal growth retardation, it has been reported that HLX expression is decreased in trophoblasts, but does not occur in any stromal cells (39, 42).

H2.0-like homeobox (HLX), which is known as Hlx in mice, has been reported to be expressed in the labyrinth layer of the placenta, secondary giant cells, and the spongiotrophoblast layer in mice. It was also stated that the spongiotrophoblast layer described here is necessary for the structural support of the placenta. Moreover, although the presence of Hlx in the mouse placenta has not been fully revealed, morphological deterioration in the spongiotrophoblast layer in the placental tissues of mutant mice has been reported; however, it was demonstrated that the layer with giant cells on top was not affected (43). In another study conducted on Hlx mutant mice, it was reported that there were defects in placental development, which caused fetal growth retardation (44, 45).

It has been observed that HLX induces an immunoreaction in cow placenta as well as in human (22) and mouse (43) placentas. The observed immunoreactivity was found to be at a similar moderate intensity in the epithelial and stromal cells of the maternal placenta, and in uninucleate and binucleate trophoblast cells in all three periods of pregnancy. Thus, in cow placenta as well as in human and mouse placentas, HLX plays very important roles in the proliferation and differentiation of trophoblasts; in the growth and development of the placenta; and in the continuity of pregnancy. In addition, it has been reported that HLX, which has a role in normal placental development and differentiation of placental cells, should be at a certain level and order. Otherwise, it has been stated that, together with the dysregulation of HLX, cytotrophoblasts proliferate and differentiate abnormally, resulting in pathological conditions such as placental choriocarcinoma (19). The findings in our study support this information and it was observed that HLX has similar densities in all parts of the placenta.

As a result, it was observed that HOXA11 and HLX proteins produced a positive immunore-

action at certain concentrations in cow uterus and placenta during pregnancy. The immunohistochemical outcomes observed in the present study suggested that HOXA11 and HLX may affect the proliferation and differentiation of cells in the uterus and placenta during pregnancy in cows, and they may contribute to the normal uterine physiology, placentation formation, and the continuity of pregnancy in cows. In addition, we think that this study will shed light on new studies to be conducted on the mammalian uterus and pregnancy physiology, and that it can contribute to the emergence of new methods for the prevention and treatment of hereditary and pathological problems of the placenta, uterus and fetus.

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IMUNOLOKALIZACIJA PROTEINOV HOXA11 IN HLX V POSTELJICI KRAVE MED BREJOSTJO

U. Topaloğlu, M. A. Ketani, M. E. Akbalık, H. Sağsöz, B. G. Saruhan, B. Bayram

Izveček: Za posteljico so poleg številnih funkcij značilne intenzivnost in karakteristike njenih hormonskih funkcij. Poročali so, da razvoj in ožiljenost posteljice pri normalni rasti ploda uravnavajo številni dejavniki, kot so rastni dejavniki, transkripcijski dejavniki in homeoboks proteini. Ta študija je bila izvedena z namenom določiti porazdelitev in morebitno fiziološko vlogo proteinov HOXA11 in HLX v maternici in posteljici bregih krav. Za vzorce tkiv smo uporabili maternice in posteljice 27 krav pasem Holstein, pridobljenih v zasebnih klavnicah. Obdobje brejosti smo izračunali na podlagi starosti plodov po formuli. Maternice smo glede na obdobje brejosti uvrstili v tri skupine po 9 vzorcev: 1 (69-89 dni), 2 (99-178 dni) in 3 (190-269 dni). Prisotnost proteinov HOXA11 in HLX v vzorcih tkiv smo preverili z imunohistokemijo. Proteini HOXA11 in HLX so bili prisotni predvsem v luminalnih in žleznih epitelijskih celicah maternice ter v epitelijskih celicah maternalne posteljice, UTC in TGC/BNC. Prisotnost HOXA11 je bila zaznavna tudi v nekaterih žilnih endotelijskih celicah. Rezultati imunohistokemije v tej študiji kažejo na morebiten vpliv proteinov HOXA11 in HLX na proliferacijo in diferenciacijo celic v maternici in posteljici krav med brejostjo in na morebitno vlogo teh proteinov pri normalni fiziologiji maternice, nastanku posteljice in vztrajanju brejosti pri kravah.

Ključne besede: proteini Hox; HOXA11; HLX; posteljica