IMMUNOHISTOLOGICAL DETECTION OF ANDROGEN RECEPTOR IN BOVINE PLACENTOMES DURING EARLY PREGNANCY

K. Pershotam, Schuler G, Bernd Hoffmann** Mare Dilly*** and Bachal Bhutto**

ABSTRACT

A study was conducted at Veterinary Clinic for Obstetrics, Gynecology and Andrology, Justus-Liebig-University, Giessen, Germany during the year 2009-10. In this study expression of androgen receptor (AR) in bovine placentomes during early pregnancy was investigated to identify the putative target cells (PTC) of placental androgens. For this purpose placentomes were collected from six healthy cows between 50 to 150 days of gestation at a local slaughter house of Giessen, Germany. These placentomes were assigned to two observational groups i.e. first and second trimester. An indirect immune peroxidase staining method was employed using the streptavidine biotin technique for signal enhancement following standard using a polyclonal primary antibody raised in rabbits against a peptide mapping at the N-terminus of the human AR was applied. Epididymal caput from a postpubertal bull was used as a positive control tissue. In bovine placentomes, irrespective from gestational age distinct to intense nuclear staining was observed in all invasive trophoblast giant cells located in the caruncular epithelium. Significant immune staining was also found throughout gestation in mature trophoblast giant cells (TGC) situated in the chorionic epithelium. This staining pattern suggests that AR expression is up-regulated during TGC differentiation. In bovine trophoblast cells the androgens and AR may be elements of an intracrine mechanism involved in the control of TGC differentiation and AR may be up-regulated by increasing androgen levels in differentiating trophoblast cells.

KEYWRDS: Bovine; placentomes; androgen receptor; estrogen; pregnancy; Germany.

***Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University, Tandojam, Sindh, Pakistan, **Veterinary Clinic for Obstetrics, Gynecology and Andrology, Justus-Liebig-University, D-35392 Giessen, Germany, ***Department of Anatomy, University of Veterinary Medicine Hannover, Bischofsholer Damm 15, D-30173 Hannover, Germany.**

INTRODUCTION

In recent years, it has been disclosed that androgens are male hormones and estrogens are female hormones. Androgens [testosterone (T) and 5α dihydrotestosterone (DHT)] control the development, differentiation, and function of male reproductive and accessory sex tissues, such as the seminal vesicle, epididymis, and prostate. Other organs and tissues such as skin, skeletal muscle, bone marrow, hair folices, and brain, are also under the influence of androgen. The principal action of androgen is to regulate gene expression through the androgen receptor (AR), which belongs to the superfamily of nuclear receptors (24).

Traditionally, progestagens and estrogens have been regarded as female sex steroids, whereas androgens were considered as the classical male hormones. However, these are produced by females and have physiological functions. Conversely, in females physiological roles of androgens have been identified beyond their function as estrogen precursors, such as regulatory functions in differentiation of granulosa cells and in oocyte maturation (25).

Androgen actions are primarily mediated by specific receptors which belong to the nuclear receptor superfamily (2, 14). Accordingly female AR knockout mice have been shown to be sub fertile (17). More recently, androgen receptors have been shown to have a second mode of action. As has been also found for other steroid hormone receptors such as estrogen receptors and androgen receptors can have actions that are independent of their interactions with DNA (5). Androgen receptors interact with certain signal transduction proteins in the cytoplasm. Androgen binding to cytoplasmic androgen receptors can cause rapid changes in cell function independent of changes in gene transcription, such as changes in ion transport. Regulation of signal transduction pathways by cytoplasmic androgen receptors can indirectly lead to changes in gene transcription, for example, by leading to phosphorylation of other transcription factors (7).

One function of androgen receptor that is independent of direct binding to its target DNA sequence is facilitated by recruitment via other DNA binding proteins. One example is Serum Response Factor (SRF), a protein which activates several genes that cause muscle growth (12). AR expression has been detected in endometrium from normal women and from women with endometriosis (19). Some studies (21) have reported relatively constant expression of AR throughout the cycle, while some other studies have

documented variation in endometrial AR with increased expression during the proliferative phase.

Due to the high estrogen output of bovine placenta, significant amounts of androgens must occur locally in the bovine trophoblast. However, a role as original regulators during bovine gestation, has not been investigated yet. In human placenta high affinity androgen binding proteins have been detected and by means of immunohistochemistry AR have been localized in decidua and trophoblast pointing to a role of androgens as local regulatory factors. Thus, in order to elucidate other possible roles of placental steroids besides unclear functions of placental progestagens and estrogens in cattle, expression of AR in bovine placentomes during early pregnancy of bovine placentomes was investigated to identify the putative target cells of placental androgens.

MATERIALS AND METHODS

Sample collection and fixation

Placentomes were collected at a local slaughter house of Giessen Germany from six healthy cows between 80 to 150 days of gestation during the year 2009-10. Gestational age was estimated according to fetal crown-rump length (13) and these animals were assigned to two observational groups: first trimester (until day 90; n=3) and second trimester (day 120-150, n=3). From each animal 3-5 medium sized placentomes were removed from the mid-region of horn that contained the fetus. Trapezoid pieces encompassing complete height of the interdigitation zone were fixed overnight in 10 percent phosphate buffered formalin. After washing in PBS and subsequent dehydration in a graded ethanol series these were embedded in a paraffin substitute (Histo-Comp-Vogel, D-35396 Gießen, Germany). Sample collection from living animals was approved by the responsible authority. (Regional Council in Giessen, Germany-record token II25.3-19c20/15cGI18/14).

Immuno-histo-chemical staining procedures and semi-quantitative assessment of immune-staining

An indirect immunoperoxidase staining method was employed using the streptavidine biotin technique for signal enhancement following standard using a polyclonal primary antibody [AR (N-20): sc-816; Santa Cruz Biotechnology, Heidelberg, Germany] raised in rabbits against a peptide mapping at the N-terminus of human AR was applied. Blocking serum,

biotinylated secondary antibody and avidine-biotin-complex were taken from Vectastain Elite ABC Kit 6101 and used following the producer's instructions (Vector Laboratories, Burlingame, CA 94010, USA).

About 5 µm tissue sections were mounted on SuperFrost-Plus slides (Menzel Glaeser, D-38116 Braunschweig, Germany), deparaffinized by two 4 minutes changes of xylene, rehydrated in graded ethanol and washed under running tap water (5 min). For antigen retrieval the rehydrated sections were preincubated in 10 mM citrate buffer (pH 6.0) for 5 minutes prior to three times 5 minutes microwave irradiation in pre-heated citrate buffer in an oven run at 560 W. After a 20 minutes cooling period slides were washed under running tap water for 5 minutes followed by a treatment of 0.3 percent hydrogen peroxide in methanol for 30 minutes to quench endogenous peroxidase activity. Then these were washed with ICC buffer (phosphate buffered saline/0.3% Triton X) for 5 minutes and covered with 10 percent blocking serum in ICC buffer to block unspecific binding sites. After draining the blocking reagent, of these primary antibody (diluted 1:500 in ICC buffer) was applied and slides were incubated for 20 hours at 4°C. These were washed with ICC buffer, covered with biotinylated secondary antibody diluted in ICC buffer and incubated for 30 minutes at room temperature. Following draining of excess antibody, sections were washed twice for 5 minutes with ICC buffer, then covered with streptavidine-peroxidase complex and incubated for 30 minutes. After washing with ICC buffer (2 changes 5 min. each), the sections were incubated with substrate (Nova RED substrate kit, Vector Laboratories, Burlingame, CA 94010). Finally the slides were washed under running tap water for 5 minutes, counterstained with hematoxylin and mounted in Histokit (Assistent, D-37520 Osterode, Germany). Negative controls were set up with serum of a non-immunized rabbit at an equal dilution as the specific primary antibody. As a positive control tissue bovine caput epididymis was used, an organ exhibiting high AR expression (1, 4, 10, 18, 25). All the slides were stained during a single run to avoid effects of inter-incubation variability.

RESULTS AND DISCUSSION

Localization of androgen receptor protein in bovine placentome and expression pattern throughout gestation

With immune-histo-chemistry, in epididymal caput from a postpubertal bull (Fig. 1) used as a positive control tissue, a highly specific staining pattern was obtained with distinct to intense predominantly nuclear staining in all cells of ductal epithelium. Moderate to intense nuclear staining was found in

the majority of peritubular smooth muscle cells and in a proportion of intertubular connective tissue cells. In negative control sections of placentomes, the primary anti-serum was replaced by serum from a non-immunized rabbit, only occasionally nonspecific staining occurred in the lumen of blood vessels and was obviously associated with serum components.

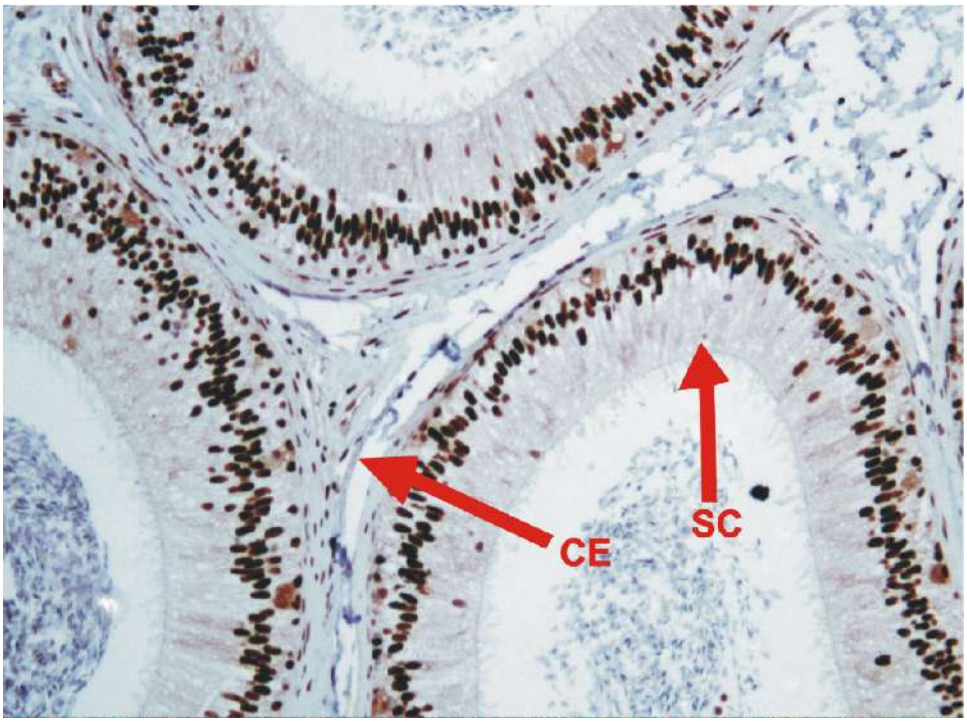

Fig. 1. Androgen receptor immunostaining in bull epidiymis (head) used as positive control. A strong immunoreactivity was observed in columnar epithelium (CE) cells and smooth cells (SC). Magnification X400.

In bovine placentomes, irrespective of gestational age distinct to intense nuclear staining was observed in all invasive trophoblast giant cells located in the caruncular epithelium (Figs. 2-3). Significant immune-staining was also found throughout gestation in mature TGC situated in the chorionic epithelium. The proportion of positive cells and staining intensity increased with stage of gestation. Immunostaining in caruncular stromal cells was moderate in 80 days pregnant animal, in bovine placentome of 120 days pregnant animal the strong specific staining was observed in both parts of bovine placentomes with higher intensity particularly in fetal part. The positive signals were higher and stronger in invasive trophoblast giant cells, moderate

staining was also visible in uninucleated trophoblast (Fig. 2). In the maternal part only caruncular stromal cells were positive with moderate staining and there was no staining in crancular epithelial cells. In bovine placentome of 140 days pregnant animal the strong specific staining was observed only in fetal part of bovine placentome.

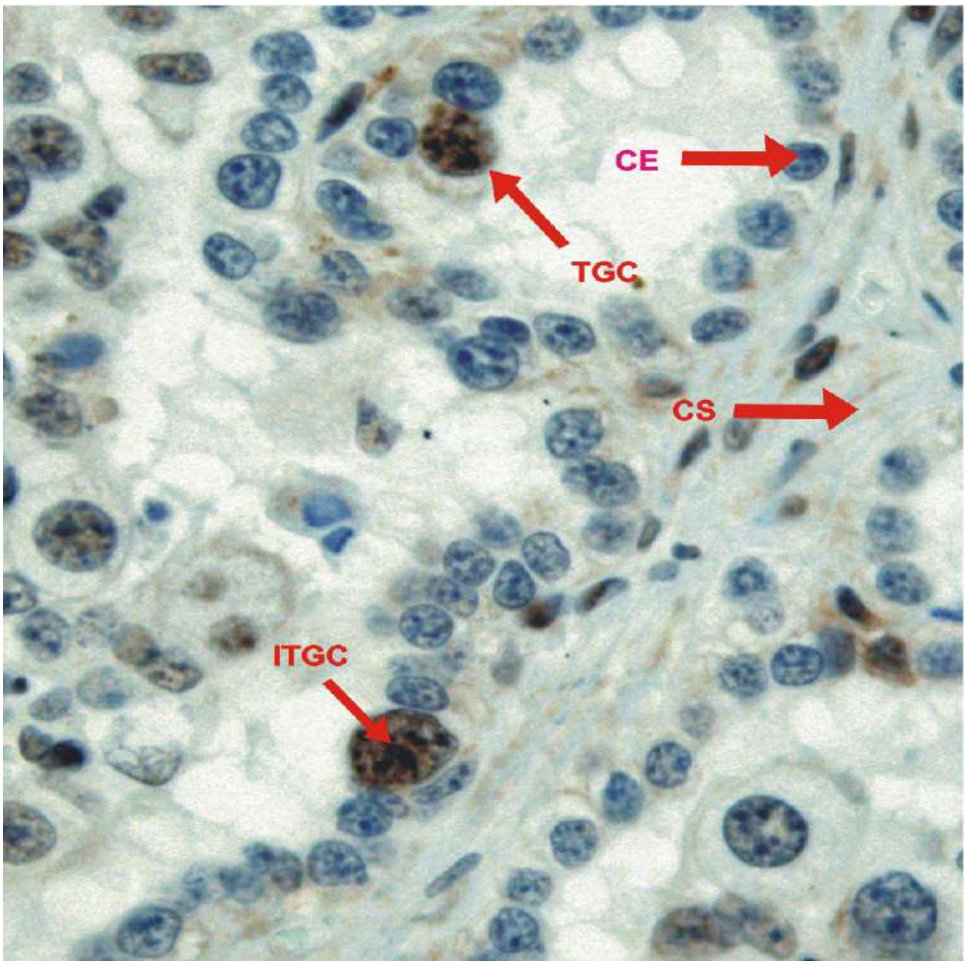

Fig. 2. Immunohistochemical detection of androgen receptor in 120 days bovine placentomes. Distinct and high positive staining is visible in the nuclei of invasive trophoblast giant cells and moderate to weaker staining are also visible in trophoblast giant cells and in crancular stromal and there is no staining caruncular epithelial cells. Magnification X400 CE= Cranucular Epithelium; CS= Cranucular Stromal; TGC= Trophoblast Giant Cells; iTGC= Invasive Trophoblast Giant Cells

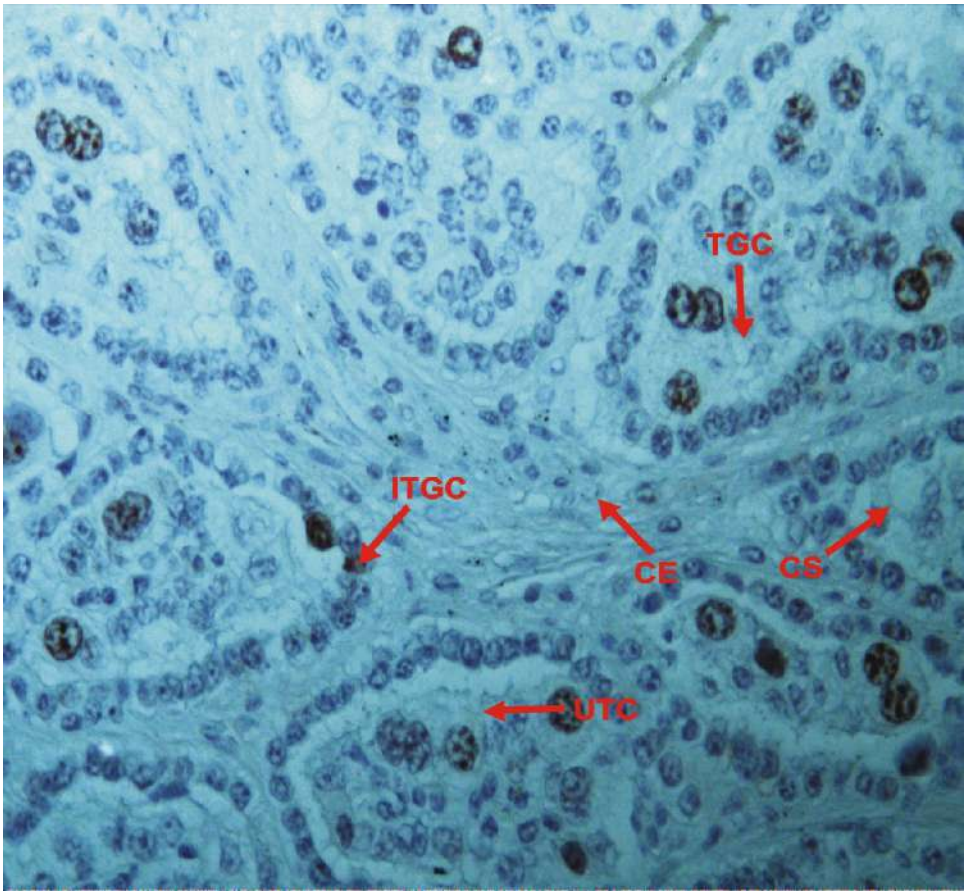

Fig. 3. Immunohistochemical detection of Androgen Receptor in placentomes from 140 days pregnant cow. Distinct and high positive staining is visible in the nuclei of invasive trophoblast giant cells and trophoblast giant cells and there is no staining in uninuclated trophoblast giant cells, caruncular stromal and caruncular epithelial cells.

The positive signals were higher and stronger in invasive trophoblast giant cells; there were no specific signals in uni-nucleated cells, whereas in the maternal part there was no positive signals in any cell type were detected.

Physiological roles of androgens in fetal and maternal part of bovine placentomes on the first view are unexpected. However, the expression (6, 9) of AR in human placenta has been suggested already in early studies (8, 20) using ligand binding assays and was later confirmed by immune-histo-chemistry in human decidua and trophoblast. In bovine placentomes, expression of AR was unequivocally demonstrated on the mRNA-level by

conventional RT-PCR and by the Taqman-based quantitative real-time RT-PCR method (11). A clearly more intensive band of exactly the same size occurred with bovine epididymis, a tissue known to highly express AR (3). Throughout early gestation, distinct to intense staining was found in nuclei of invasive TGC situated in the crancular epithelium and in two “fetal” nuclei of feto-maternal hybrid cells formed by the fusion of an invasive TGC with a crancular epithelial cell (22). Moreover, distinct nuclear staining was also found in TGC at advanced stages of differentiation before migration into the maternal epithelium. This staining pattern suggests that AR expression is up-regulated during TGC differentiation. The sequential up- and down-regulation of steroidogenic enzymes in bovine trophoblast cells during TGC differentiation (16) and the fact that in bovine placentomes the Δ4-pathway is inefficient implies that a significant synthesis of testosterone or 5α-dihydrotestosterone is only possible after entry of UTC into the TGC differentiation, when down-regulation of CYP17 and up-regulation of 3β-hydroxysteroid dehydrogenase occurs. Thus, in bovine trophoblast cells androgens and AR may be elements of an intracrine mechanism involved in the control of TGC differentiation, and AR may be up-regulated by increasing androgen levels in differentiating trophoblast cells.

CONCLUSION

The expression in trophoblast cells in placentomes of all six animals of different gestational ages provides convincing evidence that AR is widely expressed in bovine trophoblast cells. The expression in caput of epididymis further confirms the specificity of anti-body.

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