THE BOVINE PLACENTA AS A SOURCE AND TARGET OF STEROID HORMONES: ASPECTS ON THE ROLE OF ANDROGENS AND SULFONATED STEROIDS

PERSHOTAM KHATRI

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DECLARATIONS

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Pershotam Khatri

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List of abbreviations

3ß-HSD	3β-hydroxysteroid dehydrogenase-Δ5/4-isomerase
ANOVA	analysis of variance
AR	androgen receptor
BNC	binucleated cell (syn.: trophoblast giant cell, TGC)
ddH_2O	double destilled water
cDNA	complementary deoxyribonucleic acid
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	5α-dihydrotestosterone
E1S	estrone-3-sulfate
EDTA	ethylene diamine tetraacetic acid
EGTA	ethyleneglycol tetraacetic acid
ERα	estrogen receptor α
ERβ	estrogen receptror β
EST	estrogen sulfotransferase (this term is used when referring to estrogen sulfotransferase activity without exact identification of the underlying enzyme)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ICC	immunocytochemistry
IRS	immunoractive score
mRNA	messenger ribonucleic acid
PAPS	3'-phospho-adenosine-5'-phosphosulfate
PMSF	phenylmethylsulphonylfluoride
RNA	ribonucleic acid
P450c17	17α-hydroxylase-C17,20-lyase (CYP17)
P450scc	side-chain cleavage enzyme (CYP11A1)
P5S	pregnenolone-3-sulfate
PAPS	3´-phosphoadenosine-5´-phosphosulfate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	reverse transcription
SLC10A6	solute carrier family 10 (sodium/bile acid cotransporter family), member 6 (syn.: SOAT)
SOAT	sodium dependent organic anion transporter (syn.: solute carrier family 10 member 6; SLC10A6)
StS	steroid sulfatase
SULT	cytosolic sulfotransferase
SULT1E1	estrogen sulfotransferase
TGC	trophoblast giant cell (syn.: binucleated cell, BNC)
UTC	uninucleated trophoblast cell
TBE	tris boric acid EDTA
Tris	tris (hydroxymethyl)-aminomethane

1. INTRODUCTION

The placenta is the key site of feto-maternal communication regulating embryonic and fetal development and maintenance of pregnancy (Wooding et al. 1992). In the bovine placenta which is of the synepithelial type, the direct fetal-maternal contact is restricted to specialized zones called placentomes, consisting of fetal cotyledons interdigitating with maternal caruncles. Caruncular and cotyledonary epithelia are in close contact, thus allowing an efficient exchange between the fetal and maternal side (Klisch et al. 1999; Björkman 1969). Additionally, placentomes are also specialized for signal transmission between the growing fetus and the mother, and the cotyledon is an important source of auto- para- and endocrine signals presumably involved in the control of placental growth and differentiation and of other pregnancy-related processes in the maternal and fetal compartment. Immediately after the onset of placentation the bovine trophoblast starts to produce significant amounts of steroids, mainly progesterone and estrone sulfate (E1S) (Hoffmann and Schuler 2002). Although progesterone is commonly considered as the key hormone of the maintenance of pregnancy (Csapo 1956), specific functions of bovine placental progesterone have not been identified yet as throughout gestation in cattle the ovary is the predominant source of progesterone, whereas the contribution of the placenta to systemic maternal levels is negligible (Melampy et al. 1959; Stabenfeldt et al. 1970; Fairclough et al. 1975). Similarly the function of the high amounts of E1S produced in bovine cotyledons is unclear as it does not bind to classical nuclear estrogen receptors (Hähnel et al. 1973; Payne et al. 1973; Kuiper et al. 1997). Thus, sulfonation of estrogens is commonly considered as an important step in the control of estrogenic activity leading to inactivation and accelerated excretion (Brix et al. 1999). There is evidence from different experimental approaches that in bovine placenta estrogens are already sulfonated in cotyledons (Mattioli et al. 1984; Hoffmann et al. 2001), and in previous studies using immunohistochemistry (Brown et al. 1987) and in situ-hybridization (Ushizawa et al. 2007; Hirayama et al. 2008) estrogen sulfotransferase (SULT1E1) has been localized in trophoblast giant cells (TGC), where also aromatase is expressed (Schuler et al. 2006a). This co-localization of estrogen synthesis and inactivation within the same cells makes it difficult to assign a physiological role to bovine placental estrogen synthesis. However, during the last two decades increasing evidence came up that estrogen sulfates may be important substrates for the intra-tissue production of free estrogens via the activity of steroid sulfatase (STS) (sulfatase pathway; Santner et al. 1984; Reed et al. 2005). In a previous study in bovine placentomes STS was predominantly localized in the caruncular epithelium (Greven et al. 2007), where estrogen receptor α is highly expressed

(Schuler et al. 2002). Thus, a sulfatase pathway for the intracellular production of free estrogens from estrone sulfate may be active in this cell type.

Virtually no information is available on the intratissue transport of estrone sulfate. In contrast to free steroids which may penetrate cellular membranes by passive diffusion (Rosner 2006), the transmembrane passage of the much more polar steroid sulfates is commonly considered to depend on the function of so far unidentified transporters. Interestingly, the recently discovered sodium-dependent organic anion transporter (SOAT; SLC10A6) has been previously shown to efficiently mediate the cellular import of steroid sulfates (Geyer et al. 2004, 2007) and its expression has been detected in bovine placentomes (Greven 2008). Moreover, in addition to "standard SOAT", four variants deriving from the deletion of various exons and/or insertion of an additional exon have been identified. Based on the results obtained on the expression of STS and SOAT in bovine placentomes, a functional coupling of these molecules in the cell-specific provision of biologically active estrogens has been suggested (Greven 2008).

As mentioned above, so far no function has been identified unequivocally for placental progesterone and estrogens in cattle. Thus, other active products of bovine placental steroidogenesis have to be taken into account. Androgens are conventionally considered as classical male sex hormones. However, they are also produced and have regulatory functions in females as well (Burger 2002; Miller et al. 2004). Significant levels of androgens have been shown to be present in the uterine environment during pregnancy in some mammalian species, including humans, pigs and rats (Bonney et al. 1984; Stefanczyk-Krzymowska et al. 1998; Warshaw et al. 1986). In pregnant cows a slight increase of testosterone concentrations has been observed in plasma and milk throughout gestation (Gaiani et al. 1984), and local tissue concentration in the placentomes may be considerably higher. Thus, androgens might act locally within the placenta, and their effects may be controlled by aromatization to estrogens and sulfonation. However, no information is available on the expression of the androgen receptor (AR) in the bovine placenta. Consequently, putative target cells of placental androgens in bovine placentomes are still unknown.

Thus, the aims of the present studies are

- to monitor SULT1E1 expression on the protein and mRNA level in bovine placentomes throughout gestation and to characterize SULT1E1 expression in bovine trophoblast in relation to the process of TGC differentiation

- to closer characterize the expression of SOAT variants in placentomes and other bovine organs to obtain further information with resepcet to the function of SOAT as a physiologically relevant transporter of sulfonated steroids
- to identify putative target cells of placental androgens in bovine placentomes by their expression of AR and to monitor AR expression throughout gestation in bovine placentomes on the protein and mRNA level
- to monitor testosterone tissue concentrations in bovine placentomes throughout gestation

2. LITERATURE REVIEW

2.1 Gross and microscopic structure of the bovine placenta

Despite many common functional features there are striking differences between the placentae among eutherian (placental) mammal species concerning their gross and microscopic structure (Carter and Enders 2004; Enders and Carter 2004, 2006; Carter and Mess 2007). Ruminants have a cotyledonary placenta consisting of a variable number of placentomes, in which maternal and fetal tissues come into intimate contact enabling the exchange of nutrients, gases, hormones and waste products. Each of these numerous disc- or mushroom-shaped placentomes consists of a fetal component, the cotyledon, and a maternal component, the caruncle (Leiser and Kaufmann 1994; Pfarrer et al. 2001).

There is an enormous variation in placentome size and number between various ruminant species (Mossman 1987), from 4-6 large ones in deer to 100–150 rather smaller ones in cow and some antelopes (Mossman 1987, Wooding and Flint 1994). In cattle mean placentomal weight and length increase significantly during gestation, development in the non-pregnant horn is significantly different from that of the pregnant horn, with fewer, smaller, lighter placentomes (Laven and Peters 2001).

Histologically the bovine placenta is having six cellular layers that form the materno-fetal interface: on the fetal side the vascular endothelium, cotyledonary connective tissue and chorionic epithelium (trophoblast) and on the maternal side the caruncular epithelium, caruncular connective tissue and vascular endothelium (Ramsey 1982; Wooding 1992).

The chorionic epithelium basically consists of two types of trophoblast cells, the uninucleated trophoblast cells (UTC) and the mostly binucleated trophoblast giant cells (TGC; syn: binucleated cells BNC). As the weakly invasive TGC rapidly undergo apoptosis after their migration into the caruncular epithelium, there is a continuous replenishment of TGC by a high proliferative activity of UTC and their differentiation into TGC including genome multiplication. Thus a major proportion of bovine trophoblast cells are at an intermediate stage of differentiation between UTC and mature TGC (Wooding 1992; Klisch et al. 1999).

UTC are mononuclear cuboidal to columnar cells that show typical features of epithelial cells. As they are intimately connected to the caruncle by microvilli, the uptake of oxygen and nutrients and the release of waste products have been considered as their primary function. However, they have also been identified as a source of molecules with endo- or paracrine activity such as interferon τ (Bartol et al. 1985; Leaman and Roberts 1992), steroids or prostaglandins (Bartol et al. 1985; Wooding 1992; Wooding and Flint 1994; Mann et al. 1999; Schuler et al. 2008).

Mature bovine TGC have a characteristic structure quite different from the surrounding UTC (Lawn et al. 1969; Wooding and Wathes 1980; Wooding 1982; Klisch et al. 1999). It is generally accepted that bovine TGC originate from UTC by acytokinetic mitoses (Wimsatt 1951; Björkman 1968). According to Klisch et al. (1999) bovine TGC commonly arise from two consecutive endomitoses followed by an additional S-phase by each of the two tetraploid nuclei. Thus, the majority of mature bovine TGC possess two octaploid nucei. During the early stages of their development, the immature TGC are randomly scattered and located deeply within the trophectodermal layer in an intraepithelial position, such that they make no contact with either the basement membrane or the apical microvillar border of the trophectoderm (Wooding and Wathes 1980; Wooding 1984). These immature cells appear as relatively small round (or spherical) cells whose cytoplasm stain darker than the surrounding UTC. The dark staining is due to the presence of numerous cytoplasmic ribosomes in the TGC (Wango et al. 1990). TGC constitute 15 to 20 per cent of trophoblast cells at the beginning of implantation and throughout pregnancy in ruminants (Wooding and Wathes 1980; Wooding 1982, 1983). At parturition, the proportion of TGC is significantly reduced to values of about 5 percent (Gross et al. 1991; Klisch et al. 2006; Shenavai et al. 2010). Ruminant TGC are considered to have two main functions: to form the feto-maternal syncytium essential for successful implantation (Wooding and Wathes 1980; Wooding 1984; Lee et al. 1986; Wango et al. 1990) and subsequent placentomal growth at early stages of placentation and to produce and to deliver hormones such as placental lactogen (PL), prolactin related protein-1 (Zieler et al. 1990; Kessler et al. 1991; Anthony et al. 1995), and steroid hormones like estrogens (Matamoros et al. 1994, Schuler et al. 2006a) and progesterone (Reimers et al. 1985; Wango et al. 1991) throughout gestation. Moreover, ruminant TGC produce enormous amounts of pregnancy associated glycoproteins (PAGs), which are structurally related to proteinases without having proteolytic activity. Their functions are still unclear (Zoli et al. 1991, 1992; Green et al. 2000).

After implantation has accomplished, different from the sheep in bovine placentomes major feto-maternal syncytia are not present any longer. However, throughout gestation bovine TGC migrate into the maternal epithelium, where they release their hormonal products into the maternal compartment. After degranulation they rapidly undergo apoptosis. Moreover, a significant proportion of TGC fuses with single caruncular epithelial cells to form trinuclear feto-maternal hybrid cells (Wooding and Wathes 1980, Wooding 1992). The function of these processes is still unclear but is considered to serve the transport of large signal molecules across the feto-maternal barrier.

Maternal caruncular growth and differentiation starts in response to fetal signals from preformed sites of the endometrium. Maternal caruncles grow rapidly after the onset of placentation until the beginning of the last trimester, when the carunclar growth significantly decreases and finally fully stagnates during late gestation. Concomitant with caruncular growth and ingrowth of ramifying chorionic villi in the caruncle a corresponding complex system of maternal crypts is formed, which are covered by a monolayer of the mostly cuboidal caruncular epithelial cells (Prior and Laster 1979; Hradecky et al. 1988; Reynolds et al. 1990; Ferrell 1991). Starting gradually around day 250 a progressive reduction of caruncular epithelium occurs towards term, when it is significantly flattened or in some places even absent due to a substantial decrease in cell numbers (Woicke et al. 1986).

2.2 Bovine placenta as a steroidogenic organ

In addition to its role in transporting molecules between mother and fetus, the placenta is also a major temporary endocrine organ that - depending on the individual species - synthesizes a huge variety of hormones and cytokines that have major influences on ovarian, uterine, mammary and fetal physiology (Heap 1994). One class of hormones produced in the placentae of many but not all mammalian species are the sex steroids. The placentas of species exhibiting steroidogenic activity may differ widely in the spectrum of steroids produced, their quantities and profiles during gestation and synthetic pathways (Δ4- vs. Δ5 pathway) (Thomas et al. 1988; Conley and Bird 1997; Schuler et al. 1994, 2008). Moreover, due to the lack of the steroidgenic key enzyme 17α-hydroxylase-C17,20-lyase (P450c17, CYP17) in the placentae of some species, they may depend on C19-precursors provided by the mother and/or the fetus, as it is the case in humans (Diczfalusy 1969, Strauss et al. 1996) or in the horse (Allen 2001). The bovine trophoblast expresses all enzymes needed for the production of progesterone and estrogens from cholesterol: side-chain cleavage enzyme (CYP11A1, P450scc), P450c17, 3β-hydroxysteroid dehydrogenase Δ5/4-isomerase (3β-HSD) and aromatase (Schuler et al. 2006a, 2008), and the main products of bovine placental steroidogenesis occuring in maternal blood from a quantitative point of view are progesterone (Wagner et al. 1974; Hoffmann 1979) and estrone sulfate (Hoffmann et al. 1997).

2.2.1 Production of progesterone by the bovine placenta and its putative functions

During pregnancy maternal progesterone concentrations in cattle range between about 6 to 12 ng/ml in the peripheral blood. In the first three months of gestation they average 11.6 ng/ml (Schallenberger et al. 1985). In the fourth and fifth month of pregnancy a slight drop to values

about 9 ng/ml has been described. 72-24 hours before birth, a sharp drop in progesterone levels below 1 ng/ml occurs (Henricks et al. 1971; Hoffmann et al. 1977; Schallenberger et al. 1985; Eissa and el-Belely 1990). In contrast to many other species like the sheep (Thorburn et al. 1977), the horse (Holtan et al. 1979) and humans (Diczfalusy 1969), in which the placenta adopts the role as the main source of progestagens during gestation, in pregnant cattle it only contributes temporarily and to a minor extent to maternal systemic progesterone levels since the corpus luteum is the main source of progesterone throughout gestation (Estergreen et al. 1967; Day 1977; Chew et al. 1979; Johnson et al. 1981). The capacity of the bovine placenta to produce progesterone became obvious when luteolysis was induced with prostaglandin F2α or analogues, or after ovariectomy. Whereas ablation of luteal function readily induced abortion when performed before about day 150 or after day 240, in the period between a considerable proportion of cows maintained pregnancy for a longer time or even until normal term suggesting the existence of an additional source of progesterone between days 150-240 (Estergreen et al. 1967; Day 1977; Johnson et al. 1981). Moreover, the capacity of bovine placental tissues to produce progesterone was shown in vitro (Ainsworth and Ryan 1967; Wiener 1976; Reimers et al. 1985; Shemesh 1990; Schuler et al. 1994), but arteriovenous progesterone concentration differences indicate that the gravid uterus of the cow does not contribute to systemic progesterone concentrations during late gestation (Comline et al. 1974; Ferrell et al. 1983, Conley and Ford 1987). The fact that after day 240 ablation of luteal function in most cases led to immediate abortions again suggests that placental progesterone production decreases significantly during late gestation. However, measurements of placental progesterone tissue concentrations and of placental 3β-HSD activities show that these parameters only decrease at parturition (Tsumagari et al. 1994). These observations suggest that the limited capability of the bovine placenta to maintain late pregnancy in the absence of luteal progesterone is not due to a decrease in placental progesterone production but rather to an increased progesterone metabolization or an increased progesterone threshold level for the maintenance of pregnancy.

In bovine placentomes progesterone is unequivocally produced in the trophoblast (Duello et al. 1986; Myers and Reimers 1988). However, the exact cell type(s) responsible for progesterone synthesis has/have not been identified yet unequivocally. In in-vitro investigations using enriched preparations of UTCs and BNCs, progesterone production was clearly higher in the BNC fraction (Reimers et al. 1985). However, "BNC" is not a well-defined cell type as besides mature TGC it may also include TGC precursors at intermediate stages of maturation (Klisch et al. 1999). By in situ-hybridization, the mRNA specific for 3β-

HSD – the enzyme catalyzing the last step in progesterone synthesis - was localized in immature TGC but was virtually absent in UTC and mature TGC (Schuler et al. 2008). However, as in differentiating cells the emergence of a functional protein may follow the up-regulation of the corresponding mRNA with some delay (Ostermeier et al. 2002; Dadoune 2003; Lambard et al. 2004; Ballantyne et al. 1997; Charlesworth et al. 2000), the major cell type producing progesterone in the bovine trophoblast still awaits closer characterization. Progestins are key regulators in the establishment and maintenance of pregnancy (Csapo 1956, Gomes and Erb 1965, Mann and Lamming 1999). However, due to its insignificant contribution to maternal progesterone concentration, the function of placental progesterone in pregnant cows is unclear. By immunocytochemistry, in bovine placentomes progesterone receptors have been localized in the nuclei of caruncular stromal cells and caruncular vascular pericytes suggesting that these cells are rather under the control of placental than luteal progesterone (Schuler et al. 1999; Boos et al. 2000). Thus progesterone production of the trophoblast may serve the provision of high local progesterone concentrations immediately at the feto-maternal interface, which might be essential for specific actions of progesterone such as the protection of the fetus from the maternal immune system (Hansen 1998; Tibbetts et al. 1999; Hansen 2007). However, recent work (Shenavai et al. 2010) using a progesterone receptor antagonist does not support this hypothesis.

Interestingly in the bovine trophoblast steroidogenic enzymes are compartmentalized on a cellular – or more precisely – on a temporal level. P450scc and P450c17 are only expressed in UTC. 3ß-HSD and aromatase are only up-regulated during TGC differentiation, whereas P450c17 is rapidly down-regulated at a very early stage of this process (Schuler et al. 2006a, 2008). These expression patterns suggest that in UTC the steroidogenic cascade stops at dehydroepiandrosterone (DHEA). Only after entering the TGC differentiation process, 3β-HSD expression is up-regulated, and DHEA may be further converted to androstenedione and estrogens. According to this concept progesterone may only be produced if there is a carryover of the precursor pregnenolone from UTC to differentiating TGC or a leakage of this precursor from UTC to TGC. Consequently, in trophoblast cells progesterone may only be a by-product of the synthesis of estrogens or another unknown steroid (Schuler et al. 2008).

2.2.2 Production of estrogens by the bovine placenta and their putative functions

Results from the measurement of estrogen concentrations in bovine fetal fluids indicate that placental estrogen synthesis starts soon after the onset of placentation (Eley et al. 1979). Sites of estrogen production are the trophoblast cells of fetal cotyledons (Hoffmann et al. 1979;

Robertson and King 1979; Evans and Wagner 1981; Larsson et al. 1981; Gross and Williams 1988; Hoedemaker et al. 1990; Schuler et al. 1994, 2006a). Expression pattern of aromatase in bovine placentomes, which is up-regulated in trophoblast cells during TGC differentiation indicates that mature TGCs are the predominant source of estrogens in pregnant cows (Schuler et al. 2006a). The main estrogen secreted throughout gestation is estrone (E1), predominantly in its sulfoconjugated form i.e. estrone sulfate (E1S) (Hoffmann et al. 1997; Zhang et al. 1999). A rise of maternal E1S levels becomes detectable around days 100-120 of gestation. They increase continuously until late gestation to levels around 10-30 nmol/l, remain fairly constant during the last two weeks of gestation, start to decrease on the day prior to parturition and return to basal levels within 1-2 days postpartum. However, the peripartal decrease may be significantly protracted in cases of retained fetal membranes (Hoffmann et al. 1979). E1 levels initially follow a similar pattern, however, on a substantially lower level. Different from E1S, in most animals E1 concentrations continue to increase in late gestation until parturition. After birth, they return to basal levels in parallel with E1S concentration (Hoffmann et. al. 1997). Estradiol-17ß only increases significantly during late gestation. However, the primary source of this estrogen in pregnant cows is not fully clear as there is evidence of a significant estradiol-17ß production in the bovine udder de novo or by the utilization of placental precursors (Janowski et al. 2002).

Traditionally in pregnant cows estrogens have been considered as regulatory factors involved in the preparation of the birth canal for parturition (Smith et al. 1973) and in the control of mammogenesis (Schams et al. 2003), lactogenesis (Sawyer et al. 1986) and myometrial activity (Burton et al. 1987). However, this list is obviously incomplete as all of the functions mentioned are related to late gestation and parturition, whereas estrogen synthesis of the bovine trophoblast is already detectable at very early stages of gestation (Eley et al. 1979). Based on the expression pattern of estrogen receptors in bovine placentomes established by immunocytochemistry, a function as local regulators of placental growth, differentiation and functions has been suggested. Estrogen receptor alpha (ERα) was localized in the caruncle in a proportion of epithelial and stromal cells and in vascular pericytes. It was not detectable in the cotyledon (Boos et al. 2000; Schuler et al. 2002). Thus, a role of placental estrogens in the stimulation of the high proliferative activity observed in caruncular epithelium was suggested (Björckman 1969; Schuler et al. 2000; Boos et al. 2003). Also consistent with the concept of placental estrogens as local regulators, a significant correlation between estrogen tissue concentrations and proliferative activity of caruncular stromal cells was found (Schuler 2000). ERß expression was widely expressed in caruncles and cotyledons. However, as concluded

from the intensity of immunosignals, expression was generally weak with the exception of TGC, where ERβ expression was substantially up-regulated during TGC differentiation. The expression pattern of ERß in bovine TGC suggests that placental estrogens might be involved in the control of TGC differentiation via an auto- or intracrine mechanism (Schuler et al. 2005). Moreover, the detection of estrogen receptors in the fetal and maternal vascular systems point to a role of placental estrogens as regulators of angiogenesis and vascular functions. However, at current none of these suggested functions have been definitely proven. When Janowski et al. (1996) applied the estrogen receptor blocker tamoxifen to late pregnant cows, no effects on calving process could be demonstrated. However, the significance of this experiment is unclear as the extent to which the treatment was able to inhibit estrogenic effects in the presence of high estrogen concentrations occurring in late pregnant cows remains elusive.

2.2.3 Estrogen sulfotransferase expression and estrone sulfate production of the bovine trophoblast

An intriguing feature concerning bovine pregnancy-associated estrogens is the fact that, with the exception of the last month of gestation, when free estrogens increase substantially in peripheral maternal blood, sulfonated forms by far exceed the free ones (Hoffmann et al. 1997; Takahashi et al. 1997). Sulfoconjugation of estrogens abolishes receptor binding and hence receptor mediated actions (Hähnel et al. 1973). Moreover, it increases water solubility and binding of the hormone to albumine, thus limiting the distribution of the molecule in tissues. Consequently, sulfonation of estrogens is commonly considered as an important mechanism for the inactivation of estrogens also enhancing their excretion (Clarke et al. 1982). However, estrogen sulfonates may be readily converted to active estrogens by removal of the sulfate by the enzyme STS (Santner et al. 1984; Reed et al. 2005).

2.2.3.1 Classification of sulfotransferases

Sulfotransferases, which catalyze the transfer of a sulfonate group from a 3´-phosphoadenosine-5´-posphosulfate (PAPS) to an acceptor group of the substrate, are divided into two big families: (1) the membrane bound sulfotransferases located in the Golgi apparatus catalyzing the sulfonation of peptides, protein, lipids and aminoglycans and (2) the cytosolic sulftotransferases (SULTs) involved in the metabolism of a multitude of endobiotics, xenobiotics and drugs such as phenols, phenolic and neutral steroids, arylamines, primary and secondary alcohols (Chapman et al. 2004). In humans, to date a total of 13

cytosolic SULT genes have been identified, which are divided into four families: SULT1, 2, 4 and 6 (Blanchard et al. 2004; Freimuth et al. 2004). Although individual SULTs have substrate preferences, they can be quite promiscuous, so that their main biological function is often obscured. Due to substantial substrate overlap at level of subfamilies and even families (see table 1), considerable confusion occurred in early naming schemes. Only recently with modern biochemical, biophysical, and genetic characterization this issue has been resolved. The human sulfotransferase highly specific for estrogens is now named SULT1E1. It is the only SULT that exhibits affinity for estrogens in a physiological concentration range. It has a significantly higher affinity for estrogen sulfonation than other SULTs and displays a particularly high affinity for its natural substrates, estradiol-17ß and estrone, indicating an important role of this enzyme in the modulating of estrogen action (Schrag et al. 2004).

Table 1: The human cytosolic sulfotransferase family (according to Pasqualini, 2009).

SULT	Common Name	Amino Acids	Substrate Preference (endogenous)	Sequence Identities with SULT1A1
SULT1A1	P-PST/-1	295	phenols	
	TS-PST		estrogens	
	H-PST			
	HAST1/2			
SULT1A2	ST1A2	295	phenols	95.6%
	HAST4			
	TS-PST2			
SULT1A3	M-PST	295	phenols	92.9%
	TL-PST		cathecholamines	
	HAST3		estrogens	
	hEST/1			
SULT1A4			not known	99.99% homology with SULT1A3
SULT1B1	ST1B2	296	thyroid hormones	53.4
SULT1C2	HAST5	296	phenols	52.2
	SULT1C1			
SULT1C4	hSULT1C	302	not known	53.2
SULT1E1	hEST/-1	294	estrogens (high affinity)	50.1
SULT2A1	DHEA-ST	285	3ß-hydroxysteroid DHEA	34.6
	HST			
SULT2B1-v1	hSULT2B1a	350	DHEA, pregnenolone	36.3
SULT2B1-v2	hSULT2B1b	365	DHEA, cholesterol	36.9
SULT4A1-v1	hBR-STL	284	not known	34.2
SULT4A1-v2				

2.2.3.2 Properties and functions of SULT1E1

In general, the catalytic cycle of sulfonation requires the sulfonate acceptor (ROH) and the donor 3'- phosphoadenosine 5'-phosphosulfate (PAPS) to bind to a SULT, which results in the release of the sulfonate and 3´-phosphoadenosine-5´-phosphate. Although according to the common chemical nomenclature the transfer of $–SO_3^{1-}$ - as catalyzed by SULT1E1 is a sulfonation. However, compounds structured $RO\text{-}SO_3^{1-}$ are traditionally misnamed sulfates (Kuss 1994). The human SULT1E1 consists of 294 amino acids, the protein encoded by the human liver SULT1E1 cDNA is 81%, 73%, and 72% identical to the amino acid sequences of guinea pig adrenocortical, bovine placental and rat liver SULT1E1, respectively (Aksoy et al. 1994). Bovine SULT1E1 protein consists of 295 amino acids and has a maximum apparent molecular weight of 34,600 (Nash et al. 1988). As the majority of SULTs, also SULT1E1 is a homodimer in its catalytically active form. The structure and function of SULTs is reviewed in detail by Chapman et al. (2004).

As obvious from observations in knockout models, the crucial physiological role of SULT1E1 is the local control of the availability of active estrogens. In female SULT1E1 knockout mice subfertility due to placental thrombosis, placental degeneration (Tong et al. 2005) and impaired ovulation (Gershon et al. 2007) was observed. The main phenotype in male SULT1E1 knockout mice was an age-dependent leydig cell hypertrophy/hyperplasia coupled with seminiferous tubule damage, which resulted in reduced sperm motility (Qian et al. 2001; Coughtrie 2002).

2.2.3.3 Regulation of SULT1E1 expression or estrogen sulfotransferase activities

Prior to the definite characterization of individual SULTs by molecular biological methods, investigations on sulfotransferases were primariliy based on the detection of enzyme activities. Thus, due to the overlapping substrate specificities of SULTs in many cases it comes not fully clear which of the many SULTs was actually detected. Thus, in the following text the term estrogen sulfotransferase (EST) will be used in cases where only the sulfonation of estrogens was measured or results are otherwise questionable in relation to the underlying enzyme, and the term SULT1E1 will only be used where the enzyme was assessed by specific methods.

Information on the regulation of SULT1E1 is still sparse. In human and porcine endometrium, EST was up-regulated by progesterone (Meyers et al. 1983; Falany and Falany 1996). In male mice, SULT1E1 expression in testes was shown to be androgen dependent (Song 2007). In fat tissue of mice SULT1E1 is expressed in a sexually dimorphic manner and is regulated by

testosterone (Khor et al. 2008). In mouse models it has also been shown that activation of the orphan nuclear receptor liver X receptor (LXR) up-regulates SULT1E1 expression. Endogenous ligands of these receptors are cholesterol-derived oxysterols (Gong et al. 2007).

2.2.3.4 Expression pattern of SULT1E1 or estrogen sulfotransferase in general

EST activity has been reported in numerous tissues of males and females of various species including liver, kidney, brain, adrenal gland, etc. (Hobkirk 1985). However, it is also well known that marked differences of EST expression and/or activity exist in tissues depending on species, sex, age, development and physiological status (Hobkirk et al. 1983; Hobkirk and Glasier 1992; Mancini et al. 1992). The liver has been considered a primary site of steroid sulfotransferase activities, but significant activities have also been found in other organs such as in the testis of rat and man (Song et al. 1995). In male mice SULT1E1 is discretely expressed and regulated in the reproductive tract and plays a physiological role in maintaining the functional integrity of the epididymis by regulating luminal estrogen homeostasis (Tong and Song 2002). In the genital tract of bulls, SULT1E1 expression was described in the testis and epididymis (Frenette et al. 2009). In female mice SULT1E1 was highly expressed in placenta and uterus (Alnouti and Klaassen 2006). In humans SULT1E1 is widely expressed in adult and fetal organs and tissues including the placenta (Miki et al. 2002).

2.2.3.5 Expression pattern of SULT1E1 or estrogen sulfotransferase in bovine placentomes

Measurements of EST activities in bovine placentomal tissue concordantly yielded substantially higher sulfonation of estrogens in the cotyledon compared to the caruncle (Mattioli et al. 1984; Möstl et al. 1986; Hoffmann et al. 2001). Hoffmann et al. (2001) measured EST activities in caruncular and cotlyedonary homogenates fom midpregnancy until parturition, which did not change substantially during the period investigated. On a subcellular level EST activities were clearly associated with the cytosol, whereas activities in the nuclear, mitochondrial and microsomal fractions were significantly lower.

Hirayama et al. (2008) tested the hypothesis that prolonged gestation and poor signs of parturition in cows carrying somatic clone fetuses may be related to a lack of free estrogens at normal gestational length in spite of fetal maturity. Their results suggest that maternal concentrations of free estrogens in clone pregnancies did not increase sufficiently in the prepartal period to facilitate parturition due to elevated placental SULT1E1 expression levels, and they concluded that excessive estrogen sulfoconjugation is the reason for a low ratio of

active to inactive estrogens. Thus, the resulting hormonal imbalance may contribute to the lack of overt signs of readiness for parturition in cows pregnant with clones.

In order to understand the function of bovine pregnancy associated estrogens, the site of their inactivation, i.e. the sites of SULT1E1 expression on a cellular level, have to be considered. Brown et al. (1987) produced a monoclonal antibody against EST purified from bovine placentomes and localized the enzyme in the cytoplasm of TGC by immunohistochemistry without stating the stage of gestation when the tissue was collected. However, retrospectively it is doubtful if this antibody was actually specific for bovine SULT1E1 (see section 5.1).

By in situ-hybridization, SULT1E1-mRNA was also localized in TGC (Ushizawa et al. 2007; Hirayama et al. 2008). By means of real-time RT-PCR a significant increase of SULT1E1-mRNA levels was demonstrated between days 25-250 of gestation (Ushizawa et al. 2007). However, published data do not provide information on the temporospatial expression pattern of SULT1E1 in bovine placentomes on a cellular level in the course of bovine gestation and at parturition, and SULT1E1 expression in bovine trophoblast cells as a function of TGC differentiation has not been addressed. Howsoever, the co-expression of SULT1E1 and aromatase (Schuler et al. 2006a) in bovine TGC would imply that in pregnant cows E1S is a primary product of TGC. Thus, observations so far available on the localisation of SULT1E1 in bovine placentomes suggest that sulfonation of estrogens takes place in the same cells where they are produced, and which also express the β-isoform of the estrogen receptor (Schuler et al. 2005). These observations point to a role of placental estrogens as intracrine regulators during TGC differentiation. Alternatively or in addition, sulfated estrogens may be substrates for local intratissue activation of sulfated estrogens via the sulfatase pathway.

2.3 Expression of steroid sulfatase in bovine placentomes and sulfatase pathway

As sulfonation of estrogens abolishes receptor binding and hence receptor mediated actions, the predominant production of E1S by the bovine trophoblast on a first view questions the role of placental estrogens as paracrine regulators of bovine caruncular growth and differentiation via classical nuclear receptors. However, E1S may not only be considered as a mere inactivated metabolite destined for excretion. Steroid sulfates bind to albumin and have a prolonged half life in blood (up to 9 hours) compared with the much shorter half lifes of free steroids (Ruder et al. 1972). Thus, the high circulating E1S concentrations together with its prolonged half life have given rise to the view that it may act as a precursor reservoir for the local intratissue formation of biologically active estrogens via the action of STS (Santner et al. 1984; Reed and Purohit 1993; Purohit et al. 1996). During the past twenty years this

"sulfatase pathway" of estrogen formation has gained increasing interest in connection with the etiology and therapy of human hormone–dependent breast cancer in postmenopausal women, where the intratissue production of free estrogens from E1S is much more efficient than that of androgens via the aromatase route (Santner et al. 1984; Reed et al. 2005).

2.3.1 Characteristics of steroid sulfatase (STS)

StS (EC3.1.6.2, arylsulfatase C) catalyzes the hydrolysis of alkyl (e.g. DHEA sulfate, pregnenolone sulfate, cholesterol sulfate) and aryl (e.g. E1S) steroid sulfates (Reed et al. 2005). It is a member of a group of 12 different mammalian sulfatases. Early investigations suggested that arylsulfatase C and StS may be different enzymes. However, biochemical and genetic analyses confirmed that there is only one enzyme (Reed et al. 2005). The central role of placental STS for the formation of estriol in the human fetoplacental unit (Diczfalusy 1969), its abundance in the human placenta (Suzuki et al. 1992), and the virtual absence of detectable activity in cases of the inherited disorder of placental STS deficiency and recessive X-linked ichthyosis (Webster et al. 1978) have led to the enzyme from human placenta being extensively investigated (see below). Human placental STS has been purified to homogeneity and has been well characterized. Depending on the extent of glycosylation, purified human STS has a molecular mass of approximately 65 kDa (Stein et al. 1989).

The human STS polypeptide is composed of 583 amino acids encoded by a gene located on the distal short arm of the X chromosome (Xp22.3). It is composed of 10 exons spanning approximately 140 kb of DNA. The gene shares significant homology with all the other members of the sulfatase gene family. The sequence and organization of the STS gene appears to be particularly similar to that of a cluster of three sulfatase genes also located in the Xp22.3 region (Yen et al. 1988; Shapiro et al. 1989). Deficiency of STS activity produces the syndrome of X chromosome-linked ichthyosis, which is one of the most common inborn errors of metabolism in man (Shapiro et al. 1989).

The bovine StS is encoded by an 1737 bp open reading frame. The bovine StS protein consists of 578 amino acids and has a predicted molecular mass of 64.4 kDa. Like the human StS gene, the homologous bovine gene is located on the X chromosome and is comprised of 10 exons, bovine StS exhibiting 74% and 77% sequence identity to human StS on the mRNA and protein levels, respectively (Greven et al. 2007).

2.3.2 Expression and function of StS in humans and other species

StS expression has been demonstrated in a broad range of organs. However, organ and tissue distribution may vary considerably between mammalian species. In mouse it has been demonstrated in liver, testis, ovary, adrenal glands, brain, endometrium, viscera, kidney and bone (Milewich et al. 1984). In human, StS expression in lung, aorta, liver, thyroid, testis, and uterus have been reported (Miki et al. 2002). Among numerous organs tested, the highest expression was found in skin, liver and lymph nodes, the lowest one in brain tissues (Selcer et al. 2007). On a subcellular level, StS expression is associated with cellular membranes, predominantly with the endoplasmatic reticulum, golgi cisterna and to a lesser extent with plasma membrane and components of the endocytic pathway (Willemsen et al. 1988; Stein et al. 1989; Hoffmann et al. 2001). Only little information is available on the regulation of StS expression or activity. In MCF7 breast cancer cells, StS activity is up-regulated by TNFα and interleukin-6. However, this up-regulation is mediated postranslationally rather than by changes in gene transcription (Newman et al. 2000). Moreover, substrate induction of StS by exogenous E1S has been described in liver and white blood cells of ovariectomized rats (Barth et al. 2000). Accordingly, the increased up-take of substrate by organic anion transporters has been suggested as a mechanism of postranslational control of StS activity (Reed et al. 2005). STS on its part increases steroidogenic acute regulatory protein (StAR) protein expression level and stimulates steroid production (Sugawara and Fujimoto 2004).

In human placenta StS is highly expressed in the syncytiotrophoblast of chorionic villi and plays a pivotal role in the enzymatic cascade leading to the production of pregnancy-associated oestrogens (Lam et al. 1984). Due to its lack of P450c17 expression (Voutilainen and Miller 1986) the human trophoblast depends on C19-precursors to produce oestrogens which are provided by the fetal and maternal adrenals in sulfonated form (Diczfalusy 1969).

In humans, StS deficiency is a relatively common genetic disorder due to inactivating mutations of the StS gene which is located on the short arm of the X-chromosome (Hernandez-Martin et al. 1999). Apart from extremely low levels of placental oestrogen production during the fetal phase, in humans the clinical manifestation of StS deficiency is mainly characterised by a generalised desquamation of large, adherent, dark brown scales from the epidermis, and is therefore named X-linked ichthyosis (Hernandez-Martin et al. 1999). The condition originates from the accumulation of cholesterol sulfate in the outer epidermis, which prevents normal desquamation of keratinocytes (Elias et al. 2004).

2.3.3 Expression of StS in bovine placentomes

StS expression in bovine placentomes was detected by immunohistochemistry almost exclusively in the maternal part of the placentome, where it was localized in caruncular epithelial cells (Greven et al. 2007). Accordingly, StS activities were clearly higher in caruncular compared to cotyledonary homogenates (Mattioli et al. 1984; Möstl et al. 1986; Hoffmann et al. 2001). StS expression in bovine caruncular epithelial cells was clearly related to gestational age. In 100-240 day pregnant animals, expression was essentially restricted to areas adjacent to the chorionic plate and basal primary and secondary chorionic villi. In late pregnant animals expression gradually extended towards the caruncular stalk. After the onset of luteolysis and during active labour overall staining intensity had increased substantially and signals occurred ubiquitously in the flattened and partially dismantled caruncular epithelium. In addition to caruncular epithelial cells StS expression was sporadically observed in individual TGC (Greven et al. 2007).

2.3.4 The biological role of StS in bovine placentomes

The biological role of StS in bovine placentomes is still unclear but must be clearly different from its role in the human endocrine feto-placental unit, where StS located in the syncytiotrophoblast is a prerequisite for the utilization of sulfoconjugated C19-precursors provided by the fetal and maternal adrenals to produce estrogens (Diczfalusy 1969; Kuss 1994; Salido et al. 1990). Possibly, StS in caruncular epithelial cells may control locally the availability of free, active estrogens in bovine caruncles which may serve the restriction of the effects of the large amounts of pregnancy associated estrogens to a subset of estrogen responsive cells thereby avoiding detrimental systemic side effects in the maternal compartment. Accordingly, in caruncular epithelial cells StS (Greven et al. 2007) is co-expressed with ERα (Schuler et al. 2002). However, as hydrolytic activity of bovine placental StS is not limited to sulfoconjugated phenolic steroids (Schuler et al. 2008), alternatively or in addition, StS expression in the caruncular epithelium may also serve the utilization of sulfoconjugated neutral steroid precursors (e.g. pregnenolone sulfate or cholesterol sulfate) supplied with maternal blood, thus providing free steroid substrates for further metabolization in the adjacent trophoblast. This hypothesis has not been investigated so far. However, the fact that other factors acting at an early stage within the steroidogenic cascade, such as the steroid acute regulatory protein (StAR) and P450scc have been found to be expressed in the caruncular epithelium (Ben-David and Shemesh 1990; Verduzco et al. 2007) is consistent with this concept.

Moreover, the detection of high StS expression in bovine organs without quantitatively significant steroid hormone production such as the skin or gut suggests that StS may also have functions unrelated to the production of hormonally active steroids (Greven et al. 2007).

2.4 Sodium-dependent Organic Anion Transporter (SOAT, SLC10A6) and its function as a transporter of sulfonated steroids

2.4.1 Transport of free and sulfonated steroids in tissues

Free steroid hormones are commonly believed to penetrate tissues and enter cells solely by free diffusion through plasma membranes due to their lipophilic nature (free hormone hypothesis). However, the existence of cellular up-take mechanisms for carrier-bound steroids have been suggested similar to the clearence of cholesterol, which involves the recognition of carrier proteins by endocytic receptors on the surface of target cells, followed by internalization and cellular delivery of the sterols (Willnow and Nykjaer 2010). Until recently, virtually no information was available on the intra-tissue transport of steroid sulfates. As they are considerably more hydrophilic than free steroids, so far unidentified specific transport mechanisms have been postulated to be necessary for their penetration across cellular membranes. Transport systems for organic solutes comprise passive transporters, which permit passive movement of molecules across the plasma membrane down its concentration gradient, and active transporters, which use different energy coupling mechanisms (Hediger 1994).

Recently the cellular up-take of E1S into estrogen responsive cancer cells has found increasing interest in connection with the intratumoral production of active estrogens via the sulfatase pathway (Santner et al. 1984; Utsunomiya et al. 2004; Sasano et al. 2009), and a still increasing number of transporters including the sodium-dependent organic anion transporter (SOAT, SLC10A6), the organic anion transporter 6A1 (OATP6A1; syn.: organic anion-transporting polypeptide 1 (Oatp1) Eckhardt et al. 1999, Kanai et al. 1996), the organic anion transporting polypeptides B (OATP-B) (St-Pierre et al. 2002), organic anion transporter 4 (OAT-4) (Ugele et al. 2003), organic anion transporting polypeptide D (OATP-D) and organic anion transporting polypeptide E (OATP-E) (Nozawa et al. 2004) has now been identified to accept steroid sulfates as substrates exhibiting a wide range of affinities.

2.4.2 Structure and functions of the SOAT

The sodium dependent organic anion transporter (SOAT, SLC10A6) belongs to solute carrier family 10 (SLC10). The SLC10 family of sodium/bile salt cotransporters contains over 50

members in animal, plant and bacterial species. The first member of this transporter family, the Na+/taurocholate cotransporting polypeptide (NTCP; SLC10A1) was identified in 1990 by expression cloning from rat liver (Hagenbuch et al. 1990). After few years its intestinal counterpart was cloned from hamster intestinal cDNA library and was named the apical sodium-dependent bile acid transporter (ASBT; SLC10A2). Human NTCP and rat/mouse Ntcps consist of 349 and 362 amino acids, respectively, and show an overall sequence identity of >70%. In contrast to ASBT, substrate specificity of NTCP is not limited strictly to bile acids. NTCP also transports steroid sulfates such as estrone-3-sulfate (E1S) and DHEAS. They mediate sodium-coupled uptake of taurocholate and other bile acids with a Na+:taurocholate stoichiometry of 2:1 (Hagenbuch and Meier 1994; Weinman 1997).

The apical sodium-dependent bile acid transporter (Asbt; Slc10a2) was initially isolated from hamster cDNA library by expression cloning (Wong et al. 1994). Later, human ASBT, as well as the rat Asbt, rabbit Asbt, and mouse Asbt were cloned from the ileum (Wong et al. 1995; Shneider et al. 1995; Kramer et al. 1999; Saeki et al. 1999). These proteins consist of 348 amino acids and show an overall amino acid identity of >80%. Although sequence identity to the hepatic NTCP is relatively low, at 35%, all NTCP/Ntcp and ASBT/Asbt carriers transport conjugated bile acids with high affinity in a sodium-dependent manner (Wong et al. 1994, 1995; Craddock et al. 1998). In contrast to the basolateral localization of Ntcp, Asbt is highly expressed in the apical brush border membrane of enterocytes of the terminal ileum (Shneider et al. 1995). ASBT transports all major species of bile acids. However, it favors trihydroxy- over dihydroxy-bile salts and conjugated over unconjugated species.

Four more members of the SLC10 family have been identified i.e. SLC10A3, SLC10A4, SLC10A5, and SLC10A6 (SOAT) (Geyer et al. 2004; Hagenbuch and Dawson 2004). Within the SLC10 family, ASBT and SOAT are the most homologous members, with high sequence similarity (70%) and almost identical gene structures and thus have obviously emerged from a common ancestor gene (Geyer et al. 2006).

SOAT was first cloned from rat adrenal (Geyer et al. 2004) and has then been described in man (Geyer et al. 2006) and cow (Greven 2008). It is expressed in a broad spectrum of organs including brain, heart, kidney, lung, muscle, spleen, testis, adrenal gland, placenta, small intestine, and colon, with the testis exhibiting by far the highest expression in any species so far investigated. The SOAT protein consists of 370 amino acids in the rat (Geyer et al. 2004) and 377 in man (Geyer et al. 2006) and cattle (Greven 2008). It shows 42% and 31% overall amino acid sequence identity to the ileal sodium-dependent bile acid transporter (Isbt) and the Na(+)/taurocholate cotransporting polypeptide (Ntcp), respectively. SOAT is predicted to

have nine (rat) or seven (man) transmembrane domains, with an N-terminus outside the cell and an intracellular C-terminus. In functional studies using transfected Xenopus laevis oocytes or HEK293 cells, the bile acids such as taurocholic acid, cholic acid and chenodeoxycholic acid were not substrates of SOAT, but a sodium-dependent import was demonstrated for E1S (Km = 31 µM, Vmax = 5557 fmol/oocyte/30 min) and DHEA-S (Km = 30 µM, Vmax = 5682 fmol/oocyte/30 min). Thus, SOAT has been suggested as a physiologically relevant transporter of steroid sulfates (Geyer et al. 2004).

2.4.3 The bovine SOAT and its expression in placentomes during bovine gestation

SOAT-mRNA cloned from bovine placentomes showed an identical intron-extron-structure compared to the human SOAT. It is also composed of six exons and consists of 377 amino acids. The homology on the protein level was 78%. Contrary to the SOAT in other species examined so far, in the cow four variants occurred on the mRNA level in addition to the standard SOAT (variant 1) (Greven 2008). The variants are formed by the deletion of complete exons and/or the insertion of an additional exon 1´. In variant 2 exon 4 is deleted, which causes a frame shift and results in a premature stop codon, the corresponding protein of this variant is predicted to contain only 240 amino acids. In variant 3 exons 2-4 are deleted without a change in the reading frame. The predicted protein is missing 128 amino acids in comparison to variant 1 while the amino acid-sequence at the N- and C-terminus is maintained. Variants 4 and 5 are characterized by the insertion of the additional exon 1´. An additional deletion of exon 4 occurs in variant 4. However, the predicted proteins for exons 4 and 5 are identical as exon 1´ includes several stop codons. A schematic presentation of the mRNAs encoding individual bovine SOAT isoforms is included in Fig. 3 in section 3.6.

When measuring SOAT-mRNA in bovine caruncles and cotyledons between day 100 and parturition using a real-time RT-PCR system covering all identified variants, significantly higher levels were found in the maternal than in the fetal part of the placentomes. Whereas in the cotyledons they remained on a constant low level in the period under investigation, there was a substantial increase in mean SOAT-mRNA levels in the caruncles around the time of the prepartal luteolysis and at parturition (Greven 2008). Due to a high variability between individual animals, this prepartal increase was not statistically significant. The detection of significant SOAT-mRNA levels in a pure caruncular epithelial cell line suggested that the caruncular epithelium is the predominant site of SOAT expression in bovine placentomes. When measuring SOAT-mRNA concentrations in various bovine organs, the highest expression was found in testis, which was about 20fold higher compared to the expression in

placentomes, followed by skin and the adrenal gland. A lower but significant expression was also found in the liver, rumen, small intestine, lymph node, mammary gland and ovary. By transport studies using HEK 293 cells transiently transfected with bovine standard SOAT a significant sodium-dependent cellular import of DHEAS, pregnenolone sulfate (P5S) and E1S was demonstrated. The functions of bovine SOAT variants 2-5 and their specific expression patterns have not been studied so far (Greven 2008). Possibly they have a different substrate affinity and/or spectrum, may be functionally inactive or may exhibit an inverted direction of transport, as it has been described for a variant of the closely related apical sodium-dependent bile acid transporter (ASBT) similar to SOAT-variant 3 (Lazaridis et al. 2000). The results obtained for the bovine SOAT suggest that in cattle it is a physiological relevant steroid sulfate transporter, which could play an important role in the transport of pregnancy associated sulfonated steroids. The co-localization of SOAT and STS and their similar expression pattern in the bovine caruncle indicate that they are subject to similar regulatory mechanisms and that they may cooperate functionally.

2.5 Androgens and their roles in female reproduction

According to the general biochemical nomenclature, androgens are C19-steroids, which applies to the two most important endogenous androgens, testosterone and 5α-dihydrotestosterone (DHT). However, steroids with a differing number of C-atoms may exhibit significant androgenic activity, and a significant proportion of C19-steroids do not bind to the AR. Thus, from a functional point of view, androgens are synthetic or natural compounds able to bind and activate the AR (MacIndoe et al. 1981; Freeman et al. 2001; Gao and Dalton 2007).

Androgens are commonly considered as male reproductive hormones important in the induction of male sex differentiation during the fetal and neonatal phase (Levine 1971; Diamond et al. 1973) and in the control of male reproductive functions after the onset of puberty such as initiation and maintenance of spermatogenesis (Collins et al. 2003), function of epididymis (Pierrepoint and Davies 1984) and of accessory sex glands, maintenance of other secondary male characteristics and male behavior (Barkley and Goldman 1977). Moreover, they are also important metabolic hormones (Mode et al. 1984). During fetal development in males, androgens exert long-term effects which are either organizational on specific organs during a critical phase of morphogenesis (e.g. sexual differentiation of external genitalia), or programming neural functions or enzyme activities expressed later in life. At all stages of development, which extends from fetal and neonatal stages to pubertal

accomplishment, androgens also have activational effects that are immediate, multiple, reversible and dose dependent (Maguelone 1983).

As precursors for estrogen synthesis or by-products, they are also synthesized in significant amounts in female steroidogenic organs. However, due to rapid metabolism, in systemic circulation of females they are usually measured only in minimal amounts (Yin et al. 2003). Disturbed functions of steroidogenic organs in females may lead to hyperandrogenism causing hirsutism, acne, alopecia, and oligo-amenorrhea (Karrer-Voegeli et al. 2009; Yildiz 2006) and virilisation (Holt et al. 2005; Luef et al. 2002). Moreover, androgens have been shown to induce follicle atresia (Hillier and Tetsuka 1997). Adverse effect of high androgen levels on the endometrium in women may result in infertility (Tuckerman et al. 2000). Poor reproductive performance observed in women with polycytic ovarian syndrome (PCOS) may be due, in part, to the concomitant increase in both serum androgens and elevations in endometrial AR (Apparao et al. 2002). Thus any disturbance in ovarian androgen metabolism will profoundly affect the reproductive state of females and may provoke different kinds of reproductive abnormalities in women (McKenna and Cunningham 1995; Carmina et al. 1997).

On the other hand, there is increasing evidence that androgens have important regulatory functions in females under physiological conditions. Evidence for a role of androgens in female reproductive physiology of various species arises from the detection of AR in various organs involved in female reproduction such as hypothalamus, pituitary, uterus and the ovary (Pope and Cardenas 2006; Rice et al. 2007; Slomczynska et al. 2007), and from pharmacological studies of androgen action on follicle development suggesting inhibitory and stimulatory effects on follicular development depending on the developmental stage (Vendola et al. 1999; Evans et al. 1997). Accordingly, recent studies in female AR knockout mice reported decreased fertility with significantly reduced pups per litter and corpora lutea, and premature ovarian failure, thus establishing the importance of androgens in the female reproduction (Yeh et al. 2002; Matsumoto et al. 2003; Hu et al. 2004; Sen and Hammes 2010; Walters et al. 2009; Zhou 2010). AR actions have a function in the physiological growth and development of the uterus and disruption of genomic AR signaling leads to abnormal uterine development. AR -/- uteri were morphologically different from wildtype uteri and had a significant reduction in diameter, total uterine area, endometrial area, and myometrial area (Walters et al. 2009).

Reciprocal ovarian transplantation experiments to differentiate between extra- and intraovarian effects in female AR knockout mice point to neuroendocrine and local

intraovarian AR mediated actions. However, the mechanisms of androgens in female reproductive physiology are still widely unclear due to difficulties to differentiate unequivocally between direct effects of androgens or indirect effects mediated via ER after conversion of androgens to estrogens or to other metabolites with estrogenic activities such as the DHT-derived 5α-androstane-3ß,17ß-diol. Moreover, as steroid receptors these may act as transcription factors in the absence of their steroid ligand, AR functions unrelated to androgens must also be considered (Rommerts 1990; Huang et al. 2002; Bonaccorsi et al. 2006; Zhu and Kyprianou 2008).

2.5.1 Production of androgens during pregnancy in cattle and other mammalian species and putative roles of androgens during pregnancy

In addition to estrogen and progesterone, depending on the species considerable levels of androgens may also be present in the uterine environment during pregnancy, a seemingly common phenomenon among mammals of which the blastocyst and/or placenta exhibits significant steroidogenic activity, including humans **(**Bonney et al. 1984**)**, pigs (Fischer et al. 1985; Stefanczyk-Krzymowska et al. 1998) and rats (Legrand et al. 1984; Pelletier 2002). However, in these cases placental androgens are primarily considered as precursors for placental estrogen synthesis, and possible discrete functions exerted by placental bioactive androgens have been rarely addressed (see discussion in section 5.3). Similarly, it is unclear if increased androgen levels measured in maternal blood of various species (see below) have discrete functions or if they have just escaped from aromatization in placental tissues. However, a special situation has been found in the pregnant rat, where androgens produced in the placenta serve as precursors for ovarian estrogen synthesis (Jackson and Albrecht 1985).

During pregnancy levels of testosterone or other C-19 steroids in maternal blood may rise in a species-specific manner (Bamman et al. 1980; Gaiani et al. 1984; Silberzahn et al. 1984; Carlsen et al. 2006). In pregnant women, the total serum testosterone concentration increases progressively throughout pregnancy. The increase in testosterone concentration in normal human pregnancies was detectable as early as 15 days after the LH surge. However, during this early time of pregnancy testosterone was obviously primarily of luteal origin (Castracane et al. 1998). In pregnant women the maternal serum testosterone concentrations were three to four times higher than umbilical cord serum concentrations; maternal values were 100 to 140 ng/dL and cord values average 33.5 ng/dL during second half of pregnancy (Tulchinsky and Ryan 1980).

In pregnant cows, the plasma concentration of testosterone ranged between 20 and 50 pg/ml until about day 90 of pregnancy and was higher (220 pg/ml) around day 270 (Gaiani et al. 1984). Möstl et al. (1987) measured testosterone concentrations of 0.40±0.20 ng/ml during the last week of pregnancy in cows. After parturition the concentration of the androgens declined rapidly. According to Hoffmann et al. (1976) testosterone values in pregnant cows between days 247 and 273 were 0.08 - 0.55 ng/ml.

2.5.2 Expression of androgen receptors in female reproductive organs of mammalian species and putative functions

Information on AR expression in healthy female reproductive organs in different species is available for various tissues but predominantly for the ovary and uterus (Horie et al. 1992a,b). In human and macaque endometrium under normal cylic conditions, AR mRNA and protein are only expressed in stroma but not in the glands or vascular endothelium. Endometrial AR have been shown to be up-regulated by estrogens and down-regulated by progesterone (Mertens et al. 1996; Slayden et al. 2001; Slayden and Brenner 2004). Sauerwein et al. (1998) measured mRNA-levels in uterine tissue samples from normal cycling bitches and from bitches suffering from pyometra. AR-mRNA level was 3.5 fold lower in the uteri from bitches suffering from pyometra compared to uteri from healthy bitches during metestrus. However, they could not demonstrate a significant effect of the stage of ovarian cycle on uterine AR-mRNA levels in healthy bitches. A basal expression of AR in canine uterus throughout the estrus cycle has also been described by Vermeirsch et al. (2002) using immunohistochemistry. AR immunostaining was also demonstrated in the uterus of female fetuses on day 90 as well as in the uterus of 1-day-old piglets (Slomczynska et al. 2007). AR mRNA was detected in the porcine endometrium during pregnancy up to day 18 post coitum, but no transcripts were observed during 32, 50, 71 and 90 days, while AR protein was detectable in glandular epithelium and stromal cells as through day 90 of pregnancy. AR was also detected in the myometrium on all investigated days of pregnancy. However, on day 90, the immunostaining was present only in a limited number of cells (Slomczynska et al. 2007). AR in the pig endometrium has been suggested to be important for maternal receptivity for implantation (Kowalski et al. 2004). Vesanen et al. (1992) concluded from their ligand binding studies that AR is expressed in various regions of the bovine uterus and in the cervix and suggested that androgens may participate the endocrine regulation of bovine uterine and cervical functions.

Ovarian AR function is required for normal female reproduction, particularly for folliculogenesis (Shiina et al. 2006). Locally produced androgens in the ovary act via

granulosa cell (GC) ARs to modulate follicular responsiveness to gonadotrophins and thereby contribute to the paracrine regulation of ovarian function (Hillier et al. 1997). AR expression in GC has been described in various species like bovine (Hampton et al. 2004), human (Horie et al. 1992a,b; Chadha et al. 1994), monkey (Hild-Petito et al. 1991), rat (Schreiber and Ross 1976; Tetsuka et al. 1995), pig (Duda and Slomczynska 2007). GC-specific androgen receptor knockout (ARKO) mice had premature ovarian failure and were subfertile, with longer estrous cycles and fewer ovulated oocytes (Sen and Hammes 2010). Evidence for the expression of AR also in the bovine ovary comes from ligand binding studies (Vesanen et al. 1992).

Information on AR expression in placenta is limited in humans and is virtually absent in other mammalian species. First evidence for AR expression in human placenta came from early ligand binding studies, where a specific binding of natural or synthetic androgens to placental protein was found (Barile et al. 1979; Stanley et al. 1980; Hirota et al. 1981; McCormick et al. 1981, Younes et al. 1982). However, as the detection of a “receptor” by ligand binding assay is based on the observation of high affinity and low capacity binding, the definite nature of the detected binding protein remains eventually unclear. Accordingly, Macaulay et al. (1988) concluded from the results obtained with their ligand binding studies that the androgen binding site contained in the human placenta is different from the classical AR. However, later studies by means of immunocytochemistry and RT-PCR confirmed the expression of AR in the human placenta, and AR was immunolocalized in the nuclei of syncytiotrophoblasts and vascular endothelial cells (Horie et al. 1992a; Hsu et al. 2009; Uzelac et al. 2010). From their studies on dysregulation of testosterone production and AR expression in the human placenta with gestational diabetes mellitus Uzelac et al. (2010) concluded that impaired androgen signaling in the placenta may profoundly interfere with its development and function.

2.5.3 Structural and functional organization of the androgen receptor

The AR gene is a single-copy gene which in various mammalian species has been localized on the long arm of the X chromosome. Like other members of the nuclear receptor superfamily, AR has four major functional regions (Fig. 1): the N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and a hinge region connecting the DBD and LBD (Mangelsdorf et al. 1995). Two autonomous transactivation functions, a constitutively active activation function (AF-1) originating in the N-terminal and a ligand-dependent activation function (AF-2) arising in the

LBD, are responsible for the transcriptional activity of this nuclear receptor (Gronemeyer and Laudet 1995; Bevan et al. 1999; Powell et al. 2004).

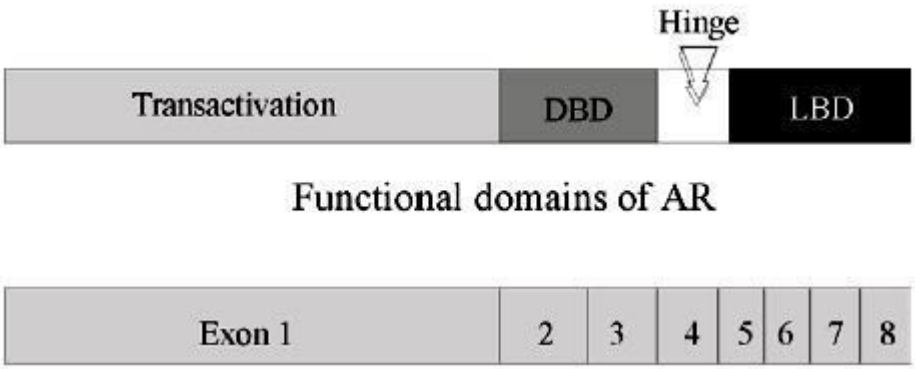


Figure 1: Functional organization of the AR and composition of the corresponding mRNA. Transactivation: n-terminal transcription activating domain; DBD: central DNA binding domain; Hinge: linker between DBD and the c-terminal ligand binding domain (LBD).

The DBD, which is highly conserved among nuclear receptors, is encoded by exons 2 and 3. The DBD includes 8 cysteine residues that form two zinc fingers (Evans 1988). The DBD of AR exhibits a high degree of amino acid sequence identity to the corresponding domains of other steroid receptors such as the glucocorticoid receptor (GR), the progesterone receptor (PR), and mineralocorticoid receptor (MR). Consequently, the four receptors recognize very similar, if not identical, hormone response elements (HREs). The two zinc fingers in the DBDs of nuclear receptors differ both structurally and functionally (Härd et al. 1990; Schwabe et al. 1990; Luisi et al. 1991). The first zinc finger contains the so-called P-box (Gly, Ser, and Val) that dictates the sequence specificity of binding to HRE (Berg 1989; Freedman 1992; Freedman and Luisi 1993). A five amino acid-residue long D-box of the GR super family is located in the N-terminal side of the second zinc finger. The D-box is important in specifying the half-site spacing requisite at the HRE (Dahlman-Wright et al. 1991). In addition, the D-box provides the entire dimerization interface for DBD-DBD interaction.

The function of the AR hinge region, defined by residues 628-669 in man, is not yet well understood. A sequence located between residues 628 and 657 within the hinge region contains a short stretch of basic amino acids that resemble the nuclear targeting signal of GR and has been described to form part of a bipartite nuclear localization signal (NLS) (Zhou et al. 1994). Wang et al. (2001) reported that AF-2 of AR LBD is inhibited by the cognate hinge

region. A mutant AR with a deletion of residues 628-646 in the hinge region exhibited transactivation activity that was more than double that of the wild-type AR.

The primary mechanism of action for ARs is to direct regulation of gene transcription (Heinlein and Chang 2004). The binding of an androgen to the AR results in a conformational change in the receptor which in turn causes dissociation of heat shock proteins, transport from the cytosol into the cell nucleus and dimerization (Tyagi et al. 2000; Marcelli et al. 2006). The AR dimer binds to a specific sequence of DNA known as a hormone response element. AR interact with other proteins in the nucleus to form a transcription complex resulting in up- or down-regulation of specific gene transcription (Smith and Toft 1993; Cleutjens et al. 1997; Pratt and Toft 2003). Up-regulation or activation of transcription results in increased synthesis of messenger RNA which in turn is transcribed by ribosomes to produce specific proteins. For example, androgens via AR up-regulate insulin-like growth factor-I receptor (IGF-IR) expression and sensitize prostate cancer cells to the biological effects of IGF-I (Pandini et al. 2005). Thus, changes in levels of specific proteins in cells is one way that ARs control cell behavior.

As AR is a transcription factor, its oncogenic functions – as mentioned above - are likely mediated through specific target genes. Prostate specific antigen (PSA), the best studied AR target gene, is thought to contribute to prostate cancer progression through its protease activity (Borgono and Diamandis 2004) and its ability to induce epithelial-mesenchymal transition and cell migration (Whitbread et al. 2006).

The transcriptional activity of AR is affected by coregulators that influence a number of functional properties of AR, including ligand selectivity and DNA binding capacity (Heinlein and Chang 2002). AR coregulators participate in DNA modification of target genes, either directly through modification of histones or indirectly by the recruitment of chromatin-modifying complexes, as well as functioning in the recruitment of the basal transcriptional machinery (Heinlein and Chang 2002). Aberrant AR coregulator activity due to mutation or altered expression levels may be a contributing factor in the progression of diseases related to AR activity, such as prostate cancer (Heinlein and Chang 2002).

2.5.4 Ligand independent actions of androgen receptor, membrane bound androgen receptors and nongenomic effects of androgens

More recently, classical nuclear ARs have been shown to have alternative modes of action. As has been also found for other steroid hormone receptors such as estrogen receptors, they can have actions that are independent of their interactions with DNA (Heinlein and Chang 2002).

ARs may interact with certain signal transduction proteins in the cytoplasm. Androgen binding to cytoplasmic ARs can cause rapid changes in cell function independent of changes in gene transcription, such as changes in ion transport. Moreover, regulation of signal transduction pathways by cytoplasmic ARs can indirectly lead to changes in gene transcription, for example, by leading to phosphorylation of other transcription factors. One function of AR that is independent of direct binding to its target DNA sequence is facilitated by recruitment via other DNA binding proteins. Finally, AR could be activated in an androgen-independent way by growth factor or cytokine signalling pathways, like those initiated by epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), keratinocyte growth factor and IL-6, which would elicit AR-mediated transcriptional activation (Culig et al. 1994; Reinikainen et al. 1996; Hobisch et al. 1998).

In addition to the intracellular (nuclear) androgen receptor (iAR) mediating genomic androgen signals resulting in receptor dimerization, nuclear translocation and subsequent activation of androgen-specific target genes (Heinlein and Chang 2004), effects of androgens have been described which are initiated at the cytoplasma membrane triggering non-genomic signals which may become manifest within minutes of androgen binding. It is now widely accepted that rapid responses to steroid hormones are mediated by at least two types of receptors: (I) a pool of classical steroid receptors associated with the plasma membrane and (II) specific G-protein coupled receptors (GPCR) unrelated to classic nuclear receptors or a receptor in close association with a GPCR. For estrogens and progesterone specific GPCR have been identified. Data from biochemical characterization of membrane androgen receptors suggest also the existence of a G-protein coupled membrane androgen receptor. However, the underlying protein has not been cloned yet (for reviews see Rahman and Christian 2007; Foradori et al. 2008, Papadopoulou et al. 2009).

3. MATERIALS AND METHODS

3.1 Tissue collection and sample preparation

3.1.1. Formalin fixed, paraffin embedded tissue for immunohistochemistry

Placentomes from healthy cows at various stages of pregnancy were collected at a local slaughterhouse. Gestational ages were estimated according to fetal crown-rump length (Rexroad et al. 1974). Additionally, placentomes were taken from three pregnant cows during elective caesarean section on day 272. From each of these animals 3-5 placentomes were removed from the midregion of the uterine horn that had contained the fetus. For comparative studies, tissue samples of various bovine organs were also collected at the slaughtherhouse and processed as described for the placentomal tissue.

Tissue samples of about 1 cm x 1 cm x 0.5 cm size were fixed overnight in 10% phosphate buffered formalin and subsequently dehydrated in a graded ethanol series. Finally they were embedded in paraffin. Formalin-fixed, paraffin embedded placentomal tissue samples prepared in the same manner from cows during the prepartal decline of progesterone (n=3) and at normal term (n=3) were available from previous studies (Schuler et al. 2005). All experiments involving living animals were performed in accordance with the relevant guidelines for the care and use of animals and with approval by the responsible animal welfare authority, the Regierungspräsidium Giessen.

3.1.2. Collection of tissue for protein and RNA isolation

Placentomes and samples from various other bovine organs and tissues were collected at a local slaughterhouse as described above. From placentomes, cotyledonary and caruncular tissue was prepared by careful manual separation in a glass dish placed on ice. Small pieces of tissues of about 2 cm x 2 cm x 1 cm size were prepared from organs/tissues, wrapped in aluminum foil, snap-frozen on dry ice and stored at -80ºC till further use. Snap-frozen placentomal tissue samples from cows during the prepartal decline of progesterone (n=3) and at normal term (n=3) were available from previous studies (Schuler et al. 2005). In these cases complete placentomes were removed during cesarean section, placed in phosphate buffered saline (PBS), taken to the laboratory within 10 min after collection, frozen in liquid nitrogen and stored at -80°C until analysis.

3.2. Immunohistochemistry

3.2.1 Immunohistochemical detection of androgen receptor

3.2.1.1 Staining procedure

An indirect immunoperoxidase staining method using a polyclonal purified rabbit antiserum raised against a peptide mapping at the N-terminus of human AR (N-20, sc-816, Santa Cruz Biotechnology, Heidelberg, Germany) was applied.

About 5 µm tissue sections were mounted on SuperFrost-Plus slides (Menzel Glaeser, MAGV Laborbedarf, Rabenau-Londorf, Germany), deparaffinized by two 4 min 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 for 5 min prior to three times 5 min microwave irradiation in pre-heated citrate buffer in an oven run at 560 W. After a 20 min cooling period the slides were washed under running tap water for 5 min followed by a treatment with 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity, then they were washed with immunocytochemistry (ICC) buffer for 5 min. Unspecific binding sites were blocked by covering with goat serum (Vector Laboratories, Burlingame, USA) diluted at a concentration of 1:67 in ICC buffer). After draining the blocking reagent, the primary antibody diluted at 1:500 in ICC buffer was applied and the slides were incubated for 20 h in a refrigerator at 4°C. They were then washed with ICC buffer, covered with the secondary biotinylated anti-rabbit antibody (from Vectastain Elite ABC Kit, PK 6101, Vector Laboratories, Burlingame, USA) diluted at a concentration of 1:200 in ICC buffer and incubated for 30 min at room temperature. Following draining of excess antibody, the sections were washed twice for 5 min with ICC buffer, then covered with streptavidin-peroxidase complex (Vector Laboratories, Burlingame, USA) and incubated for 30 min. After washing with ICC buffer (2 changes 5 min each), the sections were incubated with substrate solution (NovaRed substrate kit, Vector Laboratories, Burlingame, USA) for 8 minutes. The slides were washed under running tap water for 5 min, counterstained with hematoxylin and dehydrated in graded (70%, 96% and 100%) ethanol for 2 minutes each, 3 minutes in xylol and finally the sections were mounted in Histokit (Assisstent, Osterode, Germany). Negative controls were set up with serum of a non-immunized rabbit at an equal protein concentration as the primary antibody. As a positive control tissue bovine caput epididymis was used, as a high AR expression has been reported in this organ in other mammalian species (Tekpetey et al. 1989; Goyal et al. 1997; Zhu et. al 2000; Bilinska et al. 2004; Parlevliet et al. 2006). All the slides were stained during a single run to avoid effects of inter-incubation variability.

3.2.1.2 Quantification of immunostaining for androgen receptor using an immunoreactive score (IRS)

For the following cell types of the placentome a quantitative evaluation of immunostaining for AR was performed applying an immunoreactive score (IRS) (Remmele and Stegner 1987): invasive, mature, immature trophoblast giant cells (inTGC, maTGC, imTGC), UTC, stromal cells of chorionic villi (SCV), caruncular epithelial cells (CE) and caruncular stromal cells (CS).

UTC are small cells of the trophoblast variable in shape and form which with their basal part reside on the basal membrane of the chorionic epithelium and with their apical pole form a brush border interdigitating with caruncular eithelial cells.

Immature TGC are round or spherical, mostly binucleated cells in the trophoblast of intermediate size between UTC and TGC and are at an intermediate position between the basal membrane of the chorionic epithelium and the feto-maternal borderline. Their cytoplasm stains darker than that of surrounding mononucleate trophoblast cells, which facilitates their identification in case only one nucleus is situated in the section plane. However, as the differentiation of imTGC and UTC is frequently unconfident, these cell types were assessed as one fraction.

Mature TGC are characterized by their size, two large nuclei and copious granules in their cytoplasm. They are in contact with the caruncular epithelium, but unlike the UTC, they do not form a microvilli brush with the maternal epithelial cells.

Invasive TGC are situated in the caruncular epithelium and are in contact with its basal membrane. Moreover, they may fuse with individual caruncular epithelial cells and form three-nuclear feto-maternal hybrid cells. As they are short-lived, they may exhibit various stages of degeneration.

Staining intensity was classified as negative, weak, moderate or intense. One section from each animal was visually scanned and for each cell type percentaged distributions of the above mentioned staining categories were established. Subsequently for each staining category, the determined percentage was multiplied by the respective weighting factor, which was 0, 1, 5, and 10 for negative, weak, moderate and intense staining, respectively. Finally, the IRS was calculated as the sum of these four products divided by 100. Consequently, the IRS may range from 0 (all cells of a certain type negative) to 10 (all cells of a certain type intensely positive). The arithmetic means and standard deviations were calculated from these IRS values for each cell type of animals preassigned into the individual observational groups.

3.2.2 Immunohistochemical detection of SULT1E1

3.2.2.1 Staining procedure

For the localization of SULT1E1 on a cellular level and characterization of its expression in bovine placentomes as a function of TGC differentiation and gestational age, two immunohistochemical methods were established basically following the procedure described for AR in 3.2.1.1. The following primary antisera were used:

1) A polyclonal antiserum produced in a rabbit against recombinant bovine SULT1E1 (αbov-SULT1E1). This antiserum was a gift from Dr. Robert Sullivan, Centre de Recherche en Biologie de la Reproduction and Département d'Obstétrique-Gynécologie, Faculté de Médecine, Université Laval, Canada. Its production, purification by affinity chromatography and validation is described in a detailed manner by Frenette et al. (2009). It was applied at a dilution of 1:6000 in ICC buffer.

2) A commercial polyclonal antiserum produced in mice against human SULT1E1 (SAB 1400268, Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany; in this thesis named αhum-SULT1E1) which was applied at a concentration of 1:1000 in ICC buffer.

As secondary antibodies, 1) a biotinylated anti-rabbit antibody (from Vectastain Elite ABC Kit, PK 6101, Vector Laboratories, Burlingame, USA) at a dilution of 1:3000 in ICC buffer and 2) a biotinylated anti-mouse antibody (BA2000, Vector Laboratories, Burlingame, USA) at a dilution of 1:1000 in ICC buffer were used, respectively.

3.2.2.2 Evaluation of immunostaining for SULT1E1 using an immunoreactive score (IRS)

After immunohistochemistry using the two different primary antisera, immunostaining for SULT1E1 in UTC was evaluated semi-quantitatively basically as described in 3.2.1.2, respectively. However, in order to test for an influence of the localization, evaluation was performed separately in the chorionic plate and at defined localization of the chorionic villous tree (Fig. 2):

Primary (stem) villi: major villi with a pronounced stromal core running perpendicularly from the chorionic plate.

Secondary villi: originating directly from primary villi; characterized by narrower but significant stromal cores and, where the immediate origin from a primary villus was not visible, by an orientation more or less perpendicular to primary villi

Tertiary villi: exhibiting only minimal stromal parts surrounding central capillaries.

For each of the villous types, staining was evaluated separately in the basal (close to the chorionic plate) and apical half (adjacent to the caruncular stalk) of the interdigitation zone. One section per animal was evaluated. For each of the seven predefined localizations, mean staining intensity in UTC was classified as negative, week, moderate or intense on basis of pre-assigned photographic standards. Calculation of the IRS values was performed as described in 3.2.1.2.

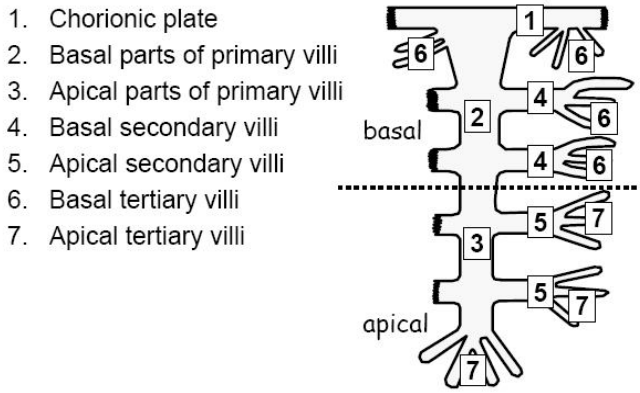


Figure 2: Schematic diagram of a bovine chorionic villus indicating the sites of semi-quantitative evaluation of SULT1E1-specific immunostaining in uninucleated trophoblast cells. The dotted line indicates the arbitrary division of the interdigitation area in a basal and an apical zone of equal height.

3.3. Preparation of total RNA from tissues

Coarse pieces of deep-frozen (-80°C) tissues wrapped in aluminum foil were broken up using a clean hammer. The tissue fragements were quickly placed in a pre-chilled mortar and powdered by a pestel under liquid nitrogen. 100 mg of tissue powder were immersed in 1 ml Trizol solution (Invitrogen, Karlsruhe, Germany) and further homogenized by three 60-120 seconds bursts on ice using an ultra turrax T25 (IKA-Werke GmbH & Co KG, Staufen Br., Germany). 200 µl chilled (-20°C) chloroform was added to the homogenized tissue, after short gentle shaking (up and down) it was kept on ice for 5 minutes followed by centrifugation at 20160 x g at 4°C for 15 minutes. After centrifugation, the uppermost of the resulting three liquid phases was taken into a fresh 2 ml round Eppendorf tube (Sarstedt, AG & Co., Nümbrecht, Germany) and 200 µl chloroform were added. After centrifugation at 20160 x g at 4°C for 15 minutes, the supernatant was taken into a fresh 2 ml Eppendorf tube and lower portion was discarded. 400 µl chilled (-20°C) isopropanol was added to the

supernatant and incubated for 1 hour at -20°C. This was followed by centrifugation at 20160 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was redissolved in 500 µl chilled (-20°C) 70% ethanol. After incubation for 10 minutes on ice followed by centrifugation at 20160 x g at 4°C for 10 minutes the ethanolic phase was discarded. This washing step was repeated. After complete draining of the alcohol, the pellet was allowed to dry at 37°C. After complete drying the pellet was solubilized in 50 µl diethylpyrocarbonate (DEPC) treated water kept in a water bath at 70°C for 10 minutes, followed by vortexing for complete solubilization. 50 U of RNAse inhibitor (Fermentas, Sankt Leon-Rot, Germany) was added and the total concentration of RNA was measured in a BioPhotometer (Eppendorf AG, Hamburg, Germany) at 260 nm in disposable cuvettes (UVette®, Eppendorf AG Hamburg, Germany) containing 2 µl of RNA stock solution and 98 µl double distilled water (ddH_2O) using 100 µl ddH_2O as a blank. Working solution aliquots adjusted to an RNA concentration of 100 ng/µl were prepared from stock solution by dilution with ddH_2O and stored at -20°C until use. The remaining RNA stock solution was stored at -80°C till further use.

3.4 DNase treatment

To eliminate genomic DNA, the RNA working solution (see previous section) was treated with DNase (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and RNase inhibitor (Fermentas, Sankt Leon-Rot, Germany) was added prior to reverse transcription. All reagents were kept on ice during pipetting. Firstly a DNase mix was prepared as stated in table 2.

Table 2: Reagents and their volumes (per sample) required for the preparation of a DNase mix used for the elimination of genomic DNA from RNA preparations.

Component	***Volume***	***Concentration of stock solution***
Incubation buffer	2 µl	
DNase 1, RNase free	1 µl	10 U/µl
RNase Inhibitor	0,25 µl	40 U/µl
total	3.25µl	

3.25 µl of DNase mix and 6.25 µl of the RNA working solution (100 ng RNA/µl) were mixed in a 0.5 ml plastic reaction tube and incubated in a thermocycler (T1 Thermocycler 49, Biometra GmbH, Göttingen, Germany) as listed in table 3.

Table 3: Incubation protocol for DNase-treatment of RNA-preparations (elimination of genomic DNA)

Temperature	***Duration***
37°C	10 minutes
75°C	5 minutes
4°C	till removal

Due to its instability, DNase treated RNA working solution had to be transcribed immediately into cDNA.

3.5 Reverse transcription

For reverse transcription (RT), the GeneAmp RNA PCR Kit (Perkin Elmer, Foster City, CA, USA) was used. The first strand cDNA was synthesized by using 1.5 µl (containing 74 ng/µl of RNA) of DNase treated RNA preparation and 8.5 µl of the RT-mastermix (Table 4) prepared according to the instructions of the kit supplier. This was pipetted in multiples according to the number of mRNA samples.

Table 4: Reagents and their volumes (per sample) required for the preparation of the RT-mastermix. Reagents and buffer were taken from the GeneAmp RNA PCR Kit (Perkin Elmer, Foster City, CA, USA).

Component	***Volume***	***Concentration of stock solution***
$MgCl_2$	2 µl	25 mM
PCR-buffer	1 µl	10x
Deoxyribonucleotidetriphosphate (DNTP) mix	4 µl	10 mM
Random hexamers	0.5 µl	50 µM
RNase inhibitor	0.5 µl	20 U/µl
Reverse transcriptase	0.5 µl	50 U/µl

A one step reverse transcription was carried out in 0.5 ml plastic reaction tubes (Biozym Diagnostik GmbH, Hessisch Oldendorf, Germany) running an automated program in a T1 Thermocycler 49 (Whatman Biometra Göttingen, Germany) as stated in table 5.

Table 5: Incubation protocol for reverse transcription

Temperature	***Duration***
21°C	8 minutes
42°C	15 minutes
99°C	5 minutes
5°C	5 minutes
4°C	till removal

3.6 Conventional reverse transcription (RT)-polymerase chain reaction (PCR)

Experiments using conventional RT-PCR were carried out for the following purposes:

1) to qualitatively confirm the expression of a target mRNA in a certain tissue
2) to check for proper operation of primer pairs designed for use in real-time RT-PCR and to confirm their specificity for the respective target mRNA.

Primer pairs were designed using Oligo Explorer (Version 1.1) and Oligo Analyzer (Version 1.1) software (Teemu Kuulasmaa, University of Kuopio, Finnland; freeware from the internet) and purchased from Eurogentec (Köln, Germany). Sequences of primer pairs are listed in table 6. For primers subsequently used in real-time RT-PCR see tables 10, 12 in sections 3.7 and 3.8, respectively. The positions of primers used for the detection of individual SOAT variants in the resepective mRNA sequence are schematically presented in Fig. 3.

Table 6: Sequences of primers (for = forward; rev = reverse) used in conventional RT-PCR, expected length of amplicons (base pairs, bp), annealing temperature (T_A) and sequence information used for primer design (accession number or reference).

Gene	***Primer***	***Amplicon length (bp)***	***T_A***	***Accession No.***
AR	for.: 5′-CAGATGGCAGTCATTCAG-3′ rev.: 5′-CTTGGTGAGCTGGTAGAAG-3′	386	56°C	XM_001253942
SOAT V-1	for. 5′-TGGATCTCAGCATCAGTATG-3′ rev.: 5′- TCATCAGCACCATACCAG 3-3′	281	56°C	EF186076
SOAT V-2	for.: 5′-TGGATCTCAGCATCAGTATG-3′ rev.: 5′- AATTGTCCTGCACCTTAAG-3′	230	56°C	EF495204
SOAT V-3	for..: 5′- ATGGATCTCAGGTGCAGGAC-3´ rev.: 5′- GACGGCCCAGGACTTAGAG -3′	322	62°C	EF495205
SOAT V-4	for.: 5′- ATGGATCTCAGCAAAGAAAG -3′ rev.: 5′- AATTGTCCTGCACCTTAAG 3′	428	55°C	EF495206
SOAT V-5	for.: 5′-CTGGTGGAGAACTGAAGAGG-3′ rev.: 5′-CTTTCATCAGCACCATACCA-3′	333	56°C	Greven (2008)
SULT1E1	for.: 5’-GAGGCAAGACCAGATGAC-3’ rev.: 5’-ACAGGCAGGTGAGACTTC-3’	229	60°C	BC102939
GAPDH	for.: 5′-CGATACTCACTCTTCTACCTTCGA-3′ rev.: 5′-TCGTACCAGGAAATGAGTTGAC-3’	82	60°C	U85042)

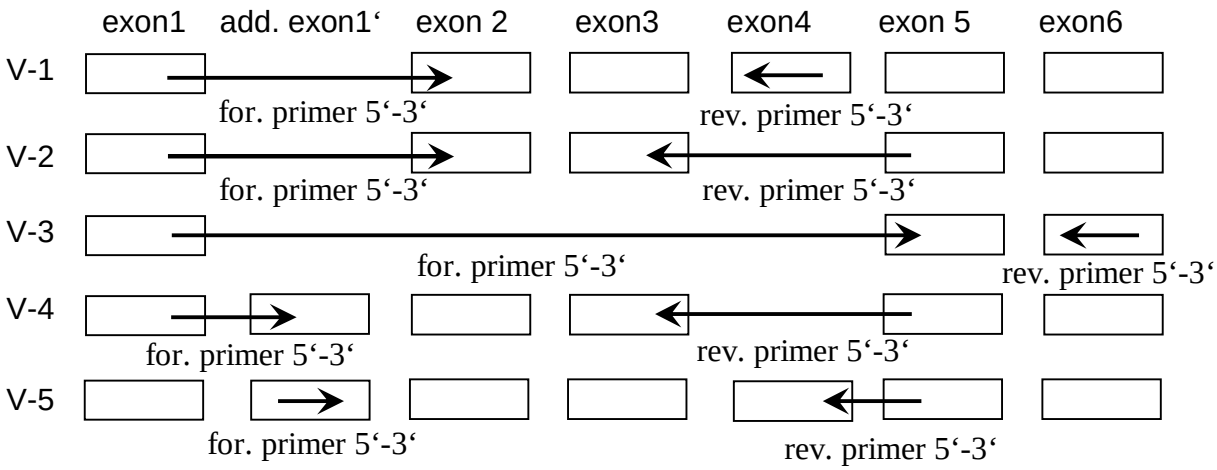


Figure 3: Position of the primers used for the detection of individual SOAT variants in the respective mRNA sequence.

3.6.1 Polymerase chain reaction

After reverse transcription, the obtained cDNA (in 10 µl) was added to 40 µl PCR mix described in table 7 and was amplified in a Personal Cycler (Biometra Göttingen, Germany) with the incubation conditions as described in table 8. Negative controls were set up using a sham cDNA preparation, in which RNA working solution was replaced by ddH_2O during the initial step of reverse transcription.

In order to validate the PCR methods for the detection of mRNA specific for individual SOAT variants and to prepare respective DNA standards, PCRs were performed using pGMET-T easy vectors (Promega GmbH, Mannheim, Germany) containing SOAT variant-specific inserts as templates. These vectors were kindly provided by Prof. Dr. Joachim Geyer, Institute of Pharmacology and Toxicology, Veterinary Faculty, Justus Liebig University Giessen. The method basically followed the above mentioned protocol. However, as 4 µl dNTP-Mix had to be added, the volume of autoclaved double distilled water in the PCR mix was reduced to 28.75 µl.

Table 7: Reagents and their volumes (per sample) required for the preparation of the PCR mix.

Component	***Volume***	***Concentration of stock solution***
$MgCl_2$	2 µl	25 mM
PCR-buffer	4 µl	10x
Forward primer	0.5 µl	20 pmol/µl
Reverse primer	0.5 µl	20 pmol/µl
Amplitaq® Gold polymerase	0.25 µl	5 U/µl
Autoclaved double distilled water	32.75 µl	
Total	40 µl	

Table 8: Incubation protocol for conventional PCR reactions

Step	***Temperature***	***Duration***	
Initial denaturing	94°C	1 minute	
Denaturing	94°C	10 minutes	39 times
Primer annealing	Depending on the individual primer pair (see table 6)	2 minutes	
Elongation	72°C	90 seconds	
Final elongation	72°C	6 minutes	
Till removal	4°C		

3.6.2 Analysis of the PCR-amplicons by agarose gel electrophoresis

The amplicons were separated and visualized on a 2% ethidium bromide stained agarose gel under UV transillumination. 1.82 g of agarose powder (Bioline GmbH, Luckenwalde, Germany) was added to 91 ml of Tris-Borate-EDTA (TBE) buffer. The mixture was then heated in a microwave at 560 W to completely dissolve the agarose powder and allowed to cool to about 60-65°C. After cooling, 1.9 µl of 1% ethidium bromide (Roth GmbH & Co., Karlsruhe, Germany) solved in ddH_2O was added and the agarose solution was mixed carefully and poured into a horizontally set gel container (Biozym Diagnostik GmbH, Hessisch Oldendorf, Germany) and allowed to solidify for about 45 minutes at room temperature.

The gel was immersed in a midi horizontal chamber (multiSUB/Biozym Diagnostik GmbH, Hessisch Oldendorf, Germany) containing TBE buffer and connected with a power supply (LKB Bromma, USA). 1.5 µl of 100 bp marker (100 ng/µl) (MBI Fermentas, Sankt Leon-Rot, Germany) and 1.5 µl loading dye (6x Loading Dye Solution; MBI Fermentas, Sankt Leon-Rot, Germany) was added to 7 µl DEPC-water and mixed thoroughly with a pipette tip. 15 µl of each PCR product and 1.5 µl loading dye were mixed with a pipette, respectively. Subsequently the 15 µl of sample mix and 10 µl marker mix (7 µl ddH_2O +1.5 µl loading dye

+ 1.5 µl DNA marker) were loaded into the wells of the agarose gel and allowed to run for 35-45 minutes under a voltage of 125 V and a current of 300 mA for the amplicons to separate. The amplicons were then visualized under UV transillumination using a UV-Transluminator fitted with a camera (Biostep GmbH, Jahnsdorf, Germany) linked to a PC equipped with software Phoretix Grabber 3.01 (Biostep GmbH, Jahnsdorf, Germany) and photographs were taken.

3.6.3 Sequencing of PCR products

In order to confirm the validity of the PCR methods for the detection and quantification of bovine SOAT variants, PCR products were separated on 1% agarose gel as described in section 3.6.2. The whole DNA band of was cut from the agarose gel and was further reduced to minimum possible size without affecting DNA band with a clean, sharp scalpel and then transferred into pre-weighed 1.5 ml colorless Eppendorf tube (Sarstedt, AG & Co., Nümbrecht, Germany). QIAEX II Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) was used for DNA extraction. From this kit, 300 µl of reagent buffer QX1 were added into a tube with 100 mg of sliced gel containing the DNA band. Then 30 µl of QIAEX II solution were added and the tube was vortexed for 30 seconds. After that, it was placed into a water bath (50°C) and the agarose was allowed to solubilize for adsorption of DNA to the QIAEX II particles for 10 min. During this period the tube was vortexed after every 2 minutes to ensure that the QIAEX II solution was in suspension. The sample was then centrifuged for 30 seconds and the supernatant was carefully removed with a pipette and discarded. The resulting pellet was washed with 500 µl of buffer QX1 solution to remove the remaining traces of agarose contaminants. It was then washed twice with 500 µl of buffer PE. After washing, the pellet was air dried for 30 minutes and then 20 µl of ddH_2O were added and the pellet resuspended by vortexing. Thereafter the samples were incubated for 5 minutes at room temperature. After centrifugation for 30 seconds at 10000 x g, the supernatant (purified DNA) was carefully taken into a new clean Eppendorf tube. Total concentration of DNA was measured in a BioPhotometer (Eppendorf AG Hamburg, Germany) in a disposable cuvettes (UVette®, Eppendorf AG Hamburg, Germany) containing 2 µl of purified DNA from PCR product and 98 µl ddH_2O using 100 µl ddH_2O as a blank. 60 ng of purified PCR product and 5 pmol (in 7 µl) of each primer pair were sent to a commercial laboratory (Scientific Research and Development GmbH, Bad Homburg, Germany) for sequencing. Data received from this laboratory were analyzed using the software BioEdit© Sequence Alignment Editor (Tom Hall, freeware from the internet).

3.7 Real-time RT-PCR for the relative quantification of mRNA specific for SOAT variants and SULT1E1 (SYBR green® method)

Relative levels of mRNA specific for SOAT variants 1, 2, 3, 5 and for SULT1E1 were determined in bovine placentomes and various organs by real-time RT-PCR (SYBR green method) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene. SYBR green is a simple and economical method to detect and quantify PCR products in real-time reactions. It binds to double-stranded DNA and emits light upon excitation. Thus, as a PCR product accumulates, fluorescence increases and becomes detectable by the fluorometer integrated in the PCR cycler. Since the dye binds to any double-stranded DNA, there is no need to design a target gene specific probe. However, since the dye does not distinguish between specific and non-specific products accumulating during PCR, follow up assays such as analysis/sequencing of the amplicon or melting curve analysis are needed to validate results.

Primer pairs (see table 10) were designed using Oligo Explorer (Version 1.1) and Oligo Analyzer (Version 1.1) software (Teemu Kuulasmaa, University of Kuopio, Finnland; freeware from the internet) and purchased from Eurogentec Köln, Germany. Preparation of total RNA, reverse transcription and DNAse treatment was performed as described in sections 3.3, 3.4 and 3.5. PCR amplification was performed using 96-well optical plates (BioRad Laboratories Inc., Hercules, CA, and Abgene Thermo Scientific UK). Each sample was analyzed in triplicate. Annealing temperatures (ranging between 50-65°C) for each primer pair for SOAT variants and SULT1E1 were optimized by temperature gradient assay using the preset program of the BioRad CFX 96 C1000 thermocyler (BioRad Laboratories Inc. Hercules CA, USA). After that, the concentration of cDNA used for real time PCR reaction was optimized for each primer pair using serial dilutions of template (cDNA). Finally the concentration of cDNA corresponding to 1/4th for SOAT variants and ½ for SULT1E1 of stock cDNA was identified as optimal. 5 µl of diluted cDNA stock solution were added to 20 µl of real-time PCR-mastermix (table 10) to yield a 25 µl PCR reaction mixture. From the 25 µl PCR reaction mixture, 23 µl was pippeted in 96 well plate and was covered with a optical sealing sheet (BioRad Laboratories Inc., Hercules, CA, USA). The plate was then transferred into a BioRad CFX 96 C1000 thermocyler (BioRad Laboratories Inc., Hercules, CA, USA). The amplification conditions were the same for the target and the reference gene i.e. denaturation for 10 min at 95°C followed by 40-45 cycles at 95°C for 15 s and 60°C for 60 s. After the completion of the amplification a melting curve was generated by increasing the temperature from 50°C to 95ºC in small increments of 0.5ºC for 5 second intervals to test

for the occurrence of non-specific products and primer dimers using BioRad CFX 96 C1000 thermocyler (BioRad Laboratories Inc., Hercules, CA, USA). Further evidence for the specificity of the products was obtained from conventional RT-PCR and subsequent analysis of the amplicons using agarose gel electrophoresis. For confirmation of the validity of SOAT variants specific methods, the PCR products were sent to a commercial laboratory (Scientific Research and Development GmbH, Bad Homburg, Germany) for sequencing as described in section 3.6.3. In order to validate the real-time RT-PCR methods, the efficiency was measured using a standard curve generated by serial dilutions of the cDNA according to the standard protocol provided by the supplier of thermocycler (BioRad Laboratories Inc., Hercules, CA, USA). The slope of the standard curve was used to determine the exponential amplification and the efficiency of the PCR reaction, which was calculated by the following equations (Rasmussen 2001):

Exponential Amplification = 10(-1/slope)

Efficiency = [10(-1/slope)] – 1

The efficiencies of the qRT-PCR amplifications were between 90% and 100% for all of the genes tested (table 9) as requested for acceptable qRT-PCR methods.

Table 9: Efficiencies of the qRT-PCR amplifications for the target genes measured by SYBR green method.

Target	***Slope***	***Amplification***	***Efficiency %***
SOAT- V1	-3.43	1.96	96
SOAT- V2	-3.58	1.90	90
SOAT- V3	-3.41	1.96	96
SOAT -V5	-3.16	1.97	97
SULT1E1	-3.60	1.90	90
GAPDH	-3.25	2.03	100

Relative gene expression values were calculated using the comparative C_T method ($\Delta\Delta C_T$ method) and reported as n-fold differences in comparison to the sample with the lowest amount of the respective target gene transcripts (calibrator) after normalizing the samples referring to the reference gene (GAPDH).

Table 10: Sequences of primers (for = forward; rev = reverse) used in real-time RT-PCR (SYBR green® method), expected length of amplicons (base pairs, bp) and sequence information used for primer design (accession number or reference).

Gene	***Primer***	***Amplicon length (bp)***	***Accession No.***
SOAT V-1	for.: 5'-TGGATCTCAGCATCAGTATG-3' rev.: 5'-AGGAGTCCACCAGCAATG-3'	243	EF186076
SOAT V-2	for.: 5'-TGGATCTCAGCATCAGTATG-3' rev.: 5'-AATTGTCCTGCACCTTAAG-3'	230	EF495204
SOAT V-3	for.: 5'-TGGATCTCAGGTGCAGGAC-3' rev.: 5'-CTTGACAACTGGGCTTCTCG-3'	230	EF495205
SOAT V-5	for.: 5'-CTGGTGGAGAACTGAAGAGG-3' rev.: 5'-CTTTCATCAGCACCATACCA-3'	333	Greven (2008)
SULT1E1	for.: 5'-GAGGCAAGACCAGATGAC-3' rev.: 5'-ACAGGCAGGTGAGACTTC-3'	229	BC102939
GAPDH	for.: 5′-GCGATACTCACTCTTCTACCTTCGA-3′ rev.: 5′-TCGTACCAGGAAATGAG TTGAC-3	82	U85042

Table 11: Reagents and their volumes (per sample) required for the preparation of the mastermix for real-time PCR (CYBR green® method). Reagents were from Abgene Thermo Scientific, UK and primers were purchased from Eurogentec, B-4102 Seraing, Belgium.

Component	***Volume (for one well)***	***Stock Solution***
ABsolute™ Blue QPCR SYBR ®	12.5 µl	2 x
Forward Primer	1.5 µl	SOAT 3.75 µM SULT1E1 1.25 µM
Reverse Primer	1.5 µl	SOAT 3.75 µM SULT1E1 1.25 µM
Autoclaved double distilled water	4.5 µl	-

3.8 Real-time RT-PCR for relative quantification of androgen receptor expression (TaqMan® method)

The TaqMan method utilizes the 5'-nuclease activity of the DNA polymerase used for PCR to hydrolyze oligonucleotides hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moeity coupled to the 3' end. They are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluorescent and the quenching molecules prevents the detection of fluorescent signal from the probe. During PCR, when the

polymerase replicates a template on which a TaqMan probe is bound, the 5'-nuclease activity of the polymerase cleaves the probe, and the fluorescence resonance energy transfer between the fluorescent and quenching dyes, which prevented fluorescent signal from the reporter dye no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage. In addition to the primer sequences, the target gene specificity of the probe sequence further increases the specificity of this method.

Preparation of total RNA, DNAse treatment and reverse transcription were performed as described in sections 3.3, 3.4 and 3.5. Primer and probes (see table 12) were designed using the Primer Express software (version 2.0, Applied Biosystems, USA) and purchased from Eurogentec, B-4102 Seraing, Belgium). Probes were labeled at the 5'-end with the reporter dye 6-carboxyfluorescin (FAM) and at the 3'-end with the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA). PCR amplification was performed on an automated fluorometer (ABI PRISM_7000 Sequence Detection System, Applied Biosystems, D-64293 Darmstadt, Germany) using 96-well optical plates. Each sample was analyzed in duplicates. For PCR 5 µl cDNA corresponding to 111 ng total RNA described in table 4 was used in a 25 µl PCR reaction mixture containing TaqMan-qPCR mastermix (Eurogentec, B-4102 Seraing, Belgium), 1.5 µl of each primer and 1 µl probe (see table 13). Amplification conditions were the same for AR and GAPDH which was used as reference gene: denaturation for 10 min at 95 °C followed by 45 cycles at 95 °C for 15 s and 60°C for 60 s.

The results were calculated using the comparative C_T method ($\Delta\Delta C_T$ method) according to the instructions of the manufacturer of the ABI PRISM_7000 Sequence Detector and were reported as n-fold differences in comparison to the sample with the lowest amount of the respective target gene transcripts (calibrator) after normalizing the samples referring to the reference gene GAPDH. Determination of efficency of the PCR amplification for AR was performed as described in section 3.7 and yielded 93%.

Table 12: Sequences of primers (for = forward; rev = reverse) and probes used in real-time RT-PCR for the measurement of relative expression levels of AR-mRNA (TaqMan® method), expected length of amplicons (base pairs, bp) and sequence information used for primer design (accession number). GAPDH was used as reference gene.

Gene	***Primer/Probe***	***Amplicon length (bp)***	***Accession No.***
Androgen receptor	for.: 5'-CACCTCTCCCAAGAATTTGG-3' rev.: 5'-TGCCTTCATGCACAGGAAT-3' probe: 5'-TGGCTCCAAATCACCCCCCAGG-3'	65	XM_001253942
GAPDH	for.: 5′-GCGATACTCACTCTTCTACCTTCG A-3′ rev.: 5′-TCGTACCAGGAAATGAGC TTGAC-3′ probe: 5′-CTGGCATTGCCCTCAACGACCACTT-3′	82	U85042

Table 13: Reagents (from TaqMan-qPCR mastermix, Eurogentec, B-4102 Seraing, Belgium) and their volumes (per sample) required for the preparation of the mastermix for real-time PCR (TaqMan® method).

Component	***Volume (for one well)***	***Concentration of stock Solution***
qPCR Master Mix	12.5 µl	2x
Forward Primer	1.5 µl	5 µM
Reverse Primer	1.5 µl	5 µM
Probe	1.0 µl	5 µM
cDNA	5.0 µl	
Autoclaved double distilled water	3.5 µl	-

3.9 Western blot analysis

3.9.1 Western blot analysis of bovine placentomal tissue for the expression of SULT1E1

Coarse pieces of deep-frozen (-80°C) cotyledonary tissue and from bovine adult and fetal liver were powdered as described above for RNA extraction in section 3.3. 200 mg of tissue powder were then immersed in 1.5 ml PBS containing PMSF (Protease Inhibitor Cocktail Tablet, Roche Diagnostics, Germany) at the concentration of one tablet in 10 ml PBS and vortexed for 2 minutes. The resulting homogenates were centrifuged at 20160 x g for 50 minutes, and the supernatants were taken and stored at -20°C till further use.

Concentration of protein was measured by BioPhotometer (Eppendorf AG, Hamburg, Germany). A 10% polyacrylamid separating gel was prepared by mixing 2.5 ml 30% acrylamide solution (Roth GmbH & Co., Karlsruhe, Germany), 2.8 ml separating gel buffer,

2.05 ml ddH_2O, 75 µl 10% SDS solution, 60 µl 10% ammonium persulfate (APS) (Roth GmbH & Co., Karlsruhe, Germany) and 6 µl TEMED (Roth GmbH & Co., Karlsruhe, Germany). The resulting solution was poured in a pre-assembled dual gel caster (Hoefer Inc. USA) and then 1 ml isopraponol was added onto the surface. The solution was allowed to solidify for 45 minutes, then the isopropanol layer on the surface was discarded and the gel surface was washed with ddH_2O. The remaining water was completely dried by help of filter paper. Polyacrylamid collecting gel was prepared by mixing 417 µl 30% acrylamide solution (Roth GmbH & Co., Karlsruhe, Germany), 313 µl collecting gel buffer, 1.73 ml ddH_2O, 25 µl 10% SDS solution, 13 µl 10% APS (Roth GmbH & Co., Karlsruhe, Germany) and 3 µl TEMED (Roth GmbH & Co., Karlsruhe, Germany). This solution was poured onto the separating gel and allowed to solidify for 40 minutes.

For polyacrylamide gel electrophoresis, 5 µg protein from placental tissue or 20 µg from fetal or adult liver were mixed with 3x loading dye, boiled for 3 minutes and then immediately placed on ice till loading on to gel. The protein was then loaded on the 10% SDS polyacrylamide gel situated in a dual gel caster fitted in a buffer chamber (Hoefer, Inc. USA) which was filled with cold (4^oC) SDS electrode buffer. At first electrophoresis was run at 300 V and 15 mA for about 15 minutes until the protein migrated into the separating gel and was then continued at 300 V and 25 mA for about 1 hour. During electrophoresis, the buffer in the chamber was kept cold constantly by running cold water through the cooling jacket of the chamber. After electrophoresis, the gel and polyvinylidene fluoride membrane (Millipore Corporation, USA) were fitted in a gel holder cassette (BioRad Laboratories, Italy) which was then placed in a trans blot tank (BioRad Laboratories, Italy). The transblot tank was filled with chilled transfer buffer, and electroblotting was performed at 100 V and 300 mA for 1 hour. During blotting the tank was constantly kept cold by the accessory cooling unit. After the blotting procedure the polyvinylidene fluoride membrane was removed from the gel holder cassette and incubated in blocking buffer (5% skim milk in PBS with 1 ml 2% thimerosal solution) overnight at 4°C to block non-specific sites. On the next day, the membrane was briefly washed with PBST buffer (0.25% Tween-20 in PBS) and then incubated for 2 hours in primary antibody solution. Primary antibodies used for this experiment were same as used for immunohistochemistry i.e. a polyclonal antiserum produced in mice raised against human SULT1E1 (αhum-SULT1E1) (SAB 1400268, Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany) at a protein concentration of 2.6 µg/ml and a polyclonal antiserum produced in a rabbit against bovine recombinant SULT1E1 (Frenette et al. 2009), at a protein concentration of 2.5 µg/ml, respectively, in blocking buffer.

The membranes were washed in PBST buffer (3 changes 7 min. each), and then incubated for 50 minutes with their respective secondary antibody i.e. biotinylated anti-mouse antibody (BA2000, Vector Laboratories, Burlingame, USA) at a dilution of 1:1000 in blocking buffer and biotinylated anti-rabbit antibody (from Vectastain Elite ABC Kit, PK 6101, Vector Laboratories, Burlingame, USA) at a dilution of 1:3000 in blocking buffer. Thereafter, the membranes were again washed in PBST buffer (3 changes 7 min. each) and then covered with streptavidin-peroxidase complex (Vector Laboratories, Burlingame, USA) for 30 minutes. After washing with PBST buffer (3 changes 7 min. each), the membranes were developed in substrate solution (NovaRed substrate kit, Vector Laboratories, Burlingame, USA) for 50 to 90 seconds. Finally the membrane was washed extensively in ddH_2O and air dried before images were captured by a digital camera (DSC-W30, Sony Corporation, Japan).

3.9.2 Western blot analysis of bovine placentomal tissue for the expression of androgen receptor

Coarse pieces of deep-frozen (-80°C) placentomal and epididymal tissue were briefly placed in liquid nitrogen, quickly enveloped in sterile aluminum foil and then reduced to small pieces by strokes with a clean hammer. The resulting tissue particles were then powdered under liquid nitrogen with a pestle in a mortar pre-chilled to -80°C. 200 mg of tissue powder were immersed in cold 1 ml PBS containing Proteinase Inhibitor Cocktail Tablet (Roche Diagnostics, Germany) (one tablet in 10 ml PBS) and homogenized by using an ultra turrax T25 (IKA-Werke GmbH & Co KG, Staufen i. Br., Germany) at highest speed bursts three times for 30 seconds on ice. Then 50 mg SDS powder was added and the samples were shortly vortexed and subsequently boiled in a waterbath for 10 minutes. Finally they were centrifuged at 1260 x g for 10 minutes at 4°C. The supernatants were taken in fresh 2 ml round Eppendorf tubes (Sarstedt, AG & Co., Nümbrecht, Germany) and the total concentrations of protein were measured using a BioPhotometer (Eppendorf AG, Hamburg, Germany) in disposable cuvettes (UVette®, Eppendorf AG, Hamburg, Germany) containing 2 µl of protein preparation and 98 µl ddH_2O using 100 µl ddH_2O as a blank.

Procedures of polyacrylamide gel electrophoresis, blotting, and immunoreaction were as described for SULT1E1 in section 3.9.1 with some minor modifications. For AR, 100 µg protein from placental and epididymal tissue were loaded onto the polyacrylamid gel. The primary antibody was the same as used in immunocytochemistry, a polyclonal antibody against human AR (rabbit polyclonal, N-20, sc816; Santa Cruz Biotechnology, Heidelberg, Germany) which was applied at dilution of 1:200 in blocking buffer. Incubation time for the

primary antibody was 2 hours, after washing the membrane was then incubated for 50 minutes with the secondary biotinylated anti-rabbit antibody (from Vectastain Elite ABC Kit, PK 6101, Vector Laboratories, Burlingame, USA) at a dilution of 1:1000 in blocking buffer. Development of polyvinylidene fluoride membrane in substrate solution (NovaRed substrate kit, Vector Laboratories, Burlingame, USA) was for 7 minutes.

3.10 Measurement of testosterone concentrations in placental tissue

Coarse pieces of deep-frozen (-80°C) placentomal tissue were wrapped in aluminum foil and broken up using a clean hammer. The tissue fragements were quickly placed in a pre-chilled mortar and powdered with a pestel under liquid nitrogen. From each sample 200 mg of tissue powder were transferred quickly into 15 ml extraction vials (Wheaton Scientific, USA) and thoroughly mixed with 3 ml toluene. The extraction vials were then placed into a rotation mixer and rotated overhead for 15 min. After a short centrifugation for a better separation between the organic and aqueous phase, the samples were placed into an ethanol/dry ice bath. After freezing of the aqueous phase the upper organic phase was decanted into test tubes (Combotest Disposable, Sarstedt, AG & Co., Nümbrecht, Germany). Again 3 ml toluene was added to the remaining aqueous phase and the extraction step was repeated. The pooled extracts in the test tubes were evaporated to dryness in a vortex evaporator (Haake Buchler GmbH, Karlsruhe, Germany) at 40°C. The extracts were redisolved in BSA buffer and introduced into radioimmunological measurement using a well-established inhouse method as previously described (Hedberg et al. 2007). Intra-assay and interassay coefficients of variation were 7.8% and 9.0%, respectively. The lower limit of detection was at 0.35 nmol/l (equivalent to 0.1 ng/ml).

3.11 Statistical evaluations

Data from gestational profiles (real-time RT-PCR, qualitative evaluation of immunostaining using IRS) were tested for an influence of the observational group by one-factorial analysis of variance, in case of significant error probability ($p<0.05$) followed by pairwise comparisons of observational groups using Tukey-Kramer test (statistical software GraphPad InStat 3.06, GraphPad Software Inc., San Diego, USA). Data from real-time RT-PCR were transformed logarithmically prior to statistical evaluation due to their obvious right-skewed distribution, and results are presented as geometric mean ($\bar{x}_g$) x dispersion factor$^{\pm 1}$, whereas data from qualitative evaluation of immunostaining using IRS are shown as arithmetic mean ($\bar{x}$) ± standard deviation (SD).

3.12 Solutions and buffers

3.12.1 Immunohistochemistry

10 mM citrate buffer

Stock solution A:	
$C_6H_8O_7$ x H_2O	21.0 g
Aqua dest.	ad 1000 ml
Stock solution B:	
$C_6H_5O_7Na_3$ x $2H_2O$	29.41 g
Aqua dest.	ad 1000 ml
Working solution:	
Stock solution A	9 ml
Stock solution B	41 ml
Aqua dest.	450 ml

Methanolic H_2O_2 solution 0.3%

Methanol	200 ml
30% H_2O_2	2 ml

Ethanol (96%)

Ethanol reinst (100%)	96 ml
Aqua bidest.	4 ml

Ethanol (70%)

Ethanol reinst (100%)	70 ml
Aqua bidest	30 ml

0.5 M EDTA

EDTA	37.22 g
Aqua bidest	ad 200 ml

ICC buffer pH 7.4:

Na_2HPO_4	1.2 g
KH_2PO_4	0.2 g
KCl	0.2 g
NaCl	8.0 g
Aqua dest	ad 1000 ml

3 ml Triton X-100 was added after adjusting pH (between 7.2-7.4)

3.12.2 Gel electrophoresis

TBE buffer (10x)

Tris ultrapure	108 g
Boric Acid	55 g
0.5 M EDTA (pH 8.0)	40 ml
Aqua bidest	ad 1000ml

TBE buffer (1x)

TBE (10x) Buffer	100ml
Aqua bidest	ad 1000ml

3.12.3 Western blot

Collecting gel buffer pH 6.8

Tris Ultrapure	7.88 g
Aqua bidest.	ad 100 ml

Adjustment of pH was performed using concentrated HCl or NaOH solution

Blocking buffer

Dry milk powder	5 g
1x PBS buffer	ad 100 ml

10x electrode buffer

Glycine	144 g
Tris-base	30 g
SDS	10 g
Aqua bidest	ad 1 liter

10x PBS stock solution

NaCl	80.0 g
$Na_2HPO_4 \times 2H_2O$	11.5 g
KCl	2.0 g
KH_2PO_4	2.0 g
Aqua bidest.	ad 1 liter

1x PBS

10x PBS stock solution	100 ml
Aqua bidest.	ad 1 liter

PBST-buffer

PBS-buffer	1000 ml
Tween 20	2.5 ml

10% aqueous SDS solution

SDS	10 g
Aqua bidest.	ad 100 ml

3x Sample buffer/loading dye

Collecting gel buffer	1.75 ml
Glycerol	1.50 ml
10% SDS	50 ml
ß-mercaptoethanol	0.50 ml
Bromphenol blue	1.25 ml

Separating gel buffer pH 8.8

Tris	23.64 g
Aqua bidest.	ad 100 ml

Adjustment of pH was performed using concentrated HCl or NaOH solution

3.12.4 Measurement of testosterone

Charcoal suspension

Charcoal (Norit A)	2.5 g
Dextran 60	0.25 g

Suspended in 500 ml aqua dest.

Phosphate buffer (pH 7.2)

KH_2PO_4	2.686 g
Na_2HPO_4	8.356 g
NaN_3	0.325 g
Aqua dest.	ad 1000 ml

Phosphate buffer with 0.1% BSA

BSA	1 g
Phosphate buffer	ad 1000 ml

3.12.5 Kits and reagents

ABC-Kit, Standard Kit: Vector Laboratories, Burlingame, USA

Agarose Multi-purpose: BIO-41025, Bioline GmbH, D-14943 Luckenwalde, Germany

ABsolute™ Blue QPCR SYBR ®: Art. Nr. AB-4219/B, Thermo Fisher Scientific Abgene House, Blenheim Road, Epsom KT19 9AP, United Kingdom

Ammonium Peroxidosulphate: Carl Roth GmbH & Co., Karlsruhe, Germany

BA2000; horse anti-mouse IgG biotinylated antibody: Vector Laboratories, Burlingame, USA

Boric acid, Pufferan® (H_3BO_3): Art. Nr.: 6943.2, Carl Roth GmbH & Co., Karlsruhe, Germany

Calcium Chloride: Merck KGaA, Darmstadt, Germany

Chloroform: Roth GmbH & Co., Karlsruhe, Germany

Dextran 60: Serva, Heidelberg, Germany

DNA-Ladder Gene Ruler 100bp, Catalogue Nr. SM0241 and SM0321: MBI Fermentas GmbH, 68789 St. Leon-Rot, Germany

Dnase 1 Rnase free 10U/µl: Roche Diagnostics GmbH, Mannheim, Germany

EDTA: Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany

Ethanol 99.6%: Roth GmbH & Co., Karlsruhe, Germany

Ethidiumbromide solution 1%: Roth GmbH & Co., Karlsruhe, Germany

Formaldehyde powder: Merck KGaA, Darmstadt, Germany

Gene Amp RNA Core Kit®: Perkin Elmer, Foster City, CA, USA /Applied Biosystems GmbH, Weiterstadt, Germany

Glycerol: Roth GmbH & Co., Karlsruhe, Germany

Histokit: Assistent, Osterode, Germany

Hydrochloric acid: Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany

Hydrogen peroxide 30 %, Art. Nr. 64271:, Merck KGaA, Darmstadt

Hematoxilin: Merck KGaA, Darmstadt, Germany

Isopropanol: Roth GmbH & Co., Karlsruhe, Germany

Methanol: Merck KGaA, Darmstadt, Germany

NovaRed substrate Kit: Vector Laboratories, Burlingame, USA

Potassium Chloride: Merck KGaA, Darmstadt, Germany

Primary antibody AR sc-816: Santa Cruz Biotechnology, Heidelberg, Germany

ProteoExtract Transmembrane Protein Extraction Kit: Novagen, Merck KGaA, Darmstadt, Germany

Protease Inhibitor Cocktail Tablet: Roche Diagnostics GmbH, Roche Applied Science, 68305 Mannheim, Germany

Rotiphorese® Gel 30: Carl Roth GmbH & Co., Karlsruhe, Germany

Rotiphorese® 10x SDS PAGE: Carl Roth GmbH & Co., Karlsruhe, Germany

RNase Inhibitor 40U/µl: MBI Fermentas GmbH, 68789 Sankt Leon-Rot, Germany

RT-PCR Core Kits: Applied Biosystems GmbH, Karlsruhe, Germany

Secondary antibody biotinylated anti-mouse BA 2000: Vector Laboratories, Burlingame, USA

Secondary antibody biotinylated anti-rabbit ABC Kit: Vector Laboratories, Burlingame, USA

Goat Serum from ABC Kit: Vector Laboratories, Burlingame, USA

Skim Milk Powder: J. M. Gabler Saliter GmbH & Co., Obergünzburg, Germany

Sodium Chloride: Merck KGaA, Darmstadt, Germany

Sodium Hydroxide: Merck KGaA, Darmstadt, Germany

Sodium hydrogenphosphate: Merck KGaA, Darmstadt, Germany

Sodium Dodecyl Sulfate, Article No. L-5750: Sigma Chemical Company, St. Louis, MO 63178, USA

Taqman® qPCR Mastermix: Eurogentec Seraing, Belgium

Thiomersal: Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany

Toulene: Merck KgaA, Darmstadt, Germany

Tween 20 (Polyoxyethylenesorbitan Monolaurate, Product No. P-1379): Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany

Tris: Roth GmbH & Co., Karlsruhe, Germany

Triton X-100: SERVA electrophorsis Heidelberg, Germany

Trizol® Reagent: Invitrogen Karlsruhe, Germany

Xylol: Merck KGaA, Darmstadt, Germany

3.12.6 Materials and equipment

ABI PRISM_7000 Sequence Detection System: Applied Biosystems, D-64293 Darmstadt, Germany

Agarose Gel Electrophoresis System (multiSub) Art. Nr. 615162: Biozym Scientific GmbH, D-31833 Oldendorf, Germany

Blotting Gel Paper 7 cm x 10 cm P7796: Sigma-Aldrich Chemicals GmbH, Deisenhofen, Germany

Biorad CFX 96 C1000 Thermal Cycler: Biorad Laboratories, Inc. 2000 Alfred Nobel Drive, Hercules, CA 94547, USA.

Centrifuge Compoact Bench Type Micro 22R: Andreas Hettich GmbH & Co. KG, D-78532 Tuttlingen, Germany

Centrifuge 0-6000 U/min: Heraeus Christ GmbH, Hanau, Germany

Dual gel caster, for 10 cm x 8 cm or 10 cm x 10.5 cm plates, SE245: Hoefer Inc., 84 October Hill Road Holliston, USA

Electrophoresis Power Supply, EPS 301: Amersham Biosciences, GE Healthcare Europe GmbH, Freiburg, Germany

Electrophoresis, Power Supply 2301 Microdrive Sr. No. 2188: LBK Bromma, USA

Eppendorf tubes 1.5 ml, 2 ml round color/colorless: Sarstedt, AG&Co., D-51582 Nümbrecht, Germany

Eppendorf Biophotometer: Eppendorf AG, Hamburg, Germany

Filter tips: 10 µl, 100 µl, 1000 µl: Nerbe plus GmbH, 21423 Winsen/Luhe, Germany

Filter tips: 10 µl, Gel 20 (20 µl): Greiner Bio-One GmbH, Frickenhausen, Germany

Gel Electrophoresis Unit Mighty Small II SE250/SE260: Hoefer Inc., 84 October Hill Road, Holliston, USA

Glass plates 10 cm x 10.5 cm: Hoefer Inc., 84 October Hill Road, Holliston, USA

Glass tubes 15 ml: Wheaton Scientific, New Jersy, USA

Glass tubes for Combotest, No./REF.86.1509: Sarstedt Aktiengesellschaft & Co., D-51558 Nümbrecht, Germany

Hand gloves UniGloves®: purchased from MAGV Laborbedarf & Laborgeräte, Rabenau-Londorf, Germany

Immobilon Transport Membrane, pore size 0.45 µm, Cat. No. IPHV0010: Millipore Corporation, Bedford, USA

Microscope Leitz DMRB with Digital Camera Leica DC300 and Leica IM-Software: Leica Microsystems GmbH, 35578 Wetzlar, Germany

Microtome RM2125RT: Leica Biosystem GmbH, Nussloch, Germany

Microtome blade Leica DB 80L: Leica Microsystems GmbH, Nussloch, Germany

Mini Trans Blot Tank with Gel Cassettes Model No. 37/S Serial Number 7300: BioRad Laboratories, Italy

Munktell Paper Sheet 200 x 500 mm 80 g/m^2: Munktell & Filtrak GmbH, Niederschlag D-09471 Bärenstein, Germany

Pap-pen, Code S2002: Dako Deutschland GmbH, D-22769 Hamburg, Germany

pH-Meter Inolab Level 1, WTW: Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany

Pipettes: 10 µl, 20 µl, 100 µl, 1000 µl: Eppendorf AG, Hamburg, Germany

QIAEX II Gel Extraction Kit, Catalog no. 20021: Qiagen GmbH, D-40724 Hilden, Germany

Reactions tubes, Art. Nr. 711098: Biozym Scientific GmbH, D-31833 Hess. Oldendorf, Germany

Reactions tubes: Biozym Scientific GmbH, D-31833 Oldendorf, Germany

SuperFrost® Slides, 24 mm x 40 mm, 24 mm x 50 mm and 24 mm x 60 mm: Menzel-Gläser, purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany

T1 Thermocycler 49: Whatman Biometra, Göttingen, Germany

Ultra turrax T10 basic: IKA-Werke GmbH & Co KG, Staufen Br., Germany

UV-Spectrometer, BioPhotometer: Eppendorf AG, Hamburg, Germany

UV-Dispoasable Uvette® 220-1600 nm: Eppendorf AG, Hamburg, Germany

UV-Transluminator: Biostep, 09387 Jahnsdorf, Germany

Water bath Typ WB-24; V220; W 550; Fabrik-Nr. 8810, max. 90°C: Medax Nagel, KG Kiel, Germany

UV-Transilluminator with Photoshop software Phoretix Grabber 3.01: Biostep, 09387 Jahnsdorf, Germany

Vortexer: Heidolph REAX control, purchased from MAGV Laborbedarf, Rabenau-Londorf, Germany

Vortex evaporator: Haake Buchler GmbH, Karlsruhe, Germany

Warm chamber: Type: 3 26, 220 Volt, 380 Watt, 50/60 Hz, 40050-IP20, Memmert, Schwabach, Germany

4. RESULTS

4.1 Expression of SULT1E1

4.1.1 SULT1E1 protein expression in bovine placentomes

4.1.1.1 Confirmation of specificity of the primary antibodies applied by western blot analysis

To confirm the specificity of the two antisera used for the detection of SULT1E1 in bovine placentomes, i.e. a polyclonal murine antiserum against human SULT1E1 (αhum-SULT1E1) and a polyclonal antiserum against recombinant bovine SULT1E1 (αbov-SULT1E1), western blot experiments were performed using a protein extract prepared from cotyledonary tissue of a parturient cow. For comparison, protein preparations obtained from two bovine fetal livers at different stages of gestation and from an adult bovine liver were included into the experiment. The two antisera applied yielded virtually identical results (Fig. 4 A and B). In cotyledonary tissue and fetal livers, a prominent band of the expected molecular size (approximately 33 kDa) was found. This band was only weak in adult liver, where the prominent band was at 72 kDa, which is consistent with SULT1E1 dimers. SULT1E1 expression was clearly higher in the cotyledon compared to fetal and adult liver.

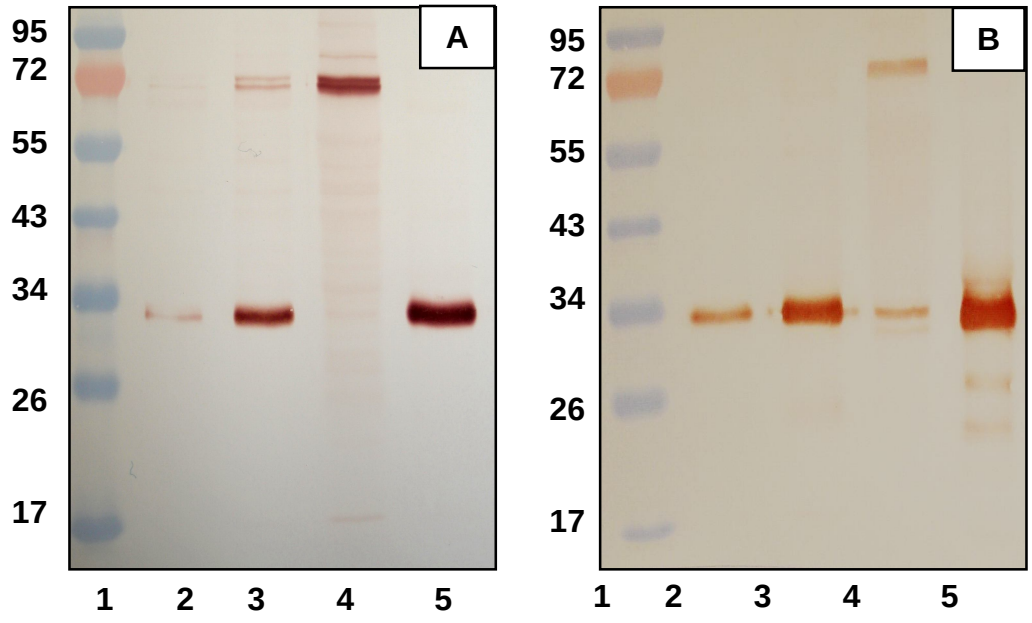


Figure 4: Western blot analysis of homogenates prepared from a cotyledon of a parturient cow and from fetal and adult livers for the expression of SULT1E1. As primary antibody, **(A)** a polyclonal murine antiserum against human SULT1E1 and **(B)** a polyclonal rabbit antiserum against recombinant bovine SULT1E1 were used. The

amounts of protein loaded per lane were 5 µg for the cotyledon and 20 µg for the livers. The expected molecular weight of monomeric SULT1E1 is about 33 kDa.
Lane 1: molecular weight marker (kDa)
Lane 2: fetal liver (day 185 of gestation)
Lane 3: fetal liver (day 210 of gestation)
Lane 4: adult liver
Lane 5: cotyledon of a parturient cow

When testing cotyledonary samples from cows at various stages of gestation and parturition in western blot experiments, again virtually identical results were obtained with the two primary antibodies used (Fig. 5). With both antibodies, a prominent band consistent with monomeric SULT1E1 (approx. 33 kDa) was detected in each of the samples investigated. This band was only weak in samples obtained from the first trimester but its intensity was significantly higher in cotyledonary protein preparations representing mid- until late gestation and parturition. With αhum-SULT1E1, only the band at 33 kDa was detected (Fig. 5A). Using αbov-bovine SULT1E1, besides the prominent band at about 33 kDa a weaker band of about the double size of SULT1E1 occurred in samples from midgestation until parturition (Fig. 5B).

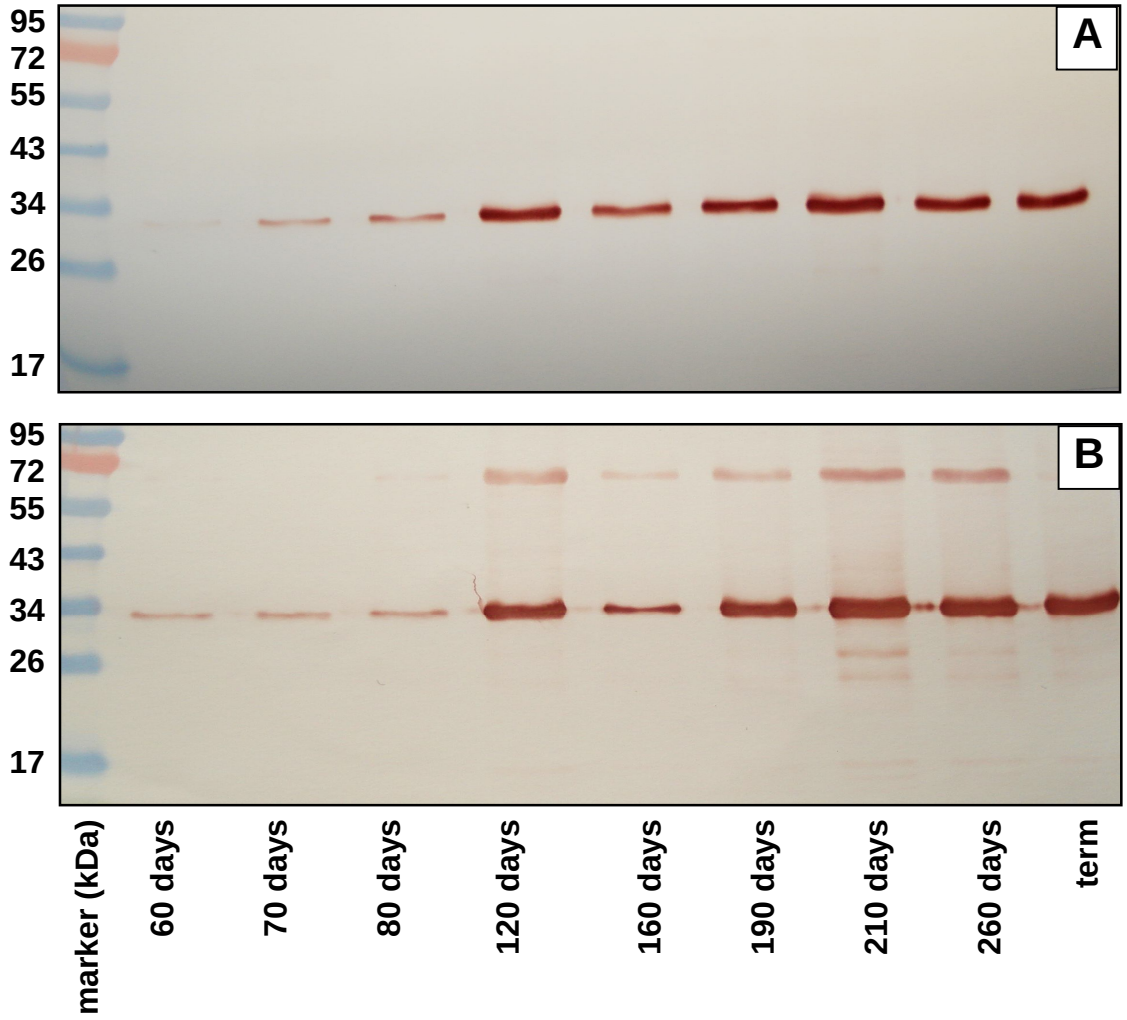


Figure 5: Western blot analysis of homogenates prepared from bovine cotyledons between day 60 of gestation and parturition using **(A)** a polyclonal murine antiserum against human SULT1E1 and **(B)** a polyclonal rabbit antiserum against recombinant bovine SULT1E1. Amount of protein loaded was 5 µg per lane. The expected molecular weight of monomeric SULT1E1 is about 33 kDa.

4.1.1.2 Expression pattern of SULT1E1 protein in bovine placentomes during gestation on a cellular level

By immunohistochemistry when using the two different primary antibodies in bovine placentomes virtually identical staining patterns were obtained (Fig. 6). Independent from the stage of gestation, significant immunostaining was only found in the trophoblast, where it was localized in the cytoplasm of UTC but was completely absent in mature TGC. In immature TGC a loss of signal intensity was observed at early stages of differentiation (Fig. 6/A3-A6;

B3 and B6). When using αbov-SULT1E1, in some of the sections of early pregnant animals (day 80, day 120) and of prepartal animals a cytoplasmic signal was also seen in the caruncular epithelium (Fig. 6/B4). However, in these cases signal intensity in caruncular epithelial cells was substantially lower compared to the UTC. With αhum-SULT1E1 no staining was observed in caruncular epithelial cells (Fig. 6/A4). No staining was observed in negative controls (Fig. 7), in which the respective primary antiserum was replaced by nonspecific immunoglobulin of the same species.

With αbov-SULT1E1 (Fig. 6/B1-7), distinct signals were found in UTC of the chorionic plate irrespective of gestational age (Fig. 6/B1). In trophoblast of chorionic villi, a gradient of staining intensity in UTC was observed with generally higher IRS in the trophoblast covering stem villi and basal secondary villi compared to the trophoblast of more apical parts of the chorionic villous tree (Fig. 8B). However, on some sections from animals at early or midgestation, intense staining was also observed at the tips of chorionic villi immediately adjacent to the caruncular stalk (Fig. 6/B2). At late gestation and at parturition, the gradient of staining intensity was less pronounced as in the distal part of the villous tree a more homogenous stronger immunostaining was observed comparable to the one in the chorionic plate and major stem villi (Fig. 6/B5 and B7; Fig. 8B). However, as for the chorionic plate and the basal parts of the chorionic villi, also for the their apical parts statistical evaluation for an influence of the observational group on IRS values applying one-factorial ANOVAs did not yield significant error probabilities. This was obviously due to the high variability of staining intensity between animals of the individual observational groups in relation to the small group sizes. With αhum-SULT1E1, a qualitatively identical staining pattern was observed (Fig. 6A/1-7; Fig. 8A). However, the tendency for an overall increase of SULT1E1 expression at the end of gestation as found with αbov-SULT1E1 was not obvious when using the primary antiserum against the human enzyme.

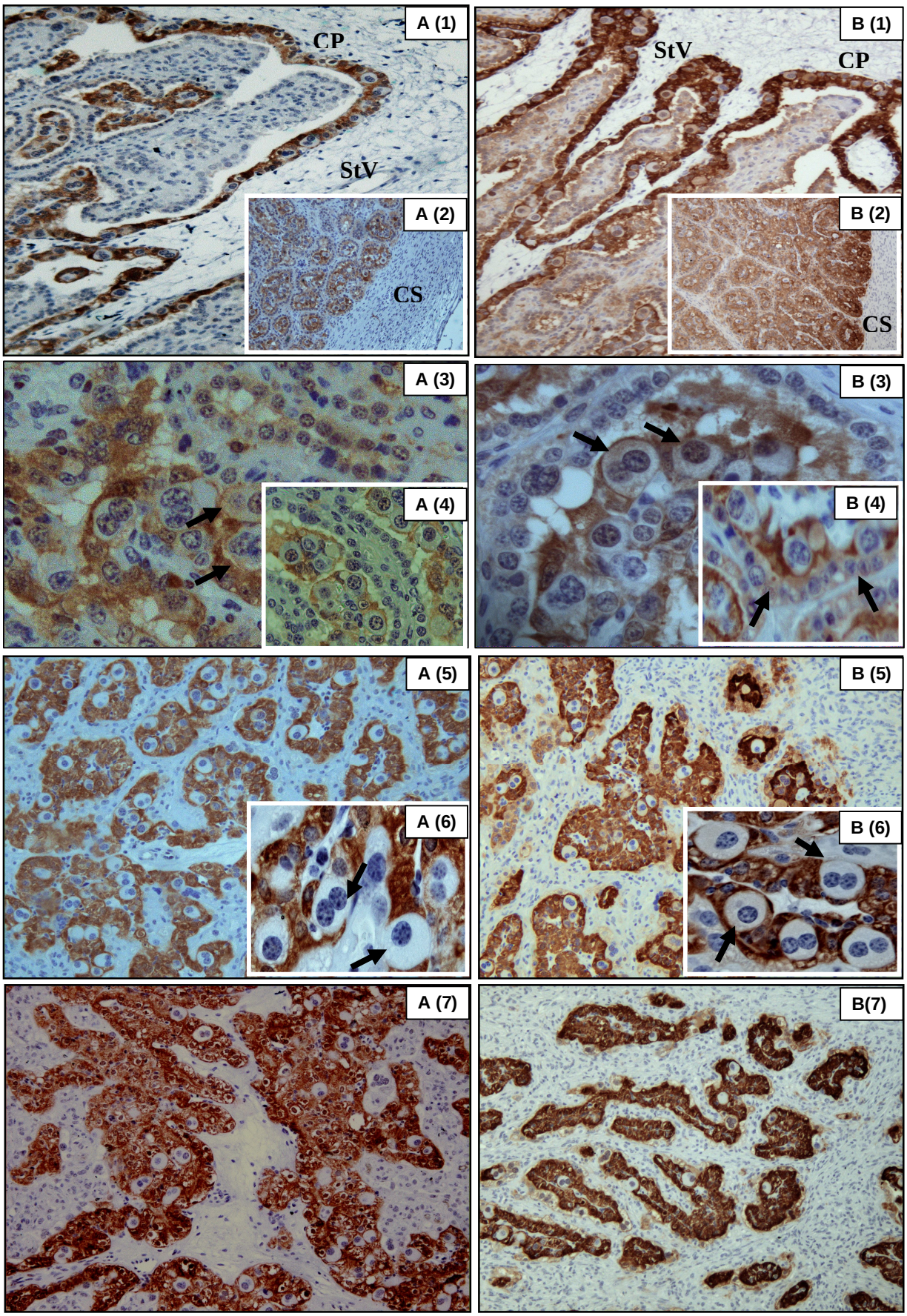

A (1)
CP
StV
A (2)
CS
B (1)
StV
CP
B (2)
CS
A (3)
A (4)
B (3)
B (4)
A (5)
A (6)
B (5)
B (6)
A (7)
B(7)

Figure 6: Localization of SULT1E1 in bovine placentomes between day 80 of gestation and parturition using **(A)** a polyclonal murine antiserum against human SULT1E1 and **(B)** a polyclonal rabbit antiserum against recombinant bovine SULT1E1. Micrographs in the same row are deriving from the same tissue block, respectively. **A1, B1**: Placentome from an 80 days pregnant cow. Intense cytoplasmic immunostaining is present in uninucleated trophoblast cells (UTC) covering the chorionic plate (CP) and the basal parts of chorionic stem villi (StV). **A2, B2:** Placentome from an 80 days pregnant cow. Pronounced immunostaining in UTC covering the tips of chorionic villi adjacent to the caruncular stalk (CS). **A3, B3**: Placentome from a 120 days pregnant cow. Strong immunostaining in UTC. Immunostaining in differentiating, immature trophoblast giant cells (TGC) - which is of weak intensity - is sporadically seen (arrows). **A4, B4:** Placentome from a 120 days pregnant cow. In addition to distinct signals in UTC, weaker immunostaining is also visible in caruncular epithelial cells (arrows). However, this was only observed with the antiserum against bovine SULT1E1 (Fig. B4) but not with the antiserum against human SULT1E1 (Fig. A4). **A5, B5, A6, B6:** Placentome from a 272 days pregnant cow: irrespective of the localization within the villous tree, intense cytoplasmic staining is visible in UTC. No immunostaining is seen in TGC at various stages of differentiation (arrows). **A7, B7**: Placentome from a parturient cow: intense cytoplasmic immunostaining is present in UTC, whereas TGC, of which the number is significantly reduced at term, are still negative. Magnification: A1, A2, A5, A7: x100; A3 A4, A6, B3, B4, B6: x400.

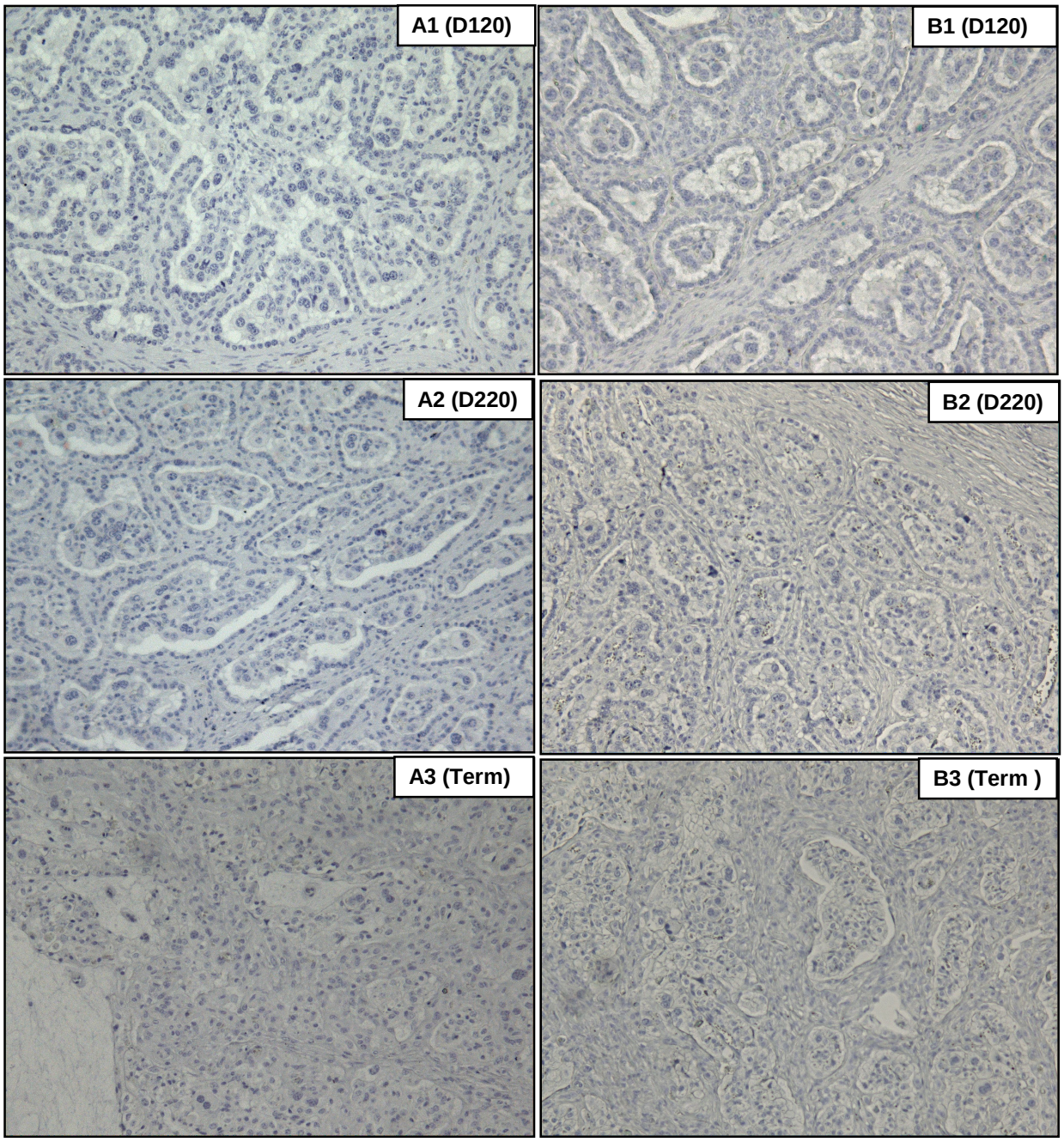


Figure 7: Negative control sections prepared from placentomes of 120 and 220 days pregnant and parturient cows. Micrographs in the same row are deriving from the same tissue block, respectively. The specific primary antibodies against human and bovine SULT1E1 were replaced **(A)** by serum of a non-immunized mouse and **(B)** by serum of a non-immunized rabbit, respectively. No staining is found in any section. Magnification: x100.

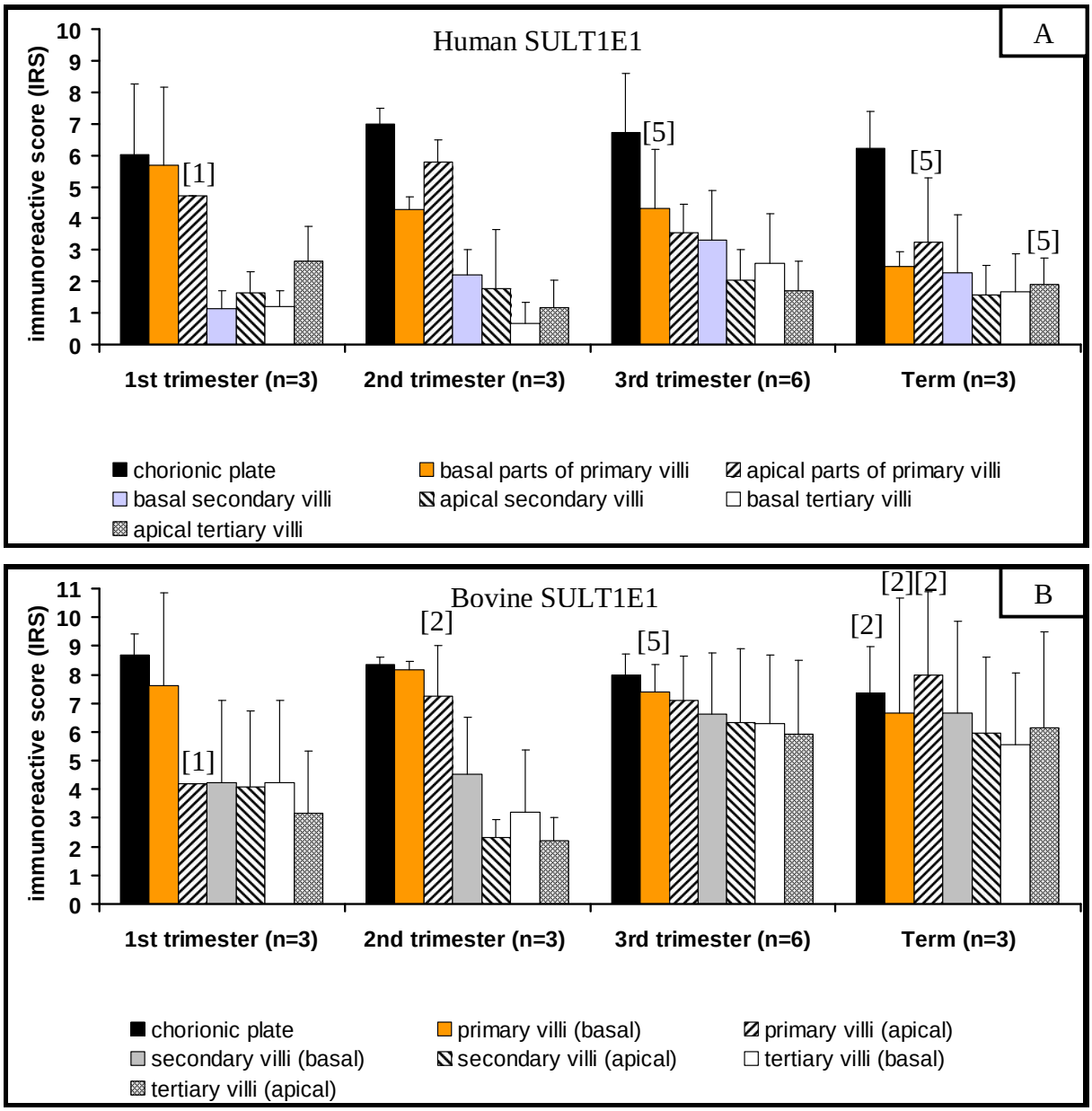


Figure 8: Results from semi-quantitative evaluation of immunostaining for SULT1E1 in uninucleated trophoblast cells of the chorionic plate and of defined localizations of the chorionic villous tree throughout gestation and at parturition presented as $\bar{x}$ and SD calculated from immunoreactive scores. Cows were assigned into four observational groups i.e. first trimester (day 60 to 90; n=3), second trimester (day 120 to 160; n=3), third trimester (day 190 to 272; n=6) and normal term (n=3). Immunostaining for SULT1E1 was performed using two different antibodies, **(A)** a polyclonal murine antiserum against human SULT1E1 and **(B)** a polyclonal rabbit antiserum against recombinant bovine SULT1E1. In a low number of cases, individual localizations were missing in the sections evaluated. In some cases, not all localizations under investigation were present in specimen from individual

animals. In these cases, the number of observations is indicated in squared brackets.

4.1.2 Relative levels of SULT1E1-mRNA in bovine placentomes during gestation

In order to measure relative SULT1E1-mRNA expression levels in the fetal part of bovine placentomes during the course of gestation and at parturition, a real-time RT-PCR method was established. To test for functionality of the primers designed for real-time RT-PCR, a conventional RT-PCR was performed using cotyledonary RNA preparations from cows at various stages of gestation and from parturient cows. In each sample, only one band of the expected size (229 bp) was detected (Fig. 9).

When measuring SULT1E1-mRNA levels by real-time RT-PCR in bovine placentomes during the course of gestation and at parturition (Fig. 10), low relative gene expression levels were found during first (2.79 x $1.34^{\pm1}$) and second trimester (2.41 x $2.33^{\pm1}$). However, they were significantly higher during late gestation (7.31 x $1.39^{\pm1}$) and at normal term (9.61 x $1.17^{\pm1}$). One-factorial ANOVA for an influence of the observational group yielded an error probability of p=0.0043.

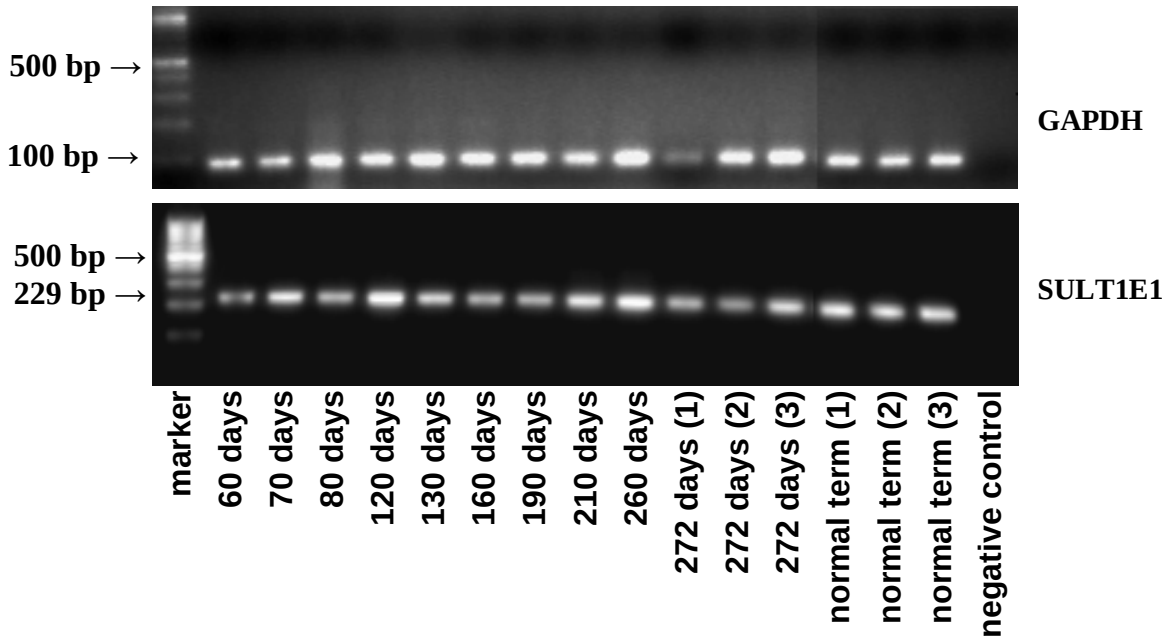


Figure 9: Analysis of RT-PCR products on agarose gel (2%) stained with ethidium bromide and visualized under UV illumination for the presence of SULT1E1 expression in the fetal part (cotyledons) of bovine placentomes obtained from cows at various stages of gestation and at normal term. The calculated size of amplicons is 229 bp. GAPDH was used as procedural and loading control. The expected size of amplicons is 82 bp. For negative control, RT-PCR was performed in the absence of RNA during reverse transcription.

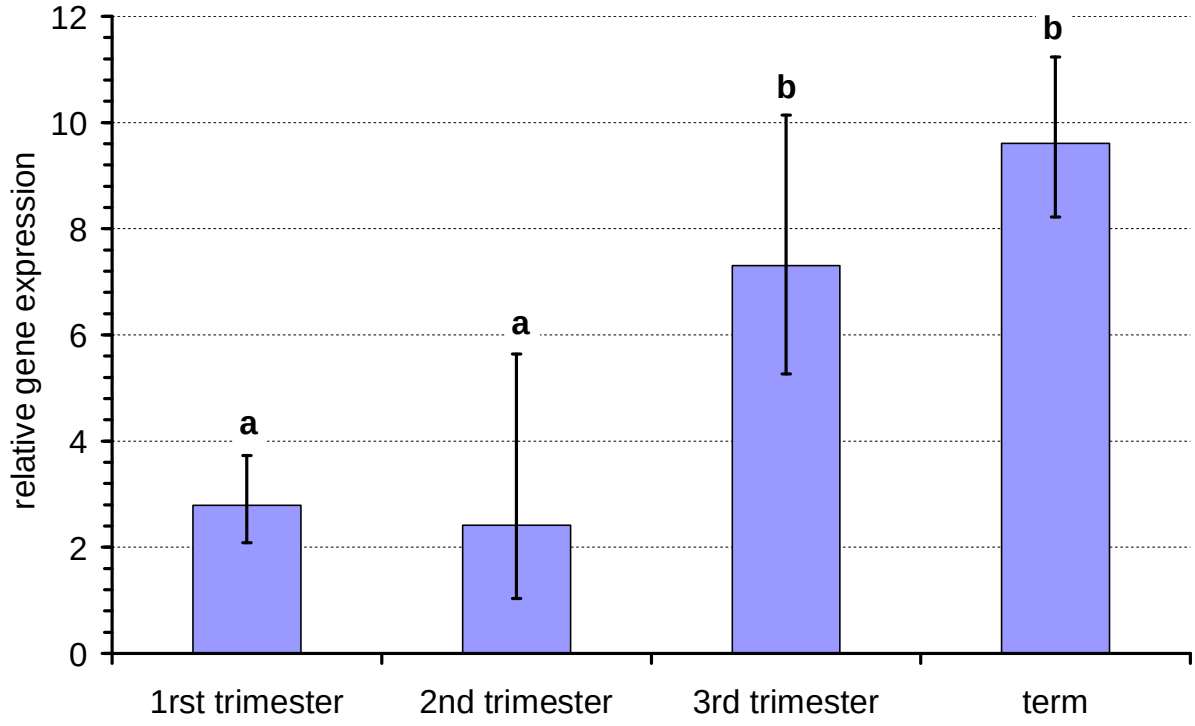


Figure 10: Relative expression of SULT1E1-mRNA in the fetal part of placentomes (cotyledons) from cows assigned into four observational groups i.e. first trimester (day 60 to 80; n=3), second trimester (day 120 to 160; n=3) third trimester (day 190 to 272; n= 6) and at normal term (n=3). Expression levels were measured by real-time RT-PCR (SYBR green method) and normalized to GAPDH used as reference gene. One-factorial ANOVA yielded a significant influence of the observational group (p=0.0043). The results are presented as geometric mean x dispersion factor$^{\pm 1}$. Bars with different superscripts are significantly different with $p < 0.05$ (pair-wise comparison of observational groups using Tukey-Kramer test).

4.1.3 Relative levels of SULT1E1-mRNA in various bovine organs

In a comparative study, various bovine organs were screened quantitatively by conventional RT-PCR for their expression of SULT1E1-mRNA. A band consistent with SULT1E1-mRNA was detected in most of the organs under investigation (Fig. 11). Only for aorta, ovarian stroma and udder no amplicon was obtained. The intensity of the band after agarose gel electrophoresis was high for placentome, fetal liver and adrenal gland. It was moderate in brain, testis, corpus luteum, epidiymis, kidney, skin, muscle, adult liver and weak in lymphatic tissue, spleen, ovarian stroma and colon.

Consistently, with real-time RT-PCR (Fig. 12) highest expression level was found in a placentome from a late pregnant cow, (2851 relative units, R.U.) followed by two fetal livers (748 and 816 R.U.). Among various organs collected from adult cattle, highest SULT1E1-mRNA-expression was found in the adrenal (144 R.U.), followed by skin (91 R.U.) and epididymis (27 R.U.). In contrast to the high SULT1E1 expression in fetal liver, in liver of adult cattle it was only minimal (21 R.U.).

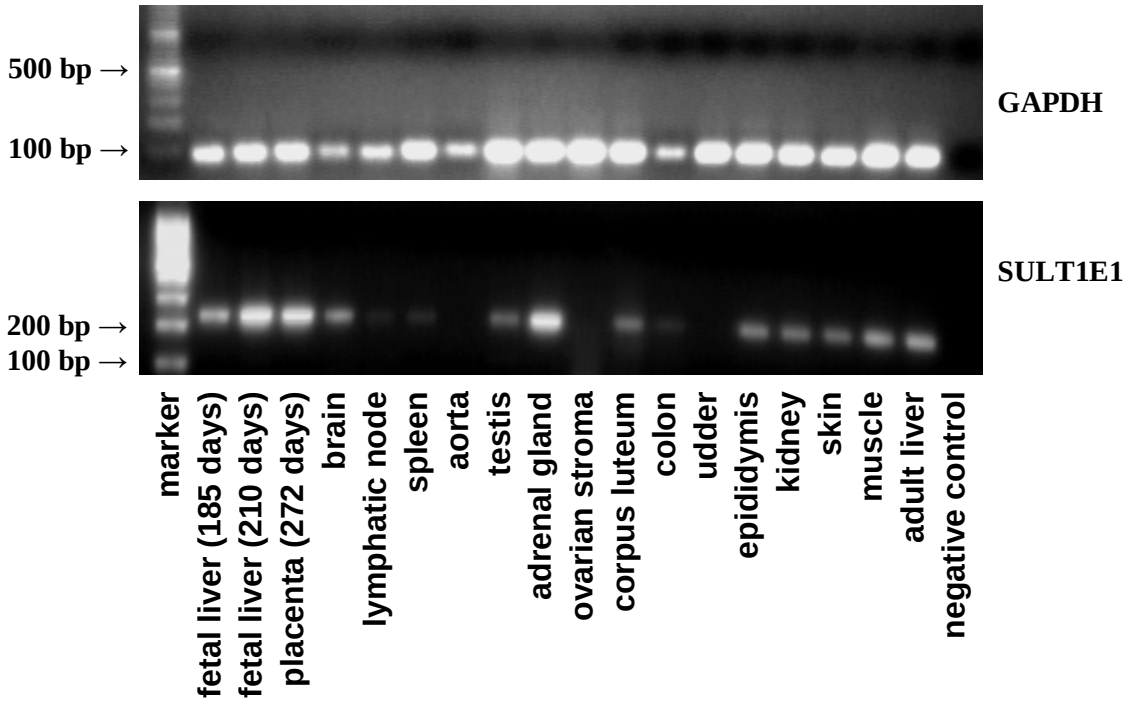


Figure 11: Analysis of RT-PCR products on agarose gel (2%) stained with ethidium bromide and visualized under UV illumination for the presence of SULT1E1 expression in various bovine organs. The calculated size of amplicons is 229 bp. GAPDH was used as a procedural and loading control; the expected size of amplicons is 82 bp. For negative control, RT-PCR was performed in the absence of RNA during reverse transcription.

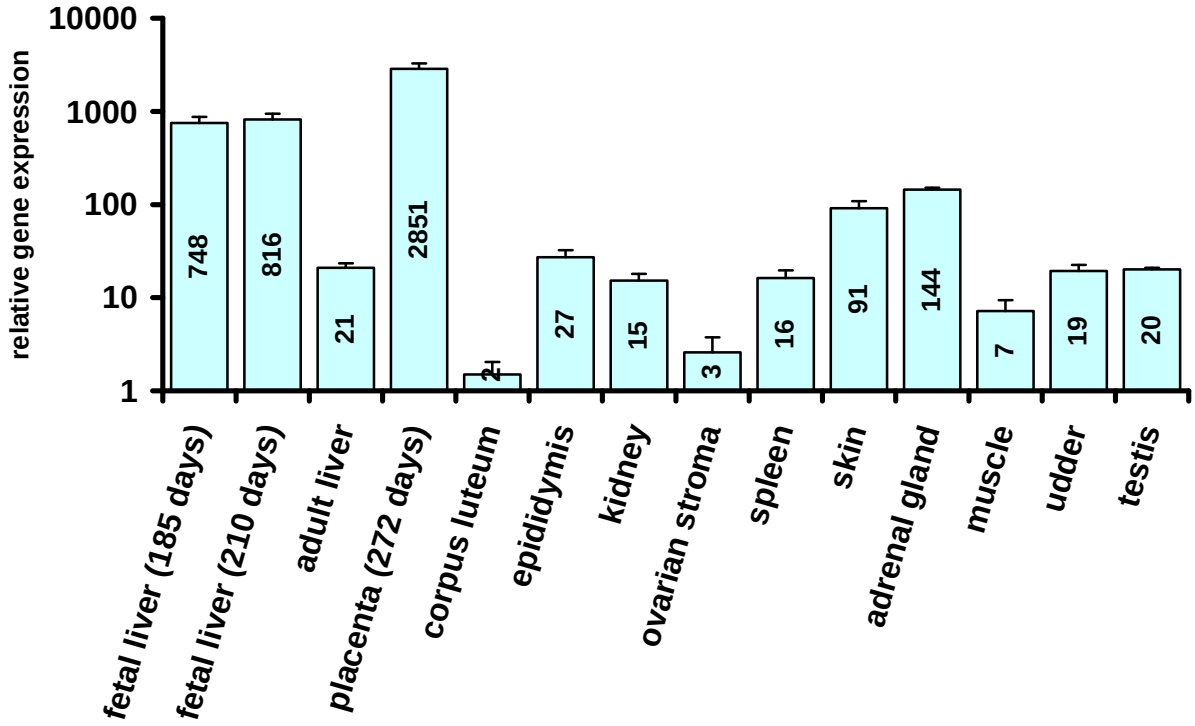


Figure 12: Relative SULT1E1-mRNA levels as measured by quantitative real-time RT-PCR (SYBR green method) in various bovine tissues. Expression levels were normalized to GAPDH used as reference gene. The results are presented as arithmetic mean plus standard deviation calculated from measurements as triplicates.

4.2 Expression of SOAT variants in placentomes and other bovine organs

4.2.1 Expression of mRNA specific for SOAT variants in bovine placentomes by conventional RT-PCR using variant specific primer pairs

Gestational profiles were established on the mRNA level by conventional RT-PCR for each SOAT variant using variant specific primers. As shown in Fig. 13, expression of variants 1,2 and 5 was found in all animals irrespective of gestational age. Variants 3 and 4 were detected in most of the placentomes but were missing in individual animals at various stages of gestation.

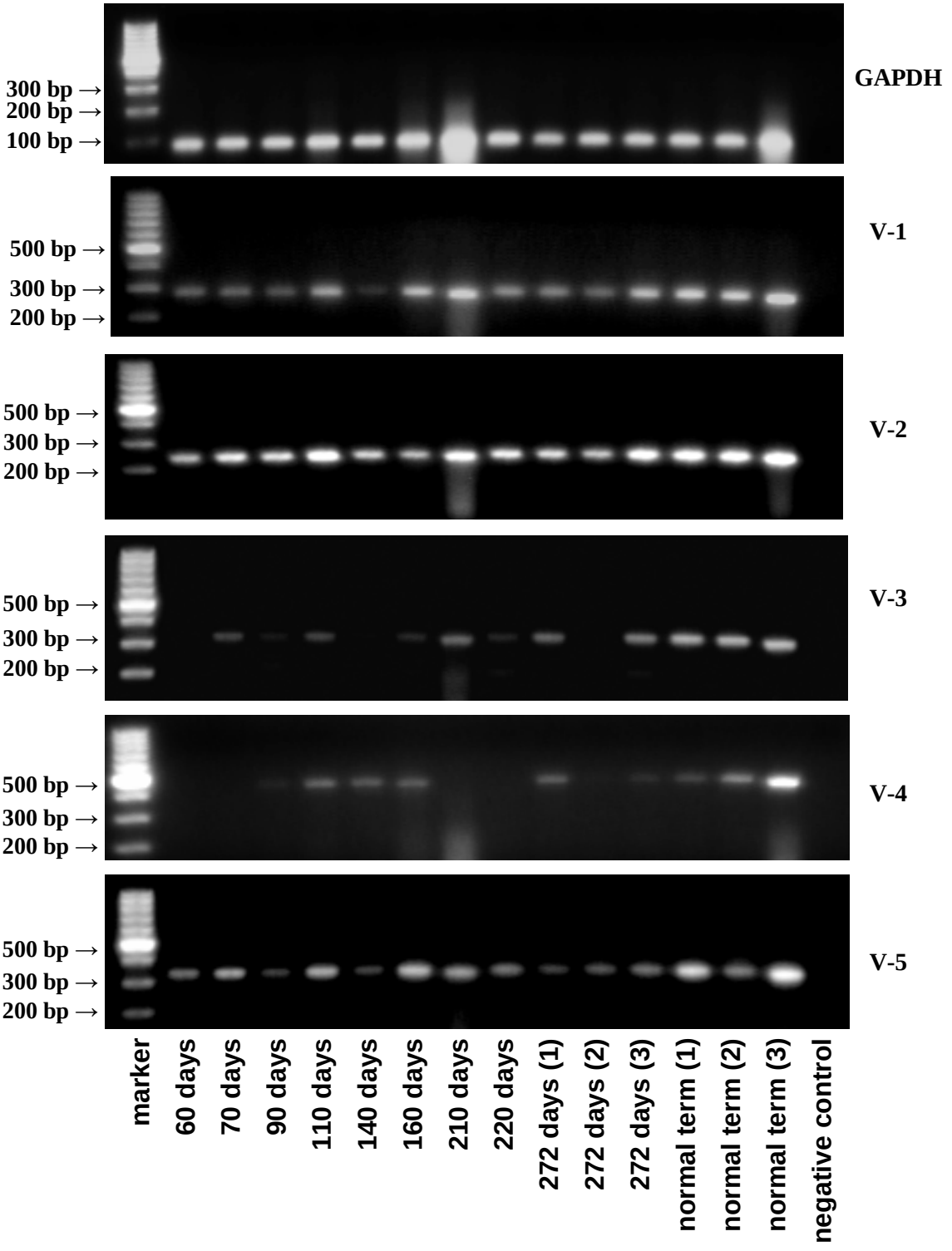


Figure 13: Analysis of RT-PCR products on agarose gel (2%) stained with ethidium bromide and visualized under UV illumination for the presence of SOAT variant expression in bovine placentomes from day 60 to day 272 of gestation using SOAT variant specific primer pairs. At term samples from the maternal part of the placentomes (caruncles) were analyzed. The calculated size of amplicons of SOAT variant 1-5 are 281, 230, 320, 428 and 333 bp respectively. GAPDH was

used as procedural and loading control (82 bp). For negative control, RT-PCR was performed in the absence of RNA during reverse transcription.

4.2.2 Relative levels of mRNA specific for SOAT variants 1, 2, 3 and 5 in bovine placentomes as measured by real-time RT-PCR

Quantitative gestational profiles in bovine placentomes were established for mRNA encoding SOAT variants 1, 2, 3 and 5 using real-time RT-PCR (SYBR green method), whereas for the measurement of SOAT variant 4 mRNA-levels no reproducible method could be established. Expression of SOAT variants 1, 2, 3 and 5 was fairly constant during gestation, whereas expression levels measured at term in the maternal part of the placentomes were considerably higher (Fig. 14).

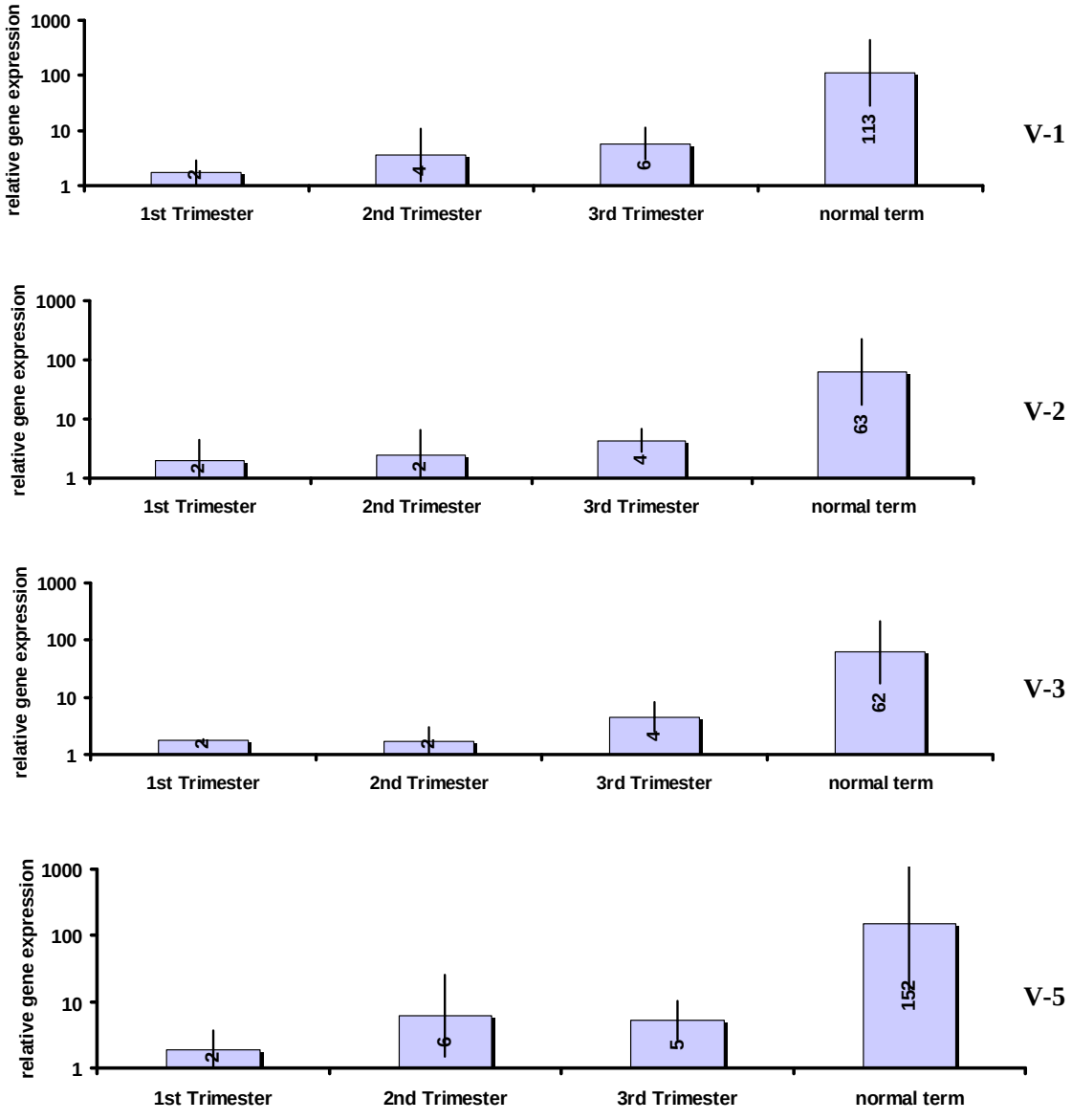


Figure 14: Relative expression levels of mRNA encoding different SOAT variants in bovine placentomes obtained from cows at various stages of gestation (first trimester: day 60-90, n=3; second trimester day: 110-160, n=3; third trimester day 210-272, n=6). At normal term (n=3), samples from the maternal part of the placentomes (caruncle) were analyzed. Expression levels were measured by real-time RT-PCR (SYBR green method) using SOAT variant specific primers and normalized to GAPDH used as reference gene. The results are presented as geometric mean x dispersion factor$^{\pm 1}$.

4.2.3 Expression of mRNA specific for SOAT variants in bovine organs by conventional RT-PCR using variant specific primer pairs

When screening various bovine organs for their expression of SOAT variants by conventional RT-PCR using variant specific primer pairs, for variants 1, 2, 3 and 5 specific bands of generally high intensity were obtained in all samples investigated (Fig. 15). For variant 4, amplicons were only obtained for 6 of 19 organs investigated, which were placentome, brain, ovarian stroma, kidney, heart and skin.

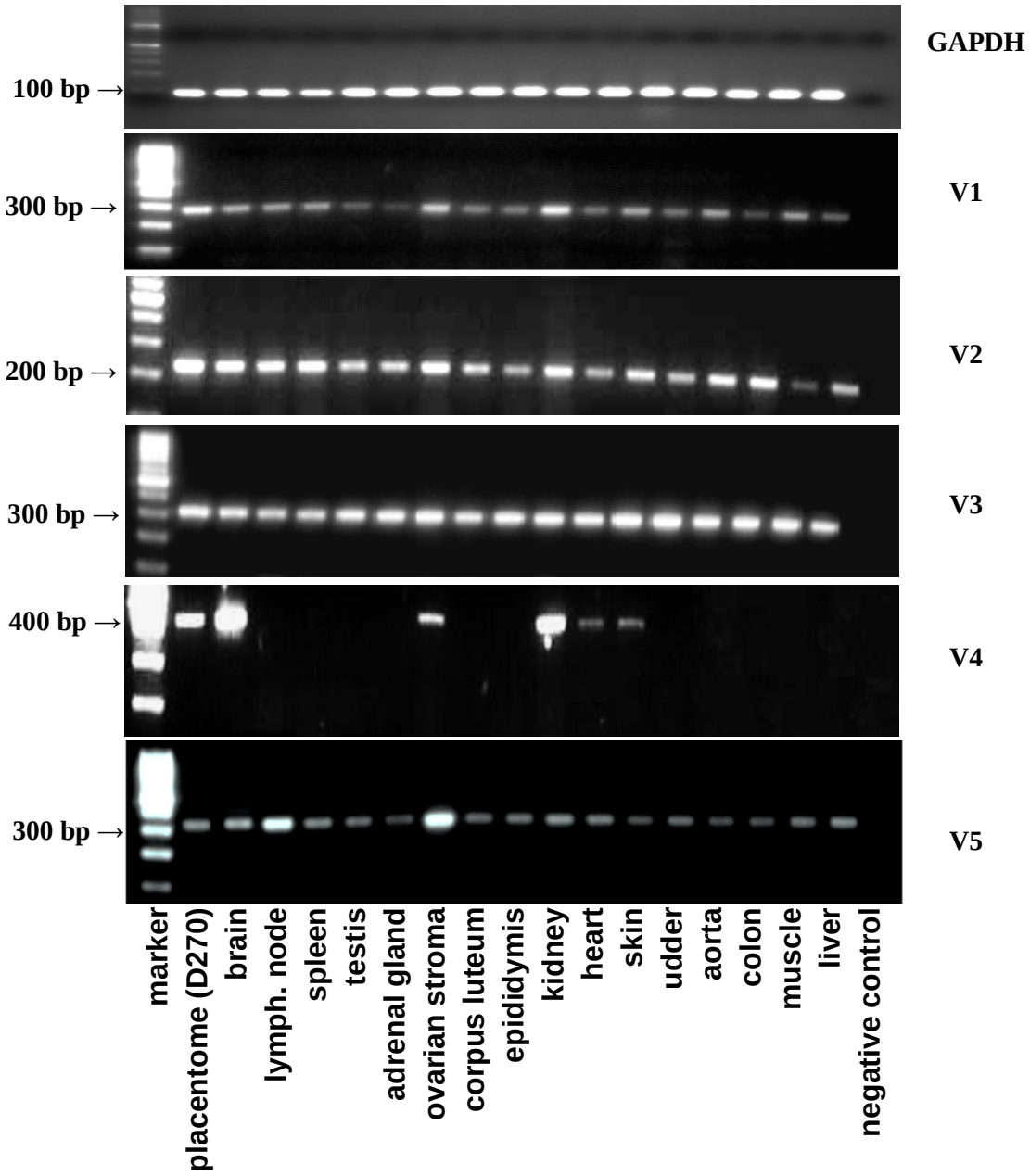
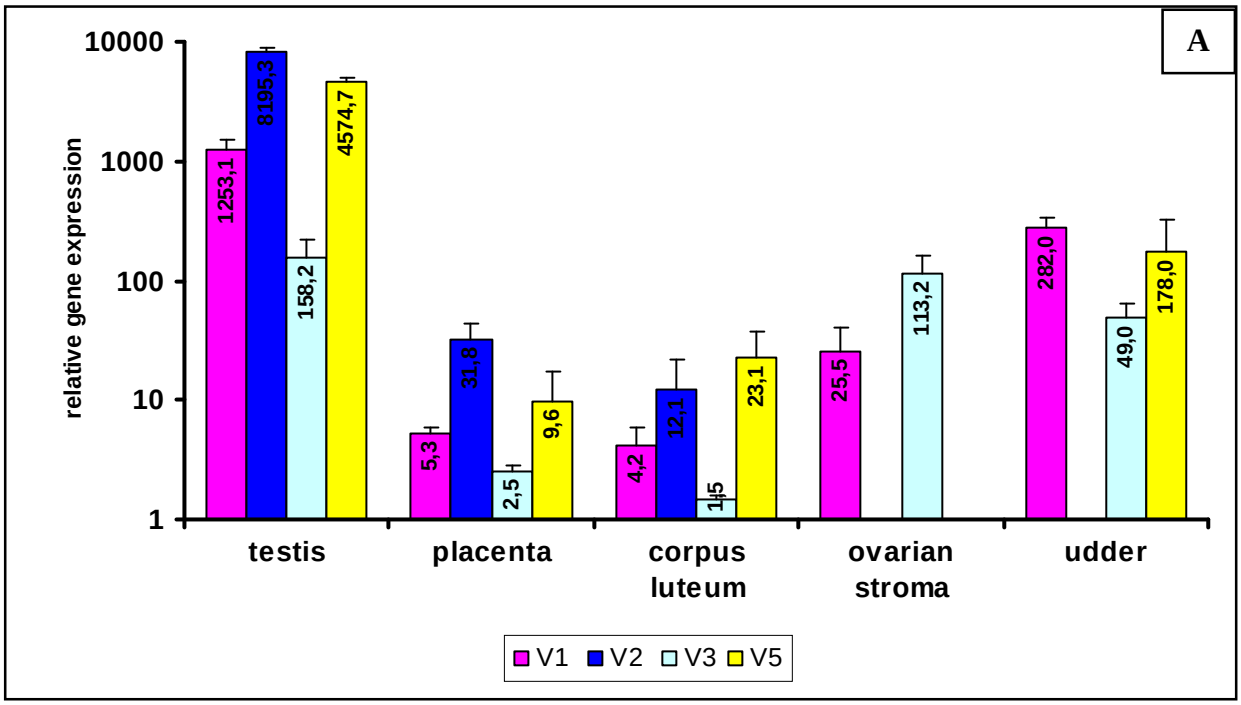


Figure 15: Analysis of RT-PCR products on agarose gel (2%) stained with ethidium bromide and visualized under UV illumination for the presence of SOAT variant expression in various bovine organs using SOAT variant specific primer pairs. The calculated size of amplicons of SOAT variants 1-5 are 281, 230, 320, 428 and 333 bp, respectively. GAPDH was used as procedural and loading control (calculated size of amplicon: 82 bp). For negative control, RT-PCR was performed in the absence of RNA during reverse transcription.

4.2.4 Relative levels of mRNA specific for SOAT variants 1, 2, 3 and 5 in various bovine organs as measured by real-time RT-PCR

When measuring relative levels of mRNA specific for SOAT variants 1, 2, 3 and 5 in various organs by real-time RT-PCR, the spectrum of detectable SOAT variants and their expression levels varied considerable between the individual organs under investigation (Fig. 16). However, for variants 1 (1253 relative units, R.U.), 2 (8185 R.U.) and 5 (4575 R.U.) expression in testis exceeded by far expression levels found in other organs. Other organs found to significantly express SOAT variant 1 were the skin (734 R.U.), the udder (282 R.U.) and the colon (256 R.U.), whereas expression in the placentome (5 R.U.) was comparably low. Besides in the testis, variant 2 was only expressed significantly in the skin (1409 R.U.). Organs with significant expression of variant 3 were the testis (158 R.U.), the ovarian stroma (113 R.U.) and the udder (4.9. R.U.). Expression of variant 5 was only measurable in a limited number of organs, which besides the testis were the udder (178 R.U.), the corpus luteum (23 R.U.) and the placenta (10 R.U.).



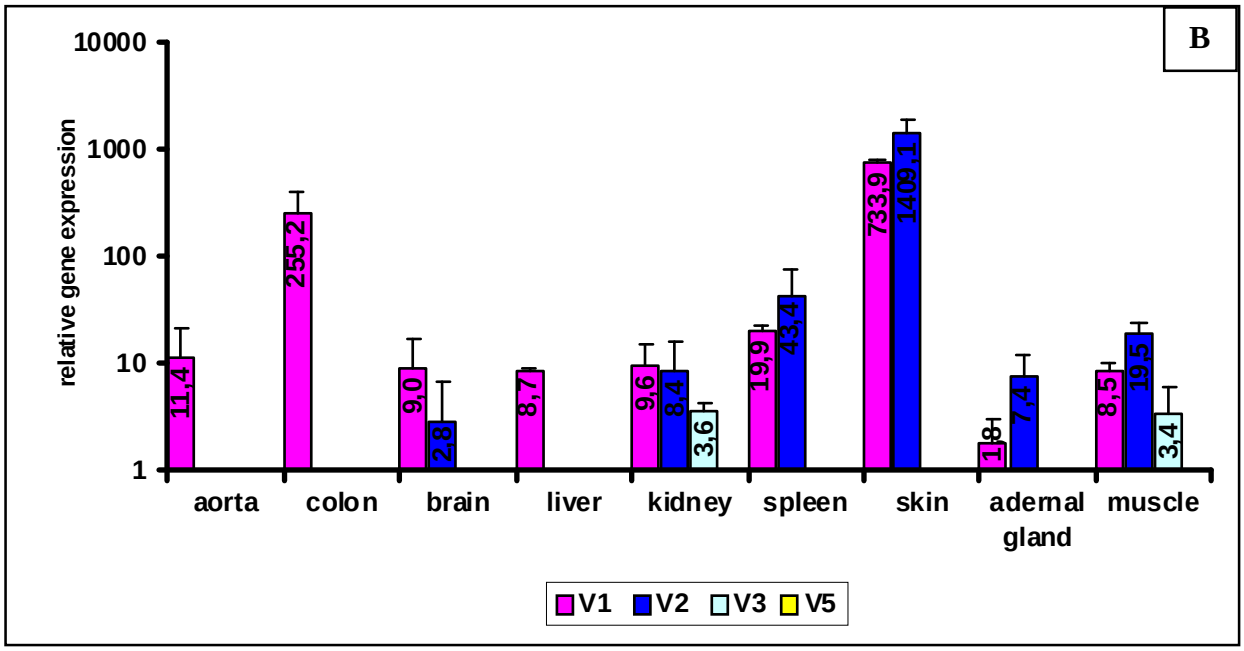


Figure 16: Relative expression levels of mRNA specific for different SOAT variants in bovine reproductive **(A)** and other **(B)** organs. Expression levels were measured by real-time RT-PCR (SYBR green method) using SOAT variant specific primers and normalized to GAPDH used as reference gene. The results are presented as arithmetic mean plus standard deviation calculated from measurements as triplicates.

4.3 Expression of androgen receptor in bovine placentomes and testosterone tissue concentrations

4.3.1 Expression of androgen receptor specific mRNA in bovine placentomes

Placentomes from 12 cows between day 50-272 of pregnancy, from three cows during the prepartal decline in progesterone and from three parturient cows were analyzed by conventional RT-PCR for the expression of AR specific mRNA (Fig. 17). In each sample, one band of the expected size (387 bp) was detected.

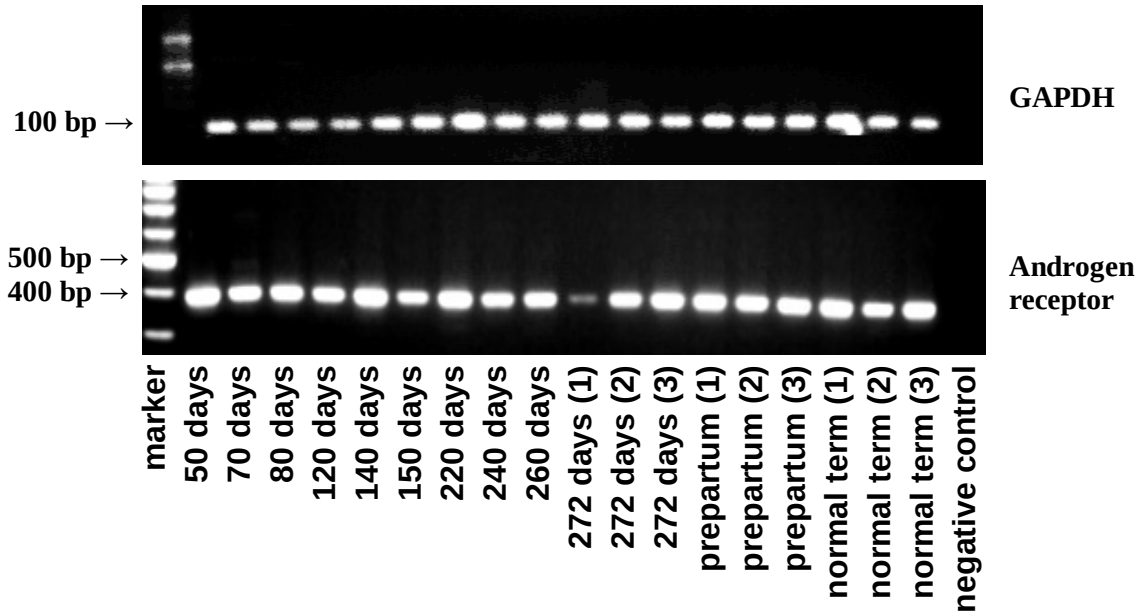


Figure 17: Qualitative detection of mRNA specific for AR in placentomes from cows at various stages of gestation and at parturition. In prepartal cows, placentomes were collected during the prepartal decline in progesterone. Expected size of the amplicons is 387 bp. GAPDH was used as procedural and loading control, the expected size of amplicons is 82 bp. For negative control, RT-PCR was performed in the absence of RNA during reverse transcription.

When measuring relative levels of androgen receptor-specific mRNA in bovine placentomes between day 100 and term by real-time RT-PCR (taqman method), mean relative gene expression values were fairly constant between 2.82-3.12 from day 100 to day 272 and rose slightly to 3.64 in prepartal cows and to 4.17 in parturient animals. However, in a one-factorial ANOVA no influence of observational group on relative gene expression was found (Fig. 18). In placentomes from cows during the second trimester, a high variability of the AR expression levels was observed.

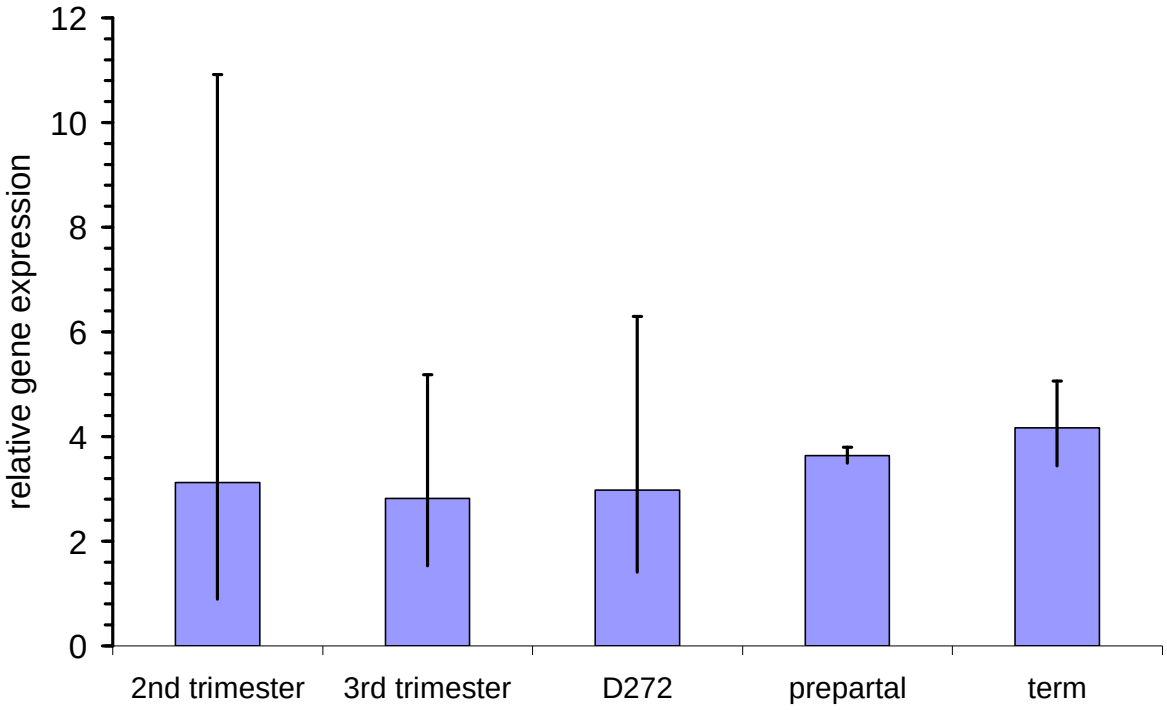


Figure 18: Relative levels of AR-specific mRNA in bovine placentomes between day 100 and term as measured by real-time RT-PCR (taqman method). Each observational group consisted of three animals. In prepartal cows, placentomes were collected during the prepartal decline in progesterone.

4.3.2 Expression of androgen receptor protein in bovine placentomes

4.3.2.1 Confirmation of the specificity of the antibody applied for the detection of androgen receptor in bovine placentomes

In western blot using a primary polyclonal antibody against human AR and a tissue homogenate prepared from a bovine placentome obtained from a cow at day 272 of gestation, one specific band corresponding to a molecular weight of approximately 110 kDa was found, which is consistent with the molecular size of AR (Fig. 19). A band of identical size was detected when a homogenate prepared from the epididymal caput of a postpubertal bull used as a positive control tissue was analyzed. The band resulting from the epididymal tissue exhibited a clearly higher intensity compared to the placentome.

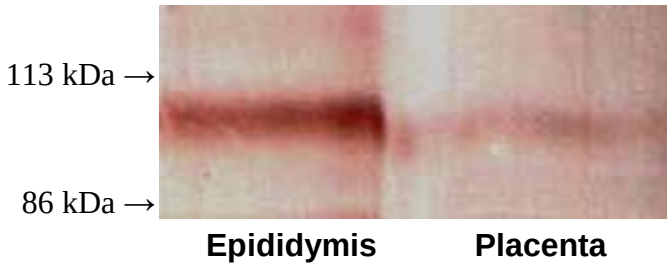


Figure 19: Western blot analysis of whole protein fractions (50 µg) prepared from a placentome of a cow at 272 days of gestation and bovine epididymal head using a polyclonal antibody against human androgen receptor.

With immunohistochemistry, in epididymal caput from a postpubertal bull (Fig. 20/A) used as a positive control tissue a highly specific staining pattern was obtained with distinct to intense, predominantly nuclear staining in all cells of the ductal epithelium. Moderate to intense nuclear staining was found in the majority of the peritubular smooth muscle cells and in a proportion of the intertubular connective tissue cells. In negative control sections of placentomes (Fig. 20/B), where the primary antiserum was replaced by serum from a non-immunized rabbit, only occasionally weak non-specific staining occurred in the lumen of blood vessels and was obviously associated with plasma components.

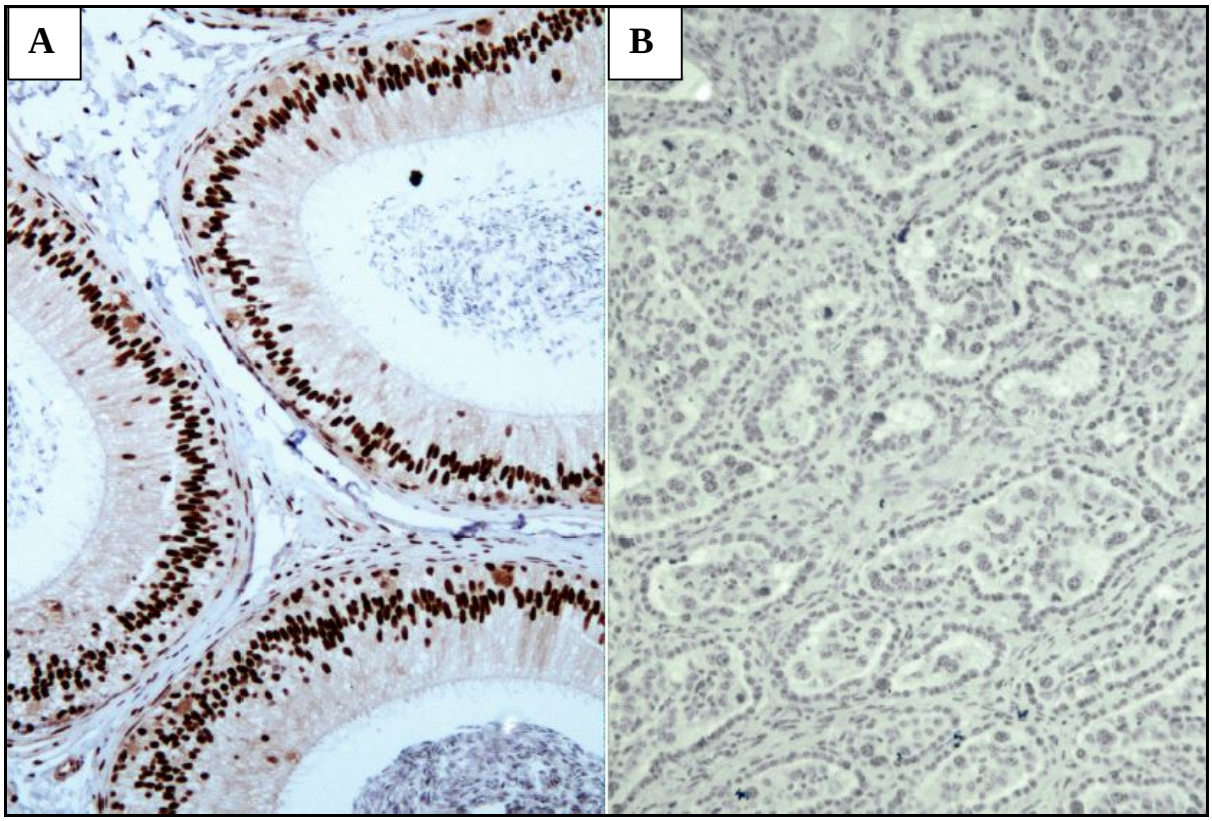


Figure 20: Immunohistochemical detection of androgen receptor in bovine tissues: results of control experiments. **A)** Immunostaining in the epididymal head of a postpubertal bull. Intense, predominantly nuclear immunostaining is visible in all columnar epithelial cells of the epididymal duct. Additionally, moderate to distinct nuclear immunostaining is also present in peritubular smooth muscle cells and in the intertubular connective tissue. Magnification x400 **B)** Placentome of a 220 days pregnant cow: negative control experiment, in which the specific primary antiserum was replaced by the serum of a non-immunized rabbit. No staining is visible in this section. Magnification: x100.

4.3.2.2 Expression pattern of androgen receptor in bovine placentomes on a cellular level

In bovine placentomes specific signals for AR were detected in trophoblast cells, cotyledonary stromal cells, caruncular epithelial cells and caruncular stromal cells (Fig. 21). Staining in stromal cells was exclusively nuclear, whereas in epithelial cell types occasionally a weak cytoplasmic signal was observed in addition to a prominent nuclear staining. Irrespective of gestational age distinct to intense staining was found throughout gestation in virtually all invasive TGC situated in the caruncular epithelium. Correspondingly, when IRS values (Fig. 22) for invasive TGC were evaluated by one-factorial ANOVA, no significant influence of the observational group was found ($p=0.6615$). Significant immunostaining was also found throughout gestation in a proportion of TGC situated in the chorionic epithelium considered mature as deduced from morphological characteristics. However, IRS values for this cell type were significantly higher at late gestation and parturition compared to early and midgestation ($p=0.0036$). Immunostaining in other cell types evaluated was also clearly related to the stage of gestation with generally low IRS values between first trimester until late gestation (day 272), when a marked increase both in the proportion of positive cells and staining intensity occurred. In placentomes from cows at day 272 of gestation, cows during the prepartal decline of progesterone and from parturient animals, AR was almost ubiquitously expressed. P-values for an influence of the observational group were 0.0026 for immature TGC/UTC, <0.001 for caruncular stromal cells and 0.001 for caruncular epithelial cells and stromal cells of chorionic villi, respectively.

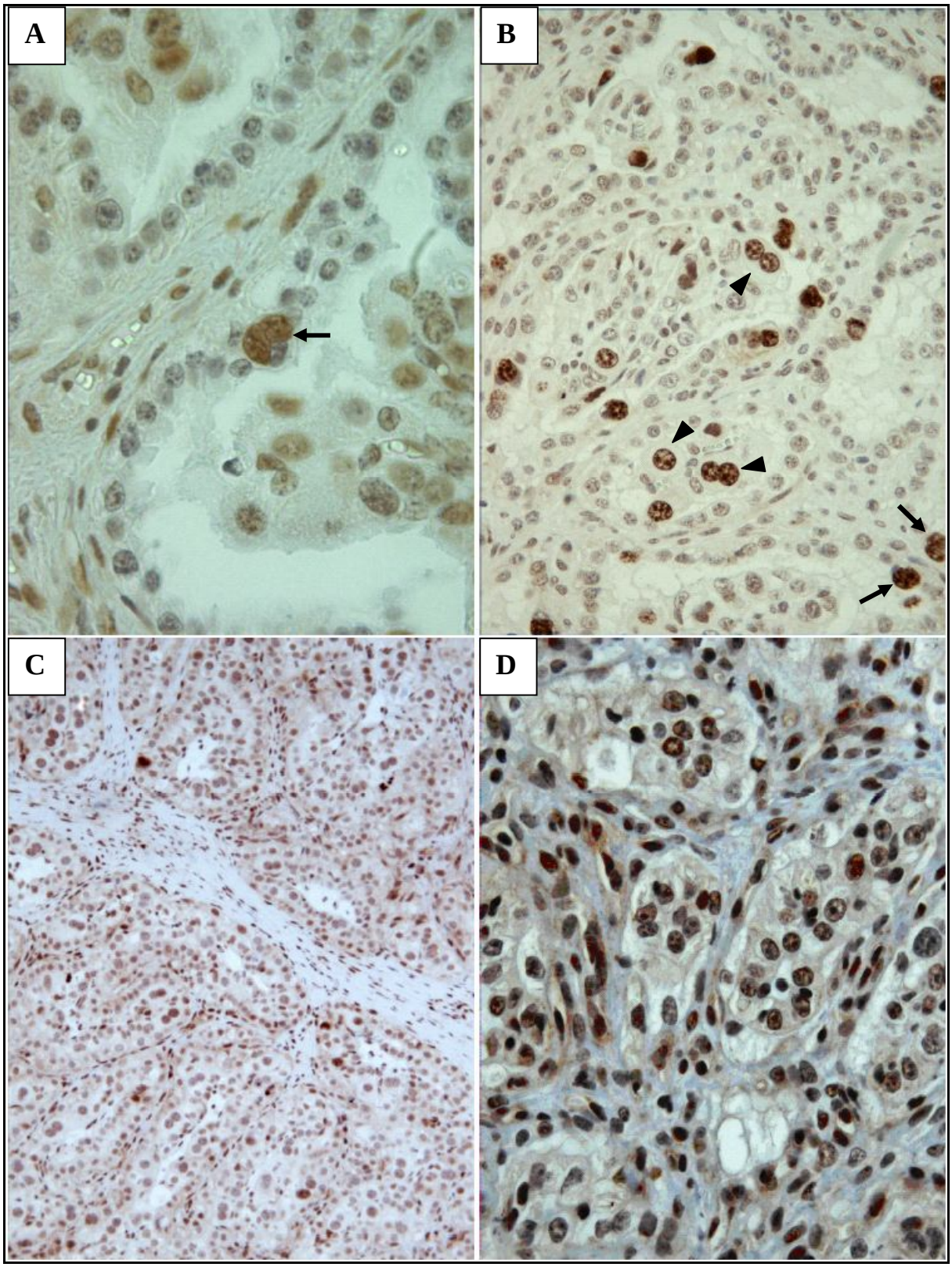


Figure 21: Immunostaining for androgen receptor in bovine placentomes. **A)** Distinct immunostaining in the two fetal nuclei of a feto-maternal hybrid cell (arrow) situated in the caruncular epithelium (day 80; magnification x400). **B)** Intense nuclear staining in mature (arrowheads) and invasive trophoblast giant cells

(arrows) (day 220; magnification: x200). **C)** Placentome of a 272 days pregnant cow. Androgen receptor specific immunostaining is present in the majority of cells (magnification: x100). **D)** Placentome of a cow during the prepartal decline of progesterone. Distinct to intense nuclear staining is found in virtually all cells (magnification: x200).

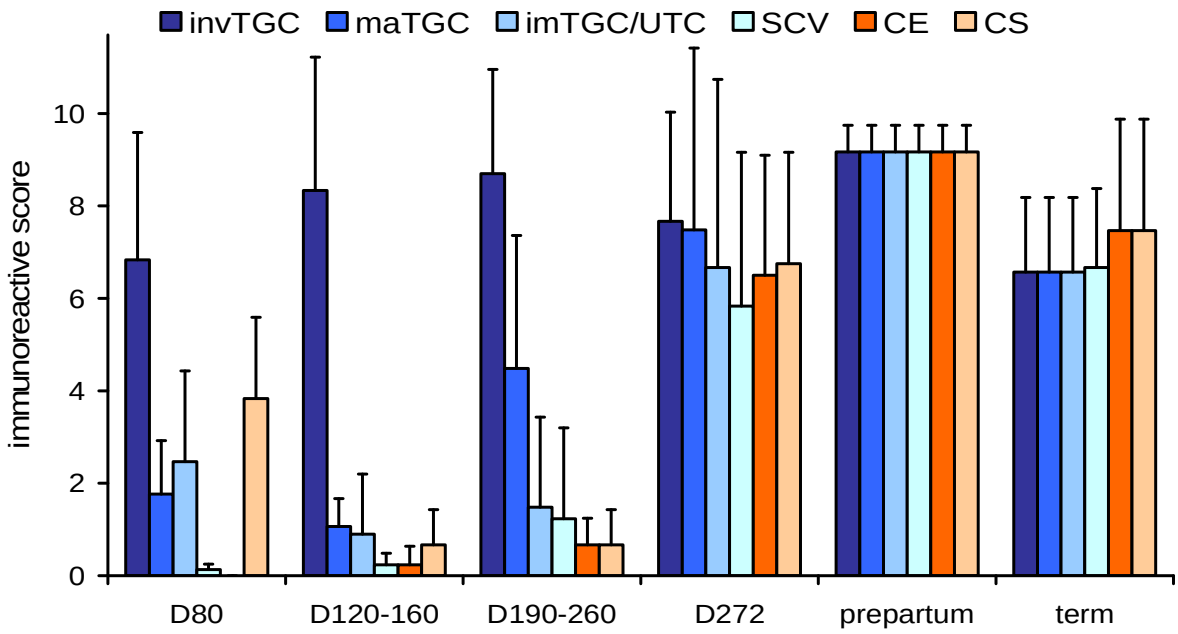


Figure 22: Quantitative evaluation of immunostaining for androgen receptor in different cell types of bovine placentomes using an immunoreactive score (IRS). Each observational group consisted of three animals and IRS values are presented as $\bar{x}$+SD. In prepartal animals tissue was collected during prepartal luteolysis.
inTGC, maTGC, imTGC = invasive, mature, immature trophoblast giant cells; UTC = uninucleated trophoblast giant cells; SCV = stromal cells of chorionic villi; CE = caruncular epithelial cells; CS = caruncular stromal cells.

4.3.3 Testosterone concentrations in bovine placentomal tissue

Testosterone concentrations measured in placentomal homogenates prepared from 12 cows between days 60-272 of gestation were all above the sensitivity of the radioimmunoassay applied (>35 pg/tube) and varied between 0.15-1.74 ng/g wet tissue (Fig. 23). Concentrations measured in placentomes of three 272 days pregnant cows were significantly higher than in placentomes of nine cows between days 60-220 ($p < 0.001$; T-test). In a correlation analysis, a significant positive correlation was found between testosterone tissue concentration and

gestational age (p = 0.002; r = 0.788). The linear regression line calculated was y = 0.0050x – 0.3484.

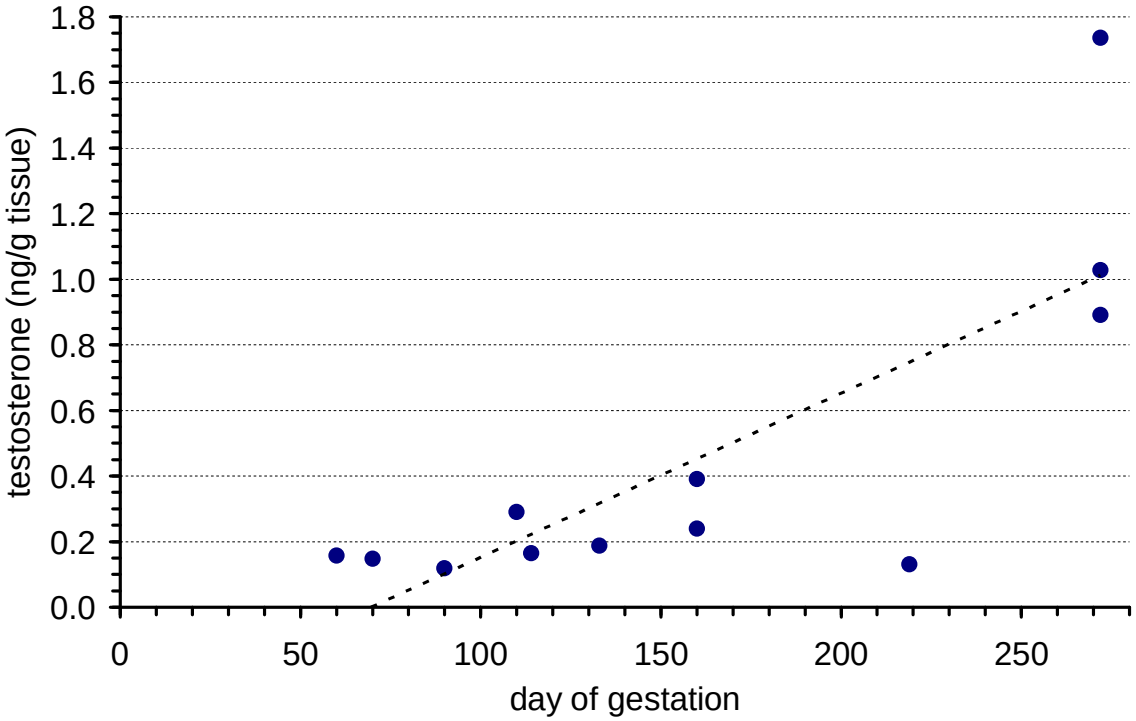


Figure 23: Testosterone concentrations measured in placentomal tissue homogenates of 12 pregnant cows between days 60 and 272 of gestation. In a correlation analysis, a significant positive correlation was found between gestational age and testosterone tissue concentration (p = 0.002; r = 0.788).

5. DISCUSSION

5.1 SULT1E1

By means of immunohistochemistry in bovine placentomes detection of SULT1E1 was virtually restricted to UTC, which clearly conflicts with results from previous studies by immunohistochemistry (Brown et al. 1987) and in situ hybridization (Ushizawa et al. 2007; Hirayama et al. 2008) suggesting the localization of SULT1E1 in TGC. However, there are several lines of evidence which, taken together, convincingly corroborate the validity of our immunohistochemical method. Firstly, antiserum αbov-SULT1E1 was produced against recombinant, highly purified bovine SULT1E1. Thus, significant cross-contamination of the antigen used for immunization with other proteins, especially of bovine origin is extremely unlikely. Moreover, to further reduce possible cross-reactivity, the antiserum was purified by affinity chromatography using recombinant bovine SULT1E1 linked to CNBr-activated sepharose (Frenette et al. 2009). In western blot, a specific band was recognized with a molecular size of approx. 33 KDa, which is consistent with monomeric SULT1E1. In placental samples with high SULT1E1 expression and in samples from adult liver a band of a molecular weight of approx. 70 kDa occurred which is consistent with SULT1E1 dimers (Adams 1991). In placentomes results from western blot and immunohistochemistry correlated well with data from real-time RT-PCR suggesting an increase of SULT1E1 expression at the end of gestation. Strong evidence for the validity of our immunohistochemical and western blot methods also comes from parallel experiments using an antiserum against human SULT1E1, which yielded virtually identical results. Even if the signals found with αbov-SULT1E1 and αhum-SULT1E1 in UTC were considerably related to cross-reaction with other members of the structurally closely related SULT family, any significant SULT1E1 expression in TGC should have been detected in addition, which was absolutely not the case. The reasons for the divergent results between this study and previous studies by other researchers are unclear and can only be suspected. The monoclonal antibody 33-11 used by Brown et al. (1987) was produced against “estrogen sulfotransferase” purified from bovine placenta. Different from the antisera used in our study, in western blot it reacted with several bands of various sizes. In a later paper of this group (Adams 1991) it was admitted that the molecule initially purified as “estrogen sulfotransferase” obviously was mainly transferrin, and no other paper could be found in which this antibody was used. Thus, there are reasonable doubts that monoclonal antibody 33-11 was really specific for SULT1E1. The fact that in bovine placentomes SULT1E1-mRNA was localized in TGC by in situ-hybridization in two studies (Ushizawa et al. 2007, Hirayama et al. 2008) can not be easily

invalidated. Basically it is possible that in bovine trophoblast a protein and its corresponding mRNA may be located in different cell types due to the permanent rapid differentiation of UTC into TGC, as in different cell types like spermatogenic cells (Ostermeier et al. 2002; Dadoune 2003; Lambard et al. 2004) and oocyte (Ballantyne et al. 1997; Charlesworth et al. 2000) a considerable lag of time has been shown between transcription of the mRNA and translation of the protein. However, in case of a significant delay between the expression on the mRNA and the protein level of a certain gene during TGC differentiation, the mRNA should be detectable at an earlier stage of differentiation than the protein and not vice versa. As the two previous studies using ISH (Ushizawa et al. 2007; Hirayama et al. 2008) were performed by collaborating groups and the probe applied is only specified in one of the papers (Hirayama et al. 2008), it remains unclear if the same method was used in both studies. In the probe used by Hirayama et al. (2008), sequences with significant homologies to bovine SULT1B1 and SULT1C1 were found. Cross-reactivity with other structurally closely related members of the SULT family (Chapman et al. 2004; Pasqualini 2009) must also be considered on the protein level. However, even if the signals found with αbov-SULT1E1 and αhum-SULT1E1 in UTC were related to cross-reaction with other members of the SULT family, any significant SULT1E1 expression in TGC should have been detected in addition, which was not the case. Finally, although ISH is generally considered highly specific, in previous studies in bovine placental tissue performed in our laboratory, in a significant number of cases considerable staining in TGC occurred after the use of sense probes applied in negative control experiments. As bovine TGC exhibit intense protein synthesis (Wooding 1992; Hoffman and Wooding 1993; Igwebuike 2006), these signals are presumably due to non-specific interaction of probes with the high amount of mRNA present in this cell type.

Until today the function of the high amounts of E1S produced in the bovine trophoblast is unclear (Schuler et al. 2008). The previously suggested localization of SULT1E1 in TGC (Brown et al. 1987; Ushizawa et al. 2007; Hirayama et al. 2008), which up-regulate aromatase (Schuler et al. 2006a) and estrogen receptor β (Schuler et al. 2005) during their differentiation, would strongly point to a predominant role of bovine pregnancy associated estrogens as intracrine regulators in trophoblast cells, as synthesis, receptor binding and inactivation of estrogens would occur in the same cell. A localization of SULT1E1 in UTC, as strongly suggested by our immunohistochemical results, is also consistent with a role of bovine pregnancy associated estrogens as intracrine regulators in TGC during differentiation. However, as inactivation of free estrogens produced in TGC would predominantly take place in UTC, there might also be some estrogenic signaling from TGC to UTC, which could serve

the cellular balance between UTC and TGC in the trophoblast. The ratio UTC/TGC is fairly constant throughout gestation at approximately 4:1 (Wooding 1983) with the exception of the immediate pre- and intrapartal phase (Woicke et al. 1986; Gross et al. 1991; Shenavai et al. 2010), when the number of TGC decreases significantly. Moreover, E1S may not only be regarded as an inactivated metabolite but may form a pool of precursors for the local production of estrogens in target cells exhibiting STS expression (sulfatase pathway; Santen et al. 1986), which could serve the limitation of the effects of placental estrogen to a pregnancy specific subset of estrogen responsive cells. Interestingly, in bovine placentomes the caruncular epithelium, which is in direct contact with the trophoblast, has been shown to express STS (Greven et al. 2007) and estrogen receptors (Boos et al. 2000; Schuler et al. 2002).

For SULT1E1 in UTC, a gradient of immunostaining was observed along the villous tree at early and midgestation with higher staining intensities at the chorionic plate and basal parts of stem villi compared to the more distal parts. At late gestation, an extension of high staining intensities also to distal parts of the chorionic villous tree occurred. Thus, SULT1E1 belongs to a considerable number of genes for which this type of expression pattern has been found in the chorionic villus tree (CYP17, CYP19: Schuler et al. 2006a, COX2: Schuler et al. 2006b,) or in its maternal counter part, the caruncular crypt system (STS: Greven et al. 2007, glucocorticoid receptor: Boos et al. 2000). Correspondingly a significant increase of SULT1E1-mRNA levels was found in late gestation, which is consistent with earlier results by Ushizawa et al. (2007).

The only noteworthy difference in the immunohistochemical staining patterns between αbov-SULT1E1 and αhum-SULT1E1 was the moderate cytosolic staining in caruncular epithelial cells occasionally observed with the antiserum against the bovine enzyme in addition to the predominant signal in UTC obtained with both antisera. The reason for this difference is unclear but may be related to different sensitivities of the two immunohistochmical methods. As caruncular epithelial cells strongly express estrogen receptor α (Boos et al. 2000; Schuler et al. 2002) and STS (Greven et al. 2007), SULT1E1 expression in this cell type could serve the limitation of estrogenic effects by free estrogens either leaking from the cotyledon or locally produced via the sulfatase pathway. Free estrogens escaping from sulfonation in the trophoblast to the fetal circulation are obviously intercepted by the relatively high SULT1E1 expression in the fetal liver. The high SULT1E1-mRNA levels measured in fetal liver in this study are consistent with high estrogen sulfotransferase activities in cytosol prepared from fetal liver in the presence of PAPS (Schuler, unpublished data). The results from the screening

of various organs for SULT1E1-mRNA from adult cattle showed that expression even in steroidogenic organs and liver was minimal in comparison to placenta and fetal liver. This result is consistent with the basal E1S levels measured in non-pregnant cows and in bulls (Schuler, unpublished data).

In conclusion, this study using two different primary antisera provides convincing evidence that, different from the results of previous studies localizing SULT1E1 in TGC, in bovine trophoblast this enzyme is predominantly expressed in UTC. This entails a revision of the concept on the availability of free and sulfonated estrogens on a cellular level in bovine placentomes.

5.2 SOAT

Different from other mammalian species investigated so far, in which only the SOAT standard form (variant 1) was detected, during cloning of bovine SOAT from placentomes four additional isoforms (variants 2-5) were identified on the mRNA level arising from the deletion of one or several exons and/or the insertion of an additional exon 1´ (Greven 2008). Whereas for SOAT variant 1 a sodium-dependent uptake of steroid-3-sulfates has been demonstrated in vitro (Greven 2008), the biological role of variants 2-5 was still unknown. It has been speculated that they may be non-functional or that they may have other functions unrelated to transport processes. For variant 3 the hypothesis was put forward that it may mediate the cellular export instead of import of substrates, as it has been described for a homologous variant of the structurally closely related apical sodium-dependent bile acid transporter (Lazaridis et al. 2000). Thus, the original aim of this part of the study was to thoroughly characterize the expression of SOAT variants on the protein and mRNA level, respectively. However, after comprehensive control experiments none of the several antibodies produced against human SOAT obtained from commercial sources or from collaborators yielded reliable results with bovine tissues in western blot and immunohistochemistry. Moreover, attempts to test for an integration into the cytoplasmic membrane of the individual SOAT variants transiently expressed in HEK cells using surface biotinylation did not yield reproducible results. Thus, only data from investigations on the mRNA level can be presented here.

Investigations by conventional RT-PCR using variant specific primers showed that in bovine placentomes 1,2 and 5 SOAT variants were widely expressed throughout gestation. However, variants 3 and 4 were missing in placentomes from some animals at various stages of gestation. Data from real-time RT-PCR methods, which could be established for variants 1, 2,

3 and 5, revealed a trend for higher expression levels at the end of gestation and at parturition, which is consistent with data from the previous study by Greven (2008), in which a similar increase of expression levels was measured using a real-time RT-PCR method not discriminating between individual isoforms.

Comparative investigations on the spectrum of SOAT isoforms in various bovine organs using variant-specific methods in conventional RT-PCR showed that - with the exception of variant 4 - all variants were detected in all samples under investigation. Thus standard SOAT and isoforms 2, 3 and 5 possess a remarkably broad distribution in bovine tissues suggesting a considerable physiological role of SOAT isoform formation in cattle. Although for each organ or tissue only one sample was included in these experiments, the results from real-time RT-PCR point to remarkable quantitative organ-specific differences in the expression of SOAT isoforms. Despite the comprehensive information on the expression of SOAT isoforms in bovine tissues obtained in this study, their physiological roles still remain unclear. SOAT variant 1, which came into the fore of our studies on bovine placental steroidogenesis because of its capacity to efficiently mediate the import of E1S in vitro (Greven 2008), has now been shown to be widely expressed in many tissues which are not considered as important source or potential target of sulfonated estrogens (sulfatase pathway). As for isoforms 2, 3 and 5, expression of SOAT variant 1 was highest in the testis. In the literature no data were found on blood levels of E1S in bulls but punctual experience from our endocrine laboratory showed that in plasma or serum of male cattle E1S concentrations are below or at the limit of detection of the assay used (0.1 ng/ml), thus questioning a role of SOAT variant 1 as a physiologically relevant transporter of E1S in male cattle. As it also efficiently mediates the cellular import of pregnenolone sulfate and DHEA sulfate (Greven 2008), one might speculate that SOAT variant 1 may be involved in the up-take of sulfonated steroid hormone precursors into Leydig cells. However, expression in other bovine steroidogenic organs which are highly active based on their steroid output per tissue weight such as the corpus luteum and adrenal is only low. Moreover, using laser capture microdissection, SOAT variant 1 expression in bovine testis was clearly higher in the tubular compartment compared to the Leydig cells (Pershotam, unpublished data), which also challenges the concept of SOAT variant 1 as a physiologically relevant transporter of steroid sulfates in the bovine testis. The roles of isoforms 2-5 remain even less clear, as in experiments using transiently transfected cells a transport function could only be demonstrated for isoform 1 but not for isoforms 2-5 (Geyer, personal communication).

5.3 Androgen receptor expression and testosterone tissue concentrations in bovine placentomes

Physiological roles of androgens in the fetal and maternal part of bovine placentomes on the first view are unexpected. However, the expression of AR in human placenta has been suggested already in early studies using ligand binding assays (Barile et al. 1979; Stanley et al. 1980; Hirota et al. 1981; McCormick et al. 1981, Younes et al. 1982), and was later confirmed by immunohistochemistry in human decidua and trophoblast (Horie et al. 1992a; Uzelac et al. 2010). 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, where in addition to specific primers further specificity is coming from the gene specific probe. Convincing evidence for the capacity of the antibody used in immunohistochemistry, which was produced against the N-terminus of human AR, to specifically detect AR in bovine tissues comes from western blot experiments. In placentomal tissue only one specific band at about 110 kDa occurred, which is consistent with the molecular weight of AR (Claessens et al. 2001, Uzelac et al. 2010). A clearly more intensive band of exactly the same size occurred with bovine epididymis, a tissue known to highly express AR (Foxley et al. 2001). Further evidence for the validity of the immunohistochemical method established to detect AR in bovine tissues comes from immunohistochemical control experiments. In negative controls, in which the primary antibody was replaced by irrelevant isotypic immunoglobulin, unspecific staining of weak intensity only occurred sporadically and was associated with plasma components present in the lumina of blood vessels. In a bovine epididymal head used as a positive control tissue, a staining pattern consistent with findings from other domestic animal species like sheep (Tekpetey et al. 1989), goat (Goyal et al. 1997), rat (Zhu et. al 2000) and horse (Bilinska et al. 2004; Parlevliet et al. 2006) was obtained.

Throughout gestation, distinct to intense staining was found in nuclei of invasive TGC situated in the caruncular epithelium and in the two “fetal” nuclei of feto-maternal hybrid cells formed by the fusion of an invasive TGC with a caruncular epithelial cell (Wooding and Beckers 1987). 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 (Schuler et al. 2008) and the fact that in bovine placentomes the Δ4-pathway is inefficient (Schuler et al. 1994) implies that a significant synthesis of testosterone or 5α-

dihydrotestosterone is only possible after the 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. A dependency of AR expression on the presence of androgens has been previously demonstrated in various cell types of the male genital tract (Zhu et al. 2000). On the other hand, up-regulation of AR in the aromatase (Schuler et al. 2006a) and ERß (Schuler et al. 2005) expressing TGC may also be stimulated by estrogens. The up-regulation of AR by estrogens has been demonstrated in endometrial stroma cells of macaques (Slayden and Brenner 2004).

Immunostaining for ARs in bovine placentomes is virtually restricted to trophoblast cells during most of the time of gestation but is almost ubiquitously detected in the immediate prepartal period and at parturition. However, this substantial overall expansion in AR expression is hardly detectable on the mRNA level. This ostensible contradiction may be explained by the fact that TGC are by far the predominant source of AR expression in the placentome, in which AR expression is constantly high throughout gestation and of which the number significantly decreases immediately prior to parturition (Gross et al. 1991; Shenavai et al. 2010). The almost ubiquitous up-regulation of AR in bovine placentomes may be related to the prepartal up-regulation of CYP17 in UTC (Schuler et al. 2006a), which may results in the increased availability of C19 precursors for the production of active androgens or estrogens in TGC or their precursors. Thus, active androgens or estrogens may “leak” in significant amounts to other cells of the placentome to stimulate AR up-regulation. Although androgen concentrations measured in placentomal tissue are low in comparison to levels usually occurring in intact males, they are clearly above the detection limit of the assay applied and must be considered sufficient to activate AR, especially when taking into account intracrine or paracrine actions. Consistently, in human villous explants the AR dependent up-gulation of the AR target gene FGF2 by 5α-dihydrotestosterone has been demonstrated, suggesting that placental AR are functional and that androgens may be important local factors in the control of placental growth and differentiation (Uzelac et al. 2010). Thus, the production of biologically inactive estrogens by the activities of aromatase in TGC (Schuler et al. 2006a) and SULT1E1 in UTC may be a mechanism to finely tune the availability of active androgens in the placentomes.

Since the discovery of steroid receptors, the concept of their roles has significantly changed from monospecific ligand activated receptors to components of the general transcription

machinery, which - among various other functions – also possess a steroid activated transcriptional activity. Thus, other functions besides effects from the classic interaction of AR with androgens must also be considered, e.g. the cross-talk of various signal cascades initiated by growth factors which may target AR (Zhu and Kyprianou 2008). Accordingly, the expression of various relevant growth factors such as fibroblast growth factors (Pfarrer et al. 2006a), vasoendothelial growth factor (Pfarrer et al. 2006b) or transforming growth factor β (Ravelich et al. 2006) has been demonstrated in bovine placentomes, and the up-regulation of AR induced by placental steroids may be a prerequisite to enable their full spectrum of effects.

In conclusion, the detection of significant AR expression and of testosterone tissue levels considered as sufficient for AR activation in bovine placentomes suggest that besides progesterone and estrogens also androgens may be active products of placental steroidogenesis in a cattle. Moreover, this concept suggests a new function for the predominant production of inactive estrogen sulfates in bovine placentomes which might serve the control of androgen activities.

6. SUMMARY

As a temporary endocrine organ, the placenta is capable of synthesizing and secreting a broad range of hormones and other bioactive molecules. Like in many other mammalian species, also in cattle the placenta exhibits a considerable steroidogenic activity with estrone sulfate (E1S) and progesterone being the main products from a quantitative point of view. However, the biological role of placental steroidogenesis in cattle is widely unclear as the placenta contributes only negligibly and temporarily to peripheral maternal progesterone blood levels, and the main estrogenic product – E1S – does not interact with classical nuclear estrogen receptors. Based on the observation that receptors for progesterone and estrogens are expressed in bovine placentomes the concept of placental steroids as local regulators of placental growth, differentiation and functions has been put forward. However, data from functional studies to corroborate this concept are not yet available, and apart from the functions of bovine placental steroids, there are still many open questions concerning synthesis and transport especially of sulfonated estrogens in cattle. Thus, this study focuses on three aspects of bovine placental steroidogenesis: 1) the expression of the estrogen-specific sulfotransferase (SULT1E1) to identify the sites of estrogen sulfonation in the pregnant cow 2) to further characterize the expression of the sodium-dependent organic anion transporter (SOAT; syn.: SLC10A6) which is considered as a putatively relevant transporter of estrogen sulfates 3) to test for the possibility that steroids other than progesterone and estrogens – e.g. androgens - are functionally important products of bovine placental steroidogenesis.

In order to localize SULT1E1 in bovine placentomes on a cellular level and to assess its expression quantitatively throughout gestation, immunocytochemical and real-time RT-PCR methods were established, respectively. In immunocytochemisty (ICC) two different primary antibodies were applied: a rabbit antiserum against bovine recombinant SULT1E1 (generously provided by Dr. R. Sullivan, Centre de Recherche en Biologie de la Reproduction and Département d'Obstétrique-Gynécologie, Faculté de Médecine, Université Laval, Quebec, Canada) and a commercial murine antiserum against the human enzyme. Specificity of these antibodies was confirmed in western blot. They yielded virtually identical results in ICC. Different from previous data published in the literature based on ICC and in situ hybridization, where SULT1E1 in bovine placentomes was localized in trophoblast giant cells (TGC), strong specific cytoplasmic staining was only found in uninucleated trophoblast cells (UTC) and SULT1E1 was rapidly down-regulated when trophoblast cells showed characteristics of TGC differentiation. Throughout gestation, distinct immunostaining for SULT1E1 was found in UTC of the chorionic plate. A gradient of staining intensity was

observed along the chorionic villous tree with a decrease of mean staining intensities between the basal parts of stem villi and the tertiary villi. With real-time RT-PCR, in the course of pregnancy a significant increase of SULT1E1-mRNA expression was found in the last trimester and at parturition ($p=0.0043$), which confirms earlier studies by other investigators. Consistent with the increase of SULT1E1-mRNA in real-time RT-PCR in immunohistochemistry an increase of overall staining intensity was detected during late gestation and at parturition with the antiserum against bovine SULT1E1 but this was not obvious with the antiserum against the human enzyme. Moreover, with the antiserum against the bovine enzyme a weak cytoplasmic signal was obtained in the caruncular epithelium in a part of placental samples in addition to the distinct signals in UTC. When screening bovine organs for SULT1E1-mRNA expression by real-time RT-PCR, highest levels were found in the placenta (2851 relative units, RU; day 272), followed by fetal liver (day 185: 748 RU; day 210: 816 RU). In adult bovine organs, SULT1E1 expression was clearly lower with highest levels in adrenal (144 RU) and skin (91 RU) and was only minimal in the remaining organs investigated including liver (21 RU).

The results give strong evidence that different from results of earlier studies the main sites of SULT1E1 expression in bovine placentomes are the UTC and not the TGC. They suggest that SULT1E1 may protect UTC from the high levels of free estrogens produced by TGC and it may be involved in the control of TGC differentiation.

The aim of the second part of this study was to further characterize the expression of the five isoforms identified so far of the SOAT in the bovine placentome and other organs. The SOAT standard form (variant 1) cloned from bovine placentome has been recently shown in vitro to efficiently mediate the cellular import of E1S and thus may be a physiologically relevant transporter for the large amount of E1S produced in the trophoblast during bovine gestation. Virtually no information was available so far on the expression pattern and functions of the remaining four isoforms. Variant-specific conventional RT-PCR methods could be established for all of the five variants. With these methods, placental tissue samples from different stages of gestation were screened for SOAT isoform expression. For comparison, a broad spectrum of other tissues and organs was also included into the study. Specific amplicons were obtained in all (variants 1, 2, 5) or most (variant 3) of the tissue samples. For variant 4, bands were only weak or absent in a considerable proportion of placental or adult tissue samples. Real-time RT-PCR methods (SYBR green) could be established for variants 1, 2, 3 and 5. Expression of all these variants did not change significantly in bovine placentomes during early and midgestation but was more than 10 fold higher in the maternal part of

placentomes from cows at normal term suggesting that their expression is up-regulated in the prepartal period. When screening various bovine organs quantitatively for expression of mRNA specific for SOAT isoforms, for variants 1, 2 and 5 expression in testis (1253 RU, 8195 RU, 4575 RU, resp.) exceeded by far expression measured in other organs or tissues. Other significant sites of SOAT-variant 1 expression are skin (734 RU), udder (282 RU) and colon (255). Besides in the testis, variant 2 was significantly expressed in the skin (1410). Highest expression for variant 3 was also found in the testes (158 RU), which – however- was only slightly higher than in the ovarian stroma (113 RU). The spectrum of measurable SOAT isoforms varied considerable between tissues and organs with testis, placenta and corpus luteum being the only organs where all four isoforms assessed were expressed in measurable levels. Expression levels in placentome for variants 1 (5.3 RU), 2 (31.8 RU), 3 (2.5 RU) and 5 (9.6) were all above the limit of detection but only minimal compared to the testis. These observations question the hypothesis of SOAT variants as important transporters of the high amounts of sulfonated estrogens in bovine placentomes. Moreover, the results suggest that in cattle standard SOAT (variant 1) and its other isoforms play an important role especially in the testis, an organ, which – however – in the bovine species does not produce considerable amounts of sulfated estrogens.

In addition to estrogens and progesterone, of which no biological role has been definitely identified yet in bovine placenta, in the bovine trophoblast androgens may also be produced and may have effects in bovine placentomes. In order to identify putative target cells of placental androgens, an immunohistochemical method was established to detect androgen receptor (AR) in bovine tissues using an antiserum raised against the N-terminus of human AR. Specificity of the primary antibody applied for bovine AR was confirmed by western blot and by control experiments using bovine epididymis as a positive control tissue. Throughout gestation, distinct nuclear signals were found in invasive TGC. As assessed by quantitative evaluation using an immunoreactive score, in TGC situated in the trophoblast, immature TGC, UTC, stromal cells of the chorionic villi, caruncular epithelial and stromal cells AR expression was low at early and midgestation but significantly increased during late gestation ($p<0.01$, resp.). Expression of AR was qualitatively confirmed on the mRNA-level by conventional RT-PCR. With real-time RT-PCR (taqman method) only a trend for an increased AR expression in the prepartal phase and at parturition was observed, which – however – was not statistically significant. Radioimmunological measurement of testosterone concentrations in bovine placental tissue yielded concentrations that must be considered sufficient to activate local ARs, and showed a significant increase ($p<0.01$) of mean

testosterone concentrations from values slightly above background level (0.1 ng/g tissue) between days 50-100 to mean concentrations of 0.9 ng/g tissue during late gestation. The results suggest that androgens may be active products of bovine placental steroidogenesis and that they may be involved in the control of TGC differentiation. However, as steroid receptors are in part constitutionally active, it cannot be ruled out the ARs detected in bovine placentomes may have functions independent from the binding of steroidal ligands.

In conclusion, results obtained in these studies provide new information on different aspects of bovine placental steroids and give starting points for new concepts on their functions.

7. ZUSAMMENFASSUNG

Die Plazenta stellt ein temporäres endokrines Organ dar, welches ein breites Spektrum verschiedener Hormone und bioaktiver Mediatoren produziert. Wie bei vielen Säugerspezies produziert sie auch beim Rind erhebliche Mengen an Steroiden, wobei aus quantitativer Sicht Progesteron und Estronsulfat die Hauptprodukte darstellen. Progesteron plazentaren Ursprungs trägt jedoch nur minimal und temporär zu den maternalen Blutspiegeln bei. Weiterhin interagiert das Hauptprodukt der plazentaren Östrogensynthese – Estronsulfat – nicht mit klassischen Östrogenrezeptoren. Daher ist die Bedeutung der plazentaren Steroidsynthese beim Rind immer noch unklar. Der Nachweis von Östrogen- bzw. Progesteronrezeptoren in den Plazentomen führte zur Hypothese, dass plazentare Steroide beim Rind nicht als Hormone im klassischen Sinn, sondern als lokale Regulatoren von Wachstum, Differenzierung und Funktionen der Plazenta selbst fungieren könnten. Eine Bestätigung dieses Konzepts durch entsprechende funktionelle Studien steht jedoch bisher noch aus, und neben der Frage nach der biologischen Bedeutung sind immer noch zahlreiche Fragen hinsichtlich Synthese und Transport der plazentaren Steroide, insbesondere der in großen Mengen gebildeten sulfatierten Östrogenen offen. Daher befassen sich diese Untersuchungen mit den folgenden drei Aspekten der plazentaren Steroidsynthese beim Rind: 1) Identifizierung des Syntheseortes der plazentaren sulfatierten Östrogene durch die Charakterisierung der Expression der östrogenspezifischen Sulfotransferase SULT1E1 auf zellulärer Ebene 2) die nähere Charakterisierung der Expression des Sodium-dependent Organic Anion Transporters (SOAT, syn.: SLC10A6) im Hinblick auf dessen Funktion als physiologisch relevanter Transsporter von sulfatierten Östrogenen 3) der möglichen Bedeutung von Androgenen als biologisch aktive Produkte der plazentaren Steroidsynthese.

Zur Lokalisierung der SULT1E1 in den Rinderplazentomen auf zellulärer Ebene und zur quantitativen Erfassung der SULT1E1-Expression im Verlauf der Gravidität wurden zwei immunhistologische bzw. eine Real-time RT-PCR-Methode etabliert. Für den immunhistologischen SULT1E1-Nachweis wurden zwei Primärantikörper eingesetzt: ein polyklonales Antiserum aus Kaninchen gegen rekombinante bovine SULT1E1 (freundlicherweise zur Verfügung gestellt von Dr. R. Sullivan, Centre de Recherche en Biologie de la Reproduction and Département d'Obstétrique-Gynécologie, Faculté de Médecine, Université Laval, Quebec, Kanada, sowie ein kommerzielles Antiserum aus der Maus gegen das entsprechende menschliche Enzym. Die Spezifität der beiden Primärantikörper für die bovine SULT1E1 wurde im Western Blot bestätigt. In der Immunhistologie ergaben sie weitgehend übereinstimmende Resultate. Im Gegensatz zu

Resultaten früherer Untersuchungen unter Anwendung der Immunhistologie sowie der In situ-Hybridisierung, in denen die SULT1E1 in den Trophoblastriesenzellen (trophoblast giant cells, TGC) lokalisiert wurden, fanden sich in den eigenen Untersuchungen starke zytoplasmatische Signale ausschließlich in den einkernigen Trophoblastzellen (uninucleated trophoblast cells, UTC). Sobald UTC morphologisch erkennbar in die TGC-Differenzierung eintraten, wurde die SULT1E1-Expression rasch herunterreguliert. Unabhängig vom Trächtigkeitsstadium war in den Plazentomen eine starke SULT1E1-Expression in den UTC der Chorionplatte nachweisbar. In den UTC der Chorionzotten fand sich ein Gradient der SULT1E1-Expression mit Abnahme der Signalintensität von den basalen Anteilen der Stammzotten zu den Spitzen der Tertiärzotten. Mittels der Real-time RT-PCR konnte über den Verlauf der Gravidität ein Anstieg der SULT1E1-mRNA-Expression in der späten Gravidität sowie unter der Geburt festgestellt werden ($p=0.0043$), was im Einklang mit früheren Untersuchungen anderer Autoren steht. In Übereinstimmung mit dem in der Real-time RT-PCR festgestellten Anstieg der SULT1E1-mRNA-Expression in der Spätgravidität und unter der Geburt wurde in der Immunhistologie unter Verwendung des Primärantikörpers gegen die bovine SULT1E1 im entsprechenden Zeitraum ein Anstieg des Farbsignals festgestellt. Dieser war jedoch bei Verwendung des Primärantikörpers gegen die menschliche SULT1E1 nicht feststellbar. Weiterhin fand sich mit dem Primärantikörper gegen die bovine SULT1E1 in einem Teil der Proben neben dem prominenten Signal in den UTC ein deutlich schwächeres zytoplasmatisches Signal im Karunkelepithel. Bei einem Vergleich der SULT1E1-mRNA-Expression in Proben verschiedener Organe – gemessen mittels Real-time RT-PCR - zeigte sich, dass beim Rind die Plazenta (Tag 272) die weitaus höchste Expression aufweist (2851 relative Einheiten, RU), gefolgt von der fetalen Leber (Tag 185: 748 RU; Tag 210: 816 RU). In Organen adulter Rinder war die SULT1E1-Expression weitaus niedriger mit höchsten Messwerten in der Nebenniere (144 RU), gefolgt von der Haut (91 RU). In den restlichen untersuchten Organen adulter Rinder, inclusive der Leber (21 RU), war sie vergleichsweise minimal.

Die Ergebnisse zeigen überzeugend, dass die bisher publizierte Lokalisation der SULT1E1 in den TGC offensichtlich nicht zutrifft, sondern dass sie in den Plazentomen des Rindes vorwiegend in den UTC exprimiert wird, wodurch diese Zellen vermutlich vor den in den TGC produzierten Östrogenen geschützt werden. Möglicherweise ist die SULT1E1 daher in die Kontrolle der Trophoblastriesenzelldifferenzierung involviert.

Ziel des zweiten Teils dieser Untersuchung war die weitergehende Charakterisierung der Expression der fünf bisher identifizierten Isoformen des SOAT in den Plazentomen und

vergleichsweise in anderen Organen des Rindes. Für die aus Rinderplazentomen klonierte SOAT-Standardform (Variante 1) konnte kürzlich in vitro gezeigt werden, dass sie effektiv den zellulären Import von Estronsulfat vermittelt, weshalb sie als physiologisch relevanter Transporter des während der Gravidität des Rindes in großen Mengen produzierten Estronsulfats infrage kommt. Über das Expressionsmuster der restlichen vier Varianten sowie über deren Funktionalität lagen bisher keinerlei Informationen vor. Für alle fünf Varianten des Rinder SOATs wurden spezifische konventionelle RT-PCR-Methoden etabliert. Unter Anwendung dieser Verfahren wurden Plazentomproben aus unterschiedlichen Trächtigkeitsstadien untersucht. Zu Vergleichszwecken wurden parallel Untersuchungen an einem breiten Spektrum von Rinderorganen bzw -geweben durchgeführt. Für die SOAT-Isoformen 1, 2 und 5 waren spezifische PCR-Produkte in allen untersuchten Proben nachweisbar. Auch die Expression der Variante 3 war in den meisten Proben nachweisbar. Banden für Variante 4 waren dagegen meist schwach oder nicht nachweisbar. Die Etablierung isoformspezifischer Real-time RT-PCR-Methoden war für die Varianten 1, 2, 3 und 5 erfolgreich. Mit ihnen konnte gezeigt werden, dass die Expression dieser Varianten in den Rinderplazentomen in der frühen und mittleren Gravidität weitgehend konstant ist, während unter der Geburt die im maternalen Teil der Plazentome gemessene Expression im Mittel um mehr als den Faktor 10 höher war. Bei vergleichenden Messungen der SOAT-Varianten-Expression in verschiedenen Rindergeweben und –organen wurde für die Varianten 1, 2 und 5 die weitaus höchsten Werte im Hoden gemessen (1253 RU, 8195 RU bzw. 4575 RU). Variante 1 wurde daneben deutlich in der Haut (734 RU), dem Euter (282 RU) und im Colon (233 RU) exprimiert. Außer im Hoden war Variante 2 in der Haut (1410 RU) deutlich nachweisbar. Für Variante 3 wurde die höchste Expression ebenfalls im Hoden (158 RU) nachgewiesen, wo sie allerdings nur unwesentlich höher war als im Ovarstroma (113 RU). Das Spektrum messbarer SOAT-Isoformen-mRNA wies zwischen den untersuchten Organen und Geweben erhebliche Unterschiede auf. Messenger-RNA für alle vier mittels Real-time RT-PCR erfassten Isoformen konnte lediglich in Hoden, Plazenta und Corpus luteum gemessen werden. In den Plazentomen war die relative Genexpression aller erfasster Varianten zwar über der Nachweisgrenze (Variante 1: 5,3 RU, Variante 2: 31,8 RU, Variante 2: 2,5 RU, Variante 5: 9.6 RU), aber nur minimal im Vergleich zur Expression im Hoden. Die im Vergleich zu anderen Organen überaus schwache Expression der SOAT-Varianten in der Plazenta stellt die postulierte Bedeutung des SOAT als physiologisch relevanter Transporter für die großen Mengen trächtigkeitsspezifischer sulfatierter Östrogene beim Rind infrage. Weiterhin lassen die Ergebnisse zur SOAT-Expression erkennen, dass dieser insbesondere im

Hoden eine besondere Rolle spielt, einem Organ, welches beim Rind jedoch keine nennenswerten Mengen an sulfatierten Östrogenen produziert.

Bisherige Vorstellungen zur Bedeutung der plazentaren Steroidsynthese beim Rind basieren auf der Annahme, dass Progesteron und/oder Östrogene deren biologisch aktive Produkte darstellen. Es besteht jedoch die Möglichkeit, dass auch andere Steroide wie z.B. Androgene, die ebenfalls im Trophoblast produziert werden, in den Plazentomen regulatorische Funktionen erfüllen. Zur Identifizierung möglicher Zielzellen plazentarer Androgene wurde ein immunhistochemisches Verfahren zur Darstellung von Androgenrezeptoren in Rindergeweben unter Verwendung eines Primärantikörpers gegen den N-Terminus des menschlichen Androgenrezeptors etabliert. Die Spezifität des Primärantikörpers für den Androgenrezeptor des Rindes wurde im Western Blot und in immunhistologischen Kontrollexperimenten bestätigt, in denen Nebenhoden eines Bullen als Positivkontrolle verwendet wurde. In den Plazentomen fanden sich während der gesamten Gravidität deutliche nukleäre Signale in invasiven TGC. Wie die quantitative Auswertung der Immunfärbung mittels eines immunreaktiven Scores ergab, war die Androgenrezeptorexpression in den reifen, noch im Trophoblasten gelegenen TGC sowie in unreifen TGC, UTC, den Stromazellen der Chorionzotten, dem Karunkelepithel und den Karunkelstromazellen in der frühen und mittleren Gravidität nur schwach, stieg aber in der Spätphase der Gravidität signifikant an (p jeweils < 0.01). Die Expression von Androgenrezeptoren in den Plazentomen wurde qualitativ mittels konventioneller RT-PCR bestätigt. Bei der Messung der Androgenrezeptor-mRNA-Expression mittels Real-time RT-PCR ergab sich ein tendenzieller Anstieg der relativen Genexpression, der jedoch nicht statistisch signifikant war. Bei der radioimmunologischen Messung der Testosteronkonzentration im Plazentomgewebe ergab sich ein Anstieg der Messwerte ($p<0.01$) von Konzentrationen geringfügig über der Nachweisgrenze des Messverfahrens (0.1 ng/g Gewebe) zwischen den Tagen 50-100 bis auf Konzentrationen um 0.9 ng/g Gewebe während der späten Gravidität. Diese Konzentrationen müssen als ausreichend angesehen werden, lokal im Gewebe vorhandene Androgenrezeptoren zu aktivieren. Diese Ergebnisse deuten darauf hin, dass beim Rind Androgene biologisch aktive Produkte der plazentaren Steroidsynthese darstellen und eine Rolle in der Steuerung der TGC-Differenzierung spielen könnten. Andererseits könnte der Androgenrezeptor auch androgenunabhängige Funktionen erfüllen, da die Aktivität von Steroidrezeptoren teilweise ligandunabhängig ist.

Die in dieser Arbeit insgesamt erhaltenen Resultate ergaben neue Informationen zu verschiedenen Aspekten der Steroidsynthese in der Rinderplazenta und erbrachten Ansatzpunkte für neue Hypothesen hinsichtlich deren funktioneller Bedeutung.

8. REFERENCES

Adams JB. Enzymic synthesis of steroid sulphates. XVII. On the structure of bovine estrogen sulphotransferase. Biochim Biophys Acta. 1991; 1076(2):282-8.

Ainsworth L, Ryan KJ. Steroid hormone transformations by endocrine organs from pregnant mammals. I. Estrogen biosynthesis by mammalian placental preparations in vitro. Endocrinology. 1966; 79(5):875-83.

Aksoy IA, Wood TC, Weinshilboum R. Human liver estrogen sulfotransferase: identification by cDNA cloning and expression. Biochem Biophys Res Commun. 1994; 200(3):1621-9.

Allen WR. Fetomaternal interactions and influences during equine pregnancy. Reproduction. 2001; 121(4):513-27.

Alnouti Y, Klaassen CD. Tissue distribution and ontogeny of sulfotransferase enzymes in mice. Toxicol Sci. 2006; 93(2):242-55.

Anthony RV, Liang R, Kayl EP, Pratt SL. The growth hormone/prolactin gene family in ruminant placentae. J Reprod Fertil Suppl. 1995; 49:83-95.

Apparao KB, Lovely LP, Gui Y, Lininger RA, Lessey BA. Elevated endometrial androgen receptor expression in women with polycystic ovarian syndrome. Biol Reprod. 2002; 66(2):297-304.

Ballantyne S, Daniel DL Jr, Wickens M. A dependent pathway of cytoplasmic polyadenylation reactions linked to cell cycle control by c-mos and CDK1 activation. Mol Biol Cell. 1997; 8(8):1633-48.

Bammann BL, Coulam CB, Jiang NS. Total and free testosterone during pregnancy. Am J Obstet Gynecol. 1980; 137(3):293-8.

Barile G, Gianì S, Montemurro A, Mango D, Scirpa P. Evidence for a testosterone binding macromolecule in human placental cytosol. J Steroid Biochem. 1979; 11(3):1247-52.

Barkley MS, Goldman BD. A quantitative study of serum testosterone, sex accessory organ growth, and the development of intermale aggression in the mouse. Horm Behav. 1977; 8(2):208-18.

Barth A, Römer W, Oettel M. Influence of subchronic administration of oestrone-3-O-sulphamate on oestrone sulphatase activity in liver, spleen and white blood cells of ovariectomized rats. Arch Toxicol. 2000; 74(7):366-71.

Bartol FF, Roberts RM, Bazer FW, Thatcher WW. Characterization of proteins produced in vitro by bovine endometrial explants. Biol Reprod. 1985; 33(3):745-59.

Ben-David E, Shemesh M. Ultrastructural localization of cytochrome P-450scc in the bovine placentome using protein A-gold technique. Biol Reprod. 1990; 42(1):131-8.

Berg JM. DNA binding specificity of steroid receptors. Cell. 1989; 57(7):1065-8.

Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol. 1999; 19(12):8383-92.

Bilinska B, Hejmej A, Pawlak M, Sadowska J, Tischner M. Immunoexpression of androgen receptors in testes of immature and adult stallions. Equine Vet J. 2004; 36,539-43.

Björkman N. Contributions of electron microscopy in elucidating placental structure and function. Int Rev Gen Exper Zool. 1968; 90: 309-71.

Björkman N. Light and electron microscopic studies on cellular alterations in the normal bovine placentome. Anat Rec. 1969; 163: 17–30.

Blanchard RL, Freimuth RR, Buck J, Weinshilboum RM, Coughtrie MW. A proposed nomenclature system for the cytosolic sulfotransferase (SULT) superfamily. Pharmacogenetics. 2004; 14(3):199-211.

Bonaccorsi L, Marchiani S, Ferruzzi P, Muratori M, Crescioli C, Forti G, Maggi M, Baldi E. Non-genomic effects of the androgen receptor and vitamin D agonist are involved in suppressing invasive phenotype of prostate cancer cells. Steroids. 2006; 71(4):304-9.

Bonney RC, Scanlon MJ, Jones DL, Reed MJ, James VH. Adrenal androgen concentrations in endometrium and plasma during the menstrual cycle. J Endocrinol. 1984; 101(2):181-8.

Boos A, Kohtes J, Stelljes A, Zerbe H, Thole HH. Immunohistochemical assessment of progesterone, oestrogen and glucocorticoid receptors in bovine placentomes during pregnancy, induced parturition, and after birth with or without retention of fetal membranes. J Reprod Fertil. 2000; 120(2):351-60.

Boos A, Janssen V, Mulling C. Proliferation and apoptosis in bovine placentomes during pregnancy and around induced and spontaneous parturition as well as in cows retaining the fetal membranes. Reproduction. 2003; 126(4):469-480.

Borgono CA, Diamandis EP. The emerging roles of human tissue kallikreins in cancer. Nat Rev Cancer. 2004; 4(11):876-90.

Brix LA, Barnett AC, Duggleby RG, Leggett B, McManus ME. Analysis of the substrate specificity of human sulfotransferases SULT1A1 and SULT1A3: site-directed mutagenesis and kinetic studies. Biochemistry. 1999; 38(32):10474-9.

Brown JM, Adams JB, Beattie CW. Production and characterization of a monoclonal antibody to bovine estrogen sulfotransferase. Hybridoma. 1987; 6(4):413-22.

Burger HG. Androgen production in women. Fertil Steril. 2002; 77 Suppl 4:S3-5.

Burton MJ, Dziuk HE, Fahning ML, Zemjanis R. Myometrial activity during natural and dexamethasone-induced parturition in the cow. Am J Vet Res. 1987; 48(1):37-44.

Carlsen SM, Jacobsen G, Romundstad P. Maternal testosterone levels during pregnancy are associated with offspring size at birth. Eur J Endocrinol. 2006; 155(2):365-70.

Carmina E, Wong L, Chang L, Paulson RJ, Sauer MV, Stanczyk FZ, Lobo RA. Endocrine abnormalities in ovulatory women with polycystic ovaries on ultrasound. Hum Reprod. 1997; 12(5):905-9.

Carter AM, Enders AC. Comparative aspects of trophoblast development and placentation. Reprod Biol Endocrinol. 2004; 2:46.

Carter AM, Mess A. Evolution of the placenta in eutherian mammals. Placenta. 2007; 28(4):259-62.

Castracane VD, Stewart DR, Gimpel T, Overstreet JW, Lasley BL. Maternal serum androgens in human pregnancy: early increases within the cycle of conception. Hum Reprod. 1998; 13(2):460-4.

Chadha S, Pache TD, Huikeshoven JM, Brinkmann AO, van der Kwast TH. Androgen receptor expression in human ovarian and uterine tissue of long-term androgen-treated transsexual women. Hum Pathol. 1994; 25(11):1198-204.

Chapman E, Best MD, Hanson SR, Wong CH. Sulfotransferases: structure, mechanism, biological activity, inhibition, and synthetic utility. Angew Chem Int Ed Engl. 2004; 43(27):3526-48.

Charlesworth A, Welk J, MacNicol AM. The temporal control of Wee1 mRNA translation during Xenopus oocyte maturation is regulated by cytoplasmic polyadenylation elements within the 3'-untranslated region. Dev Biol. 2000; 227(2):706-19.

Chew BP, Erb RE, Fessler JF, Callahan CJ, Malven PV. Effects of ovariectomy during pregnancy and of prematurely induced parturition on progesterone, estrogens, and calving traits. J Dairy Sci. 1979; 62(4):557-66.

Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, Rombauts W. Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. J Steroid Biochem Mol Biol. 2001; 76(1-5):23-30.

Clarke CL, Adams JB, Wren BG. Induction of estrogen sulfotransferase in the human endometrium by progesterone in organ culture. J Clin Endocrinol Metab. 1982; 55(1):70-5.

Cleutjens KB, van der Korput HA, van Eekelen CC, van Rooij HC, Faber PW, Trapman J. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol Endocrinol. 1997; 11(2):148-61.

Collins LL, Lee HJ, Chen YT, Chang M, Hsu HY, Yeh S, Chang C. The androgen receptor in spermatogenesis. Cytogenet Genome Res. 2003; 103(3-4):299-301.

Comline RS, Hall LW, Lavelle RB, Nathanielsz PW, Silver M. Parturition in the cow: endocrine changes in animals with chronically implanted catheters in the foetal and maternal circulations. J Endocrinol. 1974; 63(3):451-72.

Conley AJ, Ford SP. Effect of prostaglandin F2 alpha-induced luteolysis on in vivo and in vitro progesterone production by individual placentomes of cows. J Anim Sci. 1987; 65(2):500-7.

Conley AJ, Bird IM. The role of cytochrome P450 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the delta 5 and delta 4 pathways of steroidogenesis in mammals. Biol Reprod. 1997; 56(4):789-99.

Coughtrie MW. Sulfation through the looking glass - recent advances in sulfotransferase research for the curious. Pharmacogenomics J. 2002; 2(5):297-308.

Craddock AL, Love MW, Daniel RW, Kirby LC, Walters HC, Wong MH, Dawson PA. Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter. Am J Physiol. 1998; 274(1 Pt 1):G157-69.

Csapo A. The mechanism of effect of the ovarian steroids. Recent Prog Horm Res. 1956; 12:405-27.

Culig Z, Hobisch A, Cronauer MV, Radmayr C, Trapman J, Hittmair A, Bartsch G, Klocker H. Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor. Cancer Res. 1994; 54(20):5474-8.

Dadoune JP. Expression of mammalian spermatozoal nucleoproteins. Microsc Res Tech. 2003; 61(1):56-75.

Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J. Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. J Biol Chem. 1991; 266(5):3107-12.

Day AM. Cloprostenol for termination of pregnancy in cattle. A) The induction of parturition. N Z Vet J. 1977; 25(6):136–9.

Dean MA, Thomas JR. Purification and endocrine evaluation of bovine binucleate trophoblastic cells. J Tiss Cult Meth. 1988; 11(2): 83-8.

Diamond M, Llacuna A, Wong CL. Sex behavior after neonatal progesterone, testosterone, estrogen, or antiandrogen. Hormones and Behavior. 1973; 4: 73-88.

Diczfalusy E. Steroid metabolism in the human foeto-placental unit. Acta Endocrinol (Copenh). 1969; 61(4):649-64.

Duda M, Slomczynska M. Immunohistochemical localization of androgen receptor in two subpopulations of porcine granulosa cells in vitro. Reprod Domest Anim. 2007; 42(1):22-5.

Duello TM, Byatt JC, Bremel RD. Immunohistochemical localization of placental lactogen in binucleate cells of bovine placentomes. Endocrinology. 1986; 119(3):1351-5.

Eckhardt U, Schroeder A, Stieger B, Höchli M, Landmann L, Tynes R, Meier PJ, Hagenbuch B. Polyspecific substrate uptake by the hepatic organic anion transporter Oatp1 in stably transfected CHO cells. Am J Physiol. 1999; 276(4 Pt 1):1037-42.

Eissa HM, el-Belely MS. Sequential changes in plasma progesterone, total oestrogens and corticosteroids in the cow throughout pregnancy and around parturition. Br Vet J. 1990; 146(1):24-9.

Eley RM, Thatcher WW, Bazer FW. Hormonal and physical changes associated with bovine conceptus development. J Reprod Fertil. 1979; 55(1):181-90.

Elias PM, Crumrine D, Rassner U, Hachem JP, Menon GK, Man W, Choy MH, Leypoldt L, Feingold KR, Williams ML. Basis for abnormal desquamation and permeability barrier dysfunction in RXLI. J Invest Dermatol. 2004; 122(2):314-9.

Enders AC, Carter AM. What can comparative studies of placental structure tell us? - A review. Placenta. 2004; 25 Suppl A:S3-9.

Enders AC, Carter AM. Comparative placentation: some interesting modifications for histotrophic nutrition - a review. Placenta. 2006; 27 Suppl A:S11-6.

Estergreen VL Jr, Frost OL, Gomes WR, Erb RE, Bullard JF. Effect of ovariectomy on pregnancy maintenance and parturition in dairy cows. J Dairy Sci. 1967; 50(8):1293-5.

Evans RM. The steroid and thyroid hormone receptor superfamily. Science. 1988; 240(4854):889-95.

Evans G, Wagner WC. In vitro oestrogen synthesis by bovine placenta during pregnancy and induced parturition. Acta Endocrinol (Copenh). 1981; 98(1):119-25.

Evans AC, Komar CM, Wandji SA, Fortune JE. Changes in androgen secretion and luteinizing hormone pulse amplitude are associated with the recruitment and growth of ovarian follicles during the luteal phase of the bovine estrous cycle. Biol Reprod. 1997; 57(2):394-401.

Fairclough RJ, Hunter JT, Welch RA. Peripheral plasma progesterone and utero-ovarian prostaglandin F concentrations in the cow around parturition. Prostaglandins. 1975; 9(6):901-14.

Falany JL, Falany CN. Regulation of estrogen sulfotransferase in human endometrial adenocarcinoma cells by progesterone. Endocrinology. 1996; 137(4):1395-401.

Ferrell CL. Maternal and fetal influences on uterine and conceptus development in the cow: II. Blood flow and nutrient flux. J Anim Sci. 1991; 69(5):1954-65.

Ferrell CL, Ford SP, Prior RL, Christenson RK. Blood flow, steroid secretion and nutrient uptake of the gravid bovine uterus and fetus. J Anim Sci. 1983; 56(3):656-67.

Fischer HE, Bazer FW, Fields MJ. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and pseudopregnancy in gilts. J Reprod Fertil. 1985; 75(1):69-78.

Foxley GJ, Dong Q, Handelsman DJ. Quantitative reverse transcriptase polymerase chain reaction assay for mouse androgen receptor mRNA. Endocrine. 2001; 15(2):193-8.

Foradori CD, Weiser MJ, Handa RJ. Non-genomic actions of androgens. Front Neuroendocrinol. 2008; 29(2):169-81.

Freedman LP. Anatomy of the steroid receptor zinc finger region. Endocr Rev. 1992; 13(2):129-45.

Freedman LP, Luisi BF. On the mechanism of DNA binding by nuclear hormone receptors: a structural and functional perspective. J Cell Biochem. 1993; 51(2):140-50.

Freeman ER, Bloom DA, McGuire EJ. A brief history of testosterone. J Urol. 2001; 165(2):371-3.

Freimuth RR, Eckloff B, Wieben ED, Weinshilboum RM. Human sulfotransferase SULT1C1 pharmacogenetics: gene resequencing and functional genomic studies. Pharmacogenetics. 2004; 11(9):747-56.

Frenette G, Leclerc P, D'amours O, Sullivan R. Estrogen sulfotransferase is highly expressed along the bovine epididymis and is secreted into the intraluminal environment. J Androl. 2009; 30(5):580-9.

Gaiani R, Chiesa F, Mattioli M, Nannetti G, Galeati G. Androstenedione and testosterone concentrations in plasma and milk of the cow throughout pregnancy. J Reprod Fertil. 1984; 70(1):55-9.

Gao W, Dalton JT. Expanding the therapeutic use of androgens via selective androgen receptor modulators (SARMs). Drug Discov Today. 2007; 12(5-6):241-8.

Gershon E, Hourvitz A, Reikhav S, Maman E, Dekel N. Low expression of COX-2, reduced cumulus expansion, and impaired ovulation in SULT1E1-deficient mice. FASEB J. 2007; 21(8):1893-901.

Geyer J, Godoy JR, Petzinger E. Identification of a sodium-dependent organic anion transporter from rat adrenal gland. Biochem Biophys Res Commun. 2004; 316(2):300-6.

Geyer J, Wilke T, Petzinger E. The solute carrier family SLC10: more than a family of bile acid transporters regarding function and phylogenetic relationships. Naunyn Schmiedebergs Arch Pharmacol. 2006; 372(6):413-31.

Geyer J, Döring B, Meerkamp K, Ugele B, Bakhiya N, Fernandes CF, Godoy JR, Glatt H, Petzinger E. Cloning and functional characterization of human sodium-dependent organic anion transporter (SLC10A6). J Biol Chem. 2007; 282(27):19728-41.

Gomes WR, Erb RE. Progesterone in bovine reproduction. a review. J Dairy Sci. 1965; 48:314-30.

Gong H, Guo P, Zhai Y, Zhou J, Uppal H, Jarzynka MJ, Song WC, Cheng SY, Xie W. Estrogen deprivation and inhibition of breast cancer growth in vivo through activation of the orphan nuclear receptor liver X receptor. Mol Endocrinol. 2007; 21(8):1781-90.

Goyal HO, Bartol FF, Wiley AA, Khalil MK, Chiu J, Vig MM. Immunolocalization of androgen receptor and estrogen receptor in the developing testis and excurrent ducts of goats. Anat Rec. 1997; 249(1):54-62.

Green JA, Xie S, Quan X, Bao B, Gan X, Mathialagan N, Beckers JF, Roberts RM. Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. Biol Reprod. 2000; 62(6):1624-31.

Greven H. Hydrolyse und Transport sulfatierter Steroide in den Plazentomen des Rindes: Charakterisierung der Expression und der Funktionen von Steroidsulfatase (StS) und des Sodium-dependent Organic Anion Transporters (SOAT). PhD Thesis, JLU Giessen, 2008.

Greven H, Kowalewski MP, Hoffmann B, Geyer J, Rex-Haffner M, Ugele B, Schuler G. Bovine placental steroid sulphatase: molecular cloning and expression pattern in placentomes during gestation and at parturition. Placenta. 2007; 28(8-9):889-97.

Gronemeyer H, Laudet V. Transcription factors 3: nuclear receptors. Protein Profile. 1995; 2(11):1173-308.

Gross TS, Williams WF. In-vitro steroid synthesis by the placenta of cows in late gestation and at parturition. J Reprod Fertil. 1988; 83(2):565-73.

Gross TS, Williams WF, Russek-Cohen E. Cellular changes in the peripartum bovine fetal placenta related to placental separation. Placenta. 1991; 12(1):27-35.

Hähnel R, Twaddle E, Ratajczak T. The specificity of the estrogen receptor of human uterus. J Steroid Biochem. 1973; 4(1):21-31.

Härd T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR, Gustafsson JA, Kaptein R. Solution structure of the glucocorticoid receptor DNA-binding domain. Science. 1990; 249(4965):157-60.

Hagenbuch B, Meier PJ. Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. J Clin Invest. 1994; 93(3):1326-31.

Hagenbuch B, Lübbert H, Stieger B, Meier PJ. Expression of the hepatocyte Na+/bile acid cotransporter in Xenopus laevis oocytes. J Biol Chem. 1990; 265(10):5357-60.

Hagenbuch B, Dawson P. The sodium bile salt cotransport family SLC10. Pflugers Arch. 2004; 447(5):566-70.

Hampton JH, Manikkam M, Lubahn DB, Smith MF, Garverick HA. Androgen receptor mRNA expression in the bovine ovary. Domest Anim Endocrinol. 2004; 27(1):81-8.

Hansen PJ. Regulation of uterine immune function by progesterone - lessons from the sheep. J Reprod Immunol. 1998; 40(1):63-79.

Hansen PJ. Regulation of immune cells in the uterus during pregnancy in ruminants. J Anim Sci. 2007; 85(13 Suppl):30-1.

Heap RB. Paracrine and autocrine functions of the placenta: a key to the success of viviparity? Exp Clin Endocrinol. 1994; 102(3):262-8.

Hedberg Y, Dalin AM, Forsberg M, Lundeheim N, Hoffmann B, Ludwig C, Kindahl H. Effect of ACTH (tetracosactide) on steroid hormone levels in the mare. Part A: effect in intact normal mares and mares with possible estrous related behavioral abnormalities. Anim Reprod Sci. 2007; 100(1-2):73-91.

Hediger MA. Structure, function and evolution of solute transporters in prokaryotes and eukaryotes. J Exp Biol. 1994; 196:15-49.

Heinlein CA, Chang C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol. 2002; 16(10):2181-7.

Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev. 2004; 25(2):276-308.

Henricks DM, Lamond DR, Hill JR, Dickey JF. Plasma progesterone concentrations before mating and in early pregnancy in the beef heifer. J Anim Sci. 1971; 33(2):450-4.

Hernandez-Martín A, Gonzalez-Sarmiento R, De Unamuno P. X-linked ichthyosis: an update. Br J Dermatol. 1999; 141(4):617-27.

Hild-Petito S, West NB, Brenner RM, Stouffer RL. Localization of androgen receptor in the follicle and corpus luteum of the primate ovary during the menstrual cycle. Biol Reprod. 1991; 44(3):561-8.

Hillier SG, Tetsuka M, Fraser HM. Location and developmental regulation of androgen receptor in primate ovary. Hum Reprod. 1997; 12(1):107-11.

Hillier SG, Tetsuka M. Role of androgens in follicle maturation and atresia. Baillieres Clin Obstet Gynaecol. 1997; 11(2):249-60.

Hirayama H, Sawai K, Moriyasu S, Hirayama M, Goto Y, Kaneko E, Miyamoto A, Ushizawa K, Takahashi T, Minamihashi A. Excess estrogen sulfoconjugation as the possible cause for a poor sign of parturition in pregnant cows carrying somatic cell clone fetuses. Reproduction. 2008; 136(5):639-47.

Hirota K, Gomi T, Kishimoto R, Iguchi M, Hayakawa K, Nakagawa H. Androgen receptor in human placental villi. J Biochem. 1981; 89(1):153-60.

Hobisch A, Eder IE, Putz T, Horninger W, Bartsch G, Klocker H, Culig Z. Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor. Cancer Res. 1998; 58(20):4640-5.

Hobkirk R. Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. Can J Biochem Cell Biol. 1985; 63(11):1127-44.

Hobkirk R, Glasier MA. Estrogen sulfotransferase distribution in tissues of mouse and guinea pig: steroidal inhibition of the guinea pig enzyme. Biochem Cell Biol. 1992; 70(8):712-5.

Hobkirk R, Cardy CA, Saidi F, Kennedy TG, Girard LR. Development and characteristics of an oestrogen sulphotransferase in placenta and uterus of the pregnant mouse. Comparison between mouse and rat. Biochem J. 1983; 216(2):451-7.

Hoedemaker M, Weston PG, Wagner WC. Influence of cortisol and different steroidogenic pathways on estrogen synthesis by the bovine placenta. Am J Vet Res. 1990; 51(7):1012-5.

Hoffman LH, Wooding FB. Giant and binucleate trophoblast cells of mammals. J Exp Zool. 1993; 266(6):559-77.

Hoffmann B. Aspects on the formation and detection of tissue levels of anabolic steroids in domestic animals. J Steroid Biochem. 1979; 11(1C):919-22.

Hoffmann B, Schuler G. The bovine placenta; a source and target of steroid hormones: observations during the second half of gestation. Domest Anim Endocrinol. 2002; 23(1-2):309-20.

Hoffmann B, Wagner WC, Gimenez T. Free and conjugated steroids in maternal and fetal plasma in the cow near term. Biol Reprod. 1976; 15(1):126-33.

Hoffmann B, Wagner WC, Rattenberger E, Schmidt J. Endocrine relationships during late gestation and parturition in the cow. Ciba Found Symp. 1977; (47):107-25.

Hoffmann B, Bahr J, Hixon JE, Wagner WC. Observations concerning the functional status of the corpus luteum and the placenta around parturition in the cow. Anim Reprod Sci. 1979; 2: 253-266.

Hoffmann B, Goes de Pinho T, Schuler G. Determination of free and conjugated oestrogens in peripheral blood plasma, feces and urine of cattle throughout pregnancy. Exp Clin Endocrinol Diabetes. 1997; 105(5):296-303.

Hoffmann B, Falter K, Vielemeier A, Failing K, Schuler G. Investigations on the activity of bovine placental oestrogen sulfotransferase and -sulfatase from midgestation to parturition. Exp Clin Endocrinol Diabetes. 2001; 109(5):294-301.

Holt HB, Medbak S, Kirk D, Guirgis R, Hughes I, Cummings MH, Meeking DR. Recurrent severe hyperandrogenism during pregnancy: a case report. J Clin Pathol. 2005; 58(4):439-42.

Holtan DW, Squires EL, Lapin DR, Ginther OJ. Effect of ovariectomy on pregnancy in mares. J Reprod Fertil Suppl. 1979; (27):457-63.

Horie K, Takakura K, Imai K, Liao S, Mori T. Immunohistochemical localization of androgen receptor in the human endometrium, decidua, placenta and pathological conditions of the endometrium. Hum Reprod. 1992a; 7(10):1461-6.

Horie K, Takakura K, Fujiwara H, Suginami H, Liao S, Mori T. Immunohistochemical localization of androgen receptor in the human ovary throughout the menstrual cycle in relation to oestrogen and progesterone receptor expression. Hum Reprod. 1992b; 7(2):184-90.

Hradecky P, Mossman HW, Stott GG. Comparative development of ruminant placentomes. Theriogenology. 1988; 29(3):715-29.

Hsu TY, Lan KC, Tsai CC, Ou CY, Cheng BH, Tsai MY, Kang HY, Tung YH, Wong YH, Huang KE. Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies. Taiwan J Obstet Gynecol. 2009; 48(3):262-7.

Hu YC, Wang PH, Yeh S, Wang RS, Xie C, Xu Q, Zhou X, Chao HT, Tsai MY, Chang C. Subfertility and defective folliculogenesis in female mice lacking androgen receptor. Proc Natl Acad Sci USA. 2004; 101(31):11209-14.

Huang ZQ, Li J, Wong J. AR possesses an intrinsic hormone-independent transcriptional activity. Mol Endocrinol. 2002; 16(5):924-37.

Igwebuike UM. Trophoblast cells of ruminant placentas - A minireview. Anim Reprod Sci. 2006; 93(3-4):185-98.

Jackson JA, Albrecht ED. The development of placental androstenedione and testosterone production and their utilization by the ovary for aromatization to estrogen during rat pregnancy. Biol Reprod. 1985; 33(2):451-7.

Janowski T, Zdunczyk S, Podhalicz-Dziegielewska M, Rasz A, Chmielewski A. Effect of oestrogen antagonist (tamoxifen) on steroid hormone levels, maturation process of placentomes and course of late pregnancy in cows. Reprod Domest Anim. 1996; 31(2): 379-384.

Janowski T, Zdunczyk S, Malecki-Tepicht J, Baranski W, Ras A. Mammary secretion of oestrogens in the cow. Domest Anim Endocrinol. 2002; 23(1-2):125-37.

Johnson WH, Manns JG, Adams WM, Mapletoft RJ. Termination of pregnancy with cloprostenol and dexamethasone in intact or ovariectomized cows. Can Vet J. 1981; 22(9):288-90.

Kanai N, Lu R, Bao Y, Wolkoff AW, Vore M, Schuster VL. Estradiol 17 beta-D-glucuronide is a high-affinity substrate for oatp organic anion transporter. Am J Physiol. 1996; 270(2 Pt 2):326-31.

Karrer-Voegeli S, Rey F, Reymond MJ, Meuwly JY, Gaillard RC, Gomez F. Androgen dependence of hirsutism, acne, and alopecia in women: retrospective analysis of 228 patients investigated for hyperandrogenism. Medicine (Baltimore). 2009; 88(1):32-45.

Kessler MA, Duello TM, Schuler LA. Expression of prolactin-related hormones in the early bovine conceptus, and potential for paracrine effect on the endometrium. Endocrinology. 1991; 129(4):1885-95.

Khor VK, Tong MH, Qian Y, Song WC. Gender-specific expression and mechanism of regulation of estrogen sulfotransferase in adipose tissues of the mouse. Endocrinology. 2008; 149(11):5440-8.

Klisch K, Hecht W, Pfarrer C, Schuler G, Hoffmann B, Leiser R. DNA content and ploidy level of bovine placentomal trophoblast giant cells. Placenta. 1999; 20(5-6):451-8.

Klisch K, Boos A, Friedrich M, Herzog K, Feldmann M, Sousa N, Beckers J, Leiser R, Schuler G. The glycosylation of pregnancy-associated glycoproteins and prolactin-related protein-I in bovine binucleate trophoblast giant cells changes before parturition. Reproduction. 2006; 132(5):791-8.

Kowalski AA, Vale-Cruz DS, Simmen FA, Simmen RC. Uterine androgen receptors: roles in estrogen-mediated gene expressionand DNA synthesis. Biol Reprod. 2004; 70(5):1349-57.

Kramer W, Stengelin S, Baringhaus KH, Enhsen A, Heuer H, Becker W, Corsiero D, Girbig F, Noll R, Weyland C. Substrate specificity of the ileal and the hepatic Na(+)/bile acid cotransporters of the rabbit. I. Transport studies with membrane vesicles and cell lines expressing the cloned transporters. J Lipid Res. 1999; 40(9):1604-17.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology. 1997; 138(3):863-70.

Kuss E. The fetoplacental unit of primates. Exp Clin Endocrinol. 1994; 102(3):135-65.

Lam ST, Fensom A H, Coleman D, Morsman J, Nicolaides K, Rodeck CH. Steroid sulphatase activity in trophoblast samples. J Obstet Gynaecol. 1984; 5(1):26-26.

Lambard S, Galeraud-Denis I, Martin G, Levy R, Chocat A, Carreau S. Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. Mol Hum Reprod. 2004; 10(7):535-41.

Larsson K, Wagner WC, Sachs M. Oestrogen synthesis by bovine foetal placenta at normal parturition. Acta Endocrinol (Copenh). 1981; 98(1):112-8.

Laven RA, Peters AR. Gross morphometry of the bovine placentome during gestation. Reprod Domest Anim. 2001; 36(6):289-96.

Lawn AM, Chiquoine AD, Amoroso EC. The development of the placenta in the sheep and goat: an electron microscope study. J Anat. 1969; 105(Pt 3):557-78.

Lazaridis KN, Tietz P, Wu T, Kip S, Dawson PA, LaRusso NF. Alternative splicing of the rat sodium/bile acid transporter changes its cellular localization and transport properties. Proc Natl Acad Sci USA. 2000; 97(20):11092-7.

Leaman DW, Roberts RM. Genes for the trophoblast interferons in sheep, goat, and musk ox and distribution of related genes among mammals. J Interferon Res. 1992; 12(1):1-11.

Lee CS, Gogolin-Ewens K, Brandon MR. Comparative studies on the distribution of binucleate cells in the placentae of the deer and cow using the monoclonal antibody, SBU-3. J Anat. 1986; 147:163-79.

Legrand C, Marie J, Maltier JP. Testosterone, dihydrotestosterone, androstenedione and dehydroepiandrosterone concentrations in placentae, ovaries and plasma of the rat in late pregnancy. Acta Endocrinol (Copenh). 1984; 105(1):119-25.

Leiser R, Kaufmann P. Placental structure: in a comparative aspect. Exp Clin Endocrinol. 1994; 102(3):122-34.

Levine S. Sexual differentiation: the development of maleness and femaleness. Calif Med. 1971; 114(1):12-7.

Luef G, Abraham I, Trinka E, Alge A, Windisch J, Daxenbichler G, Unterberger I, Seppi K, Lechleitner M, Krämer G, Bauer G. Hyperandrogenism, postprandial hyperinsulinism and the risk of PCOS in a cross sectional study of women with epilepsy treated with valproate. Epilepsy Res. 2002; 48(1-2):91-102.

Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature. 1991; 352:497-505.

Macaulay JO, Warne GL, Krozowski ZS. Human placenta contains a high affinity R1881 binding site that is not the androgen receptor. J Steroid Biochem. 1988; 29(5):497-503.

MacIndoe JH, Woods GR, Lee FJ. The specific binding of androgens and the subsequent distribution of androgen-receptor complexes within MCF-7 human breast cancer cells. Steroids. 1981; 38(4):439-52.

Maguelone GF. Role of androgens in fetal and pubertal development. Horm Res. 1983; 18:69-83.

Mancini MA, Song CS, Rao TR, Chatterjee B, Roy AK. Spatio-temporal expression of estrogen sulfotransferase within the hepatic lobule of male rats: implication of in situ estrogen inactivation in androgen action. Endocrinology. 1992; 131(3):1541-6.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: the second decade. Cell. 1995; 83(6):835-9.

Mann GE, Lamming GE. The influence of progesterone during early pregnancy in cattle. Reprod Domest Anim. 1999; 34:269–274.

Mann GE, Lamming GE, Robinson RS, Wathes DC. The regulation of interferon-tau production and uterine hormone receptors during early pregnancy. J Reprod Fertil Suppl. 1999; 54:317-28.

Marcelli M, Stenoien DL, Szafran AT, Simeoni S, Agoulnik IU, Weigel NL, Moran T, Mikic I, Price JH, Mancini MA. Quantifying effects of ligands on androgen receptor nuclear translocation, intranuclear dynamics, and solubility. J Cell Biochem. 2006; 98(4):770-88.

Matamoros RA, Caamano L, Lamb SV, Reimers TJ. Estrogen production by bovine binucleate and mononucleate trophoblastic cells in vitro. Biol Reprod. 1994; 51(3):486-92.

Matsumoto T, Takeyama K, Sato T, Kato S. Androgen receptor functions from reverse genetic models. J Steroid Biochem Mol Biol. 2003; 85(2-5):95-9.

Mattioli M, Gaiani R, Bono G, Chiesa F. Estrone to estrone sulfate conversion and viceversa in bovine placentome. Arch Vet Ital. 1984; 35:22-6.

McCormick PD, Razel AJ, Spelsberg TC, Coulam CB. Evidence for an androgen receptor in the human placenta. Am J Obstet Gynecol. 1981; 140(1):8-13.

McKenna TJ, Cunningham SK. Adrenal androgen production in polycystic ovary syndrome. Eur J Endocrinol. 1995; 133(4):383-9.

Melampy RM, Hearn WR, Rakes JM. Progesterone content of bovine reproductive organs and blood during pregnancy. J Anim Sci. 1959; 18: 307-13.

Mertens HJ, Heineman MJ, Koudstaal J, Theunissen P, Evers JL. Androgen receptor content in human endometrium. Eur J Obstet Gynecol Reprod Biol. 1996; 70(1):11-3.

Meyers SA, Lozon MM, Corombos JD, Saunders DE, Hunter K, Christensen C, Brooks SC. Induction of porcine uterine estrogen sulfotransferase activity by progesterone. Biol Reprod. 1983; 28(5):1119-28.

Miki Y, Nakata T, Suzuki T, Darnel AD, Moriya T, Kaneko C, Hidaka K, Shiotsu Y, Kusaka H, Sasano H. Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues. J Clin Endocrinol Metab. 2002; 87(12):5760-8.

Milewich L, Garcia RL, Gerrity LW. Steroid sulfatase and 17 beta-hydroxysteroid oxidoreductase activities in mouse tissues. J Steroid Biochem. 1984; 21(5):529-38.

Miller KK, Rosner W, Lee H, Hier J, Sesmilo G, Schoenfeld D, Neubauer G, Klibanski A. Measurement of free testosterone in normal women and women with androgen deficiency: comparison of methods. J Clin Endocrinol Metab. 2004; 89(2):525-33.

Mode A, Gustafsson JA, Södersten P, Eneroth P. Sex differences in behavioural androgen sensitivity: possible role of androgen metabolism. J Endocrinol. 1984; 100(2):245-8.

Möstl E, Kiesenhofer E, Bamberg E. Östrogenproduktion in der Rinderplazenta in vitro. Zuchthygiene. 1986; 21,159.

Möstl E, Ismail MN, Choi HS, Bamberg E. Epitestosterone and oestrogen concentration in plasma of cows during induction of parturition with prostaglandin F2α. Anim Reprod Sci. 1987; 14(4):245-50.

Mossman HW. Vertebrate Fetal Membranes. Rutgers University Press, 1987; New Brunswick, New Jersey.

Myers DA, Reimers TJ. Purification and endocrine evaluation of bovine binucleate trophoblastic cells. J Tiss Cult Meth. 1988; 11:83-8.

Nash AR, Glenn WK, Moore SS, Kerr J, Thompson AR, Thompson EO. Oestrogen sulfotransferase: molecular cloning and sequencing of cDNA for the bovine placental enzyme. Aust J Biol Sci. 1988; 41(4):507-16.

Newman SP, Purohit A, Ghilchik MW, Potter BV, Reed MJ. Regulation of steroid sulphatase expression and activity in breast cancer. J Steroid Biochem Mol Biol. 2000; 75(4-5):259-64.

Nozawa T, Suzuki M, Takahashi K, Yabuuchi H, Maeda T, Tsuji A, Tamai I. Involvement of estrone-3-sulfate transporters in proliferation of hormone-dependent breast cancer cells. J Pharmacol Exp Ther. 2004; 311(3):1032-7.

Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. Lancet. 2002; 360(9335):772-7.

Pandini G, Mineo R, Frasca F, Roberts CT Jr, Marcelli M, Vigneri R, Belfiore A. Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. Cancer Res. 2005; 65(5):1849-57.

Papadopoulou N, Papakonstanti EA, Kallergi G, Alevizopoulos K, Stournaras C. Membrane androgen receptor activation in prostate and breast tumor cells: molecular signaling and clinical impact. IUBMB Life. 2009; 61(1):56-61.

Parlevliet JM, Pearl CA, Hess MF, Famula TR, Roser JF. Immunolocalization of estrogen and androgen receptors and steroid concentrations in the stallion epididymis. Theriogenology. 2006; 66(4):755-65.

Pasqualini JR. Estrogen sulfotransferases in breast and endometrial cancers. Ann N Y Acad Sci. 2009; 1155:88-98.

Payne AH, Lawrence CC, Foster DL, Jaffe RB. Intranuclear binding of 17-estradiol and estrone in female ovine pituitaries following incubation with estrone sulfate. J Biol Chem. 1973; 248(5):1598-602.

Pelletier G. Effects of estradiol on prostate epithelial cells in the castrated rat. J Histochem Cytochem. 2002; 50(11):1517-24.

Pfarrer C, Ebert B, Miglino MA, Klisch K, Leiser R. The three-dimensional feto-maternal vascular interrelationship during early bovine placental development: a scanning electron microscopical study. J Anat. 2001; 198(Pt 5):591-602.

Pfarrer CD, Weise S, Berisha B, Schams D, Leiser R, Hoffmann B, Schuler G. Fibroblast growth factor (FGF)-1, FGF2, FGF7 and FGF receptors are uniformly expressed in trophoblast giant cells during restricted trophoblast invasion in cows. Placenta. 2006a; 27(6-7):758-70.

Pfarrer CD, Ruziwa SD, Winther H, Callesen H, Leiser R, Schams D, Dantzer V. Localization of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2 in bovine placentomes from implantation until term. Placenta. 2006b; 27(8):889-98.

Pierrepoint CG, Davies P. A study of the androgenic function of the epididymis. J Steroid Biochem. 1984; 20(5):1105-11.

Pierrepoint CG, Anderson AB, Griffiths K, Turnbull AC. Metabolism of C19-steroids by foetal cotyledons from the bovine placenta at term. Res Vet Sci. 1969; 10(5):477-9.

Pope WF, Cardenas H. Androgens in female pig reproduction: actions mediated by the androgen receptor. Soc Reprod Fertil Suppl. 2006; 62:55-67.

Powell SM, Christiaens V, Voulgaraki D, Waxman J, Claessens F, Bevan CL. Mechanisms of androgen receptor signalling via steroid receptor coactivator-1 in prostate. Endocr Relat Cancer. 2004; 11(1):117-30.

Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood). 2003; 228(2):111-33.

Prior RL, Laster DB. Development of the bovine fetus. J Anim Sci. 1979; 48(6):1546-53.

Purohit A, Reed MJ, Morris NC, Williams GJ, Potter BV. Regulation and inhibition of steroid sulfatase activity in breast cancer. Ann N Y Acad Sci. 1996; 784:40-9.

Qian YM, Sun XJ, Tong MH, Li XP, Richa J, Song WC. Targeted disruption of the mouse estrogen sulfotransferase gene reveals a role of estrogen metabolism in intracrine and paracrine estrogen regulation. Endocrinology. 2001; 142(12):5342-50.

Rahman F, Christian HC. Non-classical actions of testosterone: an update. Trends Endocrinol Metab. 2007; 18(10):371-8.

Ramsey EM. The placenta - human and animal. Praeger Publishers, New York, 1982.

Rasmussen R. Quantification on the LightCycler. Rapid Cycle Real-time PCR, Methods and Applications Springer Press, Heidelberg. 2001; 21-34.

Ravelich SR, Shelling AN, Wells DN, Peterson AJ, Lee RS, Ramachandran A, Keelan JA. Expression of TGF-beta1, TGF-beta2, TGF-beta3 and the receptors TGF-betaRI and TGF-betaRII in placentomes of artificially inseminated and nuclear transfer derived bovine pregnancies. Placenta. 2006; 27(2-3):307-16.

Reed MJ, Purohit A. Sulphatase inhibitors: the rationale for the development of a new endocrine therapy. Endocr Relat Cancer. 1993; 45:51-62.

Reed MJ, Purohit A, Woo LW, Newman SP, Potter BV. Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev. 2005; 26(2):171-202.

Reimers TJ, Ullmann MB, Hansel W. Progesterone and prostanoid production by bovine binucleate trophoblastic cells. Biol Reprod. 1985; 33(5):1227-36.

Reinikainen P, Palvimo JJ, Janne OA. Effects of mitogens on androgen receptor-mediated transactivation. Endocrinology. 1996; 137(10):4351-7.

Remmele W, Stegner H. Immunhistologischer Nachweis von Östrogenrezeptoren (ER-ICA) im Mammakarzinomgewebe – Vorschlag zur einheitlichen Bewertung des Untersuchungsbefundes. Frauenarzt. 1987; 2, 41-43.

Reynolds LP, Millaway DS, Kirsch JD, Infeld JE, Redmer DA. Growth and in-vitro metabolism of placental tissues of cows from day 100 to day 250 of gestation. J Reprod Fertil. 1990; 89(1):213-22.

Rexroad Jr. CE, Casida LE, Tyler WJ. Crown-rump length of fetuses in purebred Holstein-Friesian cow. J Dairy Sci. 1974; 57:346-7.

Rice S, Ojha K, Whitehead S, Mason H. Stage-specific expression of androgen receptor, follicle-stimulating hormone receptor, and anti-Müllerian hormone type II receptor in single, isolated, human preantral follicles: relevance to polycystic ovaries. J Clin Endocrinol Metab. 2007; 92(3):1034-40.

Robertson HA, King GJ. Conjugated and unconjugated oestrogens in fetal and maternal fluids of the cow throughout pregnancy. J Reprod Fertil. 1979; 55(2):463-70.

Rommerts FG. Testosterone: an overview on biosynthesis, transport, metabolism and action. *In*: Nieschlag E, Behre HM, eds. Testosterone-Action, Deficiency, Substitution. Heidelberg, Springer. 1990; 1–22.

Rosner W. Sex steroids and the free hormone hypothesis. Cell. 2006; 124(3):455-6.

Ruder HJ, Loriaux L, Lipsett MB. Estrone sulfate: production rate and metabolism in man. J Clin Invest. 1972; 51(4):1020-33.

Saeki T, Matoba K, Furukawa H, Kirifuji K, Kanamoto R, Iwami K. Characterization, cDNA cloning, and functional expression of mouse ileal sodium-dependent bile acid transporter. J Biochem. 1999; 125(4):846-51.

Salido EC, Yen PH, Barajas L, Shapiro LJ. Steroid sulfatase expression in human placenta: immunocytochemistry and in situ hybridization study. J Clin Endocrinol Metab. 1990; 70(6):1564-7.

Santen RJ, Leszczynski D, Tilson-Mallet N, Feil PD, Wright C, Manni A, Santner SJ. Enzymatic control of estrogen production in human breast cancer: relative significance of aromatase versus sulfatase pathways. Ann N Y Acad Sci. 1986; 464:126-37.

Santner SJ, Feil PD, Santen RJ. In situ estrogen production via the estrone sulfatase pathway in breast tumors: relative importance versus the aromatase pathway. J Clin Endocrinol Metab. 1984; 59(1):29-33.

Sasano H, Nagasaki S, Miki Y, Suzuki T. New developments in intracrinology of human breast cancer: estrogen sulfatase and sulfotransferase. Ann N Y Acad Sci. 2009; 1155:76-9.

Sauerwein H, Brandstetter A, Pfaffl WM, Meyer HH, Möstl E, Handler J, Arbeiter K. Uterine androgen receptor mRNA expression in metestrous and anestrous bitches being healthy or suffering from Pyometra. Dtsch Tierärztl Wochenschr. 1998; 105(5):191-3.

Sawyer GJ, Fulkerson WJ, Martin GB, Gow C. Artificial induction of lactation in cattle: initiation of lactation and estrogen and progesterone concentrations in milk. J Dairy Sci. 1986; 69(6):1536-44.

Schallenberger E, Rampp J, Walters DL. Gonadotrophins and ovarian steroids in cattle. II. Pulsatile changes of concentrations in the jugular vein throughout pregnancy. Acta Endocrinol (Copenh). 1985; 108(3):322-30.

Schams D, Kohlenberg S, Amselgruber W, Berisha B, Pfaffl MW, Sinowatz F. Expression and localisation of oestrogen and progesterone receptors in the bovine mammary gland during development, function and involution. J Endocrinol. 2003; 177(2):305-17.

Schrag ML, Cui D, Rushmore TH, Shou M, Ma B, Rodrigues AD. Sulfotransferase 1E1 is a low km isoform mediating the 3-O-sulfation of ethinyl estradiol. Drug Metab Dispos. 2004; 32(11):1299-303.

Schreiber JR, Ross GT. Further characterization of a rat ovarian testosterone receptor with evidence for nuclear translocation. Endocrinology. 1976; 99(2):590-6.

Schuler G. Plazentare Steroide beim Rind - Biosynthese und Beziehungen zu Wachstum und Differenzierung der Plazentome. Habilitation thesis, 2000; Giessen, http://geb.uni-giessen.de/geb/volltexte/2002/908/#

Schuler G, Hartung F, Hoffmann B. Investigations on the use of C-21-steroids as precursors for placental oestrogen synthesis in the cow. Exp Clin Endocrinol. 1994; 102(3):169-74.

Schuler G, Wirth C, Klisch K, Pfarrer C, Leiser R, Hoffmann B. Immunolocalization of progesterone receptors in bovine placentomes throughout mid and late gestation and at parturition. Biol Reprod. 1999; 61(3):797-801.

Schuler G, Wirth C, Klisch K, Failing K, Hoffmann B. Characterization of proliferative activity in bovine placentomes between day 150 and parturition by quantitative immunohistochemical detection of Ki67-antigen. Reprod Domest Anim. 2000; 35(3-4):157-162.

Schuler G, Wirth C, Teichmann U, Failing K, Leiser R, Thole H, Hoffmann B. Occurrence of estrogen receptor alpha in bovine placentomes throughout mid and late gestation and at parturition. Biol Reprod. 2002; 66(4):976-82.

Schuler G, Teichmann U, Taubert A, Failing K, Hoffmann B. Estrogen receptor beta (ERbeta) is expressed differently from ERalpha in bovine placentomes. Exp Clin Endocrinol Diabetes. 2005; 113(2):107-14.

Schuler G, Özalp GR, Hoffmann B, Harada N, Browne P, Conley AJ. Reciprocal expression of 17alpha-hydroxylase-C17,20-lyase and aromatase cytochrome P450 during bovine trophoblast differentiation: a two-cell system drives placental oestrogen synthesis. Reproduction. 2006a; 131(4):669-79.

Schuler G, Teichmann U, Kowalewski MP, Hoffmann B, Madore E, Fortier MA, Klisch K. Expression of cyclooxygenase-II (COX-II) and 20alpha-hydroxysteroid dehydrogenase (20alpha-HSD)/prostaglandin F-synthase (PGFS) in bovine placentomes: implications for the initiation of parturition in cattle. Placenta. 2006b; 27(9-10):1022-9.

Schuler G, Greven H, Kowalewski MP, Döring B, Özalp GR, Hoffmann B. Placental steroids in cattle: hormones, placental growth factors or by-products of trophoblast giant cell differentiation? Exp Clin Endocrinol Diabetes. 2008; 116(7):429-36.

Schwabe JW, Neuhaus D, Rhodes D. Solution structure of the DNA-binding domain of the oestrogen receptor. Nature. 1990; 348(6300):458-61.

Selcer KW, Difrancesca HM, Chandra AB, Li PK. Immunohistochemical analysis of steroid sulfatase in human tissues. J Steroid Biochem Mol Biol. 2007; 105(1-5):115-23.

Sen A, Hammes SR. Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function. Mol Endocrinol. 2010; 24(7):1393-403.

Shapiro LJ, Yen P, Pomerantz D, Martin E, Rolewic L, Mohandas T. Molecular studies of deletions at the human steroid sulfatase locus. Proc Natl Acad Sci USA. 1989; 86(21):8477-81.

Shemesh M. Production and regulation of progesterone in bovine corpus luteum and placenta in mid and late gestation: a personal review. Reprod Fertil Dev. 1990; 2(2):129-35.

Shenavai S, Hoffmann B, Dilly M, Pfarrer C, Ozalp GR, Caliskan C, Seyrek-Intas K, Schuler G. Use of the progesterone receptor antagonist aglepristone to characterize the role of progesterone withdrawal for parturition and placental release in cows. Reproduction 2010; 140(4):623-32.

Shiina H, Matsumoto T, Sato T, Igarashi K, Miyamoto J, Takemasa S, Sakari M, Takada I, Nakamura T, Metzger D, Chambon P, Kanno J, Yoshikawa H, Kato S. Premature ovarian failure in androgen receptor-deficient mice. Proc Natl Acad Sci U S A. 2006; 103(1):224-9.

Shneider BL, Dawson PA, Christie DM, Hardikar W, Wong MH, Suchy FJ. Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter. J Clin Invest. 1995; 95(2):745-54.

Silberzahn P, Zwain I, Martin B. Concentration increase of unbound testosterone in plasma of the mare throughout pregnancy. Endocrinology. 1984; 115(1):416-9.

Slayden OD, Brenner RM. Hormonal regulation and localization of estrogen, progestin and androgen receptors in the endometrium of nonhuman primates: effects of progesterone receptor antagonists. Arch Histol Cytol. 2004; 67(5):393-409.

Slayden OD, Nayak NR, Burton KA, Chwalisz K, Cameron ST, Critchley HO, Baird DT, Brenner RM. Progesterone antagonists increase androgen receptor expression in the rhesus macaque and human endometrium. J Clin Endocrinol Metab. 2001; 86(6):2668-79.

Slomczynska M, Duda M, Burek M, Knapczyk K, Czaplicki D, Koziorowski M. Distribution of androgen receptor in the porcine uterus throughout pregnancy. Reprod Domest Anim. 2007; 43(1):35-41.

Smith DF, Toft DO. Steroid receptors and their associated proteins. Mol Endocrinol. 1993; 7(1):4-11.

Smith VG, Edgerton LA, Hafs HD, Convey EM. Bovine serum estrogens, progestins and glucocorticoids during late pregnancy parturition and early lactation. J Anim Sci. 1973; 36(2):391-6.

Song WC. The Role of Estrogen Sulfotransferase in Leydig Cells. The leydig cell in health and disease. Contem Endocrinol. 2007; III, 197-205.

Song WC, Moore R, McLachlan JA, Negishi M. Molecular characterization of a testis-specific estrogen sulfotransferase and aberrant liver expression in obese and diabetogenic C57BL/KsJ-db/db mice. Endocrinology. 1995; 136(6):2477-84.

St-Pierre MV, Hagenbuch B, Ugele B, Meier PJ, Stallmach T. Characterization of an organic anion-transporting polypeptide (OATP-B) in human placenta. J Clin Endocrinol Metab. 2002; 87(4):1856-63.

Stabenfeldt GH, Osburn BI, Ewing LL. Peripheral plasma progesterone levels in the cow during pregnancy and parturition. Am J Physiol. 1970; 218(2):571-5.

Stanley RL, Milwidsky A, Besch PK, Milwidsy A. Evidence for an androgen-binding component in the cytosol of the human term placenta. Am J Obstet Gynecol. 1980; 15; 136(8):1003-5.

Stefanczyk-Krzymowska S, Grzegorzewski W, Wasowska B, Skipor J, Krzymowski T. Local increase of ovarian steroid hormone concentration in blood supplying the oviduct and uterus during early pregnancy of sows. Theriogenology. 1998; 50(7):1071-80.

Stein C, Hille A, Seidel J, Rijnbout S, Waheed A, Schmidt B, Geuze H, von Figura K. Cloning and expression of human steroid-sulfatase. Membrane topology, glycosylation, and subcellular distribution in BHK-21 cells. J Biol Chem. 1989; 264(23):13865-72.

Strauss JF 3rd, Martinez F, Kiriakidou M. Placental steroid hormone synthesis: unique features and unanswered questions. Biol Reprod. 1996; 54(2):303-11.

Sugawara T, Fujimoto S. The potential function of steroid sulphatase activity in steroid production and steroidogenic acute regulatory protein expression. Biochem J. 2004; 380(Pt 1):153-60.

Suzuki T, Hirato K, Yanaihara T, Kadofuku T, Sato T, Hoshino M, Yanaihara N. Purification and properties of steroid sulfatase from human placenta. Endocrinol Jpn. 1992; 39(1):93-101.

Takahashi T, Hirako M, Takahashi H, Patel OV, Takenouchi N, Domeki I. Maternal plasma estrone sulfate profile during pregnancy in the cow; comparison between singleton and twin pregnancies. J Vet Med Sci. 1997; 59(4):287-8.

Tekpetey FR, Veeramachaneni DN, Amann RP. Localization of androgen receptors in ram epididymal principal cells. J Reprod Fertil. 1989; 87(1):311-9.

Tetsuka M, Whitelaw PF, Bremner WJ, Millar MR, Smyth CD, Hillier SG. Developmental regulation of androgen receptor in rat ovary. J Endocrinol. 1995; 145(3):535-43.

Thomas JL, Berko EA, Faustino A, Myers RP, Strickler RC. Human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5→4-ene-isomerase: Purification from mitochondria and kinetic profiles, biophysical characterization of the purified mitochondrial and microsomal enzymes purification from microsomes, substrate kinetics, and inhibition by product steroids. J Steroid Biochem. 1988; 31(5): 785-93.

Thorburn GD, Challis JR, Currie WB. Control of parturition in domestic animals. Biol Reprod. 1977; 16(1):18-27.

Tibbetts TA, Conneely OM, O'Malley BW. Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol Reprod. 1999; 60(5):1158-65.

Tong MH, Song WC. Estrogen sulfotransferase: discrete and androgen-dependent expression in the male reproductive tract and demonstration of an in vivo function in the mouse epididymis. Endocrinology. 2002; 143(8):3144-51.

Tong MH, Jiang H, Liu P, Lawson JA, Brass LF, Song WC. Spontaneous fetal loss caused by placental thrombosis in estrogen sulfotransferase-deficient mice. Nat Med. 2005; 11(2):153-9.

Tsumagari S, Kamata J, Takagi K, Tanemura K, Yosai A, Takeishi M. 3 beta-Hydroxysteroid dehydrogenase activity and gestagen concentrations in bovine cotyledons and caruncles during gestation and parturition. J Reprod Fertil. 1994; 102(1):35-9.

Tuckerman EM, Okon MA, Li T, Laird SM. Do androgens have a direct effect on endometrial function? An in vitro study. Fertil Steril. 2000; 74(4):771-9.

Tulchinsky D, Ryan KJ. Maternal-Fetal Endocrinology. N Engl J Med. 1980; 303:1009.

Tyagi RK, Lavrovsky Y, Ahn SC, Song CS, Chatterjee B, Roy AK. Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. Mol Endocrinol. 2000; 14(8):1162-74.

Ugele B, St-Pierre MV, Pihusch M, Bahn A, Hantschmann P. Characterization and identification of steroid sulfate transporters of human placenta. Am J Physiol Endocrinol Metab. 2003; 284(2):390-8.

Ushizawa K, Takahashi T, Hosoe M, Ishiwata H, Kaneyama K, Kizaki K, Hashizume K. Global gene expression analysis and regulation of the principal genes expressed in bovine placenta in relation to the transcription factor AP-2 family. Reprod Biol Endocrinol. 2007; 5:1-17.

Utsunomiya H, Ito K, Suzuki T, Kitamura T, Kaneko C, Nakata T, Niikura H, Okamura K, Yaegashi N, Sasano H. Steroid sulfatase and estrogen sulfotransferase in human endometrial carcinoma. Clin Cancer Res. 2004; 10(17):5850-6.

Uzelac PS, Li X, Lin J, Neese LD, Lin L, Nakajima ST, Bohler H, Lei Z. Dysregulation of leptin and testosterone production and their receptor expression in the human placenta with gestational diabetes mellitus. Placenta. 2010; 31(7):581-8.

Vendola K, Zhou J, Wang J, Famuyiwa OA, Bievre M, Bondy CA. Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. Biol Reprod. 1999; 61(2):353-7.

Verduzco GA, Miranda L, Murphy B, Quero A. Expression of steroidogenic acute regulatory protein (StAR) in caruncular and cotyledonary bovine tissues during the first half of the gestation. Biol Reprod, Special issue: Abstract book. Society for the Study of Reproduction. 40th Annual Meeting, July 21-July 25, 2007; San Antonio, Texas.

Vermeirsch H, Van den Broeck W, Coryn M, Simoens P. Immunohistochemical detection of androgen receptors in the canine uterus throughout the estrus cycle. Theriogenology. 2002; 57(9):2203-16.

Vesanen M, Isomaa V, Alanko M, Vihko R. Bovine uterine, cervical and ovarian androgen receptor concentrations. Correlation with estrogen and progesterone receptor concentrations. Acta Vet Scand. 1992; 33(4):379-86.

Voutilainen R, Miller WL. Developmental expression of genes for the stereoidogenic enzymes P450scc (20,22-desmolase), P450c17 (17 alpha-hydroxylase/17,20-lyase), and P450c21 (21-hydroxylase) in the human fetus. J Clin Endocrinol Metab. 1986; 63(5):1145-50.

Wagner WC, Thompson FN, Evans LE, Molokwu ECI. Hormonal mechanisms controlling parturition. J Anim Sci. 1974; 38(1):39-57.

Walters KA, McTavish KJ, Seneviratne MG, Jimenez M, McMahon AC, Allan CM, Salamonsen LA, Handelsman DJ. Subfertile female androgen receptor knockout mice exhibit defects in neuroendocrine signaling, intraovarian function, and uterine development but not uterine function. Endocrinology. 2009; 150(7):3274-82.

Wang Q, Lu J, Yong EL. Ligand- and coactivator-mediated transactivation function (AF2) of the androgen receptor ligand-binding domain is inhibited by the cognate hinge region. J Biol Chem. 2001; 276(10):7493-9.

Wango EO, Wooding FB, Heap RB. The role of trophoblastic binucleate cells in implantation in the goat: a morphological study. J Anat. 1990; 171:241-57.

Wango EO, Heap RB, Wooding FB. Progesterone and 5 beta-pregnanediol production by isolated fetal placental binucleate cells from sheep and goats. J Endocrinol. 1991; 129(2):283-9.

Warshaw ML, Johnson DC, Khan I, Eckstein B, Gibori G. Placental secretion of androgens in the rat. Endocrinology. 1986; 119(6):2642-8.

Webster D, France JT, Shapiro LJ, Weiss R. X-linked ichthyosis due to steroid-sulphatase deficiency. Lancet. 1978; 1(8055):70-2.

Weinman SA. Electrogenicity of Na(+)-coupled bile acid transporters. Yale J Biol Med. 1997; 70(4):331-40.

Whitbread AK, Veveris-Lowe TL, Lawrence MG, Nicol DL, Clements JA. The role of kallikrein-related peptidases in prostate cancer: potential involvement in an epithelial to mesenchymal transition. Biol Chem. 2006; 387(6):707-14.

Wiener M. Control of placental 3 beta-hydroxy-delta5-steroid dehydrogenase: comparison of enzyme characteristics in man, cow, goat, rat and Rhesus monkey. Biol Reprod. 1976; 14(3):306-13.

Willemsen R, Kroos M, Hoogeveen AT, van Dongen JM, Parenti G, van der Loos CM, Reuser AJ. Ultrastructural localization of steroid sulphatase in cultured human fibroblasts by immunocytochemistry: a comparative study with lysosomal enzymes and the mannose 6-phosphate receptor. Histochem J. 1988; 20(1):41-51.

Willnow TE, Nykjaer A. Cellular uptake of steroid carrier proteins--mechanisms and implications. Mol Cell Endocrinol. 2010; 316(1):93-102.

Wimsatt WA. Observations on the morphogenesis, cytochemistry, and significance of the binocleate giant cells of the placenta of ruminants. Am J Anat. 1951; 89(2):233-81.

Woicke J, Schoon HA, Heuwieser W, Schulz LCl, Grunert E. Morphologische und funktionelle Aspekte plazentarer Reifungsmechanismen beim Rind. 1. Mitteilung: Licht-mikroskopische Befunde. J Vet Med A. 1986; 33:660-7.

Wong MH, Oelkers P, Craddock AL, Dawson PA. Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter. J Biol Chem. 1994; 269(2):1340-7.

Wong MH, Oelkers P, Dawson PA. Identification of a mutation in the ileal sodium-dependent bile acid transporter gene that abolishes transport activity. J Biol Chem. 1995; 270(45):27228-34.

Wooding FB. The role of the binucleate cell in ruminant placental structure. J Reprod Fertil Suppl. 1982; 31:31-9.

Wooding FB. Frequency and localization of binucleate cells in the placentomes of ruminants. Placenta. 1983; 4:527-39.

Wooding FB. Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Am J Anat. 1984; 170(2):233-50.

Wooding FB. Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta. 1992; 13(2):101-13.

Wooding FB, Wathes DC. Binucleate cell migration in the bovine placentome. J Reprod Fertil. 1980; 59(2):425-30.

Wooding FB, Beckers JF. Trinucleate cells and the ultrastructural localisation of bovine placental lactogen. Cell Tissue Res. 1987; 247(3):667-73.

Wooding FBP, Flint APF. Placentation. In: Marshall's Physiology of Reproduction. 1994; III: 1, 230-466.

Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X, Xing L, Boyce BF, Hung MC, Zhang S, Gan L, Chang C. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proc Natl Acad Sci USA. 2002; 99(21):13498-503.

Yen PH, Marsh B, Allen E, Tsai SP, Ellison J, Connolly L, Neiswanger K, Shapiro LJ. The human X-linked steroid sulfatase gene and a Y-encoded pseudogene: evidence for an inversion of the Y chromosome during primate evolution. Cell. 1988; 55(6):1123-35.

Yildiz BO. Diagnosis of hyperandrogenism: clinical criteria. Best Pract Res Clin Endocrinol Metab. 2006; 20(2):167-76.

Yin D, Gao W, Kearbey JD, Xu H, Chung K, He Y, Marhefka CA, Veverka KA, Miller DD, Dalton JT. Pharmacodynamics of selective androgen receptor modulators. J Pharmacol Exp Ther. 2003; 304(3):1334-40.

Younes MA, Besch NF, Besch PK. Evidence for an androgen binding component in human placental cytosol. J Steroid Biochem. 1982; 16(2):311-5.

Zhang WC, Nakao T, Moriyoshi M, Nakada K, Ribadu AY, Ohtaki T, Tanaka Y. Relationship of maternal plasma progesterone and estrone sulfate to dystocia in Holstein-Friesian heifers and cows. J Vet Med Sci. 1999; 61(8):909-13.

Zhou ZX, Sar M, Simental JA, Lane MV, Wilson EM. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH2-terminal and carboxyl-terminal sequences. J Biol Chem. 1994; 269(18):13115-23.

Zhou X. Roles of androgen receptor in male and female reproduction: lessons from global and cell-specific androgen receptor knockout (ARKO) mice. J Androl. 2010; 31(3):235-43.

Zhu LJ, Hardy MP, Inigo IV, Huhtaniemi I, Bardin CW, Moo-Young AJ. Effects of androgen on androgen receptor expression in rat testicular and epididymal cells: a quantitative immunohistochemical study. Biol Reprod. 2000; 63(2):368-76.

Zhu ML, Kyprianou N. Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocr Relat Cancer. 2008; 15(4):841-9.

Zieler CG, Kessler MA, Schuler LA. Characterization of a novel prolactin-related protein from bovine fetal placenta. Endocrinology. 1990; 126(5):2377-82.

Zoli AP, Beckers JF, Wouters-Ballman P, Closset J, Falmagne P, Ectors F. Purification and characterization of a bovine pregnancy-associated glycoprotein. Biol Reprod. 1991; 45(1):1-10.

Zoli AP, Demez P, Beckers JF, Reznik M, Beckers A. Light and electron microscopic immunolocalization of bovine pregnancy-associated glycoprotein in the bovine placentome. Biol Reprod. 1992; 46(4):623-9.

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