

Progesterone receptor expression in the human placenta

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The presence of progesterone receptors (PR) in the human placenta has been demonstrated using the reverse transcriptase–polymerase chain reaction technique. It was observed that the amount of PR in the human placenta is less during late gestation. Electrophoretic mobility shift assays with nuclear extract isolated from the first trimester and term placenta revealed three complexes when incubated with [³²P]dCTP-labelled progesterone response element, and, in competition with unlabelled progesterone response element, the formation of all three complexes was inhibited. When supershift analysis of these complexes was carried out using antibodies which cross-react with both the A and B types of the PR or only with the B type receptor, only the A-form of PR was detected in the human placenta.

Key words: electrophoretic mobility shift assay/placenta/PR monoclonal antibody/progesterone receptor/RT–PCR

Introduction

The two most important steroid hormones produced by the human placenta are oestradiol and progesterone, the concentrations of which continuously increase throughout the course of pregnancy. The quantity of progesterone produced by the human placenta can be as much as 250–600 mg per day during late gestation (Solomon, 1994). The intracellular concentration of progesterone within the placenta is estimated to be ~7 µmol/l (Khan-Dawood and Dawood, 1984). Although progesterone is known to be indispensable for the maintenance of pregnancy, its exact role during pregnancy is not completely understood. It is known that progesterone is crucial for keeping the uterus in a quiescent state in order to prevent premature onset of labour. Apart from the uterus, the placenta itself has been suggested to be a target tissue for the action of progesterone. However, there has been no conclusive evidence for the presence of progesterone receptors (PR) in the human placenta (McCormick *et al.*, 1981; Younes *et al.*, 1981; Rivera and Cano, 1989; Padayachi *et al.*, 1990; Karalis *et al.*, 1996). This has mainly been due to the techniques employed, particularly the nuclear exchange assay, the application of which to human placenta poses considerable problems because of the high endogenous concentration of progesterone. We have demonstrated the expression of PR mRNA in the first trimester human placenta (FTHP) using the technique of reverse transcriptase–polymerase chain reaction (RT–PCR) (Shanker *et al.*, 1997). Subsequently (Rossmannith *et al.*, 1997) it has also been reported that the presence of PR in the human placenta using the technique of immunocytochemistry as well as RT–PCR at all stages of development although no binding could be demonstrated with ³H-labelled steroids. In the present paper, we report the results of our studies which provide additional

evidence to establish the presence of PR protein in the human placenta.

Materials and methods

Materials

Modifying enzymes and PCR reagents were obtained from Life Technologies Inc., Gaithersburg, MD, USA. Enhanced Chemiluminescence (ECL) detection reagents and Hybond nitrocellulose were obtained from Amersham, Life Sciences Ltd, Buckingham, UK. [^α-³²P]dCTP was obtained from Du Pont. Secondary antibody–horseradish peroxidase (HRP) conjugate and other routine chemicals were obtained from Sigma, St Louis, MO, USA. hPRA-3, which recognizes both PR types A and B, was obtained as a gift from Prof. Satyaswaroop, University of Pennsylvania, Philadelphia, PA, USA, and KC 146 which recognizes only PR type B, was generously provided by Prof. Geoffrey Greene, University of Chicago, Chicago, IL, USA.

RT–PCR

The procedures employed for the collection of human placenta, isolation of RNA, RT–PCR, and the sequences of the primers used have been described earlier (Shanker *et al.*, 1997). Primers specific for human PR were designed based on the published sequence of the human PR cDNA in order to amplify the region corresponding to amino acids 730–840 (ligand binding domain). The forward primer used was 5' GGCGGATCCGTCAAGTGGTCTAAATCATTG 3' and the reverse primer used was 5' GGCGAATTCCTGGGTTTGACTTCGTAGCCC 3'. The expected size of amplification with the set of primers used is 351 bp. To rule out the possibility that the amplification is due to contamination from uterine tissue with which placenta is closely associated and which is also a rich source for PR, every batch of RNA isolated from placenta was screened for the presence of

message for the uterine protein PP14 using specific primers. It is now well established that PP14 is an exclusively uterine protein. It should also be noted that the PP14 message could be detected even when RNA was diluted 1000-fold, and in all our RT-PCR reactions to monitor PR we employed undiluted RNA without detecting PP14 message, thus establishing that the observed amplification is indeed due to presence of PR in placenta and not due to uterine tissue contamination. Two micrograms of first trimester, or 6 µg of term, placental total RNA was used for RT-PCR; 10% of the RT reaction product was used for PCR in the case of first trimester placenta, while 20% was used in the case of term placenta.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from human placenta were prepared according to established methods (Gorski *et al.*, 1986) with some modifications in the procedure of salt extraction. All procedures were carried out in the cold, and all solutions, tubes and centrifuges were chilled to 0°C. When included, phenylmethylsulphonyl fluoride (PMSF) and dithiothreitol (DTT) were added to the buffers just prior to use. Five grams of human first trimester or term placental minces were gently homogenized in 10 ml homogenization buffer [10 mM HEPES-KOH (pH 7.6), 25 mmol/l KCl, 0.5 mmol/l spermidine, 1 mmol/l EDTA, 2 mol/l sucrose, 10% glycerol] using a motor-driven glass-teflon homogenizer until most of the cells were broken, leaving the nuclei intact. The homogenate was diluted to 28.3 ml with homogenization buffer, layered on a 10 ml cushion of the same buffer and centrifuged at 76 000 g for 30 min at -2°C in an SW28 rotor. The nuclear pellet obtained was resuspended in 1.7 ml of nuclear lysis buffer [10 mM HEPES-KOH (pH 7.6), 100 mmol/l KCl, 3 mmol/l MgCl₂, 0.1 mmol/l EDTA, 1 mmol/l DTT, 0.1 mmol/l PMSF, 10% glycerol] and KCl was added to a final concentration of 0.5 mol/l. The suspension was kept on ice for 30 min with frequent vortexing to extract DNA binding proteins. Following this, the suspension was centrifuged at 12 000 g for 10 min at 0°C and the supernatant was dialysed against two changes of dialysis buffer [25 mM HEPES-KOH (pH 7.6), 40 mmol/l KCl, 0.1 mmol/l EDTA, 1 mmol/l DTT, 10% glycerol] overnight. The dialysate was centrifuged at 12 000 g for 10 min at 0°C and the supernatant was frozen in small aliquots by placing in liquid nitrogen and stored at -70°C until further use. EMSA was performed as described (Klein-Hitpass *et al.*, 1991). Typical binding reactions contained (20 µl volume): 100–200 pg (4–5 × 10⁴ cpm) progesterin response element (PRE) oligonucleotide labelled with [α -³²P]dCTP and Klenow DNA polymerase, 250 ng poly [dIdC.dIdC, 20 µg (FTHP) or 50 µg (term placenta) nuclear extract, 25 mmol/l HEPES-KOH pH 7.6], 60 mmol/l KCl, 10% (w/v) glycerol, 2.5% (w/v) Ficoll 400, 1 mmol/l MgCl₂, 2 mmol/l DTT, 0.15 mmol/l EDTA and 0.05 mmol/l EGTA. Binding reactions were carried out at room temperature for 20 min, separated in 4.5% polyacrylamide gels, dried and autoradiographed. For antibody-mediated supershift, binding reaction mixtures were incubated for a further 20 min in the presence of 1 µg of either of the two monoclonal antibodies to the human PR, hPRA-3 or KC 146. The PRE oligonucleotide used as probe and competitor was obtained by annealing the two complementary strands 5'-ggttAAAGTCAGAACACAGTGTCTG-ATCAA-3' and 5'-ggttTTGATCAGAACACTGTGTTCTGACTTT-3'. The 5' 'ggtt' overhangs so obtained were used as templates to radiolabel the duplex by end-filling with Klenow polymerase in the presence of [α -³²P]dCTP. Protein content in the nuclear extracts was estimated according to established methods (Lowry *et al.*, 1951) using bovine serum albumin as the standard.

Western blot analysis

Nuclear extracts (200 µg protein) prepared from first trimester or term placenta were electrophoresed on 10% sodium dodecyl

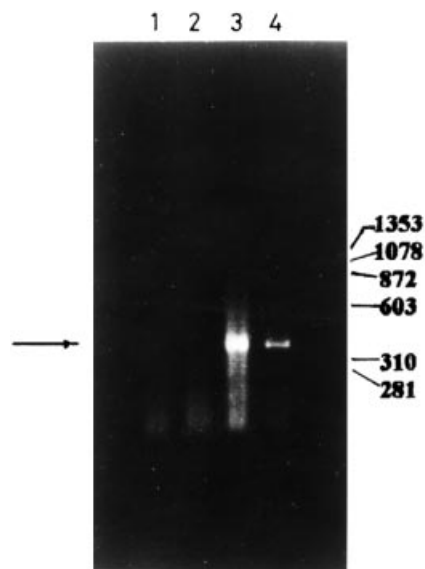


Figure 1. Demonstration of expression of progesterone receptor (PR) mRNA in first trimester human placenta (FTHP) and term human placenta by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated and reverse transcribed using Superscript preamplification (Gibco-BRL, Grand Island, NY, USA). First strand cDNA was used to carry out PCR as described in the text. PCR products were directly electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The positions of DNA markers (size in bp) are shown at the right of lane 4. Lane 1: no template control; lane 2: No RT control; lane 3: RT-PCR of FTHP RNA; lane 4: RT-PCR of term placental RNA. A 351 bp amplified product (marked by the arrow) was observed in lanes 3 and 4 but not in lanes 1 and 2.

sulphate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose as described (Towbin *et al.*, 1979). After blocking the non-specific binding sites by incubating the membrane with 5% fat-free milk, it was incubated with hPRA-3 (4 µg/ml), followed by incubation with anti-mouse IgG-HRP conjugate. Antigen-antibody complex was detected using enhanced chemiluminescence Western blotting reagents (Amersham).

Results

Expression of PR mRNA in human placenta

The RT-PCR analysis for PR mRNA using first trimester and term human placental RNA is presented in Figure 1. An amplified DNA fragment of the size 351 bp was seen in both the first trimester as well as term placental RNA. However, the quantity of PCR product formed was significantly less in term placenta than in FTHP (compare lanes 3 and 4, Figure 1), despite the fact that the quantity of RNA and first strand cDNA used for performing RT-PCR was twice the quantity in the case of term placenta as it was in the case of FTHP.

Demonstration of PR protein in the human placenta

Since the PR mRNA was detectable only by RT-PCR but not by Northern analysis (Shanker *et al.*, 1997), we felt that it was essential to demonstrate the presence of the protein. The PR is a DNA binding protein belonging to the family of steroid/thyroid/retinoid receptors and hence EMSA can be employed to demonstrate the presence of PR protein. It can be seen from

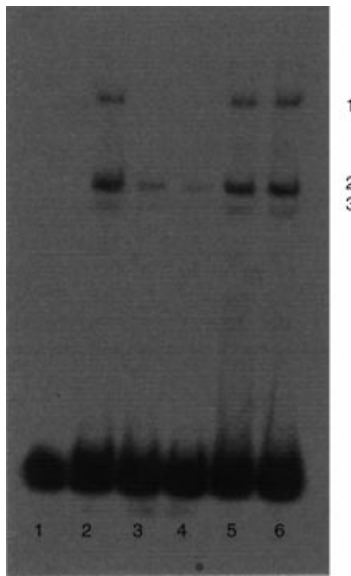


Figure 2. Electrophoretic gel mobility shift analysis (EMSA) of progesterone response element (PRE)-binding proteins in human placental nuclear extracts. Three complexes (designated as complexes 1, 2 and 3) with retarded mobility were observed (lane 2). Formation of complexes was abolished by carrying out the EMSA in the presence of 10- or 20-fold excess (lanes 3 and 4) of non-radiolabelled PRE. All three complexes were formed when EMSA were carried out in the presence of 50- or 100-fold excess (lanes 5 and 6) of an irrelevant oligonucleotide, retinoic acid response element (RARE). Lane 1: free probe; lane 2: first trimester human placenta nuclear extract; lane 3: 10-fold excess of non-radiolabelled PRE; lane 4: 20-fold excess of non-radiolabelled PRE; lane 5: 50-fold excess of non-radiolabelled RARE; lane 6: 100-fold excess of non-radiolabelled RARE.

the results presented in Figure 2 that following incubation of placental nuclear extracts with the ^{32}P -labelled PRE oligonucleotide, three retarded complexes were detected (Figure 2, lane 2, signals numbered 1, 2 and 3). All three complexes appeared to be specific in nature since the formation of each complex could be competitively inhibited by the binding reaction in the presence of excess unlabelled PRE (Figure 2, lanes 3 and 4). However, inclusion of an excess of an irrelevant oligonucleotide (retinoic acid response element, RARE) in the binding reaction had no effect on the number or the intensity of the complexes formed (Figure 2, lanes 5 and 6).

In order to identify which of the three complexes contained PR, we did supershift analysis with a monoclonal antibody to the human PR, hPRA-3, which is known to recognize both the forms of PR, i.e. PR-A and PR-B (Clarke *et al.*, 1987). It can be seen from Figure 3 that only complex 1 was supershifted by the antibody (signal numbered 1, lane 3) suggesting that it was complex 1 which contained PR. This supershift was not observed (lane 4) with an irrelevant antibody, C1C5 (which recognizes retinol binding protein), thus establishing the specificity of the antibody used. Interestingly incubation with KC 146, an antibody which recognized only the B form of PR, did not result in any supershift of the complex (lane 5). The supershifted complex numbered 2 in lane 7 of Figure 3 could represent other known or unknown members of the steroid receptor superfamily which bind to the same DNA sequence, PRE.

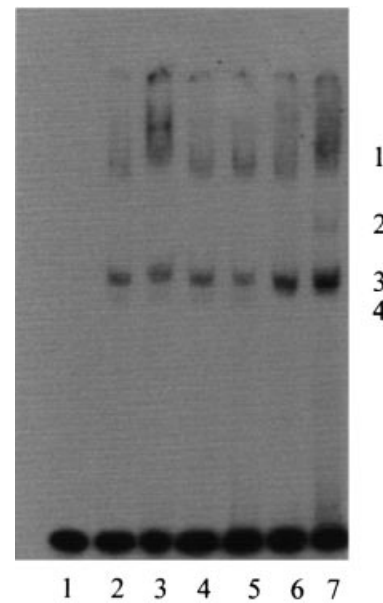


Figure 3. Supershift analysis to establish the identity of the complex containing progesterone receptor (PR) and to identify the isoforms of PR in human placenta. Following the binding reaction, samples were incubated with antibodies hPRA-3, C1C5, or KC 146 prior to electrophoresis. Supershifting of complex 1 was observed with hPRA-3 (lane 3) but not with the irrelevant antibody, C1C5 (lane 4) or with the PR-B-specific antibody, KC 146 (lane 5). Incubation with KC 146 resulted in a supershift of the PR-containing complex (signals numbered 1 and 2) in T47D cell extracts (lane 7) which were used as positive control. Lane 1: free probe; lane 2: control placental extract; lane 3: placental extract incubated with hPRA-3; lane 4: placental extract incubated with C1C5; lane 5: placental extract incubated with KC146; lane 6: control T47D cell extract; lane 7: T47D cell extract incubated with KC146.

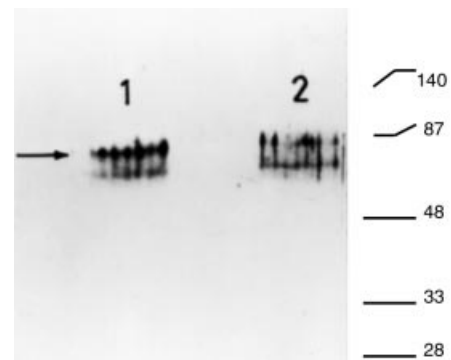


Figure 4. Western blot analysis of first trimester human placenta (FTHP) and term placental nuclear extracts. Nuclear extracts were prepared as described, subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose and subsequently processed for Western blot analysis. The positions of the molecular weight markers are indicated at the right of lane 2. The arrow at the left indicates the position of the 82 kDa human PR-A. No signals were observed in the negative control probed with normal mouse serum (data not shown). Lane 1: FTHP; lane 2: term placenta.

Western blot analysis carried out with hPRA-3 revealed the presence of an 82 kDa species corresponding to PR-A (Figure 4) and this value agrees with the size previously reported (Feil

et al., 1988). A 68 kDa species was also observed, which is likely to be a proteolytic product of PR, as has been observed in other tissues on a Western blot (Feil *et al.*, 1988). The intensity of both the signals was significantly reduced when the antibody was preabsorbed with excess of placental nuclear extract (results not shown) thus establishing the specificity of the antibody employed. It should also be noted that the quantity of PR, as judged by the intensity of the signal, was significantly less with term placental nuclear extract than with FTHP, as can be seen from Figure 4, which agrees very well with the RNA concentrations (Figure 1). Densitometric scanning of the autoradiogram shown in Figure 4 revealed that there was a 70% decrease in the level of the 82 kDa signal in term placenta as compared to FTHP.

B form of PR is not detectable in the human placenta

It can be seen from results presented in Figure 4 that only the 82 kDa size PR-A species was detectable in the placental nuclear extract. In contrast, PR-B, which has an apparent molecular weight of 116–120 kDa, was not detected by the Western blot. In order to confirm this, supershift analysis was performed with another monoclonal antibody, KC 146, which recognizes only the B form of PR (Press and Greene, 1988). It can be seen from results presented in Figure 3 (lane 5) that unlike the result with the hPRa-3, supershift of the PR-containing complex (complex I) was not observed when incubated with KC 146. However, supershift of the PR-containing complex from T47D cell nuclear extract, which is known to contain both the forms of PR, was observed with KC 146. Results of both these experiments suggested that the B form of PR was not detectable in the human placenta.

Discussion

Apart from the uterus, it has been suggested that progesterone might also exert its effects on the placenta and thereby regulate placental function. In fact, recent reports describing the effects of progesterone on the expression of chorionic gonadotrophin (CG) α and β subunit genes in the human placenta (Rao *et al.*, 1995) and corticotrophin releasing hormone (CRH) gene in human trophoblast cell cultures (Karalis *et al.*, 1996) provide sufficient evidence to suggest that placenta itself can be a target tissue for the action of progesterone, though it has not been possible to accept this suggestion with certainty due to several conflicting reports in the literature (McCormick *et al.*, 1981; Younes *et al.*, 1981; Rivera and Cano, 1989; Padayachi *et al.*, 1990; Karalis *et al.*, 1996). Although it has been reported (Karalis *et al.*, 1996) that human PR immunoreactivity could not be detected in trophoblast cells isolated from term placenta, studies (Rossmanith *et al.*, 1997) as well as our own provide definitive evidence for the presence of PR in the human placenta. We have recently established, using RT-PCR, the expression of PR mRNA in the first trimester human placenta. This conclusion was based on the results obtained by Southern hybridization, restriction mapping and DNA sequence analysis of the PCR amplified fragment (Shanker *et al.*, 1997). We have provided additional evidence in the present study that, in addition to the mRNA, PR protein is

also present in the human placenta during early as well as late gestation using gel retardation assays and Western blot analysis. Our ability to detect PR message only by RT-PCR and not by Northern analysis could be due to the fact that the transcript concentration of PR could be very low or unstable. It should be added that the RT-PCR has the added advantage of several cycles of amplification and several cases of messages not detectable by Northern analysis but detectable by PCR are known (Ma *et al.*, 1994). It is also possible that PR expression could be under a translational control so that its level of expression is adequate enough for detection by Western blot analysis. The failure to detect PR protein by Western blot (Karalis *et al.*, 1996) is perhaps due to the limitation in the quantity of protein loaded, source material or mode of detection. While Karalis *et al.* have used cytosolic extracts prepared from cultured trophoblast cells and ^{125}I -labelled protein A detection for their Western blot analysis, we have employed nuclear extracts (which are known to be enriched for nuclear receptor) prepared from placental villous tissue and enhanced chemiluminescence detection, which is a highly sensitive mode of detection, for our Western blot analyses.

The concentration of PR mRNA is much higher in FTHP than in term placenta (Figure 1) which is also reflected in the protein concentration as judged by Western blot analysis (Figure 4) or EMSA (data not shown). The extremely low concentration of PR in the term placenta could be of significance in the initiation of labour. In most mammals (except primates), the end of gestation is associated with a fall in maternal progesterone concentration which contributes to the initiation of labour. In humans and other primates, labour is initiated, although there is no fall in the concentration of progesterone. This suggests that a decline in progesterone action in late gestation, without an actual fall in the concentration of the hormone, could be an important component of initiation of labour in women. Progesterone inactivation by a specific binding protein (Westphal *et al.*, 1997), or by a local antiprogesterin (Casey and Macdonald, 1993), or by a change in PR concentrations (Khan-Dawood and Dawood, 1984) during late pregnancy has been suggested but not demonstrated. Recently it has been proposed (Karalis *et al.*, 1996) that cortisol might be the local antiprogesterin which blocks the action of progesterone to initiate labour. It has been suggested that in the human placenta, progesterone might bring about its actions by binding to the glucocorticoid receptor in the absence of PR; cortisol would compete for the glucocorticoid receptor with progesterone, thus blocking the action of progesterone resulting in the initiation of labour. However, our results demonstrate that the PR indeed are present even during late gestation, though there is a significant decline in the placental PR concentrations. Consequently, it is only justified to consider the involvement of the PR to a great extent in initiation of labour in addition to the possible involvement of glucocorticoid receptors as proposed (Karalis *et al.*, 1996). In addition, results obtained in our laboratory have demonstrated that ZK 98299, an antiprogesterin known not to have any anti-glucocorticoid activity (Koper *et al.*, 1997) (unlike the more commonly used antiprogesterin RU 486 which binds to glucocorticoid receptor), inhibits the expression of CG α and β subunit genes (Prasad,

1993), and regulates progesterone synthesis (Shanker and Rao, 1997) in the human placenta, suggesting the presence of functional PR in the human placenta. Our results demonstrate a decline in PR concentrations during late pregnancy and this agrees well with other suggestions (Khan-Dawood and Dawood 1984), who proposed that the decrease in PR at the end of gestation might be a factor contributing to the onset of labor.

PR is a ligand-inducible transcription factor belonging to the family of steroid/thyroid/retinoic acid receptors. It regulates gene expression by binding to PRE on the DNA located upstream of progesterone-responsive genes, upon activation by the binding of progesterone. The human PR exists as two major forms: PR-B is the full length receptor and is 933 amino acids long; PR-A lacks 164 residues from the *N*-terminus (Weigel, 1996). Our data suggest that only the A form of PR is detectable in the human placenta. The results obtained using nuclear extracts prepared from T47D cancer cells (known to express PR), which were used as a positive control in the gel retardation assays establish that the antibody used is specific. Besides, this antibody is a well characterised one and is reported to be specific for PR (Clarke *et al.*, 1987). It is well established that PR-A is a ligand-dependent, trans-dominant repressor of the activity of glucocorticoid, mineralocorticoid and androgen receptors, as well as that of PR-B (Tung *et al.*, 1993; Vegeto *et al.*, 1993). Studies (Tung *et al.*, 1993) reveal that when PR-B alone is present the ligands such as RU486, which are regarded as antagonists, behave as agonists. It is pertinent to note in this connection that our earlier studies (Shanker and Rao, 1997) revealed that while RU 486 stimulates progesterone synthesis in FTHP, it inhibits in the term placenta. Using anti-progesterone antibodies specific for B type as well as those that recognize both A and B type, It has been demonstrated (Wang *et al.*, 1998) that by immunohistochemistry that both types of receptors could be detected in glandular and stromal nuclei during the proliferative phase but only in stromal nuclei during the secretory phase and early pregnancy. Of importance to the present discussion is the observation that the predominant type of PR in the stromal nuclei during the secretory phase and early pregnancy is the A type, also observed by us. Additional support for the possibility that only the A form is predominantly present in certain situations comes from other studies (Viville *et al.*, 1997) who have reported that A type dominated over B type in leiomyomata. There is only other report claiming that PR-B is not detectable (Boyd-Leinen *et al.*, 1982), who reported the absence of PR-B in the oviduct of aged non-laying hens. It is suggested (Kastner *et al.*, 1990) that the differential expression of hPR forms A and B from their cognate promoters generates a system of tissue-specific regulation of progesterone action. However, the possibility that our failure to detect the PR-B in the human placenta in the present study could be due to the extremely low level of expression, which may be below the limits of detection methods employed in the study, cannot be ruled out. It has been well documented that the ratio of PR-A to -B determines the genetic response of a target cell to progesterone. Considering this, as well as our results on the differential action of RU 486 in placenta, it is possible that the presence of PR-A stimulates progesterone production

during early pregnancy, whereas, with the progression of pregnancy, in the presence of PR-A and possible presence of PR-B (not detectable by us by the present methods) RU 486 inhibits progesterone production. In a tissue such as placenta, where the ratio of PR isoforms appears to be highly in favour of PR-A, the in-vivo scenario could be very complex due to the trans-dominant repressor function of PR-A.

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