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Ovarian steroid receptors and activated MAPK in the regional decidualization in rats

Short title: Activated MAPK during in vivo decidualization

Summary sentence: The regional pattern, the cellular distribution of the steroid hormone receptors and p-MAPK3-1 as well as p-MAPK3-1 inhibition suggest a new function of p-MAPK3-1 in limiting the differentiation process of decidual tissues under ovarian steroid regulation.

Key words: rat, decidua, ESR1, ESR2, PGR, p-MAPK, hormonal antagonist

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Abstract

Though the decidua serves a critical function in implantation, the hormonal regulated pathway in decidualization is still elusive. Here we describe in detail the regional distribution and the effects of progesterone (PGR), estrogen receptors (ESR) and MAPK activation on decidualization. We showed an increase in PGR A, PGR B, ESR1, and p-MAPK 3-1 but not in ESR2, in the decidual tissue up to Day 8 of pregnancy. PGR was predominantly found in the nuclei of mesometrial decidual cells and of undifferentiated stromal cells where it colocalizes with ESR2 and ESR1. In the antimesometrial decidua, all receptors showed cytoplasmic localization. MAPK was activated exclusively in undifferentiated stromal cells of the junctional zone between the antimesometrial and mesometrial decidua and at the border of the antimesometrial decidua. Treatment with the progesterone antagonist onapristone and/or the estrogen antagonist faslodex reduced the extent of decidual tissue and downregulated levels of PGR and ESR1. The expression level of ESR2 was affected only by the progesterone receptor antagonist, while neither the antiprogestin nor the antiestrogen significantly modified the p-MAPK3-1 level. The inhibition of MAPK3-1 phosphorylation by the inhibitor PD98059 impaired the extent of decidualization and the closure reaction of the implantation chamber, and significantly down regulated ESR1. These results confirm a role of both steroid receptors in the growth and differentiation of the different decidual regions and suggest a new function of p-MAPK3-1 in regulating expression levels of ESR1, thereby keeping proliferation capacity of stromal cells and limiting the differentiation process in specified regions of decidual tissues.

Introduction

Implantation requires profound changes in the uterus for successful progression and maintenance of pregnancy. One of the most important stromal reactions to implantation in rodents is the decidualization process, which is coordinated by two basic events: first, the priming effect of the steroid hormones estradiol and progesterone and, second, the signaling interaction with the implanting blastocyst [1-2]. The importance of embryonic signaling for decidualization has been impressively shown for LIF [3] activating the HBEGF [4] and PTGS2 (COX-2) pathway. The PTGS2 derived prostacyclin as well as interleukin 11 are key regulators for decidualization [5-7]. Proliferation of the endometrial stromal cells followed by a differentiation into the decidual cells is primarily governed by both progesterone and estradiol at least in rodents [8-9]. This complex tissue remodelling is thought to ensure proper maternal-fetal interactions and to guide trophoblast invasion and placental orientation.

Both cell physiological events -proliferation and differentiation- are mainly governed by progesterone (P), a fact which has been evidenced by using antiprogestins [10-11]. P alone is able to induce uterine stromal proliferation before decidualization, and this effect is potentiated by estrogens [12]. P acts through its two receptor isoforms PGR A and PGR B, expressed from a single gene [13-14] with different responses to the ligand. Evidence that P is crucial for decidualization came from PGR deficient mice [15]. Selective ablation of PGR A and PGR B showed a distinct contribution of these two isoforms in reproduction. PGR A deficiency resulted in ovarian and uterine hyperplasia leading to infertility, while PGR B accounts for the response of the mammary gland to progesterone. The different action of both isoforms is mediated by transactivations of distinct progesterone dependent target genes [14,16].

The induction of progesterone receptors in the uterus seems to be mediated by estrogen [17-18]. In addition, estrogens amplify stromal proliferation during the pre- and peri-implantation phase in a progesterone primed uterus [19]. Estrogen mediates its function through two nuclear estrogen receptors, ESR1 (ERα) and ESR2 (ERβ), both of which exhibit a high affinity to their ligand but show differential expression pattern in tissues and cells [20]. It has been shown that besides the ESR1 protein, ESR2 is expressed in mouse as well as in rat decidual cells [21-22]. ESR1 mRNA and protein are localized mesometrially on day 6-8 in mice [21,23]. The mouse uterus exhibits ESR2 only in a small amount during implantation whereas rats show a clear localization of ESR2 at the antimesometrial side of the decidua. Mice missing ESR1 are still able to decidualize [24-25] and depletion of ESR2 does not impair uterine morphology [26]. These studies suggest that neither ESR2 nor ESR1 is necessary for the decidualization process in mice.

Recently, there was evidence from in vitro studies by our group [27] that induction of proliferation upon progestins requires both PGR and ESR2 in a stromal cell line of rat endometrium. However, both receptors need to interact in the cytoplasm for activation of the extracellular signal-regulated kinases 1 and 2 (MAPK3-1) and AKT signaling pathways. MAPK3 and MAPK1 are ubiquitous and multifunctional signalling proteins that are involved in most cellular responses to extracellular signals and function in many aspects of developmental biology [28, 29], but there is little information about the signaling cascades which govern the decidualization process in rodent endometrium in this tightly controlled temporal and spatial manner. Morphologically, the decidualization process is a highly complex event reflecting probably different functions for the different decidual compartments. The process of decidualization is strongly regionalized with two main decidual cell populations: the antimesometrial and the mesometrial part. Antimesometrial decidualization starts in stromal cells surrounding the implanting blastocyst, forming the primary decidual zone as a compact tissue with an epitheloid character expanding radially towards the muscle layers. The

mesometrial part, which never turns to a very compact tissue, develops together with the placenta and forms the decidua basalis. The two parts are clearly separated by a junctional zone, the region of the closure reaction of the uterine lumen, which is composed of non decidualized stromal cells and contains most of the vessels orientated towards the developing placenta [30]. The development of an extensive vascular network within this stromal/decidual tissue is crucial for the survival of the embryo before a functional placenta is developed [31-32]. A further non decidualized area is the region underneath the myometrium surrounding both decidual parts. The different distribution of the steroid receptors within those different parts of the decidual tissue and the undifferentiated stromal cells have already indicated that signaling cascades of the differentiation process are more complex and regionally diversified [21]. Our study defines P and E receptors mediated regionalized decidualization processes and the role of p-MAPK3-1 to separate the decidualization process in the different compartments.

Materials and Methods

Cell culture

T47D human breast cancer cells (ATCC bioresource center, VA, USA) were routinely grown in Dulbecco Modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 2mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin [33]. UIII rat normal uterine stromal cells (Dr. Cohen H., Laboratorie de Physiologie-Pharmacodynamie, Villeurbanne, France) were maintained in M199 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in humidified 95% air with 5% CO_2 [27]. Culture media were changed every 2 days.

Animal care

Adult female Wistar rats (Schering, TZH, Berlin, Germany) were housed under defined conditions with a temperature of 22 ± 1°C, an atmospheric humidity of 55 ± 10%, and a 12 h dark to 12 h light cycle. They were fed standard pellet food and provided with water ad libitum. All animal experiments were approved by the Institutional Animal Care Committees of the Instituto de Biologia y Medicina Experimental and the Universitaetsklinikum Duisburg-Essen.

Pregnancy

Mating was performed overnight with male animals and the following morning the presence of cornificated cells and sperm was evaluated by vaginal smear. The day of sperm finding was designated as 0 days post coitum (dpc). The 4 dpc pregnancies were confirmed by flushing the uterus and counting the number of blastocysts in each horn. The 6 and 8 dpc pregnancies were confirmed by the presence of implantation sites (IS).

Hormone antagonists and MAPK inhibitor treatments

The antiprogestagen Onapristone (ONA) (ZK 98299, Bayer Schering, Germany) and the antiestrogen faslodex (FAS) (ICI 182780, Tocris, Bristol, UK) were dissolved in ethanol and injected subcutaneously in sesame oil (200 µl/rat). Rats were injected with 1 mg ONA, 0.5 mg FAS, or 1mg ONA + 0.5 mg FAS per day at 6 and 7 dpc. One experimental group was injected with the MAPK inhibitor (PD98059, 5 mg/ rat/ day i.p., Lc Laboratories, USA) dissolved in 500 µl DMSO [34] on days 6 and 7 of pregnancy. Control animals were injected with 500 µl DMSO only. All treated animals were sacrified on day 8 pc. In PD98059 and DMSO experimental group, diameter of implantation size was measured (mm) with Image J 1.43 software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA). Three animals were used for each experimental approach.

Tissue Collection
Rats were killed by cervical dislocation under isofluran-anesthesia (DeltaSelect, München, Germany). The 4 dpc uterine horns were removed and cut into pieces, which were frozen in liquid nitrogen for subsequent western blot analysis. Implantation sites from 6 and 8 dpc uterine horns were dissected. The portion of uterine horn between the implantation sites was designated as interimplantation site (IIS). IS and IIS were frozen in liquid nitrogen for western blot analysis, or fixed in 4% formalin for morphologic and immunohistochemistry analysis.

Western Blot Analysis
Protein extracts were obtained from tissue samples by homogenization (Polytron PT-MR 300, Brinkmann Instruments, Westbury, NY, USA) in RIPA buffer (50mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with EDTAfree Complete Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Mannheim, Germany). Protein concentration was determined by Bradford protein assay kit (Bio-Rad Laboratories, California, USA). SDS-Page and immunoblot analysis were performed to detect progesterone receptor (PGR), estradiol receptor beta (ESR2), estradiol receptor alfa (ESR1), beta-actin (ACTB), GAPDH, phosphorylated MAPK3-1 proteins (p-MAPK3-1) and MAPK1. The following antibodies were used: rabbit polyclonal H190 anti-hPGR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); mouse monoclonal anti-hESR2 Clone PPG5/10 (DAKO Inc., Glostrup, Denmark); rabbit polyclonal MC20 anti-mESR1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); mouse monoclonal anti-hβ-Actin (Sigma-Aldrich Co., St. Lois, MO, USA), rabbit monoclonal 14C10 anti-hGAPDH (Cell Signaling Technology, Beverly, MA, USA); mouse monoclonal E10 against hMAPK3-1 (hERK1/2) phosphorylated at Thr202/Tyr204 (Cell Signaling Technology, Beverly, MA, USA); rabbit polyclonal C14 anti-rMAPK1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). A minimum of three blots from different rats were performed and the band intensities were measured with ImageQuant 3.3 program (Amersham Pharmacia Biotech, Arlington Heights, IL).

Histology
Implantation sites were fixed in 4% formalin, dehydrated in a graded series of alcohol, and embedded in paraffin. Cross serial sections of 7-10 µm were deparaffinized, rehydrated in a graded series of alcohol, and stained with haematoxylin-eosin. Photographs were taken with an Axiophot microscope (Zeiss, Jena, Germany). The area of the different regions within the implantation sites were quantified with Image J 1.43 software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) and delimited as shown in Supplemental Figure S1 (available online at www.biolreprod.org). The area of the whole implantation site was designated as 100% and the contribution of the different tissue regions were calculated relative to the entire IS.

Immunohistochemistry
For immunostaining paraffin sections (7-10 µm) were deparaffinized and rehydrated in a graded series of alcohols. After rinsing with PBS (1x5 min), the endogenous peroxidase was blocked and the sections were incubated with 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA). The sections were incubated over night at 4ºC with the primary antibodies, rinsed in PBS (3x5 min), incubated with a secondary biotinylated antibody (30 min RT), and after that with streptavidin-peroxidase complex (Millipore, Billerica, NA, USA) for 30 min. Staining was visualized with 3.3 diaminobenzidine (DAB) (Dako, Glostrup, Denmark). The following antibodies were used: rabbit polyclonal C-20 anti hPGR (SANTA Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit polyclonal H-150 anti-hESR2 (SANTA Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit polyclonal MC-20 anti-mESR1 (SANTA Cruz Biotechnology,

Inc., Santa Cruz, CA, USA); rabbit monoclonal D13.14.4E against hMAPK3-1 (ERK1/2) phosphorylated at Thr202/Tyr204 (Cell Signaling Technology , Beverly, MA, USA); rabbit polyclonal C14 anti-rMAPK1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and polyclonal Goat Anti-Rabbit Immunoglobulins/Biotinylated (Dako, Glostrup, Denmark). Controls were performed by omitting the primary antibody.

Statistical Analysis
Analysis of variance was used for statistical testing, followed by Tukey Multiple Comparison Test in figures 1 and 3, and followed by Dunnet Comparison Test in figure 2. A t-Test was performed to compare steroids receptor expression in figure 9. Differences were considered significant if $P < 0.05$. Statistical Analysis was carried out with GraphPad Prim 4.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

PGR, ESR and p-MAPK3-1 expression levels are associated with decidualization.
The aim of this project was to study the role of PGR and ESR in the activation of MAPK3-1 during decidualization in the different decidual compartments. Western blots from uteri of 4, 6 and 8 dpc pregnant rats showed that both isoforms of PGR increased from day 6 to day 8 pc, but with a stronger upregulation of PGR A compared to PGR B (Fig. 1A). While ESR1 protein expression was increased at 6 and 8 dpc, ESR2 protein levels were not modified during early pregnancy (Fig. 1A). Western blots for the activated isoform of MAPK revealed that p-MAPK3-1 levels increased up to day 8 (Fig. 1A).

The decidualization process starts in response to the implanting embryo. In order to test whether the increase in PGR, ESR2, ESR1 and p-MAPK3-1 expression was dependent on the decidualization process, we compared their expression levels in implantation sites (IS) with interimplantation sites (IIS) from 6 and 8 dpc pregnant rats. We observed significant higher levels of PGR A and PGR B, ESR1 and p-MAPK3-1 in the IS compared to the IIS from 6 and 8 dpc, while there were no differences in ESR2 (Fig. 1B). The significant increase in PGR, ESR1 and p-MAPK3-1 protein expression on day 8 compared to day 6 pc in samples of the IS is not observed in the endometrium of the IIS. These results suggest that MAPK3-1 activation and elevation of PGR, ESR1 but not ESR2 levels are related to the decidualization process. We then focused on the hormonal influence on the decidualization process at 8 dpc because of the high expression levels of steroid hormone receptors and activated MAPK3-1 in this phase.

Effects of estrogen and progesterone receptor action on the decidualization process, expression of steroid receptors and p-MAPK3-1.
Morphological changes in the decidualization process
In order to study the role of PGR, ESR, and PGR-ESR interaction on MAPK activation for decidualization, we treated pregnant rats at day 6 and 7 with the PGR antagonist onapristone (ONA) and/or the ESR antagonist faslodex (FAS). Morphology of implantation sites was analyzed at 8 dpc.

Animals treated with ONA revealed evident changes in the extension of the decidua in the implantation chamber compared to untreated control animals (Fig. 2), revealing a constriction and an elongation of the decidua in the antimesometrial part (AM). Interestingly, inner and outer myometrial layers were increased; but only in the areas in contact with antimesometrial decidua. The mesometrial decidua (M) appeared less differentiated combined with an enlarged junctional area (J) and an undifferentiated zone (UM) underlying the myometrium (Myo) (Fig. 2). FAS treated animals showed a reduced decidualization resulting in smaller implantion chambers,

whereas regionalization and morphological appearance of the decidual regions were not severely changed compared to controls (Fig. 2).

Animals treated with a combination of both antagonists presented a strong reduction in size of implantation sites due to a reduced extent of decidualization (Fig. 2). Morphology of the decidua resembled more closely the phenotype of animals treated with ONA alone, revealing a similar decidua formation at the antimesometrial pole. However, the mesometrial decidua and the junctional area have practically disappeared.

Steroid receptor and p-MAPK3-1 protein levels

In the same experimental approach, the effect of ONA and/or FAS on PGR, ESR1, ESR2 and p-MAPK3-1 protein expression in the implantation sites was analyzed on day 8 pc (Fig. 3). Levels of both, PGR A and PGR B were strongly reduced by ONA, FAS and ONA plus FAS. Downregulation of ESR2 was obvious upon ONA and ONA+FAS treatment but not after treatment with FAS alone. ESR1 showed a significant downregulation upon ONA and upon FAS and a further decrease after treatment with both antagonists. Thus, estrogen as well as progesterone antagonists, alone and in combination, decreased PGR. A synergistic effect of estrogen and progesterone antagonists was only seen on ESR1 downregulation whereas ESR2 expression seemed to be more sensitive to ONA, which diminished the progesterone action on the PR receptor. Activated MAPK3-1 levels were not significantly modified by the different antagonist treatments.

Effect of estrogen and progesterone receptor action on PGR, ESR2, ESR1 and p-MAPK3-1 expression pattern.

Next we investigated if the localization of PGR, ESR2, ESR1, p-MAPK3-1 protein expression was dependent on PGR and ESR antagonist treatments.

PGR immunohistochemistry

In control animals PGR was expressed in decidualized and non-decidualized stroma cells of the entire implantation chamber (M, AM, UM and J) with an enhanced labelling in the antimesometrial decidual region (Fig. 4). The receptor showed a nuclear localization in cells of the undifferentiated as well as of the mesometrial decidual regions but was restricted to the cytoplasm of antimesometrial decidual cells (Fig. 4, inserts).

ONA treatment resulted in a strong downregulation of PGR positive signals in all regions of the decidual tissue (Fig. 4) a fact which confirmed data from western blots. Similarly, FAS treatment resulted in a decrease in staining and revealed a more scattered distribution of PGR expression, predominantly in the mesometrial area (Fig. 4). Treatment with both antagonists, ONA and FAS, showed a similarly reduced immunolabelling of PR. However, a spot like enhancement was observed in the mesometrial region (Fig. 4). Interestingly, the subcellular localization of the receptor, which was nuclear in the undifferentiated and mesometrial zones and cytoplasmic in the antimesometrial zone, remained independent from antagonist treatments (Figure 4, inserts).

ESR2 immunohistochemistry

A strong ESR2 staining was localized predominantly in the antimesometrial decidua of control animals at 8dpc (Fig. 5). Like the PGR, ESR2 showed a nuclear localization in the cells of the undifferentiated zone and the mesometrial decidua and a shift to the cytoplasm in the antimesometrial decidua cells (Fig. 5, inserts).

ONA or FAS treatment, alone or in combination, affected neither localization nor intensity of the ESR2 signal. (Fig. 5). In addition, the intracellular localization was independent from antagonist treatment (Fig. 5, inserts).

ESR1 immunohistochemistry

Immunolocalization revealed that ESR1 was present in the entire decidua but, like PGR and ESR2, with different subcellular localization in the different decidual compartments.
ESR1 nuclear staining was strong in the mesometrial and junctional area and in some stromal cells underneath the myometrium (UM). Antimesometrial ESR1 was present in the nuclei as well as cytoplasm of differentiated decidual cells located in the border of the myometrium and in the entire antimesometrial decidua (Fig. 6).

ONA as well as FAS treatment resulted in a reduction of ESR1 positive signals in all regions of the decidual tissue and that were even more downregulated by combined ONA and FAS treatment (Fig. 6), confirming the results of the western blot analysis (Fig. 3). The subcellular location of this steroid receptor with a nuclear staining in the mesometrial decidua, in the undifferentiated stromal cells of the junctional area and underneath the myometrium, and a cytoplasmic staining in the antimesometrial region was not affected by antagonist treatment (Fig. 6, inserts).

p-MAPK3-1 immunohistochemistry
The localization of p-MAPK3-1 in 8 dpc implantation sites was restricted to the decidual cells at the lateral border of the antimesometrial decidua and some scattered cells in the primary decidual zone (Fig.7). Most of the expression was found in the undifferentiated cells of the junctional area, whereas the undifferentiated cells underneath the myometrium were not stained. The cellular localization was predominantly nuclear in all cell types (Fig. 7, inserts).

The pattern of p-MAPK3-1 immunostaining in animals treated with ONA was similar to controls but the expression in the junctional zone was reduced. The antimesometrial and the mesometrial decidua revealed less p-MAPK3-1 labeling. Interestingly, p-MAPK3-1 expressing cells were reduced to a band separating the differentiated decidua from the stromal cells underneath the myometrium (Fig. 7). FAS treatment reduced p-MAPK3-1 in the mesometrial decidua but was strongly expressed in the extended junctional area. The distribution pattern of p-MAPK3-1 after combined treatment with ONA and FAS resembled the control but with an extended size in the junctional zone (Fig. 7).

MAPK1 was homogeneously distributed throughout the decidual tissue and did not change upon the treatment with different antagonists (data not shown).

Effects of inhibition of MAPK phosphorylation on decidualization and steroid receptor expression
In order to evaluate the role for MAPK3-1 in the decidualization process the MAP2K1 (MEK1) inhibitor, PD98059, was injected on days 6 and 7 of pregnancy resulting in a significant decrease in size of implantation sites (Figure 8A) compared to the vehicle control. Histology revealed a reduced extent of the decidual tissue, in both the antimesometrial and in the mesometrial regions (Figure 8B). As evidenced by serial sections through the implantation chambers, the junctional zone was more pronounced and showed, in contrast to the controls, a completed closure reaction (Figure 8B).

Western blot analysis of steroid receptors PGR, ESR1 and ESR2 in decidual tissues of MAP2K1 inhibitor treated animals revealed a significant downregulation of ESR1 but not ESR2, and both isoforms of PGR were diminished (Figure 9).

Discussion

Interaction between ESR isoforms and PGR leading to signaling cascades inducing and regulating the decidualization process during pregnancy is not yet understood. Subsequent to our former findings on signaling in decidua and uterine stromal cell lines regulated by progesterone and/or estrogen [27, 30], we here explored the role of steroid hormones and their receptors as well as the activation of the MAPkinase MAPK3-1 in regulating the regionalized decidualization process in vivo.

While the regional and cellular distribution of PGR, ESR1 and ESR2 in the decidua did not change upon treatment with progesterone and estradiol antagonists, the levels of these proteins decreased to different extents in the decidual regions dependent on estrogen or progesterone antagonist treatment. The downregulation of the receptor levels leading to a decrease in its ligand action was associated with the extent and shape of the decidua but not with regionalization and differentiation of the decidua. This fact points to a major role of both ligands in proliferation but not in differentiation. In the rat we could confirm the investigations by Tan et al [21] about the elevation of PGR and a steady state of ESR2, but not the downregulation of ESR1 in the decidua of mice at day 8 pc. This difference could be due to species differences in the time course of the decidualization processes.

Tessier et al. [22] were the first to demonstrate the presence of ESR2 in rat decidua during pseudopregnancy whereas in mice only low expression of ESR2 has been reported [21]. Human endometrial stromal cells reveal ESR2 throughout the cycle in all endometrial compartments but mainly in the decidualized stromal cells surrounding the vessels [35], pointing to a crucial role for ESR2 in the decidualization process in humans.

Cellular receptor distribution

Our most remarkable finding was that on day 8 pc the antimesometrial decidua revealed both isoforms of the ESR and the PGR receptor in the cytosol whereas in the mesometrial decidual cells ESR and PGR were located in the nuclei. The shift of PGR and ESR from the nucleus to the cytosol in AM decidua seems to correlate with advanced differentiation of decidual cells. The undifferentiated cells of the stromal zones in the implantation chamber, junctional and underneath myometrium areas revealed ESR1, ESR2 and PGR, in the nucleus, which indicates a close interaction of the receptors. There are two possible interactions of hormone receptors according to their localization: at nuclear level, probably via DNA target genes, and at cytoplasmic level interacting with cytoplasmic kinases. Our former investigations showed that the ESR and PGR can interact with one another to activate MAPK and AKT in the cytoplasm and support proliferation [27,33] and it could be shown that activation of signaling cascades in the cytoplasm is essential for chromatin remodeling and transcriptional activation of a subset of steroid hormone target genes [36]. Our results support the idea that the non- decidualized cells which exclusively reveal activated MAPK connect the pathways of steroid hormone receptors for regulating proliferation and differentiation during the decidualization process.

Blocking of hormonal receptor actions and functional consequences

Retrieval of the steroid hormone action on the receptor starting on day 6 of pregnancy leads to a reduced decidualization, revealing that both PGR and ESR have an effect on stromal cell proliferation even when applied after day 6 of pregnancy. Inhibiting PGR or ESR activity revealed that the two decidual compartments are differently regulated by the steroid hormones although the subcellular localization of ESR and PGR receptors in the different decidual compartments is not affected. Loss of PGR action by applying onapristone does not obviously affect the antimesometrial decidual differentiation process. The decidual shape as well as the morphology of the individual cells is sustained. Since the cytosolic PGR seems no longer active

in this compartment, the antimesometrial decidual differentiation programme appears to be already established at day 6 pc. Since the mesometrial decidua is less differentiated and is continuously connected to the intermediated and undifferentiated zone, this decidual region seemed to be still under the control of the PGR. Since *Pgr* A knockout mice reveal an impaired decidualization process upon stimulation[14-15], whereas *Pgr* B knockout mice have normal implantation [16], it is more likely that PGR A drives the decidualization process. This corroborates our findings that PGR A levels increase more than PGR B during the decidualization process.

Reducing ESR1 action by suppressing ESR by FAS treatment does not obviously affect the differentiation pattern of the decidua but seems to have an effect on its extension. These findings support the former observations that primarily the proliferation of the stromal cells prior to differentiation is governed by estrogen [8].

Tessier et al. [22] investigated ESR1 and ESR2 regulation by steroid hormones and prolactin using primary cultures from deciduomata and a decidual cell line. They found a downregulation of ESR1 dependent on P which is confirmed in our experiments; however, only for the antimesometrial decidua using the onapristone approach. Furthermore, they gave evidence that ESR2 mediates estrogen induced upregulation of the progesterone receptor in the decidua. In our experiments blocking estrogen receptors did not change the amount of PGR in the mesometrial decidua but severely decreased PGR with sustaining ESR2 at the antimesometrial side. Taking into consideration that uterine decidual response is not impaired in ESR1 deficient mice [37], ESR2 seems to be the main and constant partner for PGR to drive the decidualization process.

p-MAPK3-1 mainting the extent of decidua and limiting decidualization in specified regions

A remarkable fact is that MAPK is predominantly activated in the junctional area between mesometrial and antimesometrial decidua and limits the outer part of the antimesometrial decidua.

The inhibition of MAPK3-1 resulted in impairment of antimesometrial and mesometrial decidua development showing a positive role in growth of decidual tissue for MAPK3-1.
Previous results from our laboratory showed that progestin-dependent proliferation is mediated by MAPK3-1 activation through a PGR-ESR2 preformed complex in the uterine stromal cell line UIII [27]. The junctional zone, which represents a specialized area contributing to the closure reaction and separation of the two decidual parts, does not undergo decidual differentiation despite expressing all steroid receptors. The reason could be that this area is a leading scaffold structure for developing endometrial vessels supporting the growing embryo [32], and a compact decidual tissue would impair angiogenesis. This hypothesis is supported by the colocalization with NOS2 in the endothelial cells of decidual vessels in this junctional zone, and the pregnancy loss after inhibition of NOS2 [30]. Treatment with the MAP2K1 inhibitor during decidualization resulted in reduced mesometrial decidualization and an enhanced differentiated junctional zone. The decidual phenotype suggests that impairment of the MAPK signalling pathway leads to an imbalance in proliferation of stromal cells in favour of differentiation into decidual cells, predominantly in the junctional zone. Further investigations have to clarify if angiogenesis in the junctional zone is impaired and has an impact on embryonic survival. Artificial decidualization seems not to be affected by MAPK inhibitors as shown by Scherle et al. [38]. However, they only used an initial treatment at the start of the deciduogenic stimulus and an organization into different decidual compartments with the presence of a junctional zone is missing in deciduomata.

Most obvious was the downregulation of the steroid receptors, predominantly ESR1, by inhibition of MAPK3-1 phosphorylation. Thus we cannot discriminate between a direct action of the MAPK pathway on decidualization and an indirect one by downregulating the ESR and PGR receptors. It is very well known that MAPK can act via ESR1 phophorylation [39-40] and in breast cancer cells activation of MAPK3-1 is able to regulate ESR1 expression levels but in an opposite way, that specific inhibition of MAPK3-1 resulted in an increase of ESR1 [41].

Our results suggest that the activation of MAPK3-1 in this area is probably necessary to stabilize the presence of ESR1 and its transcriptional activity via phosphorylations to promote proliferation and retarding or limiting differentiation of stromal cells into decidual cells during periimplantation.

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Figure Legends

Figure 1. Induction of PGR, ESR1, and p-MAPK3-1 during pregnancy. A) Kinetic of PGR, ESR2, ESR1 and p-MAPK3-1 protein expression during early decidualization. Western blot from 4, 6 and 8 days post coitum (dpc) extracts were analyzed with antibodies described in materials and methods. Samples from 6 and 8 dpc correspond to implantation sites (IS). PGR A, PGR B, ESR2 and ESR1 arbitrary units (AU) relative to ACTB AU and p-MAPK3-1 relative to MAPK1 AU for each stage of pregnancy were divided by the 4 dpc corresponding value. The arbitrary units of p-MAPK3 and p-MAPK1 were added up and divided by the corresponding MAPK1 AU. Data represent average ± SEM from three animals for 4, 6 and 8 dpc pregnancies and the figure also shows a representative western blot containing one sample for each stage of pregnancy. T47D: human mammary tumor cell line, Ov: rat ovary, UIII: cells from UIII rat uterine stromal cell line treated with EGF. B) Association between PGR, ESR2, ESR1 and p-MAPK3-1 expression and decidualization. Western blot from 6 and 8 dpc IS and inter implantation sites (IIS) extracts were analyzed with the antibodies described in materials and methods. Densitometric analysis was performed as in A. * $P<0.05$; ** $P<0.01$, *** $P<0.001$.

Figure 2. Morphological effect of the inhibition of ESR and/or PGR signaling in the decidualization process. A) Morphology of 8 dpc implantation sites from untreated or treated (ONA, FAS, ONA+FAS) pregnant rats was analysed in paraffin sections stained with haematoxylin-eosin. AM, antimesometrial decidua; M; mesometrial decidua; US, undifferentiated stroma; J, junctional zone between the AM and the M zone; UM, under myometrium; Myo, myometrium. B) Quantitative analysis. Table shows the percentage of tissue areas corresponding to the different regions within the implantation sites. The area of each region relative to the corresponding whole implantation site area, designated as 100%, was calculated as described in materials and methods. Data represents average ± SEM from 4-6 implantation sites from three animals for each treatment group. * $P<0.05$, ** $P<0.01$ vs untreated 8dpc corresponding region.

Figure 3. Effect of ESR and PGR antagonists on PGR, ESR2, ESR1, and p-MAPK3-1 expression. Western blot from 8 dpc implantation sites from untreated or treated (ONA, FAS, ONA+FAS) pregnant rat uterine extracts were analyzed with antibodies described in materials

and methods. PGR A, PGR B, ESR2 and ESR1 arbitrary units (AU) relative to GAPDH AU and p-MAPK3-1 relative to MAPK1 AU for each treatment group were divided by the untreated 8 dpc control values. Densitometric analysis for p-MAPK3-1 was carried out as described in Figure 1. The figure shows data representing average ± SEM from three animals for each treatment group. Representative western blot containing one sample of 8 dpc untreated, ONA, FAS and ONA + FAS treated animals are shown. ** P<0.01, *** P<0.001 vs untreated 8dpc IS.

Figure 4. PGR localization. Immunohistochemical staining for PGR on paraffin sections of complete implantation sites of uteri from 8 dpc unteated or treated pregnant animals treated with onapristone (ONA), faslodex (FAS) or ONA+FAS. Bar = 500 μm. Inserts show details of the different implantation site zones. AM, antimesometrial decidua; M; mesometrial decidua; J, junctional zone between the AM and the M zone; UM, under myometrium. Bar = 30 μm.

Figure 5. ESR2 localization. Immunohistochemical staining for ESR2 on paraffin sections of complete implantation sites of uteri from 8 dpc untreated or treated pregnant animals with onapristone (ONA), faslodex (FAS) or ONA+FAS. Bar = 500 μm. Inserts show details of the different implantation site zones. AM, antimesometrial decidua; M; mesometrial decidua; J, junctional zone between the AM and the M zone; UM, under myometrium. Bar = 30 μm.

Figure 6. ESR1 localization. Immunohistochemical staining for ESR1 on paraffin sections of complete implantation sites of uteri from 8 dpc untreated or treated pregnant animals with onapristone (ONA), faslodex (FAS) or ONA+FAS. Bar = 500 μm. Inserts show details of the different implantation site zones. AM, antimesometrial decidua; M; mesometrial decidua; J, junctional zone between the AM and the M zone; UM, under myometrium. Bar = 30 μm.

Figure 7. p-MAPK3-1 localization. Immunohistochemical staining for p-MAPK3-1 on paraffin sections of complete implantation sites of uteri from 8 dpc untreated or treated pregnant animals with onapristone (ONA), faslodex (FAS) or ONA+FAS. Bar = 500 μm. Inserts show details of the different implantation site zones. AM, antimesometrial decidua; M; mesometrial decidua; J, junctional zone between the AM and the M zone; UM, under myometrium. Bar = 30 μm.

Figure 8. Morphological effects of inhibition of MAPK3-1 phosphorylation on decidualization. Pregnant rats were injected i.p. with the MAP2K1 inhibitor PD98059 or the vehicle DMSO at day 6 and 7 pc. A) Macroscopic analysis of implantation chambers on 8 dpc in uteri of DMSO and PD98059 treated rats. Bar = 5 mm. Size of implantation sites were quantified as described in materials and methods. * P<0.01. B) Histomorphology of 8 dpc implantation sites from DMSO or PD98059 treated pregnant rats was analysed in paraffin sections stained with haematoxylin-eosin. Bar = 350 μm.

Figure 9. Effects of inhibition of MAPK3-1 phosphorylation on PGR, ESR2, and ESR1 expression. Western blot from 8 dpc implantation sites from DMSO or PD98059 treated pregnant rat uterine extracts were analyzed with antibodies described in materials and methods. PGR A, PGR B, ESR2 and ESR1 arbitrary units (AU) relative to GAPDH AU for PD98059 treatment group were divided by the DMSO treated 8 dpc control values. The figure shows data representing average ± SEM from a total of 3-5 samples corresponding to three animals for each treatment group and a representative western blot. * P<0.05.

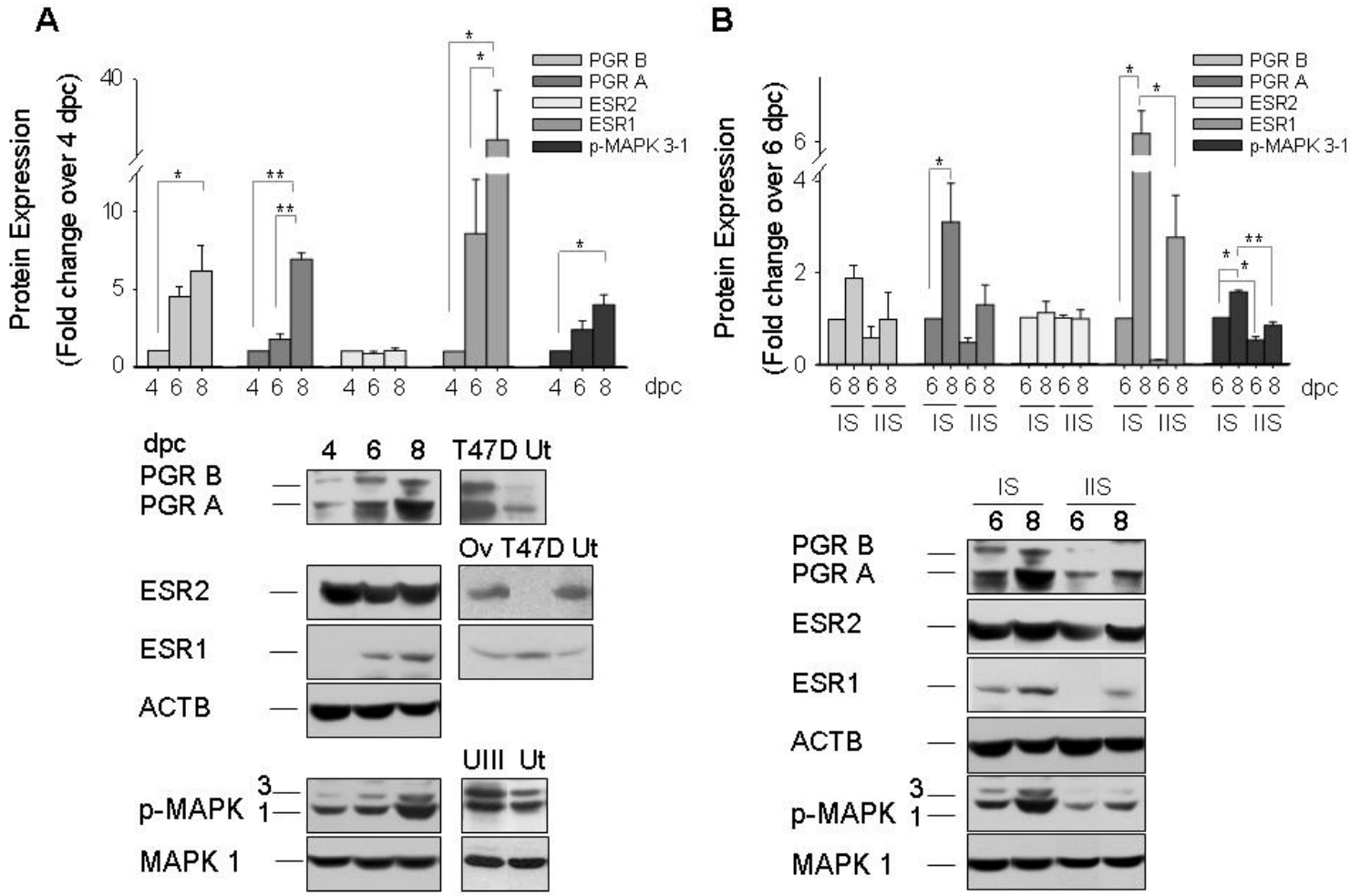
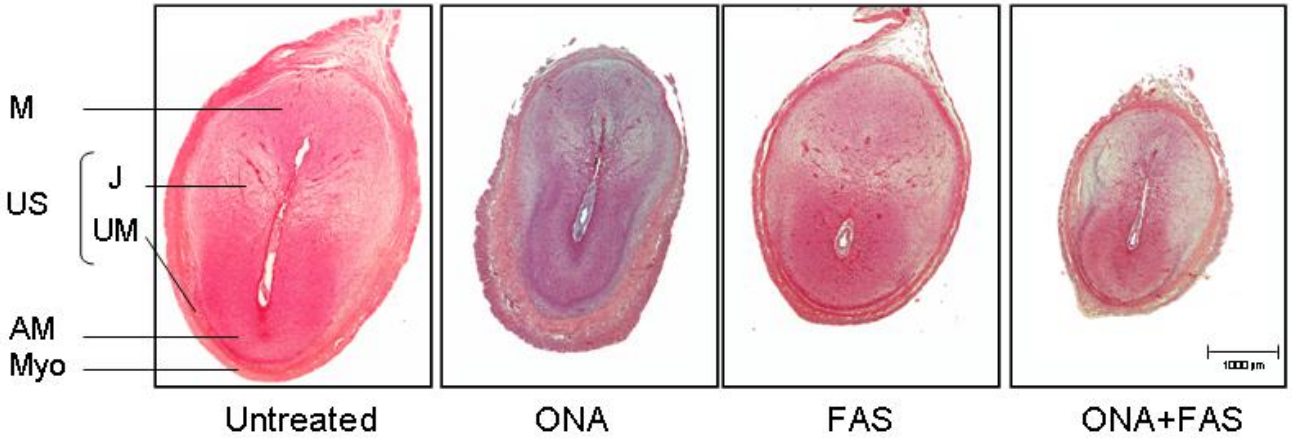


Figure 1, Vallejo et al.



Area (%)	Untreated	ONA	FAS	ONA+FAS
M	19 ± 0.7	13 ± 0.6 **	15 ± 0.2 **	10 ± 1.1 **
US	34 ± 1.6	33 ± 0.9	43 ± 2.4 **	40 ± 0.9 *
AM	25 ± 1.0	18 ± 0.3 **	22 ± 1.1 *	21 ± 0.4 **
Myo	22 ± 0.6	36 ± 0.8 **	20 ± 0.9	29 ± 0.6 **

Figure 2, Vallejo et al.

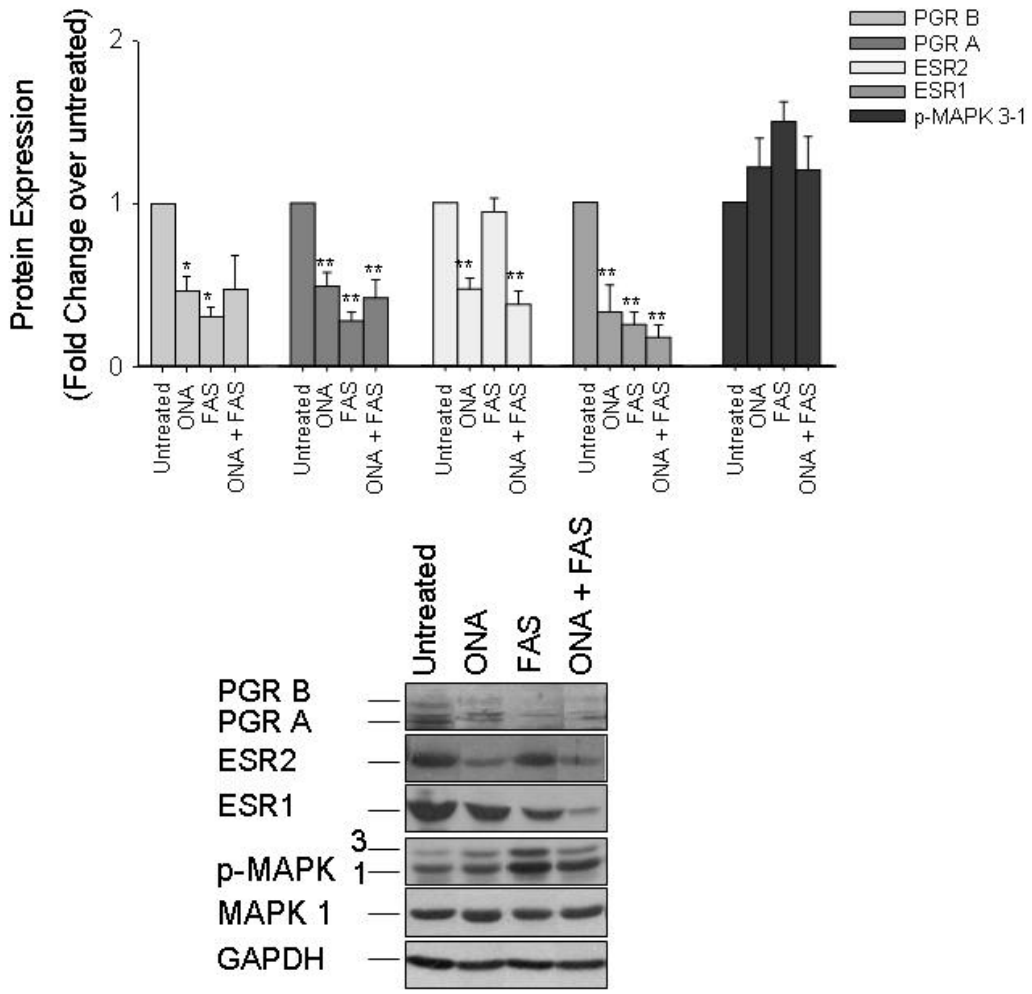


Figure 3, Vallejo et al.

PGR

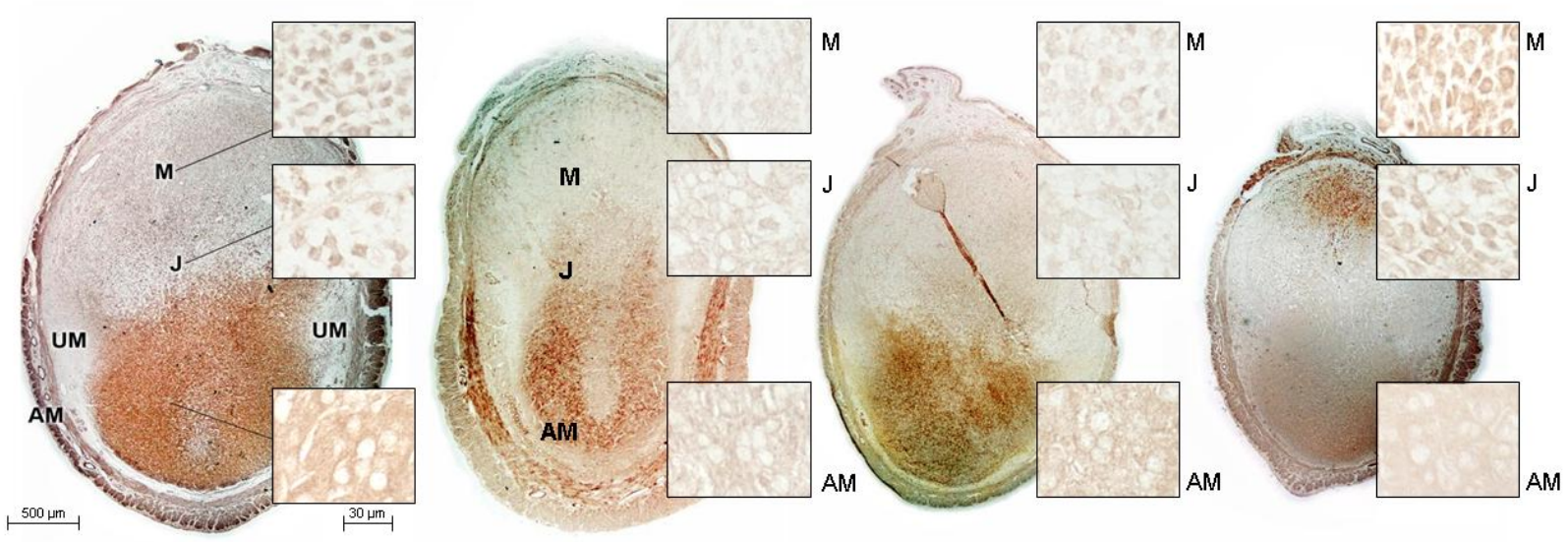


Figure 4, Vallejo et al.

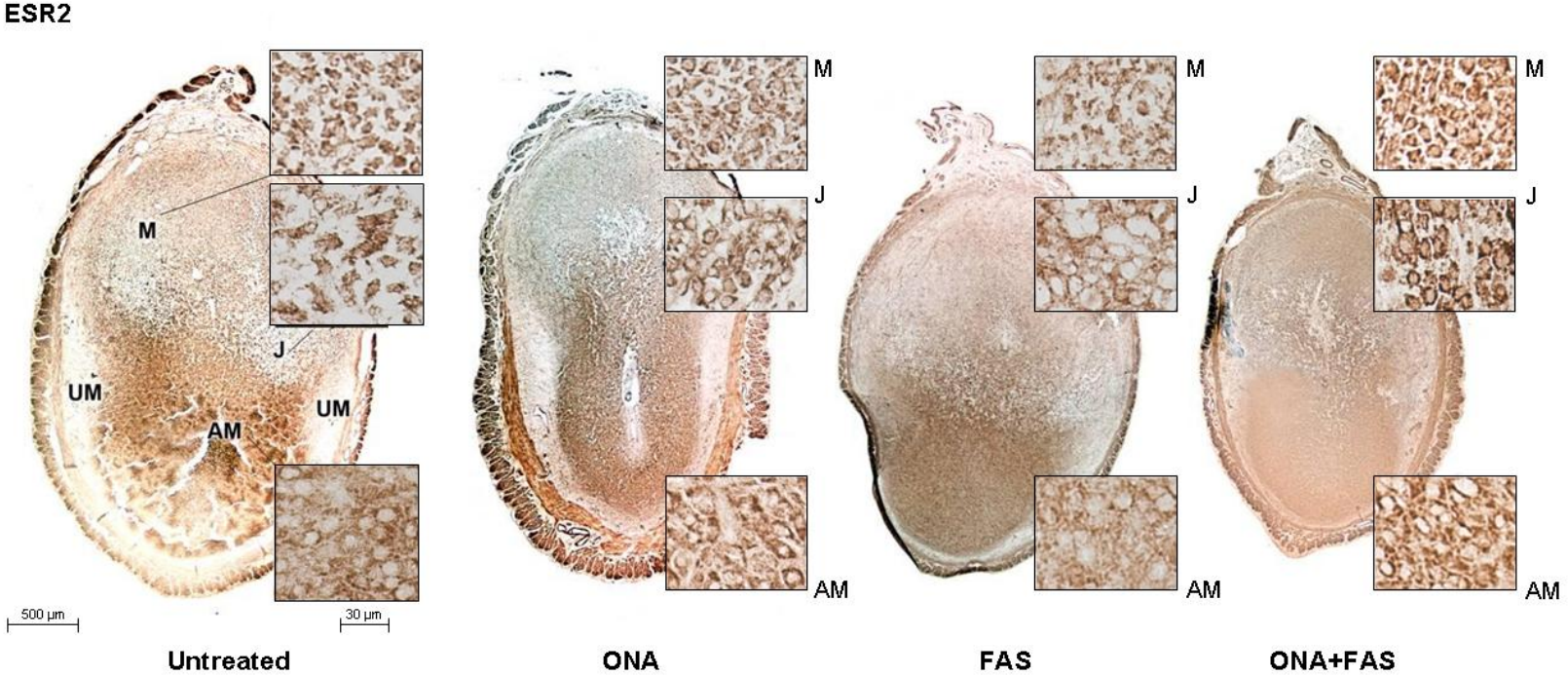


Figure 5, Vallejo et al.

ESR1

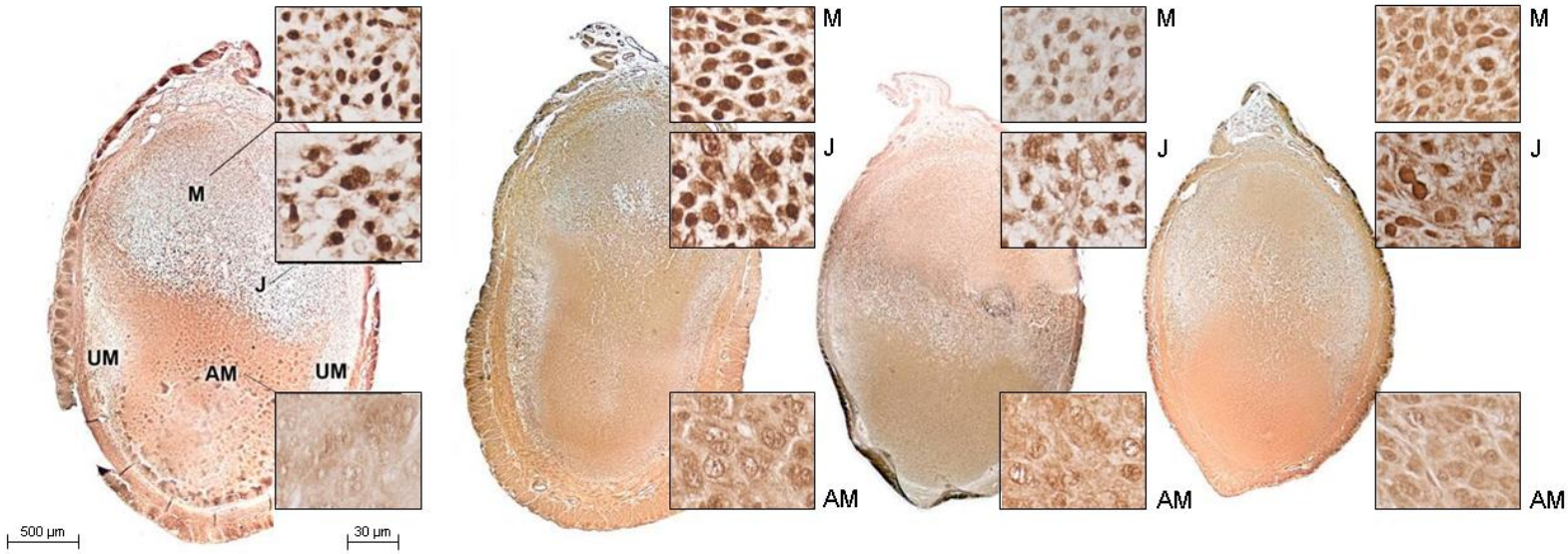
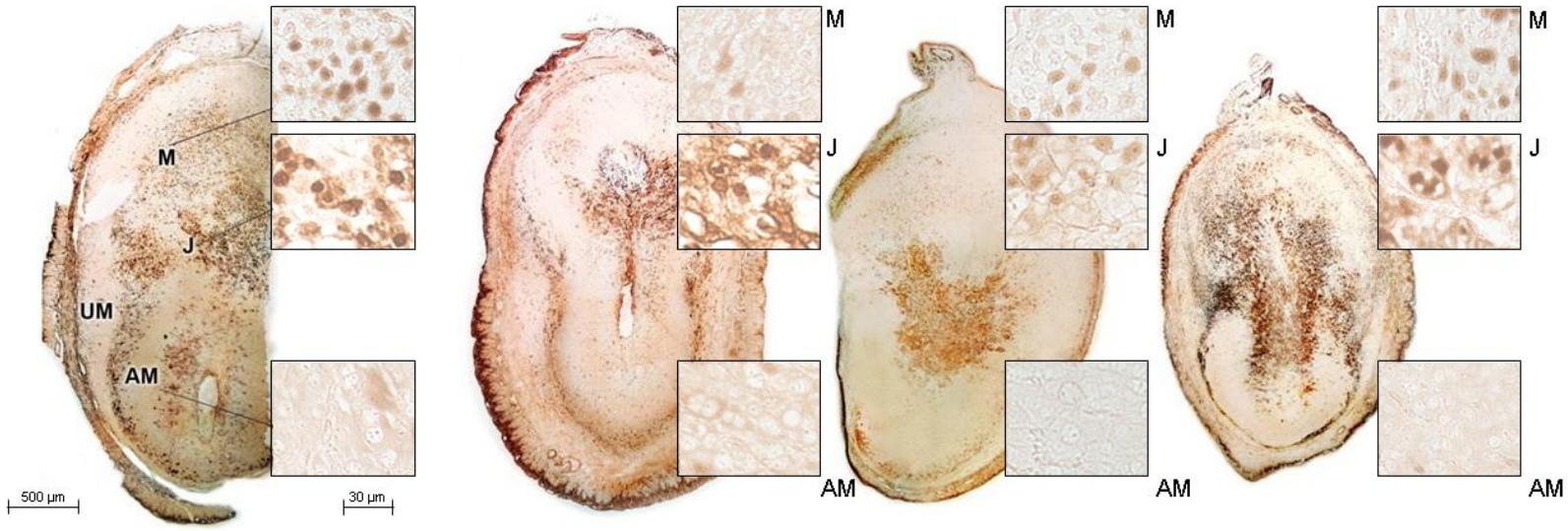


Figure 6, Vallejo et al.

p-MAPK 3-1



Untreated ONA FAS ONA+FAS

Figure 7, Vallejo et al.

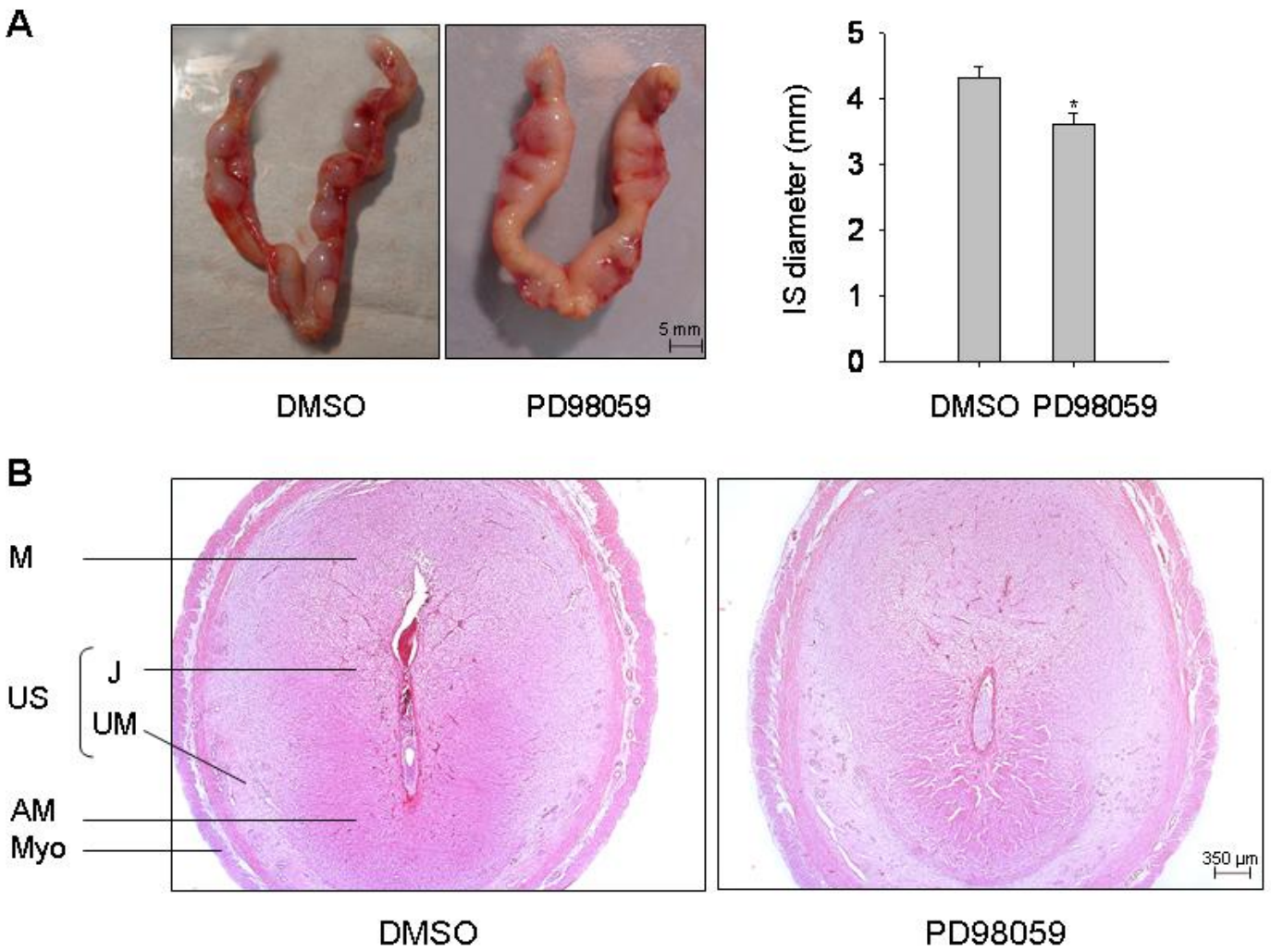


Figure 8, Vallejo et al.

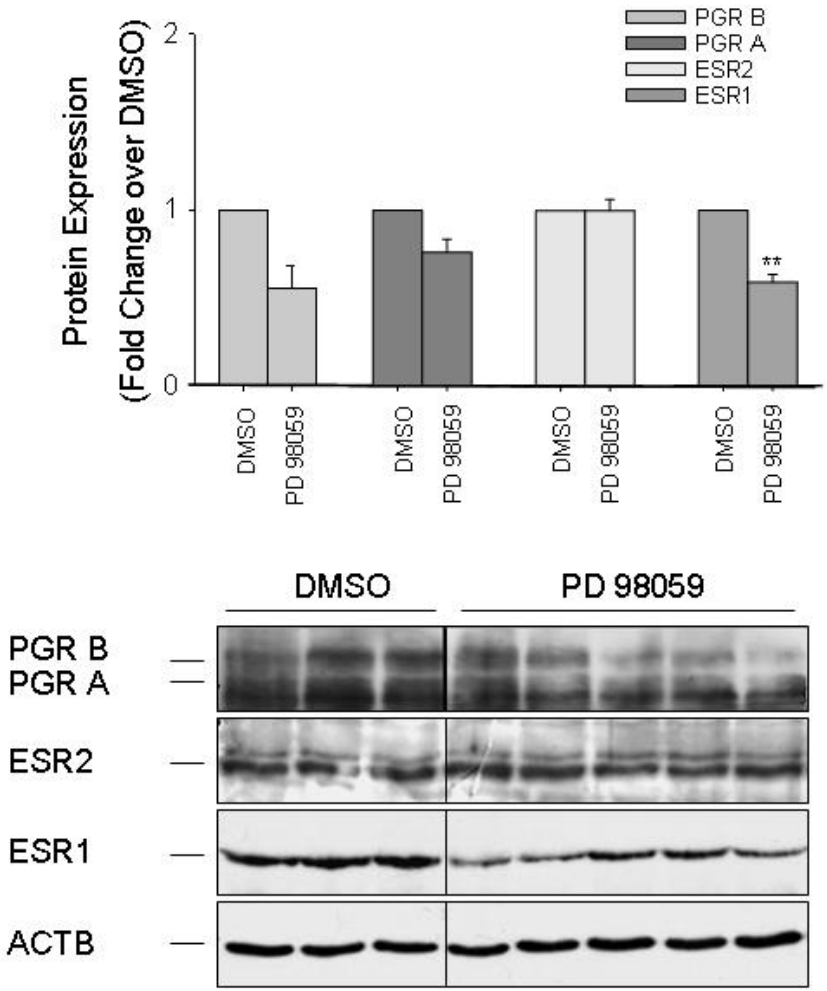


Figure 9, Vallejo et al.