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An evolutionary test of the isoform switching hypothesis of functional progesterone withdrawal for parturition: humans have a weaker repressive effect of PR-A than mice

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Abstract

Background—A decrease in maternal serum progesterone (P4) concentrations precedes the onset of labor in most placental mammals. Humans differ by maintaining high levels of P4 throughout birth. Parturition in humans probably includes mechanisms that undercut the pregnancy sustaining function of P4. One attractive hypothesis is the isoform switching hypothesis (ISH). ISH is supported by *in vivo* evidence that progesterone receptor isoform A (PR-A) inhibits PR-B and that the PR-A/PR-B ratio increases towards term.

Material and methods—Here, we test the hypothesis that isoform switching is an adaptation to high levels of P4 at term, predicting that, in humans, PR-A mediated repression of PR-B is stronger than in mouse. We use reporter assays with human and mouse PRs to detect species differences in the repressive effects of PR-A.

Results—We found that human PR-B is less sensitive to repression by human PR-A than mouse PR-B, contrary to our prediction. The difference between human and mouse PR-B sensitivity is most pronounced at PR-A/PR-B ratios typical for the preterm myometrium.

Conclusions—Our results are inconsistent with the ISH. We speculate that, instead, the lower sensitivity of human PR-B to PR-A may be relevant for the maintenance of pregnancy at high progesterone levels and increasing PR-A concentrations towards term.

Keywords

Functional progesterone withdrawal; human evolution; human parturition; isoform switching; progesterone receptor

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INTRODUCTION

One major challenge for research on human parturition is the dramatic difference in the mechanisms of labor in different mammalian species [18], with important implications for the prediction and prevention of prematurity [12,13,21,25,26]. In most mammals investigated, parturition is preceded by a decrease in serum progesterone (P4) concentrations, removing the pregnancy sustaining effects of P4 before the initiation of birth [3-5]. By contrast, systemic P4 concentrations in humans continue to increase all the way through parturition, due to P4 production by the placenta [30]. It has been inferred, therefore, that there must be a mechanism that, before the onset of labor, undercuts the pregnancy sustaining effects of P4 causing a functional progesterone withdrawal (FPW) [1].

High sustained levels of systemic P4 are found not only in humans but also in other great apes and in old world monkeys, such as the rhesus macaque [28]. It also evolved independently in the guinea pig. In humans progesterone is produced by the maternal *corpus luteum* at the beginning of pregnancy, but later the *corpus luteum* degenerates and P4 production is taken over by the placenta. Placental P4 production is probably a fetal adaptation for prolonged pregnancy [9], an interpretation based on the theory of maternal-fetal conflict as developed by David Haig [8]. This theory observes that the fitness interests of the mother and the fetus can be different, where, in general, fetal interest lies in increased resource extraction from the mother and thus longer gestational periods. By contrast, the fitness interest of the mother lies in limiting resource commitment to her current offspring and thus increasing the success chance of future pregnancies. A model of maternal-fetal conflict correctly predicts that if fetal tissues express pregnancy hormones, the mother ceases to produce them. FPW, then, can plausibly be understood as a maternal adaptation to gain control over the time of parturition when P4 production is not under her control.

The idea of FPW spawned a genome-wide search for molecular mechanisms that could explain both spontaneous term and preterm parturition [19]. FPW could have evolved in a number of ways, including by local breakdown of P4 in myometrial tissue [15,24], sequestration of P4 in the plasma by carrier proteins [11,23,29], and the activity of membrane P4 receptors, although this has not been explored to the best of our knowledge. An attractive hypothesis is that nuclear P4 receptors are involved in FPW. Nuclear P4 receptors occur in two main isoforms, PR-A and PR-B, which are splicing isoforms from the same genes [7,14]. The human PR-A is 164 amino acids shorter at the N-terminal end than PR-B. In most contexts, PR-B acts as a transcriptional activator. Co-expression of PR-B and PR-A, however, leads to a repression of PR-B activation [22,27]. Furthermore, the relative abundance of PR-B and PR-A in the human myometrium changes towards the end of pregnancy, with PR-A increasing in abundance until parturition [10,16]. These two facts led to what is known as the “isoform switching hypothesis” (ISH) of FPW [17]. The hypothesis states that the rise of PR-A levels in the myometrium towards term interferes with the uterine quiescence promoting function of PR-B and thus contributes to the readiness of the myometrium for parturition.

There are two ways PR isoform switching could have evolved: by changes in expression levels of PR-A, or in the repressive activity of the PR_A protein itself. If isoform switching

evolved by changes in the expression pattern of PR-A, then PR-A expression in term myometrium would have evolved in the human lineage, coincidental with fetal control over P4 production. Increased myometrial expression of PR-A towards the end of pregnancy, however, is not only found in humans and monkeys, but has also been reported for mice [31]. Hence, last gestational expression of PR-A is probably ancestral for the primate lineage and thus not an adaptation for FPW. Alternatively, PR-A might have evolved repressive activity to assist in preparation for parturition in spite of sustained elevated P4 levels. This hypothesis is consistent with the fact that chicken PR-A does not have repressive activity on PR-B [6]. If the chicken PR is representative of the ancestral amniote condition, then repressive activity of PR-A probably evolved some time in the mammalian lineage and it is possible that is specifically evolved as a mechanism for functional progesterone withdrawal.

Although there is some supporting evidence for the ISH as mentioned above [16,19,22,27], it is still not clear whether the expression of PR-A in term myometrium causes functional progesterone withdrawal through its repression of PR-B activity. Here, we ask whether repressive activity of PR-A is correlated with high sustained levels of systemic P4 in the human lineage. If, in fact, repression of PR-B by PR-A is a mechanism of FPW, then one would expect that this mechanism originated, or was at least enhanced, around the time the fetus took control of P4 production in the human lineage. We can test this prediction by comparing the strength of PR-A repression from human and non-primate animal. If human PR-A repression is stronger than that in the non-primate, then the comparative data would be consistent with the ISH. If not, then the evidence would contradict the evolutionary implications of the ISH, in turn weakening this hypothesis. By testing the evolutionary implications of the ISH we indirectly test whether repression of PR-B by PR-A is likely to play a role in functional progesterone withdrawal.

In this study, we compare the repressive activities of mouse and human PR-A on PR-B to test the evolutionary implications of the ISH. We found that there are differences in the repressive activities in mouse and human PRs, but the difference we found contradicts the predictions of the ISH. Human PR-B is less sensitive to repression than mouse PR-B, in particular at substoichiometric and stoichiometric PR-A/PR-B ratios typical for the preterm and prelabor myometrium. There is little difference between mouse and human PRs at PR-A/PR-B ratios typical for myometrium in labor. We conclude that changes to the PR protein are likely to be adaptive for the maintenance of pregnancy rather than for parturition.

MATERIALS AND METHODS

Experimental design for comparing PR activities across species

The choice of species for this study was influenced by both technical as well as phylogenetic considerations. Expression constructs of human and mouse PRs are relatively easy to make (see below for details). In addition, the mouse is a member of the sister taxon to primates, making this species more appropriate to compare to humans than more distantly related animals. Furthermore, an analysis of PR sequence evolution showed that adaptive modifications of the PR amino acid sequence happened in the primate lineage but not in the rodent lineage [2]; thus, mouse PR activity probably represents PR activity in the most recent common ancestor of primates and rodents. In a formal comparative study, without

additional information, at least three taxa are necessary to infer the direction of evolutionary change. In this case, however, the molecular evolution evidence suggests that the human protein is likely to be derived because of the lack of directional selection signature in the primate lineage and the lack of such a signal in the rodent lineage [2].

One challenge to a comparative study of transcription factor activity is the cell system. We used a human immortalized myometrial cell line (see below) for the main experiments and HeLa cells for control experiments and to test for cell type effects. Proteins tested in non-native cells might have lower activity than in native cell environments. Thus, the use of human cells could bias the results against the mouse, which would make it difficult to interpret what low activity of mouse PRs tested in human cells actually means. However, we found that the mouse PR-B has a higher maximal activity and PR-A repression is stronger even though we tested them in human cells. We conclude that the use of human cell lines is conservative with respect to the final result. Mouse proteins have stronger measured effects tested in human cells than human proteins, suggesting that the use of human cells is not confounding the result.

A final issue we considered concerns the amounts of expression vectors to transfect from each species. The dose-response curve of reported activity as a function of the amount of transfected PR-B expression vector differed between human and mouse genes, both in terms of the slope as well as the saturation level (Figure S1 in the supplementary material). We thus had to take care to measure the repressive effect of PR-A in a way that makes the values comparable between species. To ensure comparability we used PR-B expression construct amounts that correspond to the mid-linear range of the dose-response curve for each species. This ensured that for both species the measured PR-B activity would be equally sensitive to PR-A repression. In addition, the repressive activity was measured as the fraction of reporter activity compared to that in the absence of PR-A, leading mostly to values between 0 and 1, unless there was an activating effect of PR-A on reporter activity.

Reporter constructs and expression vectors

The progesterone responsive PGL4.36 MMTV plasmid (Promega, Fitchburg, WI, USA) and a panel of eight estrogen responsive plasmids (SwitchGear Genomics, Menlo Park, CA, USA; Product ID: ER_10-05) were the reporter constructs used in the transient transfection assays (see below). The pRL-TK plasmid (Promega) was used as an internal control.

To make the PR-A and PR-B expression constructs for human and mouse, PCRs with progesterone receptor A- and B-specific primers were performed using human and mouse ORF collections as template (Open Biosystems, Lafayette, Co, USA; human: Cat. No. OHS4559-99848151; mouse: Cat. No. MMM1013-99827146). PCR products were then cloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Two clones for each construct were sequenced.

For the estrogen receptor- α (ER- α) experiment, the human ESR1 expression construct (OriGene, Rockville, MD, USA; SKU RC226003) was used, which as the pCMV6-Entry backbone (OriGene).

Cell culture and transient transfection

HeLa cells were grown in steroid-depleted DMEM, supplemented with 5% charcoal-stripped calf serum and 1% antibiotic/antimycotic (ABAM). hTERT immortalized human myometrial cells were obtained from Dr. Ann Word (University of Texas Southwestern Medical Center, Dallas, TX, USA) and maintained in steroid-depleted DMEM/F12 medium with 10% charcoal-stripped calf serum, 20 mM Hepes, and 1% antibiotic/antimycotic (ABAM).

For transient transfections experiments involving human and mouse PR-A and PR-B, cells (HeLa and myometrial in separate experiments) were plated in 6-well plates at 70% confluency 24 h before transfection. Cells were transfected in five replicates using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA; Cat. No. 11668) according to the manufacturer's instructions. A total of 3.36 µg of DNA was transfected/well: 1.6 µg of the MMTV reporter, 0.16 µg of the pRL-TK control, and varying amount of PR-A, PR-B, and empty pcDNA vectors depending on the experiment (empty pcDNA was used to reach a total of 3.36 µg DNA for all experiments). To determine a dose-response curve for human and mouse PR-B, transfections were performed as above, but varying amounts of PR-B and pcDNA were transfected (no PR-A), in the range of 0-1 µg for PR-B (Figure S1 in the supplementary material). It was determined that 0.1 µg of human PR-B and 0.5 µg of mouse PR-B would be used in co-expression of PR-A, as these amounts yielded an activity in the mid-linear range of the dose-response curve (Figure S1 in the supplementary material). Thus, in co-expression experiments with PR-A, the amount of PR-B remained constant and PR-A and pcDNA varied.

Six hours after transfection, the media was changed to one containing 10^{-6} M medroxyprogesterone acetate (MPA), a stable progesterone analog. We chose MPA instead of P4 because of its higher chemical stability in cell culture media. The transfected cells were incubated with the hormone for 24 h. Then a luciferase assay was performed using the Dual Luciferase Assay System (Promega), according to the manufacturer's instructions.

For transient transfection experiments involving ER- α , myometrial cells were plated in 24-well plates at 70% confluency 24 h before transfection. Cells were transfected in triplicate using the Lipofectamine 2000 reagent (Cat. No. 11668) according to the manufacturer's instructions. First, to choose a reporter plasmid with the strongest response to estradiol and ER, we tested all eight estrogen responsive reporter plasmids (see above). A total of 0.67 µg of DNA was transfected/well: 0.32 µg of the reporter plasmids, 0.032 µg of the internal control pRL-TK, 0.04 µg of the ESR1 expression construct, and 0.28 µg of empty pCMV6. Six hours after transfection, the media was changed to one containing 10^{-8} M estradiol. The transfected cells were incubated with the hormone for 24 h and a luciferase assay was performed, as above.

Plasmid #8 (Product ID S114388, SwitchGear Genomics) had both the strongest response to estradiol (E2) as well as the strongest response to ER- α (Figure S2 in the supplementary material), so it was used in co-expression experiments involving ER- α and PR-A. These experiments were performed as above, except that varying amounts of PR-A and empty pCMV6 were used to measure the effect of PR-A on ER- α . Also, 6 h after transfection, the

media was changed to one containing 10^{-6} M medroxyprogesterone acetate and 10^{-8} M estradiol.

Quantitative PCR

To verify that transfection ratios correspond to mRNA levels, HeLa cells were transfected as above in 24-well plates. For mouse, 0.5 μ g of PR-A only, PR-B only, or a combination of 0.5 μ g PR-A and 0.5 μ g PR-B were transfected/well. For human, 0.1 μ g PR-A only, PR-B only, or a combination of 0.1 μ g PR-A and 0.1 μ g PR-B were transfected/well. No media change was performed after transfection, and RNA was harvested from cells 24 h after transfection using the RNA-Easy Mini RNA-Extraction Kit (Qiagen, Valencia, CA, USA) in conjunction with the RNase-Free DNase Set following the manufacturer's instructions to remove any contaminating DNA (Qiagen, Valencia). Real-time PCR was performed on these RNA templates using the TaqMan FAST Universal PCR system (Applied Biosystems, Foster City, CA, USA), using the manufacturer's instructions and primer/probe sets for human and mouse PR and for GAPDH endogenous controls.

RESULTS

Repressive activity of PR-A on PR-B activity has been mostly studied with a standard progesterone responsive element of the mouse mammalian tumor virus, MMTV [27]. To be consistent with previous work, we also used the MMTV P4 responsive element to drive luciferase activity in HeLa and myometrial cell culture. First, we compared reporter gene activity in HeLa cells transfected with mouse and human PR-B expression vectors. Consistent with previous studies we found that PR-B is an activating factor. Human and mouse PR-B, however, differed in their dose-response curve (Figure S1 in the supplementary material), with human PRB showing a steeper increase in activity but a lower saturation level than the mouse PR-B. The difference in the maximal activities of the human and mouse PR-B conflict theory [9], predicting that the human PR should be less active at the same level of P4 than the mouse PR-B.

For co-expression experiments with PR-A, PR-B transfection levels were chosen that yielded an activity in the mid-linear range of the dose-response curve of either species (see Material and methods for details). Co-expression experiments of PR-A and PR-B were done with different ratios of PR-A and PR-B expression vectors ranging from 0 to 2 (0, 0.25, 0.5, 1.0, 2.0). We used qPRR to verify that these transfection ratios correspond to the same ratios of expression at the mRNA level. Using this approach, we calculated the repressive activity of PR-A on PR-B for human and mouse by dividing the activity levels with the average reporter activity for PR-A/PRB=0, and called this value the relative receptor activity. In both HeLa and myometrial cells, the relative activity level declined with increasing PR-A/PR-B ratio (Figure 1), although the human PR-B in HeLa cells was only mildly affected within the range of PR-A/PR-B ratios tested (Figure 1B). Comparison of mouse and human relative activity levels shows that activity of mouse PR-B decreases faster than that of human PR-B. This difference is found in both HeLa and myometrial cells and is thus not cell type specific. From this, we conclude that the repressive effect of PR-A on PR-B is weaker for human PR

than for mouse PR. The difference is most pronounced at sub-stoichiometric and stoichiometric ratios of PR-A/PR-B.

Next, we tested whether the difference between the relative levels of human and mouse PR is due to a difference in PR-A repressive activity or due to a difference in the sensitivity of PR-B to PR-A. To address this question, we transfected human and mouse PRs in all combinations (Figure 2). When comparing the effect of human PR-A on both human and mouse PR-B activity, we found that the effect on human PR-B is much less than that on mouse PR-B (Figure 2A). When mouse PR-A was tested on human and mouse PR-B, again the effect on human PR-B was weaker (Figure 2B) but much less so than in the case of human PR-A. These results suggest that human PR-B is less sensitive to PR-A repression than mouse PR-B. Another interpretation of these results is that human PR-A is a less effective repressor than mouse PR-A. However, when we directly compared human PR-A and mouse PR-A on either human or mouse PR-B, we found no large or even consistent difference in the effects of the two PR-A species (Figure 2C and D). Overall, the repressive effect on mouse PR-B was stronger for PR-A from both species than on human PR-B (compare repression levels at PR-A/PR-B ratios 1 and 2 in Figure 2C and D). From these data, we conclude that the difference seen in Figure 1A and B between mouse and human repression is due to a lower sensitivity of human PR-B to PR-A than mouse PR-B.

The inference from the experiments with human/mouse PR-A/B combinations is that the difference between human and mouse repression is limited to PR-B, as it seems that there is a decreased sensitivity of human PR-B rather than a weaker inhibitory effect of human PR-A. To test this inference, we tested the effect of human and mouse PR-A on human estrogen receptor- α (ER- α), as PR-A is also known to repress the transcriptional activity of ER- α (see [8]). Various amounts of human PR-A were co-transfected with constant amounts of an estrogen-responsive reporter and an ER- α expression vector in the presence of medroxyprogesterone acetate and estradiol. At a 2:1 transfection ratio, human PR-A had a moderate inhibitory effect on the estradiol response of approximately 20% (Figure S3 in the supplementary material). However, no systematic difference was found when the same experiment was done with mouse PR-A (Figure S3 in the supplementary material). Hence, it seems likely that the difference between mouse and human in the repressive effect of PR-A is a PR-B specific effect.

DISCUSSION

It is widely accepted that functional progesterone withdrawal (FPW) is a maternal adaptation to fetal progesterone secretion [30]. However, there is no agreement on the precise molecular mechanisms responsible for EFPW. Here, we use a comparative approach to test the ISH of FPW [17].

Comparative data do not support ISH via repression by PR-A

Isoform switching probably did not evolve by the recruitment of PR-A expression in late pregnancy because this expression pattern is also known from mice [31]. In addition, PR-A alone, without PR-B, can sustain pregnancy in the mouse [20]. We inferred that if PR-A repression is the mechanism of FPW, adaptive modifications are predicted to have affected

activity of the PR proteins rather than their expression pattern. Specifically we predicted that the repressive effects of PR-A should be stronger in human than in mouse. Here, we report that we found the opposite, namely that the human PRs show weaker repressive effects than the mouse PRs (both tested in human cells). In addition, we found that the difference between mouse and human PR activities is probably due to lower sensitivity of human PR-B to PR-A rather than weaker inhibitory activity of PR-A itself. Thus, the differences in repressive activity described here are unlikely to be adaptive changes contributing to FPW.

Limitations of the current study

One limitation of this study is that activities of the PRs were tested on the promoter (MMTV) that does not play a physiological role in myometrial cells. This is the standard in this field, and we decided to conform to the experimental paradigm of the original research as physiological target genes of PR in the myometrium have not yet been identified.

A second weakness has to do with the fact that all tests were done in human cells, not in human and mouse cells which would be ideal for this type of experiment. Thus, we cannot exclude the possibility of a PR-cell of origin interaction. Nevertheless, we believe that our conclusions are conservative because we found that the mouse PR-B has both a higher maximal activity and the mouse PR-A effect was stronger compared to that of human proteins. If there was PR-cell of origin interaction one would expect the non-native protein to do less well than the native protein. Thus, we conclude that our results are likely to be real.

Finally, comparative studies usually require at least three species to infer the direction of evolutionary change. Hence, it would be desirable to test proteins from other species in the Euarchontoglires clade, i.e., the clade that unites the primate and the rodent lineage. However, the choice of a two species comparison in this study was made because of results of a previous study on PR amino acid sequence evolution. This study showed that human and chimp PRs are likely to be functionally derived, whereas the mouse PR does not show any signs of adaptive modification [2].

Interpretation of findings

In spite of the fact that the comparative evidence does not reveal molecular adaptations for FPW via isoform switching, it did reveal a difference in the repressive activity of human and mouse PR proteins. Lower repressive effects in human PRs are most noticeable at sub-stoichiometric and stoichiometric PR-A/PR-B ratios, i.e., at ratios of 0.25-1. According to detailed measurements of isoform abundance in human myometrium, Merlino and collaborators [16] found that during preterm pregnancy the PR-A/PR-B ratio is approximately 0.5, at term (but not in labor) the ratio is approximately 1, and in labor the ratio increases to 2.5. Hence, the PRA/PR-B ratios in our study that yielded the most marked effects match those found in the myometrium prior to labor. It is tempting to speculate that lower sensitivity of human PRs to repression is biologically most relevant for the preterm phases of human pregnancy, i.e., for the maintenance of pregnancy.

As both the human and the mouse PR show repression, it is likely that repression is ancestral to the primate lineage. In addition, as mice as well as humans and macaques show an

increase in the PR-A/PR-B ratio in the myometrium towards the end of pregnancy, isoform switching is also likely to be ancestral for the primate lineage. The most obvious difference among the derived primates is a sustained high level of P4 all the way through pregnancy and parturition. The group of species known to have sustained P4 levels is the great apes, including humans, and the old world monkeys, collectively known as the catarrhines [10]. The presence of substantial levels of PR-A, with strong repressive activity, in conjunction with high levels of P4 could have been a liability of the maintenance of pregnancy in ancestral catarrhines. We thus speculate that the lower sensitivity of PR-B to repression by PR-A could be a compensatory adaptation by the mother to the high levels of progesterone produced in the placenta by the fetus. This would explain why the differences in repression are most pronounced at PR-A/PR-B ratios of 1, which are the PR-A/PR-B ratios found prior to labor, whereas the repression differences between mouse and human receptors becomes smaller at higher PR-A/PR-B ratios typical for the uterus in labor [16].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Repressive effects of human PR-A on human PR-B, and of mouse, PR-A on mouse PR-B, on MMTV-Luciferase reporter construct.

(A) Results in human myometrial cells. Note that the repressive effects of mouse PR-A are stronger than those of human receptors, particularly for sub-stoichiometric/stoichiometric PR-A/PR-B ratios.

(B) Results in HeLa cells. Here, also the repressive effects are stronger for mouse than for human receptors. Standard errors are <5% and cannot be displayed in the graph (number of replicates=5).

Figure 2.

Repressive effects of all combinations of human and mouse PR-A and PR-B activity, on MMTV-Luciferase reporter construct in human myometrial cells.

(A) Effect of human PR-A on mouse and human PR-B. The apparent effect on mouse PR-B is much stronger than on human PR-B. (B) Effect of mouse PR-A on mouse and human PR-B. The effect on the human PR-B is weaker than on the mouse PR-B but less so than for the human PR-A. (C) Comparison of effects of human and mouse PR-A on human PR-B. (D) Comparison of effects of human and mouse PR-A on mouse PR-B. Standard errors are <5% and cannot be displayed in the graph (number of replicates =5). In both sets of experiments, (C) and (D), the differences between the human and mouse PR-A are small. The maximal degree of repression, however, is higher for the mouse PR-B than for the human. Overall, the results suggest that the difference between mouse and human repression is due to a lesser sensitivity of human PR-B to PR-A, rather than to a difference in the repressive activities of the mouse and human PR-A.