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Placental Expression and Molecular Characterization of Aromatase Cytochrome P450 in the Spotted Hyena (*Crocuta crocuta*)

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Abstract

At birth, the external genitalia of female spotted hyenas (*Crocuta crocuta*) are the most masculinized of any known mammal, but are still sexually differentiated. Placental aromatase cytochrome P450 (P450arom) is an important route of androgen metabolism protecting human female fetuses from virilization *in utero*. Therefore, placental P450arom expression was examined in spotted hyenas to determine levels during genital differentiation, and to compare molecular characteristics between the hyena and human placental enzymes. Hyena placental P450arom activity was determined at gestational days (GD) 31, 35, 45, 65 and 95 (term, 110), and the relative sensitivity of hyena and human placental enzyme to inhibition by the specific inhibitor, Letrozole, was also examined. Expression of hyena P450arom in placenta was localized by immuno-histochemistry, and a full-length cDNA was cloned for phylogenetic analysis. Aromatase activity increased from GD31 to a peak at 45 and 65, apparently decreasing later in gestation. This activity was more sensitive to inhibition by Letrozole than was human placental aromatase activity. Expression of P450arom was localized to syncytiotrophoblast and giant cells of mid-gestation placentas. The coding sequence of hyena P450arom was 94% and 86% identical to the canine and human enzymes respectively, as reflected by phylogenetic analyses. These data demonstrate for the first time that hyena placental aromatase activity is comparable to that of human placentas when genital differentiation is in progress. This suggests that even in female spotted hyenas clitoral differentiation is likely protected from virilization by placental androgen metabolism. Decreased placental aromatase activity in late gestation may be equally important in allowing androgen to program behaviors at birth. Although hyena P450arom is closely related to the canine enzyme, both placental anatomy and P450arom expression differ. Other hyaenids and carnivores must be investigated to determine the morphological and functional ancestral state of their placentas, as it relates to evolutionary relationships among species in this important taxonomic group.

Keywords: Aromatase; Hyena; Placenta; Letrozole; Trophoblast; Sexual differentiation; Androgen metabolism; Cloned sequence

1. Introduction

The peniform clitoris of the female spotted hyena is difficult to distinguish grossly from the male penis, suggesting that female fetuses might be hormonally virilized *in utero* [1–3]. Female spotted hyenas also have unusually high levels of androgens including androstenedione [4] which can be metabolized to testosterone by the hyena placenta during pregnancy [5]. However, aromatase cytochrome P450 (P450arom) has the potential to metabolize androgens to estrogens [6], and human female infants lacking P450arom are markedly virilized at birth [7]. Aromatase activity of normal human placentas is 20-fold higher than hyena placentas at term, indicating that the pregnant spotted hyena has a relatively lower capacity to clear testosterone and other androgens at birth [8]. Lower placental aromatase expression at term placentas may well promote fetal exposure to androgen and

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influence the development of female dominance over males that appears during the first year of life in mixed-sex litters [9,10]. However, the potential role of placental aromatization of androgens occurring during genital differentiation has not been well studied in many species, and changes in placental P450arom expression throughout gestation may be important physiologically, even in spotted hyenas.

Earlier speculation on the role of placental aromatase activity in genital morphogenesis of female spotted hyenas is based largely on mid-gestation to term (110 days) placental tissues, and how it might influence early events in sexual differentiation is therefore unclear. Recent results indicate that initial phallic development is androgen independent in both sexes. However, several features of the male penis become androgenized before gestation day (GD) 45 [11] including shape and elasticity of the tip [12], tunica albuginea thickness and insertion of the retractor penis muscles, among others [13]. Penile differentiation coincides with the development of androgen-synthetic capacity in the fetal testicular interstitium, but a similar differentiation of ovarian interstitium is delayed in female fetuses [14]. This implies that the female peniform clitoris escapes the androgen-induced masculinization experienced by litter mate male siblings. Past studies of placental androgen metabolism have been limited in number; and none have encompassed this period of genital differentiation. Therefore, we re-examined placental P450arom expression at these earlier fetal ages. The spotted hyenas placenta is hemochorial [15], more like a human than other carnivores, so which trophoblastic cells express P450arom was of interest. A full length cDNA encoding the hyena P450arom enzyme was also cloned and used for phylogenetic analysis. Since future studies plan to treat pregnant hyenas with a potent inhibitor of P450arom, Letrozole, the sensitivity of hyena and human placental enzymes to inhibition by this drug was also tested.

2. Materials and methods

2.1. Tissue collection

Placentas were collected during hysterotomy (GD 31, 35, 35, 45, 45, 45, 65, 65 and 95). A portion of tissue was preserved in 4% paraformaldahyde for 24 h, and dehydrated in a graded ethanol series prior to paraffin embedding. Fixed placentas were then sectioned to a thickness of 5 μm, and mounted on coated glass slides for immuno-histochemical examination. A second portion of placenta was frozen at −80 °C within 15 min of collection for western immunoblot analysis and enzyme activity assays. In addition to these, term human placentas were collected subsequent to normal deliveries at the Sutter General Hospital, Davis CA. The collection and use of placental tissue was considered exempt from human subject concerns by the University of California, Davis, Office of Human Research Protection. These tissues were transported to the laboratory, rinsed dissected and fragments were frozen as for hyena placental tissues.

2.2. Microsomal protein

Frozen placenta was thawed and homogenized and fractions enriched for endoplasmic reticulum (microsomes) were isolated following previously described procedures [16,17]. Briefly, tissues were homogenized in lysis buffer (0.1 M potassium phosphate, pH 7.4; 20% glycerol; 5 nM β-mercaptoethanol) with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit protease activity. Homogenates were sonicated for 5 s to disrupt protein aggregations in solution and centrifuged for 10 min at 15,000 × *g* at 4 °C. Supernatants were transferred to ultracentrifuge tubes and spun for 60 min at 100,000 × *g* at 4 °C. The supernatant was removed and the microsomal pellet was solubilized in buffer (identical to lysis buffer, but with 1 mM 3-[(3-chloramidopropyl)-dimethylammonio]-1-propanesulfonate; CHAPS) and sonicated for 3 s. Protein concentrations of microsomal solutions were determined by bicinchoninic acid protein assay following manufacturer's protocol (BCA assay kit, Pierce Chemical Co., Rockford, IL). Aliquots of microsomal protein were stored at −80 °C until use.

2.3. Aromatase activity assays

Aromatase activity of microsomal preparations was determined from tritium released from [1β-^{3}H]-androstenedione (Perkin Elmer, Boston, MA) as described by Lephart and Simpson [18] and previously validated in this laboratory [19]. In summary, 100 to 500 μg of microsomal protein preparations were incubated with 300 nM 20% tracer: 80% unlabeled androstenedione (Steraloids, Wilton, NH) at 37 °C. Reactions were initiated with a generating system, as for 17, 20 lyase activities (10 μl each of 17 mM glucose-6-phosphate, 1 mM NADPH, and 2 mM β-NADP+, and 1 μl of 1 U glucose-6-phosphate dehydrogenase; Sigma, St. Louis, MO). Additional reactions included the triazol Letrozole as a negative control to verify that the activities measured were specifically inhibited, and the sensitivity of the hyena and human placental enzymes was compared at various log dilutions (30 pM–300 nM). Reactions were stopped after 1–2 h by the addition of 30% cold TCA.

2.4. Western immunoblot

Relative level of aromatase expression was quantified by western immunoblot. Microsomal proteins were denatured and separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). From each placenta, 40 μg of microsomal protein were loaded on to gels run in an electrode buffer (50 mM Tris, 383 nM glycine, 0.1% SDS, and 0.4 mM EDTA) for 110 min at 150 V. Separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA) at 20 V for 17 h in transfer buffer (20% methanol, 20 mM Tris, 150 nM glycine). Membranes were blocked for 30 min in 20% dried milk dissolved in 0.5 M PBS with 0.1% Tween20® prior to incubation with primary rabbit antisera for 1 h at room temperature. Primary anti-aromatase antisera (rabbit antisera raised against recombinant human P450arom, courtesy Dr. N. Harada, Fujita Health University, Aichi, Japan) was diluted 1:2000 in PBS with 0.1% Tween20®. Membranes were incubated for 30 min at room temperature with donkey antirabbit IgG linked to horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) diluted 1:10,000. Immunoreactive bands were visualized by autoradiographic detection of chemiluminescence (Western Lighting, Perkin Elmer, Boston, MA). Purified recombinant human P450arom, generated as previously described [20], was used as a positive control.

2.5. Immunohistochemistry

Aromatase expression was localized to specific cell types of fixed tissue by immunohistochemistry. Immunolocalization was performed using an avidin-biotin-peroxide complex method with a VECTASTAIN Elite ABC rabbit IgG kit (Vector Laboratories, Burlingame, CA). Tissue sections were deparaffinized using CitriSolv™ (Fisher HealthCare, Houston, TX) and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked to reduce background staining by incubating tissue sections in 0.3% hydrogen peroxide:-methanol for 30 min. Sections then were washed in buffer (0.1 M phosphate buffered saline, pH 7.2 with 0.3% Triton X®) for 5 min. Aromatase immunostaining was enhanced by antigen retrieval as follows: sections were placed in Copeland jars containing 50 ml of 10X antigen unmasking solution (Vector Labs, Inc, Burlingame, CA), diluted to 1X in deionized water (final pH 6.0), and internal jar temperature was continuously monitored. Sealed jars were placed in a Presto® rice steamer heated to 95 °C for 25 min, cooled to room temperature, and rinsed in buffer. Non-specific staining was blocked by incubating sections with 1.5% normal serum for 20 min. Tissue sections were

incubated with the same primary antisera as used for immunoblot analysis at 4 °C for 16 h. Normal serum was substituted for the primary antibody in negative controls. Sections were incubated for 30 min with biotinylated secondary antibody, then 30 min with the avidin-biotin complex, visualized using an AEC (3-amino-9-ethylcarbazole) substrate (Vector Laboratories, Burlingame, CA), and counterstained with Mayer hematoxylin. Stained sections were mounted with an aqueous mounting media and covered.

2.6. Cloning hyena P450arom

Total RNA was isolated from hyena placenta (GD 65) with TRIzol Reagent (Invitrogen, Carlsbad, CA) followed by purification of mRNA (PolyAPurist, Ambion, Austin, TX). Messenger RNA (1 μg) was transcribed with 200 units M-MLV reverse transcriptase (Promega, Madison, WI) primed from Oligo dT (BD Biosciences Clonetech, Palo Alto, CA). A fragment (bp 554–1342) was amplified using 1.25 units Pfu polymerase (Stratagene), at 55 °C annealing temperature and forward (5′-GGACGTGTTGACCCTCATG-3′) and reverse (5′-TTCATCATGACCATGGCGAT-3′) primers designed on regions of homology between pig and mouse P450arom. This fragment was column purified, directly sequenced and used subsequently to design hyena P450arom specific internal primers [h696 (5′-GTCTGGTTTGAGAAGGAGAGCTTGCCACGC-3′) and h1136 (5′-GGATGATGTCATCGATGGCTATCCA-3′)]. Additional oligonucleotides were designed to prime outside the coding region at the 5′ (5′-CTGAGATCAAGCAACACAAGATG-3′) and 3′ (5′-GTGATTCAAGCTT TCTTTA-3′) ends. The 5′ primer was coupled with h696, the 3′ primer with h1136, in reactions with 2.5 units Taq polymerase (Perkin Elmer, Wellesley, MA) at 50 °C annealing temp. The resulting amplicons were subcloned into a shuttle vector pCR2.1-TOPO (Invitrogen), plasmid DNA was isolated, sequenced and the complete coding sequence was assembled.

2.7. Statistics and phylogenetic analysis

Placental microsomal aromatase activity was analyzed by regression against day of gestation for linear, polynomial or logarithmic associations, and Letrozole inhibition was tested by analysis of variance. Amino acid sequences were aligned using the Clustal W program [21]. In phylogenetic analyses, any site where the alignment postulated a gap in any sequence was excluded from all comparisons so that a comparable set of sites was used for each comparison. Phylogenetic trees of amino acid sequences were constructed by the following methods: (1) the neighbor-joining (NJ) method [22] based on the JTT distance [23]; (2) the maximum parsimony (MP) method [24]; (3) the quartet maximum likelihood method [25], using the JTT model; and (4) the Bayesian method [26] using the JTT + Γ model. The reliability of clustering patterns in NJ and MP trees was assessed by bootstrapping [27]; 1000 bootstrap pseudo-samples were used.

3. Results

Placental aromatase activity appeared to increase from about 250 pmol/mg/h in the GD 31 and 35 placentas, to averages of >700 pmol/mg/h in the GD 45 and 65 samples (Fig. 1A). A single sample from a GD 95 placenta measured less than 558 pmol/mg/h. Placentas from GD 35, 45 and 65 were from single pregnancies carrying twins and triplets, and all were mixed sex. There were no apparent differences in aromatase activities measured between placentas from male and female fetuses (Fig. 1B). Regression analysis using a second order polynomial gave the tightest fit of the available data ($y = -0.535x^2 + 71.925x - 1483.8$, $R^2 = 0.72$, $P < 0.05$). Aromatase activity in human term placental microsomes was 1777 ± 9 pmol/mg/h ($n = 3$) by comparison, and all measured aromatase activities were inhibited by Letrozole in a concentration dependent fashion. However, hyena placental aromatase

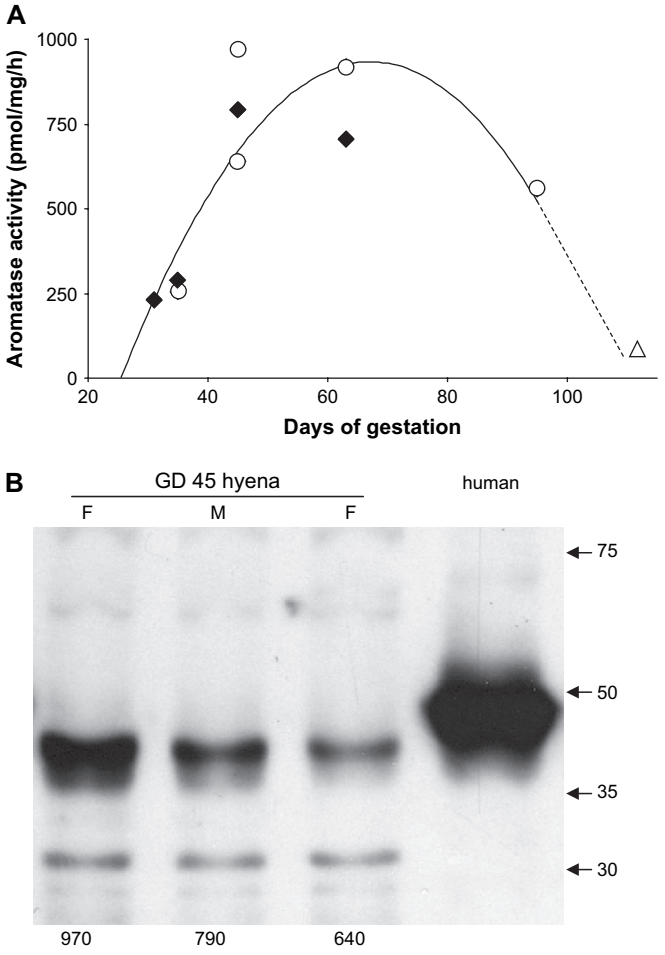

Fig. 1. Expression of aromatase cytochroem P450 (P450arom) in hyena placental microsomes. **A.** Aromatase activity (pmol/mg/h) in male (◆) and female (○) hyena placental microsomes at various stages of gestation. Procedures for microsomal protein isolation and enzyme activities from samples obtained at gestation days 31, 35, 45, 65 and 95 were as described in Section 2. Term placental enzyme activity (△) was calculated from values obtained for human term placental *microsomes* found in this study, using the fold differences between activities in hyena and human term placental *homogenates* previously determined by Yalcinkaya et al. [8]. Polynomial regression generated the fitted curve shown, extrapolated to term (dashed line) based on the predicted value. **B.** Immunoblot analysis of P450arom expression in placental microsomes from triplet GD 45 hyena fetuses (25 μg/lane) alongside human term placenta (2.5 μg). The measured aromatase activities (pmol/mg/h) for these three placentas are shown below each lane, and the sex of each fetus (M, F) is indicated above. Molecular size (kD) standards are shown to the right.

activity was more sensitive to Letrozole-induced inhibition than was human enzyme activity (Fig. 2; $P < 0.05$). The difference in Letrozole concentrations that induced 50% inhibition of vehicle control activity was six-fold higher for human (8.9 nM) than for hyena (1.4 nM) placental microsomes ($P < 0.05$).

Immunoblot analysis verified that antisera raised against human recombinant P450arom detected a prominent band of about 45 kD molecular size, lower than that of human placental P450arom (Fig. 1B). The intensity of this band corresponded well with the measured activities in microsomes

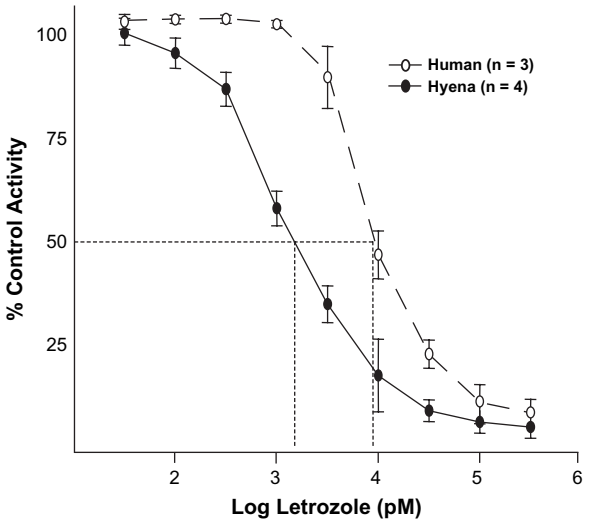

Fig. 2. Comparison of aromatase inhibitory potency of Letrozole in hyena and human placental microsomes. Inhibition of hyena (GD 65) and human term placental microsomal activity was determined at several (log) concentrations of Letrozole.

from the same fetuses (Fig. 1B). Immuno-histochemistry conducted on fixed tissues localized expression to the cytoplasm of all cells stained (Fig. 3), but the level of expression varied with stage of gestation, as expected from activity and western immunoblot analyses. At GD 31 and 35, the expression of P450arom was barely detectable in syncytial cells between the secondary and tertiary fetal villi (Fig. 3A), but was more prominent in occasional giant cells in or near the junctional zone (data not shown). P450arom expression was higher and more readily detectable in the syncytiotrophoblast of placentas at GD 45 (Fig. 3B). Giant cell expression also increased such that the cytoplasmic staining defined their cellular limits from the syncytium in some cases (Fig. 3B). Similar expression was maintained in samples at GD 65, and was still diffusely distributed throughout the placenta without any obvious regionalization (Fig. 3C). Cytotrophoblast was distinguished by its lack of P450arom expression at all stages.

Transcript isolated from a GD 65 placenta was used as template for RT-PCR using conserved primers. Amplification yielded an amplicon of expected size, and a near full-length sequence was subsequently cloned. This cDNA encoding hyena P450arom based on blast analysis, encoding a putative 503 amino acid protein which exhibited 94% and 86% identity to canine and human P450arom, respectively, at the amino acid level. Phylogenetic analysis clustered hyena and canine P450arom as a sister group to the cetartiodactyla (Fig. 4) with high bootstrap support.

4. Discussion

In contrast to perceptions from earlier reports, results of the present study indicate that, at the time of phallic differentiation, the placenta of the spotted hyena expresses P450arom at an impressive level. Adult female spotted hyenas not only have high androstenedione levels by usual mammalian standards, testosterone levels increase with [28] and during pregnancy [5]. The potential physiological impact of placental androgen metabolism on sexual differentiation will be most critical during the time of genital developmental, the first trimester of most mammals. Even at GD 31 and 35, aromatase activity exceeded 200 pmol/mg/h, not much below pig [29,30] and 100-fold higher than bovine placental microsomal activity [31], and increases thereafter. In fact, the period of genital differentiation coincides with a three-fold increase in hyena placental aromatization capacity, apparently reaching a peak around GD 45. These data are consistent with results of other studies. More estradiol than testosterone was synthesized from androstenedione infused into the uterus at GD 48 and 58, although testosterone predominated by GD 103. Even so, androgens in umbilical venous blood were still a fraction of those in uterine vein [32] suggesting that fetal exposure to maternal androgens was effectively reduced by the placenta. Thus, we propose that placental aromatase activity reduces trans-placental androgen exposure in spotted hyena fetuses during genital differentiation.

It may be equally significant that levels of placental aromatase activity may well decline as pregnancy advances from a peak around mid-gestation. Term placental material from hyenas (GD 110) was not available for this study and only a limited number of specimens were examined at each gestational stage. Therefore, conclusions relating to trends in placental aromatase activity during pregnancy must be appropriately cautious. Still, a decline of aromatase expression in hyena placenta in late gestation is indicated by the previously reported, progressive decrease in activities from GD 48–78 [32]. Furthermore, it is consistent with the very low levels (42 pmol/mg/h) measured in homogenates from several term hyena placentas [8]. Using data from Yalcinkaya et al. [8] for placental homogenates (hyena = 5% of human) to extrapolate from the activities in human term placental microsomal measured here, predicts a hyena term microsomal aromatase activity of ~80 pmol/mg/h. This estimate accords well with the regression curve fitted to the placental activities reported here, extrapolated from GD 95. However, maternal estrogens increase substantially from mid-gestation to a peak at about GD 80 and in proportion to testosterone levels [5], suggesting that substrate supply rather than placental aromatase activity limits placental estrogen synthesis up to the end of the second trimester. What impact this has on fetal steroid levels at any stage of gestation is unknown, and no data are available on fetal androgens or estrogens at term. If accurate, lower placental aromatase activities at term in spotted hyenas may be important in altering fetal exposure to androgens (and estrogens), initiating neural differentiation in late gestation that may affect post-natal behaviors [33].

Placental P450arom expression in mammals, when present, can provide a physiological barrier from, or buffer against, androgen exposure during development *in utero*. Female rat and mouse fetuses positioned between males *in utero* are also born masculinized compared to sibling fetuses next to one male or two females [34], perhaps because mice lack placental P450arom expression [35]. Conversely, virilization of female

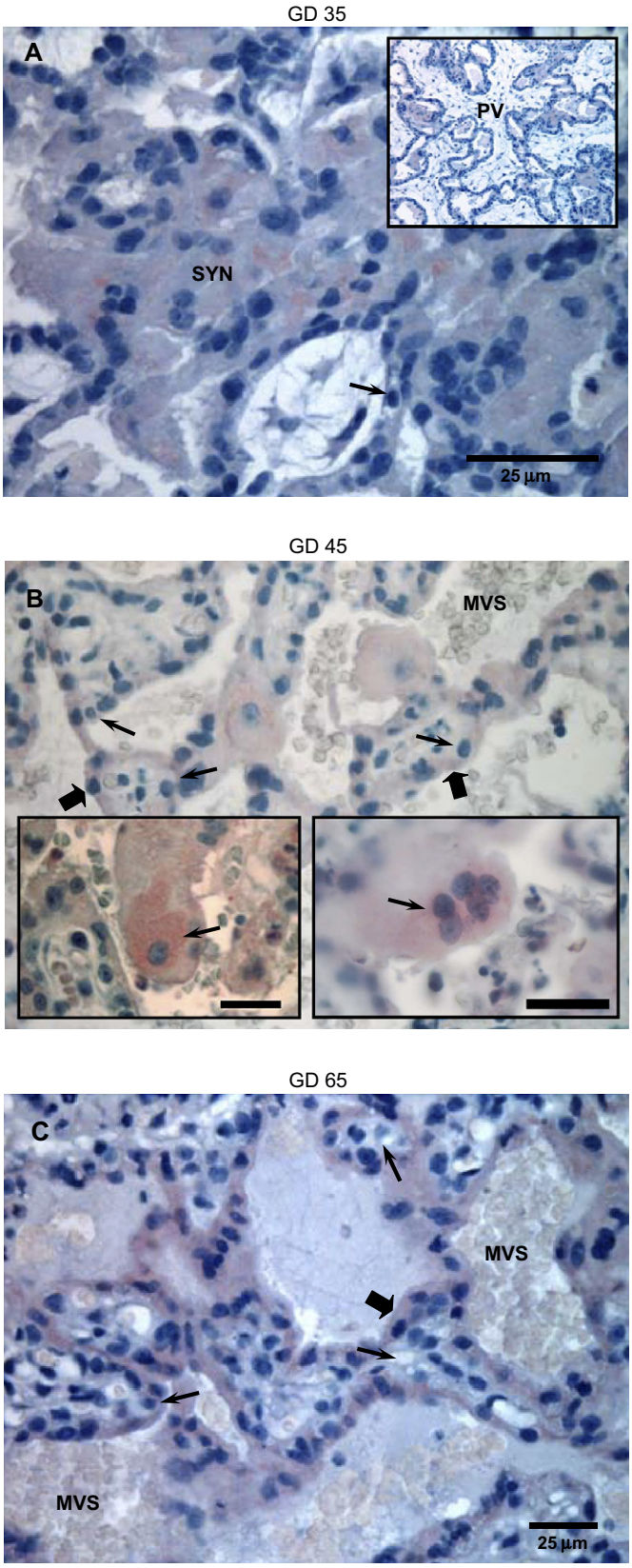

Fig. 3. Immuno-localization of expression of P450arom in hyena placenta. **A.** Gestational day (GD) 35; Expression (in red) was detected at low levels throughout the syncytiotrophoblast (SYN) forming around secondary and tertiary villi extending out from the primary villi (PV). Cytotrophoblast (arrow) can be occasionally be distinguished at the basement membrane of fetal villi by the presence of lipid inclusions. **B.** GD 45; Expression remains general throughout the syncytiotrophoblast (block arrows). Cytotrophoblast (small arrows) was sometimes distinguishable by its lack of cytoplasmic staining for P450arom. P450arom staining was most obvious in trophoblastic giant cells (lower left insert, arrow) and in trophoblast syncytium (lower right panel, arrow). The maternal vascular space (MVS) is indicated. Size bars are 25 µm **C.** GD 65; Expression remained prominent in syncytiotrophoblast (block arrows) at GD 65, but not in the cytotrophoblast (small arrows) which was sometimes surrounded by positive staining syncytiotrophoblast cytoplasm. Lipid accumulation remained obvious in some cytotrophoblast. Maternal vascular spaces (MVS) are marked.

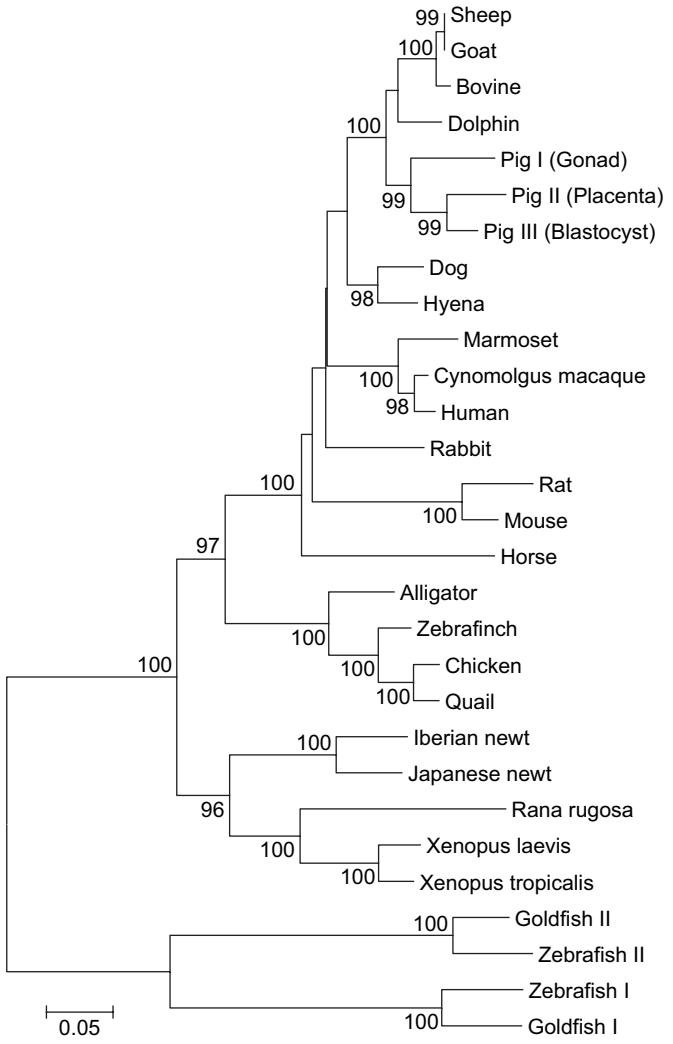

Fig. 4. Vertebrate aromatase phylogeny constructed by the neighbor-joining method based on the JTT distance with bootstrap support as indicated. Teleost sequences were used as an outgroup to root the tree. Phylogenies using MP, ML, and Bayesian methods yielded similar topologies (not shown).

piglets in male biased litters [36] may be more subtle because the porcine placenta does express P450arom [29]. Most convincingly, female infants genetically lacking P450arom expression are born severely virilized [37]. Thus, the placenta in humans and perhaps other mammals appears to insulate developing female fetuses from the effects of endogenous (fetal adrenal, male siblings *in utero*) androgens. Based on this premise, and low levels of placental aromatase activity in

hyena relative to human term placenta, Yalcinkaya et al. concluded that high maternal androgen concentrations, together with low P450arom expression, might explain masculinization of female genitalia of spotted hyenas [8]. The current data shown that aromatase activity in hyena placenta at GD 31 (233 pmol/mg/h) was not much less than the 368 pmol/mg/h measured in 12–18 week human placentas [38], and this is less than half peak activities in hyena samples at GD 45. Genital differentiation is complete in 12–14 week human fetuses [39]. Thus, levels of placental aromatase activity, within the range observed for spotted hyenas, are apparently adequate to sustain normal human female sexual development [40]. In other words, the levels of aromatase activity in the hyena placenta are substantial relative to other species like pigs and cattle, even humans, when compared at their peak, the time of gonadal differentiation. Therefore, rather than allowing androgenization as once suspected, P450arom expression may well protect female fetuses from undue masculinization in the spotted hyena as in other mammalian species.

The hemochorial character of the placenta of the spotted hyena is certainly unique among carnivores [15,41], but recent views [42] and phylogenetic analyses [43] of morphology suggest that hemochorial placentation may be an ancestral character in eutheria [44]. If so, other carnivores have derived endothelio-chorial placentation while the spotted hyena placenta has either retained it or secondarily reverted back to the ancestral state. To our knowledge, placental steroidogenesis has not been considered in this light, and is one aspect of placental function that certainly differs radically among taxa [45]. Whether or not P450arom expression and estrogen synthesis is a likely ancestral feature of the mammalian placenta, as the hemochorial type is thought to be, is unknown. It is of interest therefore that P450arom expression was localized in the syncytiotrophoblast of the hyena placenta, as it is in the hemochorial human placenta [46]. There are relatively few similar studies in other species unfortunately. However, P450arom is expressed in the binucleate cells of the bovine placenta [47] and giant cells in the placenta of camelids [48]. Although rodent placentas appear not to express P450arom [35], they do synthesize androgens, apparently by virtue of enzymes expressed in the trophoblastic giant cells [49]. P450arom expression in the hyena placenta was also observed in giant cells, evidence consistent with a trophoblastic origin of these cells. Giant cells are not observed in placentas of other carnivores, with the possible exception of the cat [50], nor is there any evidence of placental estrogen synthesis or P450arom expression in dogs [51]. Phylogenetic analysis suggests a closer relationship between the hyenidae and the felidae than the canidae [52], but there are no available data on feline placental P450arom expression. If the appearance of giant cells in the placenta of spotted hyenas and cats represents a vestige of this ancestry, then trophoblastic estrogen synthesis must have been lost in deriving the more usual (for carnivores), endothelio-chorial placenta of dogs at least.

There is no doubt that mammalian aromatases are highly homologous both functionally and structurally [6,53]. Not surprisingly, this is particularly evident within orders. The amino acid sequence of the hyena P450arom is no exception, being 94% identical with the canine P450arom coding sequence. No data are available for feline P450arom at this time. Peptide sequence of P450arom is 92% identical between rat and mouse, 94% between human and marmoset, 98% between bovine and ovine, and 100% between ovine and caprine enzymes, all of which are 503 amino acid peptides. Hyena P450arom runs at a lower molecular size than human P450arom which is known to be glycosylated [54]. In this regard, hyena P450arom is more similar to porcine gonadal and placental P450arom enzymes, both of which run at around 45 kD and neither of which are glycosylated, despite the presence of conventional glycosylation signal sequences [20]. The phylogeny of vertebrate P450arom coding sequences reported here is very similar to a careful and thorough analysis recently published [53]. It may be that the evolution and adaptive function of P450arom in mammals results more from differences in tissue-specific expression levels than from radical changes in the catalytic characteristics of the enzyme. Aromatase expression in hyena placenta, but its absence from canine placenta, is a case in point. Still, the potential for functional evolution remains even if not reflected by molecular analysis, and despite undoubtedly strong selection pressure, particularly after gene duplication. We have shown a substantial functional difference exists between two of the three paralogues of porcine P450arom, in turnover rates, novel hydroxylation products and sensitivity to inhibitors [16,19,20,29,30,55]. The hyena P450arom was clearly more sensitive to Letrozole than was human placental aromatase activity, suggesting a functional divergence at some level. Perhaps the unusually high androgen load experienced by the female hyena has driven the evolution of catalytic variance in P450arom in this carnivore, but this will require careful examination of purified enzyme protein in future studies. It will be of additional interest to examine placental morphology of other hyaenids, and other carnivore species, and whether or not they exhibit P450arom expression or have adopted other pathways for androgen metabolism. Nevertheless, the current results suggest that Letrozole will be an effective tool for further investigating the role of P450arom in anatomical and behavioral sexual differentiation of the spotted hyena.

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