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Evidence of steroid hormone activity in the chorioallantoic membrane of a Turtle (*Pseudemys nelsoni*)

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ABSTRACT

Endocrine properties of extraembryonic membranes have traditionally been viewed as a characteristic of placental amniotes. However, our laboratory recently demonstrated that this ability extends to the extraembryonic membranes of two oviparous amniotes (chicken and alligator) indicating that endocrine extraembryonic membranes are not an innovation of placental amniotes and suggesting that this could be a shared amniote characteristic. In this study, we test our hypothesis that the chorioallantoic membrane (CAM) obtained from non-archosaurian obligate oviparous amniotes such as turtles, have the potential for steroid hormone activity. To investigate synthesis of a major placental hormone, we performed explant culture and found that the turtle CAM synthesizes progesterone *in vitro* in the presence of a steroid precursor. In addition, to examine whether the CAM has the ability to respond to steroid signaling, we quantified mRNA expression of the progesterone, androgen, and two estrogen receptors. Finally, to determine if steroid receptor mRNA is translated to protein, we performed immunolocalization of the progesterone receptor. Our data demonstrate that the turtle CAM exhibits steroid synthesis and has steroid hormone signaling capabilities. To that end, steroid hormone activity has now been demonstrated in the CAMs of three oviparous species that represent three independent lineages within oviparous Reptilia that have never exhibited viviparity; thus these data support our hypothesis that endocrine activity of extraembryonic membranes is a conserved trait of Amniota.

1. Introduction

The formation of a single extraembryonic membrane during development, the yolk sac, characterizes the ancestral condition of vertebrates (Mess et al., 2003; Mossman, 1987). In addition to the yolk sac, the amniote (reptile, bird and mammal) ancestral condition is characterized by the formation of three additional extraembryonic membranes, the amnion, chorion and allantois (Mess et al., 2003). These extraembryonic membranes of the amniote egg gave rise to the placenta, which Mossman (1987) described as an apposition of extraembryonic membranes to uterine tissues "for the purpose of physiological exchange" (Mossman, 1987). Thus, it is not surprising that these membranes function in a similar manner to meet the respiratory, water and energy requirements of the developing embryo in both oviparous (egg-laying) and viviparous (live-bearing) amniotes (Mossman, 1987). Of the four extraembryonic membranes, the chorion and allantois fuse early in embryonic development to form the chorioallantoic membrane (CAM) in oviparous amniotes and this CAM in combination with maternal decidua forms the chorioallantoic placenta in viviparous amniotes (Cross et al., 2003). Both the CAM and the chorioallantoic placenta are highly vascularized structures and perform respiratory functions by providing gas exchange between the developing embryo and its external environment (Mess and Carter, 2007; Romanoff, 1960), i.e., the nest environment in oviparous species or the uterine environment in viviparous species.

We have previously stated (Albergotti and Guillette, 2011; Albergotti et al., 2009) that this conservation of basic functions of amniote extraembryonic membranes indicates that specialization of these membranes is not essential in the transition from egg-laying to live-bearing, offering a possible explanation for why most viviparous squamates (lizards and snakes) demonstrate fairly simple chorioallantoic placentae that appears to primarily serve as a respiratory organ. The CAM and chorioallantoic placenta are organs of transport, not only of gases, but also of water and other inorganic ions essential for embryonic development, such as

Abbreviations: CAM, Chorioallantoic membrane.

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calcium, potassium and phosphorus (Stewart and Terepka, 1969, as reviewed in Munro et al. (1983)). In some viviparous species, the chorioallantoic placenta takes on additional roles in the transfer of nutrients, and in the most complex placental types will transport all or the majority of nutrients the embryo requires for development (as reviewed in Blackburn (1992), Munro et al. (1983)).

Still another transport function of the chorioallantoic placenta involves the transfer of steroid hormones between maternal and fetal environments (Levitz, 1966). Yet, the chorioallantoic placenta not only transports steroid hormones, but also synthesizes and responds to an array of hormones critical for embryonic development and survival (Petraglia et al., 1996). For example, placental progesterone (P_4) is important in promoting uterine quiescence and maintaining pregnancy in guinea pigs and primates including humans (Pieber et al., 2001), initiating parturition in most mammals studied to date (Mesiano, 2001, Mesiano et al., 2002), and supporting fetal (Mark et al., 2006) and placental growth (Jojovic et al., 1998; Ogle et al., 1990) in rodents. Placental P_4 synthesis is not an exclusive characteristic of eutherian mammals as the ability to synthesize P_4 has been demonstrated in even the simplest chorioallantoic placenta of a viviparous lizard (Painter and Moore, 2005), which is simply an apposition of the CAM to the uterine epithelium without any anatomical specializations in either structure (Guillette et al., 1981). Further, placental P_4 synthesis is not an exclusive characteristic of the maternal contribution to the placenta and it is now evident that maternal and extraembryonic tissues alike contribute to steroidogenesis in mammalian (Giannoulias et al., 2005; Pasqualini, 2005; Petraglia et al., 1996) and lizard (Girling and Jones, 2003) placentae. Therefore, we hypothesized that CAMs of oviparous amniotes, sharing conserved basic functions and evolutionary history, could also perform steroidogenesis and respond to steroid signaling (Albergotti and Guillette, 2011; Albergotti et al., 2009; Cruze et al., 2012).

Albergotti et al. demonstrated that the CAM of one oviparous amniote, the chicken, shares the capability of chorioallantoic placentae to synthesize and respond to the signaling of P_4 (Albergotti et al., 2009). The chicken CAM exhibited mRNA expression of cytochrome P450 11A1 (CYP11A1) and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1), two steroidogenic enzymes involved in P_4 biosynthesis, was capable of *in vitro* P_4 synthesis, and exhibited mRNA and protein expression of the progesterone receptor (PR) detected via real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC), which indicated that steroidogenic and steroid responsive extraembryonic membranes are not exclusive characteristics of viviparous amniotes (Albergotti et al., 2009). In addition, we have demonstrated that the CAM of another oviparous amniote, the American alligator (*Alligator mississippiensis*), exhibits mRNA expression of HSD3B1, cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1) and cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), the steroidogenic enzymes critical in the synthesis of progestins, androgens and estrogens, respectively. Likewise, mRNA and protein expression of the PR and estrogen receptor 1 (ESR1) were demonstrated with RT-qPCR and IHC (Cruze et al., 2012). These studies provide evidence that the oviparous CAM of archosaurs shares the capability to synthesize steroid hormones at the molecular level and respond to steroid hormone signaling at the molecular and protein level and supports our hypothesis that steroidogenesis and steroid hormone signaling of extraembryonic membranes could be an evolutionary conserved characteristic of amniotes.

To further examine our hypothesis, we moved beyond oviparous archosaurs and investigated the potential steroid hormone activity in the CAM of a turtle, the red-belly slider (*Pseudemys nelsoni*). The placement of turtles in the amniote phylogeny remains unresolved (Zardoya and Meyer, 2001). However, birds, crocodilians, turtles (Blackburn, 1999) and the tuatara (Cree et al., 1996) represent the main lineages of extant amniotes that reproduce strictly by oviparity and the aim of our study was to investigate a third branch of the oviparous amniote phylogeny in an attempt to uncover a potentially conserved trait of amniote extraembryonic membranes. Here, we examined the capability of the turtle CAM to perform steroid synthesis and respond to steroid hormone signaling. We hypothesized that the turtle CAM has similar steroid biosynthesis properties as the chorioallantoic placenta, and therefore should synthesize hallmark placental hormones, such as P_4. To investigate steroid hormone synthesis, we examined the ability of the turtle CAM to perform *in vitro* P_4 synthesis. Likewise, we hypothesized that the turtle CAM has similar steroid signaling properties as the chorioallantoic placenta, and thus should have the capability to respond to signaling through steroid receptors. To investigate steroid hormone signaling, we examined mRNA expression coding for PR, androgen receptor (AR), ESR1 and estrogen receptor β (ESR2) in turtle CAM, which are the key receptors responding to the signaling of progestins, androgens, and estrogens, respectively. In addition, to determine if steroid receptor mRNA was translated to protein, immunolocalization of the PR was examined.

2. Materials and methods

Annual reproduction and nesting of the Florida red-belly turtle roughly coincides with that of the American alligator. It has been reported by Enge et al. (2000) and observed by our team that *P. nelsoni* routinely lay their eggs in alligator nest mounds. During the summers of 2007, 2008 and 2009, turtle eggs were collected from alligator nests located within Lake Woodruff National Wildlife Refuge (Deland, Florida) and transported to the University of Florida for incubation. Within 48 h of arrival, embryos from one or two eggs per clutch were used to determine the average embryonic stage of the clutch based on criteria described by Yntema (1968). Eggs from each clutch were incubated at a female producing temperature (FPT) of 30 °C (Ewert et al., 2004).

2.1. In vitro explant culture and radioimmunoassay

In 2008, CAMs were collected at embryonic stages 23–24. CAM explants were cut to approximately 0.1 g wet weight (mean = 0.108 g ± 0.001SEM) and were incubated at 30 °C on an orbital shaker in phenol red-free L-15 culture media (Gibco) either with (n = 18) or without (n = 21) cholesterol and cAMP as precursor. Precursor solutions and concentrations are based on (King et al., 2004). For cholesterol, 22(R)-hydroxycholesterol (Sigma) was dissolved in 95% ethanol (Fisher) to a final concentration of 10 μg/mL and combined with 1 mM dibutyryl cAMP (Sigma). After 8 h of incubation, concentration of progesterone in the culture media was quantified by solid phase radioimmunoassay as previously reported (McCoy et al., 2008). The P_4 antibody was produced by Fitzgerald Industries (PR-20) and the detectable assay range was 7.8–8000 pg/ml. To determine background of the P_4 assay, controls consisting of only cholesterol and cAMP (absence of CAM) were incubated for 8 h. In the absence of CAM, all samples were below the limit of detection of the assay (lowest limit of detection was 7.8 pg/ml). The intra-assay coefficient of variation for the P_4 assay was 10% and the inter-assay coefficient of variation was 13%. The following cross-reactivities are reported from Fitzgerald; Pregnenolone: <50.0%, Dehydroepiandrosterone: <0.03%, Hydrocortisone: <0.03%, Prednisone: <0.3%, 4-Androstenedione: <0.3%, Corticosterone: <0.4%, Spiranolactone: <0.3%, Cortisone: <0.15%, 11-Deoxycortisol: <1.0%.