Published in final edited form as:

Mol Phylogenet Evol. 2017 December ; 117: 2–9. doi:10.1016/j.ympev.2017.09.007.

Ancestral resurrection of anthropoid estrogen receptor β demonstrates functional consequences of positive selection

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Data Deposition

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KP828800

Competing financial interests

The authors declare no competing financial interests.

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Abstract

Anthropoid primates arose during the Eocene approximately 55 million years ago (mya), and extant anthropoids share a most recent common ancestor ~40 mya. Paleontology has been very successful at describing the morphological phenotypes of extinct anthropoids. Less well understood is the molecular biology of these extinct species as well as the phenotypic consequences of evolutionary variation in their genomes. Here we resurrect the most recent common ancestral anthropoid estrogen receptor β gene (*ESR2*) and demonstrate that the function of this ancestral estrogen receptor has been maintained during human descent but was altered during early New World monkey (NWM) evolution by becoming a more potent transcriptional activator. We tested hypotheses of adaptive evolution in the protein coding sequences of *ESR2*, and determined that *ESR2* evolved via episodic positive selection on the NWM stem lineage. We separately co-transfected *ESR2* constructs for human, NWM, and the anthropoid ancestor along with reporter gene vectors and performed hormone binding dose response experiments that measure transactivation activity. We found the transactivation potentials of the ancestral and human sequences to be significantly lower ($p < 0.0001$ in each comparison) than that of the NWM when treated with estradiol, the most prevalent estrogen. We conclude the difference in fold activation is due to positive selection in the NWM ER β ligand binding domain. Our study validates inferential methods for detecting adaptive evolution that predict functional consequences of nucleotide substitutions and points a way toward examining the functional consequences of positive Darwinian selection.

Graphical Abstract

Keywords

Ateles fusciceps; adaptive evolution; New World monkey; Old World monkey; exogenous; endogenous

1. Introduction

1.1 Anthropoid Origins

Great advances have been made in reconstructing early anthropoid origins. For example, the fossil remains of *Eosimias*, an early Asian anthropoid primate from the middle Eocene (48–40 mya) of China, is considered among the earliest representatives of Anthropoidea (Gebo et al., 2000), a clade whose extant members consist of apes (including humans), Old World monkeys (OWM), and New World monkeys (NWM) (Figure 1). Other early anthropoid remains have been found in the Fayum Province of Egypt, e.g., *Catopithecus*, which is believed to be a stem catarrhine that lived in Africa more than 34 mya (Kay, 2004). However, large gaps in fossil evidence and variations in interpretation still exist in the literature on anthropoid primate evolution, creating controversy especially concerning the means, origin and timing of their arrival to the New World. The recent discovery of *Perupithecus*, the earliest primate fossil in South America to date, places New World monkey ancestors in South America approximately 36 mya and supports an African origin of anthropoids in the New World (Bond et al., 2015). Morphology alone, while indispensable in reconstructing evolutionary history, does not provide information about the soft anatomy, physiology, and molecules that once existed within these and other extinct anthropoid species.

1.2 Hormone/Receptor Interactions

Estrogen receptors (ER α and ER β) are steroid receptors within the nuclear receptor (NR) superfamily, which, in humans, contains 49 extant members. The most ancient NR was also a steroid receptor and was most similar to extant ER α , with the remainder of the family, which includes ER β , mineralocorticoid, glucocorticoid, androgen, and progesterone receptors arising as the result of a series of gene duplications (Thornton, 2001; Thornton et al., 2003). The ERs bind circulating estrogens and have adaptively evolved to exploit physiological control over reproductive functions (Thornton, 2001). While several splice variants of each of the extant estrogen receptors have been described, our study focuses on the two most well characterized human isoforms (NM_001122742.1 and NM_001437.2). In humans, the genes that encode ER α (*ESR1*) and ER β (*ESR2*) are on chromosomes 6 and 14, respectively. Structurally, NRs contain five domains (Figure 2): N-terminal, DNA binding (DBD), hinge, ligand binding (LBD) and C-terminal. ER α is composed of 595 total amino acids, whereas ER β has 530 total amino acid residues with 196 residues in the LBD (Pavao and Traish, 2001; Zhao et al., 2008). Overall sequence homology between the two receptors is 47%, and 59% within the LBD alone (Barkhem et al., 1998; Enmark et al., 1997).

Estrogens that bind to these receptors are categorized as endogenous steroid hormones, meaning they are synthesized within an organism (Pike et al., 1999; Wasserman et al., 2012). Additionally, exogenous compounds such as phyto- and xeno-estrogens can bind to ERs. Upon binding of an estrogen to ER α or ER β , the receptor homodimerizes, permitting stimulative and/or repressive transcriptional control via binding to estrogen response elements (EREs) of target genes (Mosselman et al., 1996). The function of the two receptors is complicated by their ability to also act as heterodimers (sometimes composed of two different isoforms of either ER α or ER β , or one ER α and one ER β) upon binding of a hormone (Ogawa et al., 1998; Zhao et al., 2008). This enables estrogens to act as strict agonists (e.g. 17 β -estradiol), pure antagonists (e.g. ICI 162,780) or as selective ER modulators (SERMs; e.g. genistein), providing a mechanism of negative feedback helping to regulate the levels of the receptors in the system (Hall and McDonnell, 1999; Pike et al., 1999; Wasserman et al., 2012). Estrogen-like compounds produced exogenously, compete with endogenous estrogens for binding to ER β and, to a lesser extent, ER α (Barkhem et al., 1998; Kuiper et al., 1997). These include phthalates, endocrine disrupting chemicals (e.g. bisphenol A), used extensively in the plastic industry, and phytoestrogens (e.g. genistein) derived from soybeans (Jiang et al., 2013; Swedenborg et al., 2009). These interactions can cause paradoxical effects and may vary within different cell types of the same tissue (Barkhem et al., 1998).

1.3 Adaptive Evolution

Nuclear receptors were present in the common ancestor of vertebrates, and they have diversified in vertebrate lineages over hundreds of millions of years through gene duplication (Thornton, 2001). Adaptive evolution, detectable as an increase in non-synonymous nucleotide substitutions on a particular lineage above that which is expected during purifying or neutral evolution, is used as a measure of natural selection and is thought to coincide with an adaptive event (Yang, 2002). Such adaptations may increase the fitness

of the species. Detecting adaptive evolution has become a common practice in recent years, but the functional consequence of the nucleotide substitution is not often pursued (a few exceptions: (Chang et al., 2002; Jermann et al., 1995; Thornton, 2001)). As such, the selective pressure(s) that favored the non-synonymous substitutions are often unclear.

1.4 Physiological Effect

Adaptive evolution within the nuclear receptor family is believed to have allowed for exploitation of hormones resulting in an increase in control over physiological functions (Thornton, 2001). In extant anthropoid primates, the action of hormones greatly affects the development of key phenotypes and behavior (i.e. genital morphology, pregnancy, aging, aggression (Bonney et al., 1979). Whereas toxicological studies typically examine chemical effects at high concentrations (nM- μ M range), endogenous hormones are effective at much lower concentrations (pM) (Welshons et al., 2003). Studies aimed at determining the ovarian cycle of NWMs have uncovered higher circulating concentrations of steroid hormones including estradiol, the most prevalent estrogen, in these primates when compared to other anthropoid primates (Chrousos et al., 1984; Wolf et al., 1977). In NWMs, circulating estradiol levels peak at a mean concentration of 1,200 pg/ml, more than 10 times the peak level in humans (104.2 ± 11.0 pg/ml) (Chrousos et al., 1984; Romani et al., 2003; Vandenberg et al., 2012; Wolf et al., 1977). Work by Welshons *et al* has shown that at these relatively low dose concentrations, a 10-fold increase can generate significant biological effects in relation to receptor occupancy not observed at, or accurately extrapolated from, higher concentrations (a 9-fold increase at low concentrations versus a 1.1-fold increase at higher concentrations) (Vandenberg et al., 2012; Welshons et al., 2003). This is due to the ability of hormones to respond nonmonotonically whereby the slope of the dose-response curve can change signs along the range of doses tested. Intrigued by the higher circulating steroid hormone concentration in NWM we explore the functional consequence of this adaptation in NWM, by examining the evolution of ER α and ER β . Estrogen receptor β is known to function as a transcription factor in a wide range of developmental and neurological processes, and it plays a role in many cancers including breast, ovarian, endometrial, and colon cancers where overexpression has been associated with colon tumor progression (Janakiram et al., 2013). Notably, approximately 35% of captive cotton-top tamarins develop fatal colon adenocarcinomas (Brandon et al., 1989; Lowenstine, 2003). We took a retroactive approach, using sequence data from extant species to reconstruct and resurrect the ancestral crown anthropoid (ACA) ER β , which existed approximately 55–40 mya, eventually giving rise to present-day NWM and human ER β s (Figure 1) (Fleagle, 2002; Jameson Kiesling et al., 2014; MacFadden, 1990; Schrago and Russo, 2003; Wolf et al., 1977). Our inferential approach allowed us to study of the function of the ancestral receptor in the absence of the ancient molecules (hormones and their receptors), which are not preserved in fossils. We hypothesized that adaptive changes detected in the DNA sequence will have functionally altered ER β , and may coincide with environmental changes.

2. Materials and Methods

2.1 DNA sequencing and alignment

The coding regions of *ESR1* and *ESR2* were amplified from genomic DNA using polymerase chain reaction (PCR) in the following species: *Pan paniscus*, *Gorilla gorilla*, *Pongo pygmaeus*, *Hylobates lar*, *Papio anubis*, *Chlorocebus aethiops*, *Colobus guereza*, *Trachypithecus obscurus*, *Cebus apella*, *Ateles paniscus*, *Ateles fusciceps* (*ESR2* only), *Pithecia pithecia* (*ESR2* only), *Callicebus donacophilus* (*ESR2* only), and *Lemur catta* (*ESR2* only). Primer sequences are provided in supplementary table 1. Standard PCR cycling conditions were employed (Chen et al., 2008), and PCR products were sequenced using an ABI 3730 DNA Analyzer. In addition, the coding sequences of *ESR1* and *ESR2* were downloaded from publicly available databases for the following species: *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, and *Canis familiaris*. Accession numbers are provided in supplementary table 2. DNA sequences were aligned in MEGA4 using the ClustalW algorithm with amino acid sequences as a guide (Tamura et al., 2007). The alignment files are provided in supplementary documents 1 (*ESR1*) and 2 (*ESR2*).

2.2 Comparative genomics

Maximum likelihood analyses of *ESR1* and *ESR2* were performed using CODEML in the PAML4 software package (Yang, 2007). Omega (ω) values were calculated using a branch model and a branch-site model A (Zhang et al., 2005) based on the well established NWM phylogeny adapted from Perelman (Opazo et al., 2006; Perelman et al., 2011; Wildman et al., 2009; Zhang et al., 2005). The ratio of the rate of non-synonymous substitution (nucleotide substitutions resulting in new amino acids; dN) to the rate of synonymous substitution (nucleotide substitutions in which encoded amino acids remain unchanged; dS), represented by ω , can be used to measure the strength of selection acting on a protein coding gene, with $\omega < 1$ representing purifying selection, $\omega = 1$ representing neutral evolution, and $\omega > 1$ implying positive selection (Yang, 2002). These methods suggest that synonymous substitutions are generally more neutrally evolving than non-synonymous substitutions, which are more subject to the force of natural selection (Yang, 2002). We compared the free-ratio model to a one-ratio model to test whether there was ω variation along different branches in the tree. We also implemented three nested branch-models, a one ω model (all branches had the same ω), a two ω model (Figure 1, left), and a three ω model (Figure 1, right), to test for patterns of selection in NWMs (supplementary table 3). Using a likelihood ratio test (LRT), which tests nested models, the three models were tested against each other. The two ω model used the NWM stem branch and crown lineages as the foreground and all other branches as the background (Figure 1, branch tested indicated in red). The three ω model specifies the NWM stem branch and NWM crown lineages as independent foreground lineages and all other branches as the background lineage (Figure 1, branch tested indicated in blue). We separated the NWM stem branch from the other NWMs to detect whether there was a distinct signal of selection after the initial divergence of NWM from other primates. We predict that this branch will have a different ω , as divergent selection will potentially be strongest on this branch, followed by stabilizing selection to maintain any new variations in NWMs.

The branch-site model A was used to test for positively selected sites along specific lineages in the ligand binding domain of *ESR2*. LRT was used to compare the null model (fixed $\omega_2=1$) with model A that allows $\omega_2>1$ in the foreground lineage (Zhang et al., 2005). Bayes Empirical Bayes (BEB) (Yang et al., 2005), was used to detect positively selected sites. We used the Bonferroni correction procedure for multiple tests in order to reduce the false discovery rate as recommended by Yang (Yang, 2007). The Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. (dbSNP Build ID: GRCh38 Annotation Release 106) was used to determine if any of the positively selected sites were SNPs in humans, and further, whether those SNPs had been linked to any known human diseases, such as hormone resistance.

The ancestral crown anthropoid ER β sequence was determined using maximum likelihood methods implemented in PAML. The nucleotide sequence for the entire *ESR2* gene was reconstructed based on the *ESR2* sequences of all species included in the multiple sequence alignment.

2.3 ESR2 expression and reporter vector constructs

Ateles fusciceps (ID number A49736, spider monkey, NWM) placenta preserved in RNAlater (Ambion, Austin, TX) was kindly provided by Dr. Kurt Benirschke (San Diego Zoo, San Diego, CA). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and the RNeasy kit in conjunction with the RNase-Free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Isolation was performed after gross dissection of the villous tissues from the maternal membranes. The 28S/18S ratio and the RNA integrity number (RIN) were assessed using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA). An A260nm/A280nm ratio of 1.8, a 28S/18S ratio of 1.3, and a RIN of 6 were minimum requirements for inclusion in PCR and sequence analysis. Total spider monkey RNA was reverse transcribed using the BD Science SuperScript kit following the manufacturer's specifications. Human placenta cDNA was purchased from Biochain (Hayward, CA).

Total ER β cDNA from human and spider monkey was PCR amplified. The reactions were carried out using Invitrogen Platinum *pfu* Taq DNA polymerase per manufacturer recommendations (Carlsbad, CA). Cycling conditions were set at 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute, 38 cycles. Primers are listed in supplementary table 4. The ancestral crown anthropoid (ACA) hinge, LBD, and C-terminal domain ER β sequence including 5' BamHI and 3' SalI restriction sites (5'-GGATCC-3' and 5'-GTTCGAC-3', respectively) was sent to GenScript (Piscataway, NJ) for synthesis. Further (nested) amplification of the human and spider monkey yielded PCR products containing only the hinge, LBD and C-terminal domain of ER β with BamHI and SalI restriction sites as well. These amplicons were ligated into *pCMV-BD* cloning vectors at the BamHI and SalI restriction sites, which include a *Gal4* DBD, (Promega, Madison, WI) giving rise to three constructs; *pCMV-huER β* , *pCMV-ateER β* , and *pCMV-ancER β* , respectively. The constructs were sequenced for verification of the correct reading frame by Michigan State University

Research Technology Support Facility (RTSF), East Lansing, Michigan using T3 and T7 primers.

2.4 Cell culture and hormone treatment

Chinese hamster ovary (CHO-K1) cells were cultured in F-DMEM/12K medium without phenol (Mediatech, Manassas, VA), containing 10% fetal bovine serum (Sigma, St. Louis, MO). One day prior to transfection, the cells were plated onto COSTAR 24-well culture plates at a density of 1×10^5 cells per well and grown overnight to 40–60% confluence. The cells were transfected in triplicate with 20ng of one of the three constructs in conjunction with 1 μ g *pFR-luc* reporter vector, containing a 5X *Gal4* binding region, 1ng of *pRL-TK renilla* luciferase control vector (Promega, Madison, WI) and 2 μ l of Invitrogen 2000 lipofectamine (Carlsbad, CA) per the manufacturer protocol (depicted in Figure 3).

2.5 Luciferase assay and data analysis

CHO-K1 cells were treated 24 hours post-transfection with titrations of estrogen similar to human physiological concentrations with the exception of genistein; estradiol (10^{-18} to 10^{-7} M), estrone (10^{-16} to 10^{-7} M), estriol (10^{-18} to 10^{-7} M) or genistein (10^{-16} to 10^{-7} M) and incubated for 48 hours (Jones, 1996; Romani et al., 2003; Vandenberg et al., 2012). Both firefly and *Renilla* luciferase activities were detected using the Promega Dual-luciferase Reporter Assay System on the GloMax 96 microplate luminometer (Promega, Madison, WI) per manufacturer's instruction. Specific luciferase activity indicating transcriptional activation was assessed by calculating the ratio of firefly luciferase activity to the values produced from the treatment with vehicle after the removal of *Renilla* luciferase activity. Fold activation of the respective ER β regions was calculated in GraphPad Prism (version 5.0b for Mac, GraphPad Software, San Diego California USA, www.graphpad.com) by fitting each dataset to a sigmoidal dose response curve ($Y = B + ((T-B)/(1+10^{\text{LogEC50}-X}))$) where B is the Y value at the bottom plateau, T is the Y value at the top plateau, and LogEC50 – a logarithm of the effective concentration, 50% - is the X value at halfway between B and T) in conjunction with a one-site binding hyperbola ($Y = (B_{\text{max}} * X)/(K_d + X)$) where X is the ligand concentration, Y is the specific binding, B_{max} is the maximum number of binding sites, and K_d is the equilibrium dissociation constant) to determine the best curve fit for each construct and if transactivation potentials of the datasets (species) were significantly different ($p < 0.0001$) from one another.

3. Results

3.1 Purifying selection in ESR1

Upon obtaining the sequence of the coding regions of *ESR1* (the gene that encodes ER α) in 13 primate and 4 non-primate mammal species (supplementary table 2), we used comparative genomics to examine dN/dS ratios in the gene tree. An $\omega < 1$ on all branches tested indicates this gene is under purifying selection (supplementary table 3). As such, no evidence for adaptive evolution was detected in *ESR1*.

3.2 Adaptive evolution in *ESR2*

Using comparative genomics, we sequenced the coding regions of *ESR2* (the gene that encodes ER β) in 16 primate and 4 non-primate mammal species (Figure 1 and supplementary table 2) (Enmark et al., 1997).

We found the free-ratio model to be a significantly better fit to the data than the one-ratio model, ($p = 5.66E-06$). This suggests that the ratio of the rates of non-synonymous substitutions to synonymous substitutions in *ESR2* significantly varies across the tree. By implementing two nested branch-models, a two ω model (Figure 1, left), and a three ω model (Figure 1, right), we tested whether selection pressure was distinct between the NWM stem lineage and branches within crown NWMs. A three-ratio model in which rates of substitution differed in stem and crown NWMs compared to other taxa was found to be a better fit to the data than both the 1 ω and 2 ω models, demonstrating that episodic positive selection occurred in *ESR2* along the stem NWM branch ($\omega = 1.466$, $p = 2.15E-05$) (supplementary table 3), followed by purifying selection ($\omega = 0.1593$) after the diversification of NWMs from a common ancestor.

In an effort to determine if positive selection was predominantly located in a specific domain of ER β , we focused our inspection on amino acid differences between catarrhines (Old World monkeys and apes) and platyrrhines (New World monkeys). We found that many of the amino acid differences between the human and NWM lineages occur within the hinge, LBD and C-terminal domains of ER β . In order to identify specific sites (amino acids) under positive selection within the NWM ER β LBD, we used the branch-site test (Zhang et al., 2005), which shows significant evidence of sites under positive selection along the NWM stem lineage ($\chi^2 = 3.87$, $p = 0.049$). The Bayes Empirical Bayes test identified five sites that show evidence of positive selection ($p > 0.95$), 305K, 360D, 377I, 425R, and 496L in the NWM stem lineage. All of these selected sites are found in the LBD.

Interestingly, the location of three of these codons (305K, 377I, and 425R) provides evidence for a highly variable region of human *ESR2*, as they are also the location of single nucleotide polymorphisms (SNPs) in humans according to dbSNP Build ID: GRCh38 Annotation release 106. However, none of these SNPs (rs539389612, rs531227990, or rs142544449, respectively) recapitulates the NWM amino acids. In humans at the K305T SNP, the NWM encodes glutamic acid (E). The 377I SNP is synonymous in humans, but the NWM sequence encodes a valine (V). Finally, the human R425W SNP is glutamine (Q) in NWMs.

In order to design experiments to test for the functional consequences of positive selection in *ESR2* we inferred the most recent common ancestral anthropoid *ESR2* sequence using maximum likelihood methods. We then compared the reconstructed ancestral crown anthropoid (ACA) ER β to both human and NWM receptors focusing, in particular, on the hinge, LBD and C-terminal domains that are known to effect transactivation. (Bridgham et al., 2006). A total of 20 amino acid replacements (6.3%) were found when comparing the ACA ER β with the spider monkey ER β . However, only 7 amino acid differences (2.2%) were inferred between the human and ACA ER β (supplementary figure 1). These comparisons suggest the human ER β more closely resembles the ancestral ER β (97.8%),

while the extant NWM ER β has undergone more amino acid replacements, with only 93.7% conservation.

To investigate whether the detected amino acid changes on the NWM branch had any functional consequence related to hormone binding, we created three expression vectors containing the human, spider monkey and the ACA ER β region described above, and measured their transcriptional activity in Chinese hamster ovary (CHO-K1) cells when treated with estradiol, the most prevalent endogenous estrogen (Figure 3a–c). Pairwise comparisons of the transactivation potential for estradiol of each construct (shown as fold activation) are depicted in Figure 4a–c. We also treated these cells with three other estrogens (estrone, estriol, and the phytoestrogen genistein) with results shown in supplementary figure 2. These results reveal a greater transactivation potential for estradiol in the spider monkey ER β when compared to either the human ER β or the ACA ER β ($p < 0.0001$ for each comparison). The transactivation potential of ER β for estradiol has been maintained in humans since anthropoids diverged ($p = 0.5299$) whereas it has increased on the positively selected lineage leading to the last common ancestor of NWM. Conversely, the transactivation potential of ER β for estrone and genistein has increased in both humans and spider monkeys since their divergence (supplementary figure 2). Overall, the spider monkey ER β had more transactivation potential when bound to each of the various estrogens tested with the exception of genistein when compared to both the human and ACA. Taken together, these findings provide strong evidence that selective pressures resulting in positively selected nucleotide substitutions have altered *ESR2* gene function.

4. Discussion

The NRs are a highly conserved family of ligand dependent transcription factors and have functioned as such for millions of years. Throughout their evolution, increases in nucleotide substitutions would have occurred along individual lineages in response to geographic or climatic instability, predatory encounters, or other events requiring adaptation for reproductive success. Present day genomics allows us to detect the result of the changes that occurred in the past. Paleontological methods provide support for speculation on the adaptive events that caused the changes and ancestral gene resurrection allows us to study the functional consequence of the nucleotide substitutions.

4.1 Functional Relevance of Nucleotide Substitutions

Detecting adaptively evolving genes is a powerful way in which evolutionary advantages and candidate genes for disease can be identified (Burioni et al., 2009; Carrigan et al., 2015; Crespi et al., 2007; Hanson and Gluckman, 2008; Hou et al., 2009); however, it has become apparent that experimental evidence for adaptation is necessary to confirm the functional importance of inferred evolutionary changes (Nozawa et al., 2009). To determine the functional consequences of amino acid replacements in estrogen receptors we used ancestral gene resurrection (Bridgham et al., 2006; Thornton, 2001). We documented two stages of evolution in the gene that codes for ER β , *ESR2*, in NWMs, adaptive evolution on the stem NWM lineage, followed by purifying selection within NWMs. We examined the functional consequence of excess estrogen by measuring differences in ER β transactivation potential

between human, NWM, and ACA. We determined that the NWM ER β , when bound to estradiol, has greater transactivation potential than either human or ACA ER β . This implies that the ER β of ACA behaved more like the ER β in humans when bound to estradiol, and that the evolution of increased circulating endogenous estrogen levels corresponds with increased ER β estrogen transactivation potential on the NWM stem lineage. We also compared the transactivation potential of the NWM, human and ACA ER β when bound to a known phytoestrogen, genistein. We found that ACA ER β had a significantly lower transactivation potential than both the human and NWM ER β s.

It is unclear whether the change in transactivation potential is due to a single site substitution, or multiple sites working in conjunction. We identified five sites of positive selection along the NWM lineage, three of which are also the sites of SNPs (rs539389612, rs531227990, and rs142544449) in humans, although, the polymorphisms are not identical in humans and NWMs. In addition, none of the non-synonymous differences between human and NWM sequences have been, as yet, linked to any diseases (such as hormone resistance) in humans.

4.2 Arrival of Primates in the New World

When and how anthropoid primates arrived in the New World is under ongoing investigation. South America was an island continent, splitting off from Africa 100 mya (Hay et al., 1999) and Antarctica between 41 (Scher and Martin, 2006) and 31 mya (Lawver and Gahagan, 2003), until the Central American Land Bridge was formed about 3 mya (Collins et al., 1996; Leigh et al., 2014; Macfadden, 2006; Simpson, 1965). Between 35–30 mya the mean annual temperature dropped by ~5°C and the Andes Mountains, currently at 4–5km elevation, were much lower, at <1km (Isacks, 1988; Sijp and England, 2004). Thus far, the discovery of *Perupithecus* in Santa Rosa, Peru, provides the earliest evidence that the arrival of anthropoid primates in the New World occurred approximately 36 mya (Bond et al., 2015). As such, the earliest anthropoid primates, those along the stem lineage of extant NWM, encountered and endured the drastic continental and climatic changes South America was undergoing.

4.3 Adapting to the New World

Biogeographic studies based on the flora, both extant and extinct, coupled with morphologic examination of fossils found in the region, have found that upon arrival in the New World, anthropoid primates would have initially encountered widespread tropical and temperate forests that gradually converted to grasslands. (Jacobs et al., 1999). The botanical diversification coupled with their arboreal nature could have applied selective pressure on genes as the environment favored nucleotide substitutions that promoted reproductive success. This would have allowed anthropoid primates to exploit and thrive in niches toxic and/or inaccessible to other cohabiting, ground-dwelling species, i.e. marsupials, rodents, and other herbivores (Macfadden, 2006). Utilizing these niches would have involved adaptations on various levels including morphology (teeth, jaw), digestion (gut microbiome composition, energy conversion), reproduction, and the interaction of molecules (hormones and their receptors) and would have been critical for the survival of the species.

Examining the topography of dental remnants, such as those of *Perupithecus*, often the only fossil evidence that remains, has provided a useful way to infer the diets of extinct species. Understandably, while there are several methods of measuring the different aspects of dental topography, when available, comparisons using both upper and lower molar morphologies provide a more accurate dietary inference than when just one of the two teeth is available (Allen et al., 2015). However, while hundreds of rodent fossils have been found in the same locale as *Perupithecus*, the discovery of the much smaller primate fossils is much less common and future fossil discoveries may change our view of early platyrrhine diet (Bond et al., 2015).

4.4 Physiological Consequence of Environmental Pressures

Ateles, the extant New World anthropoid studied here, has been extremely successful at incorporating plants and fruits into their diets, achieving an 86% annual fruit intake (Norconk et al., 2009). Reproductive success, a necessity for species survival, can be influenced by energy levels, i.e. availability of food, and can be facilitated by chemical interactions (Ziegler et al., 2009). One possible adaptation to the consumption of such a high proportion of products rich in exogenous hormones such as phytoestrogens is to increase the level of circulating endogenous steroids. This would provide a means of diluting the biological effect of the exogenous hormones, thus reducing the threat to the reproductive success of the species, and hence survival, following consumption (Wasserman et al., 2013; Wynne-Edwards, 2001). We speculate that this type of adaptation could potentially enable *Ateles* to accommodate a higher level of circulating steroids in comparison to other anthropoid primates which correlates with a higher transactivation potential in ER β when bound to endogenous estrogens. The specific role of phytoestrogens in this relationship and whether the effect is causal has not been revealed by our data and would require further testing. In humans, high levels of circulating steroids can occur however, such a phenomenon typically results in dysfunction of the corresponding receptor, such as the effect of elevated cortisol in Cushing's syndrome. NWM, on the other hand, have consistently higher levels of circulating steroid hormones (Brandon et al., 1989). Further, the levels of phytoestrogen consumption by humans is considerably less and can be reduced by as much as 50% during food processing (Zaheer and Akhtar, 2015). While the diet of the ancestral crown anthropoid remains unknown, our results indicate that phytoestrogens (i.e. genistein) do not significantly alter transactivation of the ancestral ER β (supplementary figure 2).

Given the diverse (stimulative and/or repressive) functions of ER β , in multiple systems (endocrine, cardiovascular, neurological, etc), and the various estrogens (endogenous and exogenous) that bind to it as either agonists, antagonists, or selective ER modulators, the physiological consequences of positive selection as detailed in this work are practically innumerable, especially when considered in conjunction with the complementary and/or paradoxical functions of ER α (Barkhem et al., 1998; Enmark et al., 1997; Hall and McDonnell, 1999; Mosselman et al., 1996; Ogawa et al., 1998; Pike et al., 1999; Wasserman et al., 2012; Zhao et al., 2008).

4.5 Limitations and Future Work

While we have demonstrated that inferential methods have functional relevance, there are, admittedly, some limitations to our work. Here, we were able to resurrect the ancestral ER β , but not the ancestral estrogen or the ancestral cell in which the NR and ligand would have interacted. Unfortunately, these molecules are not preserved over time as are fossils. Instead, we employed extant estrogens and the rodent cell line CHO-K1 to control for any human or NWM cell specific interactions that could potentially skew the data. As such, we can only speculate which selective pressures fixed the changes in the LBD allowing exploitation of unused niches rich in newly encountered environments.

This work paves the way for future studies such as competitive binding assays using the estrogen antagonist progesterone. In-depth analyses involving site-directed mutagenesis may help determine which specific site(s) are driving the increase in transactivation potential. This study can be further expanded to examine the entire NR superfamily including the extant, “orphan,” receptors for which there is no known functional ligand.

5. Conclusion

Overall, reconstructing the evolutionary history of anthropoid primates has presented challenges across many fields of study each of which rely on the data gathered by another specialty e.g. biogeography depends on fossil calibrations; phylogenetic analyses depend on divergence dates. Further, each method has its own set of limitations coupled with difficulties in resolving NWM relationships because of the rapid radiations that occurred upon arrival in the New World. This study provides an additional tool that demonstrates the functional consequence of evolutionary variation in DNA sequence and contributes to the understanding of primate phylogeny. In this regard, it is important to bear in mind that extinct and extant should be considered collectively, not as distinct entities. Taken together, natural selection would have favored adaptive changes in the stem lineage leading to NWMs, with the increase of ER β transactivation potential observed in NWMs when bound to both estradiol and genistein and may have helped these primates accommodate a shift in environmental pressures that emerged after dispersal of their ancient ancestors to the New World (Fleagle, 1999; Jameson Kiesling et al., 2014).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the National Science Foundation (BCS-0827546 and BCS-0751508) as well as the Perinatology Research Branch, an intramural program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health, U. S. Department of Health and Human Services (N01-HD-3342). We would like to thank all of the sources of samples (both tissue and DNA) used herein. Specific provider information is detailed in Supplementary Table 2. AW carried out the cell culture, hormone treatment, luciferase assays and data analysis and wrote the manuscript, which all authors edited. MRM performed phylogenetic analyses, interpreted data, and wrote the manuscript. JX and CC carried out the DNA sequencing experiments and created the vector constructs. KNS and ZCH carried out analyses and interpreted data. RR provided support and expertise in every aspect of the project. DEW conceived the project and wrote the manuscript. We would like to thank Dr. Jameson Kiesling for expert graphic design.

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Highlights

- Adaptive evolution of *ESR2* was detected on the New World monkey (NWM) stem lineage.
- The ancestral anthropoid *ESR2* sequence was inferred and resurrected.
- Dose response experiments allowed functional comparison of the receptors.
- Positive selection in ER β increased the transactivation potential of the receptor.

Figure 1.

Phylogenetic relationships of mammals included in this analysis. The black circles indicate the ancestral crown anthropoid (ACA). The tree represented on the left depicts branching in a two-ratio model, red branches denote stem and crown NWMs. The tree on the right depicts branches in the three-ratio model, red branches denote stem NWMs and blue branches denote crown NWMs.

Figure 2. Structure of ERβ. Numbering indicates the C-terminal end of each domain. Shaded domains denote the region of the receptor that was transfected into CHO-K1 cells for functional comparison.

Figure 3.

a: Depiction of luciferase reporter assay. Upon estrogen (purple hexagon) binding, human ER β (*pCMV-huER β* construct depicted in pink containing a GAL4 DNA binding domain denoted by the turquoise downward arrow) enters the cell nucleus and regulates gene expression by binding to a DNA response element (dark orange band within the circular depiction of the luciferase reporter vector) triggering luciferase production quantifiable as luminescence (yellow stars). **b:** Depiction of luciferase reporter assay. Upon estrogen (purple hexagon) binding, spider monkey ER β (*pCMV-ateER β* construct depicted in brown containing a GAL4 DNA binding domain denoted by the turquoise downward arrow) enters the cell nucleus and regulates gene expression by binding to a DNA response element (dark orange band within the circular depiction of the luciferase reporter vector) triggering luciferase production quantifiable as luminescence (yellow stars). **c:** Depiction of luciferase

reporter assay. Upon estrogen (purple hexagon) binding, ancestral ER β (*pCMV-ancER β* construct depicted in green containing a GAL4 DNA binding domain denoted by the turquoise downward arrow) enters the cell nucleus and regulates gene expression by binding to a DNA response element (dark orange band within the circular depiction of the luciferase reporter vector) triggering luciferase production quantifiable as luminescence (yellow stars).

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Figure 4.

a: Luciferase production in response to estradiol. The spider monkey ER β transactivation potential (brown curve) was significantly different from the human ER β (pink curve) ($p < 0.0001$). **b:** Luciferase production in response to estradiol. The spider monkey ER β transactivation potential (brown curve) was significantly different from the ACA ER β (green curve) ($p < 0.0001$). **c:** Luciferase production in response to estradiol. The transactivation potential has been maintained between human and the ACA ($p = 0.5299$).