REVIEW

Plasticity of the zona reticularis in the adult marmoset adrenal cortex: voyages of discovery in the New World

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Abstract

Adrenarche in humans occurs at the age of 5–7 years, yet the process by which dehydroepiandrosterone (DHEA) biosynthesis in the adrenal zona reticularis (ZR) increases so dramatically remains as a matter of debate. One suggestion is that increased DHEA production by P450c17 (CYP17A1 as listed in HUGO Database) in the ZR results from a coincident fall in the expression of HSD3B, which would otherwise compete for pregnenolone substrate. Nonetheless, studies of human and rhesus adrenal show that cytochrome b5 (CYTB5) expression increases in the ZR with DHEA biosynthesis, and cloned human and rhesus P450c17 show selective increases in 17,20-lyase activity in the presence of CYTB5. The marmoset, a New World primate, expresses a fetal zone during development which regresses after birth. Adult males, however, do not develop an obvious functional ZR, while females develop a ZR in a manner that depends on their social/gonadal status. In all social and physiologic states, changes in marmoset ZR function relate directly to changes in the expression of CYTB5. Recent cloning and expression of marmoset P450c17 also show that while amino acid sequence homology is in the order of ~85% of that found in human and rhesus sequences, and basal lyase activity is low compared with rhesus, all previously described amino acids critical to human 17,20-lyase activity are completely conserved. Furthermore, the 17,20-lyase activity of the marmoset P450c17 clone is dramatically increased by addition of CYTB5. We propose that these combined data from the marmoset model provide further compelling evidence that the control of ZR CYTB5 expression is a key determinant of ZR function.
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General introduction

The inner cortical zone of the adrenal gland, the zona reticularis (ZR), is a uniquely primate attribute and has long been described in humans. More recently, the androgenic biosynthetic enzyme, P450c17(CYP17A1 as listed in HUGO Database), has been shown to be the enzyme responsible for the production of milligram quantities of dehydroepiandrosterone (DHEA) in the ZR, and is intimately involved in the process that triggers the onset of efficient C19 steroid biosynthesis (DHEA and its sulfoconjugate DS) at the time of adrenarche. The endocrine mechanisms that regulate the adrenal androgen biosynthesis, however, are still a matter of debate. This situation is not helped by the finding that while the Old World monkeys (and the Great Apes, based on circulating DHEA/DS levels) express a mature functional ZR in adulthood, the timing of the onset of ZR development and function in these species is often inconsistent with that seen in humans and is indeed inconsistent across primate species (reviewed in more detail in Conley *et al.* (2004)).

It has been argued that while chimpanzees undergo an adrenarche event process and rise in DS similar to that in humans, Old World monkeys such as rhesus macaques and baboons do not, based largely on the observation that the rise in circulating levels of DHEA/DS in rhesus and baboons is not a distinct event occurring after the regression of the fetal zone (FZ) and before puberty (reviewed in Conley *et al.* (2004) and Abbott & Bird (2009)). Recent studies, nonetheless, suggest that the onset of adrenal ZR function in rhesus monkeys begins before FZ regression is complete. Demonstrating such development of the ZR simply through a rise in circulating DS in late fetal and early postnatal life is thus confounded by a still functional FZ which is also capable of contributing to circulating DS levels (Nguyen *et al.* 2008).

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Certainly if this is the case, it suggests that an 'adrenarche' event may be common to more non-human primate species than was previously apparent, even though the developmental timing of such an event may be more variable than previously assumed. Furthermore, looking across the primate order at differential developmental or physiological states associated with the emergence of a functional ZR could both clarify the debate and perhaps help identify common underlying endocrine mechanisms regulating ZR function. One such primate that has cast particularly novel light on this question is the common marmoset (*Callithrix jacchus*), a New World monkey widely used as a model for human hypothalamic–pituitary–adrenal function (Saltzman *et al.* 1994, 1998, 2000, 2004, Pryce *et al.* 2005), stress and psychopathology (Johnson *et al.* 1996, Cilia & Piper 1997, Dettling *et al.* 2002), and hypothalamic–pituitary–ovarian function (reviewed in Abbott *et al.* (2003) and Mansfield (2003)). This review summarizes, and indeed further considers, the surprising new findings on marmoset adrenal function and their relevance to our understanding of both the biochemical onset of DHEA/DS biosynthesis in the post-term adrenal and the endocrine factors that may regulate them.

For clarity, this review is divided into two main sections. Following an overview of ZR immunohistologic zonal markers in humans and other non-human primates, the first section summarizes recent findings exploring marmoset adrenal morphology and endocrine function *in vivo* and corresponding changes in circulating steroids in males versus females, as well as in females at varying states of reproductive function. The second section then introduces the cDNA encoding marmoset P450c17 protein, (*CYP17*) and considers both sequence-based and molecular biochemical-based regulation of marmoset cytochrome P450c17 activity and particularly 17,20-lyase activity compared with the more extensively characterized human and rhesus sequences. The findings of these recent studies suggest that ZR function does indeed develop in marmosets long after regression of the FZ, but that this occurs in a manner that is both female-specific and more closely associated with gonadal function/social status than with a given developmental stage or chronological age. These findings support the notion that while marmoset P450c17 amino acid sequence is only 85% homologous to that of humans, it possesses qualitatively the same biochemical features as human P450c17 and probably provides the best and most dynamic evidence for a key role of elevated cytochrome b5 (CYTB5) as a major determinant of ZR function. The possibility that gonadal regulation of adrenal CYTB5 expression can occur, and that this effect may be fully reversible, is also considered and we propose the term 'zonal plasticity' to describe it.

Adrenal zonation and zonal function

The adult human adrenal cortex is divided into three distinct zones, the outer zona glomerulosa (ZG), the middle zona fasciculata (ZF), and the inner ZR. The ZG is the site of mineralocorticoid (i.e. aldosterone) production. Likewise, the ZF is the site of glucocorticoid (i.e. cortisol) biosynthesis, and the ZR produces C19 steroids. The latter are also commonly referred to as adrenal androgens (i.e. DHEA and DS). While all three zones initially produce steroids by conversion of cholesterol to pregnenolone (P5) in response to specific endocrine stimulation, the subsequent distinct steroid end-products are achieved by discrete distribution or zonation of expression of the subsequent steroid-metabolizing enzymes (reviewed in detail in Conley & Bird (1997) and Conley *et al.* (2004)). Among these enzymes, one single enzyme (P450c17) is capable of converting both P5 to 17-hydroxypregnenolone (17OHP5) and then 17OHP5 to DHEA (Fig. 1).

Of the three adrenocortical zones, it is the ZF and ZR zones that express the same P450c17 enzyme, yet only the ZR makes C19 steroids. Cortisol production in the ZF is associated with abundant expression of both P450c17 and HSD3B2 (Ishimura & Fujita 1997), in addition to P450c21 and P450c11. Under basal conditions at least, studies of the expressed cDNA have shown that the human P450c17 prefers P5 as a substrate over progesterone (P4) and that the hydroxylase activity is both faster and has higher affinity for binding of substrate than the 17,20-lyase reaction (Voutilainen & Miller 1986, Fevold *et al.* 1989, Brock & Waterman 1999, Flück *et al.* 2003). Since only 17-hydroxylation activity is required of P450c17 for cortisol biosynthesis, the additional presence of abundant HSD3B2 in the ZF cannot prevent this initial hydroxylase reaction, but can effectively compete for 17OHP5 and convert it to 17OHP4. This cannot be further metabolized by human P450c17 since the 17,20-lyase activity on 17OHP4 is negligible, and the competing action of HSD3B2 helps commit substrate to predominant cortisol biosynthesis (reviewed in Conley & Bird (1997)). By contrast, DHEA production in the adult human ZR requires P450c17 to perform not only the hydroxylase but also the C19 steroid-generating 17,20-lyase activity. It has previously been proposed that the relative lack of HSD3B2 expression (Endoh *et al.* 1996, Gell *et al.* 1996, 1998, Suzuki *et al.* 2000) enables this conversion of P5 to DHEA to be more efficient, but more recent observations have also shown there is also a concomitant high zonal expression of CYTB5 (a cofactor to P450c17) in the adult ZR (Suzuki *et al.* 2000). In Old World monkeys, adults also have an adrenal ZR with little or no HSD3B2 expression (Mapes *et al.* 1999), but they too exhibit intense zonal CYTB5 expression (Mapes *et al.* 1999, Dharia *et al.* 2004). Of note, studies of rhesus adrenals during neonatal development demonstrate that 17,20-lyase activity increases relatively early in a manner positively correlated with CYTB5 levels, and occurs despite concommitant increases in HSD3B expression during this period (Nguyen *et al.* 2009). Thus, we can see that while a key branch point in human and non-human primate steroid biosynthesis lies at the aforementioned junction between the P450c17 and HSD3B2 (Fig. 1), efficient DHEA production may not even require a relative fall in HSD3B2 given the additional presence of elevated levels of CYTB5.

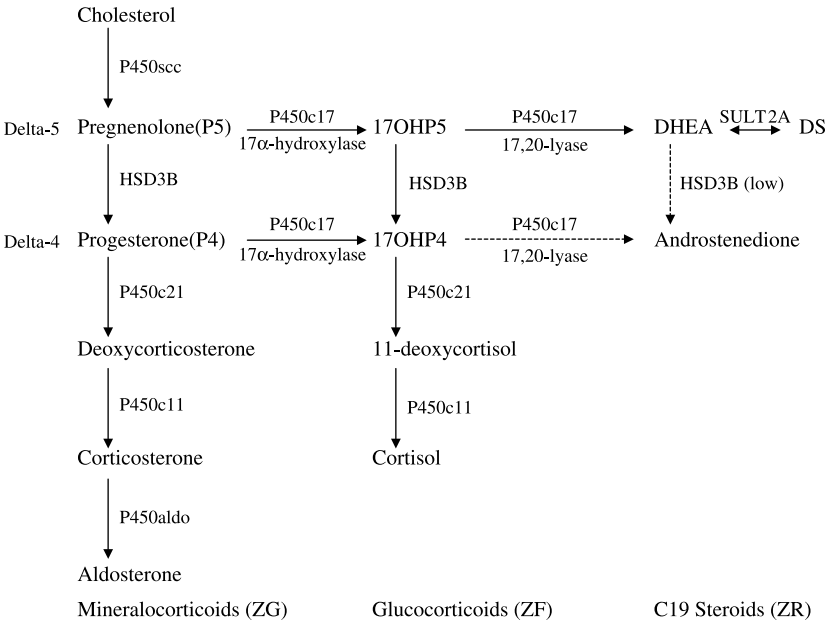

Figure 1 Human and non-human primate adrenocortical steroidogenic pathway. Dehydroepiandrosterone (DHEA) is synthesized exclusively through the delta-5-lyase pathway. The low level of HSD3B in the zona reticularis (ZR) is crucial for substantial DHEA production as higher levels of HSD3B would out-compete 17,20-lyase for 17α-hydroxypregnenolone and commence down the cortisol biosynthetic pathway. The dashed line for delta-4-lyase activity denotes its comparative absence in human and non-human primates, sheep, cows, and goats although this activity is retained in most of the other mammalian species.

Adrenal development and adrenarche

One way we can ask if elevated CYTB5 coexpression is really necessary for efficient DHEA biosynthesis is to determine whether CYTB5 coexpression appears at appropriate times in relevant DHEA-producing adrenal structures, namely the FZ, which synthesizes and releases DHEA/DS *in utero* and in the neonatal period, and in the ZR of the maturing adrenal at the time of adrenarche and beyond. Prepartum, the human fetal adrenal gland does not express a ZR but instead expresses a neocortex (also known as a definitive zone, comprising a developing ZG and ZF) and an FZ (C19 steroid-producing region) (reviewed by Pepe & Abrecht (1990), Mesiano & Jaffe (1997), Conley *et al.* (2004) and Rainey *et al.* (2004)). The FZ produces large quantities of DHEA that acts as a precursor to human placental estrogen production, since gestational levels of estriol in the human maternal circulation are derived entirely from fetal DS (recently reviewed in Rainey *et al.* (2004)). Similar to the functional adult ZR, in addition to expression of P450c17 and P450oxido-reductase (POR), this FZ is also rich in CYTB5 and relatively poor in HSD3B2 expression. At birth, the FZ begins to morphologically regress and DHEA/DS levels are seen to fall within a few months (reviewed in Conley *et al.* (2004) and Havelock *et al.* (2004)). The emergence of a functional human adrenal ZR begins around 5–7 years in a process known as adrenarche, when DHEA/DS levels begin to increase once more and the aforementioned increase in CYTB5 expression is observed in the innermost adrenal cortex, while HSD3B2 expression levels decrease (Gell *et al.* 1996, 1998, reviewed in Conley *et al.* (2004) and Rainey *et al.* (2004)). Thus, a pattern emerges in humans, and at least in some Old World monkeys such as rhesus macaques, wherein efficient DHEA production in the ZR is marked by abundant expression of P450c17, an availability of POR, and a consistent increase in CYTB5, together with the aforementioned reduction in HSD3B2.

The marmoset adrenal

The colocalized pattern of P450c17 enzyme and CYTB5 cofactor expression appears to be necessary for DHEA production in humans, and seems to be essentially the same in Old World primates. But what about New World primates such as the marmoset? Like humans, marmosets exhibit a 28-day ovarian cycle, establish complex, long-lasting social relationships, and live in small, nuclear family-based social units (reviewed in Abbott *et al.* (2003)). With regard to adrenal development and function, the FZ has been identified in the fetal adrenal by classical histology and elevated DS has been reported at birth (Levine *et al.* 1982), yet the presence of a ZR in adult marmosets has been hotly debated. Early histological investigations provided no conclusive evidence for a ZR as it was indistinct and difficult to describe

definitively. Miraglia & Moreira (1969) found morphological evidence for the presence of a ZR in marmosets, yet non-immunospecific staining methods were used and the result was subsequently debated. Levine *et al.* (1982) utilized hematoxylin and eosin staining in neonatal, infant, and adult adrenals and showed a morphologically distinct inner adrenal area at birth that regressed completely by three months of age and did not reappear upon sexual maturity at 18–21 months. Levine *et al.* (1982) also measured neonatal levels of DHEA/DS, which were high at birth but dropped precipitously by three months and rose again to clearly detectable levels (albeit not to the levels observed in humans), but only in females, at 18 months. Together, these data provide clear evidence for a functioning FZ in the fetal adrenal in the marmoset and suggest a possible sex difference in ZR functional expression upon reaching sexual maturity.

The first molecular investigation of whether male marmosets actually express a functional FZ in infancy or ZR in adulthood was undertaken only recently by Pattison *et al.* (2005), who used immunohistochemistry to determine the zonal presence of P450c17, HSD3B2, POR, and CYTB5 respectively, since these are the key proteins necessary to synthesize C19 steroids from P5. In neonatal marmoset adrenals, staining was indicative of a putative FZ, with clear staining for P450c17, reduced HSD3B2 relative to the neocortex, and clearly increased FZ levels of CYTB5 (see also Fig. 2). This was completely consistent with the previous functional reports of high DHEA/DS after birth that declined with increasing age, and so independently confirmed the observations by Levine *et al.* (1982) that neonatal marmosets exhibit a C19 steroid-secreting FZ similar to humans.

Recent investigations in primary cultures of adrenocortical cells from mature marmosets have confirmed that both acute steroidogenesis in response to forskolin (an ACTH mimetic) and/or angiotensin II, and the longer-term effects on induction of P450c17 expression of these same hormones as well as the diacylglycerol analogue 12-*O*-tetradecanoylphorbol 13-acetate (TPA) alone or in combination with forskolin, are similar to findings in rhesus monkeys and humans (Bird *et al.* 1996, Pattison *et al.* 2004). Nonetheless, while adrenal cross-sections from adult males showed P450c17 and POR protein expression throughout the cortex, they did not show the expected decrease in HSD3B2 or increase in CYTB5 in the innermost region (Pattison *et al.* 2005). Western analysis of male adrenal microsomes confirmed these data, demonstrating comparable levels of P450c17 expression to that seen in microsomes from rhesus monkeys, but diminished expression of CYTB5. HPLC analysis of the products of incubation of P5 with adrenal microsomes revealed similar 17-hydroxylase action for adult marmoset and rhesus adrenal microsomes, but greatly attenuated 17,20-lyase activity for marmosets. Consistent with this, in the same studies, adult male marmosets were treated with exogenous ACTH or dexamethasone (Dex) in an attempt to stimulate a change in circulating DHEA/DS. The ACTH challenge failed to elevate circulating DHEA/DS, and Dex treatment failed to suppress DHEA/DS, even when cortisol levels changed as expected. Together, the histochemical and functional data, both *in vitro* (microsomes) and *in vivo* (circulating steroids), provided by Pattison *et al.* (2005) definitively extended the earlier suggestion by Levine *et al.* (1982) that even though the marmoset neonate adrenal has a FZ, the adult male adrenal fails to acquire a functional ZR.

Sex- and gonad-specific changes in marmoset ZR function – evidence for zonal plasticity

The finding that marmoset males do not in fact show significant ZR function was certainly a contrast to findings in Old World non-human primates and humans, but also supported the notion that a lack of elevated CYTB5 is coincident with a lack of DHEA/DS biosynthesis. This is where the study would have ended, but for one feature of female marmosets that is somewhat unusual. Marmosets are cooperative breeders (i.e. individuals routinely provide care for other animals' offspring (Saltzman *et al.* 2009)), and females exhibit pronounced social regulation of reproductive physiology. Both in the wild and in captivity, marmosets form groups of 5–17 monkeys, with one or perhaps two reproductively active or dominant females and one reproductively active dominant male (reviewed in Abbott *et al.* (2003)). Subordinate females typically do not exhibit regular ovarian cycles, fail to ovulate, and aid in the care of dominant female's infants. Unlike some other subordination models, the cause of suppressed cyclicity in subordinate female marmosets is not stress-induced (reviewed in Abbott *et al.* (2003), Mansfield (2003) and Tardif *et al.* (2003)), as evidenced by lower cortisol levels when compared with both ovary-intact dominant females and ovariectomized females (Saltzman *et al.* 1998). Chronic suppression of hypothalamus–pituitary–ovary (HPO) function in anovulatory subordinate female marmosets is a highly reliable, repeatable and, most importantly for this review, reversible phenomenon, with a rapid onset and considerable persistence (Saltzman *et al.* 1994, 1998, 2000, 2004, 2006*a*,*b*, Johnson *et al.* 1996). Social subordination occurs as early as 1–4 days after unrelated marmosets are grouped together, and is accompanied by lowered chorionic gonadotropin (the New World primate counterpart of LH) levels in the subordinate females (Abbott *et al.* 1988). When a subordinate female is removed from the social group and singly housed or pair-housed with a male, she ovulates within 2 weeks (Abbott *et al.* 1988). If this female, however, is then introduced to a new group of unrelated adults, she may become dominant. The reversibility of social status makes the model advantageous for researchers as the formation of social groups and experiments on behavior and *in vivo* physiologic responses are not terminal. In view of this phenomenon, and indeed in light of Levine's previous studies of elevated DS in female marmosets of 18 months or older (Levine *et al.* 1982), similar studies to those in males were repeated in females by Pattison *et al.* (2007), with surprising results.

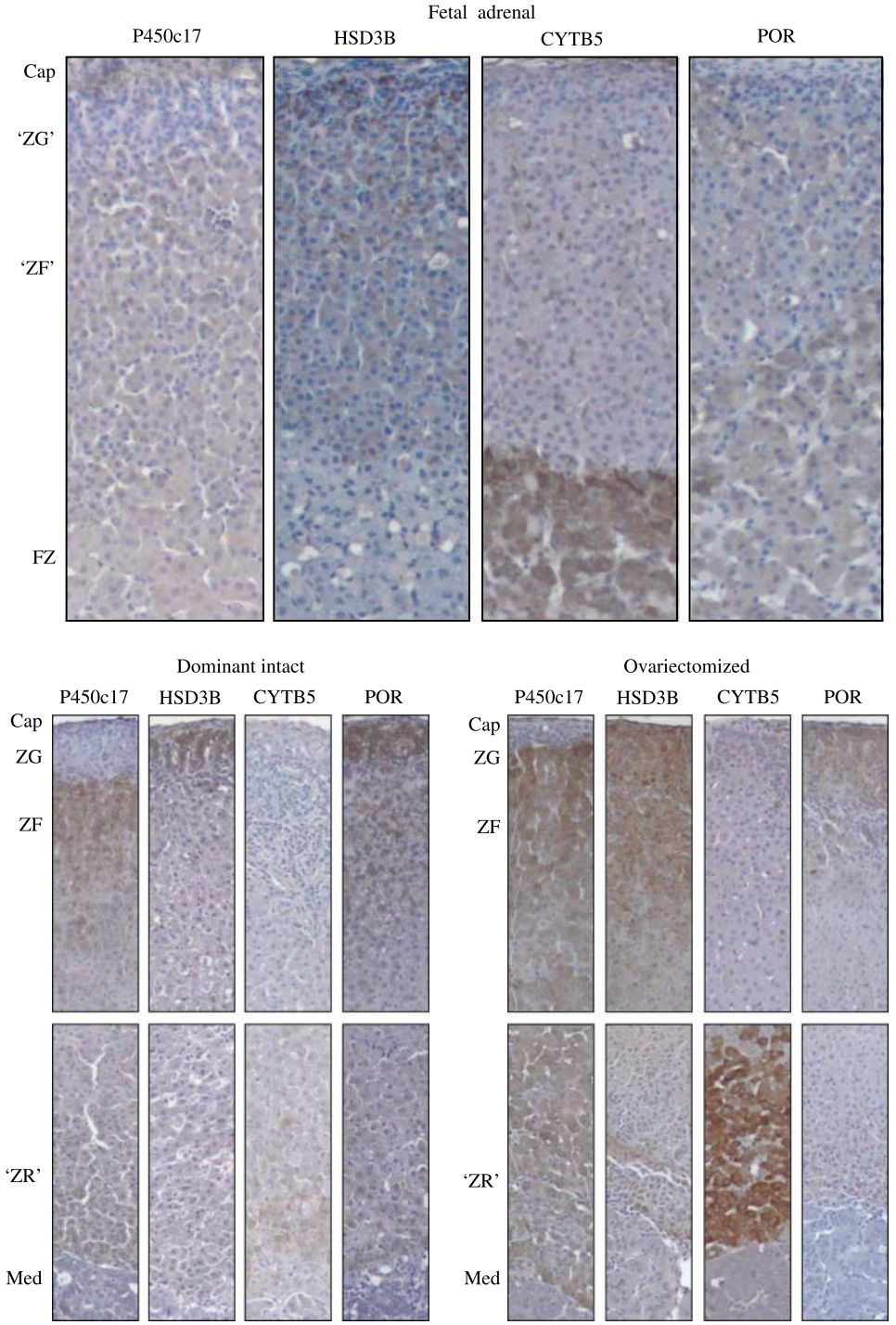

Figure 2 Zonal distribution of proteins related to DHEA biosynthesis in the marmoset adrenal cortex. Immunodetectable protein is indicated by brown stain while counterstain color is blue. (Top panels) IHC staining for steroidogenic enzymes in the neonatal marmoset adrenal, aged 1 day. Cap, adrenal capsule; 'ZG', ZG of the definitive zone/neocortex; 'ZF', ZF of the definitive zone/neocortex. (Bottom panels) Immunohistochemical analysis of adult female marmoset adrenal sections. (A) Dominant female ($n=6$; aged 24–48 months), (B) ovariectomized female ($n=6$; aged 48–84 months). Cap, adrenal capsule; ZG, zona glomerulosa; ZF, zona fasciculata; 'ZR', proposed zona reticularis; med, medulla. 10× Magnification.

Immunohistochemical analysis showed that the ZR marker CYTB5 was elevated in the innermost zone in ovary-intact, regularly cycling adult females compared with testis-intact adult males, was further elevated in adrenals from ovary-intact, anovulatory (socially subordinate) adult females, and was substantially elevated with solid/continuous staining in the innermost adrenocortical zone in ovariectomized adult females (Pattison *et al.* 2007; summarized in part in Fig. 2). As a functional test *in vivo*, following overnight Dex treatment, ovary-intact, cycling and anovulatory adult females showed higher Dex-suppressed levels of DHEA relative to adult males, but DHEA failed to increase in response to ACTH in both groups of females or in males. In direct contrast, while ovariectomized adult females exhibited initially lower Dex-suppressed DHEA levels than ovary-intact females and testis-intact males, clear increases in circulating levels of DHEA were detected after ACTH administration, suggesting an adrenal origin. The apparent differences in CYTB5 expression between groups were further verified by western blotting of adrenal microsomes, and were compared with 17,20-lyase activity in the same preparations; the two parameters were positively correlated to a high degree of significance ($P<0{\cdot}01$) across multiple treatment groups (Pattison *et al.* 2007). This correlation is particularly important since it provides the first functional data indicating that while ZR expression of CYTB5 clearly differs between adult male and adult female marmosets, and even differs with the social/physiologic state of adult females (i.e. socially dominant/cycling compared with subordinate/anovulatory compared with ovariectomized), ZR 17,20-lyase activity relates directly to the level of CYTB5 expression in the adrenal ZR regardless of sex or physiologic state of females. It was concluded by Pattison *et al.* (2007) that ovary-intact, regularly cycling adult female marmosets express a rudimentary ZR with at least a capacity for DHEA production that may well depend on the level of CYTB5 expression. The fact that expression of CYTB5 increased so dramatically on ovariectomy was also the first direct finding that, in marmosets at least, expression of ZR CYTB5 may be directly or indirectly suppressed by gonadal function. The ovary, at least in part, is thus a critical determinant of the development of a functional ACTH-responsive ZR. The most important implication of all these findings is that while adrenarche in humans may appear be a one-way event that persists through adulthood and thereafter partially declines with old age, adrenarche in marmosets is a female-specific, dynamic, and reversible phenomenon. It is for these reasons that we coin the term 'zonal plasticity' to describe such adrenocortical functional dynamics in female marmosets.

Characterization of marmoset P450c17 amino acid sequence and function

Up to this point we have considered the data characterizing the expression markers of adrenal zonation in male and female marmosets as they relate to sex and social status and the corresponding differences in circulating steroids. Although these events are not characteristic of age-related changes in ZR function in humans, coined as 'classic adrenarche', the observations of changes in CYTB5 and associated function in different marmoset goups certainly lend considerable weight to the proposal that whatever variation is seen in CYTB5 protein expression level (by histology or by the actual microsomal content determined by western blot), there is a direct correlation with the capacity to produce adrenal-derived DHEA. Indeed, variation in the expression of CYTB5 could be the primary mechanism controlling adrenal DHEA production ability in the marmoset. Nonetheless, while CYTB5 has proved to be a sound correlative marker of changes in adrenal DHEA secretion, these same studies alone present little direct evidence to suggest that variation in expression of CYTB5 is the underlying cause of changes in DHEA production in response to circulating hormones such as ACTH in the marmoset. Given that this is also the first such study in New World primates, it is all the more important to characterize both P450c17 hydroxylase and 17,20-lyase function and the role of CYTB5 in selective control of 17,20-lyase activity. While a deficiency of delta-4-lyase activity (activity on 17-hydroxyprogesterone) has been inferred in studies of marmoset adrenal microsomes (Pattison *et al.* 2005, 2007), definitive proof of this important regulatory step was still lacking. Perhaps, the biggest limitation of all was that the primary amino acid sequence was unknown for this New World primate, until very recently. As such it was not known to what extent marmoset P450c17 shares sequence homology to that in humans, great apes, or Old World monkeys. Fortunately, molecular cloning of marmoset P450c17 was recently achieved (Pattison *et al.* 2004) and so more direct examination of all these important questions was made possible for the first time in a New World primate model. The protein sequence of marmoset P450c17 and the recent characterization of its associated biochemical properties will thus form the focus of the remaining sections of this review.

Analysis of the primary amino acid sequence of marmoset P450c17

In view of the previously uncharacterized nature of the marmoset model at the biomolecular level, the recent cloning of marmoset *CYP17* mRNA by Pattison *et al.* (2004) allowed the determination of the primary sequence of marmoset P450c17 and its comparison to that of human or the previously characterized and more widely studied Old World monkeys. Since this is the first New World primate P450c17 to be entirely sequenced, it was no surprise that the similarity of the overall marmoset amino acid sequence to rhesus, its Old World monkey counterpart, was only 85·2% and to human was 82·6% (Pattison *et al.* 2004; Fig. 3). There are, nonetheless, other ways to consider the comparison, based on sequence of the secondary structures highly conserved among P450's. This latter type of analysis is necessary because P450c17 has yet to be crystallized for any species, so its structure can only be 'determined' by

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                                                  +
H17     1       MWELVALLLL TLAYLFWPKR RWPGAKYPKS LLSLPLVGSL PFLPRHGHMH NNFFKLQKKY   60
R17             ---------- ---------- -C-------- ---------- ---------- ----------
M17             ---------F --T-F----- -S-------- ---------- ---------- Q-L-------

                   +                            +   +  +          +        + +
H17     61      GPIYSVRMGT KTTVIVGHHQ LAKEVLIKKG KDFSGRPQMA TLDIASNNRK GIAFADSGAH   120
R17             ---------- ---------- ---------- --------VT ----L----- ------Y---
M17             -----L---R NK-----N-- ------M--- --------VA -I--L--KG- ------Y---

                                                                          +
H17     121     WQLHRRLAMA TFALFKDGDQ KLEKIICQEI STLCDMLATH NGQSIDISFP VFVAVTNVIS   180
R17             ---------- ---------- ---------- ---------- ---T------ ----I-----
M17             -------V-- A-S---E--- ---------- -I---V---- D--S------ --M-V-----

H17     181     LICFNTSYKN GDPELNVIQN YNEGIIDNLS KDSLVDLVPW LKIFPNKTLE KLKSHVKIRN   240
R17             -----I---- -----KIVH- -------S-G -E-----F-- --V------- ---R---T--
M17             -----S---D -A---KIVET -----VEA-G -EN---MF-- --I----S-- ---TY--L--

H17     241     DLLNKILENY KEKFRSDSIT NMLDTLMQAK MNSDNGNAGP DQDSELLSDN HILTTIGDIF   300
R17             ---T--F--- ----H----- ----V----- ---------- ---------- ----------
M17             ---N--IG-- ----H----- S---V----- K-A-----S- ---A------ -----V----

                   +                          +                +     +          +
H17     301     GAGVETTTSV VKWTLAFLLH NPQVKKKLYE EIDQNVGFSR TPTISDRNRL LLLEATIREV   360
R17             ----------- ---IV----- ---------- ---------- ----------- ----------
M17             ----------- ---IV-Y--- ---------D ---------- ---V---C- ----------

                 +           +                                    +  +       ++
H17     361     LRLRPVAPML IPHKANVDSS IGEFAVDKGT EVIINLWALH HNEKEWHQPD QFMPERFLNP   420
R17             --I------- ---------- ---------- H--------- ---------- ----------
M17             --I------- --------T- ---------- H--------- -S-*------ K--------Q

                        +           +
H17     421     AGTQLISPSV SYLPFGAGPR SCIGEILARQ ELFLIMAWLL QRFDLEVPDD GQLPSLEGIP   480
R17             ---------L ---------- ---------- ---------- ---------- --------N-
M17             --------TL ---------- ---------- --------M- ---------- -E------N-

                           +
H17     481     KVVFLIDSFK VKIKVRQAWR EAQAEGST               508
R17             ---------- ---------- --------
M17             ----M----- ---------- --------               507
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Figure 3 Sequence alignment of rhesus (r17) and marmoset (m17) P450c17 predicted AA sequences with the published human (h17) AA sequence. *Indicates three-nucleotide (one amino acid) deletion in the marmoset sequence. Crosses (+) indicate residues referred to in Table 1 as critical to activity, and grey highlighted residues indicate those also indicated in human through naturally occurring disease to be most associated with isolated lyase deficiency (See Table 1). Note no residue important for activity (indicated by +) is different in any of the three sequences. GenBank accession numbers: NM_000102, AY746983, and AY746982.

comparison to other standard reference P450's that have been crystallized. In addition, there have also been many independent efforts to implicate specific amino acids that are critical for activity, and particularly lyase activity, by both natural and artificial site-directed mutagenesis experiments. We now consider marmoset P450c17 using the information gained from these two distinct approaches for 'determining' the structure of human P450c17.

Predictions of the structure of P450c17 based on sequence: comparison of human, rhesus, and marmoset

The cytochromes P450 are mono-oxygenases containing a heme center and are so named because of the spectral signature (absorbance peak at 450 nm) of their reduced ferrous ion within their coordinated heme center when forming a complex with carbon monoxide. Many P450s exist; some metabolize drugs (xenobiotic function), others are involved in lipid breakdown in the liver, and a select group of P450s function in steroid metabolism. The P450s that contribute to steroid metabolism include side-chain-cleavage/desmolase cytochrome P450 (P450scc; CYP11A1), 17α-hydroxylase/17,20-lyase cytochrome P450 (P450c17; *CYP17*), 21-hyroxylase cytochrome P450 (P450c21; CYP21), 11β-hydroxylase cytochrome P450 (P450c11; CYP11B1), aldosterone synthase cytochrome P450 (P450aldo; CYP11B2), and aromatase cytochrome P450 (P450arom; CYP19). These steroid-metabolizing P450s can be further classified by the redox protein systems that supply electron pairs to the enzymes for their reactions. Enyzmes utilizing flavoprotein (FAD) adrenodoxin reductase and iron–sulfur protein adrenodoxin (ferrodixin; FMN) are found in the inner mitochondrial membrane and include P450scc, P450c11, and P450aldo. On the other hand, P450c17, P450c21, and P450arom all reside in the endoplasmic reticulum, or microsomal compartment, and

utilize a single flavoprotein, NADPH cytochrome P450 oxidoreductase (POR), which contains both FAD and FMN domains. P450 enzymes utilizing two redox proteins are referred to as class I enzymes, while P450s utilizing one redox protein are referred to as class II enzymes.

As P450c17 is membrane-bound, attempts to crystallize the protein in order to determine the molecular structure were unsuccessful (Lin *et al*. 1994). Thus, the linear amino acid sequence of P450c17 has to be compared with P450 forms that have been crystallized. Lin *et al*. (1994) first reported the comparison of human P450c17 to a crystallized form of P450. At that time, the only crystal structure to which human P450c17 could be compared was P450cam of *Pseudomonas putida*, a soluble bacterial P450. Lin's group, however, was able to identify several structural elements of human P450c17, including residues that line the active site. They suggested that the substrate binding pocket was sufficiently large to accommodate a C21 substrate (P5, 17OHP5, and P4) and the orientation of the lining amino acid residues allowed the 17- and 20-carbon atoms access to electrons from the heme iron. They predicted that aspartic acid 298 (D298 – conserved in the marmoset; Fig. 3) coordinated the 17α-hydroxylated group on the substrate to position C-20 over the heme iron, thereby allowing access to electrons. The structure suggested by Lin's group (1994) also appeared to explain the relative delta-4-lyase deficiency as due to relatively excessive space between the 3-keto group of 17OHP4 and the asparagine 240 (N240; the residue through which the electrons from the heme iron get transferred – also conserved in marmoset; Fig. 3). While much of their work centered on predicting crucial amino acid residues by using the crystallized structure of P450cam, Lin's group also cited examples where they, and other investigators, used linear sequence comparisons between species of P450c17 to identify other crucial amino acid residues. Specifically, they identified arginine 347 (R347) as being crucial for 17,20-lyase activity as well as mutating the rat phenylalanine 343 (F343) residue to the human threonine 343 (T343) and thus abolishing the rat delta-4-lyase activity (Kitamura *et al*. 1991, Lin *et al*. 1994). Both these residues are conserved in the marmoset (Fig. 3).

The disadvantage of comparisons made to soluble bacterial P450s stems from their designation as class I enzymes. The class I enzymes utilize a ferredoxin intermediate, much like the mitochondrial eukaryotic P450s. Cytochrome P450c17, however, is a class II enzyme served by a single redox partner protein, POR (reviewed in Bird & Conley (2002)). Most recent work has utilized the comparisons between human P450c17 and P450BM-3, a unique bacterial class II enzyme (Auchus & Miller 1999). That more recent analysis has disputed the 'bi-lobed' substrate binding pocket previously adopted using comparisons to P450cam. The new 'model' of the substrate binding pocket has the heme moiety as the floor of the pocket. The I-helix serves as one boundary wall while its opposite wall is composed of strands 4 and 5 of β-sheet 1. The two other boundary walls are made up of isoleucine 112 (I112) in the B′-C loop and the loop after the K-helix respectively. Valine residues 482 and 483 (V482 and V483), a turn in β-sheet 3, form the top of the substrate-binding pocket. Both valines are conserved in the marmoset sequence (Fig. 3) and the analysis of Auchus & Miller (1999) can be extended to include rhesus and marmoset sequences (Fig. 4).

Auchus & Miller (1999) also suggested proline 434 (P434) through isoleucine 443 (I443) formed the heme-binding site with cysteine 442 (C442) acting as the axial ligand of the heme iron. Both residues are conserved in the marmoset (Fig. 3). Additionally, the redox-partner binding site lies on the opposite side of the heme from the substrate-binding pocket. The majority of residues are arginine, resulting in a basic (i.e. positive) surface charge that interacts with the acidic (i.e. negative) surface charge of the redox partner. Geller *et al*. (1999) further showed that effectively neutralizing specific arginine residues (R347H, R358Q) selectively inhibits 17,20-lyase activity, lending further support to the importance of the positive surface charge for the redox partner binding site. A separate group, Lee-Robichaud *et al*. (1999), also looked at P450c17 residues important in 17,20-lyase activity. They, too, confirmed R347 and R358 as being key, since effectively neutralizing both to R347H and R358Q abolished 17,20-lyase activity (Lee-Robichaud *et al*. 1998). Additionally, they identified R449 as key, although no corresponding human disease related to this residue has been reported (Lee-Robichaud *et al*. 1999). Lee-Robichaud *et al*. suggested those three key arginine residues, while lying in the redox partner binding site, were required for interacting with CYTB5. All of these residues are again conserved in the marmoset sequence (Figs 3 and 4). When Lee-Robichaud *et al*. (1999) mutated the three arginine residues to lysine (i.e. positive surface charge), the P450c17 constructs regained the ability to interact with CYTB5 and that resulted in the promotion of 17,20-lyase activity.

In summary, while the crystallization of P450c17 from any species has remained elusive, much is known from sequence analysis and site-directed mutagenesis. The marmoset sequence shares only limited sequence homology overall with that for human P450c17, but it is important to note that all of the amino acids considered to date to be important to P450c17 function, and particularly CYTB5 binding (summarized in Table 1), are highly conserved in the marmoset sequence (Fig. 3).

Conservation of residues identified from natural mutations leading to isolated 17,20-lyase deficiency in humans

In addition to the anaylsis of human, rhesus, and now marmoset sequences of P450c17, independent studies have probed the amino acid sequence specifically responsible for 17,20-lyase activity by 'natural' experiments of genetic mutation and associated disease. Defined isolated 17,20-lyase

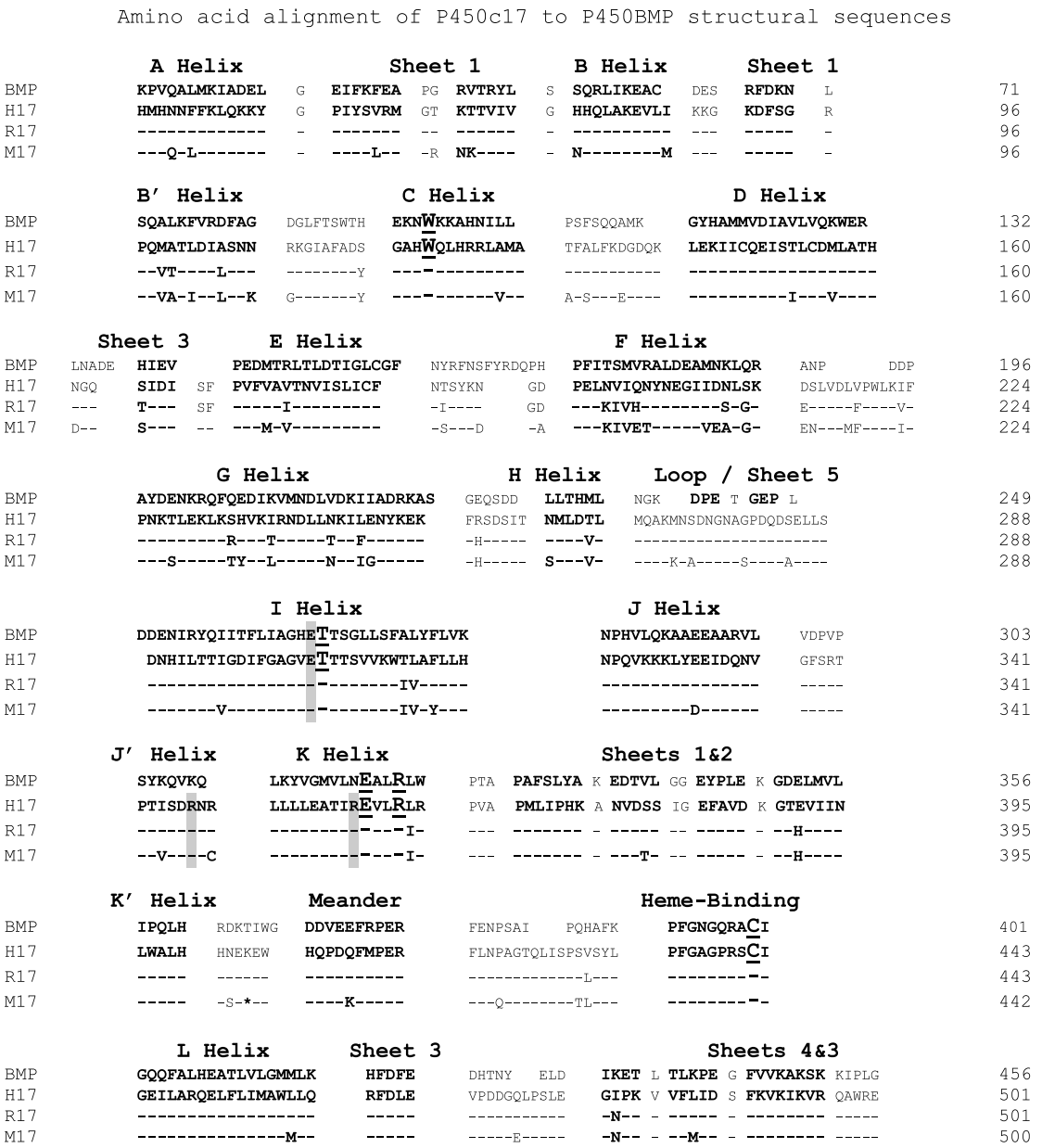

Figure 4 Alignment of human and non-human primate P450c17 sequences to a bacterial model of a class II P450. Note that the most widely conserved Class II residues underlined in the BMP sequence are fully conserved in the human and non-human primate sequences. Residues making up the known (BMP) and predicted (Human) core α-helices and β-sheets are shown in bold. *Amino acid deletion in marmoset sequence, – amino acid conserved relative to human. H-Human (GenBank accession number NM_000102), R-Rhesus (accession number AY746983), M-marmoset (accession number AY746982). Modified from alignments proposed by Auchus & Miller (1999). In addition the three amino acid positions mostly associated with human lyase activity are shown with grey highlighting and are completely conserved.

deficiency in the human population was identified only recently. Only upon sequencing and cloning of P450c17 from these patients has it been possible to definitively distinguish between complete P450c17 deficiency (i.e. 17α-hydroxylase and 17,20-lyase deficiencies) and isolated 17,20-lyase deficiency (Geller *et al.* 1997, Gupta *et al.* 2001).

Deficiencies in human CYP17 activities have been linked to mutations in its primary coding sequence (Geller *et al.* 1997, Yamaguchi *et al.* 1998, Biason-Lauber *et al.* 2000, van den Akker *et al.* 2002). Examples include mutations at arginine residue 347 to histidine or cytosine (R347H or R347C; Geller *et al.* 1997) and glutamate at residue 305 to

Table 1 Mutations in the CYP17 protein coding sequence reported with combined 17α-hydroxylase/17,20-lyase deficiency or isolated 17,20-lyase deficiency in humans. The complete list of mutations summarizes those known to effect human P450c17 hydroxylase or 17,20 lyase activity. Those mutations highlighted in bold are those associated with isolated 17,20-lyase deficiency in humans. Activities are expressed as percentages

	17α-Hydroxylase activity	17,20-Lyase activity	Site of mutation
Mutation			
P35L	38	33	Membrane
Y64S	15	NR	Membrane
G90N	<1	<1	NR
F93C	11	10	Substrate binding
R96W	25	25	NR
S106P	<1	<1	Substrate binding
F114V	2·2	<1	Substrate binding
D116V	37·7	10·7	Substrate binding
N177D	10	10	Substrate binding
E305G	Approximate to wild type	<1	Substrate binding
Y329D	5	5	NR
P342T	20	20	NR
R347H	65	<5	Redox
	44·1	<1	
R347C	13·6	<1	Redox
R358Q	65	<5	Redox
R362C	<1	<1	NR
H373L	<1	<1	Heme
W406R	<1	<1	NR
P409R	<1	<1	Substrate binding
R416C	8	10	Unknown
F417C	<1	<1	Heme
P428L	<1	<1	NR
R440H	<1	<1	Heme
R496C	<10	<10	Heme
R496H	**38**	**33**	Heme

The site of the mutation, where known, is given: membrane (membrane insertion domain); substrate binding (substrate binding domain); redox (POR/CYTB5 binding domain); heme (heme binding domain); NR (not reported) (modified from Biason-Lauber *et al.* (2000), van den Akker *et al.* (2002), Martin *et al.* (2003) and Sherbet *et al.* (2003)). See also Figs 3 and 4.

glycine (E305G; Sherbet *et al.* 2003). These specific mutations yield isolated 17,20-lyase deficiency, while all others result in partial or complete abolishment of both CYP17 enzymatic activities (Table 1). Of note, the 305, 347 and 358 residues considered important to maintaining lyase activity in the human are all highly conserved in the marmoset sequence, as they are in rhesus (Fig. 3).

Effects of CYTB5 on lyase activity in marmoset versus rhesus P450c17

Our own data (Pattison *et al.* 2005, 2007) suggest that in the marmoset, expression of CYTB5 may well contribute to DHEA production *in vivo*. It is therefore of considerable relevance that the amino acids at position 347 and 358 in human that are important for P450c17 interactions with CYTB5 are highly conserved in both marmosets and rhesus monkeys. We have recently examined for the first time the possible role of CYTB5 in selectively enhancing the 17,20-lyase activity of marmoset P450c17 compared with the better characterized human and rhesus literature and specifically, by parallel studies alongside parallel preparations of rhesus P450c17. While the rhesus *CYP17* cDNA sequence generated at University of Wisconsin–Madison by Pattison *et al.* (2004) (GenBank accession number AY746983) has one nucleotide difference from that previously published (accession number AF458332), it matches the sequence independently obtained at the University of California, Davis using different animals and reagents (A J Conley, unpublished data). Furthermore, the predicted amino acid sequences of *all* these rhesus *CYP17* cDNA's are identical.

One consideration in our own biochemical characterization of wild-type (and *c-myc* tagged) CYP17 in mammalian HEK 293 host cells in the absence or presence of added CYTB5 was that we did so in intact cells, so preserving as best we could a more physiologically relevant cellular environment. While much work has previously been published characterizing rhesus CYP17 activity on a biomolecular level, those experiments mostly utilized the CYP17 constructs transformed in yeast and cell microsomes prepared for subsequent analysis (Arlt *et al.* 2002). With such considerations in mind, we have shown in HEK 293 cells transfected only with CYP17 constructs from marmoset or rhesus adrenals, that in each case P4 substrate is quantitatively converted by intact cells to an equal amount of 17OHP4, but not to detectable amounts of androstenedione (Fig. 5). Our rhesus data concur with that of Arlt *et al.* (2002) suggesting relatively deficient delta-4-lyase activity along with human, chimpanzee, and baboon, and we now further confirm that this same relative deficiency is seen in New World marmosets. As expected, both rhesus and marmoset CYP17 convert P5 substrate to 17OHP5 and thereafter to DHEA (Fig. 5).

In addition to the aforementioned observation, that lyase activity in marmoset adrenal microsomes correlates with the microsomal CYTB5 expression, Nguyen *et al.* (2009) have confirmed that adding exogenous recombinant purified CYTB5 to male marmoset adrenal microsomes increases lyase activity to levels similar to that achieved in the rhesus adrenal. We have additionally shown that *CYTB5* cDNA co-expression in HEK293 cells also enhances the 17,20-lyase activity of both marmoset and rhesus CYP17 in a biphasic manner (Nguyen *et al.* 2009; see also Fig. 6 for comparative results with *myc* tagged CYP17, so allowing species-independent co-normalization for protein). Such biphasic activation is consistent with the pattern reported by Soucy & Luu-The (2000) for human CYP17. While Soucy & Luu-The (2000) also co-transfected CYP17 with POR and/or CYTB5, they co-transfected CYP17 with CYTB5 alone and found that CYP17 activity was attenuated above an optimal co-transfection ratio. They postulated that POR and CYTB5 may compete for electrons, thus too high a ratio of POR to CYTB5 limits the activity based on electron availability. Of note, when we compare the relative

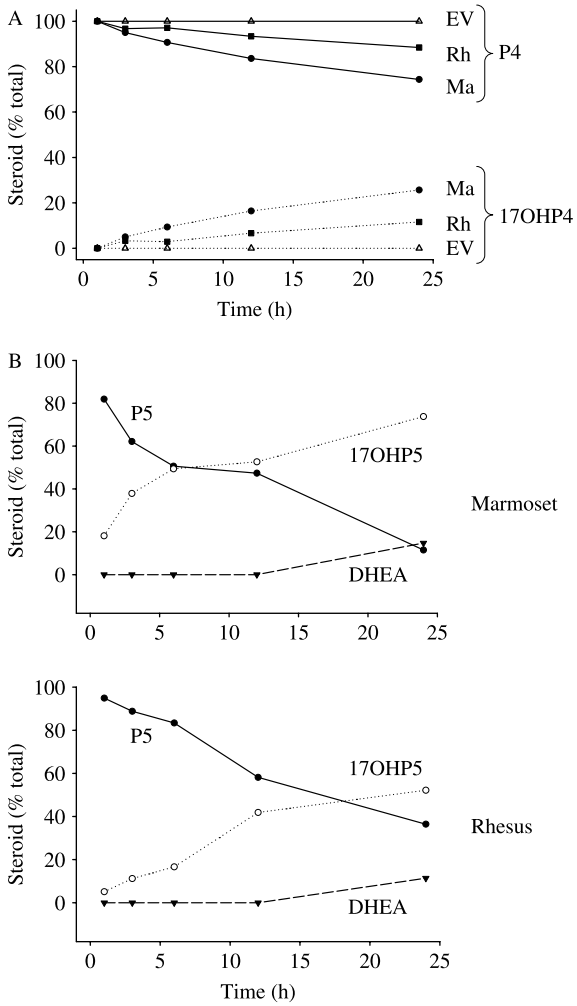

Figure 5 Metabolism of progesterone and pregnenolone by marmoset and rhesus P450c17 clones. (Panel A) Time course for progesterone metabolism. Progesterone, solid line; 17α-hydroxyprogesterone, dotted line; empty vector, open triangle; rhesus, closed square; marmoset, closed circle. HEK 293 cells were transfected in duplicate with primate *CYP17* cDNAs or empty vector (EV) for 48 h, and then incubated with 20 μM progesterone for 1, 3, 6, 12, or 24 h. Values are expressed as a percent of the total steroid products detected. Metabolism of progesterone was matched by increases in 17α-hydroxyprogesterone and no androstenedione was detected, confirming delta-4-lyase deficiency in both marmoset and rhesus *CYP17* cDNAs. (Panel B) Time course for pregnenolone metabolism. Pregnenolone, closed circles with solid line; 17α-hydroxypregnenolone, open circle with dotted line; DHEA, closed triangle with dashed line. HEK 293 cells transfected in duplicate with (A) marmoset or (B) rhesus *CYP17* cDNA for 48 h were then incubated with 20 μM pregnenolone for 1, 3, 6, 12, or 24 h. Values are expressed as the percent of total steroid products detected. Approximately, 80–85 and 60% of the pregnenolone substrate was metabolized to 17α-hydroxypregnenolone and DHEA by (A) marmoset CYP17 and (B) rhesus CYP17 respectively. No other steroid products were detected.

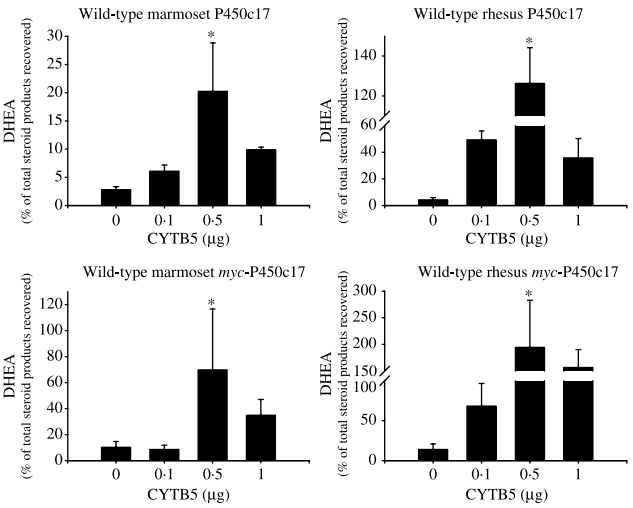

Figure 6 Increasing concentrations of co-transfected CYTB5 enhance 17,20-lyase activity. Upper panels show activity of wild-type *CYP17* cDNA clones (as indicated) in HEK293 cells, and lower panels show activity of the corresponding clones with an additional c-myc tag. Addition of CYTB5 increased 17,20-lyase activity in all four primate cDNAs to a maximal enhancement at 0·5 μg of co-transfected CYTB5 ($P<0·05$). Further addition of CYTB5, up to 1 μg, attenuated 17,20-lyase activity. Values are from $n=3$ independent replicates, 48 h in culture to allow full expression of the plasmids, with a subsequent 24 h incubation with 20 μM P5 substrate. All data are corrected for P450c17 protein expression using antisera to P450c17 (top) or myc tag (Bottom), with $P<0·05$ significance relative to control. Marmoset 17,20-lyase activity was 20–50% of that observed for rhesus CYP17 clones.

magnitude of lyase activity in the presence of added CYTB5 in our studies of intact HEK 293 cells, the level achieved for marmoset normalized using myc antibody is about 20–50% of that observed in parallel for the rhesus clones normalized using the same antibody. Thus, our combined data on marmoset P450c17 are clearly consistent with CYTB5 being a regulator of 17,20-lyase activity and yet still explain why the circulating level of DHEA/DS in female marmosets is far lower than those observed in rhesus monkeys or humans.

Summary

Just a few short years ago, marmoset adrenal physiology was not well characterized and its use as a model for human studies was still questionable. This review has summarized recent advances in our understanding of the physiological and molecular mechanisms regulating steroidogenesis in the marmoset adrenal cortex in general, and the control of 17,20-lyase activity by CYTB5, in particular. There is clear evidence that marmoset P450c17 is of similar activity, substrate specificity and function as P450c17 in rhesus monkeys and humans; its induction and expression in response to steroidogenic agonists is also similar. While it is still not possible to comment on non-protein-coding *CYP17*

gene sequences in general or the promoter in particular, it seems likely that induction of marmoset P450c17 is under similar endocrine control as in humans and rhesus monkeys.

The similarities between P450c17 enzymes across the primate order are notable, and so it may be argued that marmosets are in many ways similar to humans with regard to adrenocortical function. Nonetheless, there are also differences, namely the function of the ZR in adults and the female-specific and ovary-mediated controls of ZR function. Yet even these differences may not be as great as they would appear, given that increases in DS have been observed late in the menopausal transition among women (Lasley *et al.* 2002) and female rhesus (Shideler *et al.* 2001), when ovarian function ceases. The very fact that marmoset studies reveal ovarian related changes in adult ZR function and the additional correlation of changes in 17,20-lyase activity with zonal CYTB5 expression substantially underscore the importance of CYTB5 as a major regulator of 17,20-lyase activity *in vivo*. The question of the endocrine mechanisms and particularly the ovarian factors that control marmoset 17,20-lyase activity is now foremost on our list of future studies. It should also be stated that in spite of differences between marmoset and human adrenal function, such further studies in marmoset are not just academic. Humans and non-human primates studied to date generally exhibit an age-related decline in circulating adrenal DHEA that occurs concomitantly with decreases in cardiovascular health, immunity, and bone health (reviewed in Dharia & Parker (2004)). The mechanisms by which DHEA production in humans is turned on in the fetal adrenal, turned off in the neonatal adrenal, turned on during adrenarche and turned off with increasing age (not withstanding transient perimenopausla increases) are still unknown. The marmoset adrenal mimics the human adrenal in the fetal and neonatal period, yet adrenarche does not occur as a single remarkable event (Levine *et al.* 1982, Pattison *et al.* 2005). Indeed, the very fact that male marmosets do not express a histological or functional ZR (i.e. low CYTB5, low DHEA), while reproductively active female marmosets express a rudimentary ZR by both criteria (Pattison *et al.* 2005, 2007), and the further finding that ovariectomy in female marmosets 'turns on' a ZR phenotype associated with increasing CYTB5 expression, means that study groups can readily be created with different 'ZR functions' in a way unparalleled by other non-human primate species. Studies of marmosets alone as well as in tandem with comparative studies of rhesus monkeys provide powerful means to establish the endocrine mechanisms that both turn on and turn off adrenal ZR function/DHEA production, and at least one such mechanism involves regulation of CYTB5 expression. Fully exploring the similarities and differences between marmoset, rhesus, and human adrenocortical function may indeed be the key that is necessary to finally reveal what mechanisms underlie normal and abnormal human adrenal ZR development and function, and the relationship to gonadal function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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