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NEW RESEARCH HORIZON ***Review***

Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives

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Steroid hormones are synthesized in the adrenal gland, gonads, placenta and brain and are critical for normal reproductive function and bodily homeostasis. The steroidogenic acute regulatory (StAR) protein regulates the rate-limiting step in steroid biosynthesis, i.e. the delivery of cholesterol from the outer to the inner mitochondrial membrane. The expression of the StAR protein is predominantly regulated by cAMP-dependent mechanisms in the adrenal and gonads. Whereas StAR plays an indispensable role in the regulation of steroid biosynthesis, a complete understanding of the regulation of its expression and function in steroidogenesis is not available. It has become clear that the regulation of StAR gene expression is a complex process that involves the interaction of a diversity of hormones and multiple signaling pathways that coordinate the cooperation and interaction of transcriptional machinery, as well as a number of post-transcriptional mechanisms that govern mRNA and protein expression. However, information is lacking on how the StAR gene is regulated *in vivo* such that it is expressed at appropriate times during development and is confined to the steroidogenic cells. Thus, it is not surprising that the precise mechanism involved in the regulation of StAR gene has not yet been established, which is the key to understanding the regulation of steroidogenesis in the context of both male and female development and function.

Key words: StAR gene expression / cAMP signaling / AKAP / transcription / translation

Introduction

Steroidogenic endocrine tissues such as the adrenal gland and the gonads respond to trophic hormones and other external stimuli with a rapid surge in steroid hormone production (Leung and Steele, 1992; Saez, 1994; Cooke, 1999; Ascoli *et al.*, 2002). This acute steroidogenic response is now known to be predominantly dictated by steroidogenic acute regulatory (StAR), a rapidly synthesized labile phosphoprotein whose expression, activation and extinction is regulated by protein kinase A (PKA), PKC, as well as a host of other signaling pathways (reviewed in Stocco and Clark, 1996; Manna and Stocco, 2005). In defining StAR's role, studies have demonstrated a tight correlation between the synthesis of StAR protein and the synthesis of steroids, and StAR expression is primarily associated with steroidogenic tissues in vertebrates. Consequently, the StAR protein plays a vital role in the regulation of steroid hormones required for life itself, in the case of adrenal steroids, and for maintaining reproductive capacity, in the case of gonadal steroids (Clark *et al.*, 1994; Stocco and Clark, 1996; Manna and Stocco, 2005). The crucial role of StAR in the regulation of steroidogenesis has been obtained from patients suffering from lipoid congenital adrenal hyperplasia (lipoid CAH), an autosomal recessive disorder in which both adrenal and gonadal steroid biosyntheses are severely impaired due to mutations in the StAR gene (Lin *et al.*, 1995; Bose *et al.*, 1996). The targeted disruption of the StAR gene in mouse results in a phenotype that is essentially identical to that found in lipoid CAH in humans (Caron *et al.*, 1997; Hasegawa *et al.*, 2000). To date, dozens of nonsense and deletion mutations have been identified in the StAR gene that cause lipoid CAH in humans, and the incidence of this disease has been shown to be higher in the people of Japanese, Korean and Palestinian ancestry (Bose *et al.*, 1996; Miller and Strauss, 1999; Stocco, 2002). It has been demonstrated that the mutant StAR proteins, in contrast to the wild type, were completely inactive in promoting steroid synthesis (Lin *et al.*, 1995; Bose *et al.*, 1996).

The expression of the StAR protein is predominantly associated with the steroid-producing cells of the adrenal, ovary and testis in the adult (Clark *et al.*, 1995b, Hasegawa *et al.*, 2000; Manna and Stocco, 2005). During development, StAR mRNA has first been detected in mouse embryos at Day 10.5 (E10.5) in the urogenital ridge, a region that ultimately gives rise to the adrenals, gonads and

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a portion of the kidneys (Clark et al., 1995b). StAR expression is confined in the ovary at puberty and it has been shown to be expressed during various ovulatory phases, in the interstitum, atretic follicles, granulosa and theca and corpora luteal cells (Balasubramanian et al., 1997; Pollack et al., 1997; Ronen-Fuhrmann et al., 1998; Kerban et al., 1999; Sekar et al., 2000; Devoto et al., 2001). In the human ovary, the expression of StAR is regulated throughout the luteal phase and plays a key role in controlling luteal progesterone production during the development and demise of the corpus luteum (Devoto et al., 2002; Kohen et al., 2003; Sierralta et al., 2005). StAR mRNA is detected at E12.5 in the adrenal primordia and later is expressed in adrenal glomerulosa and fasciculata-reticularis (Pollack et al., 1997; Lehoux et al., 1998; Zhang et al., 2003). Moreover, the physiological changes in the synthesis of steroid hormones are closely linked with alterations in StAR expression, suggesting tissue-specific importance of the StAR protein during development as well as in reproduction.

In the adrenal and gonads, acute steroidogenesis is mediated by mechanisms that enhance the transcription, translation or the activity of StAR (Clark et al., 1995b; Manna et al., 2002a, 2006a; Manna and Stocco, 2005). It should further be noted that while the inhibition of the StAR protein at the level of transcription or translation results in a dramatic decrease in steroid biosynthesis in these tissues, ~10% of steroid production occurs through StAR-independent mechanisms (Lin et al., 1995; Clark et al., 1997; Manna et al., 2001). There is now a wealth of information supporting the concept that the regulation of StAR directs acute steroidogenesis, but we are still far from being able to explain why and how certain tissues express StAR and the precision with which they do so. This review will focus on the current level of understanding concerning the regulation of StAR in light of prospective research and will discuss viewpoints that may help to unravel the mechanisms governing StAR expression and steroidogenesis.

Transcription of the StAR gene

Several lines of evidence demonstrate the involvement of multiple transcription factors and signaling pathways in StAR expression and steroidogenesis (Sugawara et al., 1995; Manna et al., 2003b, 2006a, 2009a, b; Stocco et al., 2005; Martin et al., 2008); however, the regulatory mechanisms involved in cAMP-mediated StAR transcription remain unresolved. Transcriptional regulation, a key control mechanism in fundamental biological processes, is mediated by a family of cAMP-responsive nuclear factors in response to the stimulation of the adenylate cyclase signaling pathway. The transcription of the StAR gene utilizes enhancer elements to 'switch on' and silencer elements to 'switch off' gene expression (Manna et al., 2003b, 2009a; Hiroi et al., 2004; Silverman et al., 2006; Martin et al., 2008). The activation of transcription by cAMP signaling is classically mediated through the interaction of the cAMP response element (CRE)-binding protein (CREB) with a conserved CRE (TGACGTCA), or a minor variation thereof, found in the promoter region of cAMP-responsive genes (Montminy et al., 1986; Meyer and Habener, 1993; Montminy, 1997; De Cesare and Sassone-Corsi, 2000). The StAR promoter sequences of mouse (Clark et al., 1994), human (Sugawara et al., 1995) and rat (Sandhoff et al., 1998) exhibit extensive homology; however, they lack a consensus CRE and as such resemble the promoters of several steroid hydroxylase genes that are regulated by cAMP signaling

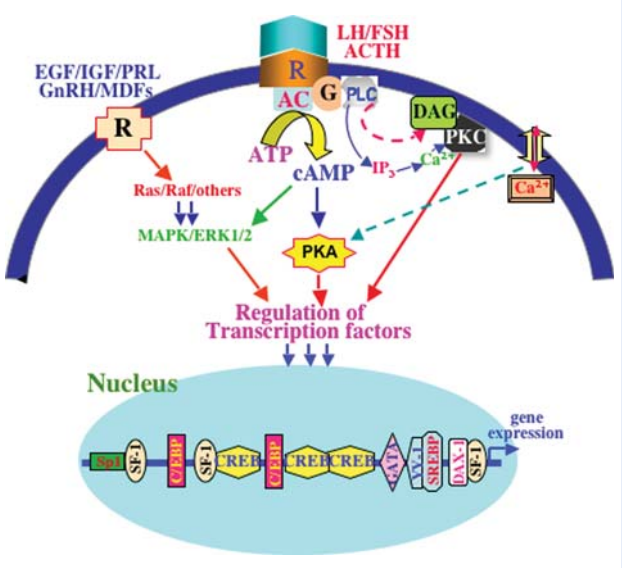

Figure 1 A schematic model illustrating multiple signaling pathways in regulating StAR gene transcription. The interaction of trophic hormones [luteinizing hormone(LH)/adrenocorticotropic hormone(ACTH)] with specific membrane receptors results in the activation of coupled G proteins (G), which in turn activates membrane-associated adenylyl cyclase (AC) that catalyzes cAMP formation from ATP. cAMP then activates protein kinase A (PKA), which results in the phosphorylation of a number of transcription factors. The concerted action of multiple transcription factors, and their interaction with most, if not all, of the cis-regulatory elements, appears to be involved in regulating StAR gene expression. cAMP-dependent mechanisms predominantly regulate StAR expression and steroidogenesis in steroidogenic tissue. The PKC pathway is involved in regulating transcription of the StAR gene. Ca^{2+} signaling has been shown to be effectively involved in modulating trophic hormone-stimulated steroidogenesis. Epidermal growth factor (EGF), insulin-like growth factor (IGF), prolactin (PRL), gonadotrophin-releasing hormone (GnRH) and macrophage-derived factors (MDFs) bind to specific membrane receptors (R), activate a cascade of protein kinases (Ras/Raf/others; MAPK/ERK) and have been demonstrated to function in the regulation of StAR expression and steroid biosynthesis.

(Waterman, 1994), suggesting the involvement of alternate mechanisms in cAMP responsiveness. In lieu of a consensus CRE, a number of positive and negative factors (which bind within the region that is ~200 bp upstream of the transcription start site) as well as signaling processes have been reported in transcriptional regulation of the StAR gene (Fig. 1) (Zazopoulos et al., 1997; Christenson et al., 2001; Shea-Eaton et al., 2002; Manna et al., 2003a, b, 2004, 2009a; Hiroi et al., 2004; Silverman et al., 2006; Martin et al., 2008).

The cAMP-responsive region of the StAR promoter contains a highly conserved overlapping motif (5′-TGACTGATGA-3′; −81/−72 bp in the mouse) that recognizes the CRE [CREB/CRE modulator (CREM)/activating transcription factor 1], activator protein 1 (AP-1, Fos and Jun) and CCAAT/enhancer-binding protein β (C/EBPβ) families of proteins (Manna et al., 2003a, 2004; Hiroi et al., 2004; Clem et al., 2005; Silverman et al., 2006; Manna and Stocco, 2008; Yivgi-Ohana et al., 2009). These proteins belong

to the basic leucine zipper (bZIP) family of transcription factors, which are structurally similar and can interact with themselves, or each other, to form selective dimers that will result in a variety of transcriptional responses (Dwarki *et al*., 1990; Hai and Curran, 1991; Masquilier and Sassone-Corsi, 1992; Millhouse *et al*., 1998; Rutberg *et al*., 1999; Manna *et al*., 2009b). The expression and activities of these proteins are mediated by a number of extracellular signals and by the phosphorylation of these proteins on several different Ser and Thr residues by multiple kinases (Abate *et al*., 1993; Chrivia *et al*., 1993; Roesler, 2001; Wilson *et al*., 2002; Johannessen *et al*., 2004; Tang *et al*., 2005; Manna *et al*., 2006b). The activity of these bZIP proteins is induced by the transcriptional co-activators, CREB binding protein (CBP) and its functional homolog p300, which possess histone acetyltransferase activity and communicate between the transcription factors and the basal transcription machinery in order to promote StAR gene transcription (Hiroi *et al*., 2004; Clem *et al*., 2005; Silverman *et al*., 2006; Manna and Stocco, 2007). While histone acetylation plays a major role in cAMP-mediated steroidogenesis, the inhibition of histone deacetylase activity by trichostatin A (TSA) is also effective in increasing StAR expression and steroid synthesis in mouse Leydig cells (Manna and Stocco, unpublished results; Clem and Clark, 2006; Liu *et al*., 2007). In addition, we have observed that TSA markedly enhances $(Bu)_2$cAMP-stimulated StAR expression and steroid synthesis, suggesting a more prominent role for histone deacetylation in steroidogenesis. However, its mechanism of action has yet to be discerned.

CREB/CREM/ATF plays a major role in a variety of physiological functions including growth and development, and is activated upon phosphorylation by a number of extracellular stimuli and environmental conditions (Nantel *et al*., 1996; Sanyal *et al*., 2002; Johannessen *et al*., 2004; Manna *et al*., 2004; Sassone-Corsi, 2005; Hogeveen and Sassone-Corsi, 2006; Kehat *et al*., 2006). Specifically, increased transcriptional activity is observed following the phosphorylation of CREB (P-CREB) at Ser133 or CREM at Ser117, events that are indispensable for their interactions with CBP/p300 (Gonzalez and Montminy, 1989; Chrivia *et al*., 1993; Kwok *et al*., 1994; Parker *et al*., 1996; Fimia *et al*., 1999). In steroidogenic cells, both PKA and PKC signaling can increase the P-CREB in a time-dependent manner, and this event correlates tightly with P-CREB and CBP's association with the proximal StAR promoter (Hiroi *et al*., 2004; Clem *et al*., 2005; Manna *et al*., 2006b; Silverman *et al*., 2006; Sugawara *et al*., 2006; Manna and Stocco, 2007). Transgenic mice expressing a non-phosphorylatable mutant of CREB (CREB-M1; $Ser^{133} \rightarrow Ala$) develop somatotroph hypoplasia and dwarfism, demonstrating the physiological relevance of CREB function in the developmental processes (Struthers *et al*., 1991). Likewise, CREB-M1 has been shown to diminish the steroidogenic response, indicating that the StAR gene is dependent on P-CREB or a similar CRE-binding protein for proper induction. Studies have shown that P-CREB and P-Fos/Jun-DNA interactions result in CBP recruitment to the StAR promoter and are associated with histone acetylase activity that facilitates chromatin remodeling and thus increases StAR transcription (Hiroi *et al*., 2004; Clem *et al*., 2005; Manna and Stocco, 2007, 2008).

The canonical CREB/ATF site (TGACGTCA) differs from the Fos and Jun consensus motif (TGACTCA) by one nucleotide, and thus overlap and/or crosstalk-affecting transcription can occur (Dwarki *et al*., 1990; Hai and Curran, 1991; Masquilier and Sassone-Corsi, 1992; Millhouse *et al*., 1998; Rutberg *et al*., 1999; Manna and Stocco, 2007). Fos and Jun play central roles in proliferation, differentiation and transformation and are regulated in a cell-type-specific manner. The c-Jun proto-oncogene, but not other Fos and Jun members, is critically involved in regulating steroidogenesis in Leydig and adrenal cells (Lehoux *et al*., 1998; Manna and Stocco, 2008). In accordance with this, mice lacking c-Jun are embryonic lethal with embryos dying between mid and late gestation, underscoring the importance of c-Jun in developmental processes (Hilberg *et al*., 1993; Johnson *et al*., 1993). The activation of either the PKA and PKC pathways increases the phosphorylation of c-Jun at Ser63 and c-Fos at Thr325, in turn recruiting CBP to the StAR promoter, and this progression of events serves to regulate StAR gene transcription (Clem *et al*., 2005; Manna *et al*., 2006b; Manna and Stocco, 2007, 2008). Phosphorylation of Fos and Jun also alters their capacity to interact with other transcription factor(s), ultimately affecting their dimerization and DNA-binding specificity (Hai and Curran, 1991; Hunter and Karin, 1992; Masquilier and Sassone-Corsi, 1992; Rutberg *et al*., 1999; Hess *et al*., 2004). Consequently, crosstalk between CREB and c-Fos/c-Jun has been demonstrated to be associated with both gain-of-function and loss-of-function on a single cis-element in fine tuning the trans-regulatory events involved in StAR gene expression (Manna and Stocco, 2007).

The overlapping CREB/ATF and Fos/Jun binding region in the mouse StAR promoter (−81/−72 bp) is also a target of C/EBPβ, a member of the C/EBP family protein. C/EBPα and C/EBPβ are expressed in steroidogenic cells, and the levels of C/EBPβ in the nucleus are increased by LH and cAMP analogs (Piontkewitz *et al*., 1996; Nalbant *et al*., 1998). Also, two putative C/EBP-binding sites have been identified in the human (Christenson *et al*., 1999) StAR promoter, and the roles of both C/EBPα and C/EBPβ are demonstrated in StAR gene transcription (Christenson *et al*., 1999; Reinhart *et al*., 1999; Silverman *et al*., 1999). In female mice, the disruption of either C/EBPα or C/EBPβ prevents normal reproductive development, leading to reduced or halted ovulation and an inability to form the corpus luteum (Piontkewitz *et al*., 1996; Sterneck *et al*., 1997). The phosphorylation of C/EBPβ at Thr325 has been shown to increase the association of C/EBPβ with the proximal StAR promoter (Tremblay *et al*., 2002; Hsu *et al*., 2008). Furthermore, CBP/p300 is involved in the trans-activation of C/EBPβ and GATA-4, which also induces StAR gene expression (Silverman *et al*., 2006). It is tempting to speculate that other factors, in addition to these bZIP proteins, may also bind to the proximal region of the StAR promoter, become phosphorylated in response to cAMP signaling (for example, SF-1, GATA-4) and enhance the recruitment of CBP/p300 to the StAR promoter.

CREB/CREM, Fos/Jun and C/EBPβ interact with each other in addition to a numerous array of transcription factors, and through these interactions, they confer a gradient of effects at the level of StAR gene transcription (Reinhart *et al*., 1999; Manna *et al*., 2003a, 2004; Clem *et al*., 2005; Silverman *et al*., 2006; Manna and Stocco, 2007). It has been demonstrated that CRE DNA-binding proteins heterodimerize with Fos/Jun and C/EBPs and result in either repression and/or activation of the transcription of several genes (Masquilier and Sassone-Corsi, 1992; Millhouse *et al*., 1998; Rutberg *et al*., 1999; Ross *et al*., 2001; Wilson *et al*., 2002). We have previously reported that CREB and c-Fos/c-Jun can form heterodimers, bind to the closely

related CRE/AP-1 sequence, alter DNA-binding affinity and result in the repression of StAR gene transcription (Manna and Stocco, 2007). On the other hand, CREB/ATF and C/EBP family members compete for binding to the elements that show sequence similarity to either the CRE or CCAAT motifs that are present within a promoter, and direct the transcription of several genes (Bakker and Parker, 1991; Liu *et al*., 1991; Wilson *et al*., 2002). It is not presently known whether C/EBPβ acts as an activator or repressor in conjunction with CREB and/or Fos/Jun with respect to StAR gene transcription but further investigation may allow us to draw such a distinction. Regardless of the trans-regulatory mechanisms involved, the 5′-flanking −81/−72 bp region of the StAR gene appears to function as a key element in the complex series of processes regulating StAR gene transcription.

Post-transcriptional processing of StAR mRNA

It is clear that the transcriptional regulation of StAR is important in dictating its tissue-specific expression, and that transcriptional events tightly integrate a number of signals in order to control the steroidogenic output in these tissues. Given that StAR mRNA levels rise rapidly in response to the cellular signals that also drive StAR protein expression and steroidogenesis, it is often presumed that StAR transcription and translation are tightly coupled in response to a single signaling event (Nieschlag *et al*., 2004; Manna and Stocco, 2007, 2008). However, it is now being discerned how a number of post-transcriptional mechanisms, such as the polyadenylation [poly(A)] of StAR mRNA and the post-translational modification of StAR protein, team together with a growing number of interacting partners to regulate StAR-mediated steroidogenesis (Fig. 2). Studies have suggested that the ability to regulate StAR mRNA stability could serve as a mechanism for maintaining or enhancing persistent levels of StAR mRNA.

The StAR gene is transcribed from a sequence ranging 3–7.5 kb in length in vertebrates (Clark *et al*., 1995a; Bauer *et al*., 2000; Manna *et al*., 2001, 2002b; Goetz *et al*., 2004). This sequence appears to span seven exons that do not appear to be differentially spliced, although an eighth splice junction following the STOP codon of StAR in the Japanese eel may give rise to an alternative 3′ untranslated region (UTR) (Affaitati *et al*., 2003). Although only one isoform of the StAR protein is observed, northern blots for StAR commonly reveal at least two predominant species of mRNA that, like a large portion of mammalian mRNAs, are derived through the use of one of many different poly(A) sites located in the 3′UTR of the StAR mRNA (Ariyoshi *et al*., 1998; Devoto *et al*., 2001; Tian *et al*., 2005). The stability and subcellular localization of a significant number of mRNAs are strongly influenced by sequences found in their 3′ UTRs, and it is likely that such mechanisms affect the post-transcriptional regulation of StAR (Guhaniyogi and Brewer, 2001; Lutz, 2008). In the rodent, StAR gene alternative poly(A) site usage results in either a shorter, more stable 1.6-kb form or a longer, but more ephemeral 3.5-kb form (Jefcoate *et al*., 2000). In humans, a 1.7-kb StAR appears as the principle transcript in steroidogenic tissues, whereas longer forms of 2.4 and 4.4 kb are observed in the adrenal and gonads, respectively (Sugawara *et al*., 1995; Clark and Combs, 1999). In addition, both the 1.7 and 4.4-kb StAR mRNAs show increased expression in the corpus luteum during early and mid-luteal phase, correlating with increased StAR protein expression and progesterone secretion (Devoto *et al*., 2001). In this manner, StAR appears similar to many other genes where longer 3′UTRs result in reduced protein expression; however, it is interesting to note that the ratio and rates at which these mRNAs are expressed is sensitive to a number of stimuli including cAMP. These observations raise a number of prospects that should be explored, as the ability to control mRNA half-life through mechanisms targeting the 3′UTR could provide steroidogenic cells an extremely sensitive set of tools for controlling StAR protein expression. It has been shown that specific regions in the longer 3′UTR of rodent StAR mRNA are responsible for destabilizing the transcript (Zhao *et al*., 2005a). Included in this region are several putative AU-rich elements (AREs), which in other acutely regulated genes can recruit factors to the mRNA that target it for rapid degradation (Barreau *et al*., 2005). Moreover, other mRNA-binding proteins can differentially stabilize such mRNAs in response to stimulation through binding to such elements. This mechanism appears feasible since the stability of the longer StAR mRNA is enhanced through PKA and MAPK pathways (Zhao *et al*., 2005b, Duan and Jefcoate, 2007). Alternatively, longer 3′UTRs in transcripts frequently possess target sites for microRNAs (miRNAs) that target the mRNA for degradation (Sandberg *et al*., 2008). Although the impact of miRNAs on StAR expression has yet to be examined, prospective miRNA sites have already been identified in the 3′UTR of rodent StAR mRNA (Duan and Jefcoate, 2007; Griffiths-Jones *et al*., 2008). Adding another layer of complexity to this is the possibility that the poly(A) sites in the StAR transcript are preferentially utilized in response to cell signaling or tissue-specific events (Lutz, 2008). The relative strength of a poly(A) site can be gauged by how rapidly the cleavage-poly(A) apparatus is assembled, and it is known that downstream sequences within an mRNA in conjunction with mRNA-binding proteins can regulate the usage of particular poly(A) signals (Chao *et al*., 1999). Therefore, how StAR mRNA stability influences steroidogenesis will be greatly improved by examining the cohort of molecules that can associate with these transcripts.

Currently, nothing is known regarding proteins that bind to StAR mRNA, although several interesting candidates that might uniquely regulate its abundance and expression can be proposed on the basis of sequences in its 3′UTR. One of the destabilizing AREs in the longer 3′UTR of the StAR gene closely resembles that found in the mRNA encoding vascular endothelial growth factor (VEGF) (Duan and Jefcoate, 2007). In the adrenal cortex, ACTH signaling through cAMP can promote the replacement of TPA-induced sequence 11b (TiS11b) with HuR at this ARE in order to stabilize VEGF transcripts and to promote its expression (Cherradi *et al*., 2006). Tis11b, a zinc finger RNA-destabilizing protein, and HuR, a nuclear-cytoplasmic mRNA shuttling protein that stabilizes transcripts, are both observed in multiple steroidogenic cells (Duan and Jefcoate, 2007), leading to the speculation of their involvement in regulating StAR gene expression. In addition to its AREs, the rodent 3′UTR in StAR also reveals putative cytoplasmic polyadenylation elements (CPEs) flanking one of the distal poly(A) signals (Dyson *et al*., unpublished observation). The recruitment of CPE-binding proteins (CPEBs) to cis-elements in the 3′UTR of mRNAs can modulate their translation in response to different stimuli, and plays an indisputable role in regulating gene expression during oogenesis and spermatogenesis

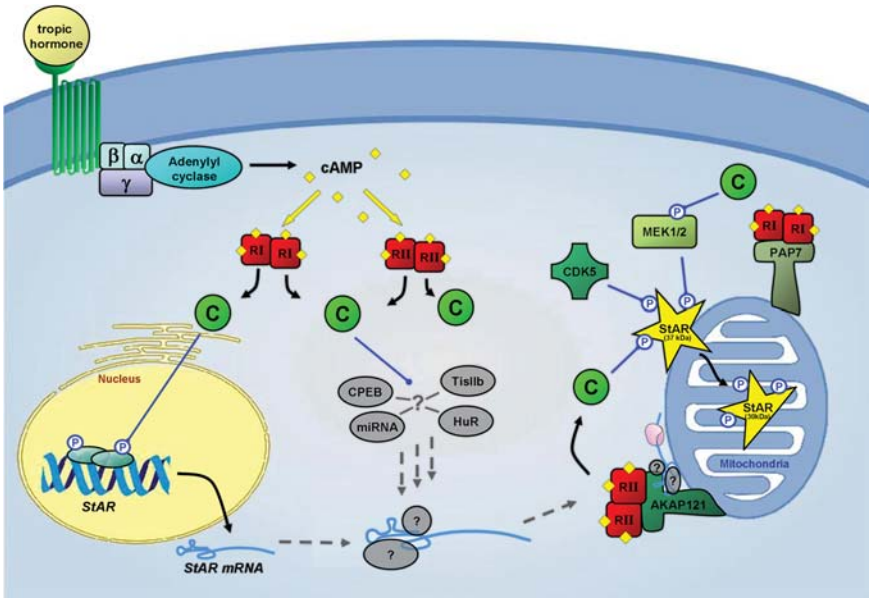

Figure 2 A model illustrating post-transcriptional and post-translational regulation of StAR. Tropic hormone activation initiates multiple signal transduction pathways known to induce StAR gene expression. Central to these pathways is the second messenger cAMP that binds to the regulatory subunits (RI and RII) of PKA resulting in the release of the active catalytic subunits (C). In addition to directing StAR gene transcription, it is predicted that PKA and other signaling events are likely to coordinate the post-transcriptional regulation of StAR mRNA, potentially through miRNAs and mRNA-binding proteins such as Tis11b, HuR and CPEB. The expression of StAR mRNA as a result of this process appears to be enhanced by A-kinase anchor protein (AKAP)121, which may recruit StAR mRNA, possibly in complex with other proteins, to the outer mitochondrial membrane, allowing it to be translated and activated on site. AKAP121 also tethers type II PKA to the mitochondria, which appears to serve a role in enhancing the activation of StAR at the outer mitochondrial membrane through the phosphorylation of Ser195 in StAR. Similarly, acyl-co-enzyme A-binding domain containing 3 (PAP7; also, ABCD3) has been demonstrated to bind type I PKA at the outer mitochondrial, also increasing steroidogenesis. The pool of AKAP-tethered PKA is likely to serve in activating mitochondrial kinase MEK1/2, which has recently been shown to activate StAR by phosphorylating Ser232. Furthermore, StAR activity in Leydig cells has been linked to its association with and phosphorylation by Cdk5; however, the specific target on StAR for this kinase remains unknown. These post-translational modifications to StAR may serve to enhance its stability or its ability to interact with other proteins necessary for cholesterol transport. Alternatively, these events may serve to prolong the duration it takes for StAR to be withdrawn into the mitochondria. The N-terminal region of full-length (37 kDa) StAR targets the protein for importation into the mitochondria and is proteolytically cleaved following StAR's translocation. Thus, although the C-terminal domain (30 kDa) of StAR possesses the intrinsic capacity to promote cholesterol transfer, it is possible that the steroidogenic potential of StAR is maximized by events that cause it to dwell longer at the outer mitochondrial membrane in contact with other proteins known to promote steroidogenesis. (Figures and dashed arrows depicted in gray represent putative targets and interactions.)

(Mendez and Richter, 2001). The observation that CPEB is also expressed in steroid-producing somatic tissues such as the hippocampus, heart and the kidney, however, poses an intriguing link to steroidogenesis and possibly to StAR expression (Gebauer and Richter, 1996; Theis *et al*., 2003; Zearfoss *et al*., 2008).

It is also possible that upon exiting the nucleus, fully processed StAR mRNA is targeted by factors that can enhance or repress its translation. Several proteins in steroidogenic cells have been shown to function in this capacity, such as mevalonate kinase, which can bind and repress the translation of the LH receptor mRNA in granulosa cells (Nair *et al*., 2008). Such a role for this kinase ostensibly allows the integration of signals for cholesterol metabolism with tropic hormone signaling, and it would be of interest to determine whether, through similar mechanisms, it could regulate StAR mRNA as well. DAX-1 has also been shown to shuttle between the nucleus and cytoplasm, where it appears to accompany nascent mRNAs into associations with ribosomes (Lalli *et al*., 2000). Given the impact that DAX-1 has on StAR transcription, it is tempting to speculate that it could also affect StAR expression post-transcriptionally (Zazopoulos *et al*., 1997; Lalli *et al*., 2000; Manna *et al*., 2009a). We have recently observed that the mitochondrial A-kinase anchoring protein 121 (AKAP121) can enhance StAR expression post-transcriptionally (Dyson *et al*., 2008). AKAP121 is unique among its family members owing to an N-terminal KH domain through which it is capable of targeting mRNAs to the mitochondria, most often by binding to unique sequences in the 3′UTRs of these mRNAs (Ginsberg *et al*., 2003). Interestingly, the translation of mRNAs bound to AKAP121 can either be repressed, as is the case for lipoprotein lipase (Unal *et al*., 2008), or enhanced, which occurs with manganese superoxide dismutase (Ginsberg *et al*., 2003). In both Leydig and granulosa cell lines, AKAP121 enhances the expression of StAR protein without appearing to alter StAR mRNA levels, and we predict that AKAP121 serves to coordinate and enhance the translation of StAR at the outer mitochondrial membrane (Dyson *et al*., 2008). The growing evidence that post-transcriptional regulation of mRNAs is necessary to complement transcription, and the ample

possibilities by which StAR could be thus regulated, present us with a number of hypotheses and it is hoped that more progress will be made in this area as continued research overcomes the technical challenges arising in the study of RNA interactions *in vivo*.

Post-translational modification of StAR

The evidence for the post-translational regulation of StAR has become more apparent, as the mechanism by which StAR binds cholesterol and mediates its transfer is subject to multiple factors that can attenuate or enhance the process. At present, a host of proteins including the voltage-dependent anion channel 1 (Bose *et al*., 2008b), the peripheral benzodiazepine receptor (PBR) (Hauet *et al*., 2005; Liu *et al*., 2006), PBR- and PKAR1A-associated protein (PAP7) (Liu *et al*., 2006), phosphate carrier protein (Bose *et al*., 2008b) and hormone-sensitive lipase (HSL) (see what follows) (Shen *et al*., 2003) are thought to functionally or physically interact with StAR. Significant exposition on how these interactions impact steroidogenesis has already been made, and would indicate that the entire complement of proteins involved in this process has not yet been fully defined (Shen *et al*., 2003; Liu *et al*., 2006; Bose *et al*., 2008b). Excellent reviews of these proteins, prospective step-by-step descriptions of how StAR may function and analyses of the physical conformation of StAR have already been made (Baker *et al*., 2007; Miller, 2007; Papadopoulos *et al*., 2007; Bose *et al*., 2008b). To better expand on this, we have herein focused on the specific post-translational modifications that alter StAR itself, and will limit our discussion to the interacting partners pertinent to these post-translational processes.

The most obvious post-translational modification to StAR is its proteolytic processing in the mitochondria. StAR is synthesized as a 37 kDa precursor-protein with an N-terminal mitochondrial-targeting sequence that is cleaved from the remainder of the protein during or after translocation into the mitochondria (Clark *et al*., 1994; Yamazaki *et al*., 2006). The N-terminal domain of StAR also destabilizes the protein in the cytoplasm, likely targeting the full-length protein for rapid removal by the proteasome, and contributing to a short half-life of the 37-kDa form (Clark *et al*., 1994; Stocco and Clark, 1996; Granot *et al*., 2003). This appears to occur in most cell types without StAR being ubiquitinylated, as inhibitors of the proteasome consistently increase StAR levels (Granot *et al*., 2007). The cleavage of the leader sequence results in a 30-kDa peptide largely defined by its cholesterol-binding domain, which shows similarity to other lipid-trafficking proteins, and is known as a StAR-related lipid-transfer (START) domain (Alpy and Tomasetto, 2005). The START domain appears responsible for the mechanics of inducing cholesterol transfer and that it functions at the outer mitochondrial membrane even in the absence of the leader sequence (Arakane *et al*., 1998; Bose *et al*., 2002b, Miller, 2007). Furthermore, the internalization of StAR into the mitochondria does not appear necessary for the transfer of cholesterol. Studies in non-steroidogenic COS (African green monkey kidney) cells demonstrate that StAR importation and cleavage proceed hand in hand, and that mitochondrial internalization serves to neutralize StAR activity by removing it from the outer mitochondrial membrane (Bose *et al*., 2002a, Yamazaki *et al*., 2006). Somewhat at odds with these findings is that the intra-mitochondrial cleavage of StAR appears linked to StAR activity in rat adrenal glands (Artemenko *et al*., 2001). In addition, the loss of the mitochondrial leader sequence, while increasing the stability of StAR, strongly diminishes its ability to promote gonadal and adrenal steroidogenesis in mice (Granot *et al*., 2003; Sasaki *et al*., 2008). Therefore, the impact that the mitochondrial targeting and processing of StAR have on steroidogenesis remains poorly defined.

It is possible that the artificial expression of StAR in non-steroidogenic tissues inaccurately models the mechanism of StAR at the mitochondria; however, the fact that StAR is properly imported and cleaved in COS and other non-steroidogenic cells suggests that any physical properties requiring proteolysis are not limited (Yamazaki *et al*., 2006; Sasaki *et al*., 2008). Alternatively, the equilibrium between the cytoplasmic and mitochondrial pathways that target StAR for degradation may be significantly altered in different cell types such that steroidogenic cells more effectively direct StAR to the mitochondria while removing it from other locations in the cell. Notably, cysteine proteases, such as those present in the lysosome, do not appear to affect the degradation of StAR precursor in hormonally responsive granulosa cells, yet they can strongly reduce StAR precursor abundance in COS cells (Tajima *et al*., 2001; Bose *et al*., 2008b). Given that StAR lacking its targeting sequence functions equivalently to full-length StAR in COS cells but not in steroidogenic cells, it is feasible that the subcellular distribution and proteolysis of StAR are more tightly controlled in steroidogenic tissues (Arakane *et al*., 1996; Sasaki *et al*., 2008). Further research focused on StAR proteolysis in steroidogenic tissues, in particular with regard to the N-terminal domain, should provide insight into how acute steroidogenesis is regulated.

The fundamental research characterizing the acute steroidogenic response to trophic hormone stimulation linked the phosphorylation of newly synthesized proteins to steroid hormone production (Garren *et al*., 1971). Subsequent research was able to rely on this feature to search for phosphoproteins unique to steroidogenic tissues that were synthesized in response to hormone treatment (Pon *et al*., 1986; Alberta *et al*., 1989; Stocco and Sodeman, 1991), and presently StAR appears to be targeted for phosphorylation by several kinases (Fig. 2). Following its discovery, the sequence of StAR confirmed the presence of at least two consensus PKA phosphorylation sites in Ser56/57 and Ser194/195, in murine and human StAR, respectively (Arakane *et al*., 1997). Of these two sites, the phosphorylation of StAR on Ser194 by PKA is essential in order to render the protein fully active in its capacity to support cholesterol transfer, and mutation of this site strongly reduces the ability of StAR to induce cholesterol transport across the mitochondrial membranes both *in vitro* and *in vivo* (Arakane *et al*., 1997; Manna *et al*., 2002a, b; Baker *et al*., 2007). Furthermore, the mutation of Ser195 is one of many point mutations in human StAR that reportedly gives rise to congenital lipoid adrenal hyperplasia (Katsumata *et al*., 2000). We have recently examined how the different isoforms of PKA regulate StAR phosphorylation (Dyson *et al*., 2009). Although both principal subtypes of PKA (either type I or type II as defined by the regulatory subunit present) are seen in steroidogenic tissues, type II PKA appears more efficient at phosphorylating StAR. This appears to be at least partly due to the presence of AKAP121, which can tether type II PKA to the outer mitochondrial membrane, and our current prediction is that type II PKA anchored to the mitochondria ensures the efficient phosphorylation of StAR prior to its importation into the mitochondria (Chen *et al*., 1997; Dyson *et al*., 2009). Another

candidate for regulating the phosphorylation of StAR is PAP7, an AKAP that binds PBR and recruits type I and possibly type II PKA to the mitochondria (Liu *et al*., 2006). PAP7 strongly enhances hormone-induced steroidogenesis, although its direct effects on StAR expression or phosphorylation have yet to be examined. Interestingly, several protein phosphatases have been reported in complex with AKAP121, such as protein phosphatase 1 and protein tyrosine phosphatase D1 (Steen *et al*., 2003; Cardone *et al*., 2004; Bridges *et al*., 2006). Multiple phosphatases have been implicated in StAR expression and steroidogenesis; thus, it may prove useful to revisit the function of these enzymes were they shown to be in complex at the mitochondria (Sayed *et al*., 1998; Castillo *et al*., 2004).

The mechanistic function of StAR's phosphorylation by PKA is not fully understood, and several prospective theories have been put forth. The ability of StAR to bind cholesterol is not affected by the phosphorylation of Ser194/195, and instead it appears that the action of PKA enhances the capacity of StAR to function at the mitochondria (Baker *et al*., 2007). Earlier studies suggest that the phosphorylation of StAR may enhance the stability of the protein, and given its short half-life in the cytoplasm, this would be effective (Clark *et al*., 2001). However, recombinant human StAR bearing an Ser195Ala mutation shows a significantly reduced ability to induce steroidogenesis when added *in vitro* to isolated mitochondria, suggesting that the intrinsic activity of StAR is affected by its phosphorylation (Baker *et al*., 2007). Another possibility is that the phosphorylation of StAR by PKA retards its importation, thereby increasing the duration of time it spends at the outer mitochondrial membrane. A similar phenomenon is observed when the mitochondrial leader sequence is lengthened to slow StAR's import (Bose *et al*., 2002a). Alternatively, the phosphorylation of StAR may enhance its interaction with other proteins at the mitochondria.

Whereas PKA is clearly moderating StAR function at the mitochondria, current evidence suggests that at least two other kinases can directly or indirectly modulate StAR phosphorylation and activity. Studies have shown that StAR itself is a substrate for the extracellular signal-regulated kinases (ERK1/2), which phosphorylate StAR at Ser232 within a highly conserved ERK1/2 docking site and enhance StAR activity at the mitochondria (Poderoso *et al*., 2008, 2009). Whereas the activation of ERK1/2 can result from PKA activity, the phosphorylation of StAR by either enzyme does not appear contingent upon the other, and at the moment it is possible that ERK1/2 and PKA activities serve analogous roles in influencing StAR. However, one notable difference is that StAR's phosphorylation by ERK1/2 requires cholesterol (Poderoso *et al*., 2008). Thus, unlike the phosphorylation of Ser194 by PKA, the action of ERK1/2 likely requires a permissive conformation of StAR to be first induced by its ligand. Hence, it will be interesting to determine whether the phosphorylation at Ser232 alters the affinity of StAR for cholesterol. More recent work examining the role of cyclin-dependent kinase 5 (CDK5) in Leydig cells has suggested that it too may alter StAR phosphorylation (Lin *et al*., 2009). The presence CDK5 in reproductive tissues has resulted in the description of several novel roles for the kinase outside of cell cycle regulation (Zhang *et al*., 1997; Musa *et al*., 2000). In Leydig cells, CDK5 is observed to physically interact with StAR, where it appears to enhance the post-transcriptional expression of StAR (Lin *et al*., 2009). Furthermore, the inhibition of CDK5 activity reduces StAR phosphorylation but not its expression. Whether CDK5 can directly or indirectly phosphorylate StAR will require additional investigation to elucidate its role in acute steroidogenesis.

Role of HSL in steroidogenesis

HSL is a multifunctional enzyme that is highly expressed in several tissues including adipose tissue, adrenal, gonads, where it is responsible for neutral cholesteryl ester hydrolase (NCEH) activity in steroidogenic cells (Osuga *et al*., 2000; Kraemer and Shen, 2002; Li *et al*., 2002; Rao *et al*., 2003). Targeted disruption of HSL in mice results in a lack of NCEH activity in the adrenal and testis, and males are sterile due to oligospermia, indicating that HSL plays essential role in regulating intracellular cholesterol metabolism (Osuga *et al*., 2000). Interestingly, the infertility observed in male HSL knockout mice is not associated with abnormal steroid biosynthesis, but is rather a direct consequence of the loss of HSL on spermatogenesis (Osuga *et al*., 2000; Chung *et al*., 2001). Several lines of evidence demonstrate that hormonal and neuronal control of HSL activity is mediated by phosphorylation of Ser residues in response to PKA signaling (Manna *et al*., unpublished results; Osterlund, 2001; Kraemer and Shen, 2002). Importantly, the hydrolysis of cholesteryl esters stored in lipid droplets serves as an important source of cholesterol, and is often necessary for optimal steroid biosynthesis (Fig. 3). Other cholesterol sources are important as well, and it can be synthesized *de novo* within the cell, or acquired from lipoprotein-derived cholesteryl esters obtained by either receptor-mediated endocytic or selective cellular uptake. In the latter process, circulating lipoproteins [high-density lipoprotein (HDL) or low-density lipoprotein (LDL)] bind to scavenger receptor class B, type I (SR-B1) and release cholesterol esters into the cells (Fig. 3) (Gwynne and Strauss, 1982; Temel *et al*., 1997; Fidge, 1999; Williams *et al*., 1999; Azhar and Reaven, 2002; Rao *et al*., 2003). Receptor-mediated endocytic uptake of lipoprotein-derived cholesteryl esters is processed via the LDL receptor in the human systems (Gwynne and Strauss, 1982; Brown and Goldstein, 1986). The roles of the Niemann–Pick C1 and C2 proteins in cholesterol trafficking via LDL receptor-mediated endocytosis and cleavage of cholesteryl esters by lysosomal acid lipase have also been reported (Watari *et al*., 2000; Gevry and Murphy, 2002).

Regardless of the source of cholesterol utilized for steroidogenesis, the transport of cholesterol from the outer to the inner mitochondrial membrane is the rate-limiting step in steroid biosynthesis and is mediated by the StAR protein (Lin *et al*., 1995; Stocco and Clark, 1996; Christenson and Strauss, 2000; Strauss *et al*., 2003; Miller, 2007). It has previously been shown that higher StAR expression and steroid production in R2C rat Leydig cells under basal conditions are due to constitutive expression of HSL and SR-B1 (Rao *et al*., 2003). It was proposed that these components are involved in the uptake of cholesterol esters from lipoproteins, thereby increasing the availability of cholesterol for steroidogenesis. Nevertheless, both *in vivo* and *in vitro* studies have demonstrated a striking correlation between hormonal induction of SR-B1 expression (associated with enhanced lipid uptake from HDL) and steroid biosynthesis in different steroidogenic cell models (Rigotti *et al*., 1996; Azhar *et al*., 1998; Manna *et al*., 2006b, 2007). Previous studies have demonstrated that the physical interaction of HSL with StAR results in an elevation in hydrolytic activity of HSL and that they both facilitate trafficking of intracellular cholesterol from lipid droplets into the mitochondria (Shen *et al*., 2003). However,

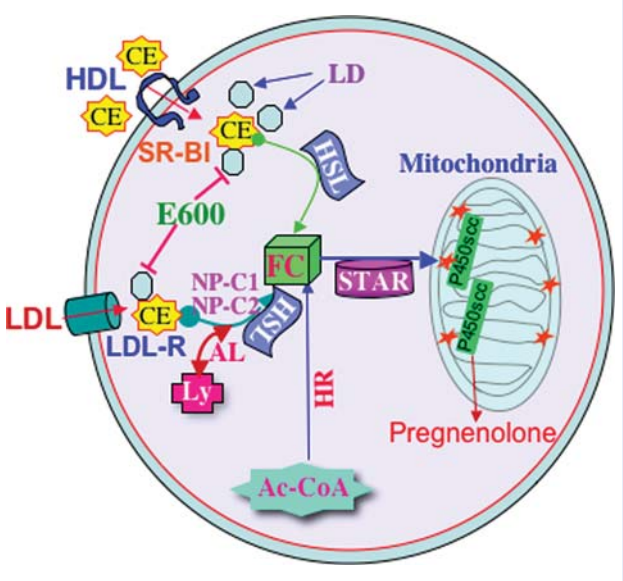

Figure 3 Metabolism of cholesteryl ester (CE) and role of hormone-sensitive lipase (HSL) in steroidogenesis. The hydrolysis of cholesteryl esters stored in lipid droplets is an important source of cholesterol for optimum steroid biosynthesis. HSL is a multifunctional enzyme that is responsible for neutral cholesteryl ester hydrolase activity. E600 blocks the release of cholesterol from lipid droplets (LD) and thus affects StAR expression and steroid synthesis. Circulating lipoproteins (HDL or LDL) bind to scavenger receptor class B, type I (SR-B1) and release cholesteryl esters into the cells. Free cholesterol (FC) for steroid production is mostly obtained in rodents via HDL-mediated cholesteryl ester internalization and followed by cleavage by HSL. However, receptor-mediated uptake of lipoprotein-derived cholesteryl esters is processed via the LDL receptor in the human systems. *De novo* synthesis of cholesterol from acetyl-co-enzyme A (AC-CoA) provides also FC for steroid synthesis. The StAR protein regulates steroidogenesis by controlling the transport of cholesterol from the outer to the inner mitochondrial membrane, the site of the cytochrome P450scc enzyme. Conversion of cholesterol is the first enzymatic step in steroid hormone biosynthesis. LD, lipid droplets; AL, acid lipase; NP-C1 and C2, Niemann–Pick C1 and C2; Ly, lysosome; HR, HMG-co-enzyme A reductase. HDL, high density lipoprotein; LDL, low density lipoprotein.

the precise mechanism through which this is accomplished remains obscure. We have recently observed that the activation of PKA pathway increases the phosphorylation but not synthesis of HSL, concomitant with StAR expression and steroidogenesis in both mouse Leydig and adrenal cells (Manna *et al*., unpublished results). In contrast, the inhibition of HSL activity by E600, which blocks the release of cholesterol from lipid droplets, markedly diminishes StAR expression and steroid biosynthesis. The decrease in the steroidogenic response in connection with HSL was also confirmed by targeted silencing of endogenous HSL using small interfering RNA, further underscoring the importance of HSL in the regulation of StAR expression and steroidogenesis. Whether members of the START domain family, i.e. StarD3 (MLN64), StarD4–StarD6, that are ubiquitously expressed in several tissues (Watari *et al*., 1997; Soccio *et al*., 2002; Zhang *et al*., 2002; Strauss *et al*., 2003) may play roles in cholesterol mobilization to the mitochondria for steroidogenesis requires additional investigation. Indeed, it has recently been demonstrated that StarD6 possesses a similar hydrophobic sterol-binding pocket and C-terminal extension to those of StAR (StarD1), and that StarD6 is fully capable in inducing StAR-like responsiveness involved in steroidogenesis (Bose *et al*., 2008a).

In summarizing these studies, we have attempted to describe how multiple signaling pathways, transcription factors and other possible regulatory elements may serve to regulate StAR-mediated steroidogenesis. Whereas considerable progress has been made towards understanding the regulatory events surrounding steroidogenesis, a complete knowledge of the molecular mechanisms involved in StAR gene transcription, translation and activation is required. Consequently, more information will be required before a clear-cut understanding of how the developmental, tissue-specific and hormone-induced StAR expression is obtained that effectively governs the synthesis of steroid hormones in different steroidogenic tissue.

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