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Review
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Review

Current knowledge on the acute regulation of steroidogenesis†

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Abstract

How rapid induction of steroid hormone biosynthesis occurs in response to trophic hormone stimulation of steroidogenic cells has been a subject of intensive investigation for approximately six decades. A key observation made very early was that acute regulation of steroid biosynthesis required swift and timely synthesis of a new protein whose role appeared to be involved in the delivery of the substrate for all steroid hormones, cholesterol, from the outer to the inner mitochondrial membrane where the process of steroidogenesis begins. It was quickly learned that this transfer of cholesterol to the inner mitochondrial membrane was the regulated and rate-limiting step in steroidogenesis. Following this observation, the quest for this putative regulator protein(s) began in earnest in the late 1950s. This review provides a history of this quest, the candidate proteins that arose over the years and facts surrounding their rise or decline. Only two have persisted—translocator protein (TSPO) and the steroidogenic acute regulatory protein (StAR). We present a detailed summary of the work that has been published for each of these two proteins, the specific data that has appeared in support of their role in cholesterol transport and steroidogenesis, and the ensuing observations that have arisen in recent years that have refuted the role of TSPO in this process. We believe that the only viable candidate that has been shown to be indispensable is the StAR protein. Lastly, we provide our view on what may be the most important questions concerning the acute regulation of steroidogenesis that need to be asked in future.

Summary Sentence

The acute regulation of steroidogenesis in the adrenal and gonads is controlled by cholesterol transfer into the mitochondria and this review covers two decades of research that has demonstrated StAR is indispensable for this process.

Key words: steroidogenic acute regulatory protein (StAR), steroidogenesis, cholesterol, mitochondria, START domain family.

Introduction: the early history

When we were asked to write this review article for the special issue of *Biology of Reproduction* commemorating the 50th Anniversary of the founding of the Society for the Study of Reproduction, a suggested topic for this review was "Steroidogenesis." As steroidogenesis encompasses a very large field and would be far beyond the scope of the parameters outlined for this issue, we have rather confined

the topic to a problem that the three of us have spent a considerable amount of time pursuing over the past years, namely, the acute regulation of steroid hormone biosynthesis. This is an interesting and important topic that spans a period of approximately six decades and has been the subject of intense investigation. The steroid hormones represent an important group of compounds that are synthesized mainly in the adrenal glands and the gonads, but are also synthesized in other tissues in the body. The adrenal steroids, the glucocorticoids and mineralocorticoids, are responsible for regulating carbohydrate metabolism, stress management, and salt balance. Testicular androgenic steroids are responsible for maintaining reproductive function and male secondary sex characteristics, while the ovarian progestins and estrogens are indispensable in maintaining female secondary sex characteristics and reproductive capacity. An additional class of steroids, the neurosteroids, are synthesized in the central nervous system and have specialized functions that appear to be limited to those tissues.

The common characteristic for the synthesis of steroid hormones in all steroidogenic tissues is the utilization of cholesterol as the substrate for steroid formation [1]. The first reaction is the conversion of cholesterol to pregnenolone and is a result of the action of the cytochrome P450 side-chain cleavage enzyme (P450scc; CYP11A1), that is part of the cholesterol side chain cleavage system that resides on the matrix side of the inner mitochondrial membrane (IMM) [2]. Pregnenolone then exits the mitochondria and is converted to progesterone and other steroids in the microsomal compartment of the cell. Also, in some steroidogenic cells, downstream steroids can re-enter the mitochondria and be converted to the final product dependent upon the complement of steroidogenic enzymes present within those cells [3]. These processes are regulated by the pituitary trophic hormones adrenocorticotropin (ACTH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). In the majority of steroidogenic tissues in the body these responses arise as a result of stimulation by the appropriate trophic hormone acting via the cAMP-protein kinase A (PKA) second messenger pathway. In addition, the mineralocorticoid-producing zona glomerulosa cells of the adrenal gland also respond to stimuli that transduce their signals through the Ca^{2+}- signaling and protein kinase C (PKC) pathways. The rapid or acute effects of hormone stimulation occur within minutes and are inhibited by protein synthesis inhibitors such as cycloheximide or puromycin. The acute effects can be distinguished temporally from slower, chronic effects that occur on the order of several hours and involve mechanisms that increase gene transcription and translation of the steroid hydroxylase cytochrome P450 and the steroid dehydrogenase enzymes involved in the biosynthesis of these steroids. The characteristics of the differences between the acute and chronic regulation of steroid hormone biosynthesis have been dealt with in greater detail in several previous review articles [1, 4–7]. The current review will focus only on the mechanisms involved in the acute phase of steroid hormone biosynthesis. The steroidogenic pathway, like many metabolic synthetic pathways, has a rate-limiting step and it was experimentally determined that the regulated and rate-limiting step in steroidogenesis was the delivery of cholesterol from the outer mitochondrial membrane (OMM) to the IMM where the CYP11A1 enzyme is located [2, 8]. A significant period of investigation ensued to determine the nature of the acutely regulated step. Early investigations indicated that ACTH could stimulate the biosynthesis of steroids [9] and most importantly, that acute steroid production had an absolute requirement for the synthesis of new proteins, as mentioned above [10–14]. Further studies demonstrated that this putative regulator protein appeared to function at the level of the delivery of cholesterol to the CYP11A1 enzyme in the IMM [15–19]. Thus, the characterization of the regulated step had a specific target on which to focus, namely, a newly synthesized protein.

Over the next few decades candidate proteins for the acute regulator emerged and included the sterol carrier protein 2 (SCP2) (reviewed in [20]), the steroidogenesis-activator polypeptide (SAP) [21, 22], the peripheral benzodiazepine receptor/translocator protein (PBR/TSPO), and the steroidogenic acute regulatory protein (StAR). Each of these proteins appeared to have characteristics that rendered them as viable candidates. However, subsequent research on SCP2 knockout mice ruled out an involvement for this protein in steroidogenesis [23]. Also, while originally characterized as a small molecular weight peptide [21, 22], SAP was later identified as a fragment of the larger glucose-regulated protein 78 (GRP78) [24]. GRP78 is a regulator of the unfolded protein response [25], and it was demonstrated that GRP78 knockout mice died at an early embryonic stage [26]. Later studies examining GRP78 conditional knockouts indicated a phenotype in oncogenic signaling [27]. Shortly after these observations, studies on SCP2 and SAP significantly waned and, in contrast, StAR and TSPO continued to be studied in great detail over the next three decades.

TSPO and StAR as candidate proteins

TSPO

The peripheral benzodiazepine receptor (PBR), now called the translocator protein (hereafter referred to as TSPO), was first described in the late 1970s (see review [28]). TSPO has a high binding affinity for benzodiazepines but was a distinctly different receptor from the central benzodiazepine receptor, the γ-aminobutyric acid type A receptor/$GABA_A$ receptor. There have been many studies attempting to characterize the pharmacological and physiological functions of TSPO utilizing in vitro steroidogenic cell lines [29, 30]. TSPO has been found to be present in many different tissues, but it appeared to be most abundant in steroidogenic cells and was localized to the mitochondria in those cells [31, 32]. In 1989–1990, studies demonstrated that treatment of adrenocortical cells and testicular Leydig cells with the TSPO ligands PK11195 and Ro5–4864 could stimulate steroid synthesis [29, 33], and expansion of these studies over the next two decades indicated that TSPO ligands could increase steroid hormone production in several different steroidogenic cell types [34–36]. These observations were further corroborated when it was reported that an intracellular TSPO-binding protein, the diazepam-binding inhibitor increased steroid synthesis in steroidogenic cells [36–39]. Knockdown of TSPO expression indicated that the presence of this protein was an absolute requirement for steroid synthesis [40, 41]. Inhibition of TSPO expression could, by itself, inhibit steroid biosynthesis thus indicating its absence could prevent steroidogenesis even in the presence of other proteins (e.g. StAR). This observation was interpreted as demonstrating that TSPO acted downstream from StAR, possibly functioning as a mitochondrial membrane cholesterol channel that received intracellular cholesterol from StAR to be transported to the IMM [34, 42]. At that time, it was not possible to confirm these observations in vivo as attempts to produce TSPO knockout mice were reported to result in early embryonic lethality [43]. Another study that appeared to strongly support the role of TSPO in steroidogenesis demonstrated that steroid production in the constitutively steroid synthesizing cell line, the R2C rat Leydig tumor cell line, occurred as a result of a higher affinity TSPO ligand-binding site in these cells [44]. In later experiments,

disruption of *Tspo* gene in R2C cells almost completely inhibited their ability to produce steroid hormones [41]. It was also reported that TSPO contained a cholesterol-binding amino acid consensus motif that was described by the authors as providing a mechanism for the binding and transport of cholesterol into the mitochondria [45]. Consideration of all these reported characteristics resulted in the authors concluding that TSPO played an indispensable role in cholesterol transfer to the IMM and thus, steroidogenesis [41, 43, 46]. This conclusion has persisted for over two decades with a number of models having been proposed as to how TSPO functioned in cholesterol transport into the mitochondria.

A more recent model that has been proposed for the role of TSPO in cholesterol transport to the IMM involves its participation in an 800 kDa protein complex [47]. Proteins in this putative complex, that was named the "transduceosome," included TSPO, the voltage-dependent anion channel 1 (VDAC1), P450scc, the ATPase family AAA domain-containing protein 3A (ATAD3A), and optic atrophy type 1 proteins. Knockdown of ATAD3A or VDAC, considered to be part of this complex, resulted in an inhibition of steroid synthesis. This model also indicated that StAR could increase steroid synthesis by mobilizing cholesterol that is bound to TSPO "polymers" present in the putative complex. Problems with this model are that native PAGE is highly prone to artifacts when dealing with transmembrane proteins, as this would include membrane vesicles that contain proteins without any rationale for associations. By providing only a short selected list of proteins without disclosing the full proteomics results [47], the study lacks necessary rigor for meaningful differentiation fact from artifact. Therefore, the existence of such a "transduceosome" complex is highly questionable. Moreover, the decrease in steroid biosynthesis with knockdown of ATAD3A and VDAC1 could be secondary to overall mitochondrial and cellular health, as transmission electron microscopy images in the same study showed severe structural disruption to mitochondria after ATAD3A and VDAC1 knockdown in MA-10 Leydig cells [47]. Unfortunately, no knockdown of TSPO was included in these experiments so assessing its absolute requirement or its function in this putative complex mediating cholesterol transfer and steroidogenesis could not be determined. Nevertheless, this model continued to receive attention and has been summarized in some recent reviews [48–50].

StAR

Orme-Johnson and colleagues originally described several rapidly induced ACTH and LH 30 kDa phosphoproteins in adrenocortical cells, and in rat corpus luteum and mouse Leydig cells, respectively [51–58]. These studies demonstrated a close relationship between the appearance of the 30 kDa proteins and steroid hormone biosynthesis and that their synthesis was cycloheximide sensitive. Our laboratory was engaged in similar studies in hormone-stimulated MA-10 mouse Leydig tumor cells and observed proteins which were identical to those described by Orme-Johnson [59–65]. These proteins were localized to the mitochondria and consisted of several different isoforms of a 30 kDa protein processed from a 37 kDa precursor protein that contained an N-terminal mitochondrial signaling sequence [53, 61]. Subsequent studies demonstrated close correlations between the synthesis of steroids and the synthesis of the 30 kDa mitochondrial proteins and thus, they represented reasonable candidates for the regulatory protein [51–65]. Purification of the 30 kDa protein, cloning of the cDNA for the precursor, and its sequencing were successfully accomplished in 1994 [66]. The nucleic acid sequence of the cDNA and the amino acid sequence of the 37 kDa protein were unique, indicating that it represented a previously undescribed protein. Expression of the 37 kDa protein in MA-10 mouse Leydig tumor cells resulted in a significant increase in steroid production in the absence of hormone stimulation. Also, expression of the 37 kDa protein in nonsteroidogenic COS-1 cells resulted in a several fold increase in pregnenolone production [67–70]. These results produced a "cause and effect" relationship between the 37–30 kDa proteins and steroid production and indicated a direct role for these proteins in hormone-regulated steroid production. As a result of these observations, this protein was named the *s*teroidogenic *a*cute *r*egulatory *p*rotein or StAR [66].

StAR is composed of a single functional domain with an α/β helix-grip fold structure containing a nine-stranded anti-parallel β-sheet forming a long hydrophobic cleft that binds cholesterol. The amino acid sequence for the StAR functional domain was used to identify a protein family, called the START domain family (START for StAR-related lipid-transfer domain) [72]. START domains bind hydrophobic lipids and StAR belongs to the mammalian START subfamily STARD1. The STARD1 subfamily has two members: StAR (STARD1) and MLN64 (STARD3). STARD3 was identified as an amplified transcript in breast cancer-derived metastatic axillary lymph nodes (MLN), and named MLN64 reflecting it was clone #64 in the screen [71]. MLN64 was the first reported protein to have a domain that shared 33% sequence identity and 53% sequence similarity with human StAR [72, 73]. STARD3 has been implicated to function in a StAR-independent mechanism to deliver cholesterol to mitochondria for steroidogenesis. Specifically, STARD3 is proposed to stimulate progesterone production by the human placenta, the one steroidogenic tissue that lacks StAR. STARD3 is a multidomain START protein with amino terminal MENTAL (MLN64 N-terminal) domain followed by a FFAT (diphenylalanine (FF)–acidic track (AT)) motif, and the carboxyl-terminal START domain. The MENTAL domain is composed of four transmembrane helices that localize STARD3 to the late endosomes and orients the START domain toward the cytoplasm. STARD3 overexpression is capable of enriching cholesterol in the mitochondria [74] or plasma membrane [75], depending on the cell type studied. The expression of only the START domain of MLN64/STARD3 is capable of stimulating progesterone synthesis in transfected COS-1 cells [76]. Again, the significance of this finding may be linked to placental steroidogenesis where high levels of progesterone are synthesized from cholesterol in the absence of StAR. In human and rodent tissues, MLN64 was detected in placenta and the START domain is processed from the larger protein, providing a possible mechanism for cholesterol transport into mitochondria in this tissue [77]. The START domain of STARD3 may function with a mitochondrial heat-shock protein to promote cholesterol transfer into mitochondria [78]. However, mice that express a STARD3 form that lacks the START domain are viable and fertile [79]; therefore, additional studies are warranted to determine the mechanism controlling cholesterol delivery into mitochondria for placental steroidogenesis.

Following the cloning of the StAR cDNA, collaborative studies with Dr W Miller and Dr J Strauss III demonstrated that mutations in the StAR gene resulted in congenital lipoid adrenal hyperplasia (lipoid CAH) [68]. Lipoid CAH is a lethal condition characterized by a nearly complete inability of the newborn to synthesize steroids. These patients have enlarged adrenals containing high levels of cholesterol and cholesterol esters and an increase in the amount of lipid accumulation in testicular Leydig cells indicating an inability to convert cholesterol to pregnenolone. In females,

the fetal ovary makes little or no steroids after the first trimester [80]. Therefore, the ovary appears to be normal until the time of puberty when it is stimulated with LH for the first time. Without StAR action, the amount of estrogen synthesized by StAR-independent action in the ovary is greatly reduced while cholesterol begins to accumulate. The suboptimal level of estrogen does result in a marginal amount of feminization that includes some breast development and vaginal bleeding [81–83]. However, the continued accumulation of cholesterol in the latter part of the cycle in the ovary eventually impairs the synthesis of progesterone and results in anovulatory cycles [83]. A recent review provides an excellent summary of the mutations in StAR that have been uncovered as of the present time [84]. These observations added compelling evidence for the essential role of this protein in the regulation of steroidogenesis since, in essence, lipoid CAH provides a human StAR knockout and the phenotype has the expected characteristics. Collaborative studies of our laboratory with Dr K. Parker and colleagues demonstrated StAR mRNA expression was tightly correlated with the appearance of steroidogenic cells and the timing of steroidogenesis in the adrenal glands and testis during embryonic development in the mouse [85]. These studies clearly demonstrated the presence of StAR transcripts in the adrenal cortex and testicular Leydig cells, but as expected, there were no StAR transcripts in the ovary during development, as estrogen is not produced in this organ until puberty, as also seen in the human female. In another study, also in collaboration with the Parker laboratory, targeted disruption of the StAR gene in mice was used to successfully produce StAR null mice [86]. StAR knockout mice had female external genitalia (regardless of sex), failed to grow normally, and died within a short period of time, presumably as a result of adrenocortical insufficiency. Serum levels of corticosterone and aldosterone were depressed, while levels of ACTH and CRH were elevated indicating impaired production of adrenal steroids with an accompanying loss of feedback regulation at the level of the hypothalamus or pituitary. The adrenal glands had an abnormal cortex that displayed elevated lipid deposits in the knockout mouse as did the Leydig cells in the testis. As seen in the human, the ovaries in the StAR knockout mice were unaffected at the time of birth, but continued to accumulate lipids as the animals entered puberty [87]. Thus, the StAR knockout mouse demonstrated characteristics similar to human lipoid CAH and further substantiated the necessity for StAR action in steroid biosynthesis.

With the availability of StAR reagents, results obtained with methodologies such as western and northern analysis, in situ hybridization, immunocytochemistry, RNAse protection assays, and RT PCR have all been used to demonstrate that StAR expression is essentially confined to steroidogenic tissues. Importantly, these studies were conducted in many different laboratories and successfully demonstrated that StAR was present in the steroidogenic cells of steroidogenic tissues. StAR expression has been demonstrated in adrenal cortical layers, adrenal tumors, ovarian theca cells, ovarian granulosa cells, ovarian corpora lutea cells, fetal mouse giant trophoblast cells, and testicular Leydig cells [88–94]. Using more sensitive technologies StAR protein has also been detected in a wider variety of tissues where its roles are, so far, not completely known [95, 96].

Characterizing the role of StAR in cholesterol transport to the IMM has proven to be refractory to elucidation even after more than two decades of effort. We originally hypothesized that import of StAR protein into the mitochondrial matrix temporarily formed contact sites between the OMM and IMM allowing the hydrophobic cholesterol to transfer between the membranes [67]. Based on the first report for a crystal structure of a START domain for STARD3/MLN64 that has a conserved structure with StAR and also binds cholesterol, Tsujishita and Hurley proposed that StAR acted as an intramitochondrial membrane shuttle to mediate cholesterol transport to the IMM by binding a one molecular of cholesterol at a time [97]. It was also proposed that StAR might alter the molecular conformation of the mitochondrial membranes to allow for the passage of cholesterol to the IMM, before it is transported to the mitochondrial matrix (reviewed in [98]). However, StAR action had to be reconsidered when it was shown that StAR need not enter the mitochondria to be active and that interactions at the OMM were sufficient for cholesterol transfer. The report that deletion of 62 amino acids at the N-terminus of StAR prevented mitochondrial import, but did not affect cholesterol transfer and steroid production [99, 100], was supported by studies that showed a TOM20-StAR fusion protein that was located at the OMM due to TOM20 and could not enter the matrix but could still induce steroidogenesis [101]. In an effort to explain what might be occurring at the level of the OMM, Dr Walter Miller and colleagues performed a series of biophysical studies, which demonstrated that StAR undergoes a conformational alteration when it binds to cholesterol [102]. This alteration, caused by an acid-induced breaking of hydrogen bonds, appears to be required for StAR activity and is a result of StAR's transition to a molten globule form [102–104]. Biochemical studies suggested that StAR interacts with a protein complex that promotes cholesterol transfer from the OMM to the IMM. Recent studies have revealed possible specific involvement of the VDAC2 and TOM22 for StAR activity at the OMM membrane [105, 106]. However, there has yet to arise a model that adequately explains the mechanism of StAR in the transfer of cholesterol to the IMM. And future studies addressing StAR's mechanism of action should include the role of StAR phosphorylation. A PKA-dependent phosphorylation at S194 (mouse) or S195 (human) is critical for StAR's function [107, 108], reviewed in [109]). This was best demonstrated using an in vivo approach wherein re-expression of wild-type StAR in StAR knockout mice restored adrenal and testis steroid production but mice expressing the StAR-S194A transgenic displayed lipid accumulation in the adrenal and testis, and low corticosterone and testosterone levels similar to StAR knockout mice [110]. Furthermore, it is likely that StAR transcription, translation, and phosphorylation are coordinated processes in the acute response to move cholesterol into the mitochondria. . In particular, data support StAR mRNA is associated with mitochondria and the mitochondrial A-kinase anchoring protein 121 (AKAP121) and type II PKA (PKAR2) [111–114]. Newly transcribed StAR mRNA is proposed to associate with AKAP121 based on the observation that a StAR mRNA-AKAP121 interaction occurred only after Bt2cAMP treatment of H295R human adrenocortical cells to increase StAR mRNA levels [114]. StAR mRNA levels but not protein levels are increased after Bt2cAMP treatment of MA-10 cells that lack AKAP121, suggesting that StAR translation is diminished when the mRNA is not localized to the mitochondria. The StAR mRNA-AKAP121-PKAR2 complex at the mitochondria warrants further examination to determine whether rapid phosphorylation of StAR S194/195 at the OMM occurs, and whether this phosphorylation promotes specific interactions with OMM/IMM proteins such as VDAC2 or TOM22.

Using the TOM20-StAR fusion protein, work performed by Dr Papadopoulos and colleagues demonstrated that knockdown of TSPO using antisense oligonucleotides resulted in an inhibition of

steroid synthesis [46]. These findings suggested cooperation between StAR and TSPO mediated mitochondrial cholesterol transport and proposed a model in which StAR carried cholesterol from cellular stores to the OMM where TSPO acted as a protein tunnel for the import of cholesterol to the IMM [42].

As further investigations added additional corroborations to the roles of TSPO and StAR in the transfer of cholesterol to the IMM in support of steroidogenesis, the mechanism whereby this occurred remained a mystery and still does. However, a major shift in this field occurred in 2014 and beyond with several reports that seriously questioned the role of TSPO in the acute regulation of steroidogenesis. The following section will summarize those studies.

TSPO is not involved in mitochondrial cholesterol import for steroidogenesis

In this section, rather than describe events in chronology, we present the most compelling pieces of evidence from precise genetic models that indicate that TSPO is not involved in steroidogenesis. In MA-10 Leydig cells, complete disruption of the *Tspo* gene using CRISPR/Cas9-mediated targeting had no effect on the extent of their ability to synthesize steroid hormones [115]. This means that in the complete absence of TSPO protein, mitochondrial cholesterol import progressed with absolutely no impediments. Of the three TSPO-knockout subclones generated, one even produced significantly higher levels of steroids compared to TSPO-intact controls. Without observing any loss of function, this result was in direct contrast to previous reports that seemed to demonstrate an indispensable role for TSPO in mitochondrial cholesterol import [41, 43, 46]. Two fundamental observations reported by this group were as follows: (1) there occurs an almost complete elimination of steroid biosynthesis in R2C Leydig cells after *Tspo* gene disruption [41]; (2) knockdown of TSPO in MA-10 cells not only inhibited their ability to synthesize steroids but also prevented StAR import into mitochondria [46], suggesting that TSPO was essential for StAR function. Not only did we find it extremely hard to reconcile recent findings with these polar opposite reports, but also recent in vivo observations in *Tspo*-deleted mice clearly supported recent in vitro results that TSPO was not involved in mitochondrial cholesterol import for steroid hormone biosynthesis.

Conditional *Tspo* deletion in testicular Leydig cells did not affect testosterone levels [116]. Global *Tspo* deletion resulted in viable fertile mice with no effects on testosterone, estrogen, progesterone, corticosterone, and aldosterone levels [117]; these TSPO knockout mice had no apparent abnormalities. A second independently generated global *Tspo* deleted mouse model confirmed this viable phenotype, and also demonstrated that mitochondria isolated from mice lacking TSPO had no defects in cholesterol import function [118]. A third independently generated global *Tspo* deleted mouse model reconfirmed this viable phenotype with no apparent abnormalities [119]. These three recent independent reports dispute the phenotypic outcome previously reported in a review article that, "Efforts to generate a PBR-negative gene knockout mouse model failed, as the animal died at an early embryonic stage suggesting that PBR is involved in basic functions necessary for embryonic development" [43]. Similarly, at the cellular level, the absolute need for TSPO in maintaining cell viability was again emphasized in review articles with claims that >70% knockdown of TSPO in cultured cells lines resulted in cell death [120, 121]. The basis for these assertions that were provided as precluding the study of steroidogenesis using gene-deleted models remains open to question, particularly because the experimental details were never published as research articles. We have carefully reviewed the history of TSPO research that includes all of the results that underlie the sustained consideration of TSPO in the mitochondrial cholesterol import model [122–124].

The Papadopoulos group subsequently generated the same $Amhr2^{cre/+}Tspo^{c\,\Delta/\Delta}$ mouse and reported that TSPO was crucial for viability and hormone-dependent adrenal steroid formation [125]. We respectfully disagree with this interpretation [126]; the low rate of $Amhr2^{cre/+}Tspo^{c\,\Delta/\Delta}$ reported as being due to embryonic lethality in this manuscript is in fact due to linkage between the *Tspo* and *Cre* loci (18.18 centimorgans apart in chromosome 15) [126]. Due to this linkage, independent assortment is not possible and there needs to be chromosomal crossover between the two loci to generate $Amhr2^{cre/+}Tspo^{c\,\Delta/\Delta}$ mice. As this can occur only at a low rate (calculated: 7.6%; observed: 4.4%), it is not indicative of embryonic mortality as classical Mendelian principles are not applicable in this context [126]. Recently, this same group extended efforts to revive a mitochondrial cholesterol transport function for TSPO in rat and human steroidogenic cells [127]; it is essential that this and similar studies receive independent validation.

Another facet to the TSPO story can be found in the field of drug development targeting this protein. This continues to be quite extensive due to significant commercial interest in small molecules that bind TSPO, mainly for diagnostic imaging of inflammatory lesions that overexpress TSPO, and testing its effects on immunomodulation [24 clinical trials in progress or completed in the USA and EU combined (Source: clinicaltrials.gov and clinicaltrialsregister.eu)]. The core mechanism of TSPO-binding drug effects on immunomodulation has been believed to be due to its putative role in steroid biosynthesis. In the existing >400 publications on TSPO-binding drug development, most studies have used the steroidogenesis model to explain outcomes without direct examination of molecular aspects of function resulting from TSPO binding. Studies in adrenocortical and Leydig cells that report effects for TSPO-binding drugs in steroidogenesis [29, 33] are often referenced; however, the pharmacology upon which this phenomenon is based is difficult to interpret (reviewed in [123]). Rigorous target validation was not considered in these studies as TSPO knockout cells were incorrectly considered to be nonviable. Recently, by using TSPO knockout Leydig cells, it was demonstrated that the effect of one prototypical TSPO-binding drug PK11195 in transient steroidogenesis, as previously demonstrated [33], persisted in the complete absence of TSPO [115]. This result is highly significant because PK11195 was used to suggest that TSPO mediates mitochondrial cholesterol import [34]; PK11195 has been used consistently to demonstrate an induction of steroidogenesis in both Leydig [33] and adrenocortical cells [29]. The finding that PK11195-induced steroidogenesis is independent of TSPO [115] provided the first evidence that the entire pharmacological basis for TSPO and steroidogenesis is likely a misinterpretation. Moving forward, it is reassuring that TSPO knockout models will allow for rigorous target validation for the putative TSPO ligands and drug discovery.

Although there are more than 200 publications on TSPO, most of these studies do not mechanistically address mitochondrial cholesterol import but rather explain the multiple effects of TSPO pharmacology by the supposition that it is involved in steroidogenesis. Aside from pharmacology, it is accurate to state that all mechanistic results specific for TSPO involvement in mitochondrial cholesterol import were reported by a single research group [37, 39–41, 44–46, 128]. The only independent study on this topic was performed by

Gavish in 1998, where it was observed that results connecting TSPO knockdown and steroidogenesis were inconclusive; no acute effects were observed [129]. Gavish and colleagues concluded that "further studies are needed to confirm the involvement of the 18-kDa PBR subunit in MA-10 Leydig cell steroid biosynthesis" [129].

For complete point-by-point examination of the history and evolution of the TSPO-steroidogenesis model and its links to physiological expression, pharmacology, binding proteins, cholesterol binding/transport, structure, genetic deletion, and human *TSPO* gene polymorphisms, see the extensive examination of literature presented in other recent reviews [122–124, 130]. Regrettably, the sheer volume of TSPO publications referencing steroidogenesis will continue to present a challenge to researchers, particularly because the physiological function of TSPO remains unestablished [131–133]. Nevertheless, it is encouraging that progress in the TSPO field, distinct from steroidogenesis research, has begun to indicate functions for TSPO particularly in fatty acid metabolism [134] and other aspects of cellular energy production [135]. These may indeed be connected to indirect effects on steroidogenic homeostasis independent of mitochondrial cholesterol import in vivo as reported in a recent study using global $Tspo^{-/-}$ mice when all steroidogenic intermediates were profiled [136].

Regulation of *STAR/Star* gene expression

STAR/Star gene expression in the adrenal cortex and gonads is under the control of the tropic hormones ACTH and the gonadotropins LH and FSH, respectively. ACTH, LH, and FSH bind to their cognate 7-transmembrane G-protein coupled receptors leading to activation of cAMP-dependent PKA, phospholipase C–diacylglycerol-PKC-inositol 1,4,5 trisphosphate (PLC-DAG-PKC-IP3), mitogen activated kinase (MAPK), and calcium signaling pathways. Activation of the PLC-DAG-PKC-IP3 and calcium signaling pathways is also the mechanism of action for angiotensin II (Ang II) and K^+-stimulated aldosterone synthesis in the adrenal zona glomerulosa. Ang II activates a G protein-coupled receptor, while K^+ activates voltage-gated calcium channels and subsequent calcium-PKC-signaling pathways. Maximal increases in steroidogenesis are observed when there is cross-talk between these pathways, yet activation of the cAMP-PKA signal transduction pathway is the major player in tropic hormone-dependent activation of steroid output. Over the past two decades the studies on *STAR/Star* gene regulation have uncovered that species-specific and cell-type-specific mechanisms control StAR expression due to differences in promoter sequence and differences in transcription factor activation and recruitment to the cAMP responsive region the *STAR/Star* proximal promoter [137–139]. Herein we focus on the sequence differences between the rodent *Star* and human *STAR* promoter to highlight how multiple trans-acting factors have been identified that work through a common promoter region to increase transcription in a species- and tissue-specific manner.

STAR/Star gene regulation is characteristic of the immediate early response genes [140]: StAR mRNA levels are increased acutely (within minutes) following tropic hormone stimulation, transcription is independent of protein synthesis (cycloheximide-insensitive), and expression is transient (transcription terminates upon removal of the stimulus). In addition, the *STAR/Star* promoter contains a TATA-box, GATA-4-, and cAMP responsive cis-acting elements, which are commonly found in highly regulated genes. The region of the *STAR/Star* proximal promoter 150 bp region immediately upstream of the transcription start site is highly conserved and is sufficient for maximal transcriptional activation of mouse, human, and rat StAR genes in the adrenal and gonads [139]. This region contains the cAMP-responsive element (CRE) which is a nonconsensus sequence that binds cAMP response element binding protein (CREB)/CREB-modulator (CREM) family members and activator protein-1 (AP-1) family members. Therefore, this element is referred to as the CRE/AP-1 site [Figure 1A] [137, 141–144].

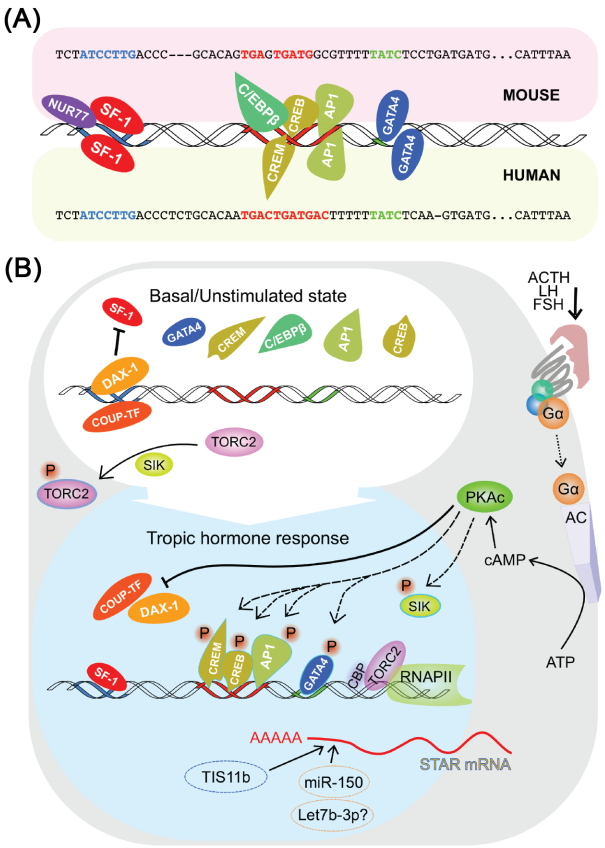

Figure 1. Transcriptional and post-transcriptional mechanisms that control the cAMP-PKA-dependent regulation of STAR expression. (A) Shown is the sequence comparison for the cAMP-responsive region of the mouse and human StAR promoter with the SF-1, CRE/AP-1, and GATA-4 elements are shown. The CRE/AP-1 is a nonconsensus CRE sequence that binds cAMP response element binding protein (CREB)/CREB-modulator (CREM) family members and activator protein-1 (AP-1) family members. (B) The left panel shows a model for maintaining low basal StAR steady-state mRNA levels. Transcriptional repression may be mediated by one or all of the following: DAX-1 and COUP-TF blocking SF-1 action, low interaction of CREB/AP-1/GATA-4 with the StAR promoter, and diminished CREB action due to lack of TORC2 interactions. Post-transcriptional repression may be mediated by promoting StAR mRNA degradation by either TIS11b or miRNAs. The right panel shows the mechanisms stimulated by cAMP-PKA signaling. Tropic hormones ACTH, LH, and FSH bind their cognate G-protein coupled receptors and activate adenylyl cyclase (AC) thereby increasing cAMP levels and promoting PKA-dependent downstream effects. Depicted is the phosphorylation of CREB/CREM, c-Fos/c-Jun (AP-1), and GATA-4 and recruitment of these transcription factors to their respective DNA elements in the StAR proximal promoter. SF-1 may be constitutively bound to the promoter with increased association after cAMP-PKA activation. Protein–protein interactions between GATA-4 and SF-1, AP-1, and CREB are proposed to stabilize the transcription complex and enhance recruitment of the coactivator CBP and RNAPII. Additional activation is mediated by loss of SIK activity and increased TORC2 association with CBP as described in the text. TIS11b levels are induced by cAMP-PKA signaling and may play a role in attenuating the acute response. The role of miRNAs in controlling StAR mRNA levels in hormone-dependent manner remains to be examined.

CREB/CREM/AP-1 are members of the basic leucine zipper (bZIP) family of transcription factors due to the bZIP motif that drives homo- and heterodimerization among family members. AP-1 is composed of members of the Fos family (c-Fos, FosB, Fra-1, and Fra-2) that heterodimerize with members of the Jun family (c-Jun, JunB, and JunD) as well as with some members of the CREB/ATF family, while Jun members can also function as homodimers [145]. The multiple heterodimer partners and the relative levels of CREB v. CREM v. AP-1 proteins contribute to the cAMP-dependent regulation of *STAR/Star* gene expression in a species or cell-type dependent mechanism. For example, CREB phosphorylation and recruitment to the *Star* CRE/AP-1 site in mouse Leydig cell cultures mediates the cAMP response yet in the adrenal CREB is not expressed and CREM isoforms bind to the CRE/AP-1 element and activate StAR transcription [146–148]. c-Jun was also shown to be a potent trans-activator of *Star* transcription in MA-10 mouse Leydig cells via binding to the CRE/AP-1 site. It is likely that c-Jun/CREB heterodimer formation explains the recruitment of these two factors to the same element in response to cAMP-PKA signaling. AP-1 family members JunB-Fos/FosB or JunB-ATF3 also are capable of stimulating *STAR* promoter-reporter gene expression in H295R human adrenocortical cells, most likely via binding to the CRE/AP-1 element [149]. Importantly, PKA-dependent phosphorylation of GATA-4, CREB/CREM, and/or c-Fos/c-Jun (AP-1) enhances both the recruitment of these factors to the *STAR* promoter and protein–protein interactions between these factors ([150], reviewed in [138, 151]). The result is enhanced recruitment of the coactivator CREB-binding protein (CBP) and RNAPII to the *STAR* promoter. However, the cAMP response is dependent on the function of SF-1 and GATA-4 (Figure 1B).

Steroidogenic factor-1 (SF-1) and GATA-4 elements flank the CRE/AP-1 site, and both SF-1 and GATA-4 binding are important for full promoter activity. The minor sequence differences between mouse and human SF-1 and CRE/AP-1 DNA elements influence the preference for transcription factor binding (Figure 1A). Mutation of the SF-1 element to block SF-1 binding located directly upstream of the CRE/AP-1 element resulted in loss of promoter activity for human *STAR* gene but not for mouse *Star* gene. This may be explained by the differences in the flanking DNA sequences between the species; the mouse *Star* promoter contains an overlapping sequence that binds NUR77 (NGFI-B (nerve growth factor induced-B) while human *STAR* promoter lacks this binding site [152–154]. Thus, SF-1 binding to this proximal element is essential for human promoter activity but not required for mouse promoter activity, likely due to NUR77 compensating for SF-1 function in the rodent [146, 155, 156]. The *Star* proximal promoter has also a highly conserved CCAAT box element that overlaps the CRE/AP-1 site and C/EBPβ can bind to this site as demonstrated by electrophoretic mobility shift assays [150, 157, 158]. In mouse Leydig and granulosa cells, activation of cAMP-PKA signaling was shown to increase C/EBPβ expression [159, 160], and C/EBPβ binding to the *Star* proximal promoter was shown to be important for transcription in mouse granulosa-luteal cells [161]. In summary, SF-1-pGATA-4 binding appears to be a common mechanism for mouse and human *STAR/Star* gene activation in multiple cell types while the homo- or heterodimer partners between CREB/CREM and AP-1 family members are dependent upon the species, cell type, and stimulus (reviewed in [137]).

Cross-talk between signaling pathways provides a fine control for StAR expression and steroidogenesis. Pharmacological inhibition of JAK2 signaling was shown to inhibit angiotensin II-stimulated StAR expression and steroid output in H295R adrenocortical cells and ACTH-stimulated increased corticosterone synthesis in the mouse adrenocortical cell line (ATC-1) or primary cultures of rat adrenocortical cells [162, 163]. *STAR*-promoter-reporter gene assays showed that STAT5 did not activate *STAR* transcription, rather JAK2 activity was necessary for increasing pCREB stability that led to transactivation of the *STAR* gene [162]. This finding highlights the central role that pCREB plays in *STAR/Star* transcription. ACTH can also act indirectly to enhance StAR expression and cortisol production. In H295R human adrenocortical cells, ACTH stimulated sphingosine-1-phosphate (S1P) secretion that works in an autocrine manner to promote cortisol synthesis via increased expression of *STAR* as well as other genes involved in steroidogenesis [164, 165]. S1P stimulation was mediated via binding to a GPCR membrane receptor linked to activating the Ca^{2+}-CamKII-ERK1/2 signaling pathway. MAPK/ERK activation promotes StAR phosphorylation so it is likely that S1P may enhance both expression and function of StAR.

Transcriptional repression also controls *STAR/Star* gene expression, although fewer studies have been devoted to this mechanism. Repressor proteins work via direct and indirect mechanisms: they bind DNA and recruit corepressor complexes (direct), or they block a trans-activator protein from binding DNA (indirect), thereby blocking assembly of a transcription initiation complex. Dax-1 (dosage sensitive sex reversal-adrenal hypoplasia congenital gene on the X chromosome gene 1), a member of the nuclear receptor superfamily, was the first reported repressor of *STAR* transcription. DAX-1 contains a strong C-terminal repressor domain yet lacks a DNA-binding domain so mediates its repressive action through indirect mechanisms [166]. For example, DAX-1 binds to a hairpin loop formed within promoters, including the *STAR* promoter [167, 168], which prevents SF-1 and/or NUR77 binding. In addition, direct SF-1-DAX-1 interactions that tether DAX-1 to the promoter via DNA-bound SF-1 results in recruitment of corepressor proteins and transcriptional repression of StAR [166, 168–170]. COUP-TF (chicken ovalbumin upstream promoter transcription factor I and II) is also capable of blocking SF-1 function through competitive binding to SF-1 elements in the *STAR* promoter [171–174]. Thus, both DAX-1 and COUP-TF can function to interfere with SF-1 activation of the *Star* promoter and represses transcription. One possible mechanism for switching from repression to activation is hormone-dependent activation of PKA resulting in decreased DAX-1 or COUP-TF expression, thereby allowing SF-1 and other factors to activate *STAR/Star* gene expression (Figure 1B). In several studies an inverse relationship between repressor protein(s) and StAR expression levels was observed [171, 175–177]. An interesting example of loss of repressor function involves an indirect mechanism that leads to enhancing CREB-mediated *Star* gene expression in the adrenal. In brief, CREB regulated transcription coactivator 2 (CRTC2), also known as co-activator transducer of regulated CREB activity (TORC2), promotes the recruitment of CBP to pCREB on target gene promoters (60). TORC2 is phosphorylated by salt-inducible kinase-1 (SIK1), an AMP-kinase family member. Phosphorylated TORC2 (pTORC2) translocates to the cytoplasm; therefore, active SIK1 represses CREB-mediated transcription via diminishing nuclear TORC2. SIK1 was first characterized as a high-salt diet-induced transcript in rat adrenals [178] and later shown to be an ACTH-responsive transcript in Y1 mouse adrenocortical cells [179]. In Y1 mouse adrenocortical cells, low basal StAR expression is proposed to be maintained, in part, by steady-state levels of active nuclear SIK1 and cytoplasmic pTORC2 that contribute to suppressed *Star* gene expression [63, 67]. To this end, ACTH stimulation results in SIK1 phosphorylation and inactivation. In vivo studies support a temporal correlation between detection of TORC2 and pCREB in

the nucleus that precedes an increase in detection of StAR transcription. Thus, one arm of the ACTH-cAMP-PKA pathway leading to acute control of *Star* transcription is the loss of SIK1 repressor action that allows for maximal pCREB-mediated transcription in the adrenal (Figure 1B) [63, 67].

In addition to transcriptional mechanisms, post-transcriptional regulation of StAR expression has been reported. It was established in early studies by northern blot analysis that two StAR transcripts are generated in a similar temporal time frame in response to cAMP-PKA signaling. A long 3.5 kb transcript and a short 1.6 kb transcript arise due to processing at different polyadenylation sites in the 3′-UTR. The short transcript is expressed at much lower levels relative to the longer transcript, although the longer transcript is subject to faster degradation. The RNA-binding protein TIS11b (zinc finger protein, Znf36L1, also known as BRF1) binds to an AU-rich destabilizing element present only in the 3.5 kb StAR 3′-UTR [180] (reviewed in [181]). The significance for targeting this particular transcript for degradation remains to be determined, yet it may represent a mechanism to attenuate the hormonal response [182]. Similar to SIK1, TIS11b is also induced by cAMP-PKA signaling in adrenal cells and an increase in both SIK1 and TIS11b would suppress StAR expression at both the transcriptional and post-transcriptional level. In this manner, the cAMP-PKA signaling remains central to modulating StAR expression and controlling tropic hormone-mediated steroidogenesis.

The role for miRNA-mediated post-transcriptional control of StAR expression in a hormone-dependent manner is a relatively new area of study. One of the challenges for future investigations of miRNAs in StAR regulation is identifying and validating functional miRNA-StAR interactions. Searching TargetScan7, miRDB, miRanda, and miRTarBase databases to identify predicted miRNA binding sites for mouse and human StAR reveals many poorly conserved sites. Studies that have characterized the differentially expressed miRNAs in Leydig cells and the adrenal have not yet revealed regulatory miRNAs that target StAR. In TM3 mouse Leydig cells after LH treatment, a total 2904 miRNAs were detected by deep sequencing, with 27 miRNAs upregulated and 2 downregulated by LH treatment [183]. No list was provided for the miRNAs that were detected in the TM3 Leydig cells, although Let-7b-3p was validated as an upregulated (threefold) miRNA. In contrast, Let-7b-3p was identified using a miRNA microarray approach as a downregulated miRNA in rat adrenal following ACTH treatment [184]. Let-7b is one of the first miRNAs proposed to regulate StAR. A putative miR-Let-7b binding site was identified in the human StAR 3′-UTR and overexpression of miR-let-7 in HEK-293 cells reduced the activity of a luciferase reporter gene containing a short segment of the human StAR 3′-UTR [185]. The authors also show that miR-let-7 overexpression in MLTC-1 mouse Leydig tumor cells decreased endogenous StAR mRNA ~20%, although the mouse StAR 3′-UTR was never tested. The focus on miR-let-7 in this study was an attempt to explain the observation that overexpression of the long noncoding RNA H19 promotes increased StAR mRNA and protein expression in both human KGN cells and mouse MLTC-1 cells. H19 was previously shown by this group to bind miR-let-7 and block its action [186]. However, StAR was never validated as a bona fide target of miR-let-7b in this study, which requires additional studies to demonstrate that mutation of 3′-UTR of human (and mouse) StAR abolishes the miRNA-mediated repression. The finding that Let-7b-3p is regulated by cAMP-PKA signaling in two steroidogenic cell types supports a more robust testing for this miRNA in controlling StAR and steroidogenesis in cell-specific context is needed. Another exciting possibility is H19 might function as a positive regulator of StAR transcription and efforts directed at H19 action in ovarian steroidogenesis seems warranted.

Lastly, the most robust study to date has demonstrated that StAR is a bona fide target for miR-150 [187]. miR-150 was shown by both RT-qPCR and in situ hybridization to be highly expressed in mouse Leydig cells relative to germ cells or Sertoli cells. Transfecting either primary Leydig cells or MA-10 mouse Leydig tumor cells with miR-150 antagomir resulted in decreased miR-150 levels, increased StAR mRNA and protein expression, and increased steroidogenesis. These results were complemented by demonstrating that a miR-150 agomir reduced StAR levels and steroidogenesis. Furthermore, modulating miR-150 levels in vivo by intratesticular injection of the agomir or antagomir recapitulated the in vitro results. Full-length mouse StAR 3′-UTR and a mutant that disrupted the miR-150 seed sequence were tested using Luciferase reporter assays in transfected MA-10 cells. The presence of miR-150 agomir decreased Luciferase activity 3-fold for the wild-type 3′-UTR but had no effect on the mutant 3′-UTR. Although the rationale for picking miR-150 to study in this context is not clear, this miRNA should get further attention as it may play a role in modulating steroidogenesis in Leydig cells via targeting StAR. miR-150 was not listed as a differentially expressed miRNA in LH-treated TM3 cells and it may reflect a mechanism to maintain low basal StAR mRNA levels.

Potential indirect mechanisms may also contribute to miRNA-mediated changes in StAR expression. One example is miR-133a targeting FOXL2, a member of the forkhead/hepatocyte nuclear factor 3 (FKH/HNF3) gene family, contributing to control of ovarian steroidogenesis. FOXL2 binds to the STAR proximal promoter (–42 bp) and represses StAR activity in the mouse ovary [188]. Foxl2 mRNA was shown to be a direct target of miRNA-133a and FSH stimulation of KGN human granulosa cells and isolated mouse granulosa cells resulted in increased miR-133a and decreased Foxl2 expression. These studies indicate the miRNA-dependent loss of the FOXL2 repressor contributes to the FSH-dependent increase in StAR expression [161, 189].

Important questions for the future

In the evolution of mitochondrial cholesterol import models over the years, the core element that has stood the test of time is StAR in that it is imperative for cholesterol import to the IMM in somatic steroidogenic cells (Figure 2). Nevertheless, precise knowledge on how this process occurs is something that remains a topic of ongoing investigations. When TSPO is removed from the steroidogenesis equation, the next model that surfaces for explaining StAR mechanism of action is the proposal of its existence as a “molten globule” in the OMM which transitions to take up cholesterol and deliver it to the IMM [103]. In support, recent research has postulated that StAR may interact with components of the mitochondria-associated endoplasmic reticulum membranes (MAMs) specific to the OMM proteins to regulate aspects of its structural folding [190] and subsequent translocation to the mitochondrial matrix [105]. However, considering that mitochondrial import of StAR may not be essential for cholesterol translocation [99, 100], this model fails to provide direct evidence for the mechanism of cholesterol import to the IMM. Moreover, the relationship between StAR and other proteins of the MAMs lacks clarity as disrupting MAMs and/or their regulators could interfere with other essential cellular functions that may indirectly influence steroidogenesis. One area that deserves more directed studies is whether StAR phosphorylation influences MAM

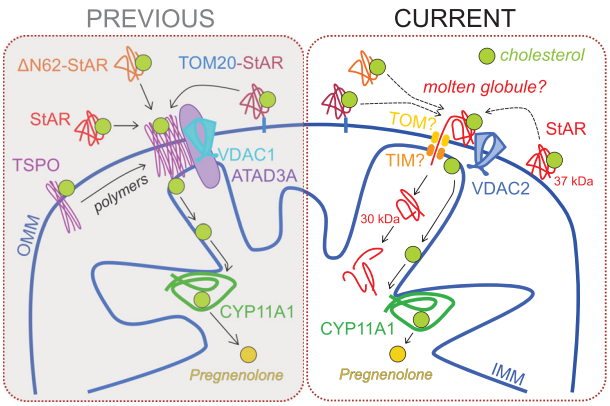

Figure 2. Current iteration for the StAR-mediated mitochondrial cholesterol import model. In previous models, it was considered that at the outer mitochondrial membrane (OMM), StAR delivered cholesterol and TSPO functioned as a channel that mediates cholesterol transport to the inner mitochondrial membrane (IMM). Function of StAR was restricted to the mitochondrial outer membrane, as deletion of its mitochondrial targeting sequence (ΔN62-StAR) or anchoring it to the OMM by fusion to TOM20 (TOM20-StAR) did not affect its function. The transport mechanism was thought to involve TSPO polymerization and association with other proteins such as VDAC1 and ATAD3A. However, these association studies dealing with transmembrane proteins were highly prone to artifacts, and recent results have definitively demonstrated that TSPO is not involved in mitochondrial cholesterol import for steroidogenesis. This fundamental shift has resulted in the current model that mitochondrial cholesterol import is entirely mediated by StAR. It is known that the active form of StAR is a 37 kDa protein that enters the mitochondrial matrix to be processed to a 30 kDa inactive form. The basis for StAR function is partially explained by evidence that StAR exists as a molten globule at the OMM, with a folded N-terminal region and less folded C-terminal region. This state allows StAR to pause as it traverses the OMM and IMM, increasing its activity in importing cholesterol during this transition. At the OMM, VDAC2 has been shown to control StAR processing and activity. Cholesterol imported to the IMM is converted to pregnenolone by CYP11A1 that resides at the matrix side of the IMM, the first enzymatic step in steroid hormone biosynthesis.

interactions, if this is indeed the mechanism for cholesterol transfer. In summary, it would seem to follow that the most important future investigations need to focus on uncovering the exact mechanisms involved in transporting cholesterol to the IMM and the role of StAR in mediating this transport. Providing this information will undoubtedly represent the next biggest advance in this area.

The question of what regulates the regulator of steroidogenesis is moving beyond transcriptional control to post-transcriptional mechanisms. The few studies to date in this area highlight that mechanisms that control steady-state StAR mRNA levels contribute to the acute tropic hormone response of steroidogenic cells to increase steroid output. The challenge is identifying the biologically relevant miRNAs that directly target StAR given that hypothesis-driven approach is limited by the lack of strong binding sites predicted for the 3′-UTR. miRNA-150 provides a good example for how a case-by-case approach to this question might work.

In extraembryonic tissues like the human placenta that do not express StAR but are still capable of producing steroids, alternate mechanisms that facilitate mitochondrial cholesterol import appear to exist. STARD3 (also known as MLN64) was proposed to mediate mitochondrial cholesterol import in the placenta [76, 191]. However, MLN64 steroidogenic function has not been directly addressed in placental cells, and its precise mechanism responsible for the claim of mitochondrial cholesterol import remains far from clear. Recent studies on this protein seem to suggest that it plays a role in endoplasmic reticulum to endosome cholesterol transport [192]. Therefore, the basis of mitochondrial cholesterol import for steroidogenesis in the human placenta is also a topic that requires fundamental exploration.

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