Original Research
Brief Communication

STARD6 is expressed in steroidogenic cells of the ovary and can enhance *de novo* steroidogenesis

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Abstract

STARD6 is a member of the StAR-related lipid transfer (START) domain family of proteins whose function thus far remains obscure. While it recently was shown to facilitate steroidogenesis in a cell-free setting, it has not been localized to steroidogenic cells of normal reproductive tissues. In a recent microarray study, we detected STARD6 mRNA in cultured porcine ovarian granulosa cells which are steroidogenic. In the present study, we examined regulation of STARD6 mRNA in porcine granulosa cultures, and found that it was not regulated by cyclic AMP, but it was reduced by combined knockdown of the transcription factors GATA4 and GATA6. We detected both STARD6 mRNA and protein in fresh granulosa cells and whole antral follicles and different stage corpora lutea of pig. The highest levels were discovered in the mid-luteal phase corpus luteum. Immunolocalization within ovarian tissues indicated robust STARD6 immunoreactivity in steroidogenic cells of the corpus luteum. Relatively lesser amounts of STARD6 signal were found in granulosa cells, theca cells, and oocytes. To test the ability of STARD6 to facilitate *de novo* steroidogenesis, non-steroidogenic COS-1 cells were co-transfected with components of the P450 cholesterol side-chain cleavage system, enabling them to make pregnenolone, and STARD6. STARD6 increased pregnenolone production by two- to three-fold over the empty vector control. In summary, STARD6 is found in the pig ovary, exhibits the strongest expression in highly steroidogenic luteal cells, and significantly enhances pregnenolone production in transfected COS cells independent of cyclic AMP treatment. Collectively, these findings indicate that STARD6 may contribute to steroidogenesis in ovarian cells, but also suggests other cellular functions that require cholesterol trafficking.

Keywords: STARD1, STARD6, ovary, steroidogenesis, corpus luteum

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Introduction

In the non-gravid individual, the gonads and adrenal cortex are the major sites for *de novo* steroidogenesis, the synthesis of new steroid hormones from cholesterol.[1] The first steroid hormone produced, pregnenolone, is derived from cholesterol by the reactions catalyzed by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) complex associated with the inner mitochondrial membrane within steroidogenic cells.[2] Pregnenolone is further modified to yield progesterone or other steroid hormones. Although P450scc carries out the rate-limiting enzymatic step for entry into the steroidogenic cascade, the transfer of cholesterol from the outer mitochondrial membrane to the inner membrane is the true rate-limiting step. This step is largely mediated by the steroidogenic acute regulatory protein (StAR or STARD1).

Initial structural examination of STARD1 led to the identification of a lipid-binding region referred to as the StAR-related lipid transfer (START) domain.[3] Genomic analyses identified 14 other mammalian proteins with START domains, which form the START domain family.[4] The STARD4 subfamily, consisting of STARD4, STARD5, and STARD6, may mediate cholesterol movement through the cytoplasm from cholesterol stores,[4,5] although STARD5 cholesterol-binding has recently been challenged.[6] An *in vitro* comparison of the START domains of STARD1-D7 found that recombinant mouse STARD6, when added to isolated pig adrenal mitochondria with cholesterol, initiated *de novo* steroidogenesis similar to or better than STARD1.[7] This finding was very exciting to the field, but was dampened by RNA data which only detected STARD6 in the germ cells of the testis but not in Leydig cells, the ovary or the adrenal.[8,9] The conclusion from these studies was that since

STARD6 was not expressed in major steroidogenic cells/tissues, it could not be involved in mediating steroidogenesis *in vivo*. Chang *et al*.[10] subsequently immunolocalized STARD6 in Leydig cells in rats under hypothyroid conditions, reopening the possibility for STARD6 involvement in steroid production.[10] Its presence in the central nervous system[11] suggests it could also facilitate neurosteroid synthesis.

In females, ovarian steroidogenesis is critical for normal reproductive function and pregnancy. In the ovary, *de novo* steroidogenesis primarily occurs in theca, luteinized granulosa, and luteal cells. Luteal cells exhibit massive *de novo* pregnenolone and progesterone synthesis due to high expression of the STARD1, CYP11A1 (encoding P450scc), and HSD3B genes.[1]

Recently, in a study examining the functions of the transcription factors GATA4 and GATA6 in cultured pig granulosa cells, we detected STARD6 mRNA by microarray.[12] In the present study, we followed up this preliminary finding to determine whether STARD6 mRNA is regulated by cyclic AMP or affected by GATA4/6 reduction in a manner similar to STARD1.[13] As STARD6 was not previously reported in granulosa cells or the ovary, we sought to identify which structures of the porcine ovary express STARD6 and whether STARD6 localizes to steroidogenic cells. In addition, we tested the ability of STARD6 to facilitate steroid synthesis in a classical COS cell assay as an indicator of its function in intact cells.

Materials and methods

Granulosa cell culture and GATA RNAi knockdown

GATA4 and/or GATA6 was knocked down in cultured ovarian granulosa cells from prepubertal gilts obtained from an abattoir as described by our lab.[13] An RNAi to firefly served as the control. Following a 72-h knockdown period in complete medium, granulosa cells were treated in serum-free medium with vehicle (water) or 8-bromoadenosine 3′,5′-cAMP (8Br-cAMP; 1 mM; Sigma, St. Louis, MO) for 6 or 24 h. GATA reduction was verified by real-time PCR and Western blotting as previously described and was typically greater than 60%.[13] Progesterone in the media and STARD1 mRNA levels were used as positive controls for 8Br-cAMP cell responsiveness and were previously reported.[13]

Real-time PCR

Total RNA isolation and real-time PCR were performed as previously described[13] with primers for pig STARD6 which amplified an 105 bp amplicon spanning exons 3-4. The primers were upstream 5′-CCGTTGCCAGCAAGGCTTC-3′ and downstream 5′-CAGGTTTGCAGAGGAAGTCA GATAG-3′ and were derived from Genbank accession no. XM_001925011.4. Porcine RPS16 mRNA served as an internal control.[13] Cultured granulosa cell mRNA quantification relative to vehicle-treated control RNAi was performed using the method of Pfaffl.[13,14] Single amplicons were initially verified by agarose gels and confirmed by single melt-curve peaks after each run. DNA sequencing verified STARD6 amplicon identity. Standard curves were used to quantify fresh cell and tissue target mRNA levels.

Western blots

Fresh follicles and corpora lutea were dissected from porcine ovaries obtained from the local abattoir as previously described.[15] Whole cell extracts were derived from at least three different animals or pools for each tissue or cell type. To reduce the presence of endogenous IgG in cell lysates, extracts were further processed using the ProteoPrep Immunoaffinity Albumin and IgG Depletion kit (Sigma). Proteins were resolved by 15% SDS-PAGE and blotted with goat anti-STARD6 (1 : 100, Santa Cruz, Santa Cruz, CA, sc-67847) primary antibody and donkey anti-goat secondary antibody (Santa Cruz, sc-2020). COS cell extracts were resolved using 4–20% gradient gels, and blotted with rabbit primary antibodies for P450scc (1 : 500, AP7899a, Abgent , Atlanta, GA), STARD1 (1 : 1500, gift of Douglas M. Stocco, Texas Tech University Health Sciences Center), STARD6 (1 : 100, AP11832b, Abgent), and actin (1 : 500, Cell Signaling, Danvers, MA). The horseradish peroxidase-coupled goat anti-rabbit IgG secondary antibody was from Zymed (65-6120).

Immunohistochemical localization of STARD6 in pig ovarian tissues

Pig ovaries were fixed immediately upon receipt in 4% paraformaldehyde and immunohistochemistry was performed using antigen-retrieval procedures previously described.[15] The primary antibody used was 1 : 200 rabbit anti-STARD6 (ARP65716, Aviva, San Diego, CA) and the secondary antibody was goat anti-rabbit IgG (1 : 600). Negative controls included omitting the primary antibody from the overnight incubation, and preabsorption of the primary antibody with excess target peptide. For preabsorption studies, STARD6 antibody was incubated overnight at 4°C with or without 200-fold molar excess of its target peptide (AAP65716, Aviva) prior to use. At least three different pigs were examined for each luteal structure. Follicles were examined on the slides containing luteal tissue and on slides from eight different peripubertal gilts exhibiting different size follicles.

COS-F2 steroidogenic assay

The expression plasmid containing the cDNA of myc-DDK-tagged human STARD6 was obtained from OriGene (Rockville, MD). Expression plasmids for human STARD1 and the F2 fusion protein, encoding all components of the P450scc system, were also utilized.[16] COS-1 cells were maintained in DMEM complete medium containing 1X non-essential amino acids, 1X anti-mycotic-antibiotic solution, and 10% newborn calf serum. When cells reached at least 50% confluency in six-well dishes, they were transfected with the plasmids (of similar molecular weights) expressing STARD6 (1 μg), STARD1 (1 μg), or empty expression vector (pcDNA3.1, 1 μg), 1 μg F2 plasmid, 0.01 μg ptk-Renilla-Luc (transfection efficiency control), and 8 μL Lipofectamine for 6 h in serum-free media in duplicate or triplicate. Following an 18-h recovery period in complete

medium, cells were changed to 1 mL fresh complete medium per well and treated with vehicle, 8Br-cAMP (0.25 mM) and/or 22 R-OH-cholesterol (5 μM) for 24 h. Media were stored and western analysis was performed on cell extracts to confirm expression of STARD1, STARD6, P450scc, and actin. Renilla luciferase activity was measured using the Dual Luciferase Assay System (Promega, Sunnyvale, CA). Pregnenolone levels in media were determined by ELISA (ALPCO Diagnostics, Salem, NH) and normalized to Renilla luciferase activity as previously described.[17]

Statistics

RNA, western, and pregnenolone experiments were repeated at least three times with three different batches of cells or tissues. Comparisons among groups for quantitative data were made using ln-transformed data by Repeated Measures ANOVA (granulosa cultures) or ANOVA (tissue and COS data) followed by Tukey's *post hoc* test.

Results

While prior studies show that 8Br-cAMP robustly induced STARD1 mRNA an average seven-fold in this granulosa cell model under similar conditions,[13] it had no significant effect on STARD6 mRNA levels (Figure 1). At both 6 h and 24 h the combined GATA4 + GATA6 knockdown reduced STARD6 mRNA levels when compared to the Control RNAi in the presence of either vehicle or 8Br-cAMP. We were unable to detect STARD6 protein in cultured granulosa cells (not shown).

We next evaluated STARD6 mRNA and protein levels in fresh granulosa cells and tissues from pig ovaries (Figure 2). STARD6 mRNA was detected in all ovarian structures and cells examined, yet mid-luteal phase (mature) corpora lutea exhibited significantly higher mRNA expression than all other tissues and cells analyzed (Figure 2(a)). STARD6 protein levels as assessed by western blot exhibited a similar tissue profile to that observed for mRNA, with the highest levels being present in mid-luteal phase corpora lutea (Figure 2(b)). Two bands were observed for most tissues. The difference between them is unclear but it could represent an unknown post-translational modification, a cleavage event,[7] or alternative splicing as suggested by our *in silico* genome analyses (data not shown).

Since fresh tissue and cell preparations contain non-steroidogenic cell types that may contribute to the STARD6 signal, we performed immunohistochemistry to determine whether STARD6 specifically localized to steroidogenic cells (Figure 3(a)–(f)). STARD6 immunoreactivity was found in oocytes (healthy and atretic), granulosa of preantral and antral follicles, some theca cells of antral follicles, and luteal cells of both early and mid-luteal phase corpora lutea. Regressed corpora lutea had some STARD6 staining only if steroidogenic luteal cells were still present (not shown). The relative staining of early and mature luteal cells was more intense than granulosa, theca, or oocytes on the same slide. Staining in early corpora lutea was mostly cytoplasmic, whereas in mature luteal cells, STARD6 staining was distributed throughout the cell including the nucleus. Oocytes, some theca, and the majority of granulosa cells also had weak nuclear staining. Preincubation of the primary antibody with its target peptide removed all signal (Figure 3(c)). In addition, omission of the primary antibody gave no positive signal (Figure 3(f)).

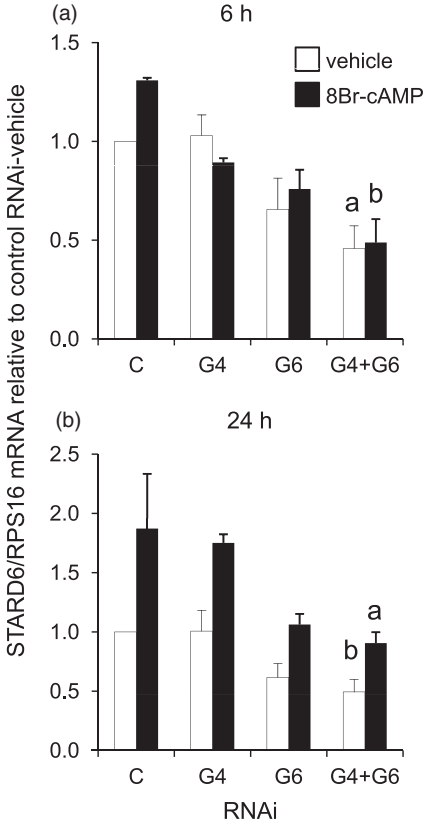

Figure 1 STARD6 mRNA expression in cultured porcine granulosa cells show that STARD6 is reduced by double GATA depletion and shows no regulation by cAMP analog. Following attachment to plates granulosa cells were transfected with the indicated RNAi molecules (C, Control; G4, GATA4, G6, GATA6, G4 + G6, GATA4 + GATA6). Vehicle or 8Br-cAMP (1 mM) treatment was for 6 or 24 h following a 72-h post-transfection knockdown period. Mean + SEM shown for 6 h, $n = 3$, and 24 h, $n = 4$ experiments. Letters indicate mRNA levels for the GATA-reduced condition were lower than the Control RNAi condition with the same treatment (vehicle or 8Br-cAMP) at the same time point, a, $P < 0.05$ and b, $P < 0.01$

Since STARD6 levels were highest in the tissue with the greatest steroidogenic capacity, we determined if STARD6 could increase *de novo* steroidogenesis by employing a classic COS cell assay (Figure 4(a)). In this assay, non-steroidogenic COS cells are transfected with the F2 plasmid which expresses a fusion protein consisting of P450scc and coenzymes ferrodoxin and ferrodoxin reductase.[16] COS cells co-expressing F2 and empty vector produced low levels of pregnenolone while co-expression with STARD6 increased pregnenolone two- to three-fold above vector-only levels. Incubation with 8Br-cAMP did not significantly alter steroid output. Positive controls STARD1 and the addition of mitochondrion-permeable 22 R-OH cholesterol to vector-only cells robustly increased pregnenolone levels far in excess of STARD6. Expression of the major proteins encoded by the transfected plasmids was verified by western blot (Figure 4(b)).

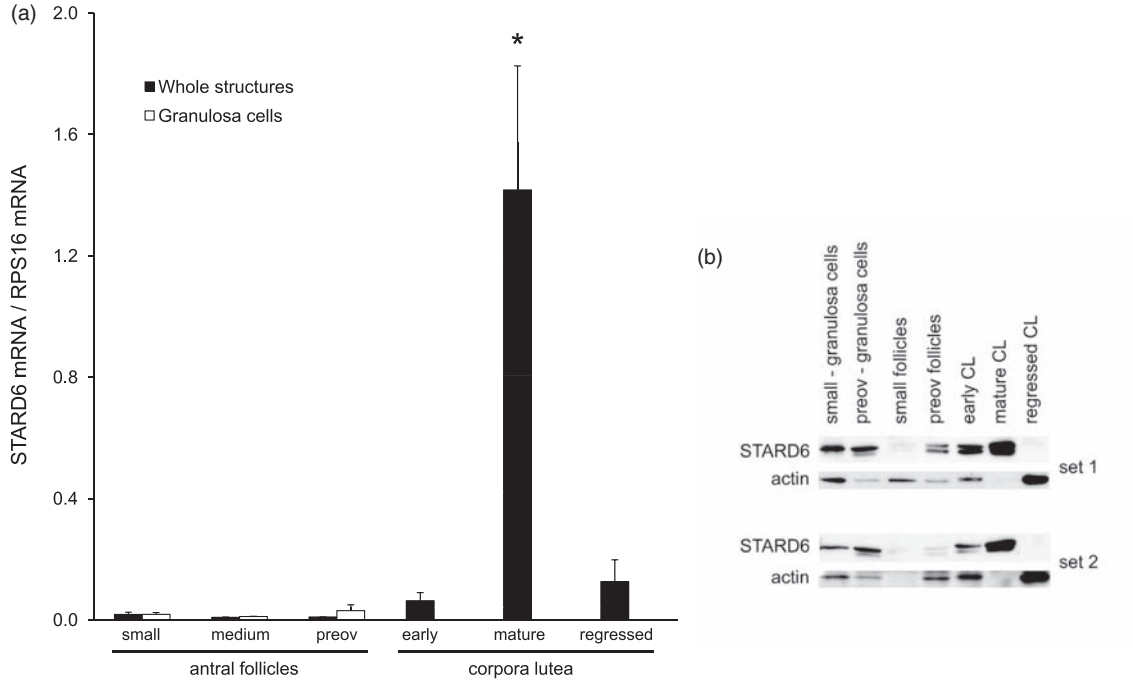

Figure 2 STARD6 mRNA and protein are highly expressed in mid-luteal phase (mature) corpora lutea. Small, medium, and preov (preovulatory) represent antral follicles of 1–2, 3–4, and 8–10 mm in diameter. Corpora lutea of the pig estrous cycle are estimated to be post-LH surges day 3–5 (early), days 8–12 (mature, mid-luteal), and days 18–23 (regressing). (a) Bars indicate mean mRNA + SEM of three independent tissue sets (from different animals). Asterisk indicates mRNA levels were higher than other tissue and granulosa cell preparations ($P < 0.5$). (b) Western blots of whole cell extracts from the indicated cells or tissues with goat anti-STARD6 or actin. The blots show the STARD6 trend for two independent tissue sets and was repeated a total of three times with similar results. Actin tended to be under-represented in mature luteal preps and over-represented in regressed luteal preps

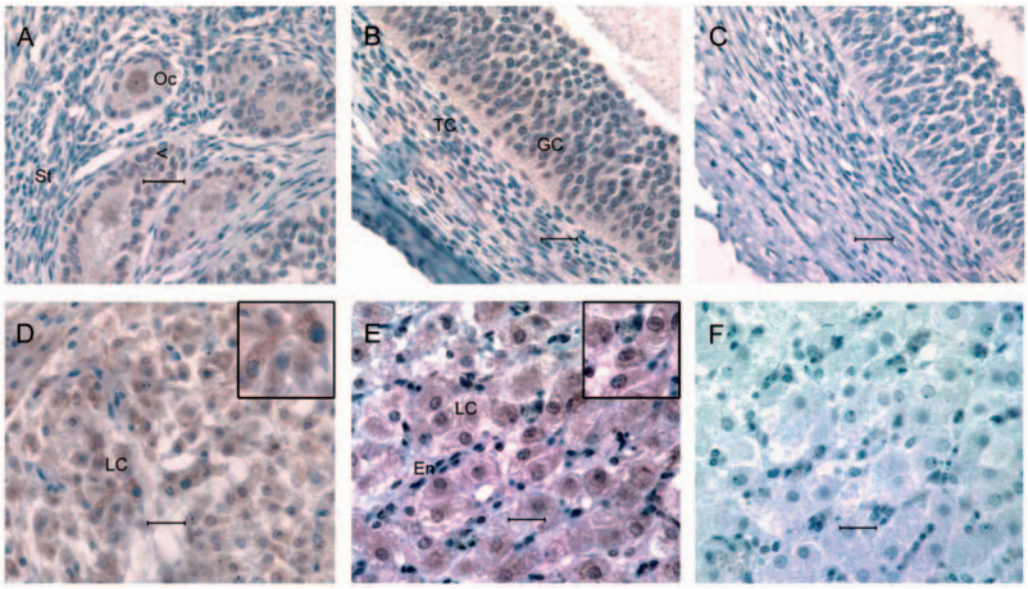

Figure 3 Immunolocalization of STARD6 in pig ovarian sections. Brown color represents positive signal. Bars represent 25 μm. (a)–(e) represent sections incubated with primary antibody to STARD6 (Aviva, ARP65716); (c) represents a negative control where the primary antibody was preabsorbed with its target peptide and (f) represents a negative control where primary antibody was omitted; (a) shows preantral follicles, Oc (oocyte), St (stroma), arrow head (<) points to positively stained granulosa cells; (b) part of the wall of a medium antral follicle showing granulosa cells (GC) and theca cells (TC); (c) negative control section similar to (b); (d) early corpus luteum with organizing luteal cells (LC); (e) mid-cycle corpora lutea with positive luteal cells and negative endothelial cells; (f) negative control section similar to section (e). Insets represent enlarged regions of same section to show cellular distribution.

Discussion

Our studies with cultured pig granulosa cells support our preliminary microarray findings[12] that these cells express STARD6 mRNA, and indicate that STARD6, unlike STARD1, is not responsive to cAMP stimulation. The transcription factors GATA4 and GATA6 regulate a number of genes involved in steroidogenesis[1] and other reproductive functions in a cAMP-dependent or cAMP-independent

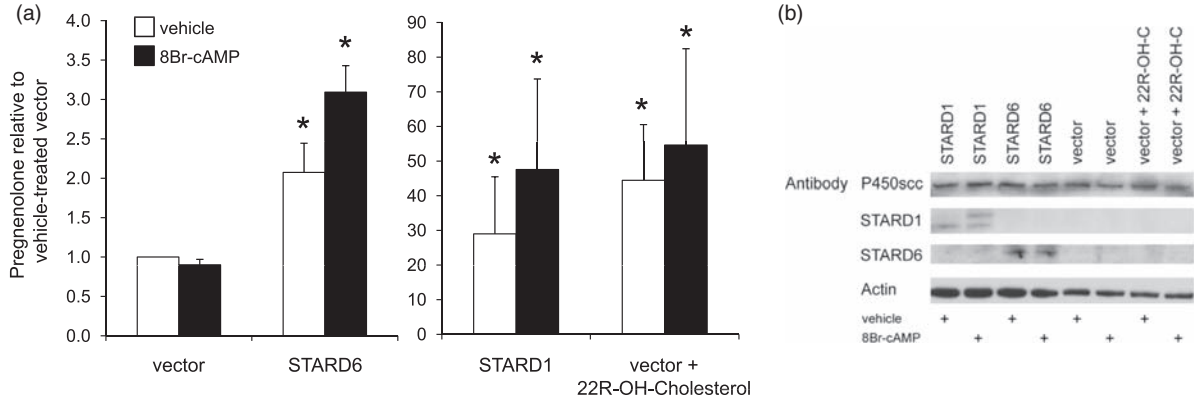

Figure 4 COS-F2 assay shows STARD6 can enhance *de novo* steroidogenesis. (a) COS cells were transfected with the F2 and indicated plasmids and cultured as described under *Materials and methods*. Pregnenolone levels were corrected for Renilla-luciferase activity to control for transfection efficiency prior to being normalized to the vector only-vehicle treated control. Bars are the mean + SEM of $n = 3$ experiments. STARD1 and 22 R-OH-cholesterol were used as positive controls and are shown separately because of the larger scale of the steroidogenic response. Asterisks indicate differences from the vector-only group with the same treatment, $P < 0.01$. 8Br-cAMP treatment (0.25 mM) or vehicle treatment and 22 R-OH-cholesterol (5 μM) treatment were present during the last 24 h of culture. (b) Western blot confirming the expression of P450scc, STARD1 (precursor and mature forms), and STARD6 in transfected COS cell lysates. Actin reflects protein loading

manner.[18,19] Combined GATA4 + GATA6 reduction significantly lowered STARD6 mRNA levels, indicating that STARD6 is either a direct GATA target gene like STARD1, or regulated by a signal dependent on these transcription factors. The fact that we could not detect STARD6 protein in cultured cells indicates that the protein if present is at low levels in this context. We speculate that the loss of STARD6 protein detection with culture of granulosa cells could reflect the need for some non-gonadotropin factor present *in vivo*, loss of some signal from other follicular cells, or be possibly due to the serum-free conditions of culture.

Unlike with cultured granulosa cells, we did detect STARD6 protein in fresh cells. The profiles of STARD6 mRNA and protein in follicles and corpora lutea taken from different stages of the estrous cycle clearly show that mid-luteal phase corpora lutea, the structure that exhibits the highest level of progesterone production,[20] also exhibited the highest level of this protein. Our RNA studies contrast that of two previous studies that failed to detect STARD6 mRNA in ovarian preparations from human and rat using Northern blots.[8,9] A major difference in our study is that we utilized RT-PCR, which is a more sensitive technique. Also, the recent commercial availability of STARD6 antibodies allowed us to further evaluate protein distribution. We do not believe ovarian STARD6 is unique to the pig, as we recently identified STARD6 transcripts in human luteinized granulosa cells by RT-PCR as well (not shown).

Our immunohistochemical studies localized STARD6 within steroidogenic cells with higher relative expression in steroidogenic luteal cells. Some STARD6 signal was observed in antral follicle theca cells which generate steroids *de novo* and in the granulosa of both preantral and antral follicles that are thought to have limited steroidogenic capacity *in vivo*.[1] When placed in culture porcine granulosa cells make measurable progesterone due to the onset of spontaneous luteinization.[21] Altogether, these correlations predict that the major role for STARD6 is in the functional corpus luteum, and that STARD6 might contribute to basal (non-cAMP-mediated) steroidogenesis. Future STARD6 knockdown in the appropriate primary cell types will be needed to investigate these possibilities.

In addition to cytoplasmic staining, we observed nuclear staining in granulosa, some theca, and luteal cells. The observed nuclear staining is consistent with its distribution in neurons and glia.[11,22] Cytoplasmic staining was also reported in developing male germ cells[8] and Leydig cells from hypothyroid rats.[10] The function of nuclear STARD6 is not understood, but might be related to the ability of START domain proteins to function as lipid sensors[5] or the recent finding that STARD6 contributes to normal mitosis.[23] More detailed studies of STARD6 subcellular localization are needed to determine if STARD6 associates with specific organelles or nuclear structures and if it does act as a lipid sensor to support cholesterol supplies for ongoing steroid production.

Using F2-transfected COS-1 cells which have previously been used to demonstrate that STARD1 can increase *de novo* steroidogenesis,[24,25] we showed that STARD6 also has this ability. Our findings are consistent with those of Bose *et al.*,[7] who found that recombinant mouse STARD6, when added to isolated pig adrenal mitochondria with cholesterol, increases *de novo* steroidogenesis. Close homologs of STARD6, STARD4, and STARD5, which similarly lack a mitochondrial targeting sequence, also increase steroidogenesis in this assay, albeit like STARD6 to a lesser extent than STARD1.[25] Co-addition of cAMP analog did not significantly augment pregnenolone production over vehicle levels. This may be due to the fact that COS cells have endogenous protein kinase A activity.[26]

In conclusion, STARD6 can now be added to the group of START domain proteins with the potential to increase *de novo* steroidogenesis in COS cells provided with the necessary steroidogenic components. STARD6 localizes to steroidogenic cells of the pig ovary, with greatest expression in the highly steroidogenic cells of the corpus luteum. The identification of STARD6 in granulosa and oocytes, as

well as its presence in the nucleus, makes it likely that STARD6 has other ovarian functions too. Among the other possible functions may be non-steroidogenic cholesterol transport between cellular compartments, participation in the endoplasmic reticulum stress response, or oxysterol production as has been proposed for other STARD4 subfamily members.[4] Finally, STARD6 mRNA, although not regulated by cAMP in cultured granulosa cells, shows a dependence on GATA4/6 transcription factors. As GATA4 and GATA6 are linked to numerous hormonal signals,[12,19] this last finding may help to identify endocrine or paracrine regulators of STARD6.

Author contributions: HAL, HSB, SRK, and YYH contributed to the design, data interpretation, and preparation of the manuscript. HAL, NEW, BS, and YYH performed the experiments. HAL analyzed the data and wrote the manuscript.

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