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short report

Cyp11a1 Deficiency Retards Cell Movement

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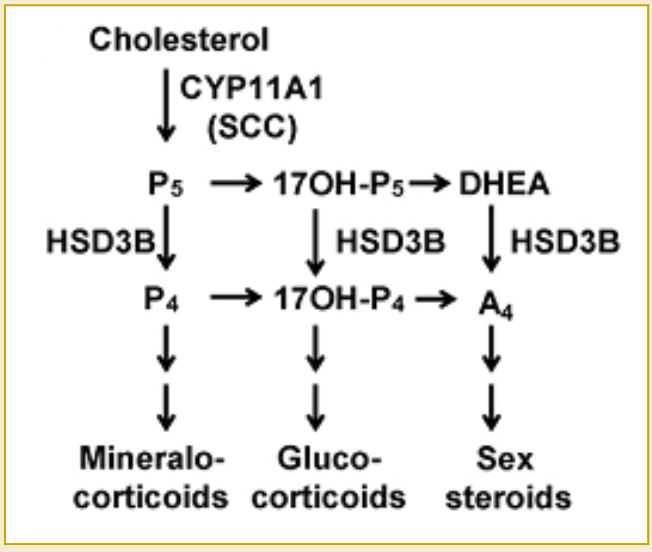
Introduction

Steroids play essential roles in physiological functions such as pregnancy, stress responses, anti-inflammatory responses, and tumorigenesis. They are also needed for embryonic and sexual development. Steroids are synthesized by endocrine glands such as the adrenal cortex and the gonads through steroidogenesis. In steroidogenesis, cholesterol is first converted to pregnenolone (P5) by cyp11a1 (SCC) (Fig. 1). After P5 synthesis the pathway diverges to produce mineralocorticoids, glucocorticoids, and sex steroids.



SCC has been shown to play a major role in zebrafish embryogenesis. In post-fertilization zygotes, blastomeres above the yolk undergo rapid cleavage, during this rapid cleavage SCC is expressed in the cytoplasm of blastomeres. During epiboly, blastomeres move downward from the animal pole to envelope the yolk. As the zygote undergoes epiboly, SCC expression is downregulated in the blastomeres. At the same time, SCC is upregulated in the yolk syncytial layer (YSL), which is an extra-embryonic layer at the forefront of the moving blastomeres. The loss of SCC expression in the YSL does not prevent epiboly but results in epibolic delay. Epibolic delay can be partially rescued by SCC and its product P5.

Figure 1: Steroidogenesis Pathway. Cholesterol is converted to pregnenolone (P5) by CYP11A1 (SCC). After P5 the pathway diverges to produce various other steroids.

P5's ability to rescue epiboly suggests that P5 or its downstream metabolites are involved in epiboly. In fact, P5 has been shown to stabilize yolk microtubules which are necessary for epiboly. P5's role in microtubule stability may also contribute to or control cell movement in other cell types.

It is not known whether the functions of SCC and P5 are conserved from zebrafish systems to mammalian systems. We showed through SCC knockdown that SCC and P5 are important for cell migration. In addition, we also began production of antibodies against zebrafish SCC protein to allow for further SCC studies.

Aims

1. Synthesize antibodies to confirm cyp11a1 protein expression and allow further experimentation.
    A. Produce recombinant cyp11a1 protein
2. Determine id Cyp11a1's and P5's role in cell movement conserved from zebrafish to mammalian systems.
    A. Knockdown Cyp11a1
    B. Cell Migration Assay

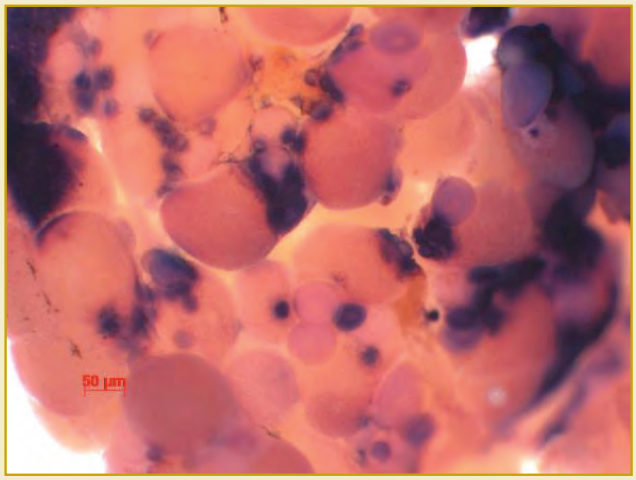


Figure 2: Zebrafish Ovary. In situ hybridization of zebrafish ovary. SCC can be found in oocyte cytoplasm. Signal becomes diluted as oocyte matures and cytoplasm increases. Location of SCC mRNA is not a guarantee that SCC is expressed. Testes and brain also show SCC in situ hybridization. Because of sample contamination, in situ staining of testes and brain was weak.

Antibody Synthesis

Recombinant Protein Induction

While wholemount in situ can provide information on the location and expression of SCC mRNA, it is not a guarantee that SCC protein is being expressed (Fig. 2). To verify protein expression, antibodies against zebrafish SCC protein are required.

A pET expression system was used to culture SCC protein for antibody production. SCC segment fragments N1, N2, and N3 were cloned into a pET29a(+) expression vector (Table1, Fig. 3). E. coli bacterial strains ER2566, DE3, pLys, and R1L were tested for ideal expression. The bacteria were transformed with recombinant SCC and grown to an OD600 = 0.8 and then induced with isopropyl β-D-1-thiogalactopranoside (IPTG) for 4 hours. After induction, proteins were separated by SDS-polyacrylamide gel electrophoresis.

The bacterial lines ER2566, DE3, and R1L showed protein expression in both the induced and uninduced conditions. The bacterial line pLys was the only bacterial line found to express protein solely in the induced condition (Fig. 4).

Optimal induction temperatures were then tested for SCC in pLys. Incubation temperatures of 16°C and 37°C were tested. The bacteria were transformed with recombinant SCC and grown to an OD600 = 0.8 before induction. During sample preparation, both the pellet and the supernatant fractions were collected from both temperature conditions to determine protein solubility. No SCC expression was observed at the 16°C condition. The SCC recombinant protein expression was found only in the 37°C temperature condition and largely in the insoluble portion (Fig. 5).

SCC and P5 Important for Cell Movement

To determine whether SCC's and P5's role in cell movement was conserved from zebrafish to mammalian systems, SCC was knocked-down in mouse Y1 adrenocortical tumor cells. Because of SCC's conversion of cholesterol to P5 in steroid production, knocking-down SCC limits SCC expression and prevents or decreases P5 expression as well.

Knockdown of SCC and P5

Knockdown was achieved by using shRNA sequences complementary to SCC mRNA. The shRNA was introduced into Y1 cells via lentivirus. Once infected the shRNA is processed by the cell to become siRNA, which binds the SCC mRNA. This leads to mRNA degradation and ultimately gene silencing.

Five shRNA sequences were synthesized: m1, m2, m3, m4, and m5. Only shRNA m1 and m4 were found to knockdown SCC expression while shRNA m5 only partially decreased shRNA expression (Fig.6).

Cell Migration

Y1 cells were infected with shRNA m1, m4, m5 and Luc shRNA against luciferase as a control. Each condition was imaged at five minute intervals for three hours. The images were compiled into a movie. Control cells infected with luciferase shRNA exhibited normal cell migration patterns. Cells infected with m1, m4, and m5 exhibited abnormal cell migration patterns. Y1 cells with reduced levels of SCC and P5 exhibited much slower movement than control cells. Reduced levels of SCC and P5 also resulted in frequent direction changes unlike their control counterparts. These differences in movement suggest that SCC and P5 do affect cell movement in mammalian systems.

Discussion

In this study we show that the role of SCC and P5 in cell movement is conserved from zebrafish to mammalian systems. SCC knocked-down cells exhibit abnormal cell movement. Because each cell within the field for all conditions came in contact with another cell in its migration path, it was not possible to perform a free cell migration quantitative analysis. Qualitatively, the data supports SCC and P5 having a role in cell migration.

Because SCC knocked-down cells exhibited frequent changes in migration direction, we posit that P5 may affect cell movement through cell polarity. P5's role in microtubule stability supports this hypothesis. The loss of P5 could negatively affect microtubules and disrupt cell polarity. A loss of cell polarity regulation may explain the constant changes in migration direction.

To further studies of zebrafish SCC, we began efforts to synthesize zebrafish SCC antibodies. Induction experiments found that the expression bacterial pLys was the only tested strain to show SCC recombinant protein expression in the induced condition. Once determined as an ideal bacterial line, temperature conditions and solubility were tested within the pLys system. It was found that the SCC recombinant proteins expressed at higher concentrations at 37˚C and were largely insoluble. Once synthesized, these antibodies will allow us to visualize SCC expression in zebrafish embryos and tissues with greater confidence than with in situ hybridization.

Materials and Methods

Cell culture and Infection – Viruses were cultured in 293FT cells plated with 9x105 cells per 6cm plate grown in 37˚C in 5% CO2. After 24 hours the cells were transfected with pMD2.G (750µg per 6cm plate), psPAX2 (250µg/6 cm plate), and shRNA

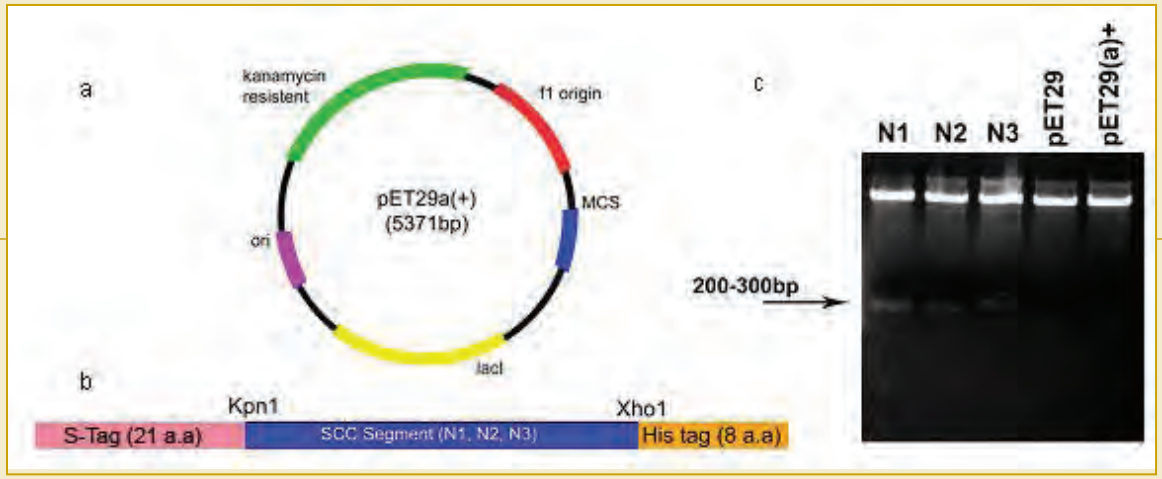


Figure 3: Zebrafish SCC protein segments (a) Segments were cloned into the pET29(a)+ expression vector with a S-Tag and a His tag for protein purification. (b) The segments were cloned in using the Kpn1 and Xho1 restrictions sites. (c) The recombinant protein visualized on 0.8% agarose gel: N1, 246 bp; N2, 248 bp; and N3, 323 bp.

Amino acid region	peptide sequence
1-82 a.a. (N1)	MARWNVTFARLDQSLSSLKNLLQVKVTRSGRAPQNS TVQPFNKIPGRWRNSLLSVLAFTKMGGLRNVHRIMVH NFKTFGPIY
81-163 a.a. (N2)	IYREKVGIYDSVYIIKPEDGAILFKAEGHHPNRINVDAW TAYRDYRNQKYGVLLKEGKAWKTDRMILNKELLLPK LQGTFVPL
210-317a.a. (N3)	ERLGLLLDNIDPEFQHFIDCVSVMFKTTSPMLYLPPGLL RSIGSNIWKNHVEAWDGIFNQADRYIQNIFKQWKENPE GNGKYPGVLAILLMQDKLSIEDIKASVTELM

Table 1: Recombinant SCC protein. Segments of zebrafish SCC protein were cloned into pET29(a)+ expression vector using Kpn1 and Xho1 restriction sites respectively.

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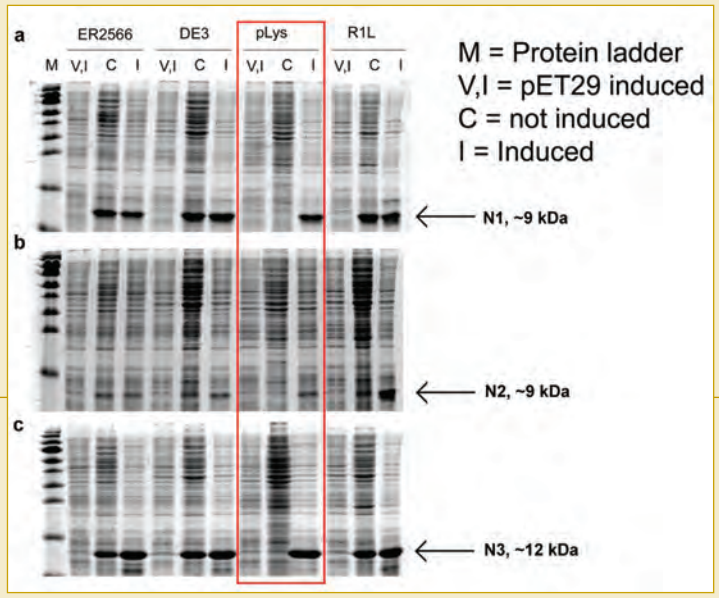

Figure 4: pLys expresses SCC segments. E. coli expression strains were transformed with SCC protein segments. The bacteria were cultured to an OD600 = 0.8 and then induced with IPTG for 4 hours. Only the pLys strain expressed recombinant protein in the induced state for all three segments. (a) N1 (b) N2 (c) N3

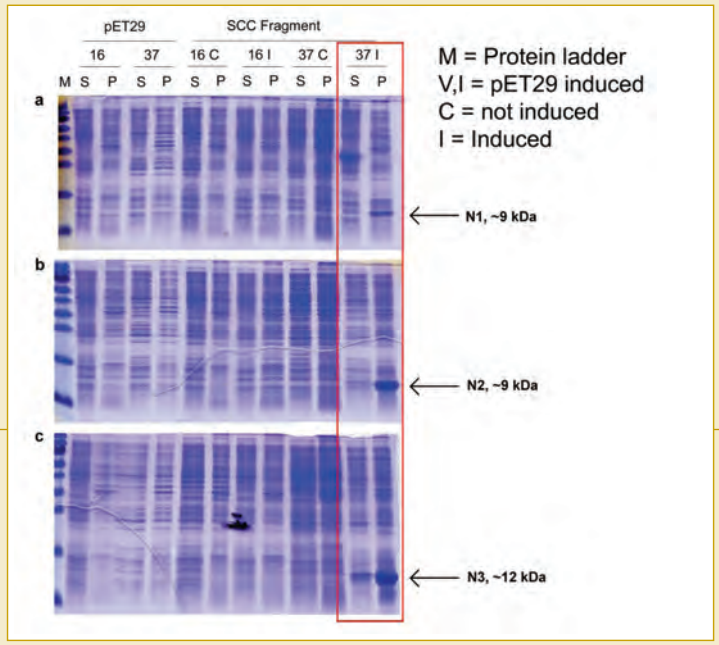

Figure 5: pLys induction conditions. Expression strain pLys was transformed with SCC protein segments. The bacteria were incubated at 16˚C and 37˚C until OD600 = 0.8 and then induced with IPTG for 4 hours. The soluble and insoluble fractions were collected. SCC is best expressed at 37˚C and found largely in the insoluble portion. (a) N1 (b) N2 (c) N3

(1µg/6cm plate) premixed in 50µl OPTI for 5 minutes and then 6µl of LMPF2000 for 15 minutes. At transfection, the medium was replaced with fresh medium. The medium was replaced again after 16 hours. Viruses were collected 48 hours post transfection. Mouse Y1 adrenocortical tumor cells were maintained in Dulbecco's modified Eagle medium (DMEM)-F12 medium supplemented with 10% fetal bovine serum. For infection, Y1 cells were plated with 5x104 cells per 6cm plate. After 24 hours the cells were infected with a viral cocktail (500µl virus, 500µl F12-FBS, and 1µl polybrene). After 24 hours of infection, the medium was replaced with puromycin (2µg/ml). After 48 hours of infection, the cells were harvested for Western blotting or filmed for a cell migration assay.

In situ hybridization – Embryos between fertilization and 48 hours post-fertilization were captured in Petri dishes, chilled on ice, and then fixed overnight in 4% paraformaldehyde/PBS buffer at 4 ˚C. Fish were chilled on ice until unconscious and then sacrificed by severing the spinal cord. Tissues were removed and fixed in 4% paraformaldehyde/PBS buffer at 4˚C. Both embryos and tissues were fixed again in 4% paraformaldehyde/PBS buffer and then washed 3 times in PBST (PBS with 0.1% Tween 20). Samples were digested in proteinase K and then washed 3 times with PBST before prehybridization in HYB+ buffer (60% formamide, 5× SSC, 500 µg tRNA, 50 µg/L heparin, and 0.1% Tween 20) for 4 hours at 70 ˚C. The P450scc(cyp11a1) probe (200 ng/mL) was incubated with the samples for over 16 hours at 70 ˚C. Samples were washed with 75%, 50%, and 25% HYB+/2× SSC buffer for 15 minutes each, followed by 2× SSC, 0.2× SSC buffer, and PBST for 15 minutes each. They were then blocked in 5% albumin in PBST for 2 hours and incubated in a 1:5000 dilution of anti-digoxigenin antibody (Fab, conjugated with AP, Roche Applied Science, Indianap-

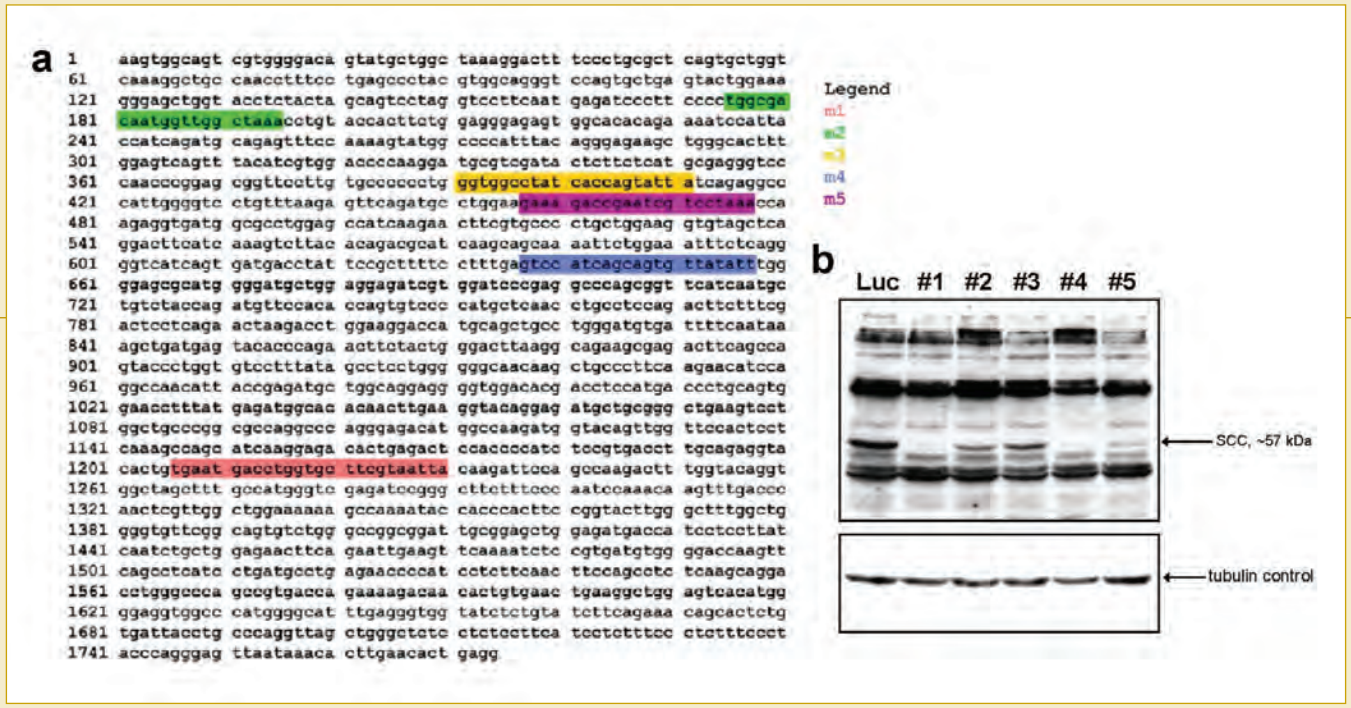

Figure 6: SCC shRNA (a) Five shRNA against SCC were generated. (b) Luc knocked-down luciferase, not normally found in Y1 cells. It was found that m1 and m4 shRNA knocked-down SCC expression with m5 only partially knocking-down SCC expression.

olis, IN, USA) at 4°C overnight. Antibodies were then removed and washed 3 times with PBST and 3 times with staining buffer (0.1 M Tris, pH 9.5, 100 mM NaCl and 50 mM MgCl2, and 1 mM levamesol). The samples were finally stained in NBT/BCIP (Roche Applied Science, Indianapolis, IN, USA) or TNBT/BCIP buffer (Chemicon, Billerica, MA, USA).

Immunoblotting and Antibodies – Cells were harvested and lysed in lysis buffer (50mM Tris (ph=7.5), 5mM EDTA, 1% Tx-100, 300mM NaCl, 50x protease inhibitor) 3 days after siRNA transfection. Equal amounts of total proteins were separated by gel electrophoresis, and then transferred to ImmobilonTM-P membrane (Millipore, Billerica, USA) via semidry transfer. Membranes were incubated with monoclonal antimouse-SCC antibodies and polyclonal anti-Flag or anti-Hsp70 antibodies in 5% milk overnight at 4°C and then with HRP conjugated secondary antibody in 5% milk for 45 minutes at room temperature. Signals were detected by chemilumiescence assays.

Expression and induction of recombinant cyp11a1 – Bacterial *E. coli* expression lines ER2566, R1L, pLys, and DE3 were transformed with recombinant cyp11a1 protein fragments were grown in 2-YT medium (tryptone 16g, yeast extract 10g, NaCl 5g in 1L H2O) with 50 μg/mL kanamycin up to an OD600 = 0.6. Cells were then induced with 1 mM IPTG at 37°C for 4 hours.

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