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Cloning and characterization of a cDNA encoding cholesterol side-chain cleavage cytochrome P450 (CYP11A1): Tissue-distribution and changes in the transcript abundance in ovarian tissue of Japanese eel, *Anguilla japonica*, during artificially induced sexual development

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Abstract

Cholesterol side chain cleavage cytochrome P450 (CYP11A1: P450scc) is a crucial steroidogenic enzyme that catalyzes an initial step in the production of all classes of steroids. A cDNA encoding Japanese eel P450scc was cloned and characterized. The cDNA putatively encoded 521 amino acid residues with high homology to those of other vertebrate forms. The recombinant P450scc produced in COS-7 cells efficiently catalyzed the conversion of 25-hydroxycholesterol into pregnenolone. By northern blot, a single P450scc transcript of approximately 3.3 kb was detected in both ovary and head kidney. Transcript levels of this enzyme significantly increased throughout ovarian development artificially induced by salmon pituitary homogenate, which suggests that gonadotropic stimuli can induce ovarian expression of the P450scc gene in teleosts, as has been reported in mammals. Furthermore, RT-PCR analysis revealed that gene expression of three steroidogenic enzymes, P450scc, P450c17 and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) show distinctly different tissue specific patterns of expression in the Japanese eel. The P450scc gene was expressed in ovary and head kidney while the sole source of the P450c17 transcript was ovary. In contrast, 3 β -HSD transcript was detected in all tissues examined, brain, liver, spleen and trunk kidney, etc. These suggest that some steroidogenic enzymes are also expressed in non-endocrine tissues and could potentially regulate the local and/or circulating steroid levels in teleosts, as they do in mammals.

Introduction

Steroid hormones modulate many physiological events including development, growth, osmoregulation and reproduction in vertebrates. In the teleost ovary, two distinct steroid hormones, estradiol-17 β and a maturation-inducing steroid, typically 17 α , 20 β -dihydroxy-4-pregnen-3-one (17 α , 20 β -DHP), are produced during reproductive cycles. It has been well documented that estradiol-17 β is essential for oocyte growth, mainly hepatic vitellogenin synthesis [1] and that 17 α , 20 β -DHP plays an important role in final oocyte maturation [2]. Pregnenolone is a sole precursor of all other steroids and is synthesized from cholesterol by the oxidative side-chain cleavage reaction catalyzed by cytochrome P450 side-chain cleavage (P450scc: CYP11A1) [3]. Therefore, P450scc is indispensable in the production both of estradiol-17 β and 17 α , 20 β -DHP in the ovary as well as in the production of other classes of steroid hormones including androgens in the testis and corticosteroids in the adrenal gland of teleosts.

Japanese eels (*Anguilla japonica*) caught from the wild have immature ovaries and further oogenesis is arrested under captive conditions. However, ovarian development can be induced by the administration of salmon pituitary homogenate (SPH) richly including pituitary hormones, such as pituitary glycoprotein hormones, growth hormone etc [4]. Since gonadotropic effects of exogenous hormones can therefore be readily assessed, the Japanese eel would be a suitable model for the study of reproductive physiology in fish ovarian tissue. In fact, SPH strongly enhances the activities of several steroidogenic enzymes [5], which is attributable in part to up-regulation of the expression of genes encoding these steroidogenic enzymes [6-8]. P450scc is assumed to be induced

during artificial induction of maturation since the titers of serum sex steroids increase during hormonal treatment [5].

The cDNA encoding P450scc has been isolated and characterized in several species of mammals and the predominant expression of this gene in both gonadal and adrenal tissues has been well documented [9-12]. Furthermore, it has been well documented in the mammalian ovary that gene expression of P450scc is mainly modulated by gonadotropins [13, 14], and insulin-like growth factor-I (IGF-I) [15, 16]. However, the gene regulation of P450scc has received little attention in fish so far although the cDNAs encoding P450scc of rainbow trout [17], zebrafish [18] and stingray [19] have been cloned. Furthermore, there are few available reports on extra-gonadal expression of this gene in fish [18, 19].

In the present study, the cDNA encoding P450scc was cloned from ovarian tissue and characterized. The expression of P450scc, and two other steroidogenic enzymes, 3 β -hydroxysteroid dehydrogenase (3 β -HSD) [20] and P450c17 [6], both in ovarian and extra-gonadal tissues were also investigated. In addition, as a first step to investigate hormonal regulation of the gene expression of P450scc in fish, changes in transcript abundance of P450scc were examined in the ovary of Japanese eels during sexual development artificially induced by treatment with gonadotropin-rich salmon pituitary homogenate.

Materials and Methods

Animal treatment, tissue collection and RNA extraction

Forty-three eels feminized by estradiol-17 β administration [21] were artificially induced with salmon pituitary homogenate (SPH) and ovarian tissues at various developmental stages were collected. Two further eels served as Ringer-injected controls. The developmental stages of ovaries were classified into pre-, early, mid-, or late vitellogenic stage, or migratory nucleus stage. The detailed procedure of hormonal treatment and the criteria defining the developmental stages of the ovary are described elsewhere [6]. Various tissues (i.e. brain, small intestine, heart, liver, spleen, head kidney, posterior kidney and ovary) were also collected from a female eel with a late vitellogenic ovary. At the time of tissue collection, fish were anesthetized with ethyl-p-aminobenzoate and sacrificed by decapitation. Tissues were immediately removed, frozen in liquid nitrogen and stored at -80 °C until analysis. Total RNA was extracted using a commercial product, ISOGEN (Nippongene, Tokyo Japan) according to the manufacturer's instructions. The RNA was subsequently enriched in poly(A)⁺-RNA with Oligotex-dT-30 (Takara, Otsu, Japan). The quantity and quality of RNA were determined by UV absorbance at 260, 280 and 320 nm wavelengths.

Cloning and sequence of Japanese eel P450scc cDNA, and Phylogenetic analysis

Complementary DNA was synthesized from oligo(dT)₁₂₋₁₈ primed poly(A)⁺-RNA from Japanese eel head kidney using Superscript II Reverse Transcriptase (Life technologies Inc, Carlsbad, CA). Eel P450scc cDNA fragments were obtained by polymerase chain reaction (PCR) using a set of primers designed from consensus sequences of other P450scc forms; forward primer: 5'-GG(TC)CC(AC)AT(ATC)TA(TC)AG(AG)GA(AG)AA-3', reverse primer: 5'-

GGGTG(AG)AG(TC)CT(ATGC)AG(ATGC)GT(TC)TC-3'. The PCR procedure consisted of 30 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min. The resultant amplicon inserted into the PCRTMII vector (Life technologies Inc.) was sequenced using an ABI 373 DNA sequencer (Perkin-Elmer, Foster City, CA). The amino acid sequence predicted from the amplicon showed high homology to highly conserved regions of other P450scc forms, thus, the product was utilized as a probe to screen a λ gt 10 cDNA library constructed from oligo(dT)-primed mRNA purified from eel ovary at the migratory nucleus stage, using Amersham's cDNA cloning system.

Approximately 500,000 phage plaques were screened with the ³²P-labeled eel P450scc cDNA fragment, using a random-primed DNA labeling kit (New England Nuclear, Boston, MA). Positive clones longer than 1.5 kb in length were subcloned into a pBluescript KS⁺ vector and bi-directionally sequenced.

A phylogenic tree of P450scc characterized to date was generated by the neighbor-joining method using the Clustal W and njplot programs [22].

Transient expression of eel P450scc in COS-7 cells

Japanese eel P450scc cDNA containing the entire open reading frame was inserted into pSG5 (Funakoshi, Tokyo, Japan), an *in vitro* eukaryotic expression vector with the SV40 promoter. COS-7 cells (Riken, Tsukuba, Japan) were dispensed into wells of 6-well tissue culture plates and subsequently transfected with a complex of eel P450scc cDNA and pSG5, using the DOSPER Liposomal Transfection Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. Empty pSG5 served as controls. COS-7 cells putatively expressing eel P450scc were incubated

for 6 or 12 hours in 2 ml of complete growth media including 10 ng ml⁻¹ of 25-hydroxycholesterol (Sigma, St. Louis, MO) as a precursor. After incubation, samples were extracted with diethyl ether and the concentrations of pregnenolone (Preg) were measured by radioimmunoassay with a specific antiserum to Preg (COSMO Bio, Tokyo, Japan) according to the manufacturer's protocol. Three replicate incubations were carried out for each experiment.

Northern blot analysis

Poly (A)⁺-enriched RNA was obtained from ovaries at various developmental stages and from head kidney of a female eel with late vitellogenic stage ovary. Northern blot analysis was conducted with RNA samples from ovarian tissue (5 µg) or head kidney (0.3 µg) with a ³²P-labeled full-length eel P450scc cDNA. Transcript abundance of P450scc in the ovary was normalized to the abundance of β-actin for quantitation [23]. The detailed procedure and conditions for hybridization, and detection of radioactive signals for each transcript are described elsewhere [6].

Analysis of the gene expression of P450scc, P450c17 and 3β-HSD by RT-PCR

Total RNA (1 µg) isolated from various tissues of a female eel at the late vitellogenic stage was primed with a random hexanucleotide primer (Life technologies Inc) and then reverse transcribed using M-MLV reverse transcriptase (Life technologies Inc) in a standard 20 µl reaction. PCR was carried out with an aliquot of this reaction (1 µl) in 50 µl of a standard PCR reaction under the following conditions: 94 °C (30 s), 60

°C (30 s) and 72 °C (1 min) for 30 cycles. The primer sets for amplification of cDNA of P450scc, 3 β -HSD and P450c17 are listed below:

P450scc (forward) 5'-TACATACCCCCTTGGCTGCTG-3'

P450scc (reverse) 5'-GCAACCTCAGTGTTTCCTTCAGG-3'

P450c17 (forward) 5'-GAAGCAGTGTGTGACTGTCCG-3'

P450c17 (reverse) 5'-CTCGTCCAGAAAGCGGGTGG-3'

3 β -HSD (forward) 5'-CCACACTGCGTCAATCATTGACG-3'

3 β -HSD (reverse) 5'-CATCACCGTAGATGTACATGGGC-3'

PCR for amplification of a β -actin cDNA fragment [23] was also performed to confirm the quality and integrity of the RNA from all tissues used. All the sets of primers were designed to generate specific amplicons spanning at least one potential intron-exon boundary in order to eliminate false-positive amplicons arising from any contaminating genomic DNA. Ten μ l of the PCR reactions were fractionated on a 2% agarose gel and ethidium bromide staining was carried out to visualize each gene specific cDNA band on the gels.

Statistics

Relative values of P450scc transcript abundance (abundance of P450scc / abundance of β -actin x 1000) were subjected to one-way analysis of variance followed by Fisher's Protected Least Significant Difference (PLSD) post hoc test. Differences were considered significant at $P < 0.05$.

Results

cDNA cloning, structural and phylogenetic analysis of eel P450scc

Eight clones were positively hybridized with an eel P450scc cDNA fragment and isolated from approximately 500,000 eel ovarian λ gt10 recombinants. Among these, four clones were putatively longer than 1.5 kbp in length and were sequenced. Three clones contained the open reading frame of Japanese eel P450scc and were identical. The longest clone (Genbank accession No. AY654741) was 2828 bp in length and was composed of 8 bp of 5'-untranslated region (UTR), 1566 bp of an open reading frame putatively encoding 521 amino acid residues and 1254 bp of 3'-UTR. Three consensus polyadenylation signals, an AATAAA and two ATTAAA, were identified in the 3'-UTR. The most distal polyadenylation signal was an ATTAAA which was located 15 nucleotides upstream from the poly (A) tail. Alignment of the amino acid residues of eel P450scc to those of the rainbow trout [17], zebrafish [18], rat [24] and human [10] forms is shown in Fig. 1. The identity of eel P450scc compared with other teleost forms and mammalian forms are over 65 % and 45-50 %, respectively. Furthermore, two domains related to enzymatic activity, heme and steroid binding domains, show higher homology than other areas (over 75 %).

Phylogenetic analysis of P450scc resulted in clear segregation into three groups, one branch containing teleost P450scc, one branch of mammalian P450scc and the stingray form (Fig.2). Eel P450scc is clustered together with other teleost forms and appears to be significantly diversified from mammalian forms.

Transient expression of eel P450scc in COS-7 cells and its enzymatic activity

COS-7 cells expressing putative recombinant proteins of eel P450scc catalyzed the conversion of 25-hydroxycholesterol into pregnenolone (Table 1). Approximately 40 % of 25-hydroxycholesterol (10 ng/ml) was converted to pregnenolone by 6 hours after incubation and thereafter, conversion ratios further increased (66 % at 12 hours). COS-7 cells transfected with pSG5 (mock control) did not show any enzymatic activity of P450scc.

Gene expression of eel P450scc in the ovary and head kidney

Northern blot analysis using poly (A)⁺-RNA from ovarian tissues and head kidney from the same fish at the late vitellogenic stage is shown in Fig. 3. The eel P450scc probe clearly hybridized to a single transcript of 3.3 kb in length in both ovary and head kidney poly(A)⁺-RNA. The intensity of signal for P450scc in head kidney was approximately 10 times stronger than that in ovarian tissues although the amount of head kidney poly(A)⁺-RNA (0.3 µg) loaded onto the gel was over 10 times less than that of ovarian tissue (5 µg).

The changes in the gene expression of P450scc in ovarian tissues during artificial induction of maturation by SPH treatment were also examined by Northern blot (Fig. 4). The transcript of P450scc was not observed in the ovary at the pre-vitellogenic stage, prior to SPH treatment. However, gene expression of P450scc was significantly stimulated with SPH and the single transcript was detected in the ovary at the early

vitellogenic stage after SPH injections. The transcript abundance further increased with ovarian development.

Tissue distribution of the steroidogenic enzyme transcripts

The differential tissue-distribution of the transcripts of three steroidogenic enzymes, P450scc, P450c17 and 3 β -HSD in the female Japanese eel is shown in Fig.5. Expression of the P450scc gene was detected in steroidogenic tissues, thus ovary and head kidney, whereas ovarian tissue was the sole source of the P450c17 transcript. On the other hand, the transcript of 3 β -HSD was observed in all the tissues examined.

Discussion

In this study, cDNAs encoding P450scc were cloned from Japanese eel ovary. The deduced amino acid sequence of eel P450scc shows high identity to other animal forms especially in two functional domains, the heme- and steroid-binding domains. Studies using site-directed mutagenesis have shown that four amino acid residues, Lys⁴²³, Lys⁴²⁵, Arg⁴⁶⁵ and Arg⁴⁶⁶, are involved in the enzymatic activity of bovine P450scc through interaction with the electron-transfer partner, adrenodoxin [25-27]. These reports also demonstrated that the bovine P450scc mutant at Arg⁴⁶⁶ resulted in complete loss of enzymatic activity although mutation of the two Lys residues showed only a partial decrease in activity. Among these residues, the two Arg are present in all P450scc forms at the corresponding position to the bovine form while the two Lys residues are not well conserved. Furthermore, recombinant eel (Table 1) and rainbow trout [17] P450scc expressed in COS cells, in which Lys residues are not completely conserved, showed

significant enzymatic activity. All these findings strongly suggest that the Arg residues are critical amino acids in the enzymatic activity of all P450scc while the Lys residues are not.

As expected on the basis of the secondary structure of eel P450scc, phylogenetic analysis clearly clustered eel P450scc together with other teleost forms and showed that teleost P450scc appear to be significantly diverged from mammalian forms. Interestingly, the P450scc of stingray, a primitive vertebrate belonging to the elasmobranch subclass, appears to be evolutionarily highly diversified from both teleost and mammalian forms of P450scc. A similar finding was previously reported for stingray P450aromatase [8]. Thus the high divergence of steroidogenic enzymes of stingrays compared with other animal forms is likely to be common.

Northern blot analysis clearly showed that there is a single transcript of approximately 3.3 kb in length in both ovary and head kidney (homologous to adrenal tissue). To the best of our knowledge, there is no evidence in any mammalian species that a transcript for P450scc expressed in gonadal tissues is different from that in adrenal tissue. These may suggest that the species of eel P450scc transcript expressed in the gonad is identical to that in the head kidney, like mammals. However, we cannot rule out the possibility that ovary and head kidney express different transcripts that possess different 5'-untranslated regions which are close or identical in size because tissue-specific expression of P450-aromatase, a member of the same gene family that P450scc belongs, is controlled in a variety of tissues by the use of tissue-specific promoters associated with alternatively spliced 5'-untranslated exons [28]. The intensity of the hybridizing signal in head kidney was much greater than that in the ovary even though

the amount of head kidney RNA used for analysis was much less than that of ovarian RNA, which suggests that the P450scc gene is more actively transcribed in the head kidney than in the ovary of Japanese eel.

Northern blot analysis revealed that the transcript abundance of ovarian P450scc drastically changed during ovarian development artificially induced by SPH treatment. P450scc transcripts were not found in ovaries at the pre-vitellogenic stage. After SPH treatment commenced, gene expression was induced in the ovaries at the early vitellogenic stage and thereafter transcript abundance further increased in conjunction with the advance of ovarian development, which has been corroborated by real-time quantitative RT-PCR developed in our laboratory [29]. In the mammalian ovary, gene expression of P450scc is highly stimulated by gonadotropin [13, 14] and IGF-I [15, 16]. It is likely that in eel ovary gonadotropin(s) controls the P450scc expression because SPH, a gonadotropin-rich source, enhances its transcription. However, IGF-I could also be a potential regulator of the gene expression of eel ovarian P450scc since it has been demonstrated that the pituitary gland expresses IGF-I in both teleosts [30] and mammals [31, 32]. It should be noted that Japanese eel is the first species of teleost in which it has been demonstrated that a hormonal agent regulates P450scc gene expression in the ovary. Further study on the gene regulation with purified hormonal reagents remains to be conducted to elucidate the detailed hormonal regulation of the steroidogenic enzymes in fish.

RT-PCR analysis demonstrated that the tissue distribution of the transcripts of three steroidogenic enzymes, P450scc, P450c17 and 3 β -HSD, is distinctly different. The transcript for P450scc was detected in ovary and head kidney while the P450c17 gene

was exclusively transcribed in the ovary. In contrast, ubiquitous gene expression of 3 β -HSD was seen in the Japanese eel. Even though the RT-PCR result is not strictly quantitative, it is apparent that the P450scc is more highly expressed in the head kidney than in the ovary, which corroborates the result obtained by Northern blot. It was unexpected that head kidney was P450c17 transcript negative because this enzyme is essential for the production of 17 α -hydroxylated corticosteroids, typically cortisol in teleosts, that is synthesized in head kidney. However, immunohistochemistry using polyclonal antibodies against eel P450scc and P450c17 recently revealed that proteins of both steroidogenic enzymes were specifically detected in the steroidogenic cells of the head kidney of Japanese eel [33]. Therefore, eel head kidney appears to express P450c17 although the disconnection between the abundance of the transcript and the protein needs to be addressed. In zebrafish, the transcripts of both P450scc [18] and P450c17 [34] were also detected in non-steroidogenic tissues by RT-PCR coupled with Southern blot, although the expression level was much less than that in steroidogenic tissues. This finding suggests that the non-endocrine tissues of Japanese eel potentially express the P450scc and P450c17 gene, however, the transcript abundance is low and below the detectable level of conventional RT-PCR as employed in this study. Multiple forms of 3 β -HSD and its expression in a variety of tissues have been demonstrated in mammals, especially in rodents [35, 36]. Therefore, the wide tissue-distribution of the 3 β -HSD transcript does not appear to be unique in Japanese eel and 3 β -HSD may be involved in reproductive physiology through the regulation of local and/or circulating steroid hormone levels in teleosts, as they are in mammals.

In this study, a cDNA encoding functional P450scc protein was isolated from the ovary of Japanese eel and the gene expression in both gonadal and extra-gonadal tissues was examined. Furthermore, developmental changes in the transcript abundance of P450scc in the ovary during artificially induced oogenesis by SPH was thoroughly examined. Much attention has been paid to the gene regulation of steroidogenic enzymes in the eel ovary by SPH, a hormonal source that potentially contains many species of pituitary peptide hormones and growth factors, which has revealed that these enzymes are differentially transcribed during the progression of oogenesis [6-8, 29] and the expression of each of them seems to be under the control of different regulatory influences. As there is no available report on the hormonal transcriptional modulation of steroidogenic enzymes in teleosts, except for those on P450aromatase [37-39], further investigation into the gene regulation of steroidogenic enzymes in Japanese eel ovary by each hormonal agent included in SPH would provide a detailed understanding of the mechanisms involved in the biosynthesis of steroid hormones in fish.

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Figure legends

Fig. 1. Alignment of the deduced amino acid sequence of eel P450scc with those of other animal species. Dashes indicate residues that are identical to the eel form. Dots indicate gaps introduced to facilitate alignment. I; the steroid-binding domain, II; the heme-binding region.

Fig. 2. Phylogenetic analysis of P450scc proteins. The analysis was performed by the neighbor-joining method using full-length protein sequences. The length of horizontal lines indicates genetic distance. The numbers beside the branches indicate bootstrap values from 1000 replicates

Fig. 3. Northern blot analysis of poly (A)⁺ RNA from ovary and head kidney of the Japanese eel. OV: ovary (5 µg), HK: head kidney (0.3 µg).

Fig. 4. Developmental changes in the relative values of P450scc transcript abundance in the ovaries of Japanese eels during artificial induction of maturation by SPH treatment. Northern blot signals of P450scc mRNA were quantified and normalized against the signal of actins. C; saline injected control, IC; initial control, EV; early vitellogenic stage, MV; mid-vitellogenic stage, LV; late vitellogenic stage, MN; migratory nucleus stage. Numbers above bars represent sample sizes. Different lettering above bars indicates significant differences between respective means.

Fig. 5. Tissue-specific expression of the gene encoding P450scc, P450c17 and 3β-HSD in the Japanese eel. Shown are the fluorescent scans of the gels stained with ethidium bromide. The integrity of the RNA from each tissue was ensured by uniform amplification of β-actin transcript.

Japanese eel	MIGRWRVCSR	AVA.PCCSSW	ALYDGGVMV	PARGQSASV	PIVRQGTKPD	SSLGIRSFNE	IPGLWKNSVA	69
Rainbow trout	-MVS-S-RS	SL-L-A-G..LP	S-.HNS-M	-V--ALS-	N-STVQN-S-	-----R-GL-	58
Zebrafish	-A--N-TLA	RLDQSLSS..LKNLLQ	KVT-S-RA-Q	N-.TVQP--K	---R-R--LL	53
Rat	-LAK.GL-L-	S-LVKS-QP.FLS	PVWQPGF...	ALTGN-AGIS	-TNSP-----	--SPGD-GWI	58
Human	-LAK.GLPP-	S-LVKGYQT.FLS	AP-EGLRRLR	VPTGE-AGIS	.TRSP-P---	--SPGD-GWL	60
Japanese eel	NLYRFWKHDG	FRNVHRIMLN	NFKNFGPIYR	EKIGFYESVN	I IKPEDAAIL	FQAEGHYPKR	LLIEAWTAYR	139
Rainbow trout	---S--L--	---I--V-VH	--NT-----	---Y-D---	---MP---	-K-----	-TV---S--	128
Zebrafish	SVLA-T-MG-	L-----VH	---T-----	--V-I-D--Y	-----G---	-K---H-N-	INVD-----	123
Rat	---H-LREN-	THRI-YHHMQ	--QKY-----	--L-NME--Y	-LD-K---T-	-SC--PN-E-	Y-VPP-V--H	128
Human	--H--RET-	THK--LHHVQ	--QKY-----	--L-NVE--Y	V-D--V-L-	-KS--PN-E-	F--PP-V--H	130
Japanese eel	DYRNHKYGLV	LKDGEDWKST	RLVLNKQVIA	PQVQENFVPL	LDEVGQDFMA	RIQGKIEKSG	NNKWTVDLSN	209
Rainbow trout	---R-----	-N---R-N	-VI--RE-IS	-K-LG----	-----V-	-VHK---R--	QD--TT--Q	198
Zebrafish	---Q-----	-E-KA--TD	-MI--ELLL	-KL-GT----	-----V-	-VHKQ--R--	QKQ--TT--TH	193
Rat	Q-YQRP I---	F-SSDA-RKD	-I---QE-M-	-DSIK-----	-EG-A---IK	VLHRR-KQQN	SG-FSG-I-D	198
Human	Q-YQRP I---	-KSAA--KD	-VA--QE-M-	-EATK--L--	--A-SR--VS	VLHRR-K-A-	SGNYSG-I-D	200
Japanese eel	ELFKYALESV	SSVLYGERLG	LLHDHIEPEA	QHFIDCISLM	FKSTSPMLYI	PPWLLRRLGA	RVMKDHVKAW	279
Rainbow trout	-----G-	-----ML-Y-N--	-----	---T-----	--AM---V--	KI-R--VE--	-----	268
Zebrafish	D--RFS----	-A-----	--L-N-D--F	-----V-V-	---T-----L	-G---SI-S	NI-KN-VE--	263
Rat	D--RF-F--I	T--VF-----	M-EEIVD--S	-R---AVYQ-	-HTSV---NM	--D-F-LFRT	KT-K--AA--	268
Human	D--RF-F--I	TN-IF--Q-	M-EEVVN--	-R--A-YQ-	-HTSV---NL	--D-F-LFRT	KT-K--VA--	270
Japanese eel	DGIFNQADRC	IQNMFRLKQ	ESGGEGRYPG	VLAILMLQGT	LSIADIKASV	TELMAGGVD	TSITLLWTLY	349
Rainbow trout	-----IY-TMR-	DTNTH-K--	---S-LMLDK	---E-----	-----	---MM----	-----	338
Zebrafish	-----I-KQW-E	NPE-N-K--	---I-LM-DK	---E-----	-----S	VTF-----	-----	333
Rat	-V--SK--EY	T--FYWD-R-	KRDFSK----	--YS-LGGNK	-PFKN-Q-NI	--ML-----	--M--Q-N--	337
Human	-V--SK--IY	T--FYWE-R-	KGSVHHD-R-	M-YR-LGDSK	M-FE---N-	--ML-----	--M--Q-H--	340
I								
Japanese eel	ELARQPELQE	ELRABIAAAR	AASQGDVMEV	LKMVPLVKAA	LKETLRLHPI	AVNLQRYITE	DVVIQNYHIP	419
Rainbow trout	---H-D--	---V-V--	QST--MLQM	---I---G-	-----V-	-S-----	EI-----	408
Zebrafish	---D--D	---S---	I-FK--MVQM	V--I--L--	-----V	-MS-P-----	-T-----	403
Rat	-M-HNLKV--	M---VL--	RQA--MAKM	VQL--L--S	I-----	S-T-----VN	-L-LR--K--	407
Human	-M--NLKV-D	M---VL--	HQA--MATM	-QL--L--S	I-----	S-T-----LVN	-L-LRD-M--	410
II								
Japanese eel	SGTLVQLGLY	AMGRDPQVFQ	RPEQYTPSRW	LQKNTR..YF	RSLGFGGPR	QCLGRRIAET	EMQLFLIHML	487
Rainbow trout	C-----	---D-P	---K-L---	-RTENQ...	-----	-----	-----	476
Zebrafish	A-----V-	---H-F-P	K---C---	ISS-RQ...	K-----	-----	---I-----	471
Rat	AK---VAS-	---ESSF-P	N-NKFD-T--	-E-SQNTTH-	-Y---W-V-	-----L	--TI--NV-	477
Human	AK---VAI-	-L--E-TF-F	D--NFD-T--	-S-DKNIT--	-N---W-V-	-----L	--TI--N--	480
Japanese eel	EKFRIENQOQ	MDTRSTFELI	ILPEKPIVLT	IRPL		521		
Rainbow trout	-N--VDK-R-	VEVH----I	L-----L--	LK--KSGQ		514	71%	
Zebrafish	-N---K-K-	IEVR-K--L	LM-----I--	-K--NASR		509	65%	
Rat amino	-N---V-SI	RDVGTK-N--	LM-----FPN	FQ--KQDLGS	TMPRKGDTV	526	47%	
Human amino	-N--V-I-HL	SDVGTN--	LM-----SF-	FW-FNQEATQ	Q	521	48%	

Fig.1

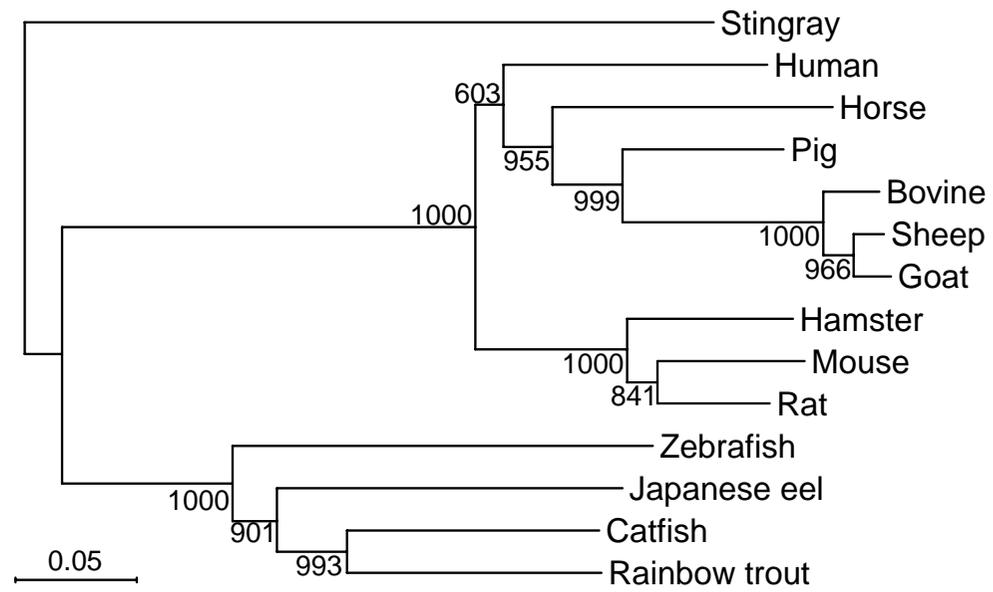


Fig.2

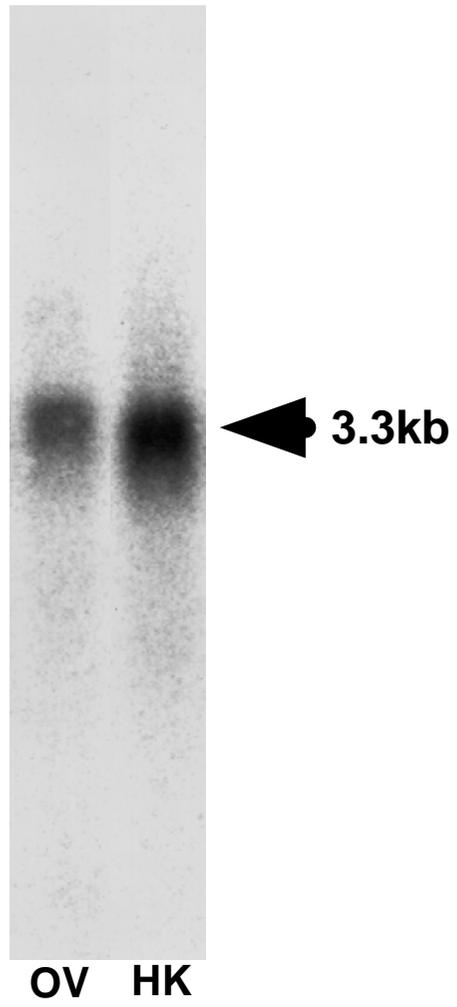


Fig.3

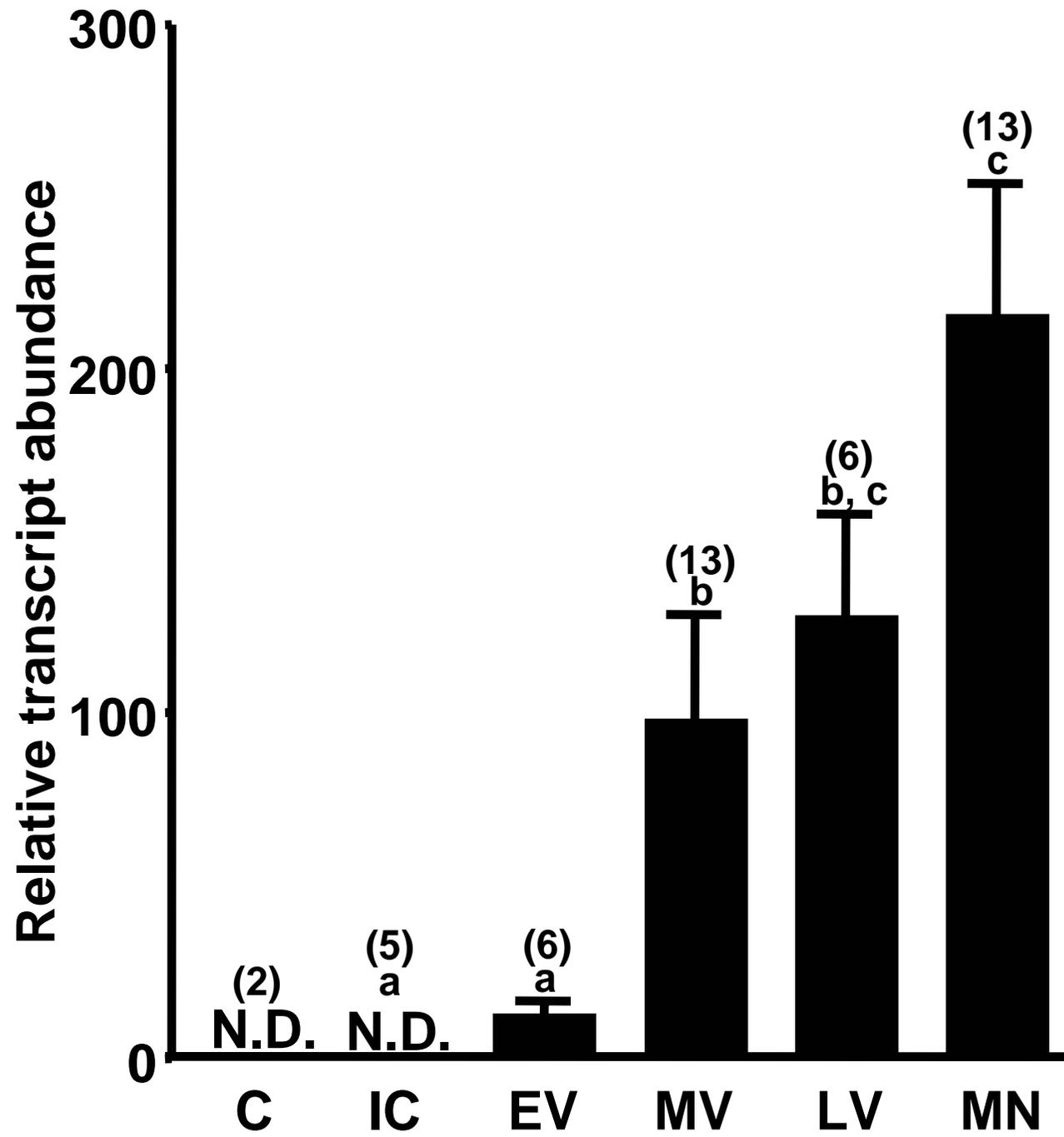
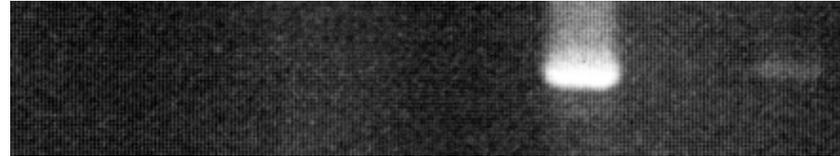
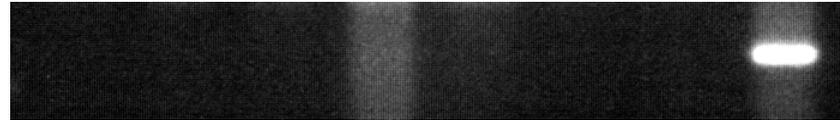


Fig.4

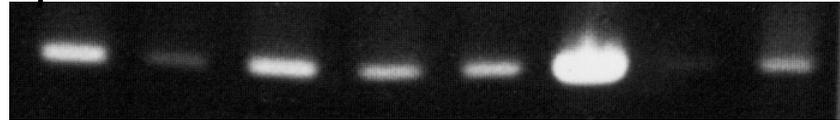
P450scc



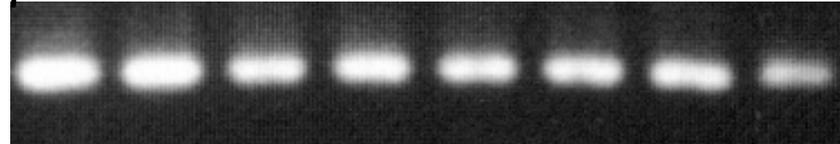
P450c17



3 β -HSD



β -actin



Brain
Small intestine
Heart
Liver
Spleen
Head kidney
Trunk kidney
Ovary

Fig.5

Table 1. Pregnenolone production from 25-hydroxycholesterol by the recombinant Eel P450scc expressed in COS-7 cells

Group	Incubation time (hour)	Pregnenolone (ng/ml)
Eel P450scc	6	3.9± 0.28
	12	6.6± 0.49
Mock	6	0.09± 0.05
	12	0.03± 0.02