

Transgenic Reporter Mice With Promoter Region of Murine LRAT Specifically Marks Lens and Meiosis Spermatocytes

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Summary

Lecithin:retinol acyltransferase (LRAT) is the major enzyme responsible for retinol esterification in the mammalian body. LRAT exhibits specific activity in the cells with active retinol metabolism where it converts retinols into retinyl esters, which represents the major storage form of retinol. Besides hepatic stellate cells in the liver, LRAT appears to have a key physiologic role in several other tissues. In this study, we generated a transgenic reporter mouse expressing green fluorescence protein (EGFP) under the control of region containing -1166 bps from promoter upstream from the putative transcriptional start site and 262 bps downstream of this start. Transgenic reporter mice exhibited specific expression in eyes and testes. In eyes, expression of EGFP-reporter is found in lens and lens epithelium and fibers from embryo to adulthood. In testes, LRAT-EGFP reporter is expressed both in Sertoli and in spermatocytes marking initiation of spermatogenesis in prepubertal mice. Our data show that the examined LRAT regulatory region is sufficient to achieve strong and selective expression in the eye and testes but not in liver and other organs.

Key words

LRAT • Transgenic mouse • Retinol • Testis • Spermatocyte • Sertoli cell • Eye • Lens

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Introduction

Retinoids plays key role in many essential physiologic functions including growth and development, reproduction, epidermal barrier functions, immune system, and vision. Vitamin A, all-trans-retinol, is delivered into metabolism from dietary uptake in the form of retinols or pro-vitamin carotenoids. Retinols are metabolized into active derivatives including 11-cis-retinal and all-trans-retinoic acid, which is essential for transcriptional gene regulation as well as vision. Retinols are also converted into retinyl esters representing the major storage form of retinol in organs such as liver, prostate, breast, kidney, and testes (D'Ambrosio *et al.* 2011, Guo *et al.* 2002, Hogarth and Griswold 2010). The liver is the most important organ for storage of retinol that is transported to other tissues with retinoid metabolism (Paik *et al.* 2004).

Two enzymes, lecithin:retinol acyltransferase (LRAT) and acyl CoA:retinol acyltransferase (ARAT) catalyze retinyl ester synthesis although LRAT appears to be the main enzyme for retinol esterification in the majority of tissues. LRAT acts together with STRA6 and the retinol binding protein (RBP4) (Kawaguchi *et al.* 2007, O'Byrne and Blaner 2013) and its activity was

found in various tissues with active metabolism of retinol including the liver, lung, eye, testis, small intestine, skin, mammary gland, and prostate epithelium (reviewed in (Liu *et al.* 2008)).

LRAT plays a key role in the liver where it is co-responsible for retinyl ester storage (Liu *et al.* 2008, O'Byrne and Blaner 2013) although this enzyme is crucial also for physiology of other organs such as testis and eyes (Batten *et al.* 2004). In testis, LRAT and retinol are essential in the process of spermatogenesis (Hogarth and Griswold 2010, Li *et al.* 2011). LRAT is expressed within Sertoli cells and maintain an optimal level of retinoid for development of spermatozoa (Shingleton *et al.* 1989). *Lrat*-deficient mice maintained on vitamin A-deficient diet resulted in meiotic failure and accumulation of undifferentiated spermatogonia in prepubertal mouse testis (Li *et al.* 2011).

In the eyes, LRAT is present in retinal pigment epithelial cells (RPE) and contributes to maintaining visual cycle by incorporating all-*trans*-retinol into retina and adjusting its concentration in the RPE (Batten *et al.* 2004, Saari *et al.* 1993). The main source of retinol is derived from dietary intake of both retinol and pro-vitamin A carotenoids. Retinols are esterified by LRAT to all-*trans*-retinyl esters that are stored in retinosomes (Perusek and Maeda 2013). In visual function, 11-*cis*-retinal functions as the chromophore for the pigments present in the rod and cone photoreceptor cells (McBee *et al.* 2001, Wald 1968). Mice lacking LRAT in RPE showed strong reduction in their ability to synthesize all-*trans* retinyl esters and a reduced light response in ERG recordings (Ruiz *et al.* 2007).

To better understand the regulation and expression of LRAT, we generated transgenic reporter C57BL/6N-Tg(LRAT-EGFP) mice expressing EGFP under the control of 5'-untranslated region from -1166 from putative transcriptional start site and 262 bps downstream sequence. The results suggest that the selected part of the regulatory 5'-untranslated region specifically drives expression of green fluorescence protein (EGFP) in eyes and testis although the liver and other organs do not show any reporter expression. In eyes, expression of EGFP-reporter is found in lens, lens epithelium and fibers. In testes, the reporter is expressed both in Sertoli and in spermatocytes marking initiation of spermatogenesis in prepubertal mice.

Material and Methods

Transgenic construct and generation of C57BL/6N-Tg(LRAT-EGFP) transgenic mice

The mouse LRAT promoter fragment -1166/+262 (numbering based on GenBank entry NM_023624, relative to the transcriptional start site), synthesized by GenScript, was excised by XbaI and BspHI from the pUC57 plasmid and inserted into the NheI and NcoI, respectively, in Sleeping Beauty (SB)-based vector, pT2/Venus was generously provided by Lajos Mátés (Mates *et al.* 2009). The resulting construct comprised LRAT promoter, EGFP, chimeric intron (from pRL/TK plasmid (Promega)) and SV40 polyA signal between transposon inverted terminal repeats. To generate transgenic mice, both construct and SB transposase mRNA were used for pronuclear microinjection into E0.5 embryos derived from C57BL/6N strain. Offspring were weaned, genomic DNA for genotyping was isolated from tail snips and 17 founders with the LRAT-EGFP transgene were identified. The F0 generation animals were bred further onto C57BL/6N background. All animal work was performed according to approval by the Animal Care Committee of the Institute of Molecular Genetics in conformity with institutional and national guidelines.

Genotyping

Genomic DNA was obtained by lysing tail snips in DNA lysis buffer (100 mM Tris-HCl pH 8.5, 200 mM EDTA, 5 mM NaCl, 0.2 % SDS, Proteinase K at 240 µg/ml) and incubated at 55 °C overnight. Samples were extracted with phenol/chloroform, the water phase precipitated by an equal volume of 96 % ethanol, rinsed in 70 % ethanol and resuspended in double-distilled water. Genotyping PCR reaction with DreamTaq Green DNA Polymerase (Thermo Scientific, Waltham, MA) was run as following: 95 °C 2 min 30 s 35 cycles, 95 °C 30 s, 59.5 °C 30 s, 72 °C 1 min, 72 °C 7 min. To detect transgenic construct following pairs of forward (F) and reverse (R) primers were used:

LRAT:

F 5'-ATCTCTCCTACGCTGGCTGT-3',

R 5'-TGCTCAGGTAGTGGTTGTCG-3';

EGFP:

F 5'-AGTCGTTGTGTGAAGCGAAG-3',

R 5'-TGCTCAGGTAGTGGTTGTCG-3'.

RNA extraction and analysis

Mouse tissues, were homogenized using TissueLyzer II in TRI-Reagent (Sigma-Aldrich, St. Louis, MO) and RNA was subsequently isolated according to the manufacturer's instructions. RNA pellets were resuspended in double-distilled water; concentration was determined by Nanodrop (Thermo Scientific). Residuals genomic DNA were removed using 1 U of DNase I (Thermo Scientific)/1 µg RNA by 15 min incubation at 37 °C, DNase was then inactivated for 5 min at 70 °C. 1 µg of RNA was reverse transcribed to cDNA by M-MLV Reverse Transcriptase (Promega) using oligo (dT) primers. RT-PCR was performed in a 10 µl reaction mixture consisting of PCR buffer (Thermo Scientific), 2 mM MgCl₂, 0.2 mM of each dNTP (Sigma-Aldrich), 0.2 mM of each primer, and 1.5 U TaqDNA polymerase (Thermo Scientific). To detect reporter expression the same EGFP primers as above were used. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control (primers: F 5'-AATCCCATCACCATCTTCCA-3', R 5'-TGGACTCCACGACGTACTCA-3'). PCR was performed using: 95 °C 3 min 41 cycles, 95 °C 20 s, 60 °C 30 s, 72 °C 30 s, 72 °C 5 min. Amplified PCR products were run on 1.5 % agarose gels containing 0.5 g/ml ethidium bromide.

Western blot analysis

Mouse tissues, frozen on dry ice, were homogenized using TissueLyzer II in Triton lysis buffer (50 mM Tris pH 7.5, 1 % Triton X-100, 150 mM NaCl, 1 % DOC, 0.1 % SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche). Protein concentration was measured with BCA (Pierce, Thermo Scientific). The amount of 50 µg of tissue homogenate was separated on 12.5 % SDS-PAGE, transferred onto the nitrocellulose membrane (Sigma-Aldrich), blocked with 3 % bovine serum albumin (BSA) in Tris-buffered saline buffer supplemented with 0.1 % Tween (PBST) and incubated overnight at 4 °C with primary antibody anti-EGFP (sc-9996, Santa Cruz Biotechnology, Texas) or anti-GAPDH (G8795, Sigma-Aldrich) diluted in 2 % non-fat dry milk/PBST. After washing the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories). Immunoreactive proteins were detected with the chemiluminescence system ECL (Rockland Immunochemical, Gilbertsville, PA) and documented by

LAS 3000 (Fuji Photo Film, Tokyo).

Embedding and sectioning

Dissected mouse tissues were fixed in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS) several hours at 4 °C, then washed three times in PBS at 4 °C for 5 min each. For cryo-embedding (Johnson *et al.* 2007), they were transferred to a solution containing 30 % sucrose in PBS and rocked overnight at 4 °C then embedded in optimal cutting temperature compound (OCT, Tissue-Tek, Sakura Finetek, Alphen aan den Rijn) in cryosection moulds (Sakura Finetek) and stored at -80 °C. Embedded tissues were cut in serial sections of 10 µm thickness using a Leica cryostat, mounted on Superfrost Plus slides (Millian, Vernier), and stored at -20 °C until further use.

Immunostaining

For immunostaining, all procedures were carried out in a humidified chamber. Slides were blocked for 1 h in blocking buffer (PBS plus 0.1 % Tween 20 or PBT containing 10 % heat-inactivated sheep serum). Sections were incubated overnight at 4 °C with primary antibody diluted in blocking buffer. Next day, samples were washed 3 × 5 min in PBT and blocked in blocking buffer for 30 min followed by a 2 h incubation at room temperature with appropriate secondary antibody diluted in blocking buffer containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; D9542, Sigma-Aldrich, 1 µg/ml in PBS). Finally, sections were washed 3 × 5 min in PBT and mounted in Mowiol (4-88, Sigma-Aldrich, used according to <http://www.niaid.nih.gov/LabsAndResources/labs/aboutlabs/rtb/biologicalImaging/protocols/Pages/mowiolPreparation.aspx>). Primary antibodies used in this study were: 1:500 anti-EGFP (A-6455, Invitrogen, Life Technologies, Carlsbad, CA), 1:500 rabbit anti-DDX4 (ab13840, Abcam, Cambridge), 1:400 mouse monoclonal anti-SCP3 (sc-74569, Santa Cruz Biotechnology). Secondary antibodies were: 1:500 anti-rabbit IgG A594 (A-11037; Invitrogen). For colocalization, the second primary antibodies were conjugated with A488 using the Zynon kit (Invitrogen). Stained sections were examined using a DMI6000 Leica confocal microscope equipped with HCX PL APO 20x/0.7 imm corr. Lbd. BL, HCX PL APO 40x/1.25-0.75 Oil CS UV lenses. Images were acquired with a Leica Camera DFC350FX R2, a high sensitivity monochrome digital camera with 1.4 megapixel resolution.

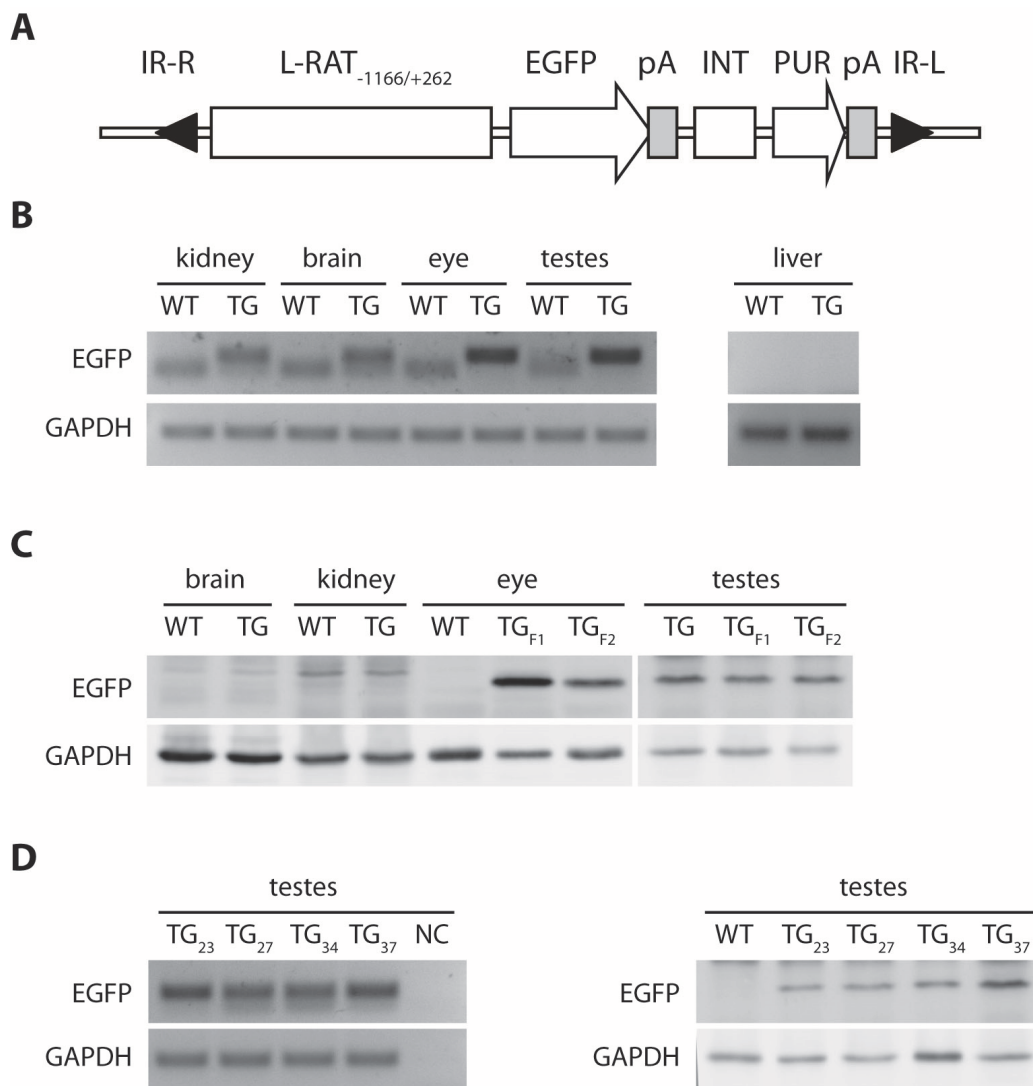


Fig. 1. Generation and analysis of LRAT-EGFP reporter mice. **A**) The LRAT-EGFP transgene contains truncated LRAT promoter (LRAT1166/+262) and EGFP cDNA followed by the chimeric intron (INT), and a SV40 poly-A signal (SV40(A)) between transposon inverted terminal repeats (IR-L, IR-R). **B**) Kidney, brain, eye, testes and liver tissue samples from wild-type (WT) and transgene (TG) mice were analyzed for EGFP transcript by semi-quantitative PCR. Note, positive EGFP PCR product corresponds to 700 bp in TG, non-specific PCR product runs at lower molecular weight in WT. GAPDH, housekeeping control. **C**) Tissue samples of kidney, brain, eye and testes were analyzed by immunoblotting for EGFP expression. GAPDH, loading control. **D**) Tissue samples of testes dissected from WT and TG mice, aged 23 (TG₂₃), 27 (TG₂₇), 34 (TG₃₄) and 37 (TG₃₇) days, were analyzed for EGFP expression by semi-quantitative PCR (left panel) and immunoblotting (right panel). NC, negative control; GAPDH, housekeeping (left panel) and loading (right panel) control.

Results

Murine LRAT promoter analysis and generation of LRAT-reporter mouse

In this study we chose proximal LRAT promoter to drive expression of EGFP based on its reported expression in hepatic stellate cells (Nagatsuma *et al.* 2009). To generate the LRAT-reporter mouse 5'-untranslated region containing altogether -1428 bp including the putative transcriptional start site and 262 bp downstream sequence was synthesized and cloned into the SB-based pT2/Venus vector between the inverted terminal repeats

(IR) replacing thus the Venus (Mates *et al.* 2009) (Fig. 1A). Our transgenic C57BL/6N-Tg(LRAT-EGFP) reporter line was generated by pronuclear co-microinjection of the transgenic constructs and transposase SB100 mRNA into zygotes derived from C57BL/6N strain. We obtained seventeen transgenic founders (three males and four females) that showed integration of the transgene. All founders were characterized using semi-quantitative PCR and Western blotting regarding the EGFP expression in organs with expected LRAT expression, i.e. in the liver, kidney, brain, eye, and testes (Fig. 1B-D). None of the founders showed detectable expression in the liver, kidney,

brain but in eyes and testis (Fig. 1B-D). The F1 and F2 lines were further analyzed using immunostaining with either anti-EGFP antibodies or direct EGFP signals (Figs 2 and 3).

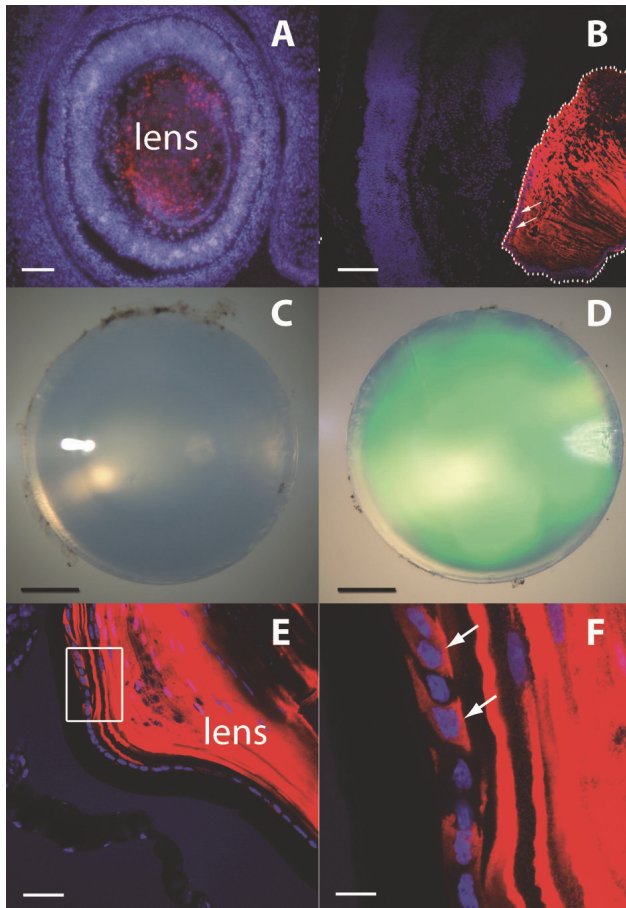


Fig. 2. Expression of EGFP protein in lens and lens epithelium of LRAT-EGFP reporter transgenic mouse from embryogenesis to adulthood. Immunohistochemical detection of EGFP (red) with nuclear counterstain (DAPI, blue). Shown are eyes from representative 14.5 dpc embryo (A), PN1 pup (B) and adult (E,F). Fresh preparation of adult lens visualized by direct fluorescence microscopy (C,D). Nuclei were stained with DAPI (blue). Dotted lines in B outline the lens. Scale bar in A, B, E = 100 μ m; C, D = 1 mm, and F = 12.5 μ m.

LRAT-EGFP reporter is expressed in eyes from embryonic stage to adulthood

EGFP expression in the eyes was obvious and bright EGFP-signal was directly observable in lenses using fluorescence stereomicroscope (Fig. 2). To characterize the expression at the cellular level, eyes were sectioned and the EGFP expression demonstrated by immunofluorescence. EGFP is observed in the lens of transgenic LRAT-EGFP reporter from 14.5 day *post coitus* (dpc) to adulthood. At higher magnification, the EGFP expression is not only observed in lens but also in

the lens epithelium and lens fibers, all of which are continued to be expressed in adult (Fig. 2A-F).

LRAT-EGFP expression in testis

LRAT has been reported to be expressed in Sertoli cells and be involved in testis development (Shingleton *et al.* 1989). Schmitt and Ong (1993) reported LRAT activity in round spermatid-enriched cells, the post meiotic germ cells, from postpubertal rat testis (Moise *et al.* 2007).

At postnatal day (PN) 7, expression of EGFP in testis of LRAT-EGFP transgenic mice is not significantly above background (not shown). By PN 14, we observed weak EGFP expression in Sertoli cells. These cells form and single cell layer encasing testicular cord, can have distinguished oval shape nucleus often indented and oriented perpendicular to the basal membrane with the strikingly noticeable nucleolus (Zolfaghari and Ross 2009) (arrowhead, Fig. 3A). Interestingly, we observed very strong EGFP expression in both nucleus and cytoplasm specifically in meiotic spermatids and not in spermatogonia. The spermatocytes usually reside further away while the spermatogonia are attached to the seminiferous membrane (arrows, Fig. 3B). This expression is in agreement with the initiation of secondary meiosis in the mouse, which begins around PN10 (Liu and Gudas 2005).

In adult testis, we detected EGFP expression strongly in spermatocytes undergoing meiosis as well as postmeiotic cells in spermatogenesis, which include round spermatids, elongating and elongated spermatids (Fig. 3B, C). EGFP expression in Sertoli cells appears to be reduced and less noticeable compare to EGFP expression in spermatocytes and spermatids (Fig. 3B).

To clarify meiotic spermatocytes in adult testis, we perform co-localization of anti-EGFP and anti-SCP3 antibodies, which stains for synaptonemal complexes protein 3, a *bona fide* marker of meiosis (Yuan *et al.* 2000). SCP3 expresses during meiosis in spermatocytes and not in spermatogonia where division is accomplished by mitosis. In the testis, SCP3 begins to express from leptotene spermatocytes to late meiotic cells when the expression ceases. LRAT-EGFP expression was detected in all SCP3 positive cells as well as the SCP3 negative postmeiosis spermatids, judged by the location of these cells near the center of seminiferous tubules. There is no EGFP expression in spermatogonia cells (arrowheads, Fig. 3C), which is in agreement with our data in PN14 aforementioned (Fig. 3C).

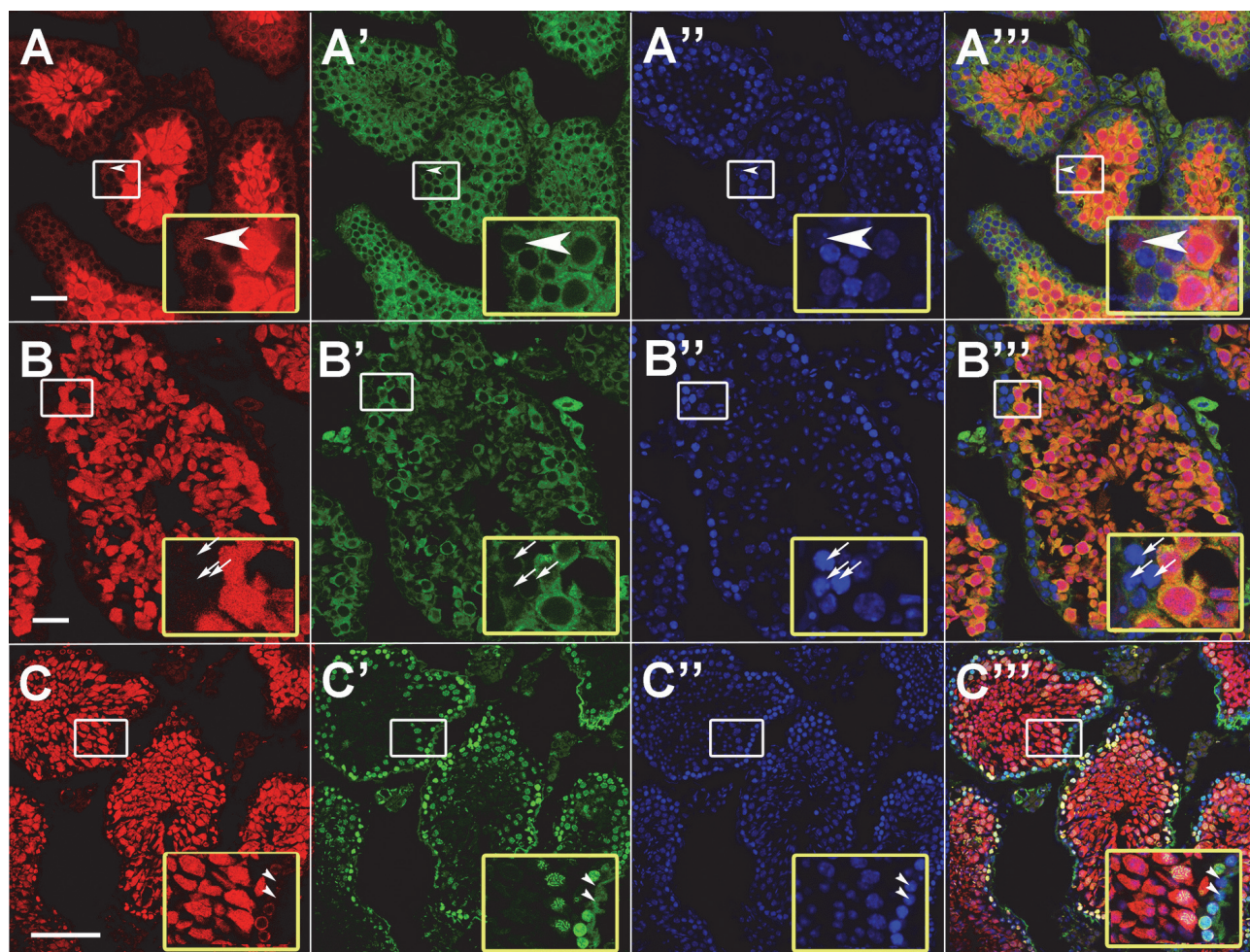


Fig. 3. Expression of EGFP protein in testis of LRAT-EGFP reporter transgenic mouse from postnatal day 14 to adulthood. Immunofluorescence staining of cross sections of tubuli seminiferi: **Panel A** is postnatal day 14, **B** and **C** are adult. Shown are anti-EGFP (red, **A**, **B**, **C**), anti-DDX4 (green, **A'**, **B'**), anti-SCP3 (green, **C'**) and DAPI nuclear staining (blue, **A''**, **B''** and **C''**). **A'''**, **B'''** and **C'''** are composite images of all channels. From postnatal day 14 to adulthood, EGFP protein (red) expression in the LRAT-EGFP reporter mouse is highly expressed in meiosis round spermatocytes (SCP3 positive) and in post meiosis spermatids (SCP3 negative) which are located more luminal. Interestingly, we detected EGFP protein in nuclei of Sertoli cells only in postnatal day 14 (arrowhead in panel A). Scale bar = 100 μ m. Insets show higher magnification view of area delineated by the white rectangles.

Discussion

LRAT is known to have the predominant enzymatic function in the liver where it is responsible for the formation of retinyl esters and essential for vitamin A storage (Batten *et al.* 2004, Liu and Gudas 2005, Moise *et al.* 2007, Zhang *et al.* 2012). We have generated transgenic mouse lines where EGFP expression is under the control of proximal LRAT promoter (-1166 to +262). Analysis of EGFP protein expression by Western blotting and immunostaining showed EGFP expression in subsets of LRAT endogenous expressing domains such as lens and testis but not in retinal epithelium, liver, lung, intestine, where endogenous LRAT is expressed. This expression pattern does not fully recapitulate the described expression of endogenous LRAT gene in spite

of the promoter region contained the necessary elements for high activity of luciferase such as TATA box, SP3 site, AP-1 site, and CAAT box, all of which are essential and well-conserved in rat, human, and mouse (Zolfaghari and Ross 2009). The differences in the expression underline the necessity of additional regulatory elements essential to drive complex expression of endogenous LRAT in tissue-specific manner.

In the eye, we showed expression of EGFP in the lens epithelium and the lens fibers which actively differentiated from equatorial zone of lens epithelium throughout life (Bhat 2001) but not in the retinal pigment epithelium (RPE). This finding is different to published reports of endogenous LRAT expression, which showed specific expression in the RPE (Batten *et al.* 2004). Lack of *Lrat* function in mice resulted in slightly shortened rod

outer segment layer in the retina and no defects in any other layer (Batten *et al.* 2004). We did not detect endogenous LRAT by our immunostaining during embryonic development or in adulthood. Collectively, this suggested that endogenous LRAT is either not expressed and/or dispensable for lens development and differentiation.

In testis, LRAT is well known to be expressed in Sertoli cells but its expression in germ cells has not been described in detail. The EGFP transgene under the control of the proximal *Lrat* promoter was also expressed in Sertoli cells, however, it was also detected in meiosis spermatocytes as well as in post meiotic cells undergoing spermatogenesis. The latter expression is in agreement with a report identifying the highest LRAT enzymatic activity occurring in microsomal and cytosolic preparations of post meiosis round spermatids as compared to Sertoli cells and other cells types in the developing rat testis (Schmitt and Ong 1993). Furthermore, male mice lacking functional *Lrat* showed oligospermia but no changes in mRNA expression of genes specific for spermatogenesis compare to wild-type (Liu and Gudas 2005). The authors concluded that lack of *Lrat* causes an absence in mature sperm and that spermatogenesis is compromised but is not completely disrupted. There was no further characterization of the oligospermia, nonetheless the data confirms the importance of LRAT in sperm formation.

Subsequent studies showed that male *Lrat* mutants embryos under the influence of a maternal vitamin A-deficient diet from the time of post conception to weaning and thereafter fed the same vitamin A deficient diet fail to undergo postnatal meiosis and

remain as undifferentiated spermatogonias (Li *et al.* 2011). Our LRAT-EGFP transgenic reporter mice exhibit expression of EGFP in meiosis spermatocyte and post meiosis spermatids and not in spermatogonia from PN14. This transgenic mouse line could be used as a negative marker to prepare spermatogonia (EGFP negative) population in isolating spermatogonial stem cells from adult mouse testis (Guan *et al.* 2006).

In summary, our transgenic mouse line expressing EGFP under the proximal LRAT promoter showed expression in the eyes and testis. This expression does not fully recapitulate reported endogenous *Lrat* gene expression, underscoring the requirement of additional regulatory elements and/or enhancers necessary to govern complex expression of endogenous LRAT. Nevertheless, this LRAT-transgenic reporter mouse might be useful to further characterize spermatogenesis.

Conflict of Interest

There is no conflict of interest.

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