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MengMeng Xu [a b], Xuehong Xu [c f], Guanliang Cao [c], Yuexin Pan [d], Odell Jones [e], Joseph L. Bryant [e], Donald Anthony [d], Haipinf He [f], Guifang Yan [g] & Chuyu Zhang [f]

[a] Gemstone Program, Department of Chemistry and Biochemistry, University of Maryland, College Park, MD, USA

[b] Pathology Department, Johns Hopkins University Hospital School of Medicine, Baltimore, MD, USA

[c] University of Maryland Biotechnology Institute, Baltimore, MD, USA

[d] Case Western Reserve University Institute of Pathology, Cleveland, OH, USA

[e] Institute of Human Virology, University of Maryland School of Medicine, MD, USA

[f] Wuhan University School of Life Sciences, Wuhan, PR China

[g] Urology Department, Johns Hopkins University Hospital School of Medicine, Baltimore, MD, USA

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MengMeng Xu[1,2], Xuehong Xu[3,6], Guanliang Cao[3], Yuexin Pan[4], Odell Jones[5], Joseph L. Bryant[5], Donald Anthony[4], Haipinf He[6], Guifang Yan[7], and Chuyu Zhang[6]

[1]Gemstone Program, Department of Chemistry and Biochemistry, University of Maryland, College Park, MD, USA
[2]Pathology Department, Johns Hopkins University Hospital School of Medicine, Baltimore, MD, USA
[3]University of Maryland Biotechnology Institute, Baltimore, MD, USA
[4]Case Western Reserve University Institute of Pathology, Cleveland, OH, USA
[5]Institute of Human Virology, University of Maryland School of Medicine, MD, USA
[6]Wuhan University School of Life Sciences, Wuhan, PR China
[7]Urology Department, Johns Hopkins University Hospital School of Medicine, Baltimore, MD, USA

Maltese-cross appearance of birefringent molecule configuration has become a typical diagnostic characteristic used to evaluate the kidney complications of Anderson-Fabry disease, an Xq22.1-linked recessive disorder. Accumulation of glycophospho- or glycosphingolipids in blood vessels seriously damages the glomerular filtration barrier and is responsible for proteinuria. Interestingly, our recent work has demonstrated that birefringent bodies also signify in the mesonephros and metanephros during kidney organogenesis, and characterized as liquid crystal in the configuration of concentric lamellation in the cytoplasm of epithelial cells and the lumen of proximal tubules. Study of this phenomenon provides prospects to understanding the molecular mechanism of Anderson-Fabry disease.

Keywords: Anderson-Fabry disease; liquid crystal; maltese-cross birefringence; mesonephros; metanephros; renal development

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Address correspondence to Xuehong Xu, University of Maryland Biotechnology Institute, 725 W Lombard St., Baltimore, MD 21201, USA. E-mail: xux@umbi.umd.edu and Chuyu Zhang, Wjhan University School of Life Sciences, Wuhan, PR China. E-mail: cyzhang@whv.edu.cn

I. INTRODUCTION

Anderson-Fabry disease, one of the most prevalent lysosomal storage disorders, is caused by a deficiency of the lysosomal enzyme alpha galactosidase A, which results in the accumulation of glycophospholipids or glycosphingolipids (GL) (globotriaosylceramide and galabiosylceramide) in the endothelial cells and smooth muscles of blood vessels [1]. This abnormal accumulation leads to an impairment of function in skin, kidney, myocardium, lung, epithelial cells of the cornea, and other tissues. As an X-linked recessive disorder located on chromosome Xq22.1, the disease affects hemizygous males, as well as both heterozygous and homozygous females. For males, the clinical features tend to be severe and onset in childhood or early adolescence. Due to X-inactivation patterns the symptoms for females could vary dramatically from virtually none to those as serious as a male patient's. Intracellular GL deposition in the myocardium, valves, and conduction system leads to an increase in ventricular wall thickness, mitral valve prolapse, electrocardiographic abnormalities such as various degrees of atrioventricular conduction block and tachycardia [2,3]. It possibly contributes to neurovascular parkinsonism [4]. However, kidney complications are the most frequent and serious effects of the disease from renal insufficiency to end-stage renal failure (ESRF) [5].

Recently, studies of Anderson-Fabry disease have been conducted using electron microscopy. Ultrastructural studies reveal that the cytoplasmic lysosomes exhibit concentric lamellation—osmiophilic myelin bodies in all types of renal cells. These myelin bodies critically damage the glomerular filtration barrier and take responsibility for disrupting the proteinuric mechanisms [6]. Furthermore, the involvement of myelin bodies with the Bowman's Capsule epithelial cells, the glomerular capillary, and the glomerular afferent arterioles contributes to the progressive loss of renal function [6]. In the urine of Anderson-Fabry patients, these myelin bodies from pathological lesions are excreted in large amounts [7]. Within the concentric lamellation-structure, myelin bodies exhibit liquid crystal characteristics and can be identified by a Maltese cross when observed between the polarizer and analyzer of polarizing microscopes. At the present time Maltese-cross lipid bodies detected with osmiophilic lamellation at the ultrastructure level and Maltese cross birefringence at the microstructure level has become one of the typical diagnostic characteristics for evaluating Anderson-Fabry disease [6,8–11]. In this paper, our recent data reveals that Maltese cross birefringence is also present in normal biological processes during the development of mesonephros and metanephros. These results demonstrate that the

birefringence bodies with the Maltese cross pattern are not only represent in the pathological GL accumulation of Anderson-Fabry disease but are also present during the physiological formation of the kidney.

II. EXPERIMENTAL MATERIALS AND METHODS

Animal and Terminology

All animals used in these experiments are covered under the regulation of University Institutional Animal Care and Use Committee (IACUC). The maintenance, use and other activities of vertebrate animals were overseen by IACUC. All experimental procedures carried out in this project were reviewed and approved prior to its initiation.

The chicken is the major vertebrate used in our experiment. Test animals were hosted mainly in the university animal facility of avian located at Luojia Domestic Animal Farm, Wuhan University School of Life Sciences. Fertilized White Leghorn eggs (*Gallus domesticus* L.) were incubated at a temperature of 37°C with relative air humidity of 60%. Experiments were conducted on required days according to experiment procedure. Age of embryos was documented as day (D) and postnatal age of chicks hatched after 21 days of incubation was documented as Postnatal (P).

Sample Preparation and Cryosection

Two procedures were used to prepare samples. (1) Smear-slide preparation: Dissected samples were taken from the embryos at different stages. Each sample was smeared on a slide and mounted with a cover slide in PBS (PH 7.4). Polarizing microscopy was then carried out immediately. (2) Cryosection: After dissected sample from the embryos, the samples were set into cryomatrix embedding agent (OCT) and placed in an aluminum foil basket. Samples were frozen by dipping the foil basket into liquid nitrogen. Frozen tissue within the OCT block were placed on the cryostat microtome and section samples were cut at thickness of 10∼30 μm. The sections for H&E staining were at thickness of 5 μm.

The samples collected using these two procedures were mounted with 20% glycerol in PBS (PH 7.2) and are ready for polarization observation.

Histology Analysis

Hematoxylin and Eosin (H&E) staining was carried out on frozen section according to our previous method [12]. After Hematoxylin

and Eosin staining, slides were dehydrated in gradients of ethanol before cover slipping with xylene based Permount medium for permanent preservation.

Microscopic observation of sample slides for histology analysis was carried out under a Nikon conventional microscope (Jnoec Ltd., Jiangnan, Assembled in Nanjing, PRC).

Polarized Light Microscopy

The samples obtained from chick prepared smear-slide and cryosection preparation were observed via two methods. Conventional observation was carried out first under non-crossed polarizer and analyzer to identify the localization of samples. This preliminary observation helped to evaluate the distribution of birefringent myelin bodies that will be further categorize by histology analysis. Once the locations were noted, optical activity of the samples was observed between crossed polarizers. Birefringence activities generated by various samples were documented for further analysis. The observation on optical activity proceeded with a XS-213A-P Polarizing Microscope (Jnoec Ltd., Jiangnan, Nanjing, PRC).

Measurement of Phase Transition

The measurements were performed using both the inverted microscope PE120 peltier system (Linkam Scientific Instruments, UK) and XS-213A-P polarizing microscope. PE120 peltier system is set to work with 5 mm aperture at a heating/cooling rate range of 0.1 to 20°C/min. Its temperature stability is ±0.1°C regulated with RS232 on temperature controller. Temperatures of phase transition between anisotropic and isotropic phases were recorded according to the change of birefringence activities of the samples between crossed two polarizing prisms.

Electron Microscope (EM) Analysis

Tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C at proximal 1 mm cubes, and then washed in 0.1 M sodium cacodylate buffer overnight at 4°C. Post-fix was conducted in osmium tetroxide (OsO_4) fresh prepared with 0.2 M sodium cacodylate buffer by mixing equal quantities of 2% aqueous OsO_4 and 0.4 M buffer). Samples were dehydrated in gradient ethanol and embed in labeled capsules with freshly prepared Epon812 after a transition of propylene oxide/Epon812 mixture and polymerize 48 hours in 60°C oven.

Before proceeding to EM, semi-thin sectioning was carried out to select the appropriate location at thickness of 1 micron using glass knives and an ultramicrotome. After the location has been identified, sections for EM were cut at around 100 nm using a diamond knife controlled by heat advance. EM was performed using a Hitachi Transmission Electron Microscope H-7000 (Hitachi Co., Tokyo).

Image Analysis

The images from conventional microscopy and polarizing microscopy were captured with a Nikon or Olympus camera. The quantification analysis of the birefringent intensity of the LCDLs was measured and constructed at different developmental stages with the image analysis software *ImageJ 1.38* (NIH, Bethesda, MD). The colors in quantification of birefringent intensity were generated by this analysis software.

III. RESULTS

Through the use of polarizing microscopy and electron microscopy, our systematic investigation revealed the existence of birefringent Maltese cross particles in mesonephros and metanephros tissues during chicken embryonic development for the first time. The characterization of this unique particle in chicken kidney would expand understanding of Anderson-Fabry disease and provide valuable information when polarizing microscopic characterization is used as clinic diagnostics to evaluate the disease complexity.

Polarizing Microscopy Revealed the Time Frame of Maltese Cross Appearances in Embryonic Development

Under polarizing microscopy, Maltese crosses are initially observed in the embryo day 8 (E8) in mesonephros. The number of these Maltese crosses increases during development of the embryo and the size of the birefringent myelin bodies continuously enlarge until E13 (Figures 1A and C). After E18, the birefringent Maltese crosses begin to vanish from observation during mesonephros regression.

In the metanephros phase, the Maltese crosses are first observed in E14 embryos. Both the number and size of the Maltese crosses increase with the development of the embryo (Figures 1B and D). The birefringent Maltese crosses can still be observed until postnatal (P) day, P2 or P3. After this stage, the Maltese crosses with liquid-crystal characteristics can rarely be detected in the matured kidney

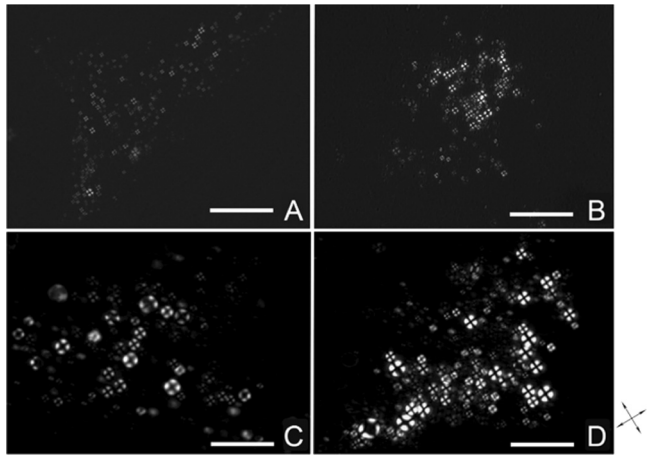


FIGURE 1 Maltese cross birefringence in chicken renal development. Panels A and C show Maltese crosses in the mesonephros of E8 and E13 embryos. In panels B and D, Maltese crosses can be observed in the metanephros of the E14 and E19 embryos. Scale bars are 10 μm.

by polarizing microscopy. Our observations revealed that birefringent Maltese crosses can also be found in the adrenal gland from E14 to E20 during embryonic development (Table 1).

Histology Analysis Demonstrates the Distribution of Maltese Cross Appearances in Kidney Tissue

We performed histological analysis on both embryonic mesonephros and metanephros tissues at different development stages including E8, E13, E18, E20 and postnatal stage P2, P6, P14. HE staining and

TABLE 1 Timed Appearance and Disappearance of the Liquid Crystal Maltese Crosses in Mesonephros and Metanephros During Chicken Embryonic Development

	Appearance of birefringence Maltese crosses	Disappearance of birefringence Maltese crosses	Duration of the Existence
Mesonephros	E8	E18	10 days (EE-ElB)
Metanephros	E14	P2, P3	9 to 10 days (E14-P2, P3)
Adrenal	E14	E20	6 days (E14-E20)

Note: E. Embryonic day; P. Postnatal day.

polarizing microscopic analysis shows that Maltese crosses are mainly distribute at two locations in chick embryonic kidney: the glomerulus (Figures 2A, B, and C) and the collecting duct connecting the kidney to the renal ureter. Unexpectedly, the Maltese crosses were observed within the wall (Figures 2D, E, and F) instead of the lumen of the ureter (LU) as indicated by arrows. The intensity of the birefringent Maltese crosses in the renal ureter is similar to that of Maltese crosses in the glomerulus (GM) (Figures 2G, H, and I).

Maltaese crosses can also be observed periodically in other parts of renal tissue including the Bowman's capsule and both the proximal and distal convoluted tubules. According to the data we documented above, we speculate that birefringent Maltese crosses are liquid crystal droplets present in renal cells and the luminal structure of kidneys. We have confirmed with further electron microscopic analysis.

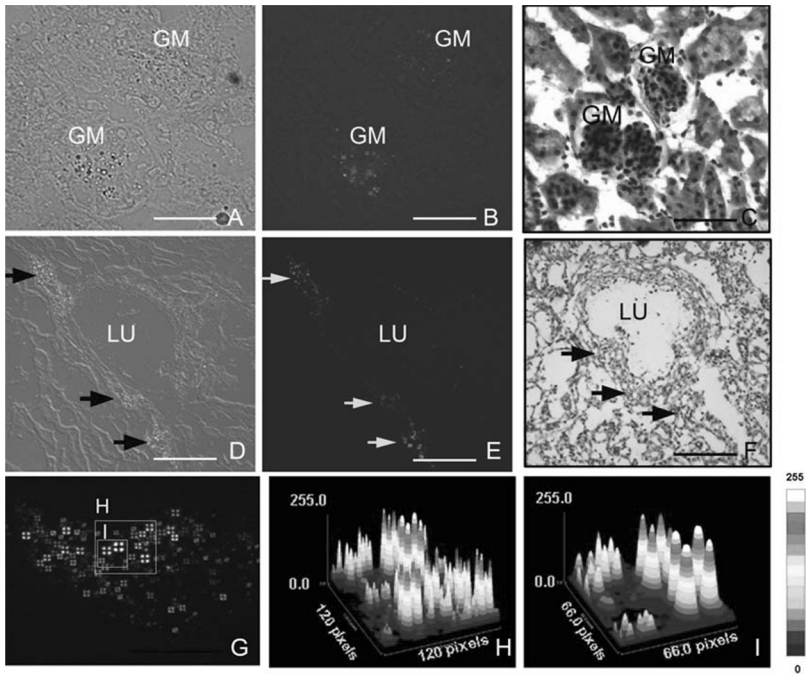


FIGURE 2 Distribution analysis of the Maltese crosses in embryonic renal tissue. The main body of the glomerulus is exhibited under non-crossed (A), crossed polarizer (B), and with HE staining (C). The collecting duct is also displayed under non-crossed (D), crossed polarizer (E), and with HE staining (F). Higher magnification (G) with its area intensity plots (H and I) displays a view of the glomerulus. Scale bars are 30 μm.

Phase Transition of Birefringent Maltese Crosses in the Chick Embryonic Kidney

During chicken development, the kidney undergoes three diverse phases: pronephros, mesonephros and metanephros. When chick is hatched, metanephros will be the matured kidney in charge of renal function. In this part of the study, we focus is on metanephros, the life-time kidney.

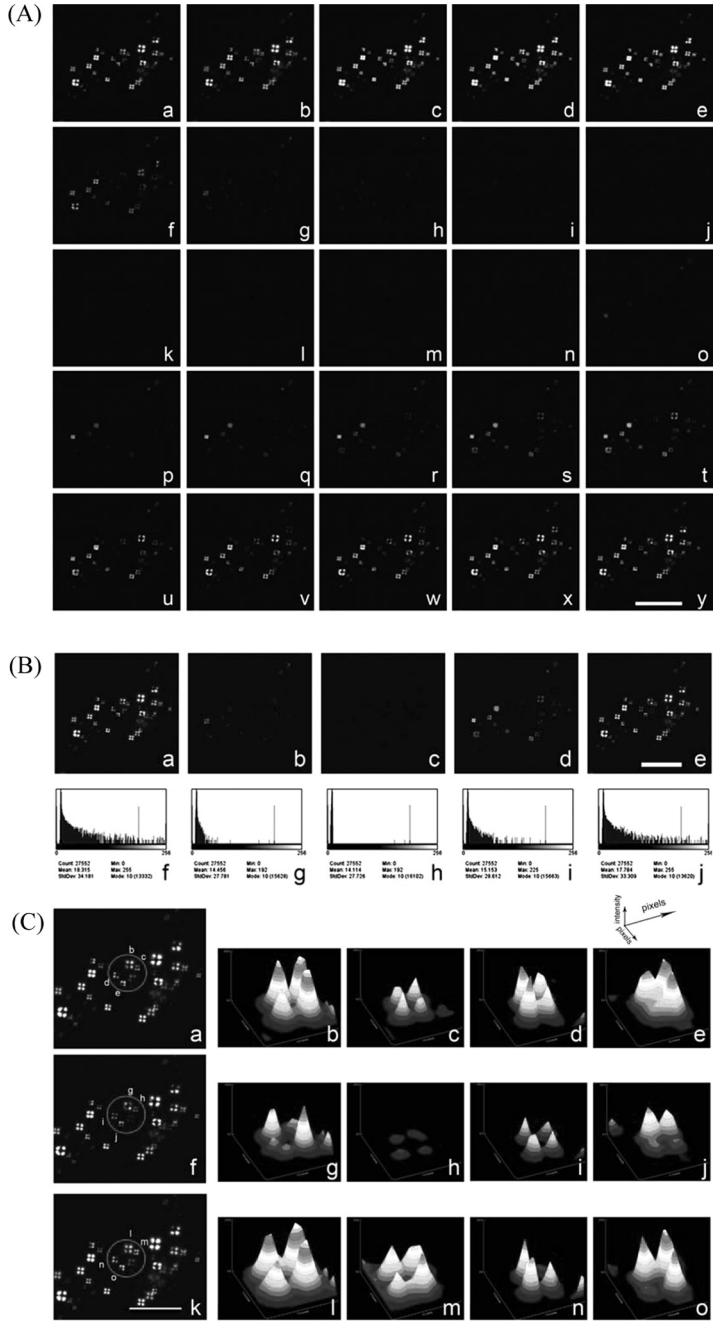
Temperature effect on Maltese cross birefringence in the glomerulus was examined under standard conditions. With temperature alteration, the birefringence underwent two processes, transforming from liquid crystal to isotropic state and upon the removal of the heat source the cross birefringence returned from isotropic state back to liquid crystal. In Figure 3A from panel a to j, we documented the transition process from Maltese cross birefringence (liquid crystalline, anisotropic state) to isotropic droplets with temperature increase. Under polarizing microscope, the phase transition of each individual Maltese cross occurs quickly and as a synchronized whole.

With temperature decrease, isotropic droplets resumed liquid crystalline status in the tissues. This process was recorded in Figure 3A from panel k to y. This process of resuming liquid crystalline status after Maltese cross transiting to an isotropic state under high temperature conditions depends on the rate of temperature-decrease. In our experiment, there was no liquid nitrogen or other vehicle used to enhance the rate. We partially plotted this temperature recovery experiment in Figure 3B (panels a to e) and their density comparison is exhibited in panel f to j. The recovery of the liquid crystal birefringence from isotropic state took more time than the duration of the initial phase transition from birefringent liquid crystal to isotropic state.

When compared to recovered Maltese crosses (Figure 3C panel f to l), liquid crystal birefringent (Figure 3C panel a to e) displayed no significant difference over all. However, the recovery for some individual Maltese crosses is not complete. The structure defects were easily observed. Eventually, the defects resumed when a longer recovery time was allowed. In this representative experiment, complete recovery took roughly ten minutes. The intensity diagrams on the right in Figure C exhibits the recovery process.

Phase Transitions Between Crystal, Liquid Crystal and Isotropic State

Cryosection was carried out using polarizing microscopy and histology analysis to identify the location of the Maltese crosses in the



metanephros during chicken development. Unexpectedly, we noticed that the Maltese crosses disappeared from polarized light after cryosection. Crystal bundles were found in both the mesonephros and metanephros. The number of the crystal bundles was close to that of the Maltese crosses (Figures 4A and B). Furthermore, we demonstrated that phrase transitions can occur between crystal, Maltese crosses liquid crystal, and isotropic state. This phenomenon is very similar to what we reported previously in the embryonic liver [13].

In Figure 4A, a representative bundle of crystals was selected to display processes of phase transition in metanephros. With temperature increase, the crystals disappeared progressively, and eventually became isotropic. This process of phase transition from crystal to an isotropic state was documented clearly in Figure 4B (panel a to j). Then the process of phase transition back from isotropic droplets to the Maltese crosses was obtained when the heated droplets were allowed to resume equilibrium at room temperature. With temperature decrease, the isotropic state became Maltese cross liquid crystalline instead of crystalline. This process proceeded rapidly under polarizing microscope (Figure 4B panel k to x). The rate of temperature decrease depended on environmental temperature. No liquid nitrogen or other source was used to increase the cooling rate in our experiments.

In Figure 4A (panels c to e), we demonstrate that both bundles of crystals and liquid crystal Maltese crosses located in glomerulus with histology analysis. These results indicate that the liquid crystal Maltese crosses transited from crystals are the same as those we observed on the smear slide of metaphase.

Electron Microscopy of Birefringent Maltese Crosses

To investigate the structure of the Maltese crosses observed during embryonic development, we performed ultrastructural studies of both mesonephros and metanephros tissues. Our data demonstrated that the Maltese cross configuration is typical of lamellar bodies. These cytoplasmic inclusion bodies display the concentric lamellation with

FIGURE 3 Thermal phase transition analysis of the liquid crystals found in embryonic renal tissues. A transition process is shown in panel A with a series of recordings from the same perspective. The phase transition (panels a to e) is exhibited in density graphs from (panel f to j) accordingly in B. Defect recovering of some Maltese crosses are showed in density graphs before (panel a to e) and after (panel f to j) the phase transition. The recovery status of Maltese crosses after 10 minutes shown in C (panel k to o). Scale bar is 10 μm.

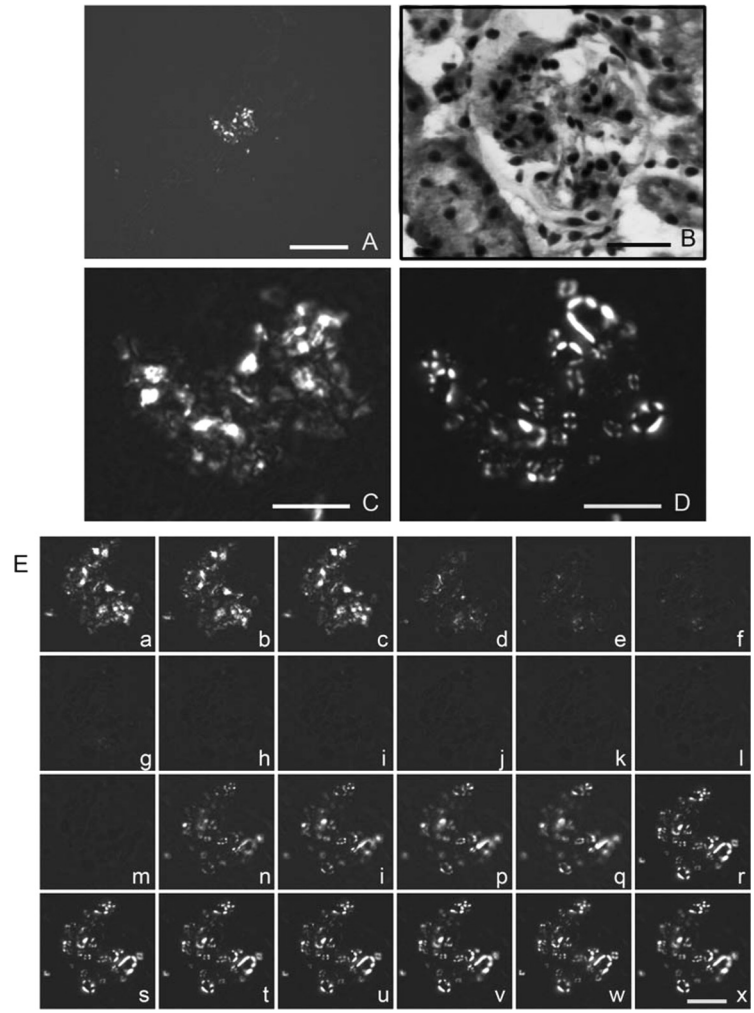


FIGURE 4 Phase transition analysis between crystal, Maltese crosses liquid crystal, and isotropic state. After cryosection, crystal bundles instead of liquid crystals were observed in the metanephros. An example of a bundle of crystals present in the tissue corresponding to glomerulus location (A and B). The bundle of crystals (C) can transit to liquid crystal (D). Phase transition processes are showed in E including the phase transition from crystal to isotropic state (panels a to j), and the phase transition from the isotropic state to liquid crystal (panels a to j). Scale bars are 40 μm in A; 15 μm in B to D; 20 μm in E.

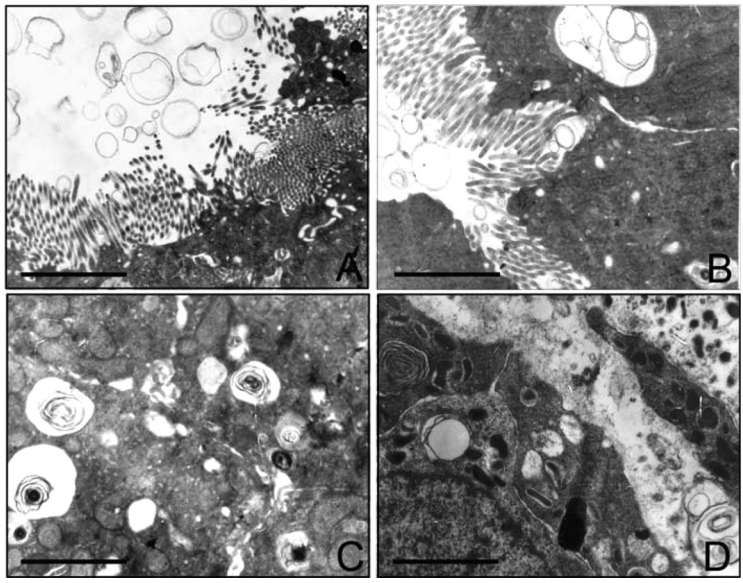


FIGURE 5 Ultrastructure analysis of the chicken embryonic renal development. The concentric lamellation are showed in both mesonephros (A and C) and metanephros tissues (B and D). Panels A and B are the lumen of proximal tubules, and panel C and D are in the cytoplasm of proximal tubular epithelial cells. Scale bars are 3 μm.

characteristic "zebra" or "onion skin" appearance (Figure 5C and D). In both mesonephros and metanephros tissues, lamellar bodies are mainly found in the cytoplasm of proximal tubular epithelial cells. They can also be detected in the lumen of proximal tubules (Figure 5A and B). Furthermore, electron microscopy reveals that although the luminal myelin bodies have significantly fewer layers of lamellation than cytoplasmic myelin bodies, the thickness of each layer of luminal myelin bodies is visibly greater.

IV. DISCUSSION

In Anderson-Fabry disease patients, deficiency of the lysosomal enzyme alpha-galactosidase A leads to progressive intracellular accumulation of glycosphingolipids in different tissues. This X-chromosome linked syndrome displays symptoms on the skin, kidneys, vascular endothelium, ganglion cells of peripheral nervous system, and heart [11]. In the heart, characteristics of this disorder mimic the morphological and clinical features of hypertrophic

cardiomyopathy with progressive left ventricular hypertrophy [14–16]. The most common and serious effect of this deficiency is kidney complications. From renal insufficiency to end-stage renal failure, patients can suffer throughout life. The intracellular and extracellular accumulation of glycosphingolipids in the renal epithelium, endothelium and other different cells is the direct cuase of these complications [6,8–11]. Enzyme replacement and enzyme enhancement therapy have proved effective for the improvement of renal and cardiac function for patients suffering from the disease.

Standard clinical diagnosis of Anderson-Fabry disease is based on some or all of the following factors, i.e., family history, history of childhood fevers in association with pain in the extremities, characteristic skin lesions (angiokeratomas), "whorled" corneal opacity, and the presence of lipid-laden cells in urinary sediment or/and biopsied tissues [17]. Among these factors, the presence of lipid-laden cells from patients is considered the most important factor for diagnosis of the disease [6,8–11]. In the cells of the sediment and tissues, the laden lipid is liquid crystalline with Maltese cross appearances. Interestingly, according to our work, these Maltese crosses are also present in the both the mesonephros and metanephros kidney during embryonic development and are, as our results demonstrated, mainly situated in the glomerulus. This distribution matches that of the Maltese cross distribution within patients with Anderson-Fabry disease.

In embryonic development of the animals, the Maltese crosses are required for kidney maturation from mesonephros to permanent metanephros kidney. The ultrastructural electron microscopy revealed that these myelin bodies are concentric laminations. These crosses have typical characteristics of liquid crystals. Although the detailed function of the Maltese crosses in renal development is unclear, the characteristics of liquid crystal provide a unique prospect to comprehending this biological process.

In *in vitro* experiments, Maltese crosses can transit from the anisotropic liquid crystal state to isotropic droplets with temperature increase. These isotropic droplets can then resume crystal structure with temperature decrease. Furthermore we found that the phase transition between anisotropic and isotropic droplets is repeatable in *in vivo* systems. The whole process has been clearly documented in our experiment results. The data indicates that these phase transitions might exist in renal development. However because mammals and birds are thermoregulation animals, the phase transition could not be controlled by temperature *in vivo*. Instead, the component withdrawing and addition in anisotropic or isotropic droplets could be the cause of phase transition. Component withdrawing could result in

phase transition from anisotropic liquid crystal state to isotropic state. Conversely the phase transition from isotropic to anisotropic liquid crystal Maltese crosses would require component addition into the isotropic droplets.

During the development of the chick, we have reported the presence of liquid crystalline droplets (LCDLs) in liver, spleen, bile, skin, brain, nerve, mesonephros, metanephros, adrenal, yolk sac, bone marrow, heart, and thyroid gland. The appearance of LCDLs in different tissues and organs occur at different embryonic stages that the LCDLs are necessary for chick development [13,18]. We hypothesize that during embryonic development there is an embryonic program for the activation of these myelin bodies in organ-genesis. The program is in charge of directing tissue or organ maturation. In kidney development, this program is activated at E8 for mesonephros and E14 for metanephros (see Table 1). After metanephros is established and ready for its permanent function, the activating program was deactivated. However, unexpected reactivation of the program in kidney during adolescence or childhood may lead to the manifestations of Anderson-Fabry disease.

V. CONCLUSION

Through the use of polarizing microscopy and electron microscopy, we demonstrated (1) the presence of Maltese crosses initially in the embryo on day 8 (E8) in mesonephros. The number of these Maltese crosses increases during development of the embryo and the size of the birefringent myelin bodies continuously enlarge until E13; (2) in the metanephros phase, the Maltese crosses are first observed in E14 embryos. Both the number and size of the Maltese crosses increase with the development of the embryo as well; (3) our ultrastructural studies demonstrated that the Maltese cross is a typical configuration of myelin bodies with concentric lamellation situated in the cytoplasm of proximal tubular epithelial cells and the lumen of proximal tubules in both mesonephros and metanephros tissues.

Birefringent Maltese crosses are not only found in mesonephros and metanephros but are also found in liver during chick development as we previously demonstrated. Therefore we hypothesize that there is an embryonic program for the activation of these myelin bodies in organ-genesis. During development of the embryo, this program facilitates kidney and liver genesis, but unexpected reactivation of the program in kidney during adolescence or childhood may lead to manifestations of the Anderson-Fabry disease. In the end, the accumulation of glycophospholipids or glycosphingolipids results in the severe

pathological progression ranging from renal insufficiency to end-stage renal failure. Therefore, investigation on the Maltese crosses in development could provide attractive prospect to understand molecular mechanism of human diseases.

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