Kidney & Blood Pressure Research

Kidney Blood Press Res 2016;41:794-801
DOI: 10.1159/000450561
Published online: November 11, 2016
Accepted: August 25, 2016

Original Paper

Gulo Acts as a *de novo* Marker for Pronephric Tubules in *Xenopus laevis*

Yajun Xie[a,b] Yuhang Liu[c] Ya Zhao[d] Hui Wang[e] Yamin Liu[f] Honglian Wang[c] Mi Li[f] Hui Zhao[e] Qin Zhou[g] Xiaoyan Lv[a]

[a]Department of Dermatology, West China Hospital, West China School of Medicine, Sichuan University, Chengdu; [b]The Institute of Genetics and Development Biology of Chinese Academy of Sciences, University of the Chinese Academy of Sciences, Beijing; [c]Laboratory of Organ Fibrosis Prophylaxis and Treatment by combine Traditional Chinese and Western Medicine, Research Center of Combine Traditional Chinese and Western Medicine, Affiliated Traditional Medicine Hospital, Southwest Medical University, Luzhou, Sichuan; [d]Department of Laboratory Medicine, the First Hospital of Xi'an, Xi'an; [e]Key Laboratory for Regenerative Medicine, Ministry of Education, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong; [f]The Division of Molecular Nephrology and the Creative Training Center for Undergraduates, The M.O.E. Key Laboratory of Laboratory Medical Diagnostics, the College of Laboratory Medicine, Chongqing Medical University, Chongqing; [g]The core facility of genetically engineered mice, West China Hospital, West China School of Medicine, Sichuan University, Chengdu, China

Key Words
Gulo • LOC495407 • Vitamin C • *Xenopus laevis* • Pronephros

Abstract
Backgrounds/Aims: Vitamin C is an antioxidant and acts as a cofactor for several key enzymatic catalytic reactions in animals. Amphibians produce vitamin C in their kidneys, as opposed to mammals that produce vitamin C in their liver. Gulo serves as a crucial enzyme for vitamin C synthesis in mammals, but the characteristics and localization of its homologous genes during kidney development in *Xenopus laevis*, an amphibian, remains unknown. ***Methods:*** We aligned amino acid sequences of Gulo across different species by using bioinformatics methods and detected patterns of expression for Gulo during kidney development by using RT-PCR and *in situ* hybridization. ***Results:*** We identified a new site on the *X. laevis* genome, LOC495407. Sequence alignment analysis indicated this fragment is highly conserved and homologous to *gulo* genes in mammals. RT-PCR and *in situ* hybridization results reveal that *X. laevis gulo* is maternally expressed during the early stages of embryonic development, particularly, in the tubules of the pronephros from the middle tail-bud stage and onward in

Y. Xie, Y. Liu, Y. Zhao and H. Wang contributed equally to this work and therefore share first authorship.

Xiaoyan Lv, MD&Ph.D.

Department of Dermatology, West China Hospital, West China School of Medicine, Sichuan University, No. 37, Guo Xue Xiang, Chengdu Sichuan 610041 (P. R. China)
Tel. +86-028-85423315, Fax +86-028-85423315 E-Mail lxiaoyan@scu.edu.cn

Kidney & Blood Pressure Research

Kidney Blood Press Res 2016;41:794-801
DOI: 10.1159/000450561
Published online: November 11, 2016

embryos. ***Conclusion:*** Gulo is a novel specific marker for pronephros tubules in *X. laevis*, and may be used as a potential marker for kidney development studies and disease diagnosis in mammals.

Introduction

Vitamin C, also known as L-ascorbic acid, is an important nutrient for human health. Vitamin C deficiency in the diet will lead to scurvy related symptoms, such as unexplainable whole-body pain, widespread bleeding of local capillary vessels, swollen gums, and many other symptoms [1, 2]. Additionally, vitamin C also serves as a co-substrate for the synthesis of some important body materials such as collagen, which is important for elasticity in blood vessels and bone [3, 4]. Moreover, it is widely believed that, as an antioxidant, vitamin C can remove reactive radicals, such as reactive oxygen species (ROS), and protect cell membranes and intracellular components from oxidative damage [5]. However, the characteristics and functions of some key genes responsible for vitamin C synthesis during embryonic development are yet to be identified.

In the animal kingdom, most species can synthesize their own vitamin C *in vivo* by using D-glucuronate as a substrate, with L-gulono-γ-lactone oxidase (Gulo) being a key enzyme for the vitamin C reaction cascade [6]. Therefore, most Gulo-deficient species are unable to synthesize vitamin C [7-9], such as humans, where *gulo* appears to exist as a pseudogene without detectable corresponding transcripts [10]. Presumably, external source of vitamin C has led to the loss of selective pressure for *gulo* during human evolution and accumulation of mutations has led to the complete elimination of gene function [7]. In contrast, the biosynthesis of vitamin C mainly takes place in the liver of many mammals, and in fish, amphibians, and reptiles, synthesis of vitamin C occurs in the kidney [6, 8]. However, the characteristic and localization of Gulo in the developing embryo of *X. laevis* remains unknown.

In this study, we use bioinformatics analysis to annotate LOC495407 in *X. laevis* and LOC101733882 in *X. tropicalis* to the homologous *gulo* gene of in mammals. We also demonstrate that *gulo* in *X. laevis* is expressed maternally as well as during the early embryonic stages in the tubules of the pronephros.

Materials and Methods

Bioinformatics analysis

The accession numbers of proteins used in this study are NP_848862 (Gulo, mouse), NP_071556 (Gulo, rat), NP_001029215 (Gulo, cow), NP_001123420 (Gulo, pig), XP_543226 (Gulo-like LOC486100, dog), NP_001088534 (LOC495407, *X. laevis*), and XP_002122023 (LOC100180753, *Ciona intestinalis*). The suspected homologous sequence of LOC495407 in *X. tropicalis* (LOC101733882 with a protein ID of XP_004915033), was identified by BLAST analysis and further revised by Hellsten et al. [11]. Multiple amino acids sequence alignment was performed with the DNAssist (version 2.2) software and the image was further processed with Adobe Illustrator CS3 software for publication. The NCBI online software BLAST was used to calculate the identity value between two different amino acid sequences. The software MEGA5 was used to generate phylogenetic trees with the neighbor-joining method based on the multiple protein alignment result produced with the software Clustal W. The domain profile of the *X. laevis* LOC495407 was predicted with the online accessible SMART protein architecture research tool) [12]. For chromosomal synteny analysis for Gulo, the mouse genome annotation version of GRCm38 and the *X. tropicalis* genome annotation version of JGI xentr draft 7.1 were referred to depict the co-linear relationship.

Whole-mount in situ hybridization

For *in vitro* synthesis of digoxigenin-labeled RNA probe, the corresponding LOC495407 probe fragment

was TA cloned into pBST-II with the following primers: forward, 5′-CCTCGACACTCATCTGAACAGCTC-3′; reverse, 5′-GTAGAAGACCTTCTCCAGATAAGC-3′. The template for probe synthesis was generated by PCR with primers flanking the T3 promoter and LOC495407 probe fragment: forward, the same as the one used for cloning above; reverse, 5′-CGTATGTTGTGTGGAATTGTGAG-3′. RNA probe was synthesized with T3 RNA polymerase (Thermo Scientific). The probe for whole-mount *in situ* hybridization reactions was generated by amplifying a partial sequence of ATP1B1 and BIP using the reference PCR primers [13, 14]. The resulting fragment was sub-cloned into a pBST-II vector.

Whole-mount *in situ* hybridization was performed according to the method previously described by [15] with the following minor modifications: the embryos of relevant stages were collected, de-jellied with 2% cysteine (pH 8.0), and fixed overnight at 4°C. Before hybridization, pigmented embryos were immersed in bleaching solution containing 3% H_2O_2. Pre-hybridization was performed for at least 6 h at 60°C. For hybridization, a 200 ng/mL probe was applied to hybridize at 60° Celsius overnight. The digoxigenin-labeled probe was recognized by a goat anti-digoxigenin antibody (Roche) at a ratio of 1:10000. Chrome was developed in BM purple AP substrate (Roche) for the instructed amount of time. Images were taken with a Zeiss stereo microscope (SteREO Discovery.V12) and further processed with Photoshop CS3 software. Vibratome sectioning was performed at 30 μm (Leica, CM1850).

RT-PCR

The RNA of the relevant stage embryos was isolated with Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with a Revert Aid First Strand cDNA Synthesis kit (Thermo Scientific). For determination of gene expression level, primers for LOC495407 were the same as those used for probe vector construction. ODC1 was used as an internal control with the following primers: forward, 5′-CAGCTAGCTGTGGTGTGGAG-3′; reverse, 5′- AGCCCATCACACGTTGGTCC-3′.

Results

In order to screen genes with pronephros specific expression from a mesonephric cDNA library database (UniGene library ID: 11985) derived from *X. laevis*, we focused on LOC495407. BLAST analysis shows that the mRNA sequence is very similar to the *gulo* genes of mice and rats (data not shown). Further to this, multiple amino acid sequence alignments across different species reveals that LOC495407 shares 76%, 74%, 75%, 73% and 72% similarity to the *gulo* genes in cows, dogs, pigs, mice, and rats, respectively (Fig. 1, 2A). Even in *C. intestinalis*, an evolutionarily lower marine invertebrate [16], we found coding sequences with notable similarity to *gulo* genes in mammals (Fig. 2A). In *X. tropicalis*, BLAST analysis with a nucleotide database for LOC495407 RNA sequence identified a gene record LOC100180753 (gene ID). Although showing highly local sequence similarity, the deduced LOC100180753 protein sequence contains a large fragment of a short repetitive motif in the middle region. Suspecting there may be errors during genome sequencing and assembly, we cloned the coding region *de novo* with a PCR-based method, which validated our hypothesis. The revised LOC100180753 transcript shows high overall sequence similarity with LOC495407 and *gulo* genes of mammals, both at RNA (data not shown) and protein levels (Fig. 1, 2A).

Bioinformatics analysis indicated that the deduced polypeptides of LOC495407 and LOC100180753 contain a FAD binding domain on its N half and a ALO (D-arabinono-1,4-lactone oxidase) domain on its C half, in line with *gulo* genes in mammals (Fig. 1, 2B and data not shown). Regarding the neighboring gene loci, there is remarkable chromosomal synteny between *gulo* in mice and LOC100180753 in the *X. tropicalis* genome (Fig. 2C). Moreover, LOC495407 and revised can be clustered into a proper evolutionary position with *gulo* genes across different species in the phylogenetic tree construction assay (Fig. 2A). Together, these findings suggest that LOC495407 and LOC100180753 (hereafter designated *Xl.gulo and Xt.gulo*) are the homologous genes of *gulo* in *X. laevis* and *X. tropicalis,* respectively, which are responsible for vitamin C synthesis.

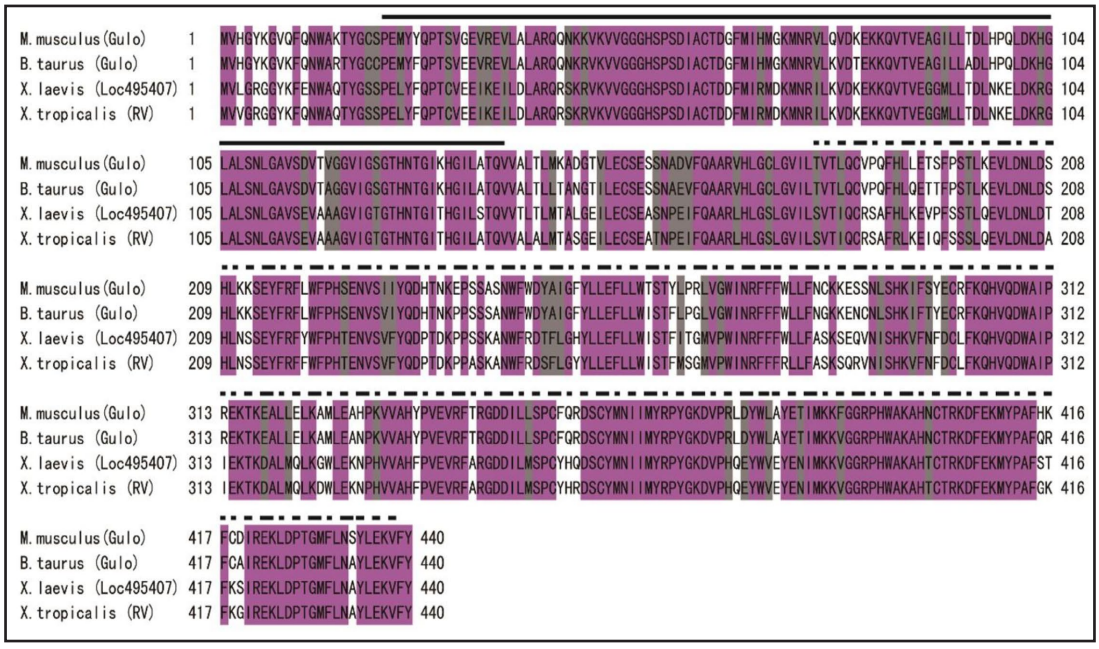

Fig. 1. Multiple amino acid sequence alignment. Protein sequences employed for alignment are Gulo in cows and mice, LOC495407 in *Xenopus laevis* and revised LOC101733882 (RV) in *X. tropicalis*. The identical amino acids are shaded in purple and conserved amino acids in grey. The black line above the sequence array indicates the FAD binding domain, and the dotted line marks the ALO domain.

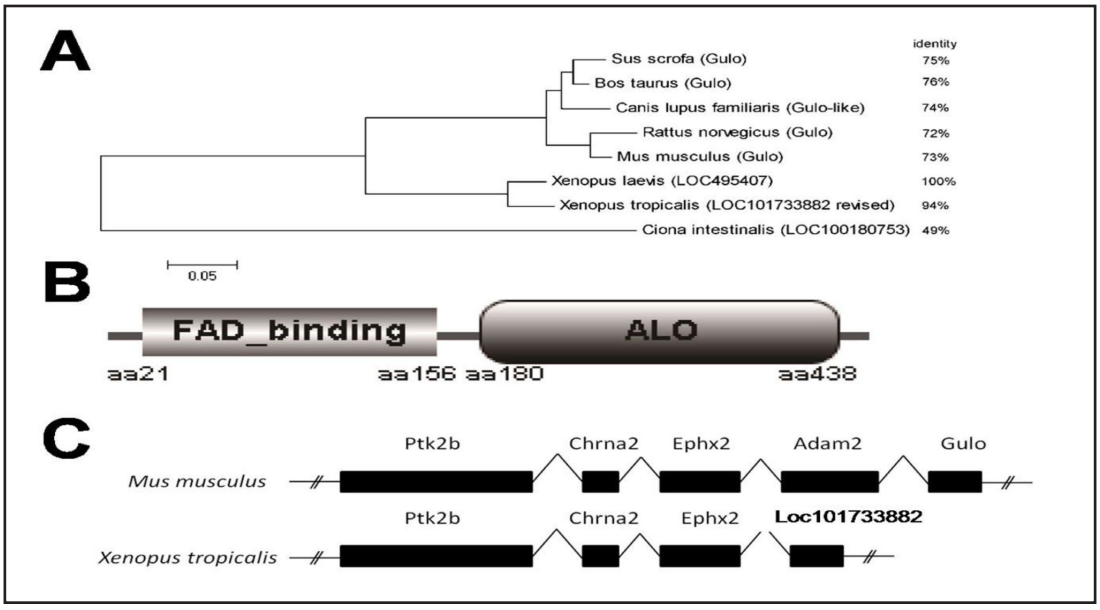

Fig. 2. Phylogenetic analysis, protein structure prediction, and chromosomal synteny evaluation of *Xenopus laevis* LOC495407. (A) Phylogenetic tree construction assay with Gulo in cows, dogs, pigs, mice, and rats, LOC495407 in *X. laevis*, LOC101733882 (revised) in *X. tropicalis* and LOC100180753 in *Ciona intestinalis*. (B) Protein structure of LOC495407 predicted by SMART protein architecture research tool. (C) Schematic diagram showing the chromosomal synteny around the genetic loci of *gulo* in mice and LOC100180753 in *X. tropicalis* which show high sequence similarity to *X. laevis* LOC495407.

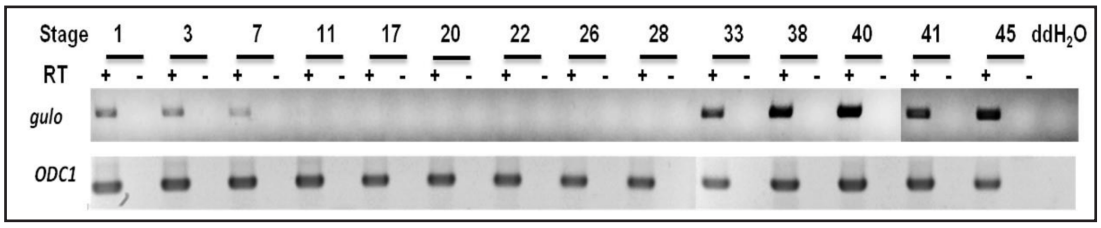

Fig. 3. Temporal expression pattern of *gulo* in developmental embryos detected by RT-PCR. Ornithine decarboxylase 1 (*ODC1*) is used as an internal control. For negative control (-), no reverse transcriptase was added to the reverse transcription reaction. RT = Reverse Transcriptase.

To determine the temporal expression pattern of *Xl.gulo* during embryonic development, RT-PCR was employed to analyze the complete RNA sequence of embryos from different developmental stages. *Xl.gulo* mRNA can be detected from embryonic stages 1 to 7, indicating that this gene is maternally expressed. However, no *Xl.gulo* transcripts can be found during the gastrula and neural developmental stages. The first instance of gene expression in zygotes was observed at the middle tail-bud embryonic stage (around stage 28), becoming more robust at the end of tail-bud development with a peak at stage 40, and was sustained at this level into the tadpole stage (Fig. 3).

To determine the spatial expression pattern of *Xl.gulo*, we performed whole-mount *in situ* hybridization. The results show that Gulo is solely expressed in the tubules of the pronephros of *X. laevis* (Fig. 4). In contrast to the RT-PCR result, no significant transcripts can be detected at stages when maternal genes dominate the transcriptome (Fig. 4A). This may be caused by the low expression level and low concentration of the maternal Gulo transcripts or sensitivity of the hybridization. As with the RT-PCR results, no expression was observed in the gastrula, neural, and early tail-bud stages of embryonic development (Fig. 4B-E). The initial hybridization signals are detected by around embryonic stage 28 and increase gradually to reach an apex by the late tail-bud stage (stages 36–40) (Fig. 4F-H and H'). This expression continues into the tadpole stage (Fig. 4I).

To identify which organ is the main location for Gulo expression during embryonic development, we selected two positive markers, ATP1B1 and BIP [13, 14], which are mainly expressed in the pronephric region (Fig. 4J-L). Our *in situ* hybridization results revealed that the location of expression for *gulo* during embryonic development is very similar to that of expression for the two positive pronephric markers. We therefore deduce that *gulo* is mainly expressed in the pronephros of *X. laevis* during embryonic development. Vibratome sectioning after hybridization shows that the signals are localized in the lateral plate layer of the pronephric domain, indicating the position of tubules, which suggests the specific expression of *gulo* in the tubule domain of the pronephros (Fig. 5).

Discussion

Recently, vitamin C was found to remodel the epigenetic landscape of cultured embryonic stage cells to a blastocyst/inner cell mass (ICM) stage-like state (low methylation state) through Tet-dependent DNA demethylation [17]. There is evidence showing that during early mammalian embryo development, the whole genome methylation landscape indeed changes dynamically [18]. However, there has been no evidence demonstrating the existence of vitamin C in gametes or early stage fertilized eggs. To our knowledge for the first time, we show that *gulo*, the key gene for vitamin C biosynthesis, can be expressed maternally. This implies the ability to produce endogenous vitamin C in the early egg and zygote blastomere stages, and not only in the tadpole and adult stages as previously reported [6, 8]. However, further studies are needed to address the *in vivo* functional relationship between vitamin C and epigenetic reprogramming.

Kidney & Blood Pressure Research

Kidney Blood Press Res 2016;41:794-801
DOI: 10.1159/000450561
Published online: November 11, 2016

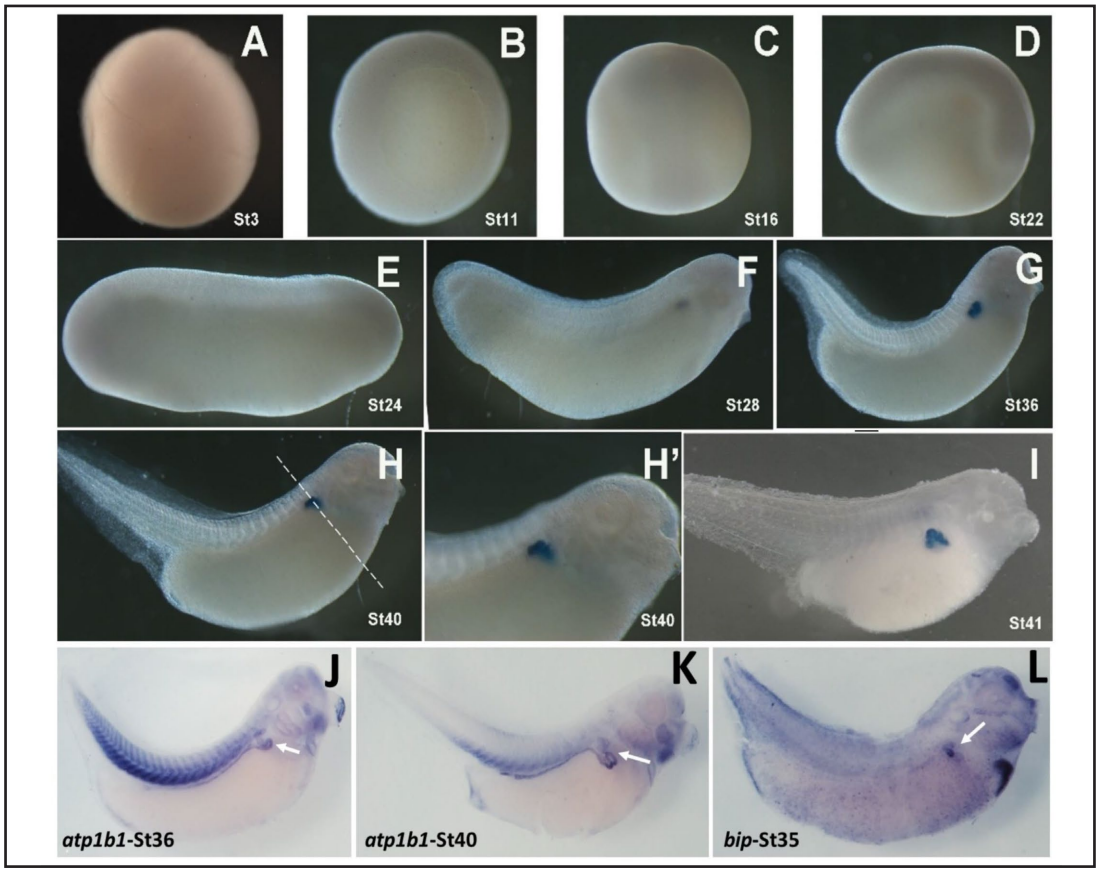

Fig. 4. Spatial expression patterns detected by *in situ* hybridization. No *gulo* expression until stage 28 (A-E) and the expression increases gradually to a high level at stage 40 (F-H), which persists into the tadpole stage (I). (H') A higher magnitude view of H. Embryo in A is viewed from animal pole, B from vegetal pole, and C from dorsal view with the head at the top of the image. Embryo in D-I is from lateral view with the head to the right of the image. The expression of two positive markers of the *Xenopus* pronephros, ATP1B1 at stages 36 and 40 (J and K) and BIP at stage 35 (L).

Furthermore, we have identified the specific activity of Gulo in pronephric tubules, implying this segment should be responsible for vitamin C production in the pronephros. However, only few studies have focused on the function of Gulo in the development of the *Xenopus* pronephros. Some studies reported the function of its synthetic product-vitamin C in kidney development or kidney-related disease. For example, in diabetic rats, vitamin C decreased lipid peroxidation and increased the activities of antioxidant enzymes in the kidneys of diabetic rats, as well as reduced urinary albumin excretion (UAE), decreased kidney weight, and glomerular basement membrane (GBM) thickness [19]. It was reported that vitamin C protects kidney function and renal arterial reactivity against renal ischemic injury in mice [20] and vitamin C can induce cardiomyocytes differentiation of murine pluripotent stem cells through the synergistic

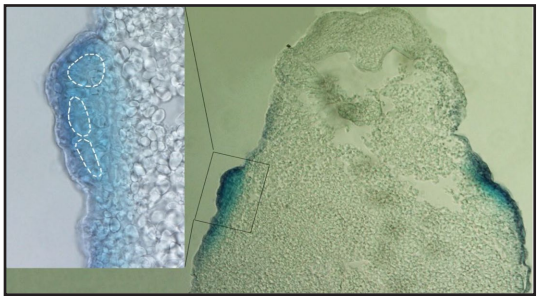

Fig. 5. The Section of H in Figure 4 showing the tubular expression domain of *gulo*. The dotted line depicts the tubule lumens.

effect of Wnt-signaling pathway [21]. Vitamin C has also been proven to function in vitamin E recycling and protect renal cells from apoptosis and senescence, increased regenerative ability of cells and fibrosis [22]. These studies suggested the potential role of vitamin C in the protection against the development of diabetic nephropathy and in the maintenance of normal development of the embryonic kidney. *X. pronephros* needs enough vitamin C during embryonic development and kidney is a main organ of *X. pronephros* to synthesize vitamin C [6, 8], however, in the early stage of *X. pronephros* embryonic development, especially in kidney function incomplete stage, how to provide sufficient vitamin C for embryonic development is a very important issue. Gulo is a key enzyme for the vitamin C reaction cascade [6] and is maternally expressed during the early stages of embryonic development, especially in the tubules of the pronephros (Fig.3 and 4), this helps to reduce the workload of the kidney and protect kidney from injury, and ensure enough vitamin C for *X. pronephros* embryonic development, especially in kidney function incomplete stage. But this speculation needs further experimental verification.

Because Gulo is a key enzyme for vitamin C synthesis, understanding its role in the development of the *Xenopus* pronephros will assist researchers in understanding the function of vitamin C in the development of the mammalian kidney, as well as kidney-related diseases. Further exploration of the etiology of vitamin C in the embryonic development of *X. laevis* will require the complete exclusion of Gulo, as vitamin C can perform its biological function at quite low dosages while sustaining normal physiological function. In addition, morpholino mediated knock-down of *gulo* will be ineffective, as it cannot completely prevent the synthesis of endogenous and maternal vitamin C.

Conclusion

Together, we conclude that Gulo can serve as a specific marker for the tubules of the pronephros and it may be involved in the synthesis of vitamin C during the early stage of embryonic development.

Abbreviations

ALO, D-arabinono-1, 4-lactone oxidase; cDNA, complementary DNA; FAD, Flavin adenine dinucleotide; Gulo, L-gulono-γ-lactone oxidase; ODC1, ornithine decarboxylase 1; ROS, reactive oxygen species; RT-PCR, reverse transcriptional polymerase chain reaction.

Disclosure Statement

The authors declare no competing financial interests.

Acknowledgements

This work was supported by funds of National Natural Science Foundation of China (31401191) to Xiaoyan Lv M.D.&Ph.D. and Science and Technology Department of Sichuan Province of China (2013JCPT002).

References

1 Magiorkinis E, Beloukas A, Diamantis A: Scurvy: past, present and future. Eur J Intern Med 2011;22:147-152.
2 Carpenter KJ: The discovery of vitamin C. Ann Nutr Metab 2012;61:259-264.
3 Englard S, Seifter S: The biochemical functions of ascorbic acid. Annu Rev Nutr 1986;6:365-406.
4 Peterkofsky B: Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 1991;54:1135S-1140S.
5 Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee JH, Chen S, Corpe C, Dutta A, Dutta SK, Levine M: Vitamin C as an antioxidant: evaluation of its role in disease prevention. J Am Coll Nutr 2003;22:18-35.
6 Gupta DS, Choudhury PK, Chatterjee IB: Synthesis of l-ascorbic acid from d-glucurono-1,4-lactone conjugates by different species of animals. Int J Biochem 1973;4:309-314.
7 Drouin G, Godin JR, Page B: The genetics of vitamin C loss in vertebrates. Curr Genomics 2011;12:371-378.
8 Chatterjee IB: Evolution and the biosynthesis of ascorbic acid. Science 1973;182:1271-1272.
9 Cui J, Yuan X, Wang L, Jones G, Zhang S: Recent loss of vitamin C biosynthesis ability in bats. PLoS One 2011;6:e27114.
10 Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K: Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. J Biol Chem 1994;269:13685-13688.
11 Hellsten U, Harland RM, Gilchrist MJ, Hendrix D, Jurka J, Kapitonov V, Ovcharenko I, Putnam NH, Shu S, Taher L, Blitz IL, Blumberg B, Dichmann DS, Dubchak I, Amaya E, Detter JC, Fletcher R, Gerhard DS, Goodstein D, Graves T, Grigoriev IV, Grimwood J, Kawashima T, Lindquist E, Lucas SM, Mead PE, Mitros T, Ogino H, Ohta Y, Poliakov AV, Pollet N, Robert J, Salamov A, Sater AK, Schmutz J, Terry A, Vize PD, Warren WC, Wells D, Wills A, Wilson RK, Zimmerman LB, Zorn AM, Grainger R, Grammer T, Khokha MK, Richardson PM, Rokhsar DS: The genome of the Western clawed frog *Xenopus tropicalis*. Science 2010;328:633-636.
12 Schultz J, Milpetz F, Bork P, Ponting CP: SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci USA 1998;95:5857-5864.
13 Li L, Wen L, Gong Y, Mei G, Liu J, Chen Y, Peng T: *Xenopus* as a model system for the study of GOLPH2/GP73 function: *Xenopus* GOLPH2 is required for pronephros development. PLoS One 2012;7:e38939.
14 Shi W, Xu G, Wang C, Sperber SM, Chen Y, Zhou Q, Deng Y, Zhao H: Heat shock 70-kDa protein 5 (Hspa5) is essential for pronephros formation by mediating retinoic acid signaling. J Biol Chem 2015;290:577-589.
15 Harland RM: *In situ* hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell Biol 1991;36:685-695.
16 Sasakura Y, Inaba K, Satoh N, Kondo M, Akasaka K: *Ciona intestinalis* and *Oxycomanthus japonicus*, representatives of marine invertebrates. Exp Anim 2009;58:459-469.
17 Blaschke K, Ebata KT, Karimi MM, Zepeda-Martinez JA, Goyal P, Mahapatra S, Tam A, Laird DJ, Hirst M, Rao A, Lorincz MC, Ramalho-Santos M: Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. Nature 2013;500:222-226.
18 Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnirke A, Regev A, Meissner A: A unique regulatory phase of DNA methylation in the early mammalian embryo. Nature 2012;484:339-344.
19 Kedziora-Kornatowska K, Szram S, Kornatowski T, Szadujkis-Szadurski L, Kedziora J, Bartosz G: Effect of vitamin E and vitamin C supplementation on antioxidative state and renal glomerular basement membrane thickness in diabetic kidney. Nephron Exp Nephrol 2003;95:e134-143.
20 Zhu YB, Zhang YP, Zhang J, Zhang YB: Evaluation of Vitamin C Supplementation on Kidney Function and Vascular Reactivity Following Renal Ischemic Injury in Mice. Kidney Blood Press Res 2016;41:460-470.
21 Ivanyuk D, Budash G, Zheng Y, Gaspar JA, Chaudhari U, Fatima A, Bahmanpour S, Grin VK, Popandopulo AG, Sachinidis A, Hescheler J, Saric T: Ascorbic Acid-Induced Cardiac Differentiation of Murine Pluripotent Stem Cells: Transcriptional Profiling and Effect of a Small Molecule Synergist of Wnt/beta-Catenin Signaling Pathway. Cell Physiol Biochem 2015;36:810-830.
22 Small DM, Coombes JS, Bennett N, Johnson DW, Gobe GC: Oxidative stress, anti-oxidant therapies and chronic kidney disease. Nephrology (Carlton) 2012;17:311-321.