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Role of the hypoxia response pathway in lens formation during embryonic development of *Xenopus laevis*

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ABSTRACT

The RING finger ubiquitin ligase seven in absentia homolog 2 (Siah2) was identified in the R7 photoreceptor cells of *Drosophila melanogaster*, and it regulates the stability of prolyl hydroxylase domains (PHDs), with a concomitant effect on HIF-1α availability in the hypoxia response pathway. We previously reported that the hypoxia response pathway contributes to eye development during the embryonic development of *Xenopus laevis*. In this paper, the role of Siah2-mediated hypoxia response pathway in eye development of *X. laevis* embryos was further characterized. *Xenopus Siah2* (xSiah2) mRNA was detected in lens tissue and xSiah2 overexpression caused a thickened lens placode, leading to loss of the optic lens. In embryos overexpressing xSiah2, lens marker gene transcription was reduced, suggesting that xSiah2 contributes to lens formation. xSiah2 overexpression decreased *Xenopus* PHD accumulation and increased *Xenopus* HIF-1α (xHIF-1α) accumulation. xHIF-1α degeneration with resveratrol restored the optical abnormality caused by xSiah2 overexpression, suggesting that the xSiah2-mediated hypoxia response pathway contributes to lens formation. Moreover, xSiah2 overexpression decreased endothelial–mesenchymal transition (EMT)-related Notch signaling-responsive genes transcription during the invasion of the lens placode. Our results suggest that the hypoxia response pathway plays an important role in the regulation of the EMT via the Notch signaling pathway during lens formation.

1. Introduction

Siah2 was identified in the R7 photoreceptor cells of *Drosophila melanogaster* [1]. Siah2 functions to target diverse protein substrates for degeneration via ubiquitination. In the hypoxia response pathway, Siah2 mediates efficient ubiquitination to regulate the stability of prolyl hydroxylase (PHD) [2]. The mammalian genome encodes three closely related PHD proteins, designed as PHD1, PHD2, and PHD3. PHD3 interacts with either PHD1 or PHD2, leading to the formation of PHD complexes. Tight regulation of the PHD complex activity and stability affects the availability of hypoxia inducible factor-1α (HIF-1α) [3]. PHD proteins require molecular oxygen to hydroxylate HIF-1α, which in turn becomes a signal for the degeneration of HIF-1α via interaction with the von Hippel–Lindau tumor suppressor protein (pVHL) ubiquitin ligase complex [4]. Available HIF-1α, after the interaction with HIF-1β [5], is a transcription factor responsible for the expression of target genes such as vascular endothelial growth factor (VEGF) gene [6].

The possible involvement of the hypoxia response pathway in the neurogenesis of vertebrates such as mice and frogs has recently been reported. HIF-1α knockout mice show defective angiogenesis as well as abnormal neurogenesis. Overexpression of *Xenopus* Siah2 (xSiah2) in *Xenopus laevis* causes the small eye phenotype [7]. This optical abnormality apparently results from a deficient lens.

Lens tissue is formed during the neurula and tailbud stages of *Xenopus* development. There are four phases of lens formation: (1) presumptive lens ectoderm (PLE) is formed in the surficial layer of the embryo during the neurula stages; (2) interaction between the PLE and anterior neural tube results in PLE thickening and development into a lens placode during the early tailbud stage; (3) the lens placode

Abbreviations: Siah2, seven in absentia homolog 2; PHDs, prolyl hydroxylase domains; HIF-1α, hypoxia-inducible factor-1α; pVHL, von Hippel–Lindau tumor suppressor protein; VEGF, vascular endothelial growth factor; PLE, presumptive lens ectoderm; EMT, endothelial mesenchymal transition; *E. coli*, *Escherichia coli*; PCR, polymerase chain reaction; pBS, pBluescriptII+; MBS, Modified Birth's Solution; NBT, nitro-blue tetrazolium chloride; SDS, sodium dodecylsulfate.

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invaginates and develops into a vesicle through the endothelial mesenchymal transition (EMT): and (4) differentiation into cellular layers occurs [8].

We previously isolated two *Xenopus* PHD (xPHD) proteins, xPHD45 and xPHD28, and characterized them during the embryonic development of *X. Laevis* [9]. In the embryonic development, the co-injection with *xPHD28* mRNA restores the small eye phenotype caused by xSiah2 overexpression, suggesting that xSiah2 contributes to eye development via xPHD. However, the function of the hypoxia response pathway in embryonic sensory organogenesis, including the lens, remains unclear. Given the importance of xSiah2 in the stability of xPHD and consequent *Xenopus* HIF-1α (xHIF-1α) levels, we asked whether the hypoxia response pathway plays a potential role in lens formation.

2. Materials and methods

2.1. Chemicals and antibodies

Resveratrol was purchased from Sigma (St Louis, MO); MMLV reverse transcriptase from Fermentas (Burlington, Canada); KOD plus DNA polymerase from TOYOBO (Tokyo, Japan); and T3, T7, and SP6 RNA polymerases and Go taq polymerase from Promega (Madison, WI). Antihuman β-actin antibody was purchased from Sigma and horseradish peroxidase-conjugated antirabbit IgG antibody was purchased from Bio-Rad (Hercules, CA). Antixenopus Siah2 antibody was prepared as follows. The first half of xSiah2 were ligated into pQE80L vector (QIAGEN, Hilden, Germany), which allows protein expression in *Escherichia coli* (*E. coli*) strains. xSiah2 peptide was then expressed in *E. coli* DH5α and purified using Ni-NTA agarose (QIAGEN). Antibodies were then raised against human PHD3, xSiah2, and human HIF-1α in rabbits using a previous described method [9,10]. Reaction of the antihuman PHD3, HIF-1α and β-actin antibodies with xPHD, xHIF-1α and *Xenopus* β-actin, respectively, was confirmed. All experiments were conducted in accordance with guidelines on the welfare of experimental animals and with the approval of the Ethics Committee on the use of animals of Kwansei Gakuin University.

2.2. Isolation of RNA and RT-PCR analysis

Total RNA extracted from 5 embryos was prepared with Isogen (Nippon gene, Toyama, Japan) according to the manufacturer's instructions. cDNA was synthesized using total RNA (1 μg) in a total volume of 10 μL with MMLV reverse transcriptase according to the manufacturer's instructions as follows: incubation at 25 °C for 15 min and at 42 °C for 60 min followed by heating at 70 °C for 10 min. Polymerase chain reaction (PCR) was performed at 94 °C for 2 min and then for a particular number of cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s in a reaction mixture containing 10 pmol of each primer, Go taq polymerase, and cDNA (100 ng). Primers, GenBank accession numbers, cycles, and sequences for PCR are shown in Table 1. The PCR products were separated by electrophoresis on a 1% agarose gel, visualized with ethidium bromide staining, and quantified by scanning densitometry using ImageJ software (version 1.36b; National Institutes of Health, Bethesda, MD). The relative mRNA transcript levels were normalized by Histone H4 (Genbank: M21286).

2.3. Isolation of xSiah2

cDNA of xSiah2 (GenBank: AF155509) was amplified by PCR. Thirty-five cycles of PCR (94 °C for 30 s, 57 °C for 30 s, and 68 °C for 90 s) were performed using the cDNA obtained from reverse transcription of total RNA from embryos as the template, KOD plus DNA polymerase and corresponding primer pairs. Primer pairs are shown in Table 2: primers 1 and 2 for xSiah2/pBluescriptII + (pBS), primers 3 and 4 for xSiah2/pCS2 +, and primers 3 and 5 for xSiah2/pQE80L. The cDNA of xSiah2 was digested with BamHI and SpeI, BamHI and EcoRI, or BamHI and HindIII and then ligated into pBS (Agilent Technologies, Santa Clara, CA), pCS2 + (RZPD, Berlin, Germany), or pQE80L (Qiagen, Valencia, CA), respectively.

2.4. Capped mRNA synthesis and micro-injection

GFP and *xSiah2* mRNAs were prepared from GFP/pCS2 + and xSiah2/pCS2 +, respectively. After the plasmids were linearized with the restriction enzyme NotI, capped mRNAs were made using a mCAP RNA synthesis kit (Promega) according to the manufacturer's instructions. Synthesized mRNAs (total 2 ng/cell) were injected into each dorsal blastomere at the two-cell stage.

2.5. Eggs and embryos of X. laevis

Unfertilized eggs of wild type and albino *X. laevis* (Watanabe Zoushoku, Hyogo, Japan) were obtained by injecting a female with 120 units of human chorionic gonadotropin (Kowa, Tokyo, Japan). The eggs were fertilized with the chestnuts suspended in 1.0 × Modified Birth's Solution (MBS) containing 0.5 mM HEPES (pH 7.5), 10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, and 0.2 mM $CaCl_2$. The chestnuts were surgically isolated from a male. The fertilized embryos were dejellied with 1% sodium thioglycollate and washed with 0.1 × MBS several times. The developmental stage of embryos was determined according to Nieuwkoop and Faber's normal table of *X. laevis* [11].

2.6. Whole mount in situ hybridization

Thirty albino embryos were fixed in fully dehydrated ethanol. Sense and antisense probes for xSiah2 were prepared from xSiah2/pBS and then linearized with SpeI or BamHI, respectively, and transcribed with T3 or T7 RNA polymerase, respectively, in the presence of digoxygenin UTP (Roche). Hybridized probes were visualized according to the Roche DIG protocol with a minor alteration that 0.45 mL of nitro-blue tetrazolium chloride (NBT) (75 mg/mL in dimethyl formamide) and 3.5 mL of 5-bromo-4-chloro-3′-indolylphosphatase *p*-toluidine salt (BCIP) (Roche) were added to 1 mL of alkaline phosphatase buffer containing 100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 50 mM $MgSO_4$, 0.1% Tween 20, and 25 mM levamisole.

2.7. Western blotting

Twenty embryos were homogenized in buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 150 mM KCl, and 100 mM PMSF, and then solubilized with sodium dodecylsulfate (SDS). The resulting solution was subjected to SDS–polyacrylamide gel electrophoresis. Proteins were blotted onto a nitrocellulose membrane and reacted with antibodies against human β-actin, human HIF-1α, human PHD, and xSiah2.

2.8. Histological analysis

Twenty embryos were fixed in fully dehydrated ethanol and embedded in paraffin. Sagittal sections were cut 10 μm thick and stained with hematoxylin and eosin.

2.9. Statistical analysis

All data are reported as mean ± SD. Statistical analysis of the data was performed by one-way ANOVA. Significance was determined by ANOVA followed by Fisher's protected least significant difference.