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Epigenetics of Vascular Formation during Zebrafish Hindbrain Development

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Abstract: The brain circulatory system is essential for the survival and development of the central nervous system in all vertebrates. Brain vessels form in a reproducible and evolutionarily conserved manner. In zebrafish, hindbrain vessels develop independently of the shear stress of blood flow but are regulated genetically. Epigenetics is another regulatory system regulating gene activation, but the epigenetic contribution to angiogenesis remains unclear. Here, we examine expression patterns of genes involved in SET1/mixed lineage leukemia (MLL) histone methyltransferase complexes. All mRNAs we tested as orthologs of molecules in the MLL complex were expressed throughout the central nervous system, including the hindbrain. We also show immunofluorescent staining of retinoblastoma-binding protein 5 (RBBP5) protein in the dorsal hindbrain, suggesting the possibility of differences in epigenetic state along the dorsal-ventral axis. Finally, we demonstrate histone methylation with non-methylated, dimethylated, and trimethylated types.

Keywords: zebrafish, hindbrain, angiogenesis, histone methylation

Introduction

Brain vascular development is a topic of intensive study. A thorough knowledge of the developmental biology of brain vessel formation is important for understanding both development of the central nervous system and cerebrovascular pathologies. Examination of the vascular anatomy of the developing zebrafish embryo reveals an initial vascular plan that is well conserved among other developing vertebrates, including humans[1)]. This conservation extends to the brain vasculature. A previous report found that in zebrafish embryos, angiogenic sprouting and invasion into the hindbrain parenchyma as hindbrain central arteries (CtA) occurs between 30 and 60 hours post fertilization (hpf)[2)]. During this stage, the dorsal hindbrain has no blood vessel. However, regional differences of the dorsal-ventral axis during vascularization have not yet been examined in detail in zebrafish or any other vertebrate model organism.

Epigenetics refers to a gene regulatory system based on DNA modifications that do not depend on the nucleotide sequence. Epigenetic regulation affects gene activity via chemical modification of histones, such as acetylation, methylation, and phosphorylation, as well as DNA methylation[3-7)]. It has been reported that epigenetic abnormalities are involved in various diseases, and that epigenetic regulation contributes significantly to ontogeny and cell differentiation[8-12)].

The retinoblastoma-binding protein 5 (RBBP5) protein is a nuclear protein 66 kD in size that binds to underphosphorylated retinoblastoma proteins[13)]. RBBP5 is also known as a component of the mammalian SET1A/SET1B histone H3-Lys methyltransferase complex[14)]. The SET1A/SET1B complex, also known as the mixed lineage leukemia (MLL) complex, is a methyltransferase that catalyzes the methylation of lysine 4 (H3K4) of histone 3. MLL proteins do not show catalytic activity by themselves, but when its catalytically active domain (SET, Su(var.)3-9, enhancer of zeste and trithorax), present at the C-terminal end, binds to WD repeat-containing protein 5 (WDR5), RBBP5, ASH2 like (ASH2L), and DPY30, the complex then exhibits

methylation activity[15, 16].

Mutations in the MLL gene are present in 5% to 10% of all cases of acute leukemia, and in an even larger proportion of infant cases. Similarly, in zebrafish, MLL genes have been reported to be important in hematopoiesis[17-19]. Zebrafish MLL1 is also involved in neurogenesis and brain tumor pathways, including neural progenitor cell proliferation, neural cell differentiation, and glial cell differentiation[20].

In this study, we examined the expression pattern of serial genes and proteins related to histone H3 methylation, and observed the methylated condition of histones during hindbrain angiogenesis.

Materials and Methods

Zebrafish

Zebrafish (*Danio rerio*) embryos were obtained from the natural spawning of laboratory lines. Embryos were raised and fish were maintained as described[21, 22]. Zebrafish used were from the EK wildtype line and the *Tg(fli1a:EGFP)*^{y1} transgenic line [23]. For imaging, embryos were treated with 1-phenyl-2-thiourea (PTU) to inhibit pigment formation[22].

Whole-mount in situ hybridization

DIG-labeled anti-sense riboprobes were synthesized using the DIG labeling kit (Roche, Indianapolis, IN). Antisense mRNA probes used were: *kmt2a (mll)*[17, 24], *kmt2d (mll2)*[24], *kmt2ba (mll4a)*[24], *kmt2bb (mll4b)*[24], *kmt2ca (mll3a)*[24], *kmt2cb (mll3b)*[24], *kmt2e (mll5)*[24], *ash2l*[19], *dpy30*[25], *wdr5*[19], *rbbp5*[26], and *kdrl*[27]. Embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 ℃ . Signals were detected with alkaline phosphatase-labeled anti-DIG antibody and BM Purple substrate (Roche).

Immunostaining

Embryos were fixed with 100% methanol for at least two days at -20℃ and were permeabilized with 0.1% Triton X-100 in PBS. Blocking was performed with Blocking Reagent (Roche). Primary antibodies (dilution 1:200) used were: mouse anti-human Histone H3 (FUJIFILM Wako Pure Chemical Corporation, #304-34781), mouse anti-human dimethyl Histone H3K4 (FUJIFILM Wako Pure Chemical Corporation, #304-34801), rabbit anti-human trimethyl Histone H3K4 (Cell Signaling Technology, #9751), and rabbit anti-RBBP5 (Cell Signaling Technology, #13171). Mouse anti-chicken myosin heavy chain (Developmental Studies Hybridoma Bank, MF20) was used as a positive control at a dilution of 1:200. Secondary antibodies (dilution 1:2000) used were: 555 anti-mouse IgG (Invitrogen, #A28180) and 555 anti-rabbit IgG (Invitrogen, #A27039).

Microscopy

RNA in situ hybridization images were captured with a DFC450C camera mounted on a Leica MZ10F stereomicroscope (LEICA). Confocal microscopy of immunostained embryos was performed using a LSM700 laser scanning confocal microscope (Carl Zeiss).

Results

MLL genes are expressed in the hindbrain

To explore the molecular profile of hindbrain development, we examined the distribution of *MLL* gene mRNAs by whole-mount RNA in situ hybridization. At around 36 hpf, CtA elongates and increases within each rhombomere[2] and histone methylation could affect this vascularization. During this CtA formation, seven *mll* genes, including *kmt2a/mll*, *kmt2d/mll2*, *kmt2ba/mll4a*, *kmt2bb/mll4b*, *kmt2ca/mll3a*, *kmt2cb/mll3b*, and *kmt2e/mll5* are expressed in the forebrain, midbrain, midbrain-hindbrain boundary, and hindbrain (Fig. 1A-G). In the hindbrain, these genes showed stronger expression in the dorsal area (indicated by arrowheads in Fig. 1A). In addition, other genes involved in the MLL complex, including *ash2l*, *dpy30*, *wdr5*, and *rbbp5*, were expressed in the central nervous system (Fig. 1H-K). The vascular endothelial cell marker kdrl was expressed in blood vessels and elongating CtA was detected in the ventral half at 36 hpf (Fig. 1L).

RBBP5 protein is localized in the dorsal hindbrain

Since the dorsal-most area of the hindbrain is avascular by 60 hpf[2], we focused on *rbbp5*, which showed obvious differences between dorsal and ventral areas. In order to see the expression patterns of the RBBP5 protein, we carried out whole-mount immunofluorescent staining. First, we investigated the cross-reactivity of anti-RBBP5 antibody on zebrafish embryos. The antibody from Cell Signaling Technol-

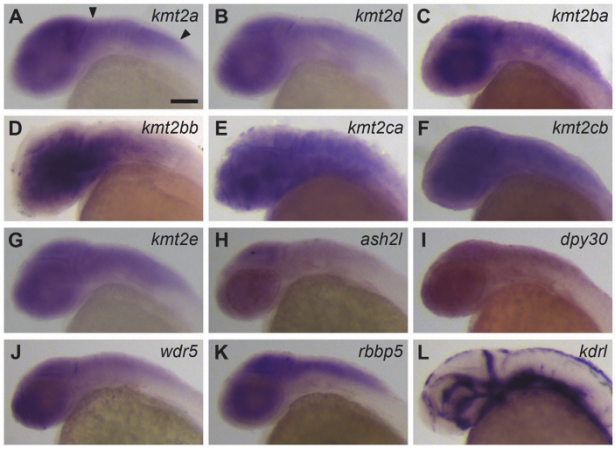

Fig. 1. Expression patterns of MLL genes. Whole-mount in situ hybridization of 36 hpf wild-type zebrafish embryos, probed for *kmt2a* (A), *kmt2d* (B), *kmt2ba* (C), *kmt2bb* (D), *kmt2ca* (E), *kmt2cb* (F), *kmt2e* (G), *ash2l* (H), *dpy30* (I), *wdr5* (J), *rbbp5* (K), or *kdrl* (L). Lateral views of head are shown, with rostral to the left. Arrowheads in A indicate anterior and posterior ends of the hindbrain. Scale bar, 100 μm.

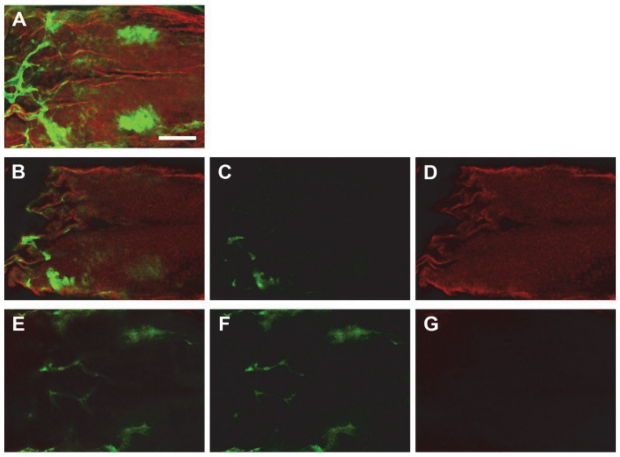

Fig. 2. Expression pattern of RBBP5 protein. Whole-mount immunofluorescent staining of hindbrain parenchymal cells with anti-RBBP5 (red), compared with blood vessels of EGFP from transgenic origin (green). 2D reconstruction of confocal image (A), single horizontal section through the dorsal hindbrain (B-D), and single horizontal section through the ventral hindbrain (E-G) of 54 hpf *Tg(fli1a:EGFP)*^{y1} embryos. Merge (B, E), EGFP (C,F), and RBBP5 (D, G). Dorsal views of head are shown, with rostral to the left. Scale bar, 50 μm.

ogy (#13171) worked well, and showed that RBBP5 was expressed broadly in the central nervous system (Fig 2A).

Next, we compared the expression levels in single planes of the dorsal and ventral areas. RBBP5 was more strongly expressed in the 13th slice from the dorsal surface (Fig. 2B-D) than in the 29th slice (Fig. 2E-G). We confirmed that the expression pattern of the RBBP5 protein was same as that of its mRNA. Altogether, we found that one component of the MLL complex was more strongly expressed in the dorsal hindbrain, which is avascular, suggesting that histone methylation by the MLL complex may be involved in maintaining avascular area and/or activating angiogenic factors in hypoxic regions.

Three types of anti-histone antibodies worked for zebrafish embryo

In order to visualize the activity of histone methylation during CtA angiogenesis in the hindbrain, we conducted whole-mount immunofluorescent staining using four types of anti-histone antibodies: (1) #304-34781 for non-methyl histone, (2) #301-34791 and #9723 for monomethyl histone, (3) #304-34801 and #9725 for dimethyl histone, and (4) #ab1012 and #9751 for trimethyl histone (#30~ were from FUJIFILM Wako Pure Chemical Corporation, #97~ were from Cell Signaling Technology, and #ab~ was from Abcam). When embryos were fixed with 4% paraformaldehyde in PBS, no staining signal was observed with any anti-histone antibodies. However, when 100% methanol was tested as an alternative fixative, #304-34781, #304-34801, #9725, and #9751 were revealed as cross-reacting. Although we were not able to obtain a working antibody for the monomethyl histone, we were able to obtain three types of anti-histone antibodies.

At 54 hpf, non-methyl type histones were ubiquitously expressed in a dot pattern, representing its subcellular localization within the nucleus (Fig. 3A). Dimethyl histone was also detected ubiquitously and concentrated in nuclei (Fig. 3B). Two different antibodies, #304-34801 and #9725, showed identical expression patterns, and we selected the stronger one for further use. Trimethyl histone was expressed ubiquitously and broadly (Fig. 3C). Negative control embryos treated only with the secondary antibody showed very low background expression (Fig. 3D). Localization in the nucleus was observed in some areas, including the epidermis.

Histone methylation is different in epidermis and the central nervous system

In order to visualize more detailed expression patterns in the hindbrain, we examined single horizontal sections using confocal microscopy. The expression of non-methyl histones was suppressed at low

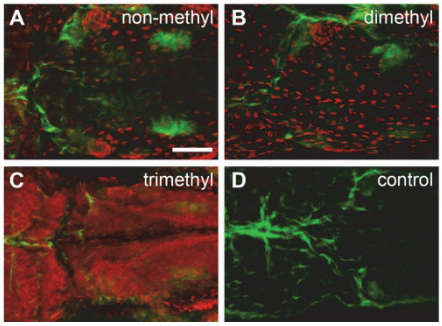

Fig. 3. Cross-reactivity of antibodies for histone methylation. Whole-mount immunofluorescent staining with anti-histone H3 antibody (red, A), anti-dimethyl histone H3 (Lys4) (red, B), anti-trimethyl histone H3 (Lys4) (red, C), and negative control (D), compared with blood vessels of EGFP from transgenic origin (green). 2D reconstructions of confocal images. 54 hpf *Tg(fli1a:EGFP)*^{y1} embryos. Dorsal views of head are shown, with rostral to the left. Scale bar, 50 μm.

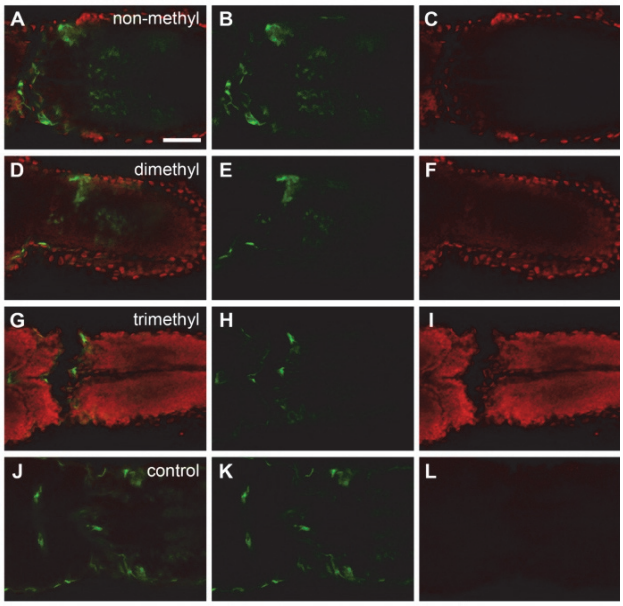

Fig. 4. Expression pattern of histone protein. Single horizontal section of confocal images of whole-mount immunofluorescent staining with anti-histone H3 antibody (red, A-C), anti-dimethyl histone H3 (Lys4) (red, D-F), anti-trimethyl histone H3 (Lys4) (red, G-I), and negative control (J-L), compared with blood vessels of EGFP from transgenic origin (green). 54 hpf *Tg(fli1a:EGFP)*^{y1} embryos. Merge (A, D, G, J), EGFP (B, E, H, K), and each histone (C, F, I). Dorsal views of head are shown, with rostral to the left. Scale bar, 50 μm.

levels in the central nervous system (Fig. 4A-C). On the other hand, boundary cells at the anterior end of the hindbrain and surface epidermal cells showed a dotted pattern, representing nuclear localized expression (Fig. 4C). Dimethyl histones also showed a similar expression pattern (Fig. 4D-F). Although the subcellular localization was different, the expression levels in the CNS were not particularly low relative to those in the epidermis (Fig. 4F). In the case of trimethyl histones, overall expression was ubiquitously strong and partially limited to nuclei (Fig. 4G-I). Negative control embryos treated only with secondary antibodies exhibited very low background expression (Fig 4J-L).

Discussion

We found that mRNA expression of genes related to histone methylation is not uniform but is strong in the cranial nervous system and especially so in the dorsal hindbrain. Since expression outside the hindbrain is not restricted to avascular tissue, it is possible that the different expression levels in dorsal and ventral portions of the hindbrain may be involved in angiogenic induction rather than the maintenance of the avascular region. Since there are multiple reports that epigenetic regulation is associated with cardiovascular disease[8, 9], this finding is consistent with the angiogenic guidance hypothesis. There is also a report that methylation status is involved in osteoarthritis in cartilage tissue, which is avascular and hypoxic[28], and thus further analysis is required.

The RBBP5 protein was expressed in the hindbrain itself but was restricted to the dorsal region. During vascular assembly in the zebrafish hindbrain, CtA sprouts from the ventral side and elongation is limited to the ventral half[2]. There may be some guidance cues for CtA formation in the dorsal and ventral regions, respectively, but the association of RBBP5 with angiogenesis has not yet been reported.

Contrary to expectations[13], the RBBP5 protein did not show nuclear localization. Because the expression region is consistent with that of the mRNA, we are confident that the antibody staining did not reveal any technical problem. RBBP5 showed high expression levels generally, and it may be the case that it is widely distributed in the cytoplasm, making nuclear localization difficult to observe. To resolve this question, it is necessary to observe at higher magnification or develop a clearer staining method.

Although there are relatively few antibodies that can be used in zebrafish, we used the methanol fixed staining method to identify five antibodies that were cross-reactive. The general staining method with 4% paraformaldehyde fixation did not yield any staining signal even when permeability was increased. Organic solvents, such as methanol, ethanol, and ac-

etone are known to reduce the solubility of proteins and flatten cells, resulting in reduced penetration in the nucleus and mitochondria. Due to this effect, the localization of the nucleus may not be visible. Fixation with trichloroacetic acid might improve the ability to visualize nuclear expression. Although technically difficult, one possible method to improve immunospecificity would be to produce an antibody using zebrafish protein as an antigen.

We did not observe any significant methylation signal in nuclei in the CNS. If these results demonstrate true characteristics of the nuclear methylation status during CtA formation, it may mean that histone methylation regulation is not involved in hindbrain angiogenesis. Knockdown and/or mutant analysis is needed to further understand what is happening here.

Finally, since monomethyl histones were not detected in this study, it is still possible that monomethyl histones are dominant in the zebrafish brain at this stage of development. Moreover, there may also be an effect of the developmental stage itself. Multiple validation is required, which could be achieved by incorporating additional antibodies, other developmental stages, and other staining methods.

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