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Notch signaling regulates the expression of glycolysis-related genes in a context-dependent manner during embryonic development

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ABSTRACT

Glycolysis, the classic pathway for producing energy, has been known to be involved in neural development. Notch signaling also contributes to neural development and regulation of glycolysis in various tissues. However, the role of Notch signaling in glycolysis-related gene regulation during neural development is poorly understood. Here, we analyzed mRNA expression patterns and levels of glucose transporters (GLUT) as well as rate-limiting enzymes in glycolysis using zebrafish *mib1*^{ta52b} mutants, in which Notch signaling was deficient at the early embryonic and larval stages. Our results indicated that in neural tissues, Notch signaling positively regulates *glut1a* and *glut3a* expression and negatively regulates *hk2* expression at the larval stage but may not regulate them during early embryonic stages. Therefore, these results suggest that Notch signaling regulates glycolysis-related gene expression in a context-dependent manner in neural tissues at different developmental stages.

1. Introduction

Glycolysis is the first step of glucose metabolism. In the presence of oxygen, cells produce ATP via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) in the mitochondria. On the other hand, rapidly proliferating cells (including developing cells, stem cells, and cancer cells) accelerate glycolysis and lactate production even under aerobic conditions, which is termed aerobic glycolysis [1,2]. In neural cells, glucose is a key substrate for maintaining energy homeostasis. Furthermore, many studies have reported the relationship between glycolysis and neural cells. Neuronal release of glutamate during synaptic activation stimulates astrocytes, which then upregulates aerobic glycolysis and increases lactate production. Lactate produced in astrocytes is released to neurons through the astrocyte-neuron lactate shuttle (ANLS). Neurons can utilize lactate rather than glucose as a source of energy (i.e., lactate is processed in the TCA cycle, and OXPHOS is accompanied by its conversion to pyruvate) [3,4]. Moreover, the glycolytic rate was increased during the proliferation of neural progenitor cells (NPCs); however, a decline in aerobic glycolysis was essential for neuronal differentiation [5]. Furthermore, glycolysis is modulated by various factors, such as the phosphatidylinositol 3-kinase (PI3K) pathway, hypoxia inducible factor (HIF), and oncogene *MYC* [1], and Notch signaling [2,13,14].

Notch signaling, which is an evolutionally conserved pathway, contributes to embryonic development, cell-fate determination, and maintenance of tissue homeostasis [6,7]. The Notch signaling pathway consists of Notch ligands (Delta and Serrate in *Drosophila*; Delta-like and Jagged in mammals) and Notch receptors. Upon binding of the ligand to receptor, the intracellular domain of Notch (NICD) is cleaved by γ-secretase. Next, the released NICD enters the nucleus, where it cooperates with the CSL (CBF1/RBPjk/RBPja/Lag1) family to regulate the transcription of Notch target genes [6,7]. Ubiquitination of Notch ligands by Mind bomb (Mib), an E3 ubiquitin ligase, is also necessary to activate Notch signaling [8–10]. It is well known that Notch signaling prevents the premature generation of primary neurons to maintain the patterning mechanisms in the developing neural cells [8,11,12].

Recent studies have suggested that Notch signaling regulates glycolysis. For example, both activated and inactivated Notch signaling enhance glycolysis in breast tumor cells [13]. Furthermore, Notch signaling promotes glycolysis through the activation of the PI3K/AKT serine/threonine kinase pathway in pre-T cells [14]. Additionally, in *Drosophila* wing discs, the mRNA expression of glucose transporter (GLUT), hexokinase (HK), and lactate dehydrogenase (LDH) is directly upregulated by Notch signaling [2]. However, it is unclear whether Notch signaling can modulate glycolysis in neural cells. Here, we show that in zebrafish (*Danio*

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rerio) embryos, Notch signaling affects the expression of glycolysis-related genes during neural development in a stage-specific manner.

2. Materials and methods

2.1. Fish maintenance and mutants

Zebrafish were raised and maintained under standard conditions with approval by the Institutional Animal Care and Use Committee at Chiba University. Zebrafish embryos were obtained from natural spawning of wild-type adults or *mib*^{ta52b} heterozygotes [8].

2.2. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from embryos 24 h post fertilization (24 hpf) or 4 days post fertilization (4 dpf), and then purified using Sepasol-RNAI Super G (Nacalai Tesque) according to the manufacturer's instructions. The cDNA synthesis was performed with the ReverTra Ace (TOYOBO). The mRNA expression levels of each gene were quantified by real-time PCR with THUNDERBIRD SYBR qPCR Mix (TOYOBO) on a LightCycler 96 System (Roche).

Results were expressed relative to the levels of reference gene *rpl-13α* [15]. Statistical analysis was performed by Student's *t*-test in GraphPad Prism 6 software (MDF).

Primer sequences are as follows

- *glut1a* (forward), 5′-AGCCAGTTTATGCCACCATT-3′
- *glut1a* (reverse), 5′-GGCCTACTCGCTCCACTATG-3′
- *glut3a* (forward), 5′-GGCGTCGTAGCCAACAAA-3′
- *glut3a* (reverse), 5′-TCAGACCACCACCAATCAGA-3′
- *hk1* (forward), 5′-ACAAGAACACCCCACGTCTC-3′
- *hk1* (reverse), 5′-GACGCACTGTTTTGTGCAAC-3′
- *hk2* (forward), 5′-CCGCAATGTGAAGTTGGAC-3′
- *hk2* (reverse), 5′-CCTTCATCTCCATCCACCAG-3′
- *pkma* (forward), 5′-TCAACTTCGCCATGGATGT-3′
- *pkma* (reverse), 5′-CAGCCAGTCAGAACGATCAC-3′
- *ldha* (forward), 5′-TCACGTGAGCAAGGAGCA-3′
- *ldha* (reverse), 5′-CGAGTTCATCGGTCAGATCC-3′
- *elavl3* (forward), 5′-CGACGCCGACAAGGCTATCAACA-3′
- *elavl3* (reverse), 5′-TTGGGCAGGCCGCTCACATACA-3′
- *rpl-13α* (forward), 5′-TCTGGAGGACTGTAAGAGGTATGC-3′
- *rpl-13α* (reverse), 5′-AGACGCACAATCTTGAGAGCAG-3′

2.3. Whole-mount in situ hybridization

Zebrafish embryos at 24 hpf or 4 dpf (raised in E3 medium with 0.003% 1-phenyl 2-thiourea (PTU) from 24 hpf to 4 dpf in order to remove pigment) were fixed in 4% paraformaldehyde overnight at 4 °C. Whole-mount *in situ* hybridization was performed as described previously [16]. The mRNA distribution was detected by 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche) using anti-DIG AP (Roche). To construct template plasmids for *in situ* probes of *glut1a*, *glut3a*, *hk1*, *hk2*, *pkma*, and *ldha*, the fragments were amplified from zebrafish cDNA by PCR and subcloned into the pBluescript vector. Probes were synthesized by T3 RNA polymerase (Promega) and DIG RNA Labeling Mix (Roche). The *elavl3* probe has been published previously [17].

2.4. mRNA and morpholino antisense oligonucleotide injection

Antisense morpholino oligo against *rbpja* (2 ng/embryo, Gene Tools OR, USA) [18] was injected into one- or two-cell stage embryos and then the embryos were raised at 28.5 °C.

3. Results

3.1. Notch signaling defects lead to increased expression of glycolysis-related genes at an early embryonic stage

To clarify whether the expression of glycolysis-related genes was regulated by Notch signaling *in vivo*, we used zebrafish *mib1*^{ta52b} mutants in which Notch signaling was deficient due to reduced Notch ligand activity [8].

First, we performed whole-mount *in situ* hybridization to evaluate the mRNA expression patterns of GLUT, glycolytic rate-limiting enzymes (hexokinase [HK], pyruvate kinase [PK]), and lactate dehydrogenase [LDH] in *mib1*^{ta52b} mutants at 24 hpf. Among these gene families, we investigated the expression of *glut1a*, *glut3a*, *hk1*, *hk2*, *pkma*, and *ldha*, which has been reported to be expressed in the nervous system of zebrafish [5,19–26].

In *mib1*^{ta52b} mutants at 24 hpf, the expression of *glut1a* was observed weakly in the brain and strongly in the pronephric duct, but there were no obvious changes in the expression levels (Fig. 1A; sibling controls, n = 18; *mib1*^{ta52b}, n = 12). On the other hand, the expression of *glut3a* was increased in the brain and spinal cord in *mib1*^{ta52b} mutants (Fig. 1B; sibling controls, n = 17; *mib1*^{ta52b} with high level expression, 100%, n = 13). The expression pattern of *hk1* was similar to that of *glut3a* except for the expression in myotomes boundaries, and its level was increased both in the brain and spinal cord in *mib1*^{ta52b} mutants (Fig. 1C; sibling controls, n = 20; *mib1*^{ta52b} with high level expression, 85%, n = 13). Furthermore, *hk2* was expressed weakly in the brain but not in the spinal cord, and its expression was not obviously different between sibling controls and *mib1*^{ta52b} mutants (Fig. 1D; sibling controls, n = 17; *mib1*^{ta52b}, n = 10). The expression of *pkma* and *ldha* was also higher in the brain and spinal cord in *mib1*^{ta52b} mutants as compared to sibling controls (Fig. 1E and F, *pkma*: sibling controls, n = 14; *mib1*^{ta52b} with high level expression, 100%, n = 10; and *ldha*: sibling controls, n = 13; *mib1*^{ta52b} with high level expression, 100%, n = 10).

We next evaluated the mRNA expression levels of these genes in sibling controls and *mib1*^{ta52b} mutants at 24 hpf using quantitative real-time RT-PCR (qRT-PCR). Although gene expression levels of *glut1a* were unchanged in *mib1*^{ta52b} mutants, those of *glut3a*, *hk1*, *hk2*, *pkma,* and *ldha* were significantly increased by 3.4-fold, 2.0-fold, 1.3-fold, 2.3-fold, and 1.5-fold, respectively (Fig. 2). Notch signaling dysfunction leads to depletion of neural progenitors and excessive formation of primary neurons [8,12,27]. Consistent with these studies, *elavl3,* an early neuronal differentiation marker, in *mib1*^{ta52b} mutants was increased 2.8-fold compared to sibling controls (Fig. 2). Taken together, the expression of *glut1a* and *hk2* might be scarce in primary neurons and not significantly altered. However, the expression of *glut3a*, *hk1*, *pkma,* and *ldha* was increased in parallel with an increase in *elavl3*-expressing neurons in *mib1*^{ta52b} mutants at an early embryonic stage.

3.2. Expression of glycolysis-related genes was differently regulated by Notch signaling at the larval stage

The results obtained from *mib1*^{ta52b} mutants at 24 hpf were inconsistent with previous studies showing that Notch signaling positively regulates glycolysis [2,13,14]. Therefore, we speculated that glycolysis-related gene expression may be regulated in a context-dependent manner during embryonic development; therefore, we next performed a similar analysis using *mib1*^{ta52b} mutants at 4 dpf at the larval stage when glucose metabolism starts to change due to a reduction in yolk-derived carbohydrates [28,29].

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