AICAR Induces Astroglial Differentiation of Neural Stem Cells via Activating the JAK/STAT3 Pathway Independently of AMP-activated Protein Kinase*§

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Neural stem cell differentiation and the determination of lineage decision between neuronal and glial fates have important implications in the study of developmental, pathological, and regenerative processes. Although small molecule chemicals with the ability to control neural stem cell fate are considered extremely useful tools in this field, few were reported. AICAR is an adenosine analog and extensively used to activate AMP-activated protein kinase (AMPK), a metabolic “fuel gauge” of the biological system. In the present study, we found an unrecognized astrogligenic activity of AICAR on not only immortalized neural stem cell line C17.2 (C17.2-NSC), but also primary neural stem cells (NSCs) derived from post-natal (P0) rat hippocampus (P0-NSC) and embryonic day 14 (E14) rat embryonic cortex (E14-NSC). However, another AMPK activator, Metformin, did not alter either the C17.2-NSC or E14-NSC undifferentiated state although both Metformin and AICAR can activate the AMPK pathway in NSC. Furthermore, overexpression of dominant-negative mutants of AMPK in C17.2-NSC was unable to block the gliogenic effects of AICAR. We also found AICAR could activate the Janus kinase (JAK) STAT3 pathway in both C17.2-NSC and E14-NSC but Metformin fails. JAK inhibitor I abolished the gliogenic effects of AICAR. Taken together, these results suggest that the astrogligenic differentiation effect of AICAR on neural stem cells was acting independently of AMPK and that the JAK-STAT3 pathway is essential for the gliogenic effect of AICAR.

Neural stem cell differentiation is controlled by intrinsic regulators and the extracellular environment. In many cases, these factors act in concert to confer potent change in cell lineages. The determination of lineage decision between neuronal and glial fates has important implications in the study of developmental, pathological, and regenerative processes (1–5). In recent years, neuronal trophic factors have been widely studied to examine their effects on neural stem cell differentiation and related mechanism. The neurotrophin family is one of the most important inducible signals for the differentiation. Among them, nerve growth factor is well known to induce neurogenesis, and neurotrophin 3 is involved in oligodendrocyte development. Another important differentiation signal family is the ciliary neurotrophic factor (CNTF)2-leukemia inhibitory factor (LIF) cytokine family, which plays a pivotal role in regulating gliogenesis in the developing mammalian central nervous system. Thus far, few small molecules were reported to have the ability to control stem cell fate. Retinoic acid is a well known compound effective in inducing differentiation of various progenitor cells including embryonic stem cells, neural stem cells (NSCs), and mesenchymal stem cells (6–8). It is not known whether and to what extent a metabolic regulator could trigger stem cell differentiation and confer new lineages.

AICAR was first reported for regulation of cellular metabolism (9), and is a well known, cell-permeable activator (10) of AMP-activated protein kinase (AMPK), a metabolic master regulator (11, 12). AMPK is activated in response to reduced energy availability (high cellular AMP:ATP ratios) and hence serves to produce a positive energy balance by switching off ATP-utilizing anabolic pathways as well as switching on ATP-generating catabolic pathways (13, 14). Activities of AICAR have been examined in various cell types (15) or even in vivo (16–18). Its effects are diverse including lipid and glucose metabolism, regulation proinflammatory response, cytokine production, cell proliferation and apoptosis. So far, most evidence pointed to its activity in activating AMPK.

We performed a series of experiments to test the role of AICAR in influencing neural stem cell differentiation. The evidence has uncovered a new activity of AICAR, namely its ability to induce astroglial differentiation via activating the JAK/STAT3 pathway independently of AMPK activation.

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MATERIALS AND METHODS

Chemicals—AICAR, Metformin, and CNTF were purchased from Sigma-Aldrich, and JAK inhibitor 1 was from Calbiochem.

Neural Stem Cell Culture—Three multiple self-renewing neural stem cells have been used in this study.

Murine immortalized neural stem cell line C17.2 (C17.2-NSC) was originally described by Snyder et al. (19). The C17.2-NSCs were maintained in Dulbecco’s modified Eagle’s medium (DMEM, from Invitrogen) supplemented with 10% fetal calf serum (HyClone), 5% horse serum (Invitrogen) and 2 mM glutamine (Invitrogen) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C and split when the cells reached ~90% confluency.

Adult primary neural stem cells were isolated from the hippocampus of post-natal (P0–P1) SD rats according to methods described previously (20), and all experiments were performed with cells that had undergone two passages. Embryonic primary neural stem cells were isolated from the cerebral cortices of E14 (embryonic day 14) SD rats. Cells were mechanically dissociated by trituration.

The neurospheres derived from primary neural stem cells (P0-NSC or E14-NSC) were maintained on uncoated 25 ml-flasks (Corning) in DMEM/F12 medium (Invitrogen) that contained N2 and B27 supplements (Invitrogen) plus penicillin (Promega, 10 ng/ml), and EGF (Promega, 10 ng/ml). The floating neurospheres were passaged by mechanical dissociation every 3–4 days.

For monolayer growth of E14-NSC, the cells were mechanically dissociated from the E14-neurospheres and plated at a density of 20 × 10^4 cells/ml on 100 μg/ml poly-L-lysine (Sigma) coated glass coverslips or 6-well plate (Corning). The monolayers maintained in DMEM/F12 medium (Invitrogen) that contained N2 and B27 supplement (Invitrogen) plus penicillin (100 μg/ml, Sigma), streptomycin (Sigma, 100 μg/ml), bFGF (Promega, 10 ng/ml), and EGF (Promega, 10 ng/ml). The floating neurospheres were passaged by mechanical dissociation every 3–4 days.

For differentiation of C17.2-NSC, 1 mM AICAR, 2 mM Metformin, or medium as control was directly added to the culture. For differentiation of neurospheres derived from primary NSC (P0 or E14), the growth factor bFGF and EGF were withdrawn (N2 and B27-supplement DMEM/F12 medium as the differentiation medium) and 1 mM AICAR, 1 mM Metformin, 100 ng/ml CNTF, or medium as control were added to the culture. For differentiation of monolayer derived from E14-NSC, the growth factors were withdrawn and 1 mM AICAR, 1 mM Metformin, 100 ng/ml CNTF, or medium as control were added to the culture after NSCs were seeded and adhered in the growth factor-contained medium for 48 h (E14 + 2 DIV, day in vitro).

Plasmid Construction and Transfection—cDNA encoding human AMPK α1, containing a mutation that alters aspartic acid 159 to an alanine (D159A) (21), was subcloned into the pCAGGS-IRES-EGFP vector (a kind gift from Dr. Ding YQ of Shanghai Institute for Biological Sciences) to generate pCAGGS-DN-AMPK-IRES-EGFP. Lipofectamine™ 2000 (Invitrogen) was used for transfection as directed by the manufacturer.

Immunocytochemistry—All cell samples were seeded on 12-mm poly-L-lysine coated glass coverslips for immunofluorescence analysis. C17.2-NSC were seeded at 5 × 10^4 cells/ml. E14-NSC were seeded at 20 × 10^4 cells/ml. Neurospheres derived from P0-NSC and E14-NSC were seeded at a density of 20–50 clones in 24-well plate. The cells were fixed in 4% paraformaldehyde and processed for immunofluorescence as described previously (20). The following antibodies were used to detect antigen: β-III tubulin (Chemicon, 1:200), Nestin (Chemicon, 1:200), O4 (Chemicon, 1:100), glial fibrillary acidic protein (GFAP) (DAKO, 1:500), Alexa dye-conjugated secondary antibodies (Molecular Probes, 1:200), Hoescht 33342 (Molecular Probes, 5 μg/ml) staining was used to label nuclei. Images were captured on an Olympus fluorescent microscope (IX51) equipped with a Coolsnaps camera spot system (Roper Scientific Inc, USA), 20× and 40× objectives. Images were combined for figures using Adobe Photoshop CS2.

Western Blot Analysis—20–100 μg of protein per lane was loaded onto a 10% SDS-polyacrylamide gel and then transferred to a Hybond-C nitrocellulose membrane (Amersham Biosciences). The membranes were processed for immunoblotting as described previously (20). The following primary antibodies were obtained from Cell Signaling Technology and used at 1:1000 dilutions unless otherwise indicated: AMPK-α, phospho-AMPKα (Thr-172), ACC, phospho-ACC (Ser-79), STAT3, phospho-STAT3 (Tyr-705), GFAP (DAKO, 1:1000), β-III tubulin (Chemicon, 1:1000), Nestin (Chemicon, 1:1000) and anti-actin (Sigma, 1:5000). Horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibody (1:5000) was purchased from Jackson ImmunoResearch. Immunoreactive bands were visualized by enhanced chemiluminescence (Amer sham Biosciences).

RESULTS

AICAR Induces Astroglial Differentiation of C17.2 Neural Stem Cells—Metabolic regulation could play a role in altering cell fate (22). Initial experiments to examine AICAR roles were performed by monitoring the morphology of the immortalized C17.2 neural stem cells in the presence and absence of the AICAR. Treatment of 1 mM AICAR for 48 h caused dramatic changes in morphology (Fig. 1A), because morphage is one of the criteria indicating the differentiation of C17.2-NSC. Immunocytochemistry result shows without AICAR treatment but in the presence of serum, nearly all C17.2 cells express Nestin, a marker for NSCs and neural progenitors (Fig. 1B, panel a), but neither the early neuronal marker β-III tubulin (Fig. 1B, panel e) nor the astrocytic marker GFAP (Fig. 1B, panel c). At 48 h of treatment with AICAR in the presence of serum, we found that 80 ± 2% of C17.2 cells displayed morphological changes and expressed GFAP (Fig. 1B, panel d). And most of them were Nestin negative (Fig. 1B, panel b). In comparison 15 ± 5% of C17.2 cells expressed β-III tubulin (Fig. 1B, panel f). Very few C17.2 cells expressed O4, a marker of the oligodendrocyte lineage (data not shown). It is obvious that after AICAR treatment, Nestin− cells decreased over time, accompanied by an increase in GFAP+ cells. Immunoblot analysis of total cell lysates of the
AICAR-treated or untreated C17.2-NSC is in agreement (Fig. 1C). The reciprocal marker appearance indicative of different cell lineage dependent on AICAR provides the evidence of the C17.2 to astrocyte transition. The AICAR activity appears dominant over that of serum.

**AICAR Induces Astroglial Differentiation of Both Adult and Embryonic Primary NSC**—To be more definitive about the effects that AICAR induced differentiation and to test any cell type difference between C17.2-NSC and primary stem cells, we exposed two kinds of native neural stem cells separated from different developmental stages to AICAR, one from post-natal rat hippocampus (P0-NSC) and the other from embryonic day 14 rat cortex (E14-NSC).

E14-NSCs were seeded and grew as monolayer system in the growth factor contained medium. At 2 DIV (day in vitro), both the bFGF and EGF were withdrawn. E14-NSCs were left untreated or treated with AICAR (1 mM) for 48 h. The quite obvious astrogliogenesis enhancement by AICAR was observed (Fig. 2, A and B): fewer Nestin+ neural stem cells were left, while fewer β-III tubulin+ early neurons and more GFAP+ early astrocytes were differentiated from AICAR-treated E14-NSC monolayer than that of untreated NSCs (Fig. 2, A, panels e–h) and control (Fig. 2, B, panels i–l), just like the results after CNTF treatment (Fig. 2, A, panels i–l and B, panels i–l). Western blot analysis further supported our results from immunocytochemistry, demonstrating that upon AICAR treatment for 2 days, expression of GFAP dramatically increased and the other two cell markers Nestin and β-III tubulin decreased compared with the untreated control cells (Fig. 2C).

As expected, the similar results were observed on the AICAR-treated P0-NSCs (supplemental Fig. S1): The promotion of astrogliogenesis by AICAR was obvious compared with the untreated control ones.

These results indicated that AICAR enhances astroglial differentiation of both primary adult NSCs (P0-NSCs) and embry...
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**FIGURE 3.** Both AICAR and Metformin activated AMPK pathway in C17.2-NSC and E14-NSC. (A) C17.2-NSC or B, E14-NSC was treated with 1 mM AICAR for 0.5, 1, 3, 6, or 12 h, cell lysates were processed for the detection of phospho-AMPK (p-Thr-172) and phospho-ACC by immunoblot. (B) Metformin at indicated concentration for indicated time. (C) Metformin at indicated concentration for indicated time. (D) Metformin at indicated concentration for indicated time.

- **AICAR Activates the AMPK Pathway in Both C17.2-NSC and E14-NSC**—AICAR, the earliest known AMPK activator, the most of its effects have been shown to be because of AMPK activation, although few reports of its AMPK-independent effects exist (23–28). To investigate if AICAR can activate the AMPK pathway in NSC as it does in other cell types, phosphorylation of AMPK and its downstream target ACC, an enzyme in the fatty acid synthesis pathway, was taken as an indicator of AMPK activation. As expected, AICAR induced the phosphorylation of AMPK and ACC in a time-dependent manner in both C17.2-NSC (Fig. 3A) and E14-NSC (Fig. 3B).

- **Metformin Also Activates the AMPK Pathway in Both C17.2-NSC and E14-NSC**—To investigate if AMPK activation is responsible for the effects of AICAR to induce differentiation on NSCs, we treated both of the two independent NSCs: C17.2-NSC and E14-NSC with another AMPK activator Metformin (29). As expected, Metformin treatment of C17.2 led to a marked increase in AMPK and ACC phosphorylation in a dose- and time-dependent manner, respectively (Fig. 3C), so did it in E14-NSC (Fig. 3D).

- **Metformin Fails to Induce the Astroglial Differentiation of Both C17.2-NSC and E14-NSC**—We then examined whether Metformin could induce NSC differentiation into astrocytes as AICAR does. Unfortunately, after 2 days treatment of 2 mM Metformin, C17.2-NSCs had no difference from the control not only on morphage (Fig. 4A) but also on marker expression (Fig. 4B). Almost all Metformin-treated C17.2 cells still highly expressed the neural stem cell marker Nestin (Fig. 4B, panel b), similar with the control (Fig. 4B, panel a). Few GFAP+ cells were observed (Fig. 4B, panels e and d). However, AICAR treated-C17.2-NSCs had significant changes in phenotype marker expression: Nestin− cells decreased (Fig. 4B, panels b) and GFAP+ cells increased (Fig. 4B, panel f). And the similar conclusion can be drawn from both the Metformin-treated primary neurospheres (Fig. 4C) and monolayer neural stem cells derived from E14-NSCs (Fig. 4D). After 2 d treatment with Metformin, E14-NSCs showed no difference from the untreated control cells in the growth factor withdrawn differentiation medium: More Nestin− neural stem cells left and more spontaneous differentiated β-III tubulin+ early neurons but fewer astrocytes than AICAR-treated NSCs.

- **Dominant-negative Mutant of AMPK Fails to Block Astroglial Differentiation**—To further determine whether the catalytic activity of AMPK is necessary for AICAR-induced astrocytic differentiation, dominant-negative AMPK (DN-AMPK) were transiently expressed (Fig. 5A) by using dicistronic vector for simultaneous green fluorescent protein expression to mark the transfected cells. To affirm the decrease of AMPK activity by dominant suppression, cell lysates from
transfected cells after 48 h of treatment with AICAR, are examined and revealed a dramatic decrease in AMPK activity (Fig. 5B). There is no obvious difference in either decreasing Nestin expression (Fig. 5C, panels b and d) or increasing GFAP expression (Fig. 5C, panels f and h), between DN-AMPK and empty vector transfection groups. These experiments reveal that reduction of endogenous AMPK activity to hinder AICAR effect on AMPK activation is no sufficient to prevent the AICAR-induced differentiation.

AICAR Induces STAT3 Phosphorylation in Neural Stem Cells but Metformin Fails—Although AICAR is the most common AMPK activator, and its most known effects have been shown to be caused by AMPK activation, here it seemed that the effect of AICAR-induced astrogliogenesis is AMPK-independent. We next studied the signaling pathways underlying the effect of AICAR on NSC differentiation. It has been reported previously that the JAK-STAT3 signaling pathway controls the onset of astrogliogenesis, and many experiments results have placed the JAK-STAT3 pathway at the center of the astrogliogenic machinery (30–34). We first investigated whether the JAK-STAT3 pathway is activated by AICAR in neural stem cells. The Western blot analysis of the E14-NSC that is treated with AICAR for 0, 5, 15, 30, 60, 180 min showed AICAR-induced activation and phosphorylation of STAT3 (Fig. 6A). In the contrast, another AMPK activator, Metformin, which activates AMPK, but fails to induce astrogliogenesis, could not activate the STAT3 pathway in NSC (Fig. 6B).

The Gliogenic Effects of AICAR Could Be Completely Blocked by the Inhibitor of the JAK-STAT3 Pathway—We used a pan-specific JAK inhibitor, JAK inhibitor I, to further determine whether the JAK-STAT3 pathway is activated by AICAR in neural stem cells. The Western blot analysis of the E14-NSC that is treated with AICAR for 0, 5, 15, 30, 60, 180 min showed AICAR-induced activation and phosphorylation of STAT3 (Fig. 6A). In the contrast, another AMPK activator, Metformin, which activates AMPK, but fails to induce astrogliogenesis, could not activate the STAT3 pathway in NSC (Fig. 6B).

FIGURE 5. Dominant-negative mutant of AMPK fails to block gliogenic effects of AICAR. A, immunoblot analysis of overexpression levels of AMPKα1 to monitor the efficiency of transient transfection C17.2-NSC with DN-AMPKα1 after 24, 48, or 96 h, empty vector of pCAGGS-IRES-EGFP (mock) as a negative control. B, immunoblot analysis of phospho-ACC to affirm the decrease of AMPK activity by dominant suppression. Cell lysates from transfected cells after 48 h treatment with 1 mM AICAR are examined and revealed a dramatic decrease in AMPK activity. C, immunochemical study of DN-AMPK-C17.2 (panels c, d, g, h) treated with 1 mM AICAR (panels d and h) for 2 days or untreated (panels c and g), using empty vector of pCAGGS-IRES-EGFP as a control (panels a, b, e, f), panels a and e is the mock-C17.2 cultured in normal growth media. Panels b and f is the mock-C17.2 treated with 1 mM AICAR for 2 days. Cells are first transfected with pCAGGS-DN-AMPK-IRES-EGFP or the empty vector. After 5 h, cultures were changed to the medium with or without 1 mM AICAR. After 2 days, cells were fixed and immunostained for Nestin (red, panels a–d) and GFAP (red, panels e–h), Hoechst nuclear staining views (blue) of the same fields in (panels a–h). Scale bar, 50 µm.

FIGURE 6. AICAR induces STAT3 phosphorylation but Metformin could not. E14-NSC were exposed to 1 mM AICAR(A) or 1 mM Metformin (B) for 5, 15, 30, 60, or 180 min. Cell extracts were prepared and analyzed by Western blot with anti-phospho-STAT3, anti-STAT3, and anti-actin. The results are representative of three independent experiments.

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**A**

| JAK inhibitor I (μM) | AICAR |
|----------------------|-------|
| 0                    | 10    |
| 50                   | 0     |
| 10                   | 50    |

**B**

| JAK inhibitor I | AICAR |
|-----------------|-------|
| -               | +     |

**C**

| DMSO          | AICAR |
|---------------|-------|
| hochest       | Nestin |
| GFAP          | Merge |

FIGURE 7. The specific inhibitor of the JAK-STAT3 pathway, JAK inhibitor I, could block gliogenic effects of AICAR. A, immunoblot analysis of STAT3 tyrosine phosphorylation in C17.2-NSC untreated, treated with 1 mM AICAR alone, JAK inhibitor I (a pan-specific JAK inhibitor, 10 μM or 50 μM) alone, or treated with 1 mM AICAR plus JAK inhibitor I (10 μM or 50 μM). C17.2-NSC were exposed to 1 mM AICAR for 3 h, JAK inhibitor I was added to the incubation medium 1 h prior to adding AICAR and remained in the medium thereafter. One representative of three independent experiments is shown. B, immunoblot analysis of GFAP expression in E14-NSC untreated, treated with 1 mM AICAR alone, 10 μM JAK inhibitor I alone, or AICAR plus JAK inhibitor I in the differentiation medium for 2 days. The results are representative of three independent experiments. C, immunofluorescence detection of E14-NSC untreated (panels a–d), treated with AICAR alone (panels e–h), JAK inhibitor I alone (panels i–l), or AICAR plus JAK inhibitor I (panels m–p) in differentiation medium. After 2 days treatment, cells were fixed and immunostained for nuclei with Hoechst 33342 (blue, panels a, e, i, m), Nestin (green, panels b, f, j, n), and GFAP (red, panels c, g, k, o). Scale bar, 50 μm.

Western blot. All above demonstrated that JAK inhibitor I can abolish the gliogenic effect of AICAR on NSC and that the JAK-STAT3 pathway seems essential for the gliogenic effect of AICAR.

**DISCUSSION**

AICAR is a most commonly utilized and quite specific agent to induce activation of AMPK, and its effects on lipid and glucose metabolism have been the hot focus of many studies. Besides, AICAR is known to exert various other effects, including regulation of proinflammatory response, cytokine production, cell proliferation and apoptosis. On neuronal cell types, AICAR has been reported to exhibit a protective effect to against apoptosis induced by fatty acids in astrocytes (35) and by glucose deprivation in hippocampal neurons (36). However, thus far there are few reports about the effect of AICAR or any other metabolic regulator on neural stem cells or other stem cells.

The present study has first demonstrated, cell-permeable small molecule, AICAR, can alter the fate of neural stem cells and directly induce astroglial differentiation of neural stem cells, reminiscent of that by neurotrophic factor CNTF.

We first observed this astroglionic effect of AICAR on an immortalized murine-derived neural stem cell line, C17.2-NSC. This NSC clone, originally derived from the external germinal layer of the neonatal cerebellum and immortalized with a recombinant retrovirus containing the v-myc oncogene (19, 37), evinces the prototypical, stable and defining features of a stem cell: self-renewal, expression of stem cell antigens, and responsiveness to various stem cell trophins and could differentiate into noncerebellar neurons, astrocytes, and oligodendrocytes both in vitro and in vivo (38–41). In our experiment, AICAR causes about 80% of these cells to differentiate into astrocytes, as specified by GFAP expression (Fig. 1).

To further confirm this result received from C17.2-NSC, we then tested AICAR on another two different primary neural stem cells derived from rat brains of different developmental stage, P0-NSCs as a model of adult neural stem cells and E14-NSCs as a model of embryonic neural stem cells. During embryonic development, the generation of three major neural cell types (neurons, astrocytes, and oligodendrocytes) in the CNS occurs sequentially, whereby almost all neurons are generated before the appearance of glial cells, with the exception of a few sites of postnatal and adult neurogenesis such as the subgranular zone of the hippocampus and the subventricular zone of the forebrain. The “neurons-first, glia-second” differentiation theme for neural progenitors can be recapitulated in culture. So E14-NSCs, which were isolated from relatively early embryonic stages (embryonic day 14), spontaneously easily give rise to neurons, whereas neural progenitors isolated from perinatal stages (P0-NSC) tend to differentiate into astrocytes. Although adult and embryonic stem cells display similar patterns of gene expression, there still are probably some distinct differences. A striking example is their response to LIF. LIF induces astrocytic differentiation in adult progenitor cells but fails to produce a similar response in embryonic day 12 cells (42). So here, we exposed both adult and embryonic NSC to AICAR (supplementary Fig. S1, Fig. 2, and Fig. 4C, panels c and f) to investigate whether the difference occurs. Furthermore, in order to exclude the effect from the different size of the seeded-neurospheres in aggregate culture systems, we also test AICAR on monolayer culture system (Figs. 2B and 4D, panels c and f). From all above the experiments, we can draw the same conclusion that AICAR obviously enhances the astroglial differentiation of both primary adult NSCs and embryonic NSCs, which strongly supported the results observed from immortalized C17.2-NSC. Besides, we found some morphological difference between AICAR-induced astrocytes and CNTF-induced astrocytes from E14-NSC. It is still unclear that the astrocytes produced by AICAR belong to type I or II astrocytes while it was reported that CNTF promote the differentiation into type I astrocytes in embryonic NSCs (25) but into type II in adult NSCs (43). And another possibility may be that AICAR could further change the morphology of the differentiated astrocytes since recently AICAR was reported to induce the stellation of primary astrocytes (44).

Although AICAR is the most common AMPK activator and its most known effects have been associated with AMPK activation, our results appear to suggest the contrary, i.e. astrocytic differentiation by AICAR is independent of AMPK (Figs. 4 and 5). Although AICAR treatment did activate AMPK activity not
only in C17.2-NSC but also in primary neural stem cells (Fig. 3, A and B), several lines of evidence suggest that this enzyme is not essential in astrogligogenic effect of AICAR. First, Metformin, even though it strongly stimulated AMPK activation in both C17.2-NSC (Fig. 3C) and E14-NSC (Fig. 3D), could not induce the astrocystic differentiation, which is confirmed not only on C17.2-NSC (Fig. 4, A and B) but also on E14-NSC (Fig. 4, C and D). Furthermore, dominant-negative mutants of AMPK were unable to prevent the AICAR-induced differentiation of C17.2-NSC (Fig. 5). We also used AMPK-specific inhibitor, compound C, to further investigate the role of AMPK, and it failed to block the astrogligogenic effect of AICAR on E14-NSC (data not shown).

JAK-STAT signaling is a critical part of the astrogligogenic machinery (25, 45). Factors that promote astrogligogenesis, including BMPs, bFGF, and Notch signaling, all require pre-activation of the JAK-STAT 46 – 49 . Therefore, we further investigated whether the astrogligogenic effect of AICAR is also dependent on JAK-STAT3. Actually, we found that AICAR treatment induced transient tyrosine phosphorylation of STAT3 (Fig. 6A), as CNTF and LIF did (25). However, Metformin could not induce the activation of STAT3 (Fig. 6B). We supposed that the difference between the ability of AICAR and Metformin to activate this signaling pathway is the reason why AICAR could induce astrogligogenesis but Metformin could not. It was confirmed by the study with a specific inhibitor of JAKs, JAK inhibitor I, which blocked not only the phosphorylation of STAT3 (Fig. 7A), but also the gliogenic effect of AICAR. JAK-STAT3 activation seems essential for gliogenic effect of AICAR (Fig. 7, B and C). Actually, there is no previous report about the effect of AICAR on the JAK-STAT pathway, the details how AICAR activates the pathway are still unclear and will be further investigated.

Recently, two published reports (27, 28) demonstrated that the inhibitory effects of AICAR on hepatic mitochondrial oxidative phosphorylation and phosphatidylcholine synthesis were independent of AMPK, respectively. They concluded that the AMPK-independent but AICAR-induced effects likely result from the accumulation of ZMP and the depletion of intracellular P. ZMP, as an AMP analogue, could directly modulate enzymes with AMP binding site such as glucokinase (50), glycogen phosphorylase (51, 52). There is also some other possibilities reported, Jhun et al. observed that AICAR but not its phosphorylated form ZMP could directly modulate enzymes with AMP binding site such as glucokinase (50), glycogen phosphorylase (51, 52). Recently, two published reports (27, 28) demonstrated that AICAR but not its phosphorylated form ZMP could directly modulate enzymes with AMP binding site such as glucokinase (50), glycogen phosphorylase (51, 52).

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