Research Article

Quadrella incana (Capparaceae) Leaf Extract Enhances Proliferation and Maintenance of Neural Stem/Progenitor Cells through Upregulating Glycolytic Flux and Redox Potential

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Neural stem/progenitor cells (NSPCs) are self-renewing, multipotent cells located in the embryonic and adult central nervous system (CNS). During embryonic development, NSPCs have the capacity to expand, self-renew, and produce neurons and glial cells of mammalian CNS [1]. Although the research on adult neurogenesis remains controversial [2], it has been known that adult neural stem cells mainly residing within the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricle wall actively produce functional neurons throughout life [3]. Extensive preclinical trials over the past few decades have suggested the potential of stem cell replacement therapy for various neurological disorders [4, 5]. Furthermore, recent clinical trials have been partially reported on neural stem cell transplantation for the treatment of ALS [6] and chronic spinal cord injury [7]. The most essential prerequisite for the success of these clinical applications is the procurement of a sufficient number of high-quality neural stem cells.

Several mechanisms have been suggested to underlie the homeostatic regulation of NSPCs. Among these, metabolic control of NSPCs has been extensively studied. Compared to differentiated neurons, NSPCs are highly reliant on the anaerobic glycolytic pathway for energy production and survival. Under normoxic conditions, LDH activity and lactate production were found to be enhanced in NSPCs compared to neurons [8]. During neuronal differentiation from NSPCs, the expression of most glycolysis genes, particularly glucose transporters encoded by Glut1/3 as well as Ldha encoding lactate dehydrogenase A, was reduced. Consistent with this result, the levels of glycolysis intermediates were dramatically
lower in neurons compared to NSPCs. In contrast, the metabolic regulators PGC-1α and ERRγ were significantly increased during neuronal differentiation to maintain the expression of TCA and mitochondrial respiratory complex genes [9]. The metabolic conversion from aerobic glycolysis to mitochondrialOXPHOS is essential for neuronal differentiation. FoxO3-null NSPCs displayed a reduction in mitochondria-localized HK2 via downregulated AKT signaling, leading to a diminished proliferative potential [10]. PINK1-deficient NSPCs showed mitochondrial defects and elevated glycolysis, and the differentiation/mutation of newborn neurons in PINK1−/− mice was clearly compromised [11]. Constitutive expression of the core glycolytic genes, HK2 and LDHA, was found to induce neuronal cell demise, in Nicaragua, and identified in 2014 by Indiana Coronoado of the Herbarium, National Autonomous University of Nicaragua at Leon. A voucher specimen (accession number KRB 0058006) of the retained material is preserved at the herbarium of KRB. The dried and refined whole plant of Quadrella incana (53 g) was extracted with 11 of 99.9% (v/v) methanol with repeated sonication (15 min) and resting (2 h) for 3 days at 45°C. The resultant product was filtered with nonfluorescent cotton and concentrated by rotary evaporator (N-1000SWD, EYELA) under reduced pressure at 45°C. A final total of 8.55 g methanol extract of Quadrella incana was obtained upon freeze-drying. Dried Quadrella incana leaf was dissolved in DMSO at a stock concentration of 100 mg/ml.

2.2. Preparation of Quadrella incana (Kunth) Ilitis & Cornejo (Capparaceae) Leaf Extract. Quadrella incana (Kunth) Ilitis & Cornejo was collected in Ocotal city, Nueva Segovia province, in Nicaragua, and identified in 2014 by Indiana Coronoado of the Herbarium, National Autonomous University of Nicaragua at Leon. A voucher specimen (accession number KRB 0058006) of the retained material is preserved at the herbarium of KRB. The dried and refined whole plant of Quadrella incana (53 g) was extracted with 11 of 99.9% (v/v) methanol with repeated sonication (15 min) and resting (2 h) for 3 days at 45°C. The resultant product was filtered with nonfluorescent cotton and concentrated by rotary evaporator (N-1000SWD, EYELA) under reduced pressure at 45°C. A final total of 8.55 g methanol extract of Quadrella incana was obtained upon freeze-drying. Dried Quadrella incana leaf was dissolved in DMSO at a stock concentration of 100 mg/ml.

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2.3. Protein Preparation and Immunoblot Analysis. Cells were disrupted directly with laemmli buffer (60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue), followed by sonication and heat denaturation at 95°C. Samples were fractionated by SDS-PAGE and transferred to a PVDF membrane. After blocking membranes with 5% nonfat dried milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 30 min, the membrane was washed with TBST and incubated with antibodies against Bmi1 (1 : 1,000, Bethyl Laboratories), βII tubulin (1 : 1,000, Abcam), phospho-Histone 3 at Ser 10 (1 : 1,000, Cell Signaling), β Actin (1 : 5,000, Sigma Aldrich), GAPDH (1 : 1,000, Cusabio), Fubp1 (1 : 1,000, Abcam), Sox2 (1 : 1,000, Cusabio), Prdx3 (1 : 1,000, Bethyl Laboratories), phospho-mTOR (1 : 1,000, Cell Signaling), total mTOR (1 : 1,000, Cell Signaling), FoxO1 (1 : 1,000, Thermo Fisher Scientific), FOXO3 (1 : 1,000, Thermo Fisher Scientific), phospho AKT (1 : 1,000, Thermo Fisher Scientific), and total AKT (1 : 1,000, Cell Signaling) overnight at 4°C. The next day, membranes were washed three times (10 min each) with TBST and incubated with horseradish peroxidase-conjugated antimouse (1 : 10,000, Bethyl Laboratories) or antirabbit antibodies (1 : 5,000, Bethyl Laboratories) for 1 hour. Membranes were washed with TBST, and signals were detected with D-Plus™ ECL Femto system (Dongin LS). Quantification of Western blots was performed with ImageJ.

2.4. Immunofluorescence. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100/PBS for 15 min each at room temperature. After blocking samples with 2% BSA/PBS for 30 min, cells were subjected to immunofluorescence staining with anti-Nestin (1 : 100, Novus Biologicals), anti-FoxO1 (1 : 200, Thermo Fisher Scientific), and anti-βIII tubulin (1 : 200, Abcam) primary antibodies overnight at 4°C. The next day, cells were washed with PBS and incubated with Flamma®552- or Flamma®488-conjugated goat antirabbit IgG (Bioacts) or goat antimouse IgG (Bioacts)

2. Materials and Methods

2.1. Neural Stem Cell Preparation and Differentiation. Primary NSPCs were isolated from the brain of day 10.5 mouse embryos and maintained in N2 culture medium supplemented with 20 ng/ml EGF and bFGF. For differentiation induction, NSPCs were dissociated into single cells using TrypLE (Life Technologies) and plated on polyornithine

and fibronectin-coated plates in an N2 culture medium including 1% fetal bovine serum (FBS) and B27 Supplements (Life Technologies) without growth factors for 3 days.
GraphPad Prism software (version 6, San Diego, CA) was used for all statistical analyses. Differences were considered significant when *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

3. Results

3.1. Quadrella incana Leaf Extract Enhanced the Proliferation and Stemness of NSPCs. To discover potential bioactivities related to NSPC maintenance, we first performed phytochemical screening with crude extracts from seven plant species. To focus on the proliferation and stemness of NSPCs in this study, we used NSPCs derived from early-stage (E10.5) mouse embryo, which retains a high proliferative capacity. Although NSPCs are multipotent cells that differentiate into neurons, astrocytes, and oligodendrocytes, E10.5 NSPCs give rise to neurons, not glial cells, after short-term culturing. Primary NSPCs were incubated with seven different extracts at the concentration of 100 μg/ml for 3 days under a proliferating condition. Interestingly, *Quadrella incana* leaf extract significantly upregulated the protein level of Bmi1, which promotes NSPC self-renewal and proliferation [19, 20]. Consistently, we found the induction of the mitotic marker,
phospho-Histone 3 at Ser 10 upon *Quadrella incana* leaf extract treatment. In contrast, differentiated neuronal marker βIII tubulin was downregulated upon treatment with this extract, suggesting that *Quadrella incana* leaf has a suppressive effect on spontaneous differentiation of NSPCs (Figure 1(a)). Morphologically, the average sphere size of cultured NSPCs was much larger in *Quadrella incana* leaf extract-treated cells than vehicle-treated cells (Figures 1(b) and 1(c)). Notably, we observed some percentage of cells attached to the bottom of the plate when they were kept in proliferating media including DMSO for 3 days. Arrows indicate cells attached to the bottom of the plate.

3.2. *Quadrella incana* Leaf Extract Did Not Alter the Differentiation of NSPCs. Given the stemness-maintaining function of *Quadrella incana* leaf extract under a proliferating condition, we next tested whether this plant extract could fully suppress the differentiation of NSPCs. To this end, primary NSPCs were differentiated for 3 days in the presence of DMSO or *Quadrella incana* leaf extract. Immunostaining for the neuron-specific marker βIII tubulin demonstrated that the morphology and density of neuronal cells were not altered by *Quadrella incana* leaf extract (Figure 2(a)). Consistent with this data, *Quadrella incana* leaf extract did not change the level of βIII tubulin protein accumulation under the differentiation condition (Figure 2(b)). As shown in Figure 1(a), *Quadrella incana* leaf extract lowered βIII tubulin protein level under the proliferating condition, confirming that it had an inhibitory effect on spontaneous differentiation of NSPCs. Consistent with the immunoblotting result, *Quadrella incana* leaf extract downregulated Tubb3 mRNA expression in the proliferating condition but showed a negligible effect on Tubb3 transcripts in
the differentiation condition (Figure 2(c)). Together, our data suggests that *Quadrella incana* leaf could suppress unintended differentiation of NSPCs but could not fully block the induced differentiation of NSPCs.

### 3.3. Glycolytic Flux and Redox Homeostasis Are Important for the Proliferation of NSPCs.

Increasing evidence suggests that oxidative glycolysis and redox balance are critical mechanisms contributing to the proliferation and stemness of NSPCs. To confirm this, we treated NSPCs with the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) and the oxidative stress inducers sodium nitroprusside (SNP) and hydrogen peroxide. Indeed, the average sphere size of cultured NSPCs was significantly reduced when glycolysis was inhibited or oxidative stress was induced (Figures 3(a) and 3(b)). Notably, when we challenged proliferating NSPCs with SNP, most cells tended to adhere to the bottom of the plate, suggesting that excessive nitric oxide donated by SNP caused cells to lose their sphere-forming capability. Although 2-DG treatment lowered lactate levels as expected, neither SNP nor...
hydrogen peroxide reduced lactate production, indicating that oxidative stress inducers decreased the proliferative potential of NSPCs independent of glycolytic inhibition (Figure 3(c)).

3.4. Quadrella incana Leaf Extract Contributed to the Redox Homeostasis of NSPCs. Given the importance of glycolytic flux and redox balance for the homeostasis of NSPCs, we explored the critical factors contributing to the proliferation and stemness maintenance of NSPCs. A previous study reported that FoxO family members cooperatively modulate NSPC proliferation and self-renewal [21]. Among FoxO genes, FoxO1 and FoxO3 are highly expressed in undifferentiated NSPCs, and they seem to favor NSPC proliferation over neuronal differentiation [10, 22]. Furthermore, these two genes are well-known critical factors in redox regulation [23].

Based on these evidences, we tried to determine protein levels of FoxO1 and FoxO3 in NSPCs treated with Quadrella incana leaf extract. Interestingly, Quadrella incana extract clearly upregulated FoxO1 in NSPCs. However, FoxO3 abundance was not altered by the extract treatment (Figure 4(a)). Although FoxO1 expression was not induced by a lower concentration (20 μg/ml) of the extract, treatment with 100 μg/ml did increase the levels of FoxO1 protein (Figure 4(b)) and mRNA (Figure 4(c)). FoxO1 immunostaining also showed that FoxO1 was accumulated in Quadrella incana leaf extract-treated NSPCs (Supplementary Fig 2A). Because FoxO1 was reported to maintain stemness by upregulating the Notch pathway in adult NSPCs [22], we tested whether FoxO1 induction by the extract also positively controls the Notch pathway. However, Quadrella incana leaf extract failed to increase the Csl-mediated Notch reporter activity (Supplementary Fig 2B). In addition, the expression level of Hes1, one of the main target genes of the Notch pathway, was unchanged by treatment with the extract (Supplementary Fig 2C), suggesting that FoxO1 accumulation by Quadrella incana leaf extract showed a negligible effect on the Notch pathway.

We next tested whether Quadrella incana leaf extract indeed elevated the ROS scavenging capacity of NSPCs through FoxO1 induction. Among FoxO1 target genes, Prdx3 is known to have a prominent role in maintaining the cellular redox balance and mitochondrial functions [24, 25]. In addition, Sod2, another transcriptional target of FoxO1 [24], was reported to scavenge oxygen radicals in NSPCs, leading to cell survival [12]. As expected, FoxO1 accumulation by Quadrella incana leaf extract resulted in the upregulation of Prdx3 and Sod2, suggesting that the extract possibly enhances the redox potential of NSPCs (Figures 4(d) and 4(e)). In line with these results, the relative ratio of reduced to oxidized glutathione (GSH/GSSG) was increased by Quadrella incana leaf extract, suggesting that the extract possibly upregulated the reducing capacity of NSPCs (Figure 4(f)). Consistently, the extract dramatically suppressed the accumulation of reactive oxygen species inside NSPCs in a dose-dependent manner (Supplementary Fig 3). Together, our results demonstrate that Quadrella
Figure 4: Continued.
incana leaf extract could suppress oxidative stress through FoxO1 accumulation, contributing to the redox homeostasis of NSPCs.

3.5. Quadrella incana Leaf Extract Activated Lactate-AKT-mTOR Axis by Upregulating Glycolytic Flux. In addition to FoxO1, we also found that Fubp1 was upregulated by Quadrella incana leaf extract in a dose-dependent manner (Figures 4(a)-4(c)). A previous study demonstrated that Fubp1 enhances the lactate production, leading to AKT-mTOR activation [26, 27]. Given that oxidative glycolysis is important for the energy metabolism of NSPCs, we first checked the lactate production, the end product of glycolysis, in Quadrella incana leaf extract-treated NSPCs. Interestingly, intracellular and extracellular lactate levels were dramatically increased upon Quadrella incana leaf extract treatment (Figure 5(a)). Clearly, the extract reversed the lactate level that was inhibited by 2-DG (Figure 5(b)). However, the expression of Myc, a major coordinator of glycolysis, was unchanged by extract treatment, suggesting that other regulators including Fubp1 would be involved in the upregulation of glycolytic activity (Figure 5(c)). Consistent with the upregulated lactate production, the levels of main glycolytic genes such as Glut1, Glut3, Hk1, Hk2, Pkm1, and Ldha appeared to be downregulated upon differentiation (Supplementary Fig 4). Therefore, the upregulation of glycolytic genes by the Quadrella incana leaf extract would contribute to the maintenance of undifferentiated status by enhancement of lactate synthesis.

As previously reported, lactate can activate the AKT-mTOR axis [27]. To test whether Quadrella incana leaf extract induces the AKT-mTOR axis, phosphorylated levels of AKT and mTOR were determined. As expected, the phosphorylation level of each protein was clearly increased, mirroring that upregulated lactate synthesis presumably contributed to the activation of the AKT-mTOR axis (Figure 5(d)). In line with this result, mitotic marker phospho H3 was upregulated by Quadrella incana leaf extract. However, inhibition of PI3K-AKT and mTOR pathway clearly reduced phospho H3 level, confirming that Quadrella incana leaf extract contributes to the NSPC growth through AKT-mTOR axis (Supplementary Fig 5). Together, our data collectively suggests that Quadrella incana leaf extract enhances the glycolytic flux, leading to the lactate-AKT-mTOR axis activation.

4. Discussion

Recent preclinical and clinical studies raised a possibility that grafted NSPCs may replace degenerated cells in neurodegenerative conditions. To use NSPCs in stem cell therapies, the
Figure 5: *Quadrella incana* leaf extract upregulated glycolytic pathway, leading to the activation of lactate-AKT-mTOR axis. (a) Relative intracellular or extracellular lactate levels in proliferating NSPCs after treatment with DMSO or 100 μg/ml *Quadrella incana* leaf extract. The relative lactate levels of DMSO-treated cells were set to 1. (b) Relative extracellular lactate levels in proliferating NSPCs in the presence or absence of 2-DG after treatment with DMSO or 100 μg/ml *Quadrella incana* leaf extract. The relative lactate levels of DMSO-treated cells were set to 1. (c) mRNA levels of Myc and glycolytic genes (Glut1, Glut3, and LHDA) in proliferating NSPCs treated with DMSO or 100 μg/ml *Quadrella incana* leaf extract were measured by qRT-PCR. Values are means ± s.e.m. The mRNA levels of DMSO-treated cells were set to 1. (d) Western blot analysis of phospho mTOR and phospho AKT in proliferating NSPCs after treatment with DMSO or 100 μg/ml *Quadrella incana* leaf extract for 3 days. Levels of phospho mTOR and phospho AKT were normalized to total mTOR and total AKT, respectively. The relative band intensities of phospho mTOR and phospho AKT proteins are shown on the right panels. The intensities of DMSO-treated sample were arbitrarily set as 1.
securement of many healthy cells should be a key prerequisite. Given the importance of glycolytic flux and redox balance in the proliferation and stemness of NSPCs, we showed that *Quadrella incana* leaf extract contributed to the maintenance of NSPCs by upregulating glycolysis and redox potential (Supplementary Fig 6).

In this study, we focused on two genes, FoxO1 and Fubp1. While FoxO1 is highly expressed in the proliferating NSPCs, it is downregulated in the early phase of neuronal differentiation [22]. Although FoxO1 was reported to antagonize the differentiation and maintain the homeostasis of adult NSPCs through the cooperation with Notch pathway, our results demonstrated that FoxO1 accumulation upon *Quadrella incana* leaf extract treatment did not enhance the Notch activity. Instead, our study found that the increased FoxO1 upon *Quadrella incana* leaf extract treatment upregulated its target antioxidant genes Prdx3 and Sod2. Therefore, *Quadrella incana* leaf seemed to support the proliferative potential of NSPCs through regulating the cellular redox balance. Previously, we demonstrated that Fubp1 increases the lactate production, leading to the activation of the AKT-mTOR axis, by regulating hexokinase genes. Although we showed that *Quadrella incana* leaf extract promoted the accumulation of Fubp1 in NSPCs and induced the lactate-AKT-mTOR axis, the extract enhanced the expression of Glut1, Glut3, and Ldha, rather than hexokinase genes. Therefore, it would be possible that molecular mechanisms or target gene sets of Fubp1 could be cell-type dependent. Otherwise, it would also be possible that another key glycolysis regulator would be involved in the *Quadrella incana* leaf-mediated lactate-AKT-mTOR axis elevation.

Alterations in NSPC homeostasis followed by reduced generation of newborn neurons are associated with several neurological or neurodegenerative diseases, such as Alzheimer’s disease, dementia, and depression. Given the therapeutic capacity of NSPCs, a fundamental understanding of how NSPC fate is tightly regulated would be required. In the present study, we suggested that metabolic flux and redox homeostasis would be critical factors for NSPC cell fate decision. Although we found that *Quadrella incana* leaf extract enhances the NSPC proliferation through regulating these critical factors in vitro, we could not verify the biological function of the extract in NSPC proliferation in vivo, because pharmacokinetic characteristics of this extract were not analyzed yet. If the in vivo physiological function of this extract on NSPC homeostasis is verified in the future, it will be possible to use the *Quadrella incana* leaf extract clinically to boost NSPC proliferation followed by efficient neurogenesis.

Although we did not identify a specific molecule responsible for the bioactivity of *Quadrella incana* leaf extract on NSPCs, there may be positive interactions between components in the crude extract of a given plant. Our findings may be useful for obtaining a sufficient quantity of clinically available NSPCs for therapeutic strategies against acute brain injury or long-term degenerative conditions.

**Data Availability**

The data used to support the findings of this study are included within the article.

**Conflicts of Interest**

The authors declare no conflict of interest.

**Authors’ Contributions**

D.Y.K. made the conceptualization. M. K. and J. H. C. worked the methodology. M. K., J. H. C., T. J. K., S. M. L., and H. J. K. did the validation. M.K., J. H. C., and D. Y. K. assessed the formal analysis. M. K., J. H. C., T. J. K., S. M. L., and H. J. K. evaluated the investigation. I.C. and D.K.Y. gathered the resources, , M. K. and D.Y.K. wrote the original draft preparation and the review and editing and processed the visualization. D.Y.K. performed the supervision, project administration, and funding acquisition. Min-gyu Kang and Jin-Hwa Cho both authors contributed equally to this work.

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**Supplementary Materials**

Supplementary Figure 1. *Quadrella incana* leaf extract facilitates NSPCs proliferation. (A) Cell growth ability was analyzed using CCK-8 assay in proliferating NSPCs treated with DMSO, 40 μg/ml or 100 μg/ml *Quadrella incana* leaf extract for 3 days. Statistical significance was determined by one-way ANOVA. (B) Immunofluorescence analysis of phospho histone 3 (pH 3) at Ser10 (red) abundance in DMSO- or *Quadrella incana* leaf extract-treated NSPCs. Nuclear DAPI (4’,6-diamidino-2-phenylindole) staining is shown in blue. Scale bars = 100 μm. (C) The quantification of pH 3-positive cell proportions in DMSO- or *Quadrella incana* leaf extract-treated NSPCs as in (B). Supplementary Figure 2. Upregulated expression of FoxO1 by *Quadrella incana* leaf extract did not activate Notch pathway. (A) Enhanced expressions of FoxO1 and Nestin were determined. FoxO1 staining is shown in green and neural stem cell marker Nestin is shown in red. Nuclear DAPI (4’,6-diamidino-2-phenylindole) staining is shown in blue. (B) CSL induced luciferase reporter activity was measured in NSPCs with vehicle or 100 μg/ml *Quadrella incana* leaf extract treatment. (C) Quantitative real-time PCR (qRT-PCR) of Hes1 mRNA in NSPCs with vehicle or 100 μg/ml *Quadrella incana* leaf extract treatment under proliferating condition (prol) or differentiating conditions.
condition (diff). Supplementary Figure 3. Quadrella incana leaf extract suppresses accumulation of reactive oxygen species in NSPCs. NSPCs were treated with DMSO or the indicated concentration of Quadrella incana leaf extract. ROS accumulation was assessed by CellROX staining and fluorescence microscopy 24 hrs after treatment. Scale bars = 200 μm. Supplementary Figure 4. Expression changes of main glycolytic genes during NSPC differentiation. Relative expressions of indicated genes in undifferentiated neural stem/progenitor cells (prol) and differentiated neural cells (diff) were measured by qRT-PCR. Supplementary Figure 5. Quadrella incana leaf extract upregulated AKT and mTOR pathway, leading to the proliferation of NSPCs. (A) Western blot analysis of phospho mTOR, phospho AKT, and phospho H3 in proliferating NSPCs. Levels of total proteins were also detected for normalization. 1 μM LY294002 and 20 nM rapamycin was treated to block the PI3K-AKT pathway and mTOR pathway, respectively. (B) The relative band intensities of phosphor proteins are shown. Supplementary Figure 6. A graphical presentation of NSPC homeostatic regulation by Quadrella incana. (Supplementary Materials)

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