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Sonic hedgehog stimulates glycolysis and proliferation of breast cancer cells: Modulation of PFKFB3 activation

Xin Ge [a], Pengwei Lyu [a], Yuanting Gu [a], Lin Li [a], Jingruo Li [a], Yan Wang [a], Linfeng Zhang [a], Chao Fu [b], Zhang Cao [a, *]

[a] Department of Breast Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China
[b] Department of Ultrasonography, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China

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ABSTRACT

Sonic hesgehog (Shh) signaling has been reported to play an essential role in cancer progression. The mechanism of Shh involved in breast cancer carcinogenesis remains unclear. The present study sought to explore whether Shh signaling could regulate the glycolytic metabolism in breast cancers. Overexpression of the smoothed (Smo) and Gli-1 was found in human primary breast cancers. The expressions of Shh and Gli-1 correlated significantly with tumor size and tumor stage. In vitro, human recombinant Shh (rShh) triggered Smo and Gli-1 expression, promoted glucose utilization and lactate production, and accelerated cell proliferation in MCF-7 and MDA-MB-231 cells. Notably, rShh did not alter 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) expression but augmented PFKFB3 phosphorylation on ser^{461}, along with elevated fructose-2,6-bisphosphate (F2,6BP) generation by MCF-7 and MDA-MB-231 cells. This effect could be dampened by Smo siRNA but not by Gli-1 siRNA. In addition, our data showed the upregulated expressions of MAPK by rShh and elevatory PFKFB3 phosphorylation by p38/MAPK activated kinase (MK2). In conclusion, our study characterized a novel role of Shh in promoting glycolysis and proliferation of breast cancer cells via PFKFB3 phosphorylation, which was mediated by Smo and p38/MK2.

1. Introduction

The Hedgehog (Hh) family secreted proteins includes Sonic (Shh), Indian (Ihh), and Desert (Dhh) [1], a group of proteins firstly found to be involved in animal development [2]. Hh pathway activation is initiated by the binding of these Hh ligands. Then, the cell surface receptor Ptch family (Ptch1, Ptch2) and the smoothened (Smo) transmit the signal into the cell, following with the signal transduction into the nucleus by the Gli-family factors (Gli-1, Gli-2) [3–5]. Deregulation of the Shh signaling pathway has been shown to affect developmental process, tissue regeneration, stem cell renewal and cancer pathology [6]. In human hepatocellular carcinoma (HCC), Shh signaling pathway mediates cancer cells invasion and metastasis by increasing MMP-9 expression via ERK pathway [7]. Moreover, overexpression of Shh was found in breast cancer tissues and survival analysis suggested that Shh overexpression was a poor prognosis indicator for breast cancers [8]. These evidence characterized that Shh overexpression was a critical event in breast carcinogenesis [8,9]. In addition, Shh activation induced transcription of hexokinase 2 (HK2) and pyruvate kinase M2 (PKM2) and caused a robust increase of glycolytic metabolism in medulloblastoma [10]. The process is mediated by the canonical activation of the Gli transcription factors [10]. Nevertheless, the exact role of Shh signaling in breast cancer remains unclear.

The Warburg effect is a description of metabolism in malignant tumors. Cancer cells are inclined to produce energy by glycolysis rather than by oxidative phosphorylation (OXPHOS) [11]. The Warburg phenotype had stronger associations with triple negative breast carcinomas (TNBC) than luminal breast cancers, suggesting a correlation between metabolic phenotype and the biology of breast cancer [12]. Furthermore, the metabolic phenotype of breast cancer stem cells (BCSCs) and their differentiated progeny was also investigated [13]. BCSCs consume more glucose, produce less lactate, and have higher ATP content compared to their differentiated progeny [13].

* Corresponding author. Department of Breast Surgery, The First Affiliated Hospital of Zhengzhou University, No.1 Jianshe Dong Road, Erqi District, Zhengzhou 450052, China.
E-mail address: zzzhangcao@126.com (Z. Cao).

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Fructose-2,6-bisphosphate (F2,6BP), an allosteric activator of Phosphofructokinase-1 (PFK-1), plays a key regulation role in glycolytic flux [14,15]. Steady state levels of F2,6BP are maintained by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) family enzymes, especially by PFKFB3 [16]. Some anti-oncogenes may modulate PFKFB3 expression. In the embryonic fibroblasts, phosphatase and tensin homolog (PTEN) knock-out was shown to significantly raised the protein levels of PFKFB3 and concentrations of F2,6BP [17]. PFKFB3 expression has been demonstrated to contribute to tumor growth and metastasis. Heterozygous deletion of the PFKFB3 gene has been found to reduce both the glucose metabolism and growth of Ras-transformed tumors in syngeneic mice [18].

In this study, we sought to explore whether Shh signaling could regulate the glycolytic metabolism in breast cancers. We found that Shh and Gli-1 were overexpressed in breast carcinomas and their expressions were correlated with critically clinicopathological factors. In vitro, Shh stimulated glycolysis and proliferation of breast cancer cells via PFKFB3 activation, which was mediated by Smo and p38 MAPK/MK2.

2. Materials and methods

2.1. Collection of human samples

Samples of breast tumor tissues and normal breast tissues ($n = 117$) were obtained from the patients (mean age 52.4 years, range 27–59 years) enrolled in the First Affiliated Hospital of Zhengzhou University between 2011 and 2014. Human samples were prepared and quickly frozen in liquid nitrogen. The study was reviewed and approved by the Ethical Committee of the First Affiliated Hospital of Zhengzhou University and carried out in according with the declaration of Helsinki. Written informed consent was obtained from each patient.

2.2. Cell culture and treatment

Breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Invitrogen) under a 37 °C, 5%CO_2 circumstance.

For human recombinant Shh (rShh; Novoprotein, Summit, NJ, USA) treatment, 1×10^4/well breast cancer cells were treated with 0, 5, 10 μg/mL rShh for 1 h and then prepared for further analysis. To inhibit the activity of MAPK in MCF-7 and MDA-MB-231 cell lines, a list of specific inhibitors including SB203580 (10 nM), PF3644022 (10 nM), UO126 (10 nM), LY294002 (10 nM) were added into the medium and incubated for 30 min.

2.3. Immunohistochemistry staining

Tumor tissues and normal control samples were detected with anti-Smo (1:400; Santa Cruz Biotech, Santa Cruz, CA, USA) and anti-Gli-1 (1:300; Santa Cruz Biotech) polyclonal antibody. Image acquisition was performed with a microscope (Olympus, Tokyo, Japan) and assessed by a pathologist. Immunostaining intensity (1,weak; 2, moderate; 3, intense) and the ratio of positive cells (0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, >75%.) were calculated in at least five fields (400×). The scores of each sample were multiplied to give a final score of 0, 1, 2, 3, 4, 6, 8, 9, or 12.

2.4. siRNA transfection

For Smo expression and Gli-1 expression silencing, small interfering RNAs (siRNAs) for control (non-specific siRNA) and Smo or Gli-1 siRNA were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA), MCF-7 and MDA-MB-231 cells were transfected with control, Smo or Gli-1 siRNA using Lipofectamine 2000(Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, cells were harvested for further analysis.

2.5. RNA extraction and qRT-PCR

Total RNA was extracted from tissues and breast cancer cells using Trizol reagent (Takara, Dalian, China). For PFKFB3 expression analysis, the cDNA was generated using HiScript 1st Strand cDNA Synthesis Kit (Vazyme; Nanjing, China). Expression quantification of the target genes (Smo, Gli-1 and PFKFB3) was carried out on a 7500 Fast Dx Real-Time PCR System (Applied Biosystems) with HiScript® Q RT SuperMix for qPCR(Vazyme; Nanjing, China). The primers including forward: 5′-CTG GTA AGA GGA CGT GCA GA-3′, reverse: 5′-AGG GTG AAG AGC GTG CAC TG-3′ (for Smo); forward: 5′-TCA CTA CTA GAG TCC CAA CC-3′, reverse: 5′-GCA GAT GTG AAG CGA TAT GA-3′ (for Gli-1); forward: 5′-AGA ACT TCC ACT CTC CCA CCC AAA-3′, reverse: 5′-AGG GTA GTG CCC ATT GTT GAA GGA-3′ (for PFKFB3) and forward: 5′-GAG CTA CGA GCT GCC TGA CG-3′, reverse: 5′-CCT AGA AGC ATT TGC GGT GG-3′ (for β-actin) were used. All experiments were repeated for three times.

2.6. Western blot

Total proteins were extracted and separated by SDS-PAGE, and western blot analysis was performed according to standard procedures. β-actin was used as the reference gene for protein quantification. Monoclonal antibody for Smo (1:1000, Santa Cruz Biotech), Gli-1(1:1000, Santa Cruz Biotech), PFKFB3 (1: 1500, Sigma–Aldrich, St. Louis, MO, USA), p-p38 MAPK (1:1000, Sigma–Aldrich), p-MK2 (1:1500, Santa Cruz Biotech), p-p42/p44 MAPK (1:1000, Santa Cruz Biotech), p-ERK1/2 (1:1500, Santa Cruz Biotech), p-Akt (1:1500, Santa Cruz Biotech) and β-actin (1:2000, Abcam, Cambridge, UK) were employed for the immunodetection. Polyclonal antibodies including anti-p-PFKFB3 (ser478) and anti-p-PFKFB3 (ser461) were obtained according to a previous report [19]. Goat anti-mouse IgG (1:10 000) was used as the second antibody following with enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ, USA) detection.

2.7. Glucose utilization assay

Glucose Assay Kit (Abcam) was employed to assay the concentration of D-glucose in medium of MCF-7 and MDA-MB-231. Glucose utilizition by the cells was calculated as the difference between the starting glucose concentration and the final glucose concentration in medium. All experiments were repeated for three times.

2.8. Lactate and F2,6BP measurements

Lactate levels were measured using a lactate oxidase-based colorimetric assay read at 540 nm according to the manufacturer's instructions (Beyotime, Wuxi, China) and normalized to cell numbers. Before the measurement of total intracellular F2,6BP, 1×10^4 cells were trypsinized and washed twice with PBS. The F2,6BP concentration was normalized to total cellular protein as measured by the bicinchoninic acid (BCA) assay. All experiments were repeated for three times.

2.9. Cell proliferation assays

To evaluate cell proliferation, 1×10^4/well breast cancer cells were treated with 0, 5, 10 μg/mL rShh for 1 h before plating into

wells of 96-well plates. At 1d, 2d, 3d and 4d after transfection, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, -diphenyltetrazolium bromide (MTT) assay following the manufacturer's protocol (Beyotime, Wuxi, China). All experiments were repeated for three times.

2.10. Statistical analysis

Data were represented as means ± SD. All tests were performed with SPSS17.0 software (Chicago, IL, USA). The potential association between Smo expression or Gli-1 expression and clinicopathologic factors for each patient was calculated by Chi square test. Difference comparison was carried out using one-way ANOVA analysis following with post-hoc tests. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Clinical examination of Shh and Gli-1 expression in human breast cancers

To evaluate the functions of Shh and Gli-1 in human breast cancers, the expressions of Shh and Gli-1 proteins between primary breast carcinomas and normal control were compared by immunohistochemistry. The results showed significant differences between breast carcinomas and normal control in shh and Gli-1 expressions (Fig. 1A–D). Furthermore, we also analyzed the correlations between shh protein or Gli-1 protein and clinicopathological factors of breast cancers (n = 117) by Chi square methods. We observed that the expressions of Shh and Gli-1 had a significant correlation with tumor size and tumor stage (Table 1). The results might suggest a role of shh and Gli-1 in breast cancer carcinogenesis.

3.2. Shh triggers glycolysis and proliferation of breast cancer cells

Cancer cells preferentially employ aerobic glycolysis for energy production, a phenomenon known as the Warburg effect [11]. We then sought to delineate if sonic hedgehog signaling was involved in the modulation of glycolytic metabolism in breast cancer cells. Recombinant Shh (rShh, 0, 5, and 10 μg/mL) was added into the cultures of MCF-7 and MDA-MB-231 cells and incubated for 1 h. After that, Smo and Gli-1 expression as well as the glucose utilization and lactate production were measured in these cancer cells. Compared with the control (0 μg/mL rShh), rShh treatment markedly upregulated Smo and Gli-1 expression (Fig. 2A–D) and boosted the rate of glucose utilization (Fig. 2E) and lactate production (Fig. 2F) in MCF-7 and MDA-MB-231 cells. Meanwhile, the proliferation of MCF-7 and MDA-MB-231 cells was also assessed after rShh exposure. Our data exhibited a significant promotion of MCF-7 and MDA-MB-231 cells proliferation by rShh exposure (Fig. 2G and H).

3.3. Shh regulates PFKFB3 activation in breast cancer cells

As Shh signaling was shown to enhance glycolysis in breast cancers, we next speculated that the expression or the activity of pfkfb3, a citical regulator of glycolysis process, was influenced by

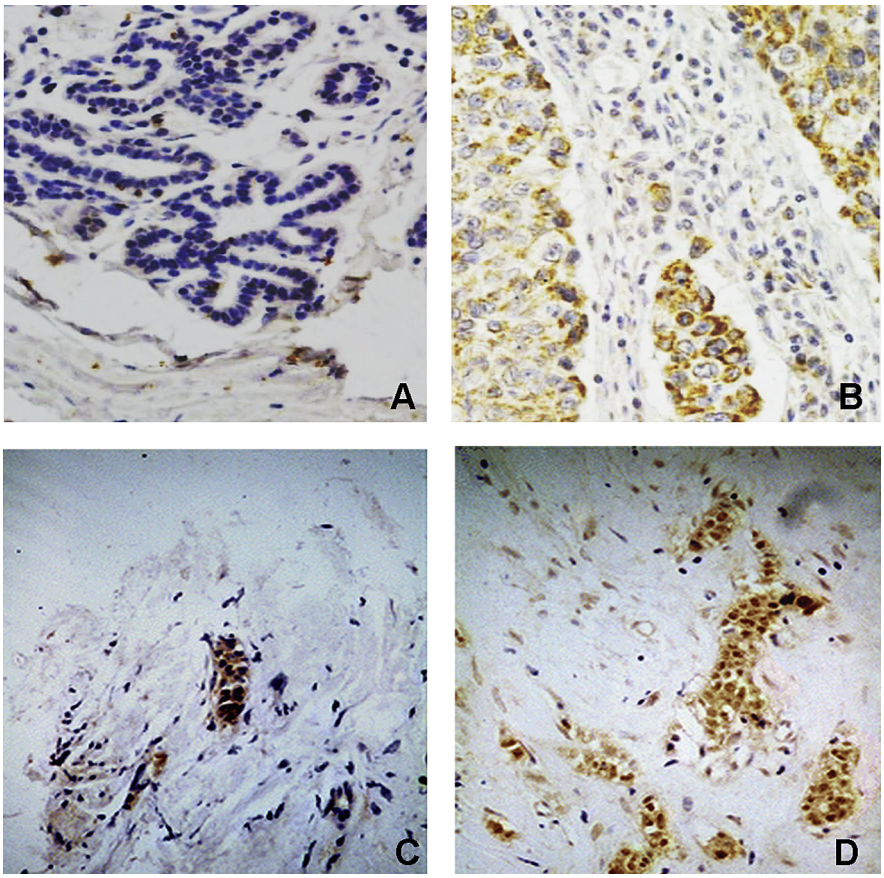

Fig. 1. Immunohistochemistry staining of human breast tumor tissues and normal breast tissues. A. Sonic hedgehog (Shh) protein expression in normal tissues; B. Shh protein expression in breast tumor tissues; C. Gli-1 protein expression in normal tissues; D. Gli-1 protein expression in breast tumor tissues. 400×.

Table 1
Correlation of clinicopathological factors with Shh mRNA or Gli-1 mRNA expression in breast cancer patients (n = 117).

	Shh(+) n(%)			Gli-1(+) n(%)		
	Low	High	p	Low	High	p
Age						
<50	36/65 (55.4)	29/65 (44.6)	0.709	31/65 (47.7)	34/65 (52.3)	0.135
≥50	27/52 (51.9)	25/52 (48.1)		32/52 (61.5)	20/52 (38.5)	
Tumor size						
<50 mm	41/74 (55.4)	33/74 (44.6)	0.032	45/74 (60.8)	29/74 (39.2)	0.026
≥50 mm	15/43 (34.9)	28/43 (65.1)		17/43 (39.5)	26/43 (60.5)	
Tumor stage						
I	14/21 (66.7)	7/21 (33.3)	0.041	16/21 (76.2)	5/21 (23.8)	0.030
II	18/47 (38.3)	29/47 (61.7)		20/47 (42.6)	27/47 (57.4)	
III	9/27 (33.3)	18/27 (66.7)		11/27 (40.7)	16/27 (59.3)	
IV	6/22 (27.3)	16/22 (72.7)		8/22 (36.4)	14/22 (63.6)	
Grade						
I	15/28 (53.6)	13/28 (46.4)	0.791	19/28 (67.9)	9/28 (32.1)	0.776
II	24/52 (46.2)	28/52 (53.8)		32/52 (61.5)	20/52 (38.5)	
III	19/37 (51.4)	18/37 (48.6)		22/37 (59.5)	15/37 (40.5)	
Node metastasis						
Yes	37/55 (67.3)	18/55 (32.7)	0.672	31/55 (56.4)	24/55 (43.6)	0.992
No	45/62 (72.6)	17/62 (27.4)		35/62 (56.5)	27/62 (43.5)	

Low indicates immunoreactivity score <5; High indicates immunoreactivity score ≥5.

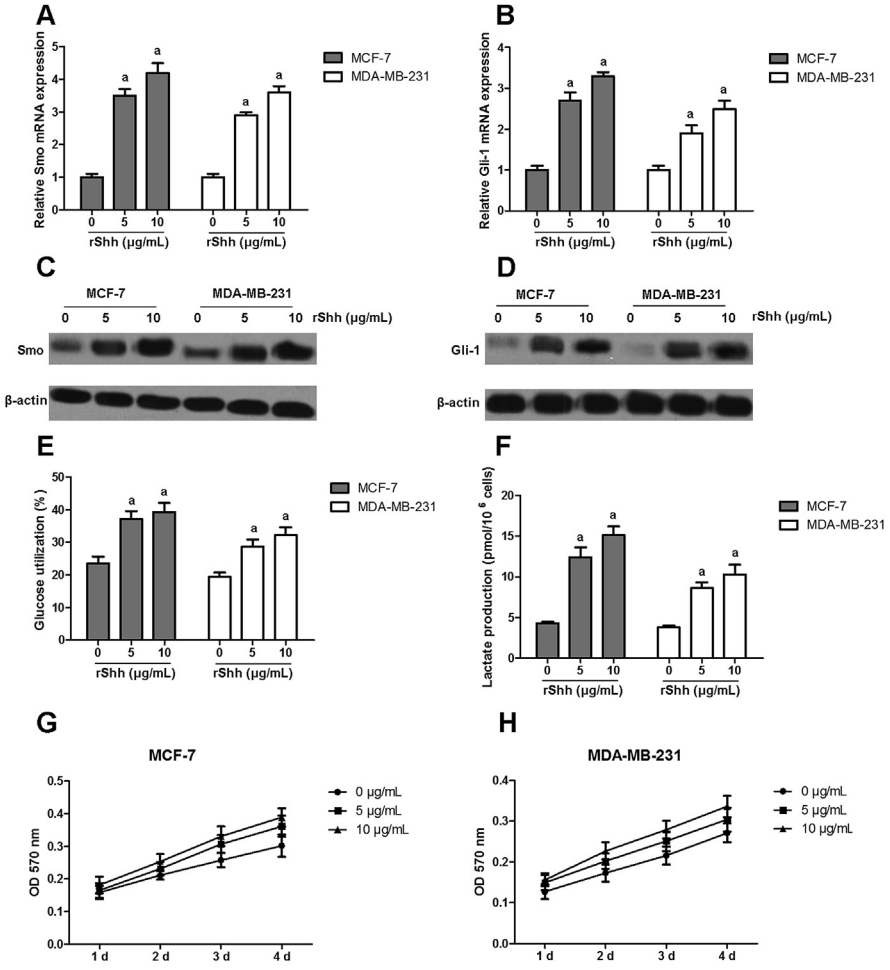

Fig. 2. Glycolysis and proliferation assay in MCF-7 and MDA-MB-231 cells after human recombinant Shh (rShh) stimulation. A, B: Quantification of the smoothened (Smo) and Gli-1 mRNA expression in MCF-7 and MDA-MB-231 cells treated with 0, 5, 10 μg/mL rShh for 1 h; C, D: Immunoblot detection of Smo and Gli-1 protein expression in MCF-7 and MDA-MB-231 cells; E. Glucose utilization assay in MCF-7 and MDA-MB-231 cells; F. Determination of lactate production in MCF-7 and MDA-MB-231 cells; G, H: Evaluation of cell proliferation of MCF-7 and MDA-MB-231 by MTT. [a] compared with 0 μg/mL rShh group, $p < 0.05$.

Shh signaling. Various doses (0, 5, and 10 μg/mL) of rShh were added into the cultures of MCF-7 and MDA-MB-231 cells and the expression of PFKFB3 mRNA and protein was analyzed by qRT-PCR and Western blot. Compared with the control, both of PFKFB3

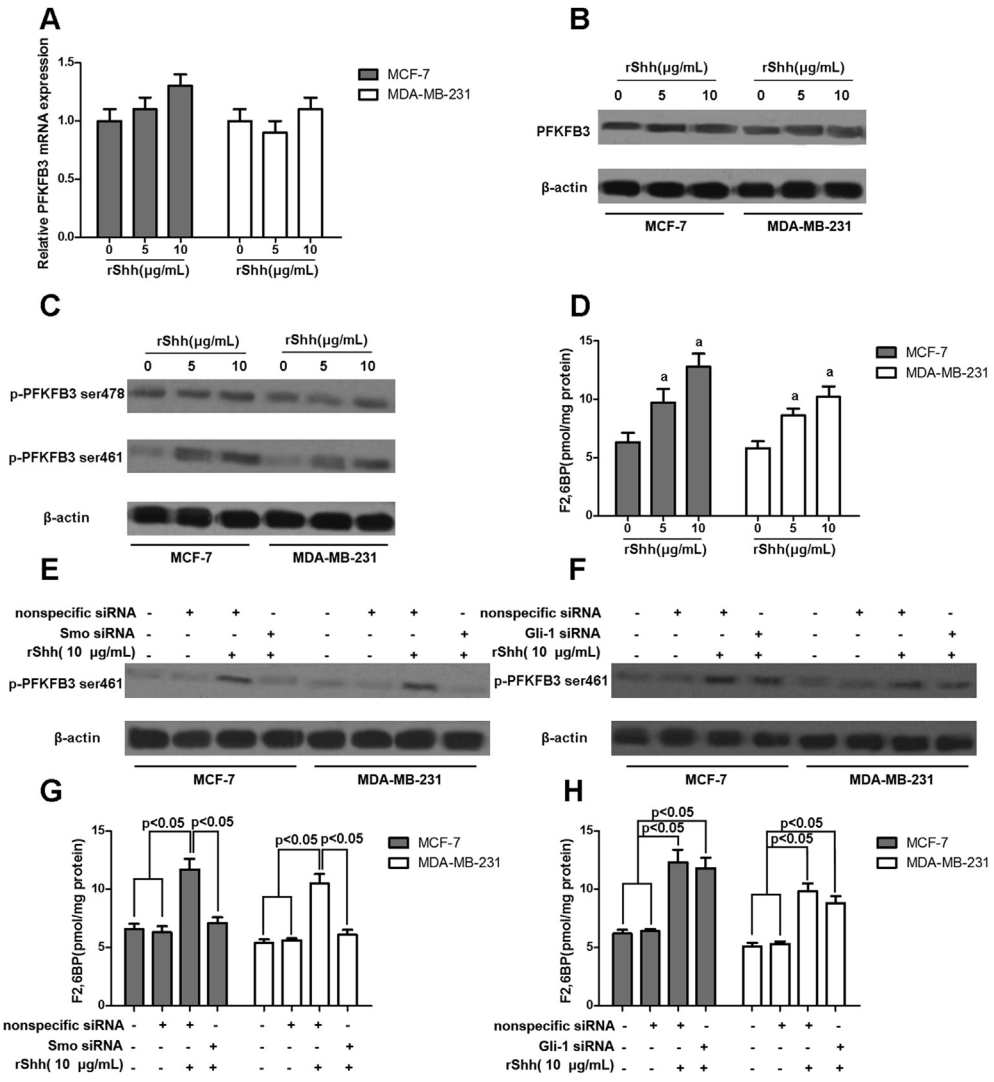

Fig. 3. Activation of 6-phosphofructo-2-kinase (PFKFB3) in MCF-7 and MDA-MB-231 cells by rShh treatment. A, B: Quantification of PFKFB3 mRNA and PFKFB3 protein in MCF-7 and MDA-MB-231 cells treated with 0, 5, 10 μg/mL rShh for 1 h; C. Immunoblot detection of p-PFKFB (ser478) and p-PFKFB3 (ser461) expression in MCF-7 and MDA-MB-231 cells; D. Measurement of Fructose-2,6-bisphosphate (F2,6BP) generation in MCF-7 and MDA-MB-231 cells; E, F: Immunoblot detection of p-PFKFB (ser461) expression in MCF-7 and MDA-MB-231 cells after nonspecific, Smo siRNA or Gli-1 siRNA trasfection; G, H: Measurement of F2,6BP generation in MCF-7 and MDA-MB-231 cells after nonspecific, Smo siRNA or Gli-1 siRNA trasfection; [a] compared with 0 μg/mL rShh group, $p < 0.05$.

mRNA and protein levels were not altered by rShh (Fig. 3A and B). Notably, the expression of phosphorylated PFKFB3 (ser^{461} not ser^{478}) was upregulated after rShh treatment (Fig. 3C). Subsequently, an increasing of F2,6BP production, the substrate of PFKFB3, was also observed in rShh treated cancer cells (Fig. 3D). Furthermore, transfection of Smo siRNA rather than Gli-1 siRNA into MCF-7 and MDA-MB-231 cells abrogated phosphorylated PFKFB3 expression (Fig. 3E and F) as well as F2,6BP production (Fig. 3G and H) induced by rShh (10 μg/mL). These results indicated that Shh did not influence the expression of PFKFB3 but led to PFKFB3 activation via Smo in breast cancer cells.

3.4. p38/MK2 signaling is implicated in PFKFB3 activation

Evidence has shown that PFKFB3 is phosphorylated by MAPK signaling [20] and there is an intercourse between Shh signaling and MAPK signaling [21]. Therefore, we next examined the activation of p38 MAPK/MK2, p42/p44 MAPK/ERK, PI3K/Akt signaling in breast cancer cells after rShh treatment. In the results, phosphorylation of p38 MAPK, MK2, p42/p44 MAPK, ERK, and Akt was significantly augmented by rShh exposure (Fig. 4A). To further clarify PFKFB3 phosphorylation by MAPK signaling, a list of specific inhibitors including SB203580 (p38 MAPK inhibitor), PF3644022 (MK2 inhibitor), UO126 (p42/p44 MAPK inhibitor), LY294002 (PI3K inhibitor) were employed in our study. Conditioning of SB203580 and PF3644022 but not UO126 or LY294002 resulted in decreasing level of PFKFB3 phosphorylation (Fig. 4B) and F2,6BP production (Fig. 4C) in MCF-7 and MDA-MB-231 cells. These results revealed that p38 MAPK/MK2 was responsible for PFKFB3 phosphorylation induced by Shh in breast cancer cells.

4. Discussion

Aberrant expression of Shh expression and Shh signaling activation were observed in various malignant cancers [22,23]. In breast carcinomas, Shh protein expression was significant higher than that in normal breast tissues [9]. Shh expression in breast carcinomas was correlated significantly with early clinical stage [9].

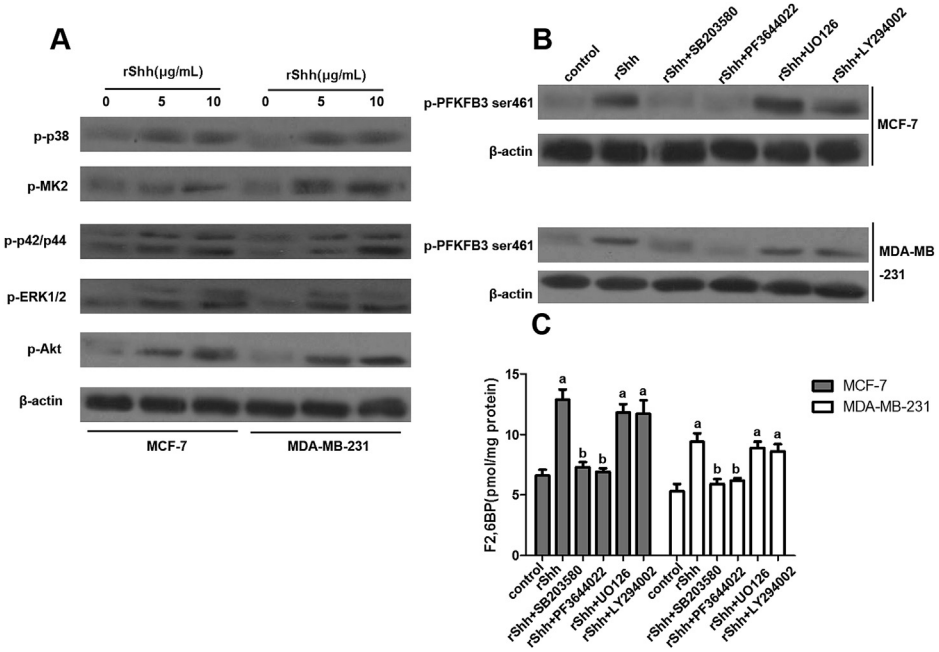

Fig. 4. MAPK activation and PFKFB3 phosphorylation in MCF-7 and MDA-MB-231 cells by rShh treatment. A. Immunoblot detection of p-p38, p-MAPK-activated protein kinase 2 (MK2), p-p42/p44, p-ERK1/2, p-Akt expression in MCF-7 and MDA-MB-231 cells treated with 0, 5, 10 μg/mL rShh for 1 h; B. p-PFKFB3 (ser461) expression of MCF-7 and MDA-MB-231 cells treated rShh (10 μg/mL), SB203580 (10 nM), PF3644022 (10 nM), UO126 (10 nM), or LY294002 (10 nM); C. Measurement of F2,6BP concentration in MCF-7 and MDA-MB-231 cells. [a] compared with control, $p < 0.05$; [b]compared with rShh group, $p < 0.05$.

Investigators also demonstrated that the downstream of Shh, Gil-1 was crucial for hypoxia-induced epithelial–mesenchymal transition (EMT) and invasion of breast cancer cells and may be a therapeutic target for intervention of breast cancer metastasis [24]. In this study, we compared the expression of Shh protein and Gli-1 protein between breast carcinomas and normal tissues. Enhanced Shh and Gli-1 expressions were found in the breast cancer tissues. Shh protein mostly located nears the plasma membrane while Gli-1 protein located in the nucleus. The correlations between Shh expression or Gli-1 expression and clinicopathological variables were also analyzed. These data showed positive correlations between Shh expression or Gli-1 expression and tumor size and tumor stage, providing a robust hint that Shh signaling might play a role in breast carcinogenesis.

For in vitro study, the study here employed a recombinant protein-rShh to simulate the effect of Shh signaling on breast cancer. In the breast cancer cell lines, rShh significantly boosted Smo and Gli-1 expression as well as the glucose utilization and lactate production. Accompanying with the accelerated glycolytic process, enhanced proliferation of breast cancer cells was also observed. This evidence characterized the regulation role of Shh signaling in glycolysis and proliferation in breast cancer cells.

Emerging evidence has protruded the role of PFKFB3 in tumor cell growth [25]. Forced expression of PFKFB3 could enhance glycolysis in breast cancer cells, leading to increased lactate production and cell proliferation [26]. Furthermore, enhanced phosphorylation of PFKFB3 protein was found in human tumor cells, vascular endothelial cells, and smooth muscle cells [26]. In our investigation, rShh exposure did not influence the expression of PFKFB3 mRNA and protein in MCF-7 and MDA-MB-231 cells. In contrast, the phosphorylation of PFKFB3 (ser^{461}) along with F2,6BP production, was markedly raised by rShh treatment. Further analysis found that inhibition of Smo but not Gli-1 expression abrogated PFKFB3 (ser^{461}) phosphorylation by rShh conditioning. Thus our study first demonstrated that Shh might contribute to PFKFB3 (ser^{461}) phosphorylation via Smo.

Previous study demonstrated that PFKFB3 phosphorylation at Ser461 was mediated by MK2 [20]. Also, the ERK (extracellular-signal-regulated kinase)/RSK (ribosomal S6 kinase) pathway might contribute to PFKFB3 phosphorylation [19]. Therefore, our study sought to delineate the pathway involved in PFKFB3 phosphorylation by rShh stimulation. Signaling pathways of the insulin growth factor (IGF), placental growth factor, PI3K/AKT/mammalian target of rapamycin (mTOR), WNT, and Notch pathways have been identified to interact with Shh signaling [27]. Shh has also been shown to regulate myoblast proliferation and differentiation via the MAPK/ERK and the PI3K/Akt signaling pathways [28]. The p38 MAPK activity was shown to play a central role of in modulating Shh-mediated gene transcription in astrocytes [21]. The current study thus examined the intercourse between Shh signaling pathway and MAPK signaling pathway. The expressions of p-p38 MAPK, p-MK2, p-p42/p44 MAPK, p-ERK, and p-Akt were analyzed in MCF-7 and MDA-MB-231 cells which were exposed to rShh in prior. Under this condition, p-p38 MAPK, p-MK2, p-p42/p44 MAPK, p-ERK, and p-Akt were all upregulated by rShh. As following, the inhibitors including SB203580 (for p38 MAPK), PF3644022 (for MK2), UO126 (for p42/p44 MAPK), and LY294002 (for PI3K) were used in the screening of the executor responsible for PFKFB3 phosphorylation. We found that treatment of SB203580 and PF3644022 remarkably reduced PFKFB3 phosphorylation and F2,6BP production, which suggested the role of p38 MAPK/MK2 in PFKFB3 activation.

Collectively, the study reported here demonstrated that Shh signaling might contribute to PFKFB3 activation via Smo and p38 MAPK/MK2, causing accelerated glycolytic process and cell proliferation in breast cancer cells. Therefore, our study might provide more details in elucidating metabolic transition of breast cancers.

Conflict of interest

The authors declare no conflict of interest.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.052.

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