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# Lactate dehydrogenase-A is indispensable for vascular smooth muscle cell proliferation and migration

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ABSTRACT

The proliferation and migration of vascular smooth muscle cells (VSMCs) have been implicated in the pathogenesis of atherosclerosis. Increased aerobic glycolysis is a key feature of cellular phenotypes including cancer and immune cells. However, the role of aerobic glycolysis in the atherogenic phenotype of VSMCs remains largely unknown. Here, we investigated the role of lactate dehydrogenase-A (LDHA), which is a key enzyme for glycolysis, in the proliferation and migration of VSMCs. Activation of primary rat VSMCs with fetal bovine serum (FBS) or platelet-derived growth factor (PDGF) increased their proliferation and migration, glycolytic activity, and expression of LDHA. Wound healing and transwell migration assays demonstrated that small interfering RNA-mediated knockdown of LDHA and pharmacological inhibition of LDHA by oxamate both effectively inhibited VSMC proliferation and migration. Inhibition of LDHA activity by oxamate reduced PDGF-stimulated glucose uptake, lactate production, and ATP production. Taken together, this study shows that enhanced glycolysis in PDGF- or FBS-stimulated VSMCs plays an important role in their proliferation and migration and suggests that LDHA is a potential therapeutic target to prevent vessel lumen constriction during the course of atherosclerosis and restenosis.



## 1. Introduction

Characteristic metabolic reprogramming in proliferative cells has emerged as a crucial player in determination of their phenotypes and signal transduction processes [1]. Although mainly studied in cancer models, growth factors influence cell proliferation by altering intracellular metabolism; therefore, several key enzymes that regulate glycolysis or mitochondrial respiration have been proposed as targets for the treatment of cancer [2,3]. A Warburg-like increase in the glycolytic rate also appears to be inherent to non-malignant cells such as immune and skeletal muscle cells [4–6]. Several recent studies suggested that metabolic reprograming in vascular smooth muscle cells (VSMCs) participates in the complex regulation of their proliferation and migration [7–9].

VSMC proliferation and migration are involved in physiological processes such as development, wound healing, and angiogenesis [10]. However, once tissue repair is complete, the continuation of VSMC proliferation and migration due to excessive mitogens such as growth factors in the cellular microenvironment induces pathogenic vessel lumen constriction during the course of atherosclerosis and restenosis [11,12]. For this reason, extensive research has focused on elucidating the intracellular mechanisms involved in the regulation of VSMC proliferation and migration [13–15]. Recent studies showed that enhanced glycolysis may be required for

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platelet-derived growth factor (PDGF)-induced VSMC proliferation, supported by increased glycolytic enzyme expression, lactate production, and glucose utilization [7,8].

Modulating VSMC metabolism via anti-glycolytic therapies may be a means to treat atherosclerosis and restenosis because increased glycolysis is associated with the proliferation of VSMCs and cancer cells [8]. The enzyme lactate dehydrogenase-A (LDHA) is of particular interest because it catalyzes the nicotinamide adenine dinucleotide-dependent reduction of pyruvate to lactate, an essential step for regenerating nicotinamide adenine dinucleotide, which is needed to maintain glycolysis and other metabolic activities [16–18]. However, the role of LDHA in VSMC proliferation and migration has not been investigated. Therefore, we aimed to examine the role of LDHA in the proliferation and migration of VSMCs.

## 2. Material and methods

### 2.1. Immunohistochemical staining of balloon-injured rat carotid arteries

The rat carotid artery balloon-injury method was described previously [19]. Rat arterial tissue was fixed in 4% paraformaldehyde and embedded in paraffin. For immunohistochemical staining, paraffinized aorta sections were deparaffinized with xylene and ethanol. Endogenous peroxidase was blocked by treatment with 3% $H_2O_2$ for 15 min and then samples were incubated with an anti-LDHA antibody (Cell Signaling Technology, 1:100). Blocking of endogenous peroxidase and protein detection were performed using an Ultravision LP Detection System HRP Polymer kit (Lab Vision, Fremont, CA, USA) according to the manufacturer's protocol.

### 2.2. Cell culture

Rat aortic smooth muscle cells were cultured using a transplant method as described previously [20]. Cells were isolated from a 4-week-old Sprague-Dawley male rat (Hyochang Science, Daegu, Korea). The trimmed aorta was washed with sterilized cold phosphate-buffered saline (PBS) and sliced into pieces measuring 1–3 $mm^2$. The pieces of aorta were attached to dishes and cultured in low-glucose Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) for 2 weeks at 37 °C in 5% $CO_2$. The medium was changed every day. Cells were maintained in Dulbecco's Modified Eagle Medium containing 5.5 mM glucose and supplemented with 10% FBS. Cells at passage 4–6 were used in all experiments.

### 2.3. Western blot analysis

Cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM $Na_3VO_4$, 1% NP-40, and protease and phosphatase inhibitors. Protein samples were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked with 5% skimmed milk prepared in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with each primary antibody overnight at 4 °C. The primary antibody against LDHA (1:1000) was purchased from Cell Signaling Technology (Beverly, MA, USA), and that against β-actin (1:5000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were thrice washed with TBST and incubated with a HRP-conjugated mouse (Santa Cruz) or rabbit (Cell Signaling Technology) secondary antibody. HRP was detected using ECL reagent (Bionote, Suwon, Gyeonggido, Korea).

### 2.4. LDHA activity

LDHA activity was determined by measuring the consumption of NADH in 20 mM HEPES-$K^+$ (pH 7.2), 0.05% bovine serum albumin, 20 μm NADH, and 2 mM pyruvate using a microplate reader (excitation, 340 nm; emission, 460 nm) as previous described [21,22].

### 2.5. siRNA transfection

For gene silencing, cells were transfected with 50 nM scrambled siRNA or siLDHA (Bioneer, Daejeon, Korea) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### 2.6. Cell counting

Non-transfected VSMCs and those transfected with scrambled small interfering RNA (siRNA) or siRNA targeting LDHA (siLDHA) for 24 h were serum-starved for 18 h and incubated with 10% FBS or 20 ng/ml rat recombinant PDGF (R&D Systems, Minneapolis, MN, USA) with or without 60 mM sodium oxamate (Sigma, St. Louis, MO, USA). Cells were trypsinized, stained with trypan blue solution, and counted with a hemocytometer.

### 2.7. Migration assay

For the wound healing assay, a wound was generated by scratching VSMCs with a 200 μl pipette tip. When the wound had closed, cells were washed thrice with PBS, fixed in 4% paraformaldehyde, washed thrice with PBS, and stained with 0.05% crystal violet. For the transwell migration assay, cells were seeded in a membrane transwell with a pore size of 8 μm (Corning Incorporated, New York, NY, USA). After being washed thrice with PBS, cells were fixed in methanol. Cells inside the transwell were removed with a swab. Migrated cells were washed thrice with PBS, stained with Mayer's hematoxylin (Sigma), washed with PBS, and dried completely.

### 2.8. Glucose uptake, lactate, and ATP assays

Glucose uptake was measured using a Glucose Uptake Colorimetric Assay Kit (Biovision, Milpitas, CA, USA). Lactate and ATP were measured using assay kits (Bioassay Systems, Hayward, CA, USA) according to the manufacturer's protocol.

Determination of the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR).

Primary VSMCs were seeded in a Seahorse XF24 plate, serum-starved for 18 h, and incubated with or without PDGF or sodium oxamate for 24 h. Then, the basal extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured and automatically calculated using a Seahorse XF24 analyzer (Seahorse Bioscience, North Billerica, MA, USA).

### 2.9. Statistical analysis

All values are presented as the mean ± SEM. Statistical analysis was performed using the two-tailed Student's t-test. $p < 0.05$ was considered statistically significant.

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### 2.10. Study approval

All animal procedures were approved by the Institutional Animal Care and use Committee of Kyungpook National University.

## 3. Results

LDHA expression is upregulated in the neointima of a balloon-injured rat carotid artery and in PDGF- or FBS-stimulated VSMCs.

To elucidate the role of LDHA in the proliferation and migration of VSMCs, we first measured the expression levels of LDHA in the balloon injury-induced neointima and growth factor-stimulated VSMCs. Immunohistochemical staining showed that LDHA expression was significantly higher in the neointimal region of rat carotid arteries after balloon angioplasty than in the arteries of sham-operated control mice (Fig. 1A). Consistently, LDHA protein level and activity were markedly higher in PDGF- or FBS-stimulated VSMCs (Fig. 1B-D).

### 3.1. Inhibition of LDHA suppresses PDGF- or FBS-stimulated VSMC proliferation and migration

In the next series of experiments, we evaluated whether the observed enhancement of LDHA expression upon balloon injury or growth factor stimulation plays a crucial role in VSMC proliferation and migration by knocking down LDHA using siRNA. The successful knockdown of LDHA in FBS- or PDGF-stimulated VSMCs was confirmed by western blot analysis and by measuring LDHA activity (Fig. 2A–D). As reported previously [7,8], treatment of VSMCs with FBS or PDGF significantly increased their proliferation, but this was prevented by siLDHA (Fig. 2E and F). We examined the effects of siLDHA on FBS- or PDGF-stimulated VSMC migration by performing

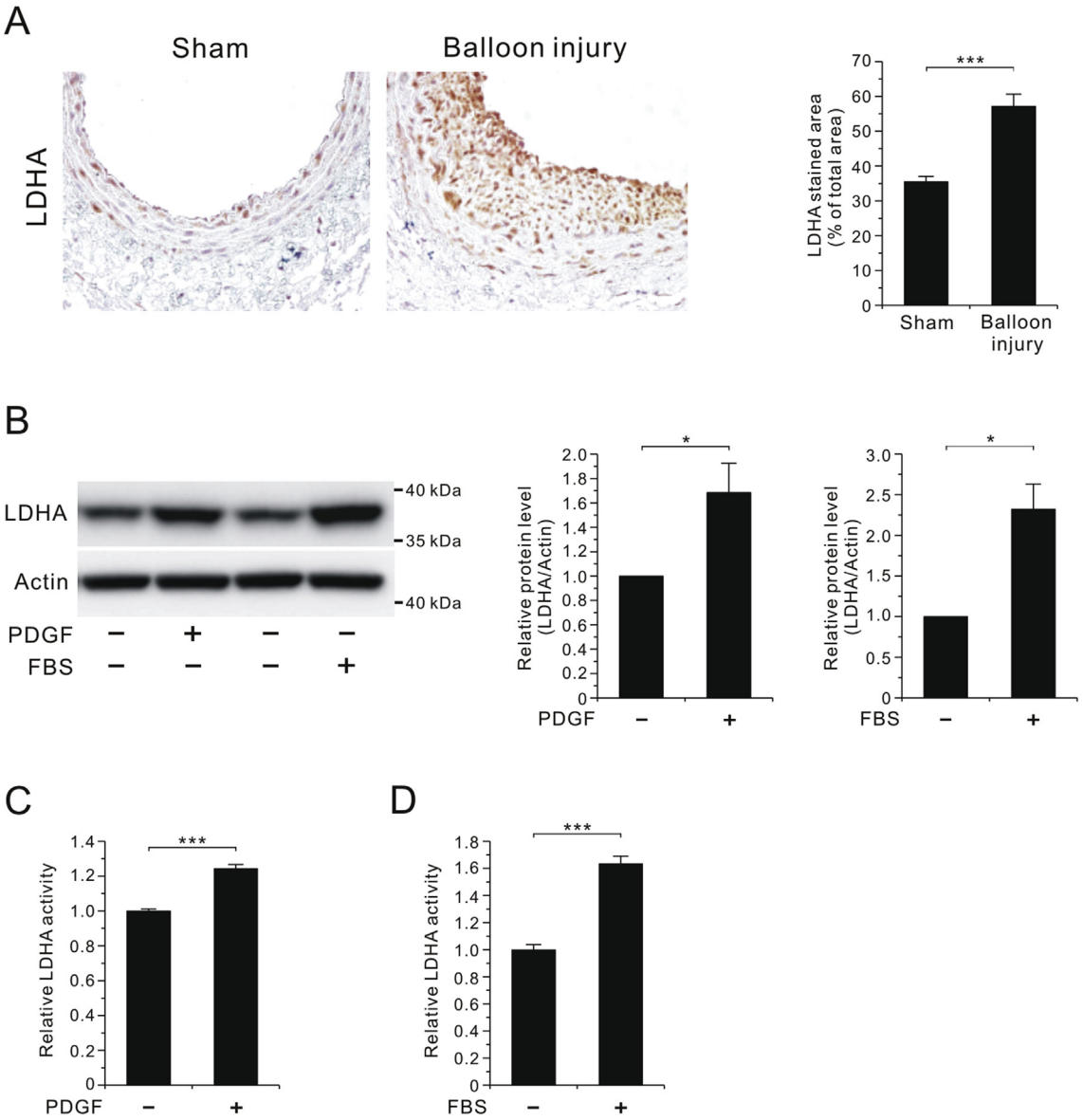


**Fig. 1. LDHA levels in the balloon injury-induced neointima and growth factor-stimulated VSMCs.** (A) Sham and balloon-injured rat common carotid arteries were immunohistochemically stained with an anti-LDHA antibody (left). Quantification of the LDHA-stained area (right). Data are expressed as the mean ± SEM (n = 6). ***p < 0.001. (B) Western blot analysis showing the LDHA protein levels in PDGF- and FBS-treated VSMCs (left). Primary rat VSMCs were serum-starved for 18 h and then treated with 20 ng/ml PDGF or 10% FBS for 24 h. Quantification of relative LDHA levels (right). Data are expressed as the mean ± SEM (n = 3). *p < 0.05. (C and D) LDHA activity in PDGF-treated (C) and FBS-treated (D) VSMCs. Data are expressed as the mean ± SEM (n = 6). ***p < 0.001.

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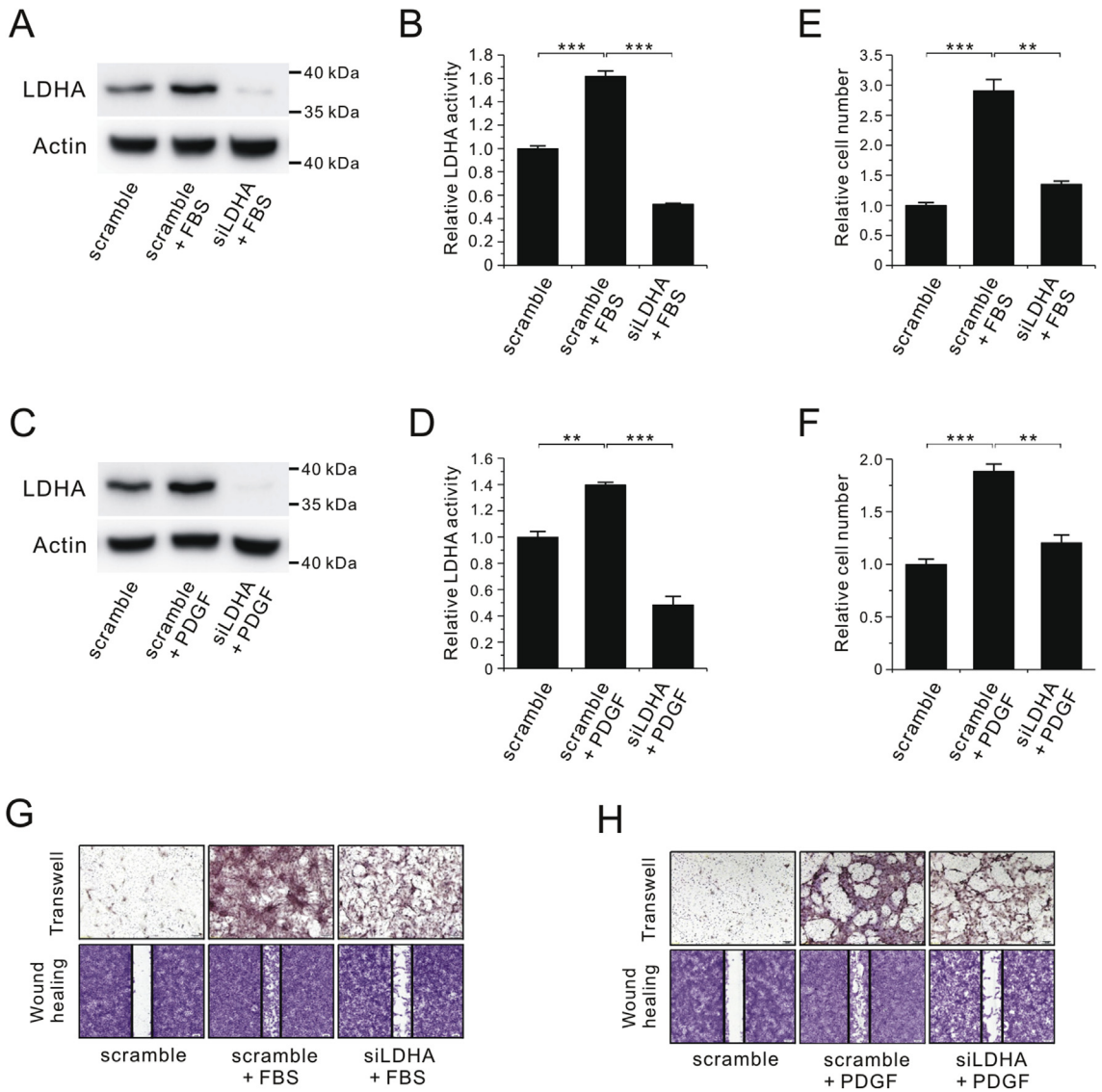


**Fig. 2. siLDHA suppresses FBS- or PDGF-induced VSMC proliferation and migration.** (A–D) Western blot analysis (A and C) and LDHA activity (B and D) showing the knockdown efficiency of siLDHA. Data are expressed as the mean ± SEM (n = 3). **p < 0.01 and ***p < 0.001. (E and F) Relative cell numbers showing the effect of siLDHA on FBS-stimulated (E) or PDGF-stimulated (F) VSMC proliferation. Data are expressed as the mean ± SEM (n = 3). **p < 0.01 and ***p < 0.001. (G and H) Transwell assay (upper) and wound healing assay (lower) showing the effects of siLDHA on FBS-stimulated (G) or PDGF-stimulated (H) VSMC migration. Primary rat VSMCs were transfected with scrambled siRNA or siLDHA for 24 h, serum-starved for 18 h, and then treated with 10% FBS or 20 ng/ml PDGF.

the transwell migration and wound healing assays. siLDHA treatment significantly suppressed the FBS- and PDGF-induced increases in VSMC migration (Fig. 2G and H).

We further confirmed the role of LDHA in the FBS- and PDGF-induced increases in VSMC proliferation and migration by using a pharmacologic inhibitor of LDHA, oxamate. Oxamate treatment blocked FBS- and PDGF-stimulated LDHA activity (Fig. 3A and B). Consistent with the results obtained with siLDHA-treated cells, oxamate treatment significantly suppressed VSMC proliferation and migration induced by FBS or PDGF (Fig. 3C–F).

### 3.2. LDHA inhibition reduces PDGF-stimulated glycolysis in primary rat VSMCs

To understand the metabolic role of LDHA in VSMC proliferation and migration, we examined the effects of LDHA inhibition on glucose metabolism in PDGF-stimulated VSMCs. As expected, PDGF treatment significantly increased glucose uptake (Fig. 4A) and increased lactate production and ATP production (Fig. 4B and C). Moreover, PDGF treatment increased both the ECAR and OCR, as measured with an XF analyzer (Fig. 4D and E). Collectively, these data indicate that glycolysis and glucose oxidation were increased in PDGF-stimulated VSMCs due to enhanced glucose uptake. However, the PDGF-induced increases in glucose uptake, lactate and ATP production, and the ECAR and OCR were markedly reduced when LDHA activity was suppressed by oxamate (Fig. 4).

## 4. Discussion

The present study showed that upregulation of LDHA and increased glycolysis in growth factor-stimulated VSMCs play an important role in their proliferation and migration. Stimulation of VSMCs with PDGF led to an increased glycolytic rate accompanied by elevated cellular glucose uptake and lactate production. It also

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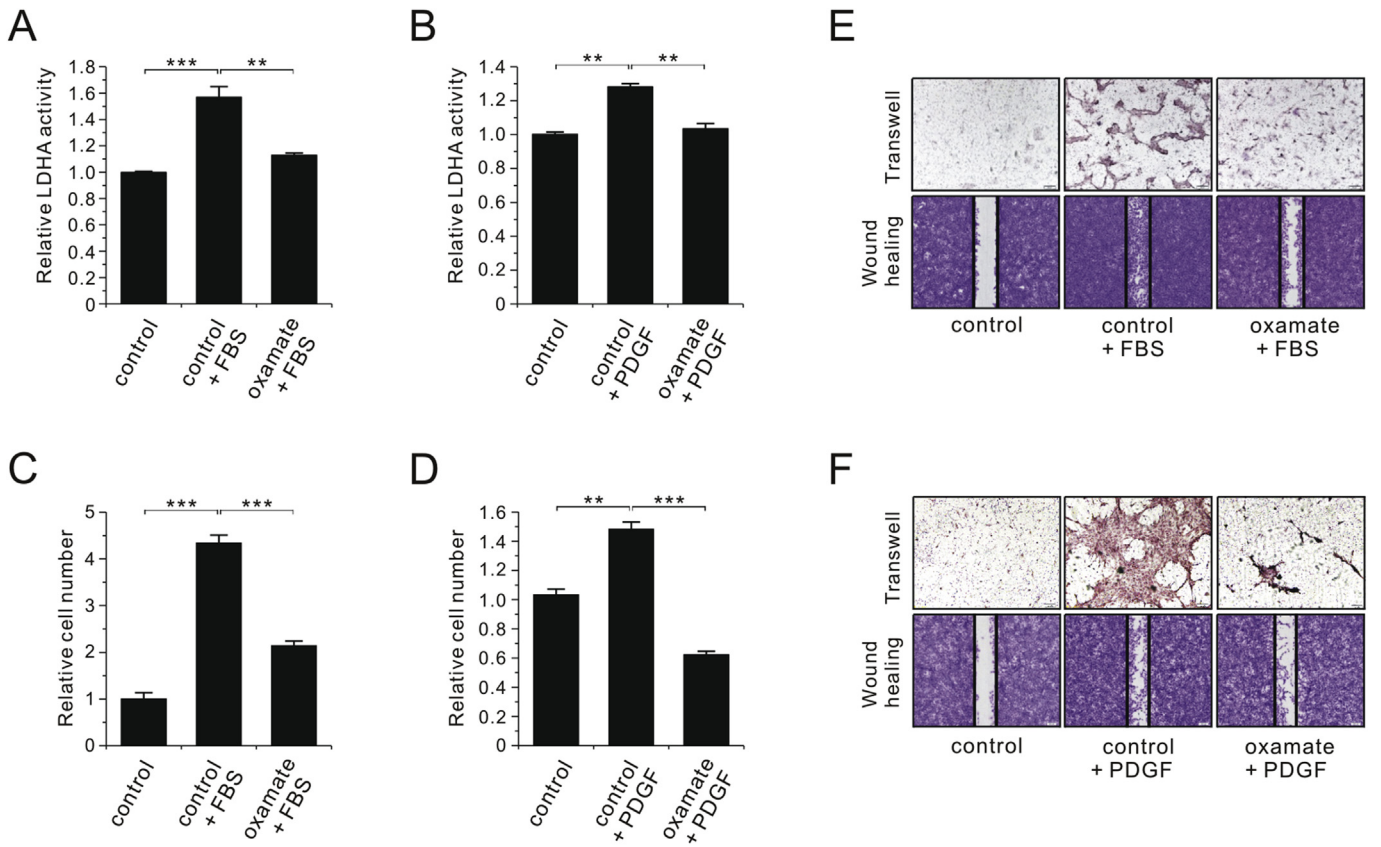


**Fig. 3. Pharmacologic inhibition of LDHA suppresses FBS- or PDGF-stimulated VSMC proliferation and migration**. (A and B) LDHA activity showing the effect of oxamate on LDHA activity in FBS-stimulated (A) or PDGF-stimulated (B) VSMCs. Data are expressed as the mean ± SEM (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$. (C and D) Relative cell numbers showing the effect of oxamate on FBS-stimulated (C) or PDGF-stimulated (D) VSMC proliferation. Data are expressed as the mean ± SEM (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$. (E and F) Transwell assay (upper) and wound healing assay (lower) showing the effect of oxamate on FBS-stimulated (E) or PDGF-stimulated (F) VSMC migration. Primary rat VSMCs were serum-starved for 18 h and then treated with 10% FBS or 20 ng/ml PDGF with or without 60 mM sodium oxamate.

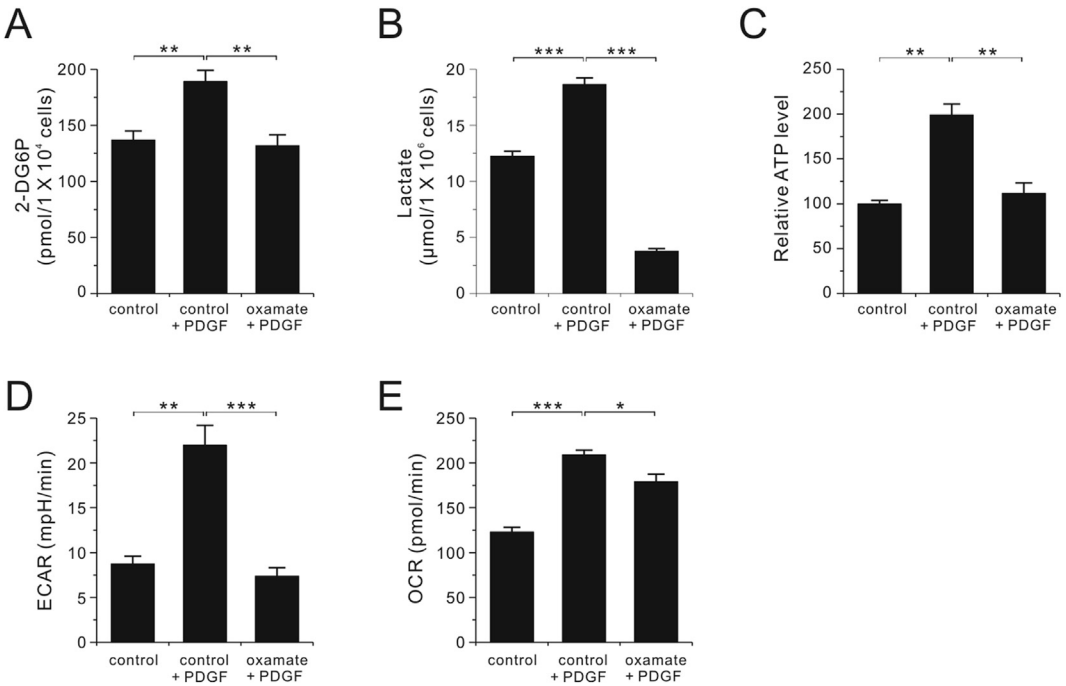


**Fig. 4. Inhibition of LDHA reduces glucose uptake and glycolysis in PDGF-stimulated VSMCs**. (A) Glucose uptake level, (B) lactate level in media, (C) ATP level, (D) ECAR, and (E) OCR in VSMCs. Primary rat VSMCs were serum-starved for 18 h and then treated with 20 ng/ml PDGF with or without 60 mM sodium oxamate for 24 h. Data are expressed as the mean ± SEM (n = 4 (A), n = 3 (B and C), and n = 5 (D and E)). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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enhanced glucose oxidation and ATP production in VSMCs. However, inhibition of LDHA significantly suppressed PDGF-stimulated glycolysis and glucose oxidation, and also markedly blunted VSMC proliferation and migration under FBS or PDGF stimulation.

Increased aerobic glycolysis appears to be a cellular response to diverse biologic cues and needs and is not restricted to cancer cells [4–9]. Several publications have reported that increased glycolytic flux due to growth factors is critical for the bioenergetic shift that occurs during VSMC proliferation and migration [9,23]. Unlike most differentiated cells, VSMCs maintain phenotypic plasticity switching between a 'differentiated' (also termed “contractile”) and a 'dedifferentiated' (also termed “synthetic” or “proliferative”) state [24]. Recent studies showed that glucose metabolism is a primary regulator of VSMC phenotype and proliferative capacity [7,8,25]. Treatment of VSMCs with PDGF significantly increases glycolytic flux, cell proliferation, and the expression of osteopontin and vimentin, markers of the synthetic VSMC phenotype [7]. Inhibition of glycolysis does not cause cell death, but decreases proliferation and prevents the upregulation of osteopontin and vimentin gene expression [30]. Although there has been much progress in understanding the molecular mechanisms that regulate VSMC phenotype switching in recent years, more work is required to understand the direct effects of metabolic changes on VSMC proliferation and migration. Here, we show that LDHA levels were significantly higher in balloon-injured neointima and growth factor-stimulated VSMCs. Moreover, PDGF-treated VSMCs showed increased lactate production and ECAR. However, our study also showed that PDGF-treated VSMCs had higher OCR, suggesting that proliferative VSMCs constitute a state of increased glycolysis and glucose oxidation. Therefore, the reprogramming of glucose metabolism in proliferative and migratory VSMCs needs to be clarified.

LDHA has been suggested as an attractive target for cancer therapies that affect the metabolism of cancer cells by decreasing aerobic glycolysis and suppressing growth [17]. When the balance of flux across the LDHA pathway is perturbed, cellular homeostasis is disrupted, resulting in a loss of metabolic efficiency and a new metabolic phenotype in various cancer cells [26–28]. Inhibition of LDHA in cancer cells significantly reduces glucose consumption, resulting in lower glucose uptake and glycolytic rates [28]. Since the pentose phosphate pathway, a representative glucose-derived biosynthetic pathway, and glycolysis share several common metabolites, a decrease in the glycolytic rate affects the flux balance of pentose cycle intermediates by decreasing the contribution of the G6PDH pathway to lactate production [29]. Thus, LDHA inhibition alters flux through affluent pathways to the pyruvate pool [29]. Because increased glycolysis is characteristic not only of cancer cells but also of non-cancer cells [2,4–6], LDHA could be used as a target for the treatment of atherosclerosis caused by the excessive proliferation of VSMCs. Our data demonstrating a decrease in glucose uptake, ATP production, and ECAR in response to inhibition of LDHA is agreement with the results of previous work on cancer cells in other contexts. Accordingly, this study showed that inhibition of LDHA abrogated growth factor-stimulated VSMC proliferation and migration, suggesting that enhancement of glycolysis by LDHA is essential for VSMCs to acquire their full proliferative and migratory potential.

In summary, we have shown that glycolysis regulated by LDHA is important not only for the proliferation but also the migration of VSMCs. Although the precise molecular mechanism by which LDHA functions in VSMC proliferation and migration remains to be investigated, targeting LDHA may be a novel therapeutic option to treat diseases related with uncontrolled VSMC proliferation and migration such as atherosclerosis and restenosis, thereby extending pharmacological research of LDHA from cancer to vascular biology.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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