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Extracellular acidification synergizes with PDGF to stimulate migration of mouse embryo fibroblasts through activation of p38MAPK with a PTX-sensitive manner

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ABSTRACT

The elucidation of the functional mechanisms of extracellular acidification stimulating intracellular signaling pathway is of great importance for developing new targets of treatment for solid tumors, and inflammatory disorders characterized by extracellular acidification. In the present study, we focus on the regulation of extracellular acidification on intracellular signaling pathways in mouse embryo fibroblasts (MEFs). We found extracellular acidification was at least partly involved in stimulating p38MAPK pathway through PTX-sensitive behavior to enhance cell migration in the presence or absence of platelet-derived growth factor (PDGF). Statistical analysis showed that the actions of extracellular acidic pH and PDGF on inducing enhancement of cell migration were not an additive effect. However, we also found extracellular acidic pH did inhibit the viability and proliferation of MEFs, suggesting that extracellular acidification stimulates cell migration probably through proton-sensing mechanisms within MEFs. Using OGR1-, GPR4-, and TDAG8-gene knock out technology, and real-time qPCR, we found known proton-sensing G protein-coupled receptors (GPCRs), transient receptor potential vanilloid subtype 1 (TRPV1), and acid-sensing ion channels (ASICs) were unlikely to be involved in the regulation of acidification on cell migration. In conclusion, our present study validates that extracellular acidification stimulates chemotactic migration of MEFs through activation of p38MAPK with a PTX-sensitive mechanism either by itself, or synergistically with PDGF, which was not regulated by the known proton-sensing GPCRs, TRPV1, or ASICs. Our results suggested that others proton-sensing GPCRs or ion channels might exist in MEFs, which mediates cell migration induced by extracellular acidification in the presence or absence of PDGF.

1. Introduction

A variety of disorders are accompanied by acidification of microenvironments. These disorders include, but are not limited to solid tumors and inflammations including atherosclerosis [1], asthma [2], infection [3]. Tumor hypoxia [4] and the accumulation of inflammatory cells [1] cause the formation of acidic microenvironments within solid tumors and inflammatory lesions. Acidic microenvironments in turn exert largely influential effects on the development of tumors and inflammations. For example, extracellular acidic pH has been reported to promote the invasion and metastasis of melanoma cells, and also to promote local invasion of human breast cancer cells and human colon cancer cells [5].

Ovarian cancer G protein-coupled receptor 1 (OGR1), G protein-coupled receptor 4 (GPR4), T-cell death associated gene 8 (TDAG8), and G2 accumulation (G2A) share 40%–50% homology with each other and form the OGR1 family of GPCRs. These OGR1 family receptors have been shown to be specific receptors for lysolipids, such as sphingosylphosphorylcholine and lysophosphatidylcholine. In 2003 Ludwig et al., however, first showed that OGR1 and GPR4 sense extracellular protons or pH and are coupled to G-proteins to stimulate intracellular signaling pathways [6]. Shortly after

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Ludwig et al. made their discovery, G2A and TDAG8 were also shown to sense extracellular proton concentrations. Thus, OGR1, GPR4, G2A and TDAG8 are unique GPCRs that recognize both lipids and protons as ligands. In addition to these proton-sensing GPCRs, some ion channels have also been reported to be modulated by extracellular acidification, including proton-sensing capsaicin-sensitive transient receptor potential vanilloid subtype 1 (TRPV1) [7] and acid-sensing ion channels (ASICs) [8].

Cell migration is essential for normal embryonic development, immune system function, angiogenesis, tissue repair and regeneration, but it is also conversely associated with inflammatory disease, vascular impairment, and tumor cell invasion. Cell migration is a complex process that involves multiple intracellular signal pathways. Of the signal pathways involved, the GPCRs/Gi/p38MAPK signal cascade exerts its effects on cell migration by remodeling the actin cytoskeleton. Previous studies have shown that the migration of vascular smooth muscle cells [9], prostate cancer PC3 cells [10], and glioma cells [11] induced by LPA are mediated at least partly via the Gi/p38MAPK pathway.

In the present study, we aimed to examine the effects of extracellular acidification on intracellular signaling pathways of MEFs. Our observation is that this is the first time to detect and document the effects of extracellular acidification on intracellular signaling pathways in MEFs.

2. Materials and methods

2.1. Materials

YM-254890 was generously provided by Dr. M.Taniguchi (Astellas, Tsukuba, Japan). The mouse G2A (Mm00490809)-, OGR1 (Mm01335272)-, TDAG8 (Mm00433695)-, GPR4 (Mm00558777)-, and GAPDH (4352932E)-specific probes for real-time PCR were obtained from TaqMan gene expression assays from Applied Biosystems (Foster City, CA). The sources of all other reagents were described previously [12].

2.2. Ethics statement

All animal procedures were performed in strict accordance with the guidelines of the Animal Care and Experimentation Committee of Gunma University (Maebashi, Japan), and all animals were bred in the Institute of Animal Experimental Research of Gunma University. The protocol was approved by the Animal Care and Experimentation Committee of Gunma University (Permit Number: 11-019). Diethyl ether anesthesia of experimental mice was used as the method of sacrifice, and all efforts were made to minimize suffering.

2.3. $TDAG8^{Tp/Tp}$, $OGR1^{geo/geo}$, and GPR4 knock-out mice

$TDAG8^{TP/TP}$ mice were obtained by backcrossing to C57BL/6 mice more than five generation from TM88ICR mice, which contains a transposon insertion in the *tdag8*. All the information of $TDAG8^{Tp/Tp}$ [13] and $OGR1^{geo/geo}$ [14] mice is the same as that described previously. The MEFs of $GPR4^{-/-}$ were provided by Prof. F. Okajima and Dr. C. Mogi at Gunma University (Maebashi, Japan). The $GPR4^{-/-}$ mouse will be published elsewhere.

2.4. Mouse embryo fibroblasts isolation and culture

We prepared wild-type and OGR1-, GPR4-, TDAG8-gene-knockout MEFs from mice of C57BL/6 genetic background. MEFs were isolated from the 13.5 days embryos of mice, and methods essentially the same way as previously reported [15].

2.5. Cell migration assay

The migration experiment was performed as described previously [16].

2.6. Western blotting

The detailed method is the same as described previously [17].

2.7. Quantitative PCR using real-time TaqMan technology

Total RNA was isolated by using RNAisoPlus (Takara, Japan) according to instructions from the manufacturer. After DNase I (Promega) treatment to remove possible traces of genomic DNA contaminating in the RNA preparations, 5 μg of total RNA was reverse transcribed using random priming and multiscribe reverse transcriptase according to instructions from the manufacturer (Applied Biosystems). To evaluate expression level of OGR1, TDAG8, G2A and GPR4 mRNAs, quantitative PCR was performed using real-time TaqMan technology. The expression levels of the target mRNAs were normalized to the relative ratio to the expression of GAPDH mRNA. Each real-time PCR assay was performed at least three times, and the results were then expressed as mean ± SEM.

2.8. Cell viability assay

MEFs were subcultured in 48-well plates. When cells had reached 30%–40% confluence, the culture medium was changed to HEPES-buffered DMEM with different pH (pH 7.6, pH 7.2, pH 6.8 and pH 6.4) containing 0.1% BSA in the presence or absence of 20 ng/ml PDGF, then incubated for forty-eight hours. The living cell numbers were measured by MTT assay.

2.9. BrdU (5-bromo-2′-deoxyuridine) incorporation assay

Cell Proliferation was measured by BrdU incorporation assay using BrdU cell proliferation kit from Cell Signaling. MEFs were plated in 6-well plates. When cells had reached 30%–40% confluence, culture medium was changed to HEPES-buffered DMEM with different pH containing 0.1% BSA in the presence or absence of PDGF. MEFs were incubated for twenty-four hours. Cells were then pulsed with 30 uM BrdU for 60 min, fixed and followed by immunodetection of the incorporation of BrdU label. Absorbance was read at 660 nm to determine cell proliferation.

2.10. Data presentation

All experiment procedures were performed in duplicate or triplicate. The results of multiple observations are presented as means ± SEM from more than two different batches of cells unless otherwise stated. Statistical significance was assessed by ANOVA, the student t-test or the paired t-test by SPSS13.0 software; values were considered significant at $p < 0.05$ (*).

3. Results

3.1. Extracellular acidification synergizes with the signals induced by PDGF to modulate MEFs migration

We first examined whether growth factors, such as PDGF, EGF, and IGF-1, induced the migration of MEFs; if so, then whether extracellular acidification imparts significant effects on induction of MEFs migration. Our results showed that at pH 7.6, PDGF significantly enhanced cell migration; however EGF and IGF-1 had no

significant effect on MEFs migration (Fig. 1A). Our results also showed that extracellular acidification induced the migration of MEFs, and largely enhanced PDGF-induced migration of MEFs. Moreover, as the extracellular pH became more acidic, the numbers of cell migration and PDGF-induced cell migration were both significantly increased (Fig. 1B).

Previous study has shown that PDGF positively induced MEFs migration. Our results found that extracellular acidic pH stimulates both MEFs migration and PDGF-induced MEFs migration. As shown in Fig. 1A and B, when pH 6.8 HEPES-buffered DMEM was mixed with PDGF, cell migration was greater than with pH 6.8 adjustments or PDGF alone. To validate whether the increasing effects induced by pH 6.8 and PDGF on MEFs migration are additive or not, we analyzed all the results of 34-time independent migration assays in our study using the paired t-test. Statistical analysis confirmed that pH 6.8- and PDGF-induction increasing effects on MEFs migration were not additive ($t = 7.72$, d.f. $= 33$, $P = 0.000$), and when pH 6.8 and PDGF were mixed together, the number of migrating cells was approximately 50% greater than the sum of migrating cell numbers induced by pH 6.8 and PDGF alone (Data not shown). These findings indicate that signaling pathways initiated by extracellular acidification interact with signaling pathways initiated by PDGF to facilitate directed cell migration.

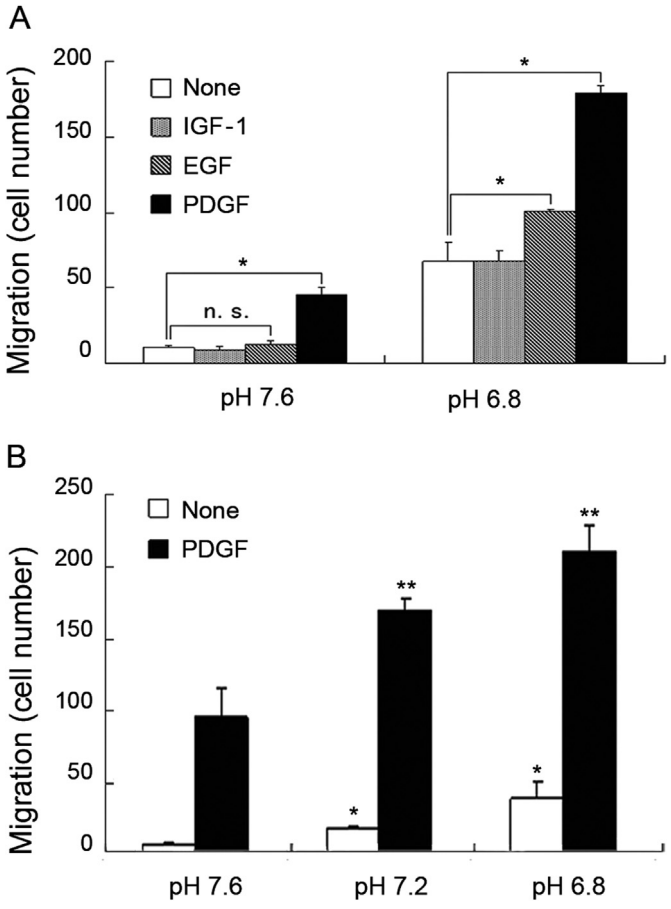

Fig. 1. Extracellular acidification stimulates chemotactic migration and largely enhances PDGF-induced migration in MEFs. (A) Normal MEFs, which were prepared from WT mice, were subjected to migration assay. The cells were stimulated with HEPES-buffered DMEM (pH 7.6 or pH 6.8) in the presence or absence of 10 ng/ml IGF-1, 10 ng/ml EGF and 20 ng/ml PDGF. *Statistically significant. n.s. Not significant. (B) We examined the ability of cell migration at pH 7.6, pH 7.2 and pH 6.8 buffers in the presence or absence of 20 ng/ml PDGF. As shown in B, pH 7.2 and pH 6.8 enhanced the cell migration and PDGF-induced cell migration compared to pH 7.6 with PDGF or without PDGF. Data are the mean ± SEM from more than two independent experiments (n = 3 for each experiment). *$p < 0.05$ versus pH 7.6; **$p < 0.05$ versus pH 7.6 + PDGF.

3.2. Extracellular acidification induced cell migration and enhanced PDGF-induced cell migration in MEFs through proton-sensing mechanism

We wondered if the effects of extracellular acidification on cells migration and PDGF-induced cell migration are due to the changes of the cell's overall viability and proliferation. Therefore we performed MTT assay and BrdU incorporation assay. As shown in Fig. 2A, extracellular acidification inhibited the viability of MEFs in the presence or absence of 20 ng/ml PDGF, when the cells were incubated for forth-eight hours (number of observation was four). BrdU incorporation assay showed that extracellular acidification inhibited the proliferation of MEFs (Fig. 2B).

3.3. Extracellular acidification stimulates MEFs migration and PDGF-induced MEFs migration through the Gi/p38MAPK signal pathway

Based on our current study results described, we began to further research the mechanisms of extracellular acidification-induced MEFs migration in the presence or absence of 20 ng/ml PDGF. By way of migration assay with inhibitors, we found extracellular acidification-induced MEFs migration (with or without PDGF) was completely suppressed when cells were pre-incubated with 50 ng/ml PTX, a specific inhibitor for G_i protein, which suggests that G_i signal pathway was involved in acidification-induced MEFs migration in the presence or absence of PDGF (Fig. 3A). Meanwhile, we also found that YM254890, a specific inhibitor for G_q protein, has no effect on pH6.8-induced cell migration with or without PDGF (Fig. 3B). Western-blotting showed that the protein expression of p38MAPK at pH 6.8 was greater than at pH 7.6 (Fig. 3C and D) and the protein expression of p38MAPK at pH 6.8 containing 20 ng/ml PDGF was also significantly greater than at pH 7.6 with 20 ng/ml PDGF (Fig. 3E and F). Our study results identified extracellular acidification stimulates MEFs migration and PDGF-induced MEFs migration through Gi/p38MAPK signal pathway, suggesting that a pH receptor might be involved in the mechanism.

3.4. The known proton-sensing GPCRs, TRPV1 and ASICs, were unlikely to be involved in the effects of acidification on MEFs migration and PDGF-induced MEFs migration

Using real-time Taqman PCR, we examined which OGR1 family receptors are expressed in MEFs. As shown in Fig. 4A, MEFs express mRNAs of OGR1, GPR4, and TDAG8. To prove which proton-sensing GPCR might be involved in the regulation of acidic pH on cell migration and PDGF-induced cell migration, we used MEFs derived from TDAG8-, GPR4-and OGR1-KO mice, respectively and compared the acidification effect on cell migration and PDGF-induced cell migration with that in normal MEFs derived from WT mice (Fig. 4B and C). Our results showed that extracellular acidification also enhanced cell migration and PDGF-induced cell migration in TDAG8-, GPR4-and OGR1-KO MEFs. There were no statistically significant differences between three proton-sensing GPCRs-KO cells and normal cells. Since G2A mRNA is not expressed in MEFs, our study results suggested that known proton-sensing GPCRs, i.e., TDAG8, GPR4, OGR1 and G2A are not involved in the increasing effects of acidification on cell migration and PDGF-induced cell migration.

Moreover, cross-talk between the signal induced by acidic pH and signal induced by PDGF resulted in the amplification of cell migratory response. The numbers of pH 6.8 enhanced PDGF-

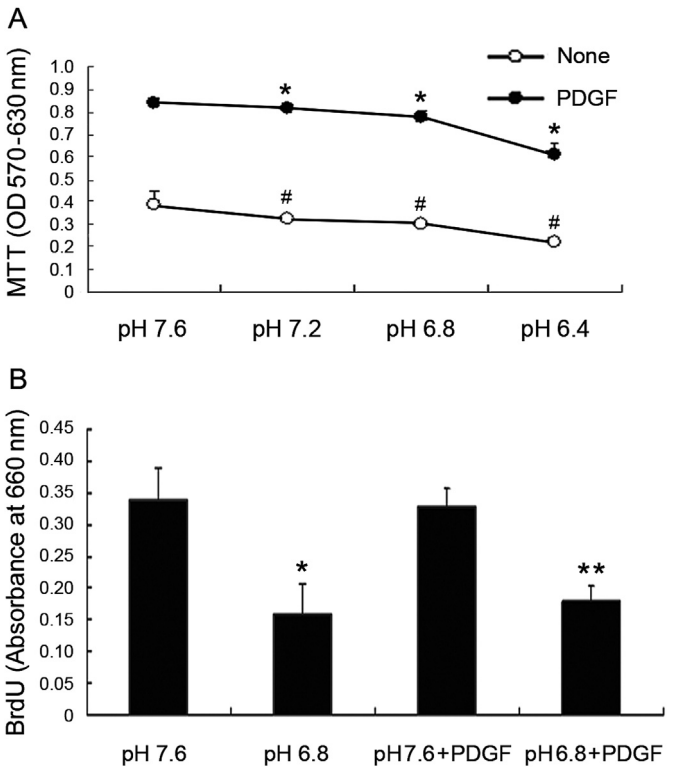

Fig. 2. Extracellular acidification inhibited the viability and the proliferation of MEFs. (A) MEFs were cultured at the indicated pH values in the presence or absence of 20 ng/ml PDGF for 48 h. Cell viability was then evaluated by a 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in MATERIALS AND METHODS. Data are expressed as differences in the absorbance at 570 nm from that at 630 nm $^{*}p < 0.05$ versus pH 7.6 + PDGF; $\#p < 0.05$ versus pH 7.6. (B) MEFs were cultured at pH 7.6 and pH 6.8 in the presence or absence of 20 ng/ml PDGF for 24 h. Cell proliferation was then evaluated by BrdU incorporation assay. Absorbance was read at 660 nm to determine cell proliferation. Data are the mean ± SEM from more than two independent experiments (n = 3 for each experiment). $^{*}p < 0.05$ versus pH 7.6; $^{**}p < 0.05$ versus pH 7.6 + PDGF.

induced cells migration were 50%–70% greater than the sum of migrating cell numbers induced by pH 6.8 and PDGF alone in three genes knock-out MEFs (Data not shown).

Previous studies have shown that the capsaicin-sensitive TRPV1 channels and amiloride-sensitive ASICs can be modulated by acidic pH. We first used capsaicin as a test agent in migration assay and aimed to examine whether proton-sensing TRPV1 channel is involved in the acidification-induced action. As shown in Fig. 4D, there shows no significant difference between capsaicin test and controls. The results verified that proton-sensing TRPV1 channel is not involved in the acidic effect on migration and PDGF-induced cell migration. We then additionally used amiloride, a blocker of ASICs, to examine whether ASICs play a significant role in our study. Results showed that ASICs channel has no effect on MEFs migration induced by extracellular acidification in the presence or absence of 20 ng/ml PDGF (data not shown). Taken together, our results indicated that known proton-sensing GPCRs, TRPV1, and ASICs, might not be involved in acidic effects on MEFs migration and PDGF-induced MEFs migration.

4. Discussion

Hypoxia is a characteristic of solid tumors, results from an imbalance between O_2 supply and its consumption. Major causative factors of tumor hypoxia are abnormal structure and function of the microvessels supplying the tumor, increased diffusion distances between nutritive blood vessels and the tumor cells, and a reduced transport capacity of the blood due to the presence of disease-related anemia. The hypoxic conditions preclude the cells from running their mitochondrial respiratory chain. To satisfy their energy demands, tumor cells switch to a glycolytic metabolism resulting in an increased consumption of glucose and an extensive production of lactate and protons, which causes intracellular and extracellular acidosis in tumor cells. To prevent major cytosolic acidification and to cope with abnormal extracellular acidification, tumor cells developed numerous defense mechanisms that also affect their migratory machinery [4]. Metastasis is the most deadly aspect of cancer, and the extent to which a tumor metastasizes correlates with the tumor cells' migratory activity. Hence, studying the effects of extracellular acidification on the cell migration will contribute to our further understandings of the relationship between tumor metastasis and its acidic microenvironments, and understanding these relationships will provide valuable scientific evidence to further identify new targets for the treatment of related disease.

Up to now, only a few papers discussed the relationship between extracellular acidification and cell migration. The direct effects of extracellular acidification on migration in a variety of cell types as well as their underlying mechanisms still remain unknown. In our study, we investigate specifically the effects of acidic-pH on MEFs migration. Our results indicated that extracellular acidification stimulates MEFs migration at least partly through activation of p38MAPK with a PTX-sensitive manner either by itself or synergistically with PDGF.

Previous studies have reported that some signals may interact with each other through cross-talk. For example, LPA synergistically induced COX-2 expression, PGT2 production and MKP-1 expression under acidic pH through crosstalk between LPA receptors and OGR1, and the signals initiated by LPA synergizes with signaling pathways initiated by PDGF to facilitate directed cell migration. Our study verified that the signals induced by extracellular acidification have a cross talk with signals induced by PDGF. This is the first indication that signals induced by extracellular acidification interact with signals induced by PDGF and induce cell migration.

Accumulating evidences validated that a number of disorders are accompanied by acidification of microenvironments, such as solid tumors and inflammatory diseases including atherosclerosis [1], asthma [2], infection [3]. Acidic microenvironments also further enhanced the invasion and metastasis of tumors as well as the development of inflammations partly through activation of cell migration. PDGF and PDGF receptors have been reported to correlate with a variety of diseases through activation of cell migration, which include tumors, restenosis, accelerated atherosclerosis in the transplanted heart, cardiac fibrosis and other fibrotic diseases characterized by fibroblasts activation [18]. Our present study validate that there is a synergistic effect between extracellular acidic pH and PDGF on the induction of MEFs migration. Whether synergism of acidic microenvironments and PDGF on MEFs migration exists in other cell types and whether this synergism largely accelerated the onset and the progression of diseases accompanied by extracellular acidification or by increasing concentration of PDGF remains to be explored. The results will provide new insights into the elucidation of the underlying mechanisms and the treatment or the relief of these related disorders.

To date, OGR1 family of GPCRs (TDAG8, GPR4, OGR1, and G2A), TRPV1, and ASICs channels have been reported to sense acidic pH and stimulate intracellular signaling pathways. Accumulating evidences are showing that known proton-sensing GPCRs or ion channels mediated cell signaling pathway involved in migration. For example, GPR4 regulated tumor cell migration and extracellular

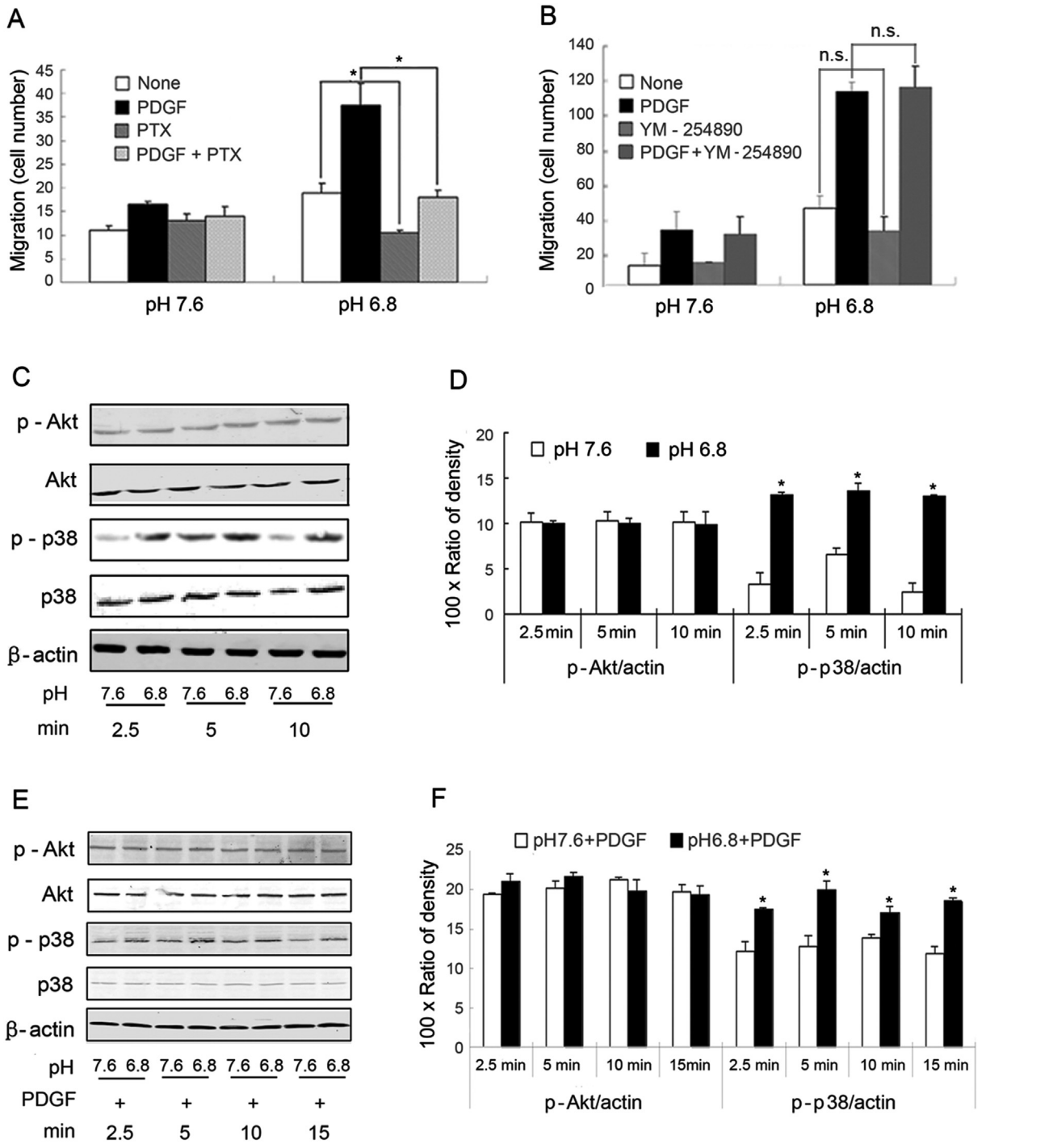

Fig. 3. Extracellular acidification stimulates MEFs migration and PDGF-induced MEFs migration via a Gi/p38MAPK signal pathway. (A) MEFs were pretreated with G_i protein inhibitor (pertussis toxin, PTX, 50 ng/ml, 12 h) to induce cell migration with or without 20 ng/ml PDGF. PTX significantly suppressed pH 6.8-induced cell migration with or without PDGF. (B) MEFs were pretreated with G_q inhibitor YM-254890 (100 nM, 30 min) to induce cell migration. YM-254890 has no effect on acidic pH-induced cell migration with or without 20 ng/ml PDGF. Data are the mean ± SEM from more than two independent experiments (n = 3 for each experiment). *Statistically significant. n.s. Not significant. (C, E) The cells were pretreated with pH 7.6 or pH 6.8 buffers in the presence or absence of 20 ng/ml PDGF for the indicated time, and then analyzed by Western blot for detection of phosphorylated/unphophorylated p38MAPK, and phosphorylated/unphophorylated Akt. Western blot showed p38MAPK was activated by pH 6.8 in the presence or absence of PDGF. (D) showed related amount of protein from (C). *$p < 0.05$ versus corresponding pH 7.6 at the same point time. (F) showed related amount of protein from (E). *$p < 0.05$ versus corresponding pH 7.6 + PDGF at the same point time.

proton-promoted metastasis of breast cancer [19], and OGR1 suppressed cell migration of MCF7 breast cancer cells through a Gα12/13-Rho-Rac1 pathway [20]. Nevertheless, using real-time Taqman PCR, OGR1-, GPR4-and TDAG8-KO technology, and migration assay with inhibitors, we found OGR1 family receptors, TRPV1, and ASICs were unlikely to participate in the increasing effects of acidic pH on MEFs migration and PDGF-induced MEFs migration, suggesting that not the known proton-sensing GPCRs, but an unknown pH receptor might be involved in the effects of extracellular protons on MEFs migration.

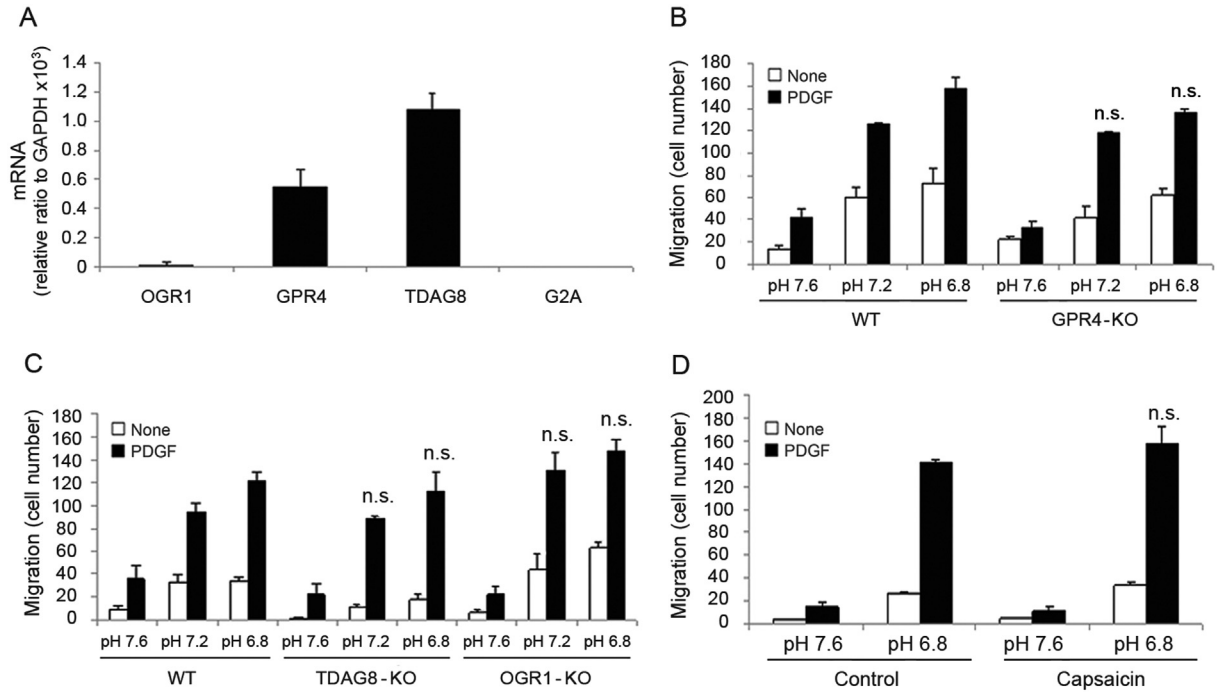

Fig. 4. MEFs sense extracellular acidification was not regulated by known proton-sensing GPCRs, TRPV1 or ASICs. (A) mRNA expression of OGR1, GPR4, TDAG8, and G2A in MEFs derived from WT mice. The respective receptor mRNA content is expressed as the relative ratio to GAPDH mRNA content. (B, C) Cell migration was induced in the presence or absence of PDGF (20 ng/ml) at the indicated pH values in MEFs derived from WT mice, GPR4-KO mice, TDAG8-KO mice, and OGR1-KO mice, respectively. (D) MEFs derived from WT mice were induced to migration at the indicated pH with or without an agonist of TRPV1 (capsaicin, 10 μM), in the presence or absence of PDGF (20 ng/ml). Data are the mean ± SEM from more than two independent experiments (n = 3 for each experiment). n.s. Not Significant. Effects of GPR4-,TDAG8-, OGR1-KO and capsaicin were not significant.

In conclusion, our present study investigated the effects of extracellular acidification on intracellular signaling pathways of MEFs. We have concluded that extracellular acidic pH stimulated MEFs migration and PDGF-induced MEFs migration at least partly through activation of p38MAPK with a PTX-sensitive manner. Signaling pathways initiated by acidic pH synergize with signaling pathways initiated by PDGF to facilitate directed cell migration. MEFs sense acidic environments might not be regulated by known proton-sensing TRPV1, ASICs, or proton-sensing GPCRs. Our study results suggested that other proton-sensing GPCRs or ion channels might exist in MEFs, which mediates cell migration induced by acidic pH.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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