



## Molecular and cellular pharmacology

## Hypoxia-induced upregulation of Orai1 drives colon cancer invasiveness and angiogenesis

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## ABSTRACT

In colon cancer, hypoxia promotes metastasis and angiogenesis, but little is known about the mediators of these effects. Here, we reported that expression of Orai1 is up-regulated in colon cancer cells in response to hypoxia, and the increase in Orai1 is mediated by Notch1 pathway. We also showed upregulation of Orai1 contributes to hypoxia-induced invasion and angiogenesis, and inhibition or downregulation of Orai1 reverses these effects. Mechanistic study revealed that upregulation of Orai1 by hypoxia potentiates store-operated  $\text{Ca}^{2+}$  entry (SOCE), and then causes activation of nuclear factor of activated T cells isoform c3 (NFATc3) in colon cancer cells. Furthermore, expression of Orai1 was correlated with tumor metastasis in patients. These results identify Orai1 as a novel target gene of hypoxia and reveal the role of Orai1 signaling in regulating hypoxia-induced invasiveness and angiogenesis under hypoxic conditions. Strategies to target this signaling might be developed to treat colon cancer metastasis and angiogenesis associated with hypoxia.

## 1. Introduction

Colon cancer is the third most common malignancy in men and the second in women worldwide (Ferlay et al., 2010; Cunningham et al., 2010; Hanahan and Weinberg, 2011). Colon cancer is crucial cause of cancer-related death with more than 600,000 deaths per year (Ferlay et al., 2010; Cunningham et al., 2010; Hanahan and Weinberg, 2011). The incidence rate of colon cancer has been rising fast in Asian countries (Sung et al., 2005). Tumor development and metastasis are the main causes of mortality in patients with colon cancer (Hanahan et al., 2011; Chaffer and Weinberg, 2011). The pathogenic mechanisms underlying colon cancer progression and metastasis appear to be a complex process. Targeted therapies and immunotherapy are still far from satisfactory for advanced-stage colon cancer patients (Hanahan et al., 2011; Sung et al., 2005; Chaffer and Weinberg, 2011; Tørring et al., 2017; Stintzing et al., 2017). Thus, there is an urgent need to explore the molecular mechanism of colon cancer development and metastasis. Hypoxia, known as low oxygen concentration, is a microenvironmental hallmark of most solid cancers (Semenza, 2012). There is increasing evidence that hypoxia has been identified as an inherent impediment to cancer therapy due to its multiple contributions to chemoresistance,

angiogenesis and invasiveness properties (De Bock et al., 2011; Vaupel et al., 2004; Brown and Giaccia, 1998). Thus, hypoxia is always associated with therapy resistance and poor prognosis. A considerable amount of effort has been made to explore the mechanisms controlling hypoxia and invasiveness/angiogenesis properties in human colon cancer, but the molecular components involved in linking them are still not entirely clear. Therefore, understanding the signaling underlying colon cancer to hypoxia is critical for developing new therapies.

Intracellular  $\text{Ca}^{2+}$  is one of most important second messengers regulating various physiological and pathophysiological processes in cancer cells, such as migration, invasion and angiogenesis (Prevarskaya et al., 2011; Villalobos et al., 2017). Store-operated  $\text{Ca}^{2+}$  entry (SOCE) is a crucial  $\text{Ca}^{2+}$  entry pathway, in which depletion of intracellular stores stimulates  $\text{Ca}^{2+}$  entry from extracellular milieu (Prakriya et al., 2006; Chen et al., 2016). Orai1 and STIM1 are two important molecular determinants of SOCE, in which STIM1 serves as a  $\text{Ca}^{2+}$  sensor in endoplasmic reticulum whereas Orai1 is a critical pore-forming component for  $\text{Ca}^{2+}$  permeation (Prakriya et al., 2006; Chen et al., 2016). Interestingly, the literatures showed that Orai1 is overexpressed in many types of cancer including cervical cancer, liver cancer, gastric cancer and glioblastoma (Vashisht et al., 2015; Xia et al., 2016).

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Moreover, Orai1-mediated SOCE is reported to play a key role in several types of cancer, but identity of the inducing factor remains unclear. Overexpression of Orai1 elevates human gastric cancer cell migration and invasion (Xia et al., 2016). In breast cancer cells, Orai1 regulates cell migration and metastasis (Yang et al., 2009). Orai1 plays an important role of cell invasion in human melanoma cells (Sun et al., 2014). However, the contributions of Orai1 to hypoxia-induced invasiveness and angiogenesis are still unknown in colon cancer.

Here, we uncovered a mechanism by which hypoxia up-regulates Orai1 and thereby promotes invasiveness and angiogenesis of colon cancer cells which involves Notch1/Orai1/SOCE/NFATc3 signaling. In addition, Orai1 is correlated with tumor metastasis in patients. Our findings bring insight into Orai1 signaling during hypoxia-induced invasiveness and angiogenesis in colon cancer.

## 2. Material and methods

### 2.1. Ethics statement and patients

The experiments involving clinical samples were approved by the Medical Ethics Committees of Jiangnan University. The human colorectal cancer samples (n = 71) were from Affiliated Hospital of Jiangnan University. Informed consent was requested as anonymous specimens and was given by all human participants in this study. Patients were recruited between 2006 and 2009.

### 2.2. Reagents and antibodies

Fetal bovine serum (16000-044) and Leibovitz's L-15 (41300) were purchased from Gibco Invitrogen. McCoy's 5A medium (M4892), CoCl<sub>2</sub> (C8661) and anti-Orai1 antibody (O8264) were obtained from Sigma. SKF96365 (1147), FK506 (3631) and thapsigargin (1138) were purchased from Tocris Bioscience. AnCoA4 (D00175902) was purchased from Calbiochem; DAPT (S2215) was purchased from Selleckchem; Alexa Fluor 488 Donkey Anti-Rabbit IgG (R37118) antibody was obtained from Life Technologies Corp. Anti-NFATc3 (sc-8405) and anti-ATCB antibodies (sc-47778) were obtained from Santa Cruz Biotechnology. Anti-Notch1 (#3608) and anti-NICD (#4147) antibodies were purchased from Cell Signaling Technology. Anti-HIF1α antibody (610958) was obtained from BD Biosciences. Anti-TATA binding protein antibody (TBP; ab51841) was obtained from Abcam.

### 2.3. Cell culture

The human colon cancer HCT-116 (CCL-247), SW480 (CCL-228) and HMEC-1 (CRL-3243) cells were purchased from American Type Culture Collection (ATCC). Cell lines were maintained in McCoy's 5A or Leibovitz's L-15 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. All experiments were carried out in cells between passage 10 and 20. Cells were cultured at 37 °C, in 95% O<sub>2</sub> and 5% CO<sub>2</sub> in a humidified incubator. To establish hypoxic conditions, HCT-116 or SW480 cells were cultured in 100 µM CoCl<sub>2</sub> (Chigurupati et al., 2010).

### 2.4. Real-time PCR analysis

Total RNA was isolated by Trizol (Life Technologies; 15596018). Reverse transcription polymerase chain reaction (RT-PCR) was performed with PrimeScript™ RT reagent Kit (Takara; RR037A) according to the manufacturer's instructions. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in 7500 Fast Real-time PCR system (Applied Biosystems). The sequences of primers used were as follows: ACTB forward 5'-CACCATTGGCAATGAGCGG TTC-3', reverse 5'-AGGTCTTTGCGGATGTCCACGT-3'; Orai1 forward 5'-GCCCTTCGGCCTGATCTTTAT-3'; reverse 5'-TGGAACGTCCGGTCA GTCTTAT-3'; HEYL forward 5'-TGGAGAAAGCCGAGGTCTTGCA-3',

reverse 5'-ACCTGATGACCTCAGTGAGGCA-3'; HES1 forward 5'-GTGG GTCCTAACGCAGTGTGTC-3', reverse 5'-GTCAGAAGAGAGAGGTGGGCTA -3'.

### 2.5. siRNA and plasmid transfection

The pool siRNAs against human Notch1 or NFATc3 were obtained from Santa Cruz. In brief, cells were transfected with 100 nM of each siRNA duplexes using DharmaFECT transfection reagent according to the manufacturer's protocol (Xie et al., 2014). The two short hairpin RNA (shRNA) constructs against human Orai1 (shOrai1#1 and shOrai1#2), shRNA-resistant Orai1 (Rescue) and Orai1<sup>G98A</sup> were used as described previously (Wang et al., 2015).

### 2.6. Western blotting analysis

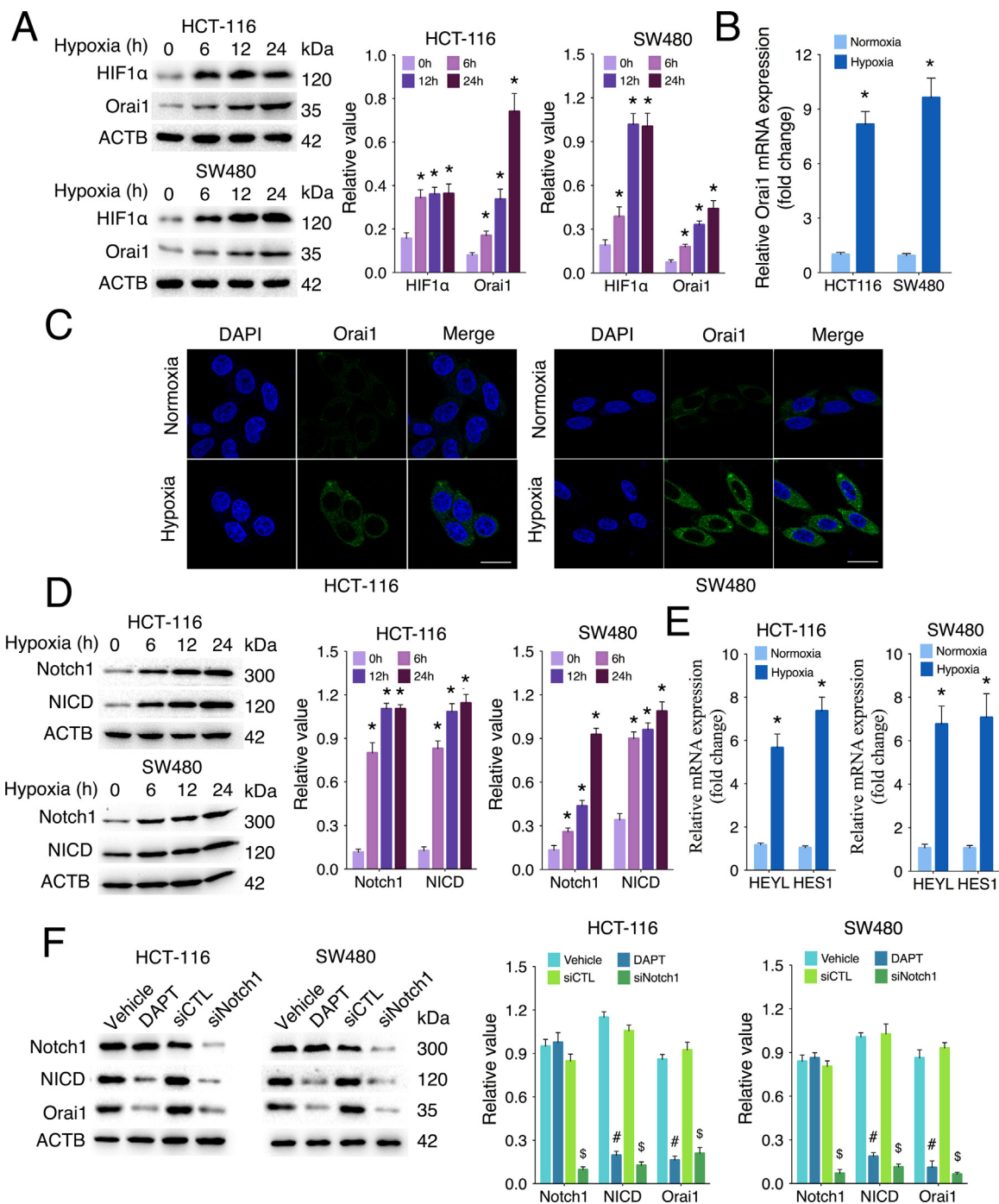
Cultured cells were harvested and followed by gentle shaking in 100–200 µl ice-cold freshly prepared lysis buffer [20 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM DTT, 1 mM sodium orthovanadate (Sigma, S6508), 1 µg/ml leupeptin (Sigma, L2884), 1 mM phenylmethylsulfonyl fluoride (Sigma, P7626)] for 60 min on ice. The lysate was centrifuged at 13,700g for 20 min at 4 °C. The nucleic fractions were prepared using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Biotech) according the manufacturer's instructions. The supernatant was collected and the protein concentration was determined by Pierce protein concentrators (Thermo). For Western blotting, protein samples were calibrated to equal amounts, boiled with SDS-PAGE loading dye and loaded at ~20 µg into each lane of polyacrylamide gel and separated by a SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane, and the membrane was then immersed in a blocking solution containing 5% BSA and 0.1% Tween-20 in PBS for 1 h at room temperature with constant shaking. Proteins were blotted with the primary antibodies at 4 °C overnight with constant shaking. Then washed 3 times for 15 min each in TBST at room temperature, immunodetection was accomplished with incubation of secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h, followed by reaction with Chemiluminescence HRP substrate (WBKLS0500, Millipore). The relative band intensity was quantified using the ImageJ software (NIH).

### 2.7. Immunofluorescence

Cells were grown on slides and cultured in normoxic or hypoxic conditions. After that, cells were washed 3 times with PBS, fixed with 4% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and then blocked with 0.5% BSA in PBS for 15 min. Slides were incubated with primary antibodies at 4 °C overnight, followed by incubation with appropriate secondary fluorescently labeled antibodies for 1 h at room temperature. All antibodies were diluted with 0.5% BSA in PBS. Slides were mounted with Vectashield mounting medium and images were taken with an Olympus FV1000 laser scanning confocal microscope.

### 2.8. SOCE measurement

Cultured cells were loaded with Fura-2/AM in normal Tyrode's solution. For store depletion, cells were pretreated with 4 µM thapsigargin for 15 min in Ca<sup>2+</sup>-free Tyrode's solution, which contained in mM: 140 NaCl, 5.4 KCl, 1 MgCl<sub>2</sub>, 5.5 glucose, 0.2 ethylene glycol tetraacetic acid (EGTA), and 5 HEPES, pH 7.4. Ca<sup>2+</sup> influx was initiated by the addition of 2 mM Ca<sup>2+</sup> to the bath. Fura-2 fluorescence signals were measured using dual excitation wavelengths at 340 and 380 nm using an Olympus fluorescence imaging system. Change in Ca<sup>2+</sup> was displayed as change in Fura-2 ratio (Wang et al., 2015).



**Fig. 1.** Hypoxia induces Orai1 expression through Notch signaling in colon cancer cells. **A.** Representative images and summary data from western blotting showing the time course of HIF1α, Orai1 and ACTB expression in HCT-116 and SW480 cells exposure to hypoxia. **B.** Real-time PCR for Orai1 mRNA in hypoxic colon cancer cells. **C.** Representative images from immunofluorescence of Orai1 in hypoxic colon cancer cells. Scale bars, 25 μm. **D.** Representative images and summary data from western blotting showing the time course of Notch1, NICD and ACTB expression in hypoxic colon cancer cells. **E.** Real-time PCR for HEYL and HES1 mRNA in hypoxic colon cancer cells. **F.** Representative images and summary data from Notch1, NICD, Orai1 and ACTB protein levels in colon cancer cells with DAPT (75 μM) for 2 h or specific siRNA for 24 h before exposure to hypoxia. Values are means ± S.E.M. of four to six experiments. \*,  $P < 0.05$ , compared to 0 h (normoxia); #,  $P < 0.05$ , compared to Vehicle; \$,  $P < 0.05$ , compared to siCTL.

## 2.9. Intracellular $Ca^{2+}$ measurement

A Fluo-4 NW Calcium Assay kit (Invitrogen) was used to measure intracellular  $Ca^{2+}$  on the SpectraMax i3x Multi-Mode plate reader (Molecular Devices) following manufacturer's protocols as previously described (Chigurupati et al., 2010).

## 2.10. Migration and invasion assays

Cell migration and invasion assays were determined using the transwell chamber (Corning). Briefly, cells were harvested, washed, resuspended and then seeded into the upper chamber in serum-free medium. The medium containing 10% FBS was placed in the lower chamber and the cells were further incubated for the indicated time. Cells migrated through the membrane were fixed with 4%

paraformaldehyde and stained with crystal violet. The number of migrated cells was counted in 5 randomly selected microscopic fields and photographed. Cell invasion was determined with Matrigel matrix (BD Biosciences) coated on the upper surface of the transwell chamber. Cells were seeded, fixed, stained and counted as described above.

### 2.11. Cell attachment and detachment assays

Cell attachment and detachment assays were done as described previously (Wang et al., 2009). Briefly, the cells were seeded in 24-well plates at  $5 \times 10^4$  per well. For the attachment assay, one hour after seeding, unattached cells were removed and the attached cells were counted with the trypan blue staining. For the detachment assay, after 24 h incubation, the cells were incubated with 0.05% trypsin for 3 min to detach the cells from the culture plates.

### 2.12. Endothelial cell tube formation assay

The tube formation assay was performed as previously described (Zhang et al., 2016). Briefly, HMEC-1 cells were seeded in the matrigel-coated culture plates and suspended in conditioned medium collected from HCT-116 or SW480 cells with different treatments.

### 2.13. Immunohistochemistry

Immunohistochemistry was performed as previously described (Zhang et al., 2017). The paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed by heating slides for 10 min in pH 6.0 sodium citrate buffer at a sub-boiling temperature. The slides were incubated with the primary antibody at 4 °C in a humidified chamber. Subsequently, the tissue sections were incubated with the GTVision III Detection System/Mo&Rb Kit (Gene Tech Company Limited). All the slides were analyzed by pathologists blind to the samples. The German semi-quantitative scoring system (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) and the percentages of stained cells (0% = 0, 1–24% = 1, 25–49% = 2, 50–74% = 3, 75–100% = 4) were used to assess the results. The final immune reactive score was determined by multiplying the intensity score by the percentage score, ranging from 0 to 12.

### 2.14. Statistical analyses

We determined the correlations between Orai1 expression and NFATc3 expression using the Pearson correlation test. Statistical analysis was performed using two-tailed Student's *t*-test for comparisons between two groups or one-way ANOVA for comparisons between multiple groups. All statistical analyses were carried out with the GraphPad Prism software version 5.0. Data were expressed as mean  $\pm$  standard error of the mean (S.E.M.) of at least three independent experiments. *P* value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Hypoxia induces Orai1 expression through Notch signaling in colon cancer cells

Recently, several studies report the involvement of Orai1 in tumor progression and metastasis (Xia et al., 2016; Sun et al., 2014; Yang et al., 2009). To understand the influence of hypoxia on Orai1 expression, we exposed the cell lines HCT-116 and SW480 to the hypoxia-mimicking compound CoCl<sub>2</sub> and quantified Orai1 proteins by western blotting (Chigurupati et al., 2010). As control for the hypoxic effect, HIF1 $\alpha$  protein expression was increased under hypoxia (Fig. 1A). The increased protein expression of Orai1 was time dependent in the case of

HCT-116 and SW480 exposed to hypoxia (Fig. 1A). Quantitative real-time PCR was used to determine Orai1 mRNA expression and the results were consistent with Orai1 proteins (Fig. 1B). Immunofluorescence staining also confirmed the increase in Orai1 proteins in hypoxic colon cancer cells (Fig. 1C). Notch1 expression has previously been shown in colon cancer cell lines and clinical samples (Zhang et al., 2014; Gao et al., 2011). Because Notch signaling plays a key role in the regulation of cell fate decision and metastasis, we assessed Notch activity in colon cancer cells under hypoxia (Artavanis-Tsakonas et al., 1999). A time dependent increase in Notch1 and NICD (activated form of Notch1) protein was observed following hypoxic exposure (Fig. 1D). To further confirm the activation of the Notch signaling, transcriptional alterations of Notch target genes HEYL and HES1 were detected. We found that HEYL and HES1 mRNA expressions were enhanced after the hypoxic switch (Fig. 1E). To characterize whether hypoxia-induced Orai1 expression requires the activation of Notch signaling in colon cancer cells, pharmacologically inhibition of Notch signaling was undertaken using DAPT, a small molecule Notch signaling inhibitor. Notch1 siRNAs were used to knockdown the expression of Notch1. DAPT and gene silencing of Notch1 suppressed hypoxia-induced Orai1 protein expression (Fig. 1F). These results demonstrate that hypoxia mediates Orai1 expression in a Notch signaling dependent manner.

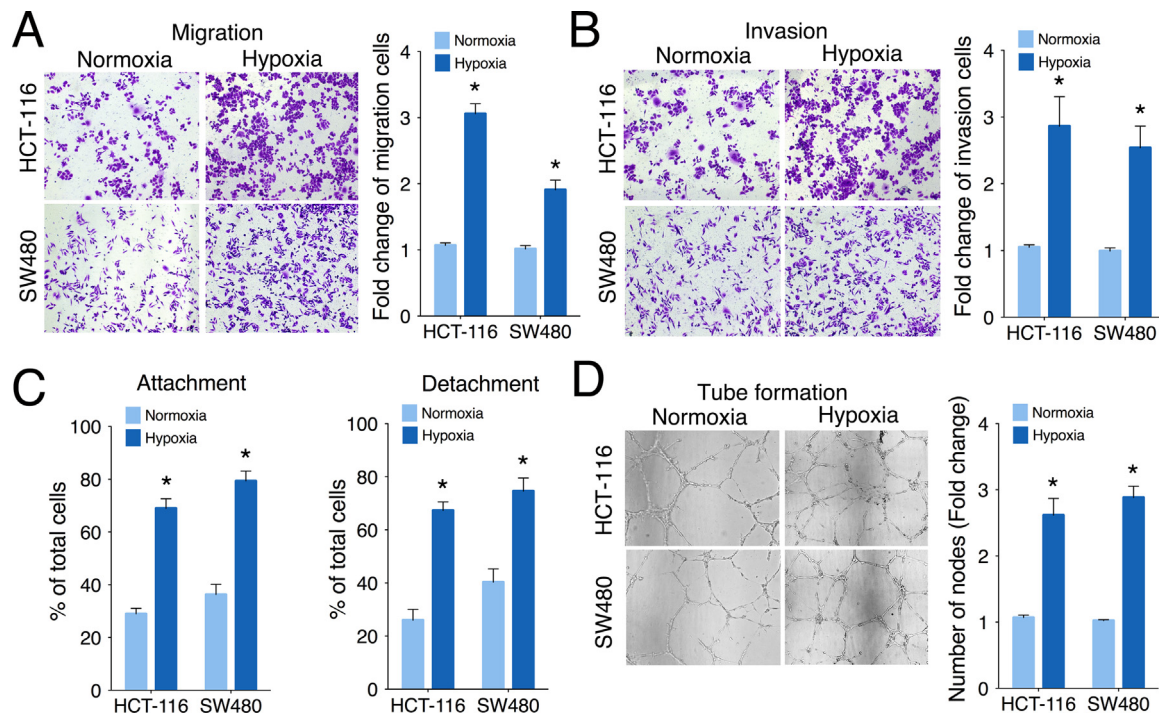
### 3.2. Orai1 mediates hypoxia-induced invasiveness and angiogenesis in colon cancer cells

Given the positive correlation between hypoxia and Orai1 expression, we try to determine whether Orai1 mediates hypoxia-induced invasiveness and angiogenesis in colon cancer cells. For this purpose, we performed the migration and invasion assays in normoxic and hypoxic colon cancer cells. Hypoxia promoted cell motility in HCT-116 and SW480 cells (Fig. 2A and B). We also found that hypoxic colon cancer cells have increased capacity for attachment and detachment (Fig. 2C and D). To determine the tumor angiogenesis under hypoxia, we measured the degree of angiogenic induction in HMEC-1 cells grown in conditioned medium harvested from hypoxic colon cancer cells using tube formation assay. The results showed that hypoxia increased angiogenesis (Fig. 2D). The knockdown effects of Orai1-specific shRNAs were verified in normoxic and hypoxic colon cancer cells (Fig. 3A). Down-regulation of Orai1 reduced tumor invasiveness and angiogenesis and these effects could be rescued by cotransduction with a shRNA-resistant Orai1 construct (Rescue) (Fig. 3B to F). AnCoA4 is used as an Orai1 inhibitor and recombinant Orai1<sup>G98A</sup> can be used as a dominant-negative construct of Orai1 (Wang et al., 2015). AnCoA4 and Orai1<sup>G98A</sup> also decreased hypoxia-induced invasiveness and angiogenesis (Fig. 3B to F). Notably, DAPT and knockdown of Notch1 were markedly decreased capacity for hypoxic colon cancer cells invasiveness and angiogenesis (Fig. 3B to F). Together, these data demonstrate that hypoxia-induced cell invasiveness and angiogenesis is dependent on Orai1.

### 3.3. Orai1-regulated SOCE activation is critical for hypoxia-induced invasiveness and angiogenesis in colon cancer cells

Orai1-regulated SOCE is a predominant Ca<sup>2+</sup> influx pathway and SOCE has been shown to have a critical role in tumor progression. To measure the SOCE, normoxic or hypoxic colon cancer cells in Ca<sup>2+</sup> free solution were incubated with 4  $\mu$ M thapsigargin (TG) for 15 min to deplete intracellular Ca<sup>2+</sup> stores, followed by adding 2 mM Ca<sup>2+</sup> to the solution. Importantly, SOCE was markedly increased in hypoxic colon cancer cells (Fig. 4A). However, TG-induced Ca<sup>2+</sup> release was similar in normoxic and hypoxic condition (Fig. S1). Orai1 shRNAs or Orai1<sup>G98A</sup> transduction and AnCoA4 incubation decreased TG-induced SOCE in hypoxic colon cancer cells (Fig. 4B). DAPT or knockdown of Notch1 also inhibited TG-induced SOCE (Fig. 4B). Moreover, SKF96365, an inhibitor of SOCE, resulted in reduced TG-induced SOCE in colon





**Fig. 2.** Hypoxia increases cell invasiveness and angiogenesis in colon cancer cells. A. Migration capacity of colon cancer cells exposure to hypoxia 24 h or normoxia was examined by transwell assay. B. Invasion capacity of colon cancer cells exposure to hypoxia 28 h or normoxia was examined by matrigel invasion assay. C. Summary data from attachment and detachment assay in hypoxic colon cancer cells. D. Representative images and summary data from tube formation showing the degree of angiogenic induction in HMEC-1 cells grown in conditioned medium harvested from HCT-116 or SW480 cells under hypoxia or normoxia. Values are means  $\pm$  S.E.M. of four to six experiments. \*,  $P < 0.05$ , compared to normoxia.

cancer cells exposed to hypoxia (Fig. 4B). We also investigated the basal  $[Ca^{2+}]_i$  by using Fluo-4 fluorescence spectrophotometry. After exposing to hypoxia for 12 h, basal  $[Ca^{2+}]_i$  were remarkably increased (Fig. S2). AnCoA4 and Orai1 shRNA decreased basal  $[Ca^{2+}]_i$  in HCT-116 and SW480 cells under hypoxia (Fig. S2). To address the potential mechanism by which Orai1 mediates hypoxia-induced invasiveness and angiogenesis, we investigated the effect of downstream of SOCE on cell motility and angiogenesis. Pharmacologic inhibition of SOCE reduced hypoxic colon cancer cells migration, invasion, attachment and detachment (Fig. 4C to F). In line with the results described above, number of branch points was decreased in conditioned medium harvested from hypoxic colon cancer cells with SKF96365 treatment (Fig. 4G). These results suggest that Orai1-regulated SOCE activation is critical for hypoxia-induced invasiveness and angiogenesis in colon cancer.

### 3.4. NFATc3 is the target of Orai1/SOCE signaling in hypoxic colon cancer cells

Recently, reports showed that Orai1-regulated SOCE activates NFATc3, a  $Ca^{2+}$ -dependent transcription factor implicated in tumor processing (Liu et al., 2018; Lee et al., 2016; Kar and Parekh, 2015). Thus, we validated the role of Orai1 in NFATc3 activation in hypoxic colon cancer cells. Hypoxia enhanced NFATc3 activation, as considerably increased in translocation to the nucleus (Fig. 5A). Because  $Ca^{2+}$  is critical for NFATc3 activation, we investigated whether Orai1-regulated SOCE evaluates NFATc3 activation following the hypoxic switch. Orai1 shRNAs markedly inhibited the accumulation of NFATc3 in the nucleus and shRNA-resistant Orai1 construct reversed the effect of Orai1 shRNAs (Fig. 5B). Consistent with this, DAPT, AnCoA4 and SKF96365 also decreased hypoxia-induced NFATc3 activation (Fig. S3). Next, we determined the effect of NFATc3 knockdown or NFAT inhibition on the ability of hypoxia-induced invasiveness and angiogenesis. The knockdown effects of NFATc3 siRNAs were confirmed by

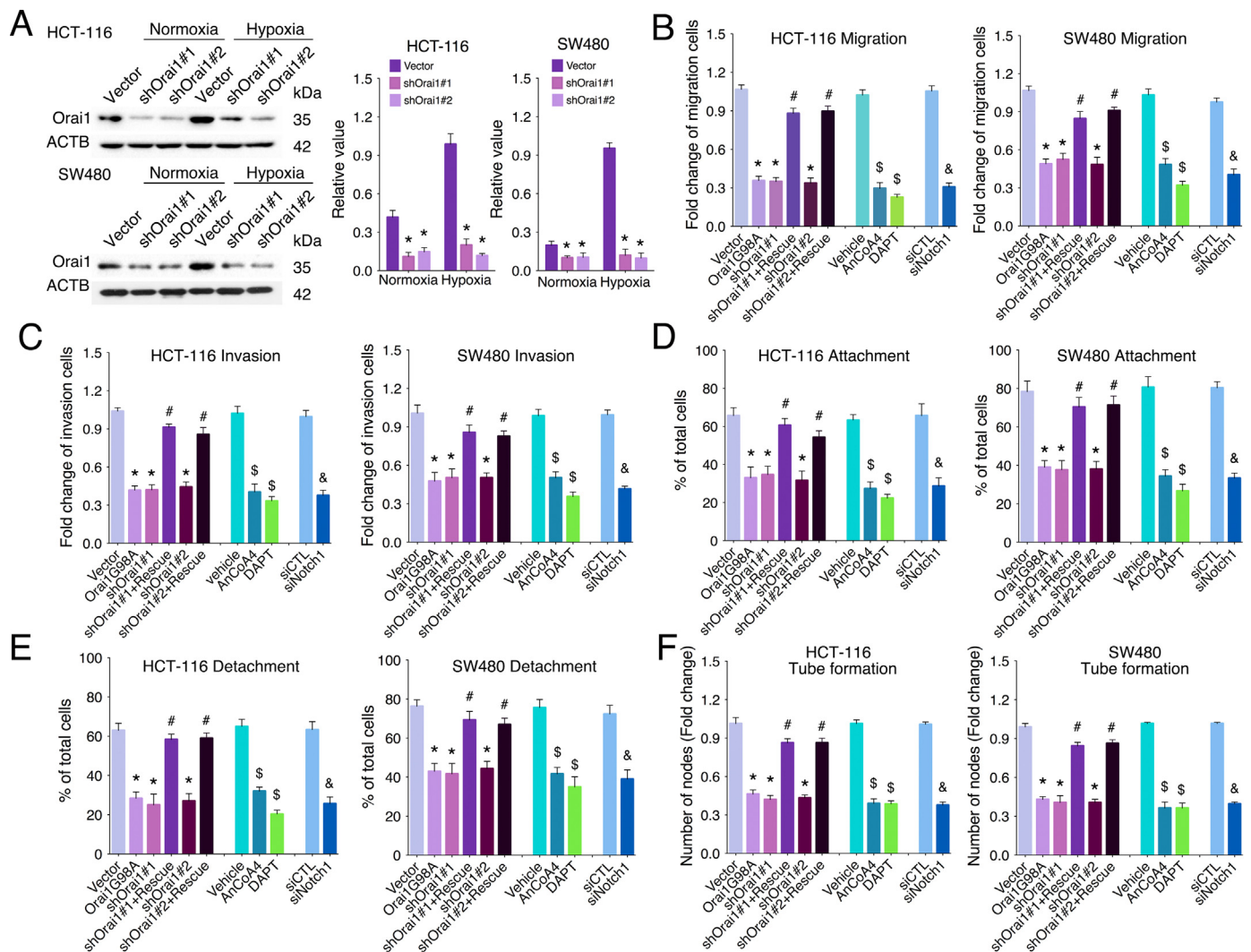
NFATc3 protein levels (Fig. S4). Consistent with the effect of SOCE inhibition, pharmacologic inhibition or gene-silencing of NFATc3 suppressed hypoxia-induced invasiveness and angiogenesis (Fig. 5C to G). Collectively, these data suggest that orai1 drives invasiveness and angiogenesis via SOCE/NFATc3 signaling in hypoxic colon cancer cells.

### 3.5. Orai1 expression is linked to tumor metastasis in clinical samples

To determine whether the Orai1 expression is also seen in clinical samples, 71 colorectal cancer samples with or without local metastasis to the lymph-nodes were collected (Table S1). We performed Orai1 and NFATc3 immunohistochemistry on these samples. We found that the 24 colorectal cancer samples with local metastasis (N1–2) displayed significantly elevated expression of Orai1 and NFATc3 compared to 47 colorectal cancer samples without local metastasis (N0) (Fig. 6A and B). In addition, the expression of Orai1 showed a markedly positive correlation with the expression of NFATc3, which is consistent with the above mentioned analysis in vitro (Fig. 6C). Taken together, the expression of Orai1 detected in patient derived tumor samples indicates that Orai1 plays a critical role in colon cancer metastasis.

## 4. Discussion

Colon cancer is most common malignant tumor in human and tumor metastasis is the major cause of mortality (Hanahan and Weinberg, 2011; Ferlay et al., 2010; Cunningham et al., 2010; Sung et al., 2005). Hence, understanding the mechanism controlling metastasis is critical to improving colon cancer survival. Orai1-mediated SOCE has been confirmed and this process is recognized to regulate cancer cell proliferation and invasion (Xia et al., 2016; Sun et al., 2014; Yang et al., 2009). Research on the role of SOCE under hypoxia is limited. Our study was the first to reveal the role of hypoxia-induced Orai1 on invasiveness and angiogenesis in colon cancer. Here, we showed that upregulation of Orai1 expression is dependent on Notch activation in

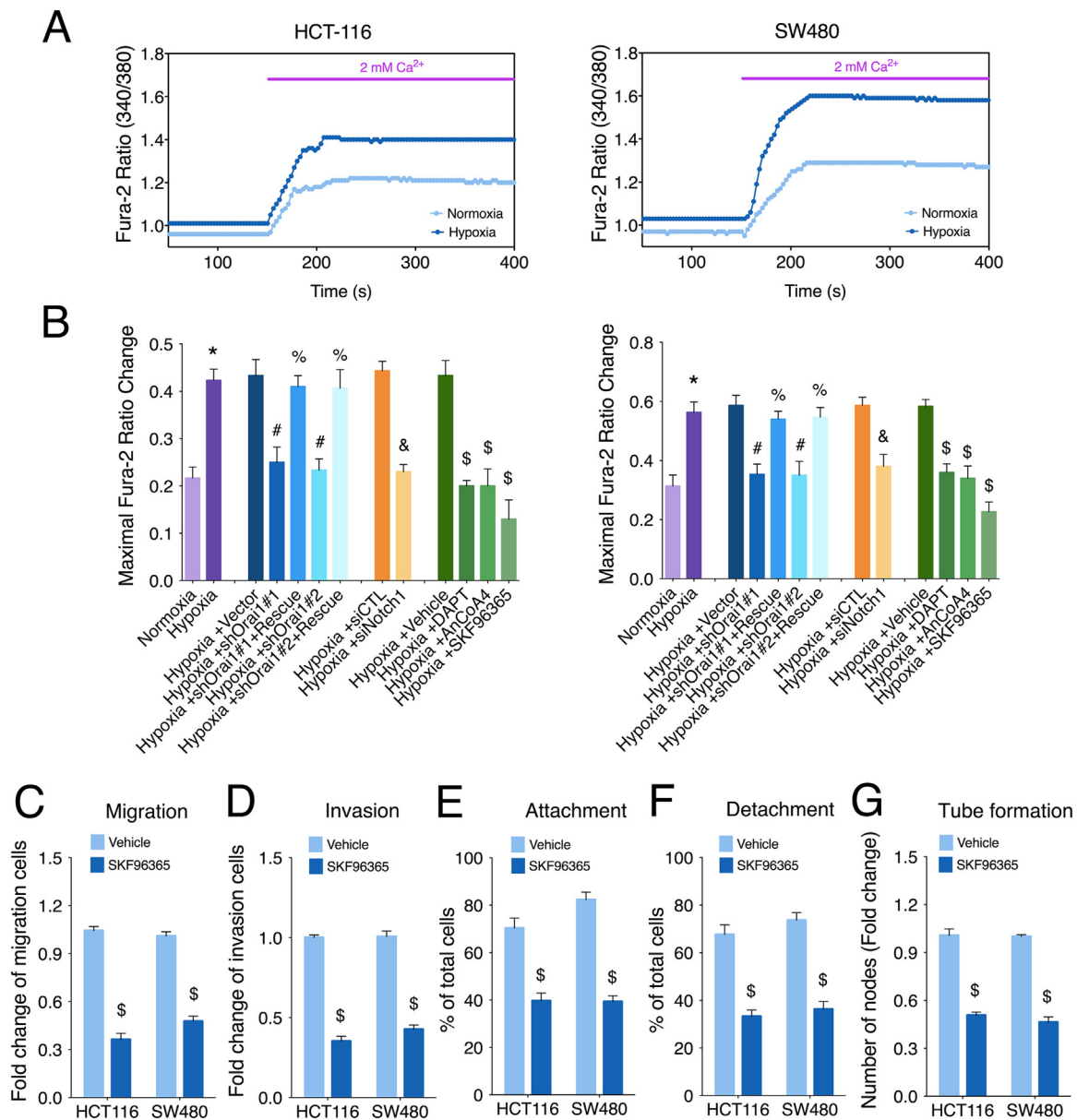


**Fig. 3.** Orai1 mediates hypoxia-induced invasiveness and angiogenesis in colon cancer cells. **A.** Representative images and summary data from western blotting showing the expression of Orai1 and ACTB in hypoxic or normoxic colon cancer cells with Orai1 shRNAs. **B.** Summary data from migration assay showing colon cancer cells with empty vector (Vector), Orai1<sup>G98A</sup>, Orai1-shRNA, shRNA-resistant Orai1 (Rescue) for 24 h or DAPT (75  $\mu$ M), AnCoA4 (10  $\mu$ M) for 2 h, or Notch1 siRNAs for 24 h before exposure to hypoxia. **C.** Summary data from invasion assay in colon cancer cells described as in B. **D.** Summary data from attachment assay in colon cancer cells described as in B. **E.** Summary data from detachment assay in colon cancer cells described as in B. **F.** Summary data from tube formation assay in colon cancer cells described as in B. Values are means  $\pm$  S.E.M. of three to six experiments. \*,  $P < 0.05$ , compared to Vector; #,  $P < 0.05$ , compared to shOrai1 #1 or #2; \$,  $P < 0.05$ , compared to Vehicle; &,  $P < 0.05$ , compared to siCTL.

hypoxic colon cancer cells. Blockade of Orai1 inhibited the hypoxia-induced invasiveness and angiogenesis in colon cancer. SOCE/NFATc3, as the downstream of Orai1, supports hypoxia-induced invasiveness and angiogenesis. We also found significant positive correlation between Orai1 and Notch1 in clinical samples with and without local metastasis. Therefore, our study shed light on an essential role of Notch1/Orai1/SOCE/NFATc3 in regulating hypoxia-induced invasiveness and angiogenesis (Fig. 6D).

Low oxygen environment has been shown to be associated with progression and metastasis for colon cancer (Bristow and Hill, 2008; Sung et al., 2005). HIF1 $\alpha$  represents key mediator of the transcriptional response to hypoxia and play an important role in cancer proliferation and poor clinical outcome (Yang et al., 2008; Keith and Simon, 2007). Here, HIF1 $\alpha$  protein levels were increased in colon cancer cells exposed to hypoxia. Similarly, Notch1/NICD expressions were enhanced in response to hypoxia. In addition, Notch target genes HEYL and HES1 were also elevated. The Notch-target genes that mediate the development of aggressive type in colon cancer are still little known. In this study, we found that Orai1 expression was significantly up-regulated under

hypoxia. Knockdown of Notch1 or pharmacological inhibition of Notch signaling blocked the hypoxia-induced increase in Orai1 expression, suggesting that Notch1 activation is responsible for the upregulation of Orai1 after hypoxia switch. Future study is needed to clarify whether Notch1 directly mediates Orai1 transcription. Previous studies showed that Orai1 plays an essential role in breast and gastric cancer cell migration (Xia et al., 2016; Yang et al., 2009). Therefore, to determine the role of Orai1 in regulating hypoxia-induced aggressive type, we examined the effects of pharmacological inhibition or gene silencing of Orai1. In line with the previous notion, colon cancer cells showed a remarkable increase in migration under hypoxia. Block the function or knockdown of Orai1 reduced migration and invasion, suggesting Orai1 leads to invasion in colon cancer cells in response to hypoxia. It has been reported hypoxia supports tumor progression through angiogenesis, which is associated with tumor metastasis (Sui et al., 2017; Xiang et al., 2017; Loges et al., 2009). Importantly, Li et al. (2011) showed that Orai1 plays a role in regulating vascular endothelial growth factor-induced tube formation in human umbilical vein endothelial cells. We found that inhibition of Orai1 signaling suppresses hypoxia-induced



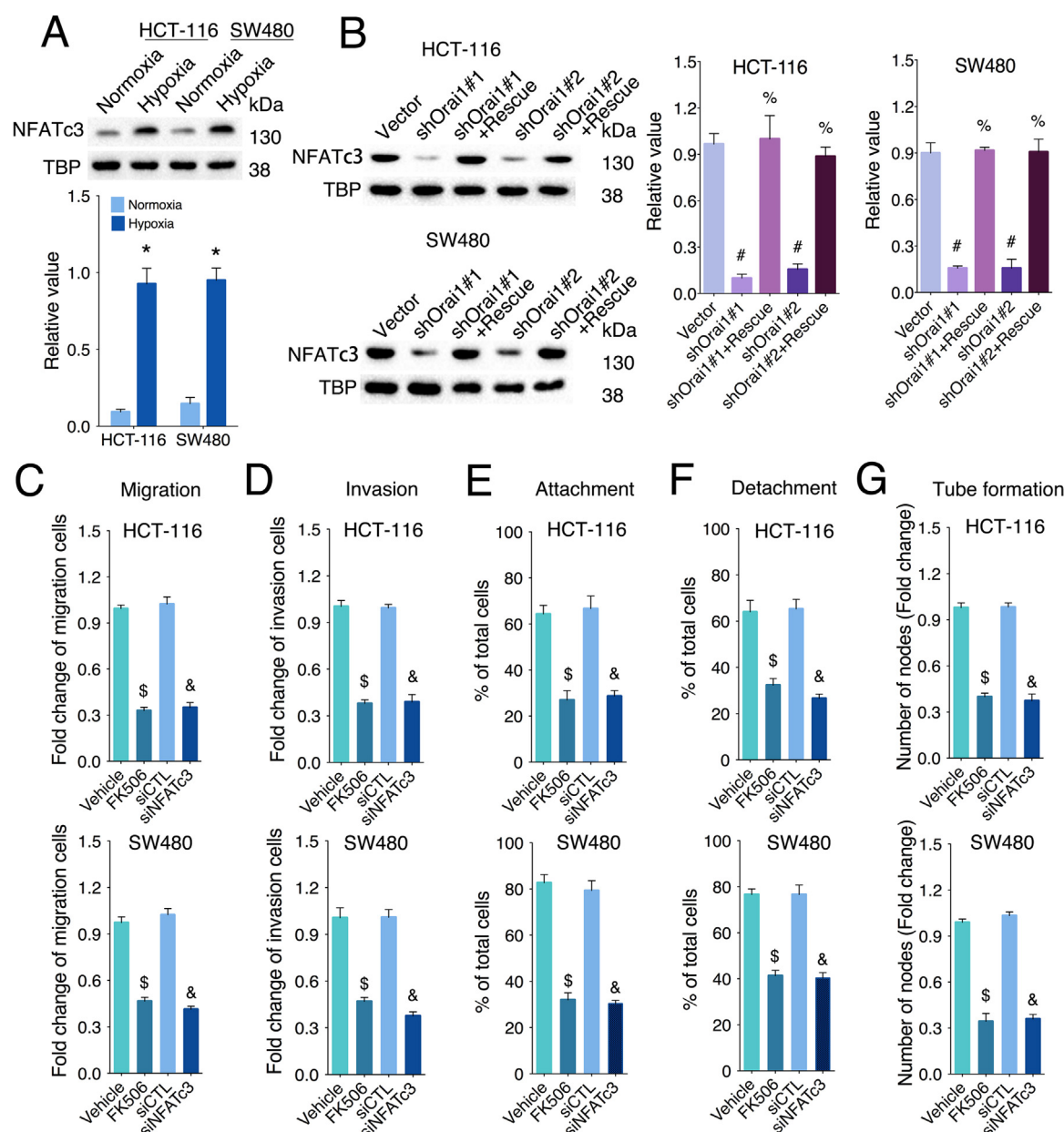
**Fig. 4.** Orai1-regulated SOCE activation is critical for hypoxia-induced invasiveness and angiogenesis in colon cancer cells. A and B. Representative traces and summary data of SOCE measurement in colon cancer cells with empty vector (Vector), Orai1<sup>G98A</sup>, Orai1-shRNA, shRNA-resistant Orai1 (Rescue) for 24 h or DAPT (75  $\mu$ M), AnCoA4 (10  $\mu$ M), SKF96365 (10  $\mu$ M) for 2 h, or Notch1 siRNAs for 24 h before exposure to hypoxia. C and D. Summary data from migration or invasion assay in colon cancer cells described as in B. E and F. Summary data from attachment or detachment assay in colon cancer cells described as in B. G. Summary data from tube formation assay in colon cancer cells described as in B. Values are means  $\pm$  S.E.M. of four to six experiments. \*,  $P < 0.05$ , compared to Normoxia; %,  $P < 0.05$ , compared to shOrai1 #1 or #2; #,  $P < 0.05$ , compared to Vector; \$,  $P < 0.05$ , compared to Vehicle; &,  $P < 0.05$ , compared to siCTL.

angiogenesis. Our findings imply that Orai1 is critical for hypoxia-induced invasiveness and angiogenesis.

Previous studies have suggested that  $\text{Ca}^{2+}$  signaling responds extracellular stimuli and regulates various fundamental cellular functions including migration (Villalobos et al., 2017; Chen et al., 2016; Prevarskaya et al., 2011; Prakriya et al., 2006). SOCE is a main  $\text{Ca}^{2+}$  entry pathway, which is regulated by Orai1 (Prakriya et al., 2006). To test whether Orai1-mediated SOCE is critical for hypoxia-induced invasiveness and angiogenesis, we examined effect of hypoxia on TG-induced SOCE. Here, we found that hypoxia increased TG-induced SOCE in colon cancer cells, which was suppressed by knockdown of Orai1. Blocking the function of Orai1 by Orai1<sup>G98A</sup> or AnCoA4 also inhibited the increase in TG-induced SOCE in colon cancer cells under hypoxia. Notably, inhibition of SOCE by SKF96365 blocked hypoxia-induced invasiveness and angiogenesis. Therefore, Orai1-mediated SOCE is

responsible for colon cancer cells invasiveness and angiogenesis under hypoxia. However, the molecular target of Orai1-mediated SOCE that was involved in these processes is unknown. NFAT family is  $\text{Ca}^{2+}$ -dependent transcription factors, which are mediated through binding  $\text{Ca}^{2+}$  to calcineurin. It is also known that NFAT signaling is vital for tumor cell invasion (Yoeli-Lerner et al., 2005). Previously, Orai1-mediated SOCE has been shown to stimulate NFATc3. Activation of Orai1 is required for translocation of NFATc3 to the nucleus (Kar and Parekh, 2015). Recent evidence indicates that Orai1/NFATc3 signaling mediates cancer stemness in oral/oropharyngeal squamous cell carcinoma (Lee et al., 2016). However, the role of NFATc3 in hypoxic colon cancer cells invasiveness and angiogenesis has not been reported. Here, we found that hypoxia promotes NFATc3 to migrate from cytoplasm to the nucleus. Suppression of Orai1 inhibited NFATc3 translocation in response to hypoxia. Knockdown of NFATc3 or inhibition of NFAT





**Fig. 5.** NFATc3 is the target of Orai1/SOCE signaling in hypoxic colon cancer cells. A. Representative images and summary data from western blotting showing NFATc3 and TBP expression in colon cancer cells with empty vector (Vector). B. Representative images and summary data from western blotting showing NFATc3 and TBP expression in colon cancer cells with empty vector (Vector), Orai1-shRNA, shRNA-resistant Orai1 (Rescue) for 24 h before exposure to hypoxia. C and D. Summary data from migration or invasion assay in colon cancer cells with FK506 (1  $\mu$ M) for 2 h or NFATc3 siRNAs for 24 h before exposure to hypoxia. E and F. Summary data from attachment or detachment assay in colon cancer cells described as in C. G. Summary data from tube formation assay in colon cancer cells described as in C. Values are means  $\pm$  S.E.M. of four to six experiments. \*,  $P < 0.05$ , compared to Normoxia; #,  $P < 0.05$ , compared to Vector; %,  $P < 0.05$ , compared to shOrai1#1 or #2; \$,  $P < 0.05$ , compared to Vehicle; &,  $P < 0.05$ , compared to siCTL.

signaling decreased hypoxia-induced invasiveness and angiogenesis, suggesting activation of NFATc3, the downstream of Orai1-mediated SOCE, plays an important role in colon cancer cells invasiveness and angiogenesis after the hypoxia switch. Importantly, expressions of Orai1 and NFATc3 were higher in clinical samples with local metastasis. In addition, we found statistically significant positive correlations between Orai1 and NFATc3 expression in clinical samples.

## 5. Conclusion

Our findings showed that hypoxia-induced invasiveness and angiogenesis are dependent on upregulation of Orai1 in colon cancer cells. Orai1 expression is positively correlated with metastasis in patients.

Our findings reveal a novel role of Orai1 in controlling colon cancer malignancy under hypoxia.

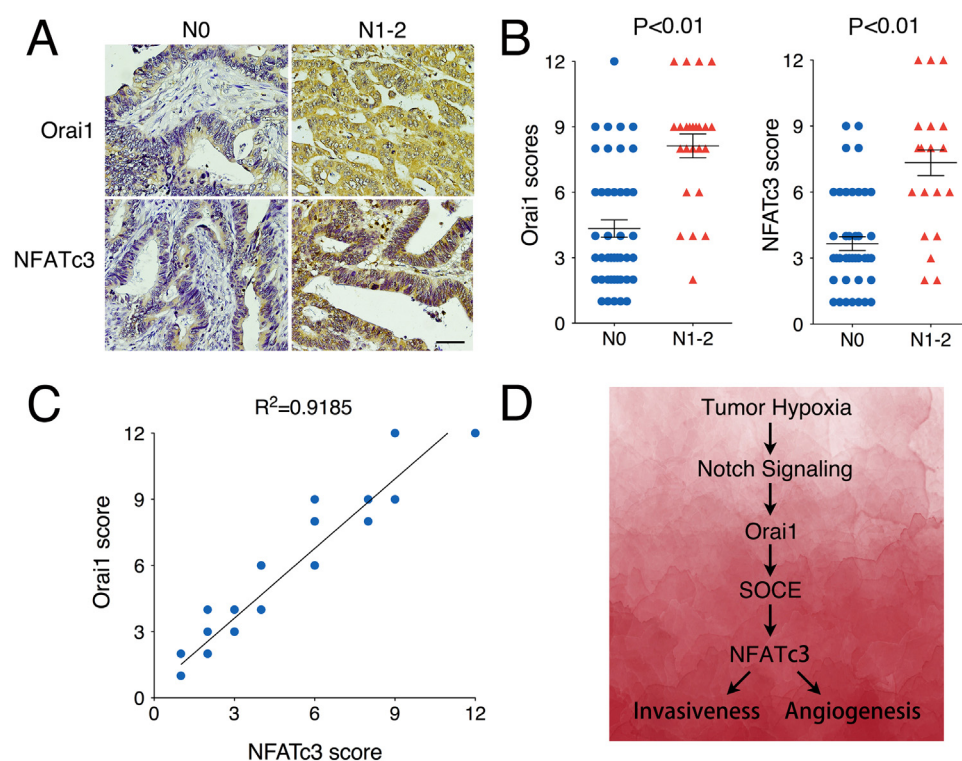
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## Declarations of interest

The authors declare no competing financial interests.





**Fig. 6.** Orai1 expression is linked to tumor metastasis in clinical samples. **A** and **B.** Representative images and summary data from immunohistochemical staining of Orai1 or NFATc3 in N1-2 (with lymph node metastasis,  $n = 24$ ) and N0 (without lymph node metastasis,  $n = 47$ ) patients. **C.** Pearson correlation of Orai1 expression with NFATc3 ( $n = 71$ ). Data were analyzed using Pearson correlation test. (Scale bars: 50 μm). **D.** Model of the regulation of Orai1 signaling in hypoxia-induced invasiveness and angiogenesis in colon cancer. Arrows represent promotion events.

## Author contribution statement

X.Y.L., X.W., H.K., Y.W., F.Y., and L.F. performed experiments. P.Z., J.J. and X.M. designed experiments. X.Y.L. and P.Z. did the statistical analysis. X.Y.L. and P.Z. wrote the manuscript

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ejphar.2018.05.008>.

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