Abbreviations List

KD: kinase-dead

PDK1: 3-phosphoinositide-dependent protein kinase-1

PH: pleckstrin-homology

$PI(3,4)P_2$: phosphatidylinositol 3,4-bisphosphate

$PI(3,4,5)P_3$: phosphatidylinositol 3,4,5-trisphosphate

PI3K: phosphoinositide 3-kinase

shRNA: short hairpin RNA

WT: wild-type

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Figure Legends

Figure 1. D-3 phosphoinositides are necessary for invadopodia formation.

(A) MDA-MB-231 cells transfected with GFP, GFP-Akt-PH wild-type (WT), or GFP-Akt-PH R25C mutant constructs were cultured on fluorescent gelatin-coated coverslips for 7 h and imaged by confocal microscopy. Arrowheads denote degradation sites on the gelatin matrix. (B-D) Degraded areas on the gelatin matrix (B), the percentage of cells with invadopodia (C), and the relative number of invadopodia per cell (D) were quantified for transfected cells as described in the *Materials and Methods*. (E) MDA-MB-231 cells stably expressing GFP-Akt-PH WT were cultured on fluorescent gelatin-coated coverslips for 3 h, stained for F-actin, and observed by confocal microscopy. Inserts are magnified images of the boxed regions. Arrowheads denote invadopodia where GFP-Akt-PH signals were accumulated. Data in (B), (C), and (D) are represented as mean (+SEM) of 4 independent determinations. * $p < 0.01$, and ** $p < 0.005$ by Student's *t*-test.

Figure 2. Class I PI3K catalytic subunit p110α is an essential regulator of invadopodia formation.

(A) Real-time quantitative PCR analysis of the expression of PI3K isoforms in MDA-MB-231 cells. The relative mRNA levels of PI3K isoforms normalized with the mRNA levels of cyclophilin B are shown. (B and C) MDA-MB-231 cells were transfected with siRNAs targeting individual PI3K isoforms for 48 h and the expression profiles of PI3K isoforms were determined by RT-PCR (B) and immunoblot analyses (C). Cyclophilin B (Cycl) and β-actin were used as internal controls. (D) MDA-MB-231 cells transfected with the indicated siRNAs were cultured on fluorescent gelatin-coated coverslips for 7 h, and the degraded areas on

matrices for 7 h and stained with phalloidin to visualize invadopodia. Arrowheads denote the gelatin degradation sites. (E-G) Gelatin degradation activity (E), the percentage of cells with invadopodia (F), and the number of invadopodia per cell (G) were determined in p110α cell lines. (H) Cells expressing E545K or H1047R p110α were examined for gelatin degradation in the presence or absence of PIK-75 (100 nM). (I) Cells expressing E545K or H1047R p110α were cultured on fluorescent gelatin matrices for 4 h and stained with anti-HA antibody to visualize localization of E545K and H1047R p110α. Inserts are magnified images of the boxed regions. Arrowheads denote colocalization of the HA signals with the gelatin degradation sites. (J) Cells labeled with CellTracker Green were analyzed for invasion through Matrigel-coated Transwell inserts for 24 h. Data in (E), (F, G, and H), and (J) are represented as mean (+SEM) of 7, 6, and 3 independent determinations, respectively. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Student's *t*-tests.

Figure 5. PDK1 and Akt are essential downstream effectors of p110α for invadopodia formation.

(A) MDA-MB-231 cells were transfected with control or 2 distinct PDK1 siRNAs for 48 h and used for immunoblotting to determine the amount of PDK1. (B-D) Cells transfected with the control or PDK1 siRNA were cultured on fluorescent gelatin-coated coverslips for 7 h. Degraded areas on the gelatin matrix (B), the percentage of cells with invadopodia (C), and the number of invadopodia per cell (D) were quantified for transfected cells. (E) Cells were transfected with control or two different sets of siRNAs targeting *Akt1*, 2, and 3 for 48 h and used for immunoblotting analysis with anti-pan-Akt antibody. (F-H) Degraded areas on the gelatin matrix (F), the percentage of cells with invadopodia (G), and the number of invadopodia per cell

(H) were quantified for siRNA-transfected cells. (I) Cells stably expressing E545K or H1047R p110α were transfected with indicated siRNAs for 48 h and tested for invadopodia activities for 7 h. (J) MDA-MB-231 cells plated onto fluorescent gelatin-coated coverslips for 4 h were stained with anti-Akt or anti-PDK1 antibody. Inserts are magnified images of the boxed regions. Arrowheads denote accumulation of Akt and PDK1 signals at the gelatin degradation sites. Data in (B, G, and H), (C, D, and I), and (F) are represented as mean (+SEM) of 6, 4, and 3 independent determinations, respectively. * $p < 0.02$, and ** $p < 0.005$ by Student's *t*-tests.

Figure 6. Pharmacological inhibition of PDK1 and Akt blocks invadopodia formation.

(A and B) MDA-MB-231 cells were serum-starved overnight and treated with inhibitors, 10 μM of OSU-03012 for PDK1 (A) or 20 μM of Akt inhibitor VIII (Akt i VIII) for Akt (B) for 1 h. The cells were subsequently stimulated with 8 nM of EGF for 10 min and used for immunoblotting to determine the phosphorylation status of Akt (p-Akt). (C) MDA-MB-231 cells were cultured on fluorescent gelatin-coated coverslips for 7 h in the presence of various inhibitors, including OSU-03012, Akt inhibitor VIII, and Calphostin and GF109203X for PKC. The degraded areas on the gelatin matrix were quantified. (D and E) Dose-response curves of gelatin degradation obtained in the presence of increasing concentrations of OSU-03012 (D) or Akt inhibitor VIII (E) are shown. (F) Representative images of MDA-MB-231 cells treated with OSU-03012 (10 μM) and Akt inhibitor VIII (20 μM) are shown. Arrowheads denote the gelatin degradation sites. (G-J) The percentage of cells with invadopodia (G and I) and the relative number of invadopodia per cell (H and J) were quantified for cells treated with 10 μM of OSU-03012 (G and H) or 20 μM of Akt

inhibitor VIII (I and J). (K) Cells expressing E545K or H1047R p110α were examined for gelatin degradation in the presence of OSU-03012 (10 μM) or Akt inhibitor VIII (20 μM). Data in (C, I, and J) and (G, H, and K) are represented as mean (+SEM) of 6 and 4 independent determinations, respectively. * $p < 0.02$, and ** $p < 0.005$ by Student's t-tests.

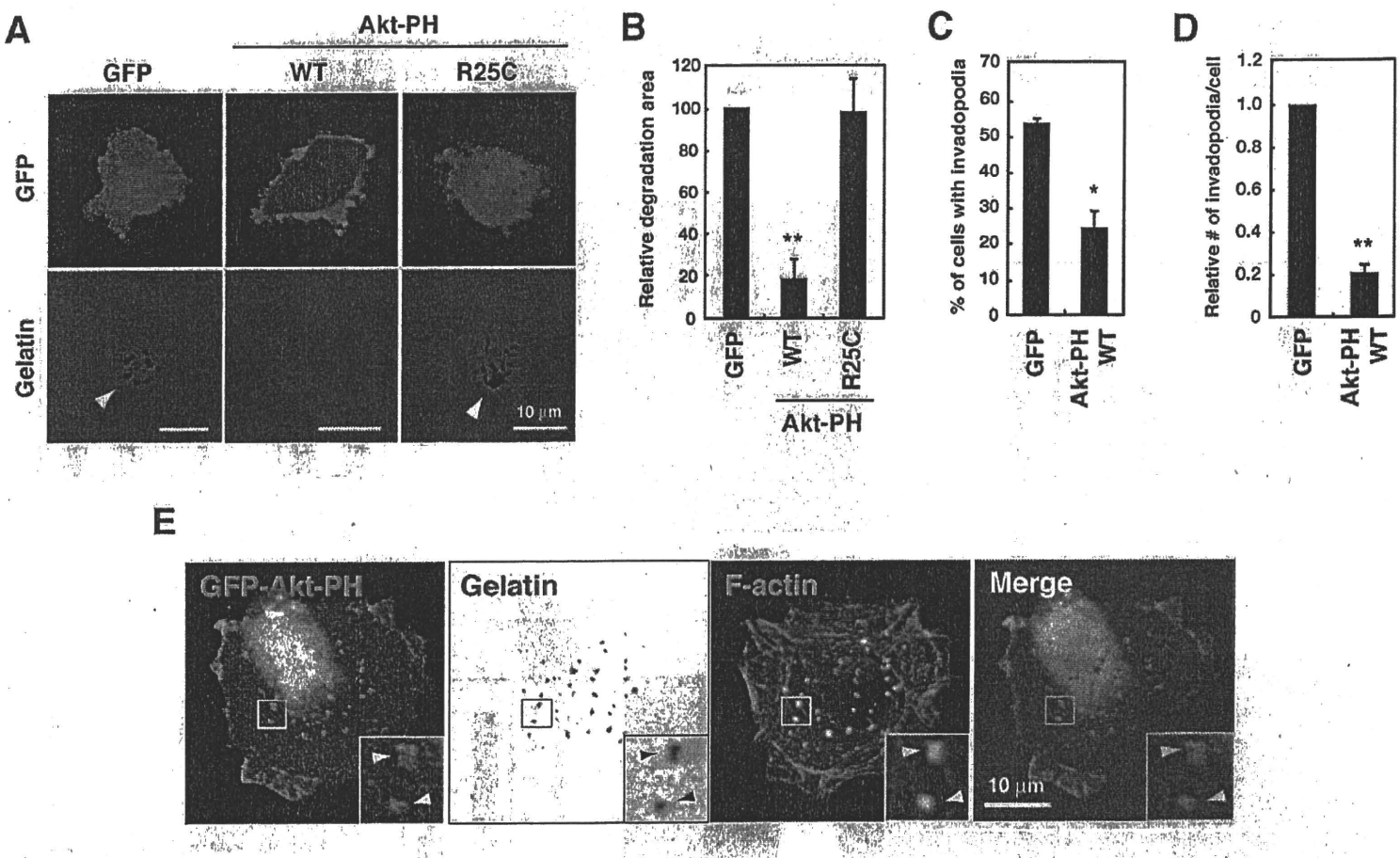
Figure 7. Expression of Akt constructs affects invadopodia formation.

(A) MDA-MB-231 cells stably expressing HA-tagged wild-type (WT), kinase-dead (KD), or myristoylated constitutively active (Myr) Akt1 were analyzed by immunoblotting. (B-D) Cells stably expressing the Akt constructs were cultured on fluorescent gelatin-coated coverslips for 7 h and stained for F-actin. Degraded areas on the gelatin matrix (B), the percentage of cells with invadopodia (C), and the number of invadopodia per cell (D) were quantified. Data are represented as mean (+SEM) of 6 (B) and 4-8 (C and D) independent determinations. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Student's *t*-tests. (E) Representative images of cells expressing the Akt constructs. Arrowheads denote the gelatin degradation sites. (F) Cells expressing WT or Myr Akt1 were cultured on fluorescent gelatin matrices for 3 h and stained with anti-HA antibody and phalloidin. Inserts are magnified images of the boxed regions. Arrowheads denote localization of the HA signals at invadopodia.

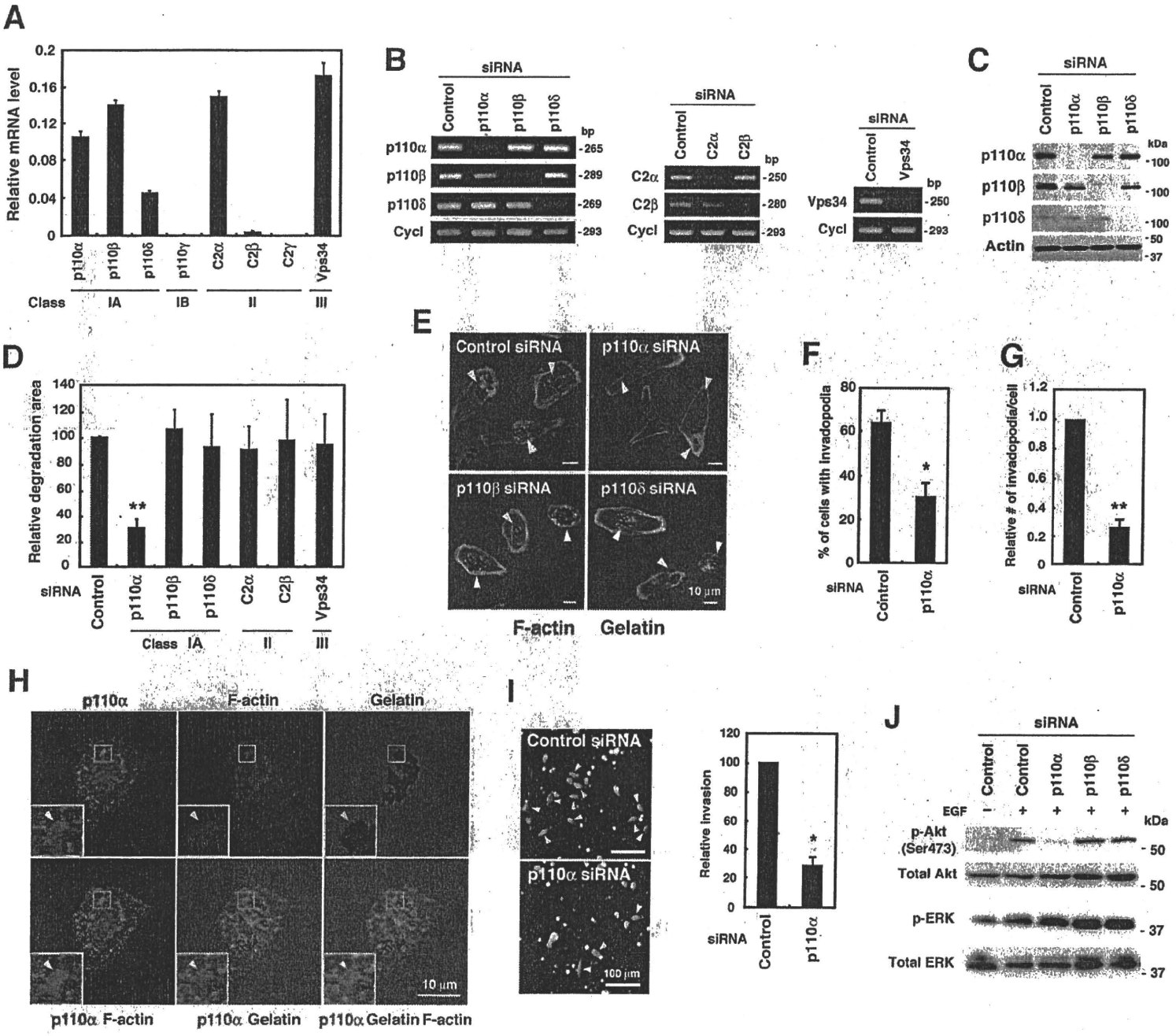
Figure 8. A model of the function of PI3K signaling in invadopodia formation and cell invasion.

p110α that is activated downstream of growth factor receptors produces the signaling lipid $PI(3,4,5)P_3$ in order to regulate invadopodia formation and cancer cell invasion. $PI(3,4)P_2$ that is generated via dephosphorylation of $PI(3,4,5)P_3$ by

synaptojanin-2 (SJ2) may regulate invadopodia formation through Tks5/N-WASP axis in parallel with $PI(3,4,5)P_3$. PDK1 and Akt are activated by both $PI(3,4,5)P_3$ and $PI(3,4)P_2$, and act as mediators of the PI3K signaling pathway for invadopodia formation.



Yamaguchi et al. Fig. 1



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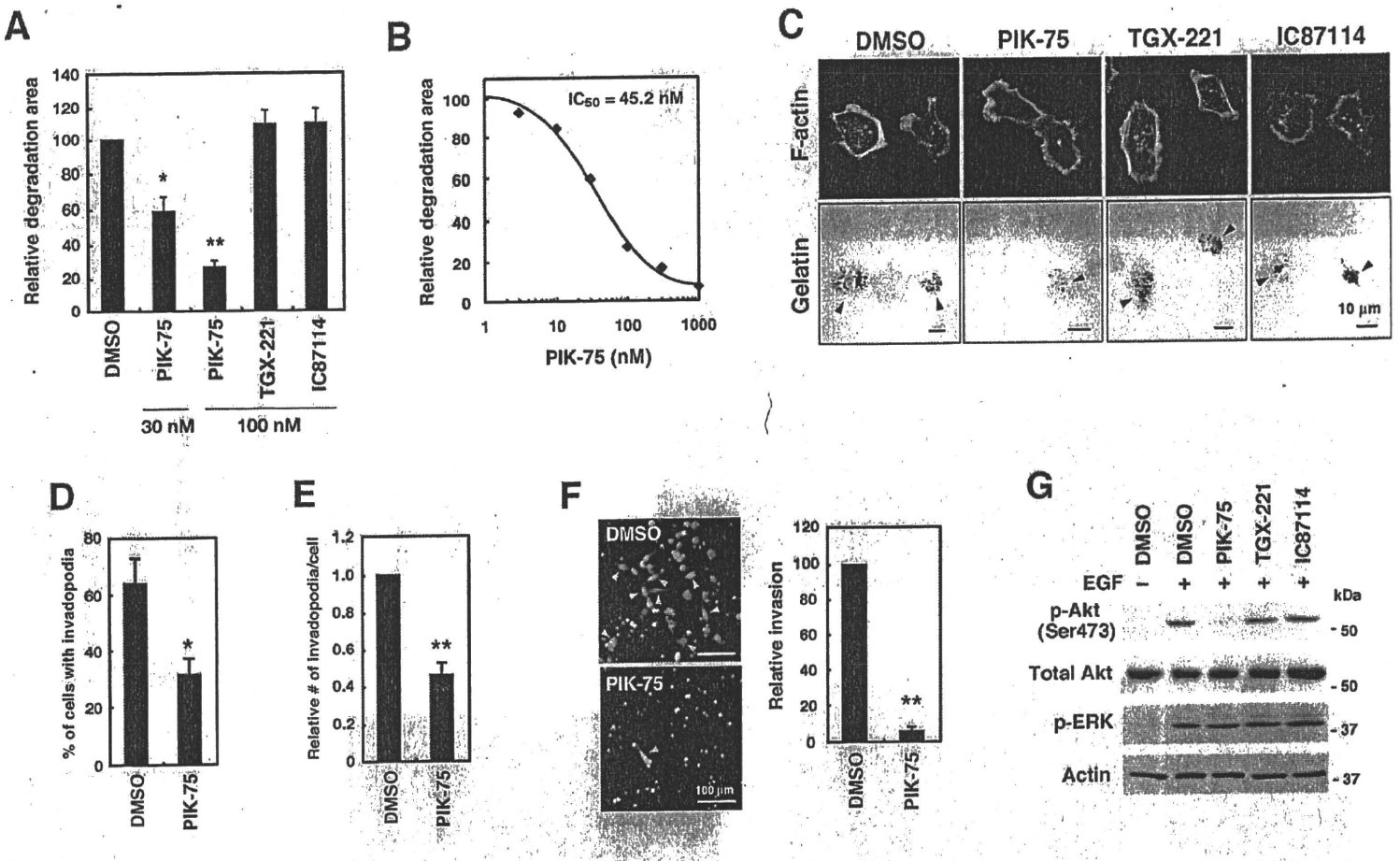

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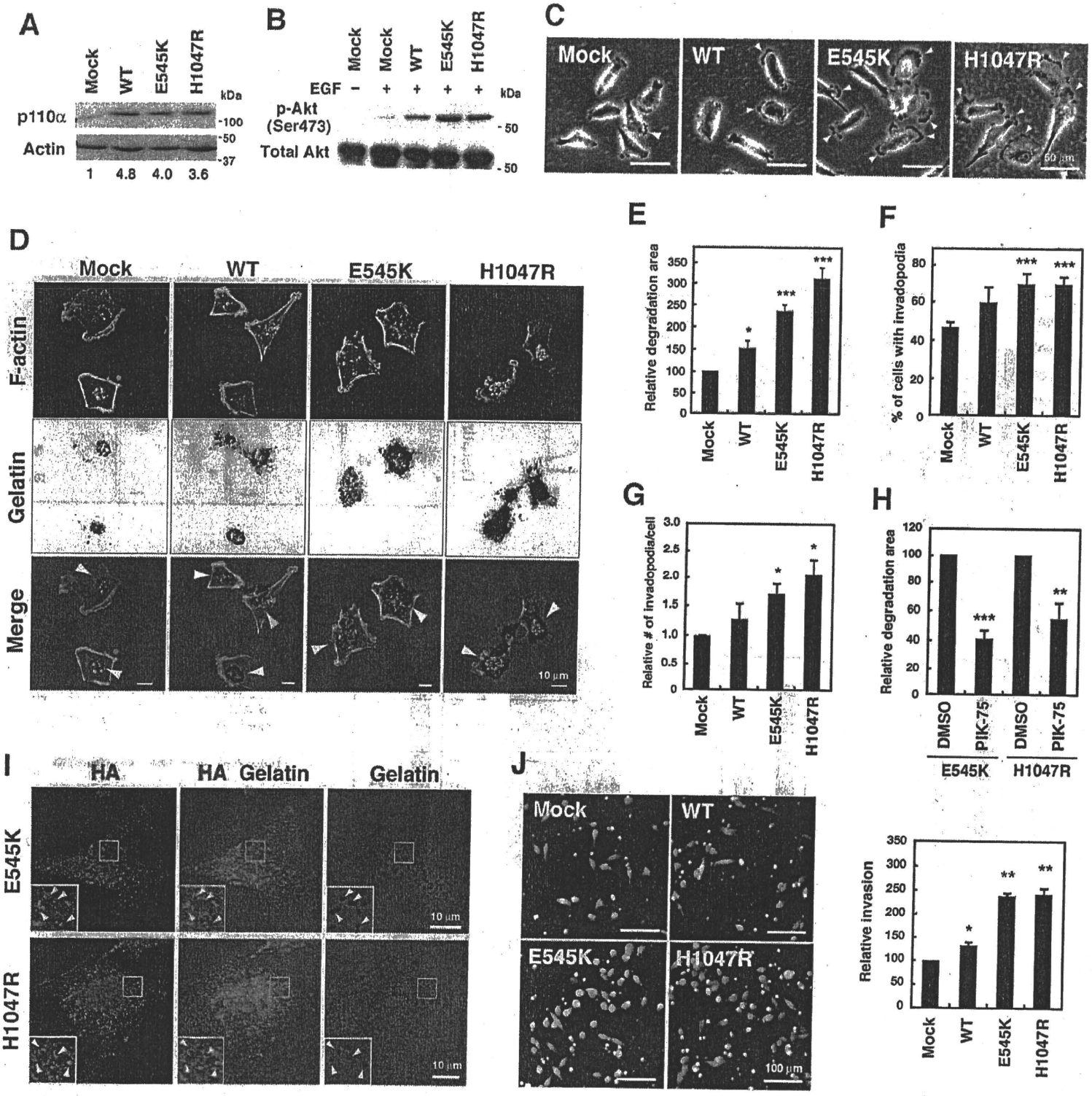
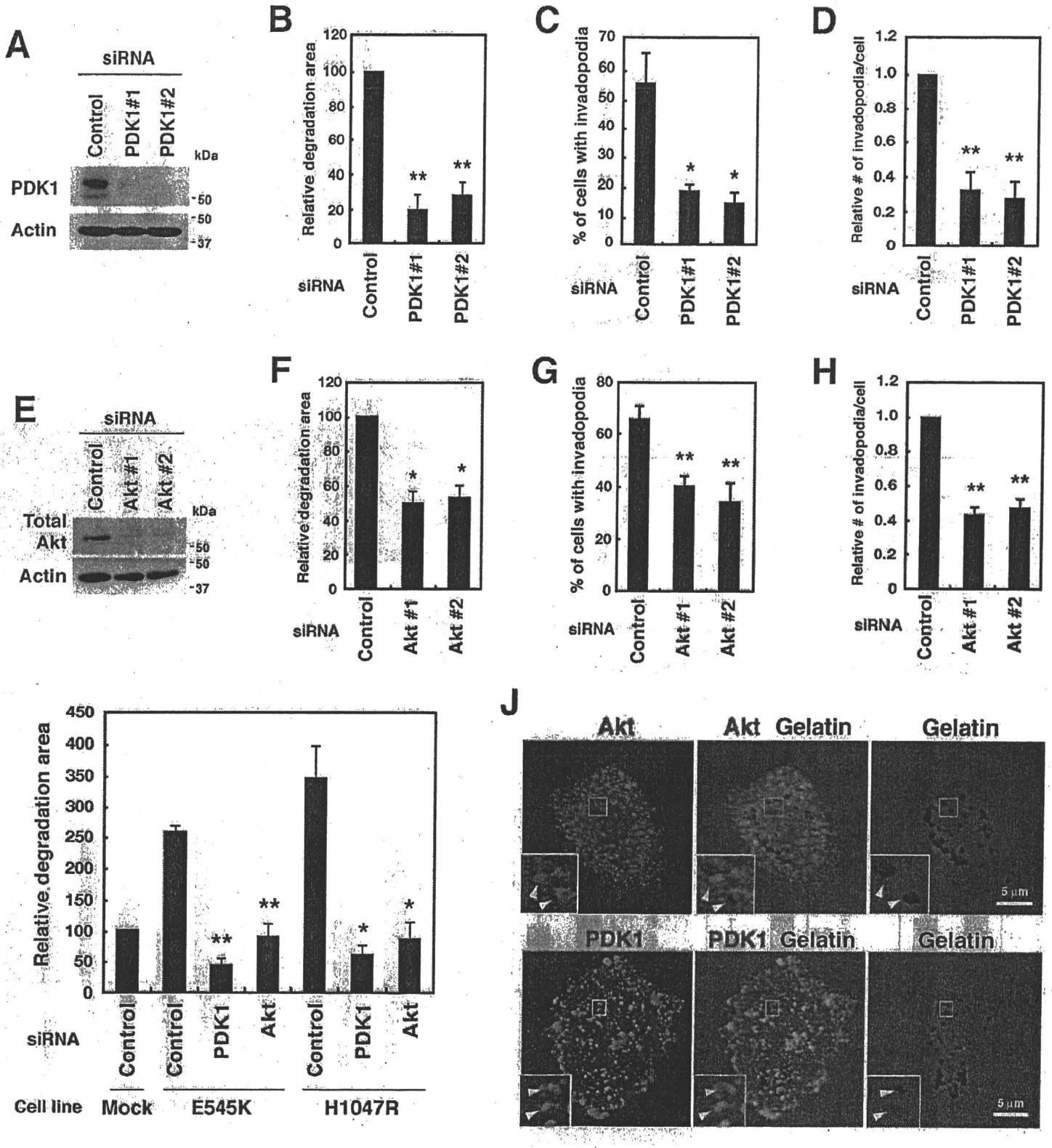


Yamaguchi et al. Fig. 4



Yamaguchi et al. Fig. 5