Warfarin blocks Gas6-mediated Axl activation required for pancreatic cancer epithelial plasticity and metastasis

Amanda Kirane[1,2]*, Kathleen F. Ludwig[2,3]*, Noah Sorrelle[2,4], Gry Haaland[6], Tone Sandal[6], Renate Ranaweera[6], Jason E. Toombs[1,2], Miao Wang[1,2], Sean P. Dineen[1], David Micklem[7], Michael T. Dellinger[1,2], James B. Lorens[6] and Rolf A. Brekken[1,2,5,#]

[1]Division of Surgical Oncology, Department of Surgery, [2]Hamon Center for Therapeutic Oncology Research, [3]Division of Hematology/Oncology, Department of Pediatrics, [4]Cell Regulation Graduate Program, and [5]Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75390-8593 USA

[6]Department of Biomedicine, Centre for Cancer Biomarkers, Norwegian Centre of Excellence, University of Bergen, Bergen Norway

[7]BerGenBio AS, Bergen, Norway

[#], corresponding author; *, equal contribution

Running title: *Warfarin inhibits Axl-mediated tumor progression*

Corresponding author:

Rolf A. Brekken, PhD
Hamon Center for Therapeutic Oncology Research
University of Texas Southwestern Medical Center
6000 Harry Hines Blvd.
Dallas, TX 75390-8593
Tel: 214.648.5151; Fax: 214.648.4940
rolf.brekken@utsouthwestern.edu

Key words: warfarin, Axl, Gas6, metastasis, pancreatic cancer, EMT

Figures, 4; Supplemental Information: Figures, 6; Table, 2

Abstract

Repurposing 'old' drugs can facilitate rapid clinical translation but necessitates novel mechanistic insight. Warfarin, a vitamin K "antagonist" used clinically for the prevention of thrombosis for over 50 years, has been shown to have anti-cancer effects. We hypothesized that the molecular mechanism underlying its anti-tumor activity is unrelated to its effect on coagulation, but is due to inhibition of the Axl receptor tyrosine kinase on tumor cells. Activation of Axl by its ligand Gas6, a vitamin K-dependent protein, is inhibited at doses of warfarin that do not affect coagulation. Here we show that inhibiting Gas6-dependent Axl activation with low dose warfarin or with other tumor-specific Axl targeting agents, blocks the progression and spread of pancreatic cancer. Warfarin also inhibited Axl-dependent tumor cell migration, invasiveness and proliferation while increasing apoptosis and sensitivity to chemotherapy. We conclude that Gas6-induced Axl signaling is a critical driver of pancreatic cancer progression and its inhibition with low dose warfarin or other Axl targeting agents may improve outcome in patients with Axl-expressing tumors.

Introduction

Vitamin K "antagonists" have been associated anecdotally with anti-tumor and anti-metastatic effects in preclinical and clinical studies since the 1960's (1-3). Results from dedicated clinical studies designed to evaluate the anti-metastatic activity of warfarin have been variable, in part due to complications associated with full anti-coagulation. The anti-cancer effects of warfarin are generally attributed to thromboembolic inhibition, although the molecular mechanism has not been elucidated. The Axl receptor tyrosine kinase is associated with aggressive cancer and poor patient outcome in several malignancies including pancreatic cancer (4). Since warfarin blocks vitamin K-dependent γ-carboxylation of glutamic acids (5) and the γ-carboxyglutamic acid-rich (GLA) domain of Gas6 is required to induce Axl tyrosine kinase activity (6-8), we hypothesized that the anti-tumor activity of warfarin could be due to inhibition of Gas6-mediated Axl activation on tumor cells. Warfarin potently inhibits Gas6-dependent Axl activation (9) at an IC_{50} ≈0.6 nM, a concentration well-below that required to achieve anti-coagulation (5, 10). Here, we exploited this differential effect to determine if low dose (1.5- 3.0 μM) warfarin treatment impedes pancreatic cancer progression by inhibiting Axl signaling independent of anti-coagulation.

Materials and Methods

Cell lines

Human pancreatic cancer cell lines AsPC-1, Panc-1, Capan-1, and Mia PaCa-2 were obtained from ATCC (Manassas, VA), the murine cell line Pan02 was obtained from the DCTD tumor repository maintained by the NCI at Frederick. C5LM2 is a variant of Panc1 developed in our laboratory that was generated through 2 passages of growth in vivo and culture of liver metastases and has been characterized previously (11). C5LM2, AsPC-1, Panc-1, Pan02, and Mia PaCa-2 lines were grown in DMEM, Capan-1 in was grown in IMDM, all cell lines were grown in a humidified atmosphere with 5% CO_2, at 37˚C, and have been DNA fingerprinted for provenance using the Power-Plex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained by ATCC and were confirmed to be free of mycoplasma (e-Myco kit, Boca Scientific).

Animal studies

All animals were housed in a pathogen-free facility with 24-hour access to food and water. Experiments were approved by, and conducted in accordance with, an IACUC approved protocol at UT Southwestern. LSL-$Kras^{G12D}$; $Cdkn2a^{lox/lox}$; $p48^{Cre}$ (KIC) mice were generated as previously described (12). Four- to 6-week-old female NOD/SCID and C57/Bl6 mice were obtained from an on campus supplier. A total of 1x10ˆ6 AsPc-1, Panc-1, Mia Paca2, Capan-1, C5LM2, Mia shLuc and Mia shAxl or 5x10ˆ5 Pan02 cells, were injected orthotopically as described (12). Mice with established tumors, as determined by sonography were randomized to receive normal drinking water or water containing 1 mg/L (~3.0 µM) warfarin for experiments in immunocompromised mice and 0.5 mg/L (~1.5 µM) in experiments in immunocompetent animals with or without gemcitabine 25 mg/kg twice weekly depending on experimental design. KIC mice were treated with warfarin 4 weeks starting at 3 weeks of age. For all experiments, warfarin containing water was replenished every 3 days. For Mia Paca2 tumor bearing mice, additional conditions of gemcitabine +/- 10C9 (250 µg ip 2x/week) were conducted. Mice bearing Panc-1, Capan-1, C5LM2 and Mia Paca2 tumors were sacrificed after 6 weeks of therapy. AsPc-1 tumor bearing mice received 4 weeks of therapy and Pan02 tumor

bearing mice 3 week of therapy. ShRNA lines were allowed to grow for 8-10 weeks. For all therapy experiments primary tumor burden was established by weighing pancreas and tumor en bloc. Metastatic incidence was determined by visual inspection of the liver and abdominal cavity and confirmed by H&E of liver sections. Tissues were fixed in 10% formalin or snap-frozen in liquid nitrogen for further studies. C5LM2 cells were injected intrasplenically to establish liver metastases, tumors were allowed to grow for 24 weeks and mice were randomized to receive either normal drinking water or warfarin (1 mg/L) starting 48 hours prior or 48 hours after tumor cell injection. Liver tumor burden was determined by liver weight.

Histology and tissue analysis

Formalin-fixed tissues were embedded in paraffin and cut in 6 µm sections. Sections were evaluated by H&E and immunohistochemical analysis using antibodies specific for vimentin (Phosphosolutions), endomucin, E-cadherin, (Santa Cruz), phospho-histone H3 (Upstate), cleaved caspase-3 (Cell Signaling). Negative controls included omission of primary antibody and immunofluorescence evaluation was conducted as described (12). Necrotic area was determined by quantification of percent viable tumor area on low magnification of tumor sections by H&E.

Statistics

Data were analyzed using GraphPad software (GraphPad Prism version 4.00 for Windows; GraphPad Software; www.graphpad.com). Results are expressed as mean± s.e.m. or s.d. Data were analyzed by t-test or ANOVA and results are considered significant at $p < 0.05$.

Additional methods are described in the supplemental material.

Results and Discussion

We evaluated the efficacy of low dose warfarin (0.5-1 mg/L of drinking water) as a single agent in five murine models of pancreatic ductal adenocarcinoma (PDA) (**Fig. 1A and B**). Low dose warfarin therapy was administered when animals had established intrapancreatic tumors as measured by sonography. Treatment with low dose warfarin reduced primary tumor growth in in a syngenic model (Pan02, **Fig. 1A**), a spontaneous genetic PDA model (*KIC*, **Fig. 1A**) but had little effect on the growth of human tumor xenografts (Panc1, AsPC1, Capan-1, **Fig. 1A**). Importantly, low dose warfarin consistently and potently inhibited metastatic burden (**Fig. 1B and Supplementary Table 1**) in four of the five PDA models. Expression analysis revealed that warfarin-sensitive tumors expressed detectable levels of Axl, while the nonresponsive Capan-1 tumors did not (**Fig. 1C-E**). Furthermore, Gas6 was expressed at detectable levels in most PDA cell lines (data not shown and (4)), indicative of autocrine Axl activation. To evaluate the effects of selective Axl inhibition on PDA we used a stable retroviral shRNA approach. Axl knockdown completely suppressed the growth of orthotopic Mia PaCa-2 tumors (**Fig. 1F**). Extended in vivo growth of shAxl Mia Paca-2 cells in an independent experiment resulted in 4 of 7 mice developing tumors. These tumors were subsequently found to express Axl (**Supplementary Fig. 1**). To validate tumor-selective inhibition of Axl activity in the treatment setting we developed a function-blocking human-specific anti-Axl monoclonal antibody, 10C9 (**Supplementary Fig. 2**). Treatment of established orthotopic Mia PaCa-2 tumors with 10C9 blunted primary tumor growth and potently suppressed metastases (**Fig. 1G**). These results support the notion that low dose warfarin inhibits pancreatic tumor progression in manner dependent on tumor cell Axl expression.

To determine the effect of warfarin on Gas6-induced Axl signaling in PDA we evaluated phosphorylated Axl (pAxl) and downstream signaling via the PI3K-Akt signaling pathway (13). Warfarin prevented γ-carboxylation of Gas6 in vitro (**Fig. 2A**) inhibited basal pAxl levels in Panc-1 cells, an effect that was rescued by addition of exogenous vitamin K (**Fig. 2B**). The effect of warfarin on pAxl was validated in Mia PaCa-2 and Panc-1 immunocytochemistry (**Supplementary Fig. 3**). Further warfarin or BGB324, a specific inhibitor of Axl tyrosine kinase

activity (14) inhibited phosphorylation of Axl in Panc-1 cells (**Fig. 2C**). Consistent with these results, treatment of Panc-1 cells in vitro with 10C9 resulted in decreased Axl and p-Axl levels (**Supplementary Fig. 2C and D**). Further, warfarin inhibited Gas6-induced activation of AKT in Panc1 cells in vitro (**Fig. 2D**). Additionally, the effect of low dose warfarin treatment on Panc-1 xenografts was consistent with the effects on Axl signaling in vitro. Warfarin treatment substantially suppressed the level of pAxl and pAkt in Panc-1 tumors (**Fig. 2E**), decreased expression of phosphorylated histone H3, a marker of proliferation, and elevated cleaved caspase 3, and tumor necrosis (**Supplementary Fig. 4**) and increased the level of cleaved Parp (**Fig. 2E**). Low dose warfarin also reduced intratumoral microvessel density (**Supplementary Fig. 4D**) consistent with the reported pro-angiogenic activity of Axl (15).

Axl has been associated with enhanced tumor cell migration and metastatic invasiveness (16). Warfarin reduced basal and Gas6-induced cell migration (scratch assay) in an Axl-dependent manner (**Fig. 2F**). Furthermore, tumor cell sphere formation and invasiveness in 3D culture was inhibited by warfarin and shRNA knockdown of Axl in Mia PaCa-2 cells (**Fig. 3A-C**). Warfarin also inhibited anchorage independent growth of Axl-expressing cells (**Fig. 3D**) and inhibited liver colonization of Panc-1 cells after intrasplenic injection regardless of whether warfarin was administered pre or post (48 hrs) tumor cell injection (**Fig. 3E**).

We also evaluated whether Axl inhibition with warfarin or 10C9 augmented the efficacy of gemcitabine, the frontline therapy for pancreatic cancer. Warfarin treatment had no effect on the IC_{50} of gemcitabine on Axl-negative cells lines (Capan-1 and Mia PaCa-2 shAxl) in vitro. However, low dose warfarin potentiated the anti-proliferative effect of gemcitabine, reducing the IC_{50} 8.4 and 211 fold in AsPC-1 and Panc-1 cells, respectively. Warfarin also lowered the gemcitabine IC_{50} in Mia PaCa-2 and Pan02 cells (**Supplementary Table 2**). In vivo blockade of Axl activation with low dose warfarin or 10C9 augmented gemcitabine reduction of primary tumor growth and dramatically improved metastatic control (**Fig. 3F and G**).

Metastasis and drug resistance are linked to induction of epithelial-to-mesenchymal transition (EMT) gene programs in pancreatic cancer (17). Axl expression is elevated in tumor cells by EMT and correlated with mesenchymal marker proteins such as vimentin (16). Mia PaCa-2 cells display an EMT-like phenotype under basal conditions (18). We found that treatment of Mia PaCa-2 cells with warfarin for 48 hrs in vitro reduced pAxl levels, surface Axl expression and the mesenchymal markers Zeb1 and vimentin, while elevating the expression of the epithelial marker E-cadherin (**Supplementary Fig. 5**). Treatment of Panc-1 cells in vitro with TGFβ and collagen I, conditions that induce EMT , enhanced Axl expression and activation, an effect that was blocked by addition of warfarin (**Fig. 4A**). Consistent with these results, Zeb1 and nuclear β-catenin levels, another mesenchymal marker, were significantly reduced by warfarin indicative of phenotypic reversal (**Fig. 4A**). Furthermore, Gas6 addition to Panc1 cells in culture increased the expression of vimentin and Zeb1, an effect that was blocked by 10C9 (**Fig. 4B**). Additionally, we identified that exposure to TGFβ and collagen induced Axl expression in Capan-1 cells (Capan-EMT), which correlated with increased expression of transcription factors (Zeb1, Snail, Twist) that drive EMT. The EMT-dependent induction of Axl in Capan-1 established autocrine activation via endogenous Gas6. Correspondingly, the Capan-EMT cells were sensitive to treatment with warfarin leading to decreased Axl expression, upregulated E-cadherin and increased cleaved caspase-3 levels (**Supplementary Fig. 6**). Finally, we found that low dose warfarin treatment of PDA Panc1 xenografts reduced expression of vimentin and elevated the expression of E-cadherin, results consistent with the observed EMT reversal in vitro (**Fig. 4C**).

Our data show that warfarin exerts its anti-cancer effects by inhibiting Gas6-mediated Axl activation on tumor cells. We found that Gas6-Axl signal transduction is required to maintain epithelial-mesenchymal plasticity traits of aggressive pancreatic tumors comprising tumorigenicity, invasiveness, survival, drug sensitivity and metastasis. EMT gene expression patterns are apparent early in pancreatic cancer development, associated with inflammatory pre-malignant lesions, and drive early metastatic spread. Inflammatory mediators such as TGFβ that induce EMT transcription factor-mediated gene reprogramming are prominent in malignant pancreatic cancer. Consistent with this, Axl expression is elevated by EMT

transcription factors in breast and lung epithelial cells (16, 19, 20). Further, Axl expression is associated with EMT gene signatures in drug resistant NSCLC and a requisite effector of EMT-related acquired resistance to various therapeutics (19). The wide spread expression of Axl in advanced cancer from diverse cellular origins suggests that tumor cell associated Axl is a fundamental contributor to malignant progression. Inhibition of Axl signaling is associated with loss of malignant traits including cell migration and metastasis (16). Congruently, we show that low dose warfarin treatment and tumor-specific Axl selective targeting potently block metastasis in several models of PDA. This is associated with a loss of mesenchymal protein expression and EMT transcription factor expression that result in decreased proliferation and increased apoptosis.

Our results demonstrate that low dose warfarin-mediated Axl inhibition is effective as an anti-cancer agent without associated complications from anti-coagulation. These results strongly suggest that the anecdotal anti-tumor effects observed clinically with coumarin-based anti-coagulants are due in part to specific inhibition of Gas6-mediated Axl activation on tumor cells. These results are consistent with recent studies that show γ-carboxylation of Gas6 is required for Gas6-mediated Axl activation (21). Further, Paolino et al (10) demonstrated that low dose warfarin treatment (0.5 mg/L in drinking water) inhibits Gas6-mediated activation of TAM receptors, Tyro3, Axl and Mer (aka Mertk) on natural killer (NK) cells, leading to enhanced NK cell anti-tumor activity in a murine mammary adenocarcinoma (4T1) model system. We have previously shown that tumor selective Axl inhibition is sufficient to block metastasis in the 4T1 model (20). Hence, the effects of systemic Axl inhibition may exert anti-tumor effects through tumor and host-response dependent mechanisms. On the other hand, while each of the animal models we employed has an intact NK compartment, we did not observe any anti-tumor activity in Axl-negative Capan-1 cells suggesting minimal NK cell anti-tumor activity in these models. Taken together, our results of tumor-selective Axl inhibition in multiple settings suggests that inhibition of tumor cell Axl tyrosine kinase activity is a critical determinant for the observed efficacy of warfarin in cancer patients.

Acknowledgments

The authors thank Drs. Alan Schroit, Thomas Wilkie and John Mansour for critical comments on the text and the members of the Brekken and Lorens laboratories for advice and helpful discussion.

Grant Support

References

1.   Brown JM. A study of the mechanism by which anticoagulation with warfarin inhibits blood-borne metastases. Cancer Res. 1973;33:1217-24.

2.   McCulloch P, George WD. Warfarin inhibition of metastasis: the role of anticoagulation. Br J Surg. 1987;74:879-83.

3.   Schulman S, Lindmarker P. Incidence of cancer after prophylaxis with warfarin against recurrent venous thromboembolism. Duration of Anticoagulation Trial. The New England journal of medicine. 2000;342:1953-8.

4.   Song X, Wang H, Logsdon CD, Rashid A, Fleming JB, Abbruzzese JL, et al. Overexpression of receptor tyrosine kinase Axl promotes tumor cell invasion and survival in pancreatic ductal adenocarcinoma. Cancer. 2011;117:734-43.

5.   Nakano T, Kawamoto K, Kishino J, Nomura K, Higashino K, Arita H. Requirement of gamma-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells. The Biochemical journal. 1997;323 (Pt 2):387-92.

6.   Hafizi S, Dahlback B. Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. FEBS J. 2006;273:5231-44.

7.   Hasanbasic I, Rajotte I, Blostein M. The role of gamma-carboxylation in the anti-apoptotic function of gas6. Journal of thrombosis and haemostasis : JTH. 2005;3:2790-7.

8.   Varnum BC, Young C, Elliott G, Garcia A, Bartley TD, Fridell YW, et al. Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Nature. 1995;373:623-6.

9.   Tsou WI, Nguyen KQ, Calarese DA, Garforth SJ, Antes AL, Smirnov SV, et al. Receptor tyrosine kinases, TYRO3, AXL, and MER, demonstrate distinct patterns and complex regulation of ligand-induced activation. J Biol Chem. 2014;289:25750-63.

10.   Paolino M, Choidas A, Wallner S, Pranjic B, Uribesalgo I, Loeser S, et al. The E3 ligase Cbl-b and TAM receptors regulate cancer metastasis via natural killer cells. Nature. 2014;507:508-12.

11.   Melisi D, Ishiyama S, Sclabas GM, Fleming JB, Xia Q, Tortora G, et al. LY2109761, a novel transforming growth factor beta receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis. Mol Cancer Ther. 2008;7:829-40.

12.   Ostapoff KT, Cenik BK, Wang M, Ye R, Xu X, Nugent D, et al. Neutralizing Murine TGFbetaR2 Promotes a Differentiated Tumor Cell Phenotype and Inhibits Pancreatic Cancer Metastasis. Cancer Res. 2014.

13.   Sawabu T, Seno H, Kawashima T, Fukuda A, Uenoyama Y, Kawada M, et al. Growth arrest-specific gene 6 and Axl signaling enhances gastric cancer cell survival via Akt pathway. Mol Carcinog. 2007;46:155-64.

14.   Holland SJ, Pan A, Franci C, Hu Y, Chang B, Li W, et al. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. Cancer Res. 2010;70:1544-54.

15.   Ruan GX, Kazlauskas A. Axl is essential for VEGF-A-dependent activation of PI3K/Akt. The EMBO journal. 2012;31:1692-703.

16.   Gjerdrum C, Tiron C, Hoiby T, Stefansson I, Haugen H, Sandal T, et al. Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival. Proc Natl Acad Sci U S A. 2010;107:1124-9.

17.   Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, et al. EMT and dissemination precede pancreatic tumor formation. Cell. 2012;148:349-61.

18.   Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, et al. Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. Cancer Res. 2009;69:5820-8.

19.   Wilson C, Ye X, Pham TQ, Lin E, Chan SM, McNamara E, et al. AXL inhibition sensitizes mesenchymal cancer cells to anti-mitotic drugs. Cancer Res. 2014.

20.   Byers LA, Diao L, Wang J, Saintigny P, Girard L, Peyton M, et al. An epithelial-mesenchymal transition gene signature predicts resistance to EGFR and PI3K inhibitors and identifies Axl as a therapeutic target for overcoming EGFR inhibitor resistance. Clin Cancer Res. 2013;19:279-90.

21.   Lew ED, Oh J, Burrola PG, Lax I, Zagorska A, Traves PG, et al. Differential TAM receptor-ligand-phospholipid interactions delimit differential TAM bioactivities. Elife. 2014;3.

Figure Legends

Figure 1. Warfarin inhibits tumor progression in Axl expressing cell lines. A, Primary tumor burden after therapy with warfarin. Therapy was initiated when implanted tumors were visible by ultrasound (~10 mm^3) and consisted of control (normal drinking water) or warfarin, administered in the drinking water at 0.5 mg/L (immunocompetent mice: Pan02 (n=4, control; 3, warfarin), KIC (n=10, control; 8, warfarin)) or 1 mg/L (Panc-1 (n=10, control; 8, warfarin), AsPC-1 (n=8, control; 6, warfarin), Capan-1 (n=10, control; 7, warfarin)) and continued for 2-4 weeks until control mice were moribund. Therapy in *KIC* mice was initiated at 3 weeks of age and continued for 4 weeks. **B,** Metastases were determined grossly upon sacrifice and confirmed by histological evaluation of the liver. Metastatic burden is normalized to mean number of metastases in control treated animals and is displayed as a fold change. Incidence of metastasis is also indicated. **C,** Murine pancreatic cancer cells express Axl by flow cytometry. **D, E,** Expression of Axl message and protein by human pancreatic cancer cell lines. **F,** shRNA-mediated knock-down of Axl suppresses growth of orthotopic Mia PaCa-2 tumors (n=8, shLuc; 7, shAxl). Tumor volume determined by serial ultrasound. **G,** Inhibition of Axl with mAb 10C9 reduces tumor growth and suppresses metastasis of MiaPaCa-2 tumors (n=7, control; 8, 10C9). Therapy with mAb 10C9 (250 μg 2x/week) was initiated when tumors were established as above and persisted for 4 weeks. All results were compared by unpaired two-tailed t-test with Welch's correction, actual p values are shown. Error bars indicate s.e.m.

Figure 2. Warfarin inhibits Axl signaling in vitro and in vivo. A, HEK293 cells engineered to stably express recombinant Gas6 were grown in the presence of Vitamin K or Vitamin K + warfarin. Gas6 levels and γ-carboxylation were assayed by immunoblotting conditioned media. Conditioned media from untransfected HEK293 cells was used as a negative control. **B,** Panc1 cells were grown overnight in media with 1% serum with no additions (NT), warfarin (2 μM) or BGB324 (2 μM). Lysates were probed for total Axl (tAxl) and phosphorylated Axl (pAxl). **C,** Panc1 cells were grown in the presence of control media, Vitamin K, warfarin or warfarin + Vitamin K. The level of phosphorylated Axl (pAXL, red) was determined by immunofluorescence. **D,** Panc1 cells were grown overnight in media with 1% serum with no additions (control), warfarin (1 μM), Gas6 (1.3 nM) or Gas6 + warfarin. Lysates were probed for phosphorylated Akt (pAkt) and actin. **E,** Lysates from Panc1 tumors harvested from mice treated with control or warfarin were probed for expression of tAxl, pAxl, actin, pAKT, tAKT, and cleaved Parp. **F,** The effect of warfarin on cell migration was assessed by a 'scratch' assay. Monolayers of the indicted cells were wounded with a pipet tip. The cells were incubated in media containing 2% serum +/- warfarin (2 μM) or media containing 2% serum + Gas6 (1.3 nM) +/- warfarin. Wound closure was monitored at 16 hrs and is reported as % wound closure. *p<0.05, ***p<0.001 by ANOVA, Bonferroni's MCT.

Figure 3. Axl inhibition reduces colony formation and enhances chemotherapy. A-C, parental Mia PaCa-2 cells or Mia PaCa-2 cells stably transfected with shRNA targeting Axl (Mia shAxl) were grown as spheroids in matrigel for 7 days in the presence or absence of warfarin (200 ng/ml), n=4/condition. Mia PaCa-2 cell colonies form large stellate colonies characteristic of invasive tumor growth. Colonies and cognate cell projections were imaged (**A**) with a Nikon Phase contrast microscope using 40X and 200X magnification. Mean total colony number (**B**) and total colony area +/- s.d. (**C**) reflective of invasive growth were calculated using Image J image analysis. Scale bar, 100 μm. ****, $p<0.001$ vs Mia PaCa-2 NT; ##, $p<0.01$; ###, $p<0.005$. by ANOVA with Tukey's MCT. **D,** soft agar colony formation for AsPC-1, Mia PaCa-2, and Capan-1 cells grown in normal growth media in the presence or absence (Control) of warfarin (2 μM) for 14 days. Mean +/- s.d. colonies/ hpf is shown. Unpaired two tailed t-test with Welch's correction. **E,** liver metastases were quantified after intrasplenic injection of C5LM2 cells. Animals (10/group) were treated with normal drinking water, warfarin (1 mg/L) beginning 48 hours prior to (pre-op) or 48 hours following tumor cell injection (post-op) and then continued on warfarin therapy until time of sacrifice. ***, $p<0.005$; ****, $p<0.001$ vs control; #, $p<0.05$ vs post-injection treatment group by ANOVA with Tukey's MCT. **F - I,** Mice bearing established orthotopic C5LM2 (**F, G**) or Mia PaCa-2 (**H, I**) were treated with saline (control), gemcitabine (Gem), Gem + warfarin (Gem + War). Mice bearing Mia PaCa-2 tumors were also treated with warfarin alone (War), Gem + 10C9. Mice were sacrificed when control treated animals were moribund and primary and metastatic burden was determined. Primary tumor weight +/- s.d. (**F, H**) and fold change in metastases +/- s.d. (**G, I**) is shown. The incidence of metastasis in each group is shown as a %. **, $p<0.01$; ***, $p<0.005$; ****, $p<0.001$ vs Control; [##], $p<0.01$ vs Gem by ANOVA with Tukey's MCT.

Figure 4. Warfarin inhibits Axl-dependent maintenance of EMT. A, the expression level of pAxl, Zeb1 and nuclear β-catenin in Panc1 cells in vitro was measured by immunofluorescence under normal culture conditions or after growth on collagen matrix and treatment with TGF-β (20 ng/ml) to induce epithelial to mesenchymal transition, with or without warfarin (2 μM). p-Axl was normalized to total Axl area. **B,** Panc1 cells were treated with either SFM, recombinant Gas 6 (100 ng/ml) or Gas6 following pretreatment with 10C9 (mAb anti-Axl). Transition to a mesenchymal phenotype was characterized by changes in vimentin and nuclear Zeb1 expression determined by immunofluoresence. (**A,B**) Data are displayed as mean ± SEM and represent 5 images per chamber, with assay performed in triplicate. % area per image was normalized to cell number. Images were analyzed using Elements software. *, $p<0.05$; ****, $p<0.001$ by ANOVA with Tukey's MCT. **C,** Paraffin embedded sections of Panc-1 tumors were analyzed by immunofluorescence for markers of EMT. Representative images of E-Cadherin and Vimentin are shown. Total magnification, 200X; scale bar, 100 μM. Images were analyzed using Elements software; quantification of % area fraction is shown. Data is displayed as mean ± s.d. and represent 5 images per tumor with 5 animals per group analyzed. ****$p<0.0001$ by t-test.
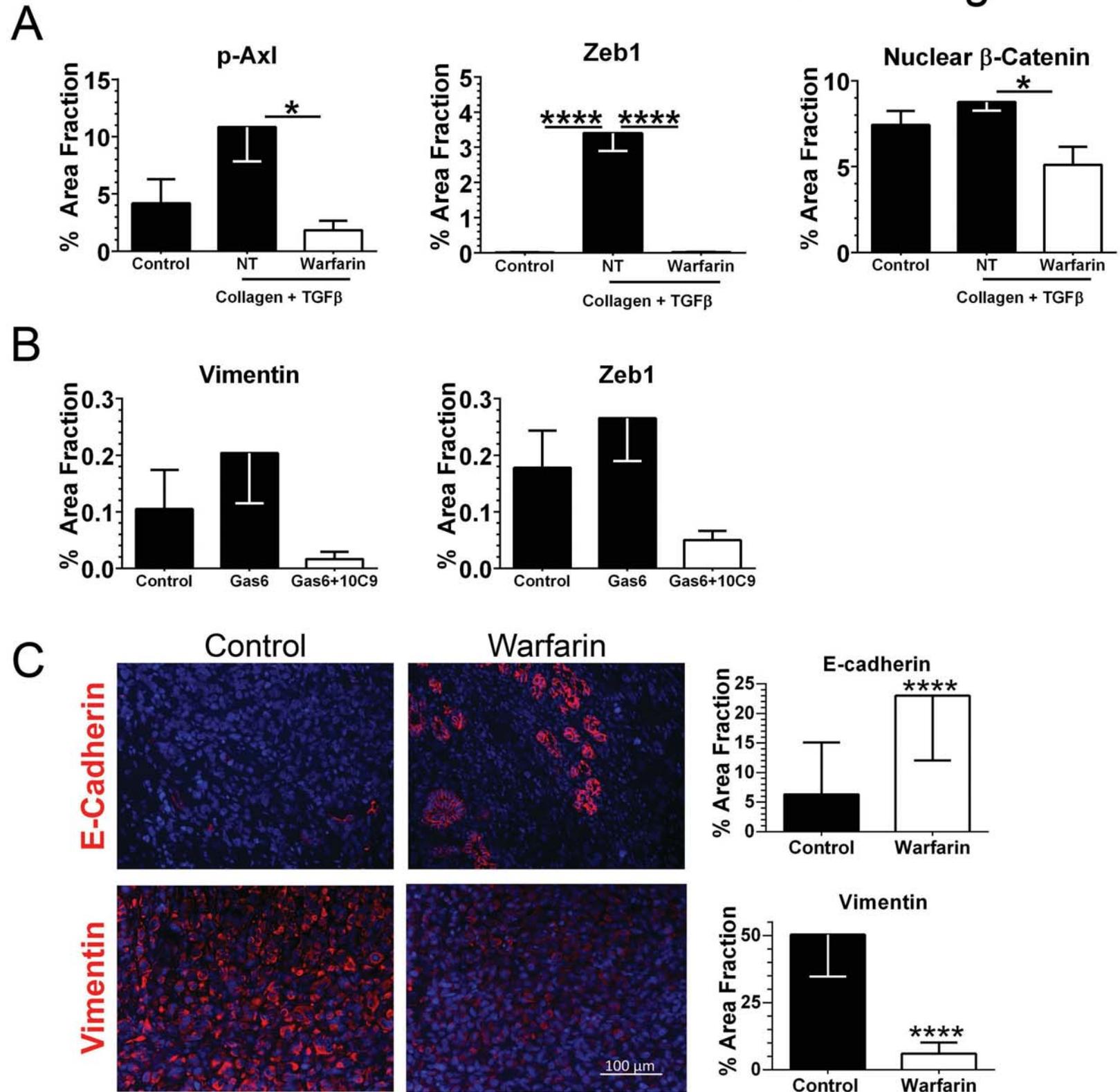
Kirane et al Figure 1
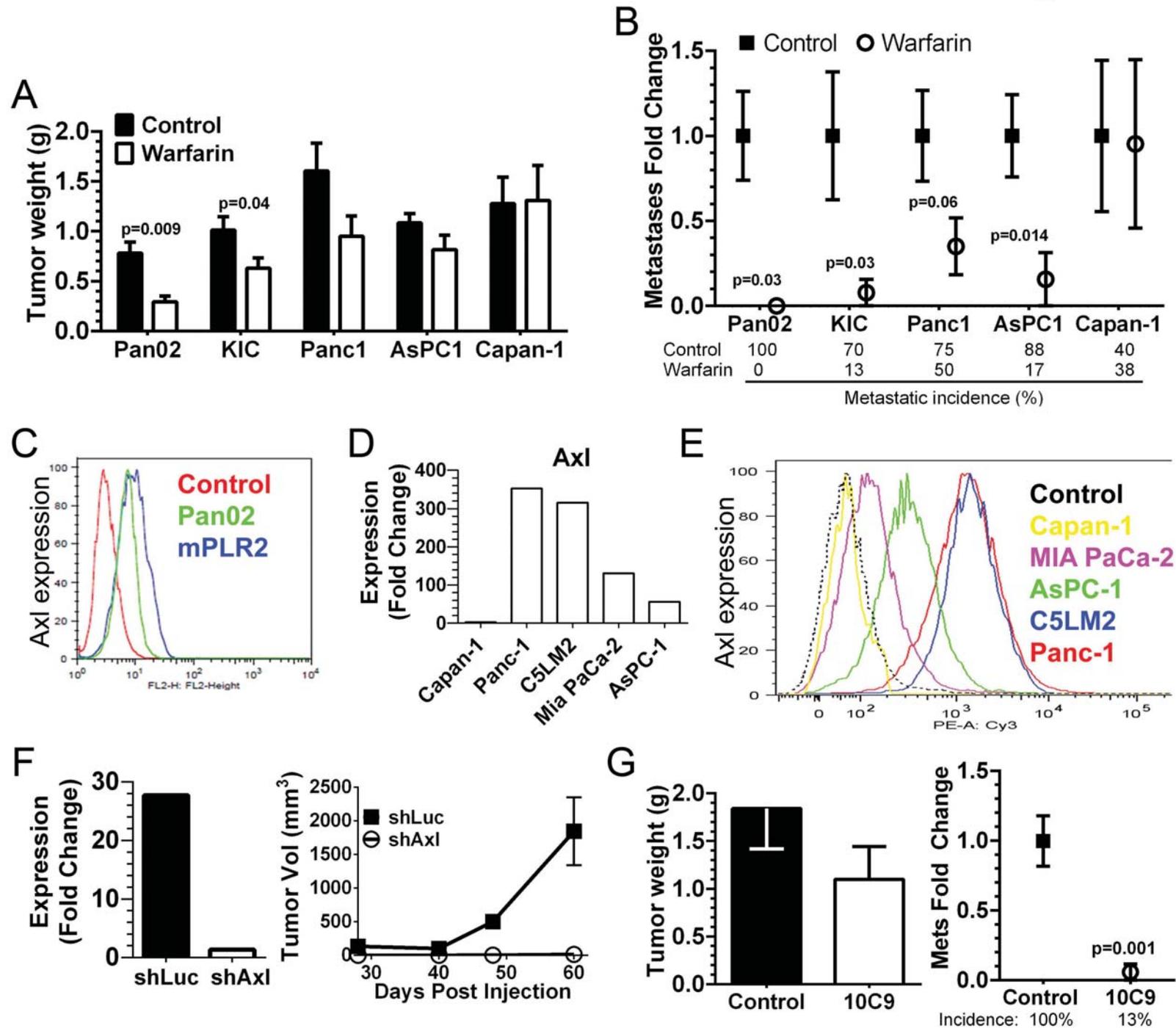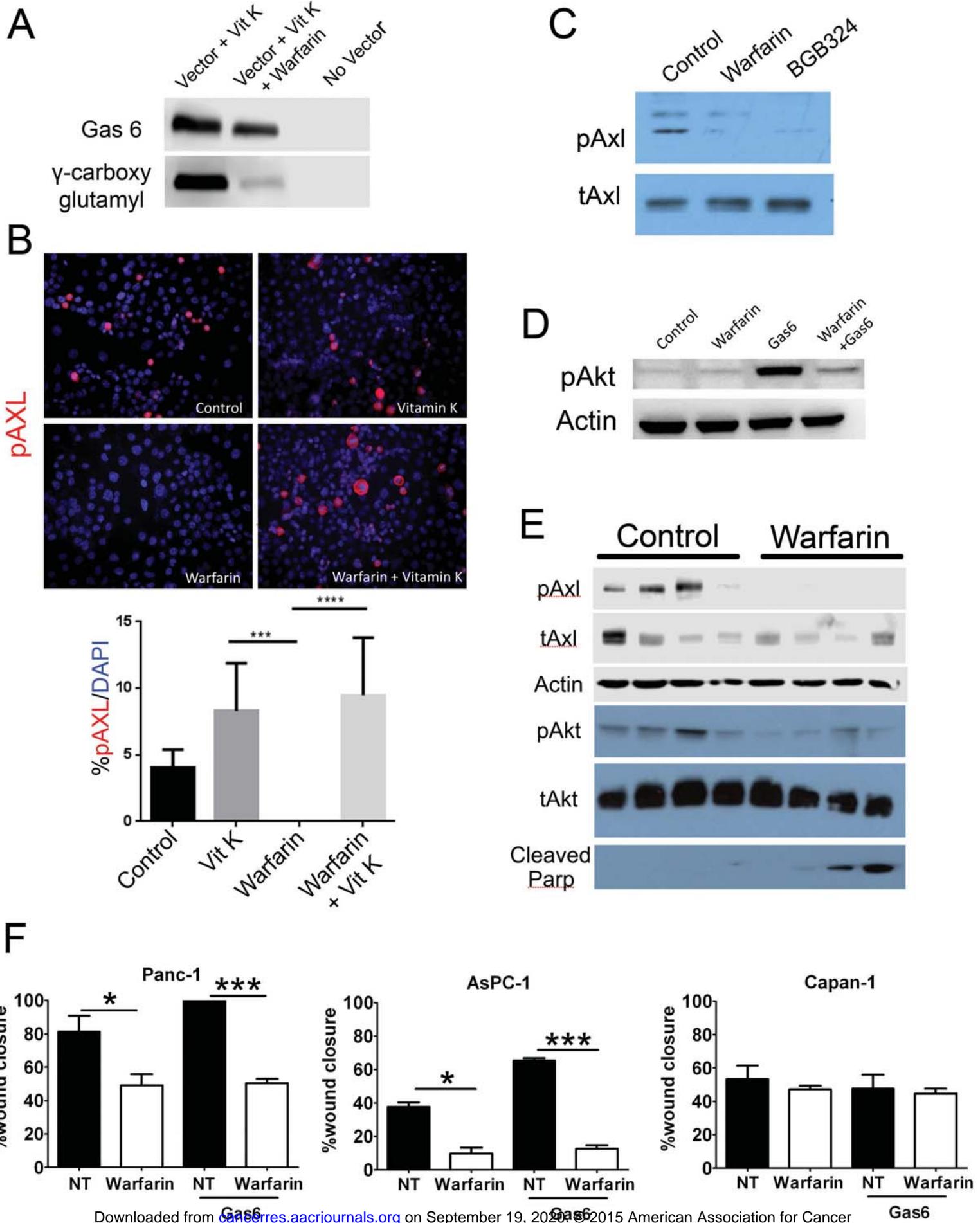
Kirane et al Figure 3
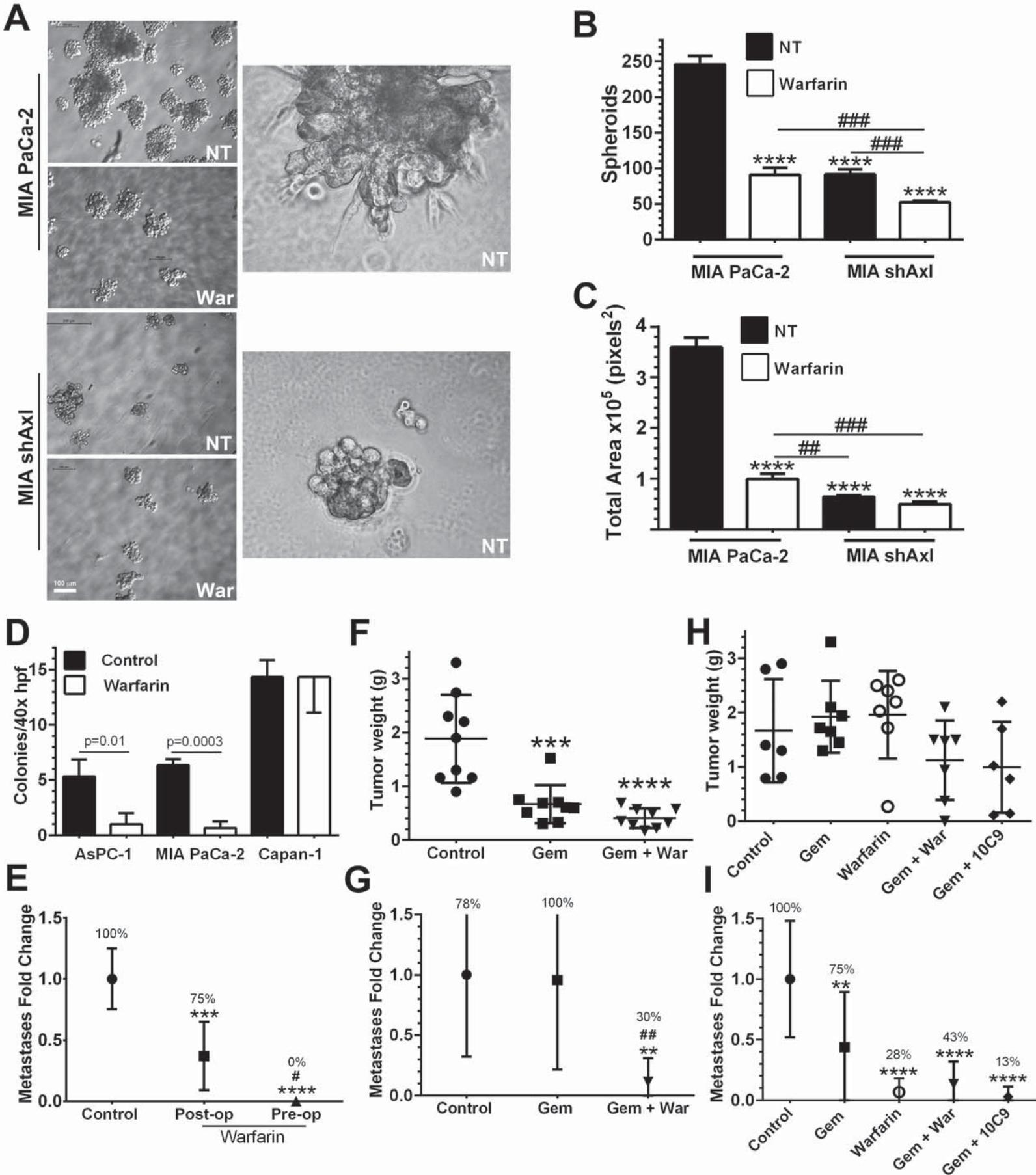
Kirane et al Figure 4

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Warfarin blocks Gas6-mediated Axl activation required for pancreatic cancer epithelial plasticity and metastasis

Amanda Kirane, Kathleen F Ludwig, Noah Sorrelle, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-2887-T
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2015/07/27/0008-5472.CAN-14-2887-T.DC1
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2015/07/23/0008-5472.CAN-14-2887-T. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.