Kruppel-like factor 4 signals through microRNA-206 to promote tumor initiation and cell survival

C-C Lin  
*West Virginia University*

S B. Sharma  
*West Virginia University*

M K. Farrugia  
*West Virginia University*

S L. McLaughlin  
*West Virginia University*

R J. Ice  
*West Virginia University*

*See next page for additional authors*

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Authors
C-C Lin, S B. Sharma, M K. Farrugia, S L. McLaughlin, R J. Ice, Y V. Loskutov, E N. Pugacheva, K M. Brundage, D Chen, and J M. Ruppert

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Tumor cell heterogeneity poses a major hurdle in the treatment of cancer. Mammary cancer stem-like cells (MaCSCs), or tumor-initiating cells, are highly tumorigenic sub-populations that have the potential to self-renew and to differentiate. These cells are clinically important, as they display therapeutic resistance and may contribute to treatment failure and recurrence, but the signaling axes relevant to the tumorigenic phenotype are poorly defined. The zinc-finger transcription factor Kruppel-like factor 4 (KLF4) is a pluripotency mediator that is enriched in MaCSCs. KLF4 promotes RAS-extracellular signal-regulated kinase pathway activity and tumor cell survival in triple-negative breast cancer (TNBC) cells. In this study, we found that both KLF4 and a downstream effector, microRNA-206 (miR-206), are selectively enriched in the MaCSC fractions of cultured human TNBC cell lines, as well as in the aldehyde dehydrogenase-high MaCSC sub-population of cells derived from xenografted human mammary carcinomas. The suppression of endogenous KLF4 or miR-206 activities abrogated cell survival and in vivo tumor initiation, despite having only subtle effects on MaCSC abundance. Using a combinatorial approach that included in silico as well as loss- and gain-of-function in vitro assays, we identified miR-206-mediated repression of the pro-apoptotic molecules programmed cell death 4 (PDCD4) and connexin 43 (CX43/GJA1). Depletion of either of these two miR-206-regulated transcripts promoted resistance to anoikis, a prominent feature of CSCs, but did not consistently alter MaCSC abundance. Consistent with increased levels of miR-206 in MaCSCs, the expression of both PDCD4 and CX43 was suppressed in these cells relative to control cells. These results identify miR-206 as an effector of KLF4-mediated prosurvival signaling in MaCSCs through repression of PDCD4 and CX43. Consequently, our study suggests that a pluripotency factor exerts prosurvival signaling in MaCSCs, and that antagonism of KLF4-miR-206 signaling may selectively target the MaCSC niche in TNBC.

A potential mediator of the MaCSC phenotype is the pluripotency factor Kruppel-like factor 4 (KLF4). This zinc-finger transcription factor promotes the formation of induced pluripotent stem cells from adult somatic cells and can have both antitumorigenic and protumorigenic roles in a context-dependent manner.16-20 The capability of KLF4 to exert protumorigenic influences may reflect its role as a prosurvival stress response factor.21-28 In support of a protumorigenic role, KLF4 promotes epithelial transformation in vitro, escape from RAS-induced senescence and skin tumor initiation in transgenic mice.16,29,30 Furthermore, loss-of-function studies reveal that KLF4 promotes cell survival following radiation-induced DNA damage, and promotes the tumorigenicity of colorectal CSCs-enriched spheroid cells.26,31

In human breast cancer, KLF4 promoter demethylation and KLF4 protein expression indicate an unfavorable prognosis.32-34 KLF4 expression is positively correlated with tumor size, advanced grade and stage.35 We previously identified microRNAs, including microRNA-206 (miR-206) and miR-21, as direct transcriptional targets of KLF4 that promote RAS-extracellular signal-regulated kinase (ERK) signaling in triple-negative breast cancer (TNBC) cells.36,37 Although on its own each miR exerts only subtle

1Department of Biochemistry, West Virginia University, School of Medicine, Morgantown, WV, USA; 2Program in Cancer Cell Biology, West Virginia University, Morgantown, WV, USA and 3Division of Preventive Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA. Correspondence: Professor JM Ruppert, Department of Biochemistry, West Virginia University, School of Medicine, PO Box 9142, 1 Medical Center Drive, Morgantown, WV 26506, USA.
E-mail: mruppert@hs.c.wvu.edu

These authors contributed equally to this work.

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influences on RAS-ERK pathway activity, the coexpression of miR-206 and miR-21 potently represses the expression of pathway inhibitors including RASA1 and SPRED1. Furthermore, miR-206 directly represses KLF4 translation, constituting a feedback loop.36

In this study, we observed elevation of KLF4 and miR-206 in the P+/E+ and ALDH^{high} MaCSC fractions. In TNBC cells, both KLF4 and miR-206 were critical for cell survival and in vivo tumor initiation. We identified the tumor-suppressor programmed cell death 4 (PDCD4) as a potential mediator of cell survival by miR-206. Furthermore, in TNBC cells we demonstrated the miR-206 regulation of a previously validated transcript, the gap junction protein connexin 43 (CX43/GJA1).38

Consistent with the elevated levels of miR-206 in MaCSCs, PDCD4 and CX43 levels were decreased. Supporting functional roles downstream of KLF4 and miR-206, suppression of either PDCD4 or CX43 led to anoikis resistance, an intrinsic property of CSCs.7,39-43 Finally, further documenting a prosurvival role, miR-206 promoted chemoresistance of TNBC cells against paclitaxel or doxorubicin. Our studies identify KLF4 and miR-206 as functional MaCSC markers that mediate cell survival. Consequently, KLF4 and/or miR-206 may be therapeutically targeted to selectively cripple MaCSCs in TNBCs.

RESULTS

miR-206 is highly expressed in basal-like breast cancers and MaCSCs

KLF4 protein levels correlate with an aggressive phenotype in breast tumors.32,33,35 Similar to KLF4, miR-206 was increased in human tumors of advanced histological grade (Figure 1a, left panel). Consistent with studies that identified upregulation of miR-206 in ER+ breast tumors, miR-206 levels were elevated in TNBCs compared with both ER+ and HER2+ human subgroups (Figure 1a, right panel).44,45 Enrichment of miR-206 was similarly observed in murine basal-like mammary tumors (Figure 1b). Compared with normal mammary tissues or tumors arising in the luminal MTMT_{Neu} model,46 we observed upregulation of both KLF4 and miR-206 in basal-like tumors derived from the C3(1)/TAg mammary model,46 we observed upregulation of both KLF4 and miR-206 in basal-like tumors derived from the C3(1)/TAg (C3(1)/TAg) genetically engineered mouse model (GEMM) (Figure 1c). These results are consistent with the direct regulation of miR-206 by KLF4 as previously reported.37

MaCSCs are enriched in the triple-negative subgroup of breast cancer and are thought to contribute to the aggressive behavior of these cancers.47-49 Similar to human and murine mammary carcinoma cells displaying high ALDH activity,1,50-52 MDA-MB-231 TNBC cells displaying the P+/E+ surface marker profile represent TICs.12 For SUM159PT cells, CD44+/CD24-/ESA- subset was previously identified as TICs.53 As the P+ phenotype is a surrogate for the CD44+/CD24- profile, the P+/E- SUM159PT cells are likely to represent MaCSCs.12

We analyzed KLF4 and miR-206 levels in flow-sorted sub-populations of MDA-MB-231 cells (Figure 1d, left panel). Compared with non-MaCSCs (that is, P/E), miR-206 and KLF4 were increased in the P+/E- sub-population (Figure 1d, middle panels). Using P+/E- cells, we profiled the expression of other genes associated with stem-like cell phenotypes.5,14,18,19 Compared with P/E cells, the expression of CD44, MYC, SOX2, NANOG, ZEB1 and SNAI2 was upregulated in P+/E- cells, whereas CD24 and POU5F1 (OCT3/4) expression were decreased (Figure 1d, right panel). Similarly, the P+/E- fraction of SUM159PT cells displayed elevated levels of KLF4 and miR-206, and showed a similar stem cell marker profile as the MDA-MB-231 cells (Figure 1e). These results associate KLF4 and miR-206 with the MaCSC phenotype in human breast cancer models.

KLF4 and miR-206 are enriched in MaCSCs derived from human patient-derived xenografts (PDxGs) and the C3(1)/TAg GEMM

KLF4 was similarly consistently elevated in lineage-negative (Lin-)ALDH^{high} MaCSCs isolated from human mammary tumor tissues that were passaged as PDxGs (Figure 2a). miR-206 was upregulated in three of these four cases. Notably, none of these tumors displayed an appreciable CD44+/CD24- MaCSC population (data not shown), consistent with the variable expression of these markers in patient samples.10,54,55

Tumorspheres are enriched for MaCSCs.7,8 Compared with cells grown in adherent (two-dimensional (2D)) monolayers, tumorspheres formed from the Lin- cells of C3(1)/TAg mammary tumors showed elevated levels of KLF4 and miR-206 (Figure 2b). ALDH^{high} cells from other mammary cancer GEMMs were previously shown to have properties of MaCSCs.50,52 Similar to the human tumors, Lin-/ALDH^{high} cells of C3(1)/TAg mammary tumors also had increased KLF4 and miR-206 relative to ALDH^{low} cells (Figure 2c). These results identify KLF4 and miR-206 as MaCSC markers and potential mediators of MaCSC malignant properties.

KLF4 and miR-206 can promote MaCSC abundance

To determine the effect of KLF4-miR-206 signaling on MaCSC abundance, we depleted KLF4 in MDA-MB-231 cells using two distinct lentiviral short hairpin RNA constructs (Figure 3a, left upper panel). Consistent with previous studies, miR-206 was suppressed following KLF4 knockdown (Figure 3a, left lower panel). In addition, P+/E- cell abundance was modestly decreased upon KLF4 depletion (Figure 3a, middle and right panels). Conversely, gain-of-function experiments showed that exogenous KLF4 promoted both miR-206 levels and the abundance of P+/E- cells (Figure 3b).

We next sought to determine whether miR-206 could have a causal role downstream of KLF4 to regulate MaCSC abundance. As expected, transfection of miR-206 mimic into MDA-MB-231 cells elevated the miR-206 level as detected by quantitative reverse transcription and PCR (qRT-PCR; Figure 3c, left upper panel). In addition, the level of KLF4 was suppressed, attributed to direct regulation of KLF4 protein translation by miR-206 (Figure 3c, left lower panel).36 Despite the reduced levels of KLF4, miR-206-transfected cells displayed higher P+/E- cell abundance relative to the control cells (Figure 3c, right panel). Similar regulation of P+/E- cell abundance by miR-206 was observed for SUM159PT cells (Figure 3d). These results establish miR-206 as a potential effector of KLF4 for regulation of MaCSC abundance.

To determine whether miR-206 can promote the MaCSC phenotype, we assayed by limiting dilution the capability of miR-206-transfected MDA-MB-231 cells to initiate tumors in vivo. Consistent with an increased number of P+/E- cells, miR-206-transfected cells formed tumors more efficiently in NOD/SCID-gamma (NSG) mice compared with control cells (Figure 3e; 2 × 10^3 cells, P = 0.0022). These results implicate miR-206 as an effector of KLF4 that promotes tumor initiation.

Endogenous KLF4 and miR-206 promote tumor cell survival and in vivo tumorigenesis

We next examined the impact of endogenous KLF4-miR-206 signaling on tumor initiation. Depletion of KLF4 reduced the tumor initiation rate of MDA-MB-231 cells in athymic nude mice (Figure 4a, left panels). This decrease in tumor incidence was reflected by the reduced mean tumor volume for all animals combined (Figure 4a, middle panels). Indicating that the major effect of KLF4 in this setting is restricted to tumor initiation, analysis of the tumor-positive subset revealed little difference in
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the tumor growth rate between KLF4-depleted cells and the control (Figure 4a, right panels).

To study the role of endogenous miR-206 during in vivo tumorigenesis, we analyzed the tumorigenicity of MDA-MB-231 cells treated by in vitro transfection of anti-sense oligonucleotides (anti-miR-206). Compared with cells transfected with the control, anti-miR-206 treatment reduced both tumor incidence and tumor growth (Figure 4b). As an indicator of successful transfection, KLF4 expression was increased (Figure 4c, left panel). Effects on tumor growth were not likely attributed to differences in cell proliferation rates, as anti-miR-206 had little effect (Figure 4c, right panel).

The critical role of endogenous miR-206 for tumor initiation following orthotopic injection, despite its minimal effects on cell proliferation or MaCSC abundance, pointed to a potential role in regulating cell survival. We therefore assayed for resistance to cell death following matrix deprivation (anoikis), an intrinsic property of CSCs. Indeed, consistent with our previous report that analyzed two human TNBC cell lines, anti-miR-206 transfection sensitized several human TNBC models and a murine basal-like mammary cancer model (that is, M6 cells) to anoikis (Figure 4d, left panel). Consistent results were obtained when anoikis was analyzed by poly ADP ribose polymerase (PARP) cleavage (Figure 4d, right panel). In support of a prosurvival role for endogenous miR-206, depletion of KLF4 sensitized TNBC cells to anoikis (Figure 4e). These results suggest that endogenous KLF4 exerts a prosurvival effect by induction of miR-206.

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Figure 1. KLF4 and miR-206 are selectively expressed in basal-like mammary cancers and in the MaCSC population. (a) miR-206 levels were analyzed by microarray in 98 primary human breast tumors. The Gene Expression Omnibus (GEO) accession number is indicated. (b) miR-206 levels were analyzed by microarray in 42 mammary tumors from GEMMs. The GEO accession number is indicated. (c) Klf4 and miR-206 expression was evaluated in normal mammary tissues from FVB/N mice and in primary tumors arising in the MMTV-Neu and C3(1)/Tag GEMMs. RNA levels were determined by qRT–PCR. (d) MaCSCs were isolated from MDA-MB-231 cells by sorting using PROCR (P) and ESA (E) as described. Transcript levels were analyzed in P+/E+ and P−/E− cells. (e) MaCSCs were isolated from SUM159PT cells and analyzed similarly as described above for MDA-MB-231 cells. For these cells, the P+ profile was used as a surrogate for CD44+/CD24−. (*P < 0.05; **P < 0.01; ***P < 0.001).
miR-206 suppresses the translation of the tumor-suppressor 
PDCD4 and promotes tumor cell survival. We previously reported that RAS-ERK signaling, a prosurvival pathway, is maintained in TNBC cells by KLF4, in part through its regulation of miR-206.37 In contrast to the prominent effect of miR-206 on tumor initiation and cell survival, on its own this miR has only limited effects on ERK activity.37 We therefore sought to better understand how endogenous miR-206 can promote anoikis resistance.
The tumor-suppressor PDCD4 was identified as a potential miR-206 targeted transcript by multiple miR-target prediction tools.37,56 PDCD4 is a negative regulator of RAS-ERK-AP1 signaling and protein translation, and promotes breast cancer cell apoptosis.37–39 We therefore analyzed PDCD4 as a miR-206-regulated transcript.

Consistent with regulation of PDCD4 by miR-206, KLF4 depletion in MDA-MB-231 cells increased PDCD4 expression (Figure 5a, left panel). Similarly, although anti-miR-206 treatment elevated PDCD4, transfection of miR-206 mimic was suppressive (Figure 5a, middle and right panels). Direct regulation of PDCD4 by miR-206 was determined using translational reporter assays. Fragments of the PDCD4 3′ UTR containing two putative miR-206-binding sites (denoted WT-A and WT-B; Figure 5b) were cloned downstream of the open reading frame of firefly luciferase (luc). Relative to the controls, in MDA-MB-231 cells miR-206 mimic repressed WT-reporter luc activity by 72%, and anti-miR-206 induced the reporter by 1.9-fold (Figure 5c). Reporter regulation by miR-206 was abolished by mutation of site WT-A, but not by mutation of site WT-B, thus identifying site WT-A as a functional miR-206-binding site (Figure 5b and c). In agreement with previous studies, PDCD4 depletion in TNBC cells promoted resistance to anoikis, with little or no effect on 2D proliferation (Figure 5d).

Consistent with miR-206 regulation of PDCD4 in MaCSCs, the P′/E′ sub-population of MDA-MB-231 cells exhibited decreased levels of PDCD4 mRNA and protein compared with non-MaCSCs (Figure 5e). In TNBC cells, the depletion of PDCD4 was not sufficient to alter the abundance of the P′/E′ fraction (Figure 5f). These results appear to support a selective role of PDCD4 for suppression of tumor cell survival.

miR-206 promotes cell survival by suppressing CX43 in MaCSCs. Our identification of miR-206 regulation of PDCD4 led us to seek additional targets of this miR that may be important for promoting cell survival. DIANA-miRPath analysis identifies gap junction signaling as the top-ranked miR-206-regulated pathway (P = 2.58 × 10–10).50 Among the targeted gap junction proteins, CX43 is a validated miR-206-regulated transcript, as previously shown in muscle cells.38,61 CX43 is deficient in human breast tumor cells and MaCSCs, and may exert a tumor-suppressor role.52–68

Consistent with its regulation by miR-206 in breast cancer cells, CX43 was increased in KLF4-depleted MDA-MB-231 cells (Figure 6a, left panel). Similarly, inhibition of miR-206 led to elevated CX43 levels, and transfection of miR-206 mimic was suppressive (Figure 6a, middle and right panels). In TNBC cells, the activity of a translational reporter containing the CX43 3′ UTR was induced by 1.5-fold following anti-miR-206 treatment, and suppressed by 53% following transfection of miR-206 mimic (Figures 6b and c). Supporting the direct regulation of CX43 by miR-206 in breast tumor cells, mutation of site A (mut206-A) abolished regulation by miR-206 (Figure 6c). Similar to PDCD4 depletion, suppression of CX43 in TNBC cells promoted resistance to anoikis, with only subtle effects on cell proliferation (Figure 6d).

miR-206 confers chemoresistance in TNBC cells. Consistent with the promotion of cell survival by miR-206 as determined by anoikis assays, TNBC cells transfected with miR-206 mimic were more resistant to paclitaxel or doxorubicin (Figure 7a). Furthermore, inhibition of the endogenous miR-206 moderately sensitized TNBC cells to either agent (Figure 7b). Collectively, these results link pluripotency factor signaling and the enhanced cell survival of MaCSCs, supporting roles of KLF4-miR-206 signaling for breast tumor cell survival, chemoresistance, and tumor initiation through the repression of PDCD4 and CX43 (Figure 7c).

**DISCUSSION**

CSCs were first identified in hematopoietic malignancies and subsequently in solid tumors such as breast cancer.3–5,6,69 Despite substantial progress, questions remain regarding the relationship of CSCs to the adult stem cells of normal tissue, and the nature of the signaling pathways that regulate CSC properties.6 Despite this uncertainty, it is clear that CSCs represent a highly malignant subpopulation of tumor cells with the capability to resist therapy.3,5

In TNBC cells, KLF4 directly regulates miR-206 transcription, and depletion of KLF4 consistently results in loss of the vast majority of miR-206.36,37 In this study, we identified KLF4 and miR-206 as critical promoters of breast tumor cell survival. Both factors were preferentially expressed in the MaCSCs purified from 2D cell culture models of TNBC, from tumorspheres cultured in 3D, from human PDXs and from primary mouse mammary cancers. As shown by anti-miR treatment of TNBC cells, endogenous miR-206 directly repressed the translation of the tumor suppressors PDCD4 and CX43 and promoted tumor cell survival, chemoresistance and in vivo tumor initiation. Immunoblot analysis of MaCSCs revealed suppressed levels of both PDCD4 and CX43. Mirroring the role of endogenous miR-206, depletion of each tumor suppressor did not alter the abundance of CSCs, but instead enhanced tumor cell survival consistent with previous reports.57,70

miRs can act as critical factors for regulating the abundance and/or survival of MaCSCs.71–74 In skeletal muscle, miR-206 is important for repression of PAX7 during stem cell differentiation, and for muscle regeneration following injury.75–79 In a mammary cancer context, miR-206 expression is elevated in ER tumors, which are enriched for MaCSCs.44,45,47–49 In agreement with previous studies, we observed that miR-206 is upregulated in human breast cancers that display a higher grade, in human TNBCs and in basal-like mammary tumors derived from the C3(1)/TAg GEMM (Figure 1a and c). Multiple previous studies have reported that enforced expression of miR-206 can suppress tumor cell proliferation, invasion or

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**Figure 2.** KLF4 and miR-206 are enriched in ALDH<sup>H<sub>lo</sub></sup> MaCSCs derived from human PDXs and the C3(1)/TAg GEMM. (a) KLF4 and miR-206 levels were measured in MaCSCs purified in replicate manner from four cases of human mammary carcinoma passaged as xenografts in mice (PDXs), Purified lineage-negative (Lin) cells were sorted based on ALDH activity. Fluorescence was analyzed in the presence of the ALDH substrate BAAA and in presence/absence of the ALDH inhibitor DEAB. The number of xenografted tumors that were analyzed for each case is indicated below the column data (bars, s.e.m.), n.s., not significant. (b) Lin<sup>−</sup> cells were isolated from the spontaneous mammary tumors arising in C3(1)/TAg females (N = 3). Cells from each animal were grown as either adherent monolayers (2D) or in suspension (3D) for 7–10 days. Photomicrographs (left) depict the morphology of cultured cells. RNA was extracted from 2D or 3D cell cultures and KLF4 and miR-206 levels were determined. (c) KLF4 and miR-206 levels were measured in the ALDH<sup>H<sub>lo</sub></sup> tumor cells isolated from C3(1)/TAg animals (N = 3, see panel b). ALDH<sup>H<sub>lo</sub></sup> tumor cells derived from other GEMMs of mammary cancer have been demonstrated to be enriched for TICs (<sup>13</sup>*P < 0.05; **P < 0.01; ***P < 0.001).
Figure 3. KLF4 and miR-206 promote MaCSC abundance. (a) MDA-MB-231 cells were transduced with lentiviral vectors expressing KLF4 short hairpin RNAs or a non-targeting control (Ctl). KLF4 protein expression was analyzed by immunoblot (left upper panel). β-Actin served as a loading control. miR-206 levels were measured by stem loop qRT–PCR (left lower panel). The cell surface marker profile of the transduced cells was analyzed by flow cytometry (representative scatter plot, middle panel; column data, right panel) (N = 3; bars, s.e.m.). (b) MDA-MB-231 cells were transduced with a retroviral vector encoding KLF4 or empty vector (Ctl). KLF4 and miR-206 levels were analyzed in these cells (left panels) and the MaCSC abundance was determined by flow cytometry. (c) MDA-MB-231 cells were transfected with either miR-206 mimic or control oligonucleotides (Ctl) and then analyzed as in the previous panels. (d) SUM159PT cells were transfected with the indicated miR-mimics and then analyzed as in the previous panels. (e) MDA-MB-231 cells were transfected with miR-206 mimic or control. The indicated number of cells were mixed with matrigel (50% (vol/vol) in DMEM) and injected into NSG mice. Tumor initiation was measured at 4 weeks post-injection (**P < 0.01; ***P < 0.001).
Figure 4. Endogenous KLF4-miR-206 signaling promotes in vivo tumorigenesis and cell survival. (a) KLF4-depleted and control MDA-MB-231 cells were orthotopically injected into athymic nude mice. Tumor initiation and tumor size were determined twice per week using digital calipers (right panels; bars, s.e.m.). (b) MDA-MB-231 cells were transfected with the indicated anti-miRs. Briefly, cells were subjected to sequential transfections in vitro. At 2 days post-transfection, the cells were injected into athymic nude mice. Tumor incidence and growth were measured as described above. (c) Residual transfected cells (see panel b) were directly lysed for immunoblot analysis (left panel) or else placed in culture for 2D cell proliferation analysis (right panel, ATPlirse; N = 6, bars, s.d.). Post-tf, post-transfection. (d) TNBC cells were transfected with either anti-miR-206 or anti-miR-Ctl and then deprived of matrix for 24 h. Anoikis was measured by Trypan blue exclusion (left panel, N = 3, bars, s.d.). In parallel, cells were assayed by immunoblot analysis of cleaved PARP. Cyclohexamide (CHX) treatment served as a positive control for induction of cell death. (e) Anoikis was measured in KLF4-depleted MDA-MB-231 cells or control cells by Trypan blue exclusion (N = 3, bars, s.d.), by flow cytometric analysis of propidium iodide (PI)-stained cells (N = 3, bars, s.d.), and by analysis of cleaved PARP. n.s., not significant (*P < 0.05; **P < 0.01; ***P < 0.001).
metastasis. These tumor-suppressor-like effects of miR-206 may result from higher level enforced expression of the exogenous miR. In this study, suppression of endogenous miR-206 blocked tumor initiation, and moderate (fivefold) overexpression of exogenous miR-206 promoted initiation in a limiting dilution assay. In addition, we observed that

![Figure 5](image_url)

Figure 5. miR-206 suppresses the translation of the tumor-suppressor PDCD4. (a) PDCD4 levels were determined by immunoblot analysis of the indicated cells. (b) Alignment of the PDCD4 3’UTR region indicating two potential miR-206 binding sites, WT-A and WT-B. The miR-206 seed sequence is underlined. Mutated miR-206 binding sites in the PDCD4 3’UTR that were utilized in translational reporter assays are indicated (mt206-A and mt206-B). (c) For analysis of PDCD4 protein translation, MDA-MB-231 cells were co-transfected with reporters in combination with either miR-mimic (left panel) or anti-miR (right panel). The normalized activity of the reporters relative to empty luc vector was analyzed 24 h post-transfection (N=3; bars, s.e.m.). (d) PDCD4 was depleted in the indicated TNBC cells and PDCD4 levels were determined by immunoblot (upper panels). Cells were suspended in 3D culture for 24 h, and anoikis was measured by flow cytometric analysis of propidium iodide (PI)-stained cells (middle panels; N=3; bars, s.e.m.). Following 4 days of 2D culture, the relative cell number of PDCD4-depleted cells and control cells was determined by the ATPlile assay (N=6; bars, s.d.). (e) PDCD4 mRNA and protein expression was analyzed in the indicated sub-populations of TNBC cells. Non-MaCSCs comprises the P+/E- and P-/E- subgroups. The immunoblot data correspond to one of the three independent experiments that analyzed mRNA levels (N=3, bars, s.e.m.). (f) MaCSC abundance was analyzed in PDCD4-depleted TNBC cells and control cells (N=3; bars, s.d.); n.s., not significant (*P<0.05; **P<0.01; ***P<0.001).
either exogenous or endogenous miR-206 could promote malignant properties including tumor cell survival and drug resistance. Depletion of endogenous KLF4 suppressed in vivo tumor initiation by MDA-MB-231 cells in athymic nude mice, yet had little effect on the growth rate of established tumors. Similarly as observed for KLF4, transient inhibition of endogenous miR-206 by anti-miR-206 transfection suppressed tumor initiation in vivo but did not alter the in vitro proliferation or the MaCSC abundance. These results suggest that endogenous KLF4 can signal through miR-206 to promote tumor initiation, probably by impacting cell survival rather than MaCSC abundance. In contrast, exogenous KLF4 or miR-206 promoted MaCSC abundance, mirroring the role of exogenous KLF4 for generation of induced pluripotent stem cells.18,19 It will be interesting to determine whether miR-206 similarly influences the generation of induced pluripotent stem cells.

In this study, we have identified endogenous KLF4 and a downstream effector, miR-206, as functional markers and prosurvival factors that are enriched in MaCSCs. Prosurvival signaling by miR-206 was attributed to direct regulation of PDCD4 and CX43, and miR-206 enhanced the chemoresistance of TNBC cells.

Figure 6. KLF4-miR-206 signaling suppresses CX43 in MaCSCs. (a) CX43 expression was analyzed in shKLF4 cells and control cells by immunoblot. Similarly, CX43 expression was analyzed in cells transfected with the indicated miR mimic or anti-miR. (b) Alignment of CX43 3’UTR region, indicating two previously validated miR-206 binding sites, WT-A and WT-B.38 The miR-206 seed sequence is underlined. The mutation generated in miR-206 binding site A is indicated (mt206-A). (c) For analysis of CX43 protein translation, MDA-MB-231 cells were cotransfected with reporters in combination with anti-miR (left panel) or miR-mimic (right panel). The normalized activity of the reporters relative to empty luc vector was analyzed at 24 h post-transfection. (d) CX43 expression was assayed in CX43-depleted TNBC cells and control cells (upper panels). Cells were suspended in 3D culture for 24 h, and anoikis was measured by flow cytometric analysis of propidium iodide (PI)-stained cells (middle panels, N=3; bars, s.e.m.). Following 4 days of 2D culture, the relative cell number of CX43-depleted cells and control cells was determined by the Assay for Drug Response (AdR) assay (lower panels, N=6; bars, s.d.). (e) CX43 mRNA and protein expression was analyzed in the indicated sub-populations of MDA-MB-231 cells (N=3; bars, s.e.m.). Non-MaCSCs were composed of the P+/E- and P-/E- subgroups. n.s., not significant (*P<0.05; **P<0.01; ***P<0.001).
Our study, therefore, provides a rationale for miR-206-directed antago-miR therapy for the sensitization of the MaCSCs.74,84–88

MATERIALS AND METHODS

Cell lines, cell culture and drug treatments
MDA-MB-231 cells were provided by Katri S Selander (University of Alabama at Birmingham, AL, USA), SUM159PT cells were provided by Gary L Johnson (University of North Carolina at Chapel Hill, NC, USA) and M6 mammary carcinoma cells derived from the C3(1)/SV40 TAg mouse model were provided by Jeffrey E Green (NIH). HCC1143 cells were from ATCC (Manassas, VA, USA). Cells were maintained as subconfluent monolayers as previously described.36,37

For chemoresistance experiments, cells were treated with the indicated doses of paclitaxel (Sigma, St Louis, MO, USA) or doxorubicin (Merck, Billerica, MA, USA) for 72 h. Cells were treated with cycloheximide (Sigma) at 20 μg/ml for 24 h. Cell proliferation was determined using the ATPlite Luminescence Assay System (PerkinElmer, Waltham, MA, USA).

Retroviral transduction
Suppression studies utilized the following pGIPZ lentiviral shRNAmir plasmids (V2LHS_28277 – shKLF4-1, V3LHS_410934 – shKLF4-2, V3LHS_411731 – shCX43-1, V3LHS_411733 – shCX43-2, V3LHS_366084 – shPDCD4-1, V3LHS_366087 – shPDCD4-2; GE Dharmacon/Open Biosystems, Lafayette, CO, USA). The retroviral vector pBABEpuro-HA-KLF4 and viral transduction was previously described.36 Cells were selected using puromycin (1 μg/ml).

Plasmid construction
pMIR-REPORT firefly luciferase vector was purchased from Ambion (Austin, TX, USA), pRL-TK Renilla luc reporter was obtained from Promega (Madison, WI, USA).

Immunoblot analysis and antibodies
Cell extracts for immunoblot analysis were prepared as previously described.26 PARP cleavage assays were performed as recommended (Roche, Indianapolis, IN, USA). Following electrophoresis, proteins were
transferred onto nitrocellulose membranes and probed with the indicated antibody: KLF4 (Santa Cruz Biotechnology, Dallas, TX, USA), PDCD4 (Rockland Immunodiagnostic, Philadelphia, PA, USA), CX43 (Sigma), PARP (Roche) or β-actin (Santa Cruz Biotechnology). Bound antibodies were detected using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA).

Animal studies
Female athymic nude mice (Crl:Nu(Ncr)-Foxn1tm1, Charles River, Frederick, MD, USA) were obtained at 6–8 weeks of age. In all, 2 × 10^6 cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM) and injected into the fourth mammary fat pad. For tumor initiation/limiting dilution assays, NSG (NOD.Cg-Pkrckdscl II2gmr1WJf/SzJ; Jackson Lab, Bar Harbor, ME, USA) were obtained at 6–8 weeks of age. Tumor cells were suspended in DMEM containing matrigel (50% (vol/vol) and injected into the fourth mammary fat pad. Tumors were measured semeweekly using digital calipers. Tumor volume was determined by \( \text{V} = \frac{1}{2}\pi \times (L_1 \times L_2)^2 \), where \( L_1 \) and \( L_2 \) are the long and short axes, respectively. Animal procedures were performed under an approved protocol.

Isolation of mammary carcinoma cells from tumors
Human mammary cancer tissue was passaged as PDXs in NSG mice. HCl-001 and HCl-002 were obtained from Alana L Welm, University of Utah, and HCl-012 and HCl-027 were obtained from the West Virginia University Tissue Bank. PDX tumors and tumors arising in female C3(1)/Tg mice were harvested upon reaching a size of 1–2 cm^3. Tissue was minced and suspended in DMEM/F12 containing Gentle Eagle’s medium (Sigma) and incubated with streptavidin-conjugated microbeads (Miltenyi Biotec) before magnetic cell sorting as recommended by manufacturer.

For analysis of PROCR/ESA expression, cells were blocked with 10 g/ml normal human immunoglobulin G (R&D System, Minneapolis, MN, USA) in ice cold staining buffer (phosphate-buffered saline supplemented with 0.5% (wt/vol) bovine serum albumin and 2 mM EDTA) and incubated with streptavidin (BD Bioscience, San Jose, CA, USA). Anti-mouse-CD31 (clone I3D3; BD Bioscience). Brieﬂy, cells were suspended in ice cold staining buffer (phosphate-buffered saline supplemented with 0.5% (wt/vol) bovine serum albumin and 2 mM EDTA) and incubated with streptavidin (BD Bioscience, San Jose, CA, USA). Anti-mouse-CD31 (clone I3D3; BD Bioscience). Brieﬂy, cells were centrifuged and the returning cell pellet was washed twice with staining buffer before addition of 10 mg/ml heparin, 20 ng/ml epidermal growth factor, 20 ng/ml fibroblast growth factor and 1% (wt/vol) methylcellulose. For analysis of anoikis, cells were suspended in culture as previously described.36 Cell death was analyzed by propidium iodide staining and flow cytometry (Invitrogen, Carlsbad, CA, USA), by Trypan blue exclusion, or by analysis of cleaved PARP.

Statistical analysis
Data were analyzed using either the unpaired t-test (two-tailed), or else one-way analysis of variance followed by Tukey’s multiple comparison ad hoc post-test. Tumor volumes were analyzed using two-way analysis of variance with a Bonferroni post-test. Tumor initiation was analyzed using a \( \chi^2 \) test. Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Differences were considered significant when the analysis yielded \( P < 0.05 \).
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Supplementary Information accompanies this paper on the Oncogenesis website (http://www.nature.com/onzc).