DDR2-regulated arginase activity in ovarian cancer-associated fibroblasts promotes collagen production and tumor progression

Favour A Akinjiyan  
Washington University School of Medicine in St. Louis
Zainab Ibitoye  
Washington University School of Medicine in St. Louis
Peinan Zhao  
Washington University School of Medicine in St. Louis
Leah P Shriver  
Washington University School of Medicine in St. Louis
Gary J Patti  
Washington University School of Medicine in St. Louis

See next page for additional authors

Follow this and additional works at: https://digitalcommons.wustl.edu/oa_4

Part of the Medicine and Health Sciences Commons

Please let us know how this document benefits you.

Recommended Citation
Akinjiyan, Favour A; Ibitoye, Zainab; Zhao, Peinan; Shriver, Leah P; Patti, Gary J; Longmore, Gregory D; and Fuh, Katherine C, "DDR2-regulated arginase activity in ovarian cancer-associated fibroblasts promotes collagen production and tumor progression." Oncogene. 43, 3. 189 - 201. (2024).  
https://digitalcommons.wustl.edu/oa_4/3076

This Open Access Publication is brought to you for free and open access by the Open Access Publications at Digital Commons@Becker. It has been accepted for inclusion in 2020-Current year OA Pubs by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Authors
Favour A Akinjiyan, Zainab Ibitoye, Peinan Zhao, Leah P Shriver, Gary J Patti, Gregory D Longmore, and Katherine C Fuh
Ovarian cancer has poor survival outcomes particularly for advanced stage, metastatic disease. Metastasis is promoted by interactions of stromal cells, such as cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME), with tumor cells. CAFs play a key role in tumor progression by remodeling the TME and extracellular matrix (ECM) to result in a more permissive environment for tumor progression. It has been shown that fibroblasts, in particular myofibroblasts, utilize metabolism to support ECM remodeling. However, the intricate mechanisms by which CAFs support collagen production and tumor progression are poorly understood. In this study, we show that the fibrillar collagen receptor, Discoidin Domain Receptor 2 (DDR2), promotes collagen production in human and mouse omental CAFs through arginase activity. CAFs with high DDR2 or arginase promote tumor colonization in the omentum. In addition, DDR2-depleted CAFs had decreased ornithine levels leading to decreased collagen production and polyamine levels compared to WT control CAFs. Tumor cell invasion was decreased in the presence CAF conditioned media (CM) depleted of DDR2 or arginase-1, and this invasion defect was rescued in the presence of CM from DDR2-depleted CAFs that constitutively overexpressed arginase-1. Similarly, the addition of exogenous polyamines to CM from DDR2-depleted CAFs led to increased tumor cell invasion. We detected SNAI1 protein at the promoter region of the arginase-1 gene, and DDR2-depleted CAFs had decreased levels of SNAI1 protein at the arginase-1 promoter region. Furthermore, high stromal arginase-1 expression correlated with poor survival in ovarian cancer patients. These findings highlight how DDR2 regulates collagen production by CAFs in the tumor microenvironment by controlling the transcription of arginase-1, and CAFs are a major source of arginase activity and L-arginine metabolites in ovarian cancer models.

**Graphical Abstract**

Ovarian cancer has poor survival outcomes particularly for advanced stage, metastatic disease. Metastasis is promoted by interactions of stromal cells, such as cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME), with tumor cells. CAFs play a key role in tumor progression by remodeling the TME and extracellular matrix (ECM) to result in a more permissive environment for tumor progression. It has been shown that fibroblasts, in particular myofibroblasts, utilize metabolism to support ECM remodeling. However, the intricate mechanisms by which CAFs support collagen production and tumor progression are poorly understood. In this study, we show that the fibrillar collagen receptor, Discoidin Domain Receptor 2 (DDR2), promotes collagen production in human and mouse omental CAFs through arginase activity. CAFs with high DDR2 or arginase promote tumor colonization in the omentum. In addition, DDR2-depleted CAFs had decreased ornithine levels leading to decreased collagen production and polyamine levels compared to WT control CAFs. Tumor cell invasion was decreased in the presence CAF conditioned media (CM) depleted of DDR2 or arginase-1, and this invasion defect was rescued in the presence of CM from DDR2-depleted CAFs that constitutively overexpressed arginase-1. Similarly, the addition of exogenous polyamines to CM from DDR2-depleted CAFs led to increased tumor cell invasion. We detected SNAI1 protein at the promoter region of the arginase-1 gene, and DDR2-depleted CAFs had decreased levels of SNAI1 protein at the arginase-1 promoter region. Furthermore, high stromal arginase-1 expression correlated with poor survival in ovarian cancer patients. These findings highlight how DDR2 regulates collagen production by CAFs in the tumor microenvironment by controlling the transcription of arginase-1, and CAFs are a major source of arginase activity and L-arginine metabolites in ovarian cancer models.

**Oncogene** (2024) 43:189–201; https://doi.org/10.1038/s41388-023-02884-3

A full list of author affiliations appears at the end of the paper.

Received: 17 May 2023 Revised: 21 October 2023 Accepted: 30 October 2023
Published online: 23 November 2023
INTRODUCTION
The process of cancer progression is supported by replacement of normal tissue matrix with tumor-associated matrix which is primarily produced by cancer-associated fibroblasts (CAFs). A major structural component of the tumor extracellular matrix (ECM) is fibbrillar collagens. Their abundance, fiber orientation, and architecture have been shown to be associated with pro-tumorigenesis in multiple cancers [1–8]. This interplay between CAFs and fibrillar collagens has been shown to facilitate tumor growth and metastasis in ovarian cancer [9–11].

The receptor tyrosine kinase, Discoidin Domain Receptor 2 (DDR2), is a non-integrin collagen receptor that acts as a sensor of ECM fibrillar collagens. The action of DDR2 in CAFs can influence fibrillar collagen mRNA levels and can mechanically remodel tumor ECM collagen fibers via integrin regulation [5]. High expression of DDR2 in experimental mouse tumor models leads to increased tumor metastasis, and in various human tumors is associated with poor survival [12–17].

L-arginine is a nonessential amino acid that is cleaved by arginase to urea and L-ornithine. L-ornithine can then be further metabolized by ornithine decarboxylase (ODC) to polyamines or by ornithine aminotransferase (OAT) to form proline. Proline is a critical and abundant amino acid in the biosynthesis of collagens. Arginase has largely been studied in the immune response in myeloid cells as well as modulating T cell immunity [18–20]. In tumors, arginase is highly expressed in tumor-associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs) which deplete available arginine leading to T cell impairment [21]. However, the regulation and functional role of arginase expression by CAFs has not been extensively evaluated. Here we report that DDR2 regulates collagen protein production by CAFs in the tumor microenvironment by controlling the transcription of arginase-1. Our findings reveal how CAFs are a major source of arginase activity and L-arginine metabolites in ovarian tumors and that DDR2 and arginase in CAFs may be a target in ovarian cancer.

RESULTS
DDR2-null mice have decreased ovarian tumor burden
Immunohistochemical evaluation of advanced stage, human ovarian cancer specimens demonstrate that high expression of DDR2 in tumors is associated with poor survival [22]. Since DDR2 is predominantly expressed by mesenchymal cells, we asked whether presence of DDR2 in ovarian cancer-associated stromal cells impacts ovarian tumor burden in mouse models. To do so, we utilized a previously published intraperitoneal tumor model by introducing three mouse ovarian tumor cell lines into the intraperitoneal cavity of adult female Ddr2−/− or control WT C57BL/6 mice (i.e., a syngeneic mouse tumor model). The three ovarian cancer cell lines were: (1) ID8TB−/− (mouse ovarian surface epithelium cell line [23]), (2) BPPNM (fallopian tube epithelial-derived cell line [24]), and (3) KPCA (fallopian tube epithelial-derived cell line [24]). ID8TB−/− and BPPNM tumor cell lines expressed DDR2, while KPCA did not (Supplementary Fig. S1A). In all three experimental settings, ubiquitous Ddr2−/− recipient mice developed significantly less tumor burden than the WT mice, and the ID8TB−/− model in Ddr2 KO mice had increased survival and decreased ascites compared to the Ddr2 WT mice (Fig. 1A–D, Supplementary Fig. S1B). These data indicated that the presence of DDR2 in stromal cells or the host, in general, impacted ovarian cancer burden, regardless of tumor cell DDR2 expression status.

Arginase-1 mRNA expression is decreased in tumors from Ddr2−/− mice
To determine how tumor-associated stromal expression of DDR2 affected tumor burden, we performed targeted mRNA expression profiling of ID8TB−/− tumors dissected from WT and Ddr2−/− mice using the Nanostring nCounter Tumor Signaling 360 panel which includes 760 genes and 20 internal reference genes. Volcano plot analysis revealed that arginase-1 (Arg1) mRNA level, in particular, was dramatically decreased in tumors from Ddr2−/− hosts (Fig. 2A, Supplementary Fig. S2A, Supplementary Table 2). Quantitative PCR on mRNA isolated from omental ID8TB−/− tumor nodules, different than those used for Nanostring analysis, confirmed that Arg1 mRNA was indeed decreased in ID8TB−/− and BPPNM tumor nodules from Ddr2−/− hosts (Fig. 2B). Related Arg2 mRNA level was also decreased in tumor nodules (Fig. 2C). Arginase enzyme activity in whole tumor extracts and serum from tumor-bearing mice was also significantly decreased in Ddr2−/− mice (Fig. 2D, E). Finally, when fixed tumor slices were immunostained for arginase-1 protein, tumors from Ddr2−/− hosts had decreased arginase-1 expression (Fig. 2F).

The action of DDR2 in CAFs controls arginase activity
To determine which cell(s) in the host tumor stromal compartment expressed DDR2, we first interrogated published human and BPPNM mouse ovarian cancer single-cell RNA sequencing datasets [24]. In both, DDR2 was found to be primarily expressed in CAFs but was also present in some tumor cell clusters (Fig. 3A, B, Supplementary Fig. S3A and B, Supplementary Tables 3 and 4). Notably, in both samples, none of the identified immune cell clusters expressed DDR2 mRNA.

Based on these results, we examined three distinct validated human omental CAF cell lines for DDR2 expression [25], and all expressed DDR2 (Fig. 3C and Supplementary Fig. S3C). When DDR2 expression was shRNA-depleted in all CAF cell lines, to varyng degrees (Fig. 3C and Supplementary Fig. S3C), Arg1 mRNA expression decreased (Supplementary Fig. S3D) as did cellular arginase activity (Fig. 3D and Supplementary Fig. S3E). Importantly, these changes in Arg1 expression and activity were rescued, to levels approximating that in WT CAFs by expressing a RNAi-resistant isoform of DDR2 in hCAF68 cells depleted of Ddr2 (Fig. 3C, D).

To determine if DDR2 regulates Arg1 expression and arginase activity in omental CAFs in vivo, we made use of CAFs from the mouse ID8TB−/− syngeneic ovarian tumor model. CAFs from WT and Ddr2−/− tumor-bearing mice were isolated as previously published [26]. Following negative selection to deplete immune cells with an anti-CD45 antibody and epithelial cells with an anti-EpCAM antibody, remaining stromal cells were immortalized using SV40 large T virus. Similar to human omental CAF cell lines, mouse CAFs from ID8TB−/− tumors in Ddr2−/− mice had decreased Arg1 mRNA levels (Supplementary Fig. S3F). Arg1 expression, and arginase activity (Fig. 3E, F) which was increased upon constitutive Arg1 overexpression in Ddr2−/− CAFs (Fig. 3E, F).

Given the findings that Arg1 is expressed in Ddr2−/− CAFs, we used another in vivo approach to identify whether other cell populations expressed Arg1. We performed single-cell mRNA sequencing (scRNAseq) on ID8TB−/− mouse tumors dissected from WT mice (Fig. 4A, Supplementary Table 5). Using established tumor, CAF, and immune markers [24] (Supplementary Fig. S5A), we identified two tumor, three CAF and four immune cell clusters (Fig. 4A). Violin plot analysis of the various cell clusters present revealed that DDR2 was expressed in the three PDGFRα+ CAF clusters and one tumor cell subpopulation (Fig. 4A, B). Arg1 mRNA expression was present in two of the three Ddr2−/− CAF clusters as well as in two immune cell clusters and one tumor cell cluster (Fig. 4B).

Next, we performed multiplex immunohistochemistry analysis on ID8TB−/− tumors from WT and Ddr2−/− mice for expression of Arg1 and various tumor stromal cell type markers (CAF + PDGFRα; macrophage + F4/80). Arg1 expression was present in 30% of cells expressing the CAF marker protein PDGFRα (Fig. 4C–E). In tumors from Ddr2−/− mice, the proportion of Arg1-positive CAFs (%Arg1+ and PDGFRα+) was significantly decreased compared to WT mice (Fig. 4C). This was not a result of overall decreased CAF
populations in tumors from Ddr2−/− mice as the proportion of PDGFRα+ CAFs were similar between tumors from WT and Ddr2−/− mice (Fig. 4D). The proportion of Arg1-positive macrophages (%Arg1+ and F4-80+/F4-80+) and Arg1-positive tumor cells (%Arg1 and CK8+/CK8+) were similar between tumors from WT and Ddr2−/− mice (Supplementary Fig. S4B–G). Flow analysis of single-cell suspensions from ID8TB−/− tumors in WT hosts revealed that 17.4% of total cells were CAFs (e.g., PDGFRα positive) (Supplementary Fig. S4H, Supplementary Table 6) while 20% of cells isolated from tumors were CD45+ immune cells (Supplementary Fig. S4H).

Taken together, these accumulated cell line and in vivo data indicate that Arg1 is expressed in Ddr2+ CAFs, and Ddr2 regulates arginase-1 protein levels and arginase activity. Moreover, in this mouse ovarian tumor model, CAF-derived Arg1, as opposed to immune cell- or tumor cell-derived Arg1, was likely a significant contributor to overall arginase activity in ovarian cancer.

### Ovarian tumor omental CAFs with high DDR2 and ARG1 expression promote in vivo omental colonization

Omental colonization can be part of tumor progression in ovarian cancer. To determine if DDR2-regulated arginase-1 activity in CAFs impacted tumor cell colonization in vivo, we co-injected WT mice intraperitoneally with syngeneic luciferase-positive KPCA tumor cells (low DDR2 expression and low arginase activity) (Supplementary Fig. S1A, Supplementary Fig. S5A) with various luciferase-negative mouse omental CAF cell lines from WT or Ddr2−/− mice. After 5 days, mice were sacrificed, omentum digested, and luciferase assay performed which reflected the amount of KPCA tumor cells that had colonized the omentum (Supplementary Fig. S5B). When KPCA cells were co-injected with WT mouse CAFs, there was a significant increase in omental colonization by KPCA cells (Fig. 5). Compared to WT CAFs, when Ddr2−/− CAFs were used, there was significantly less tumor cell colonization (Fig. 5). Mice co-injected with Ddr2−/− Arg1OE CAFs had increased omental colonization compared to those co-injected with Ddr2−/− CAFs. This data suggested that in vivo DDR2 and Arg1 expressing CAFs might impact early steps of omental colonization or the proliferation of tumor cells after attachment.

### SNAIL protein was detected at the promoter region of arginase-1 gene

The action of DDR2 in CAFs appeared to regulate Arg1 expression at the transcriptional level (Fig. 2A, B). We have previously shown...
that SNAIL (SNAI1), an EMT inducing transcription factor that promotes tumor cell migration and invasion [27], is regulated by the action of DDR2 in tumors, post-transcriptionally [28]. SNAI1 can act as both a transcriptional repressor and activator [29–31].

We confirmed that SNAIL protein level was indeed decreased in Ddr2-depleted CAFs (Fig. 6A). To determine if SNAIL protein could impact Arg1 transcription, we performed chromatin immunoprecipitation (ChIP) experiments to determine if SNAIL was present at the promoter region of the endogenous Arg1 gene. In human ovarian tumor CAFs, SNAIL protein was detected at the promoter region of the human Arg1 gene, while in Ddr2-depleted CAFs, there was less SNAIL detected (Fig. 6B, Supplementary Fig. S6A). To confirm this finding, we constitutively overexpressed SNAIL1 in DDR2-depleted CAFs (shDDR2 SNAIL OE) (Fig. 6C) and performed quantitative PCR for Arg1. Arg1 mRNA levels were increased in shDDR2 SNAIL OE CAFs compared to DDR2-depleted CAFs (shDDR2). In control experiments, we confirmed nuclear localization of SNAIL in shDDR2 SNAIL OE CAFs (Supplementary Fig. S6B). This suggested that DDR2-regulated SNAIL1 expression impacts Arg1 transcription in ovarian CAFs.

DDR2-dependent arginase activity in CAFs is important for ovarian tumor collagen protein production and secretion

Arg1 is a central cytosolic enzyme controlling cellular L-Arginine metabolism. Arg1 cleaves L-Arg to generate urea and L-Ornithine. L-Ornithine is subsequently metabolized to generate L-Proline and polyamines [32–34] (Fig. 7A). To determine if DDR2 signaling impacted L-Arginine metabolite production in ovarian tumor CAFs, we generated a series of human omental CAF cells: (1) DDR2-expressing WT control (shSCRM), (2) DDR2-depleted (shDdr2), (3) DDR2-depleted and constitutively overexpressing Arg1 (shDdr2 Arg1OE) and (4) a transfection control empty vector.
In Ddr2-depleted CAFs, both intracellular and secreted L-Arginine levels, as determined by a standard biochemical assay, were increased (Fig. 7B). Compared to WT CAFs, L-Ornithine levels were significantly decreased in Ddr2-depleted CAFs (Fig. 7C).

In breast tumor CAFs, the action of DDR2 has been shown to contribute to the production of collagens, by affecting mRNA synthesis [13]. However, whether DDR2 signals could also regulate the production of collagen proteins, and if so, how has not been addressed. When ovarian tumor nodules from WT and Ddr2−/− mice were stained for fibrillar collagens with trichrome blue, the amount of detected fibrillar collagen in tumors from Ddr2−/− mice was significantly decreased (Fig. 7D). In addition, cultured DDR2-depleted human omental CAFs expressed decreased collagen1α1 protein as detected by immunofluorescence (Fig. 7E). We next determined the L-Proline content in human omental CAF cell lines using a standard biochemical assay. Ddr2-depleted CAFs had decreased L-Proline content (Fig. 7F). In collagen proteins, much of
the proline exists in its hydroxylated form, hydroxyproline [35]. In Ddr2-depleted CAFs, there was also decreased cellular hydroxyproline levels to that present in control WT CAFs (Fig. 7G). Constitutive Arg1 overexpression in Ddr2-depleted CAFs increased the amount of procollagen 1α1 secreted to levels produced by control WT CAFs (Fig. 7H). In other control experiments, siRNA-mediated depletion of Arg1 in WT CAFs resulted in decreased procollagen 1α1 secretion (Supplementary Fig. S7C, D).

While hydroxyproline is a sensitive marker for collagen level in cells, it is not a direct measure of collagen synthesis since hydroxyproline residues may be elevated due to collagen synthesis and degradation. Newly synthesized triple helical procollagen is secreted into the extracellular space where additional cleavage and crosslinking occurs to form mature collagen fibers [36]. To determine if DDR2 signals affected collagen protein synthesis and secretion, we measured secreted procollagen 1α1 levels in the culture media from various CAFs. Ddr2-depleted CAFs secreted less procollagen 1α1 compared to WT CAFs (Fig. 7H). Constitutive Arg1 overexpression in Ddr2-depleted CAFs (shDDR2 Arg1OE) increased the amount of procollagen 1α1 secreted to levels produced by control WT CAFs (Fig. 7H).

Fig. 4 Identification of a subpopulation of DDR2-expressing CAFs that express arginase-1 in vivo. A UMAP plot showing showing cell clusters for ID8TB−/− tumors in WT mice. B Violin plots showing expression of Ddr2, Arg1 and Pdgfra in cell clusters for ID8TB−/− tumors in WT mice. C Analysis of multiplex immunohistochemistry on ID8TB−/− tumor slices showing the percent of double positive Arg1+ and Pdgfra+ cells as a proportion of all Pdgfra+ cells in tumor slices (n = 7 mice). Entire tumor slice was analyzed in Halo. D Analysis of multiplex immunohistochemistry on ID8TB−/− tumor slices showing the percent of Pdgfra+ cells in tumor slices (n = 7 mice). Entire tumor slice was analyzed in Halo. E Representative images of multiplex immunohistochemistry ID8TB−/− tumor slices from WT and Ddr2−/− mice stained for Pdgfra, Arg1 and hematoxylin (n = 7 mice). Scale bar = 50 μm. In all panels, Student's t-test was used for statistics **p < 0.001, ***p < 0.001, ns = p > 0.05.
with $^{13}$C C6-labeled L-arginine and after 72 h intracellular collagen peptides were isolated following cell lysis and subjected to mass spectrometry. This type of experiment does not distinguish the peptides were isolated following cell lysis and subjected to mass spectrometry. This type of experiment does not distinguish the

**DISCUSSION**

**DDR2 signaling in multiple preclinical tumor models has been shown to impact tumor progression and metastasis, and high DDR2 expression in human tumor specimens has been associated with worse clinical outcomes [12–14, 16]. We found that although DDR2 is expressed by a subset of tumor cells in human tumor specimens, the majority of DDR2 expression was in CAFs (Fig. 3A, B). DDR2 was not expressed by bone marrow derived myeloid and immune cells (Fig. 3A, B). We have shown in omental

Ovarian tumor progression is dependent upon tumor cell attachment to and invasion through the basement membrane with subsequent interaction with CAFs. Given our findings that DDR2 inactivated CAFs have lower L-Ornithine levels and this can lead to a decrease in polyamines, we asked whether DDR2-regulated arginase activity in CAFs impacted polyamine production. To do this, we biochemically determined the total polyamine level in CAFs cells (+/− DDR2) and their secreted media. Ddr2-depleted CAFs had decreased intracellular and extracellular polyamine levels compared to WT control (Fig. 8A). We next performed mass spectrometry on conditioned media produced by human CAFs (+/− DDR2). Ddr2-depleted (shDDR2) CAFs produced decreased levels of spermidine and putrescine compared to WT control (shSCR) (Fig. 8B).

Media secreted by Ddr2-depleted human ovarian tumor CAFs leads to decreased ovarian tumor cell invasion and migration compared to media secreted by Ddr2-expressing CAFs [25]. We confirmed this result in Matrigel invasion assays in Boyden chambers using two human ovarian tumor cell lines (Tyknu; OVCA8B) and CAF conditioned media (CM) added to the lower well (Fig. 8C, and Supplementary Fig. S8A, B). CM from shDdr2 Arg1OE CAFs rescued this defect (Fig. 8C). CM from Arg1-depleted CAFs did not support ovarian tumor cell invasion through Matrigel (Fig. 8D, and Supplementary Fig. S7D, S8C and S8D). We also performed Matrigel invasion assays using CM from WT CAFs pretreated with the arginase inhibitor, CB1158 [38]. CB1158 was removed from CM prior to invasion assay using a 10 kDa molecular cutoff filter. We observed a dose-response inhibition of tumor cell invasion through Matrigel when conditioned media from CAFs treated with an arginase inhibitor was added (Fig. 8E, and Supplementary Fig. S8E and S8F).

Polyamines are polyatomic molecules that can contribute to cellular proliferation and invasion [39, 40]. To determine if DDR2-dependent (Arg1-dependent) polyamine production specifically could contribute to ovarian tumor cell invasion, we added exogenous spermidine or putrescine to CM from Ddr2-depleted CAFs and repeated the Boyden-chamber Matrigel invasion assays. Both polyamines rescued the tumor cell invasion defect of CM from Ddr2-depleted CAFs (Fig. 8F, and Supplementary Fig. S8G and S8H).

In sum, these data indicated that the presence of DDR2 in omental ovarian tumor CAFs controlled polyamine production, likely through DDR2-regulated Arginase-1 production. Moreover, polyamine production by CAFs could support ovarian tumor cell invasion through Matrigel.

**High stromal ARG1 expression in ovarian cancer correlate with poor overall survival**

We have previously shown that high stromal expression of DDR2 protein in human ovarian tumors correlates with worse overall survival [22]. Given that DDR2-regulated arginase activity in CAFs affected ovarian cancer tumor collagen production, we asked whether stromal arginase-1 expression correlated with ovarian cancer patient survival. We quantified stromal arginase-1 expression in a human ovarian cancer tumor microarray by immunohistochemistry and correlated stromal arginase-1 protein expression with survival outcomes. Patients with high stromal DDR2 and high stromal arginase-1 expression had median overall survival of 23 months whereas patients with low stromal DDR2 and low stromal arginase-1 had a median overall survival of 171 months (Supplementary Fig. S9A). We then performed a multivariate analysis for DDR2 and arginase-1 controlling for known clinical factors that influence survival. We identified that advanced stage or high stromal arginase-1 were associated with poor survival in ovarian cancer patients (Supplementary Fig. S9B).
CAFs that the presence of DDR2 affects production of collagen and other secreted ECM proteins at the level of their mRNA production or stabilization by mechanisms that have not been explored previously. DDR2 can also impact the cell intrinsic regulation of the collagen binding functions of CAFs that remodel the ECM, such as the collagen fibrillar matrix. Thus, in the absence of DDR2 in CAFs, this results in a tumor ECM that is less permissive for metastatic spread.

Other examples of CAF regulation of collagen protein production include pyrroline-5-carboxylate reductase 1 (PYCR1) and transforming growth factor beta (TGF-β) [41–43]. For example, PYCR1 is highly expressed in CAFs from patients with breast cancer, and this key enzyme is needed for proline synthesis that supports collagen production [41]. TGF-β activated fibroblasts have been found to upregulate production of both proline and glycine to support collagen production in lung cancer [42, 43]. We observed an increase in DDR2 expression in CAFs cultured on polymerized collagen compared to those cultured on plastic (Supplementary Fig. 7E). Thus, it is possible that there could be a feedback loop mechanism of signal amplification where the activation of DDR2 leads to increase in collagen production which further causes DDR2 activation. Further studies are necessary to fully elucidate the role of fibrillar collagen in the amplification of DDR2’s signals.

Fig. 6 DDR2’s regulation of arginase-1 is dependent on SNAIL1 transcriptional activity. A Western blot of DDR2-expressing (shSCRM) and DDR2-depleted (shDdr2) CAFs with the indicated antibodies. B Chromatin immunoprecipitation-qPCR (ChIP-qPCR) of Arg1 expression using SNAIL or IgG control antibodies on shSCRM and shDdr2 CAFs. E-cadherin expression was used as a positive control. ChIP-qPCR of E-cadherin expression using SNAIL or IgG control antibodies on shSCRM and shDdr2 CAFs (n = 3 replicates). C Quantitative PCR assay showing DDR2, SNAIL and Arg1 gene expression using RNA from shSCRM, shDDR2 and DDR2-depleted SNAIL-overexpressing CAFs (shDDR2 SNAIL OE). In all panels, ****p < 0.0001, ***p < 0.001, **p < 0.01, ns = p > 0.05.

Our data suggests that CAFs are a major source of arginase activity and L-arginine metabolites in ovarian cancer. This may suggest that CAFs play a significant role in modulating immune cell activity through metabolic availability of arginine and its metabolites. In melanoma, fibroblasts have been found to suppress cytotoxic T lymphocyte activity through increased arginase activity [48, 49]. A prior study showed that tumor cell DDR2 plays a role in response to anti-PD1 therapy [50]. Given our findings on the importance of CAF DDR2, it is possible that fibroblast DDR2 may also contribute to anti-tumor immunity.

This study utilized ovarian mouse and human tumor cell lines as well as CAFs that were cultured from the omentum of patients with metastatic ovarian cancer and mouse CAFs isolated from tumors. We also utilized intraperitoneal models of metastatic colonization similar to Walton et al. and Iyer et al. [23, 24].
Fig. 7 DDR2-dependent arginase activity in CAFs promotes increased collagen synthesis. A Schematic showing arginine metabolism by arginase into L-ornithine and other downstream products. B Arginine assay showing levels of intracellular and extracellular arginine in DDR2-expressing (shSCRM) and DDR2-depleted (shDdr2) CAFs ($n = 3$). C Ornithine assay showing levels of intracellular ornithine in DDR2-expressing (shSCRM) and DDR2-depleted (shDdr2) CAFs ($n = 3$). D Representative images of modified Masson's trichrome stain for collagen (blue) from ID8 Trp53$^{-/-}$ Brca2$^{-/-}$ tumors from Ddr2 WT and Ddr2 KO mice ($n = 6$ mice). Scale bar = 50 μm. One tumor slice per mouse was used (6 total tumor slices per group). Percent area of collagen was quantified using the entire tumor slice in Halo. E Representative images of collagen immunofluorescence stain in DDR2-expressing (shSCRM) and DDR2-depleted (shDdr2) CAFs ($n = 3$ replicates, 12 images per group). F Proline assay showing levels of intracellular proline in DDR2-expressing (shSCRM) and DDR2-depleted (shDdr2) CAFs ($n = 3$ replicates). G Hydroxyproline assay measurement of DDR2-expressing (shSCRM), DDR2-depleted (shDdr2), DDR2-depleted and constitutive arginase-1 overexpressing (shDdr2 Arg1OE) and DDR2-depleted empty vector control (shDdr2 EV) CAFs ($n = 3$ replicates). H Procollagen1α1 assay measurement of conditioned media from shSCRM, shDdr2, shDdr2 Arg1OE and shDdr2 EV CAFs ($n = 3$ replicates). In all panels, Student's $t$-test was used for statistics ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns = $p > 0.05$. 

Oncogene (2024) 43:189 – 201
A limitation of our study is that we used mice with host global DDR2 knockout for our in vivo tumor burden studies. Prior work has shown that DDR2 plays a role in bone development, lipolysis, and ECM deposition in bone and heart [51–55], so it is possible that DDR2’s role in other cell types contributes to the observed tumor burden phenotype. To clarify the specific role of fibroblast DDR2 in tumor progression, we performed the omental colonization assay and determined that CAF DDR2 and arginase affects the early steps of tumor progression.

In conclusion, our work uncovered that DDR2 signals can regulate collagen protein synthesis and secretion by CAFs from human and mouse ovarian tumors. It does so by controlling the transcription of arginase-1 and thus arginase activity. This transcriptional regulation could occur in a SNAIL1-dependent manner as DDR2 stabilizes SNAIL1 protein levels [28] (Fig. 5A), and SNAIL1 protein was detected at the Arg1 promoter in CAFs. DDR2-dependent arginase activity in CAFs appeared to be critical for collagen deposition in ovarian tumors and could explain how...
DDR2 regulates tumor ECM fibrillar collagen production and mechanical properties. This work further supports the importance of targeting the tumor microenvironment in cancer progression.

**MATERIALS AND METHODS**

**Cell lines and culture**

ES2 cells were obtained from NCI and maintained in McCoy’s 5A (modified) medium ( Gibco 16600082) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin (Penstrep) ( Gibco 15140122). Tyknu cells were maintained in DMEM Medium ( Gibco 11965084) supplemented with 10% FBS and 1% Penstrep. Tyknu cells were a gift from Dr. Erinn Rankin. ID8 Trp53−/− Brca2−/− (ID8TB−/−) cells were a gift from Dr. Iain McNeish [23] and were maintained in DMEM with 4%FBS, 1% insulin-transferrin-selenium (ITS) ( Gibco 41400045) and 1% Penstrep. KPCA and BPPP4M cells were a gift from Dr. Robert Weinberg [24] and were maintained in DMEM with 4%FBS, 1% ITS, 2 ng/ml epidermal growth factor (EGF) (Sigma E9644-2MG) and 1% Penstrep.

Human CAFs were isolated from the omentum of chemo naïve patients with advanced stage, high-grade serous ovarian or fallopian tube cancer, validated, and maintained as previously described [25, 26]. Patients provided written consent for sample collection and use. Our study was approved by the Washington University Institutional Review Board (IRB 201309050). Mouse tumors were collected, and RNA was extracted using RNeasy Mini Kit (Qiagen 74104) and cDNA was prepared using the SuperScript IV kit (Invitrogen 00-5523-00). Antibodies used are listed in Supplemental Table 1. Additional details are in supplemental methods.

**Immunohistochemical staining and image analysis**

Tissues were fixed in formalin for 24 h, embedded in paraffin after graded ethanol dehydration, and sectioned into 5-μm sections using a microtome. FFPE sections were stained for Hematoxylin & Eosin (Thermo Fisher) and Modiﬁed Masson’s Trichrome (Diagnostic Biosystems KT034) according to manufacturer’s instructions. After dewaxing and epitope retrieval, tissues were allowed to invade for 48h. Polymerized gel was removed from the field at 20x. Additional details are in supplemental methods.

**Patient survival and multivariate analysis**

Patients provided written consent prior to inclusion in study. Washington University’s Institutional review board gave approval for this study (IRB 201709191). At the time of tumor debulking surgery, samples were collected from patients with advanced stage, high-grade serous ovarian or fallopian tube cancer and used to create an ovarian cancer tissue microarray. Clinical characteristics and survival information were collected from patient charts. Overall survival was determined via Kaplan-Meier analysis using time of death or date of last patient follow-up. The log-rank test was used for analysis and to differentiate the overall survival between patient groups. Using the ergodicity search (25%~75%), patients were sorted into two groups with low vs. high Arg1 and DDR2 expression and determined the log-rank P-values of overall survival and difference cutoff values. The value where the most significant P-value was determined to be the optimal cutoff level. Survival curves were calculated using the Kaplan-Meier method.

**Single-cell dissociation, flow cytometry, and single-cell RNA sequencing**

Omental ID8TB−/− tumors from WT mice were collected and dissociated in media containing 1 mg/ml collagenase III (Worthington LS004182), 1 mg/ml hyaluronidase (Worthington LS002592), and 0.2 mg/ml DNase Type IV (Sigma D5025). Mechanical dissociation was performed for 1 min at 500 rpm using the gentlemacs dissociator (Miltenyi Biotec 130-096-427) and enzymatic digestion was performed for 30 min at 37 °C and 150 rpm. Cells were filtered using a 70 μm filter and red blood cells were lysed using RBC lysis buffer (Biolegend 420301). Dead cell removal was performed (Miltenyi Biotec 130-090-101). Cells were resuspended in FACS buffer (PBS, 1 mM EDTA, 4% FBS) and counted. Fc receptors were blocked to reduce non-specific staining and cell surface staining was performed per manufacturer’s recommendation. After cell surface staining was complete, cells were fixed and permeabilized and intracellular staining was performed (Invitrogen 00-5523-00). Antibodies used are listed in Supplemental Table 1. Flow cytometry data was collected on a Cytek Aurora (4L 16UV-16V-148-BR configuration). After single-cell dissociation and dead cell removal, cells were centrifuged and resuspended in PBS + 0.1%BSA, and final concentration was adjusted to 1000 cells/μl and sent for scRNA processing (10X Genomics). Additional details are in supplemental methods.

**Polyamine detection by mass spectrometry (MS)**

Protein lysates, collected in 9 mol/l urea and 0.075 mol/l Tris, pH 7.6, were sonicated twice for 15 s and spun down at 10,000 g for 10 min. Protein concentration was quantified using a Bradford protein quantification assay and samples were normalized to 100 μg. Lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes and blocked for 30 min at room temp in 10% milk in 1X TBST. Membranes were incubated overnight in antibodies (see Supplemental Table 1) at 4 °C on a shaker, washed thrice with 1X TBST, and incubated in HRP-conjugated secondary antibody. After three additional washes in TBST, membranes were developed with ECL (Thermo Fisher 34095).

**Patient survival and multivariate analysis**

Patients provided written consent prior to inclusion in study. Washington University’s Institutional review board gave approval for this study (IRB 201709191). At the time of tumor debulking surgery, samples were collected from patients with advanced stage, high-grade serous ovarian or fallopian tube cancer and used to create an ovarian cancer tissue microarray. Clinical characteristics and survival information were collected from patient charts. Overall survival was determined via Kaplan-Meier analysis using time of death or date of last patient follow-up. The log-rank test was used for analysis and to differentiate the overall survival between patient groups. Using the ergodicity search (25%–75%), patients were sorted into two groups with low vs. high Arg1 and DDR2 expression and determined the log-rank P-values of overall survival and difference cutoff values. The value where the most significant P-value was determined to be the optimal cutoff level. Survival curves were calculated using the Kaplan-Meier method.
Arginine tracing experiment

DDR2-expressing and DDR2-depleted CAFs were cultured in media with or without labeled arginine for 72 h, lysed and subjected to mass spectrometry analysis. Additional details are in supplemental methods.

Chromatin immunoprecipitation

3 × 10⁶ DDR2-expressing and DDR2-depleted CAFs were used for ChIP assay (Abcam ab500) following manufacturer’s protocol. Additional details are in supplemental methods.

DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available in the GEO repository accession code GSE242830.

REFERENCES


ACKNOWLEDGEMENTS

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health R01CA234553 (KCF) and R01CA223758 (GDL), 2R01HD00849-28 (KCF), F30CA271621 (FAA), American Cancer Society RSG-19-080-01-TBG (KCF), Cancer Frontier Fund 8002-88 (KCF), OCRZ Liz Tilberis Fund (KCF), Washington University Ovarian Cancer Research Innovation Fund (FAA). We also thank Iain McNeish for providing us with the ID8 Trp53−/−/Broa2+/− cells. We thank the Genome Technology Access Center at the McDonnell Genome Institute. Proteomic analyses were performed by the Mass Spectrometry Technology Access Center at McDonnell Genome Institute (MTAC@MGI). REDCap was supported by Clinical and Translational Science Award (CTSA) Grant (UL1TR000448) and Siteman Comprehensive Cancer Center and NCI Cancer Center Support Grant P30 CA091842.

AUTHOR CONTRIBUTIONS

Conceptualization, FAA, GDL, and KCF; Data curation, FAA and ZI; Formal analysis, FAA and PZ; Funding acquisition, FAA, GDL, and KCF; Investigation, FAA, ZI, and LPS; Methodology, FAA, LPS, GJP, GDL, and KCF; Project administration, GDL and KCF; Resources, GDL and KCF; Supervision, GDL and KCF; Validation, FAA and ZI; Visualization, FAA, ZI, PZ, and LPS; Writing—original draft, FAA; Writing—review & editing, FAA, ZI, PZ, LPS, GJP, GDL, and KCF.

COMPETING INTERESTS

The Longmire laboratory receives funding from Pfizer-CTI, San Diego CA, and Centene Corporation, St. Louis MO.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41388-023-02884-3.

Correspondence and requests for materials should be addressed to Katherine C. Fuh.

Reprints and permission information is available at http://www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

This is a U.S. Government work and not under copyright protection in the US; foreign copyright protection may apply 2023

1Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO 63110, USA. 2Center for Reproductive Health Sciences, Washington University, St. Louis, MO 63110, USA. 3ICCE Institute, Washington University, St. Louis, MO 63110, USA. 4Department of Medicine (Oncology), Washington University, St. Louis, MO 63110, USA. 5Department of Chemistry, Washington University School of Medicine, St. Louis, MO 63110, USA. 6Center for Metabolomics and Isotope Tracing, Washington University School of Medicine, St. Louis, MO 63110, USA. 7Department of Obstetrics and Gynecology & Reproductive Sciences, University of California San Francisco, San Francisco, CA 94143, USA. 8These authors contributed equally: Gregory D. Longmore, Katherine C. Fuh. 9email: katherine.fuh@ucsf.edu