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# TWIST1 induces expression of Discoidin Domain Receptor2 (DDR2) to Promote Ovarian Cancer Metastasis

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## Abstract

The mesenchymal gene program has been shown to promote the metastatic progression of ovarian cancer; however, specific proteins induced by this program that lead to these metastatic behaviors have not been identified. Using patient derived tumor cells and established human ovarian tumor cell lines, we find that the Epithelial-to –Mesenchymal Transition inducing factor TWIST1 drives expression of Discoidin Domain Receptor 2 (DDR2), a receptor tyrosine kinase (RTK) that recognizes fibrillar collagen as ligand. The expression and action of DDR2 was critical for mesothelial cell clearance, invasion and migration in ovarian tumor cells. It does so, in part, by upregulating expression and activity of matrix remodeling enzymes that lead to increased cleavage of fibronectin and spreading of tumor cells. Additionally, DDR2 stabilizes SNAIL1, allowing for sustained mesenchymal phenotype. In patient derived ovarian cancer specimens, DDR2 expression correlated with enhanced invasiveness. DDR2 expression was associated with advanced stage ovarian tumors and metastases. *In vivo* studies demonstrated that the presence of DDR2 is critical for ovarian cancer metastasis. These findings indicate that the collagen receptor DDR2 is critical for multiple steps of ovarian cancer progression to metastasis, and thus, identifies DDR2 as a potential new target for the treatment of metastatic ovarian cancer.



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Supplementary Information

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

**Keywords**

Mesothelial cell clearance; receptor tyrosine kinase; collagen receptor; fibronectin; Snail1

## Introduction

Unique from other malignancies of epithelial origin, the metastatic cascade of ovarian tumors commonly occurs via the peritoneal circulation, and the majority of metastases are confined within the abdominal peritoneal cavity[50]. After tumor cell detachment from the primary site in the ovary, tumor cells must attach to the mesothelial cells covering the peritoneal and pleural organs, then clear the mesothelial layer to invade through the underlying basement membrane and extracellular matrix (ECM) to form metastatic implants[19]. Thus, for efficient metastasis, ovarian tumor cell interaction with the surrounding ECM and subsequent remodeling of the ECM is critical[5, 20]. Identifying cellular and molecular targets important for one or more of these steps could have tremendous clinical impact by enabling the development of therapeutics that block these critical steps.

The ability of ovarian cancer cells to adopt a mesenchymal gene program has been shown to promote ovarian cancer metastasis[10]. Induction of epithelial-to-mesenchymal transition (EMT) in ovarian cancer cells through expression of EMT transcription factors TWIST1, SNAIL1, or ZEB1 promotes mesothelial cell clearance[10]. These factors also promote the ability of an ovarian cancer cell to invade the basement membrane and extracellular matrix, and form metastatic implants[4, 37, 42]. While these EMT transcription factors induce a mesenchymal program crucial for ovarian cancer metastasis, the specific proteins whose expression is regulated during EMT that functionally mediate mesothelial cell attachment, clearance, and tumor cell invasion are not fully appreciated.

Mesenchymal properties of the developing cranial mesoderm are regulated by Twist1 transcriptional targets that influence cell-matrix interactions. Among the mesenchymal genes transcriptionally regulated by TWIST1 in this developing tissue is Discoidin Domain receptor 2[1]. TWIST1 binds to the 5'-regulatory region of Ddr2 inducing its expression in this tissue. Discoidin domain receptor 2 (DDR2) is a unique receptor tyrosine kinase (RTK) that has been shown to promote the metastasis of multiple cancers[6, 8, 17, 32, 33, 43, 45, 47, 51]. Unlike most RTKs that utilize soluble growth factors as ligands, fibrillar collagens serve as the ligand for DDR2[34, 40]. This provides for unique cell-ECM interactions. Fibrillar type I collagen is the major structural element of the ovarian stroma and has been shown to increase four-fold in ovarian cancer[26]. DDR2 is a mesenchymal gene not typically expressed by normal epithelium[2], however, its expression is activated in many cancer cells of epithelial origin as they progress to invasive and metastatic cancers[39]. DDR2 has been shown to stabilize the EMT transcription factor SNAIL1 in tumor cells that have undergone EMT, thereby maintaining mesenchymal cell behaviors, including invasion and migration[32, 43, 51].

In orthotopic transplant and spontaneous genetic models, depletion or deletion of *Ddr2* in tumor cells prevents metastasis *in vivo* in breast[8, 51] and prostate[47] cancer models. The role of DDR2 in promoting invasion and metastasis has been ascribed to its regulation of a number of different molecular effectors, including upregulation of MT1-MMP activity via a

SNAIL1 mediated pathway[43, 51]. In addition, the expression and activity of various matrix remodeling enzymes, such as matrix metalloproteinases (MMPs) and lysyl oxidases is influenced by the presence and activation of DDR2[8, 22]. Furthermore, while DDR2 itself does not mediate strong adhesive contacts, it has been shown to have an adhesion promoting role through enhancement of an integrin activation state[16]. Whether DDR2 contributes to ovarian cancer metastasis is not known.

In this study, we show that TWIST1 regulates DDR2 expression in ovarian cancer cells. We find that the presence of DDR2 in ovarian tumor cells is critical for mesothelial cell clearance, and tumor cell invasion and migration, in part through promotion of ECM remodeling. We also demonstrate that the action of DDR2 in ovarian tumor cells is critical for ovarian tumor metastasis *in vivo*. As such, DDR2 represents a potential therapeutic target to modulate numerous pathways critical for metastatic ovarian tumor progression.

## RESULTS

### TWIST1 regulates DDR2 Expression in Ovarian Cancer Cells

The EMT transcription factor TWIST1 has been shown to transcriptionally induce expression of Ddr2 in developing mesenchymal neural tissue[1]. Ovarian cancer cells have been shown to adopt a mesenchymal program during tumor progression, which influences tumor cell-matrix interactions and remodeling that promote metastasis[11]. Therefore, we asked whether the TWIST1-DDR2 pathway was present in ovarian cancer cells. In a collection of ovarian cancer cell lines, the expression of DDR2 and TWIST1 was highly correlated, particularly in cell lines with metastatic potential (Fig 1A). To determine if TWIST1 regulated DDR2 expression in these ovarian cancer cells, TWIST1 expression was depleted using shRNA. In A2780 and ES2 ovarian tumor cells, this resulted in decreased DDR2 mRNA (Fig S1A) and protein level (Figure 1B). In contast, depletion of DDR2 had no effect on TWIST1 levels (Figure 1B).

TWIST1 expression is induced during TGF-β-induced EMT[44]. Therefore, we asked whether TGF-β-induced TWIST1 expression in ovarian tumor cells resulted in DDR2 expression. Treatment of epithelial OVCAR3 ovarian cancer cells (which do not express TWIST1 or DDR2 at baseline) with 2 ng/mL of TGF-β induced EMT, as assessed by expression of α-SMA (Figure 1C). Both TWIST1 and DDR2 expression were induced as well (Figure 1C) and maintained under continuous exposure to TGF-β. In OVCAR3 cells, shRNA-mediated TWIST1 depletion did not affect EMT induction in response to TGF-β, as as seen by expression of α-SMA (sup fig 1B, C). However, in the absence of TWIST1, DDR2 was not expressed (sup fig 1B, C). These data indicate that as ovarian cancer cells progress to more invasive, mesenchymal phenotypes, TWIST1 promotes DDR2 expression.

### DDR2 Promotes Mesothelial Cell Clearance

Upon detachment of ovarian tumor cells from the primary site, the layer of mesothelial cells lining the peritoneal cavity must be cleared by the tumor cell for successful attachment[19]. Constituitive TWIST1 overexpression in ovarian tumor cells promotes their capacity for mesothelial cell clearance[10]. Therefore, we asked whether expression of DDR2, induced by

TWIST1, could be responsible for mediating mesothelial cell clearance. We adapted an ovarian tumor spheroid mesothelial cell clearance assay[9] and confirmed that ES2 cells readily clear the mesothelial monolayer, as previously reported[10] (Figure 1D and E). Genetic depletion of DDR2 in ES2 cells significantly reduced their mesothelial cell clearance ability (Figure 1D and E) to an extent similar to that observed when TWIST1 was depleted[10] (Supplemental figure 1D and E).

These results indicated that the action of DDR2 in ovarian tumor cells that have adopted an invasive, mesenchymal phenotype, is largely responsible for their capacity to clear mesothelial cells.

## DDR2 Contributes to Invasion and Migration of Ovarian Cancer Cells

Subsequent to clearing the mesothelial layer, ovarian cancer cells must invade and migrate through the underlying basement membrane (BM) and extracellular matrix (ECM) for successful metastases. As TWIST1 mediated EMT is known to contribute to these behaviors in other cancers[14, 25, 41, 48], we sought to determine whether the action of DDR2 in ovarian tumor cells contributed to these tumor cell functions. Matrigel has been used extensively as a BM surrogate, and DDR2 depleted cells were significantly impaired in their ability to invade through Matrigel coated transwells (Figure 2A and B, Supplemental figure 2A–D). To assess ECM invasion/migration, ovarian tumor cells were embedded in 3D Collagen I gels. DDR2 depleted cells were significantly impaired in their ability to invade 3D type I collagen (Figure 2C and D), as shSCRM cells formed invasive protrustions in 3D collagen, while shDDR2 cells remained rounded. We also observed decreased cellular migration of shDDR2 cells through uncoated Boyden chambers (Supplementary Fig. S2C and S2D). There were no differences in proliferation between control and DDR2-depleted ovarian tumor cells (Supplementary Fig. S2E and F). In sum these results indicated that the action of DDR2 in ovarian tumor cells contributed to critical tumor cell functions within the metastatic cascade, such as mesothelial cell clearance, BM invasion and ECM invasion/migration, but did not influence tumor cell proliferation.

## DDR2 activation leads to increased SNAIL1 protein level in ovarian cancer cells

We have shown that DDR2 is a transcriptional target of TWIST1 in ovarian cancer cells, and its activity serves to mediate many of the pro-metastatic phenotypes associated with the TWIST1 mesenchymal program. DDR2 activation in response to collagen I stimulation has also been shown to stabilize the EMT transcription factor SNAIL1 in multiple cancer cell types, thereby sustaining mesenchymal cell features and tumor cell invasion and migration[32, 35, 43, 51]. Moreover, constitutive SNAIL1 expression can promote mesothelial clearance in ovarian cancer[10]. Therefore, we asked whether DDR2 regulated SNAIL1 levels in human ovarian cancer cells, and in addition to carrying out TWIST1 mediated functions, could contribute to maintaining a mesenchymal phenotype. Using phosphorylation of DDR2 as a measure of its activation, we confirmed that when ES2 or A2780 ovarian tumor cells were added to Collagen I coated plates, DDR2 was activated (Supplemental figure 3A). Under these stimulating conditions, SNAIL1 protein was increased in control cells (Figure 3A and B). This was dependent upon the presence of DDR2 signaling, as DDR2 depletion in those same cells abrogated the increase in SNAIL1 following collagen I stimulation (Figure

3A and B). These data indicated that DDR2 activation by collagen I serves to stabilize SNAIL1 protein levels in ovarian cancer cells.

## DDR2 regulates expression and activity of extracellular matrix remodeling enzymes

Matrix remodeling enzymes such as matrix metalloproteinases (MMPs) and lysyl oxidases (LOX) are known to be key contributors to ovarian tumor cell invasion and progression, as well as mesothelial clearance[5, 13, 20]. Multiple reports have linked DDR2 activity with increased MMP and LOX expression[8, 27, 28, 46]. Furthermore, DDR2 has been shown to influence expression of the MMP activating enzyme, MT1-MMP (MMP14), downstream of SNAIL1 stabilization[51], as well as activation of MT1-MMP in response to collagen[24] in various cell types. As collagen I induced DDR2 activation led to increased SNAIL1 protein in ovarian cancer cells (Figure 3A and B), we asked whether the level and activity of matrix remodeling enzymes in ovarian tumor cells was dependent upon the action of DDR2. Quantitative RT-PCR analysis of select matrix remodeling enzymes showed that overall there was a reduction in the expression of these enzymes in ES2 and A2780 ovarian tumor cells depleted of DDR2, even though the expression profile of matrix remodeling enzymes differed between the tumor cell lines (Figure 3C). In ES2 cells, DDR2 depletion led to decreased MMP2, MMP7, MMP13, and LOXL2 mRNA level (Figure 3C). In A2780 cells, MMP1, MMP3, and LOXL2 mRNA levels were reduced in DDR2 depleted cells (Figure 3D). Although mRNA level of MT1-MMP (MMP14) was not influenced by the presence of DDR2 in unstimulated cells, Western blot analysis of ES2 and A2780 cells showed that active MT1-MMP protein level increased in response to collagen I stimulation (Figure 3A and B), and this increase was abolished when DDR2 was depleted (Figure 3A and B). Finally, in the ES2 cell line, gelatin zymography showed that MMP-2 level and activity was reduced in DDR2-depleted cells compared to control ES2 cells (Figure 3E).

These results indicated that DDR2 activation in ovarian tumor cells regulated expression and activity of matrix remodeling enzymes. These enzymes could facilitate migration, invasion, and mesothelial cell clearance.

## DDR2 contributes to Fibronectin cleavage by ovarian cancer cells and promotes ovarian cancer cell spreading on Fibronection

Fibronectin (FN) cleavage and remodeling has been shown to be important for the ability of ovarian cancer cells to clear the mesothelial cell layer covering the peritoneum during the early stages of metastasis[19, 20]. As MMPs are efficient at FN cleavage[20, 53] and DDR2 modulates expression and activity of MMPs in ovarian cancer cells (Figure 3), we asked whether the presence of DDR2 in ovarian tumor cells led to more efficient cleavage of FN. Cell free media from collagen stimulated ES2 or A2780 shSCRM cells was capable of rapid cleavage of human FN (Figure 4A–C). When DDR2 was depleted in these cells, there was a delay in the cleavage of FN, as quantified by the amount of remaining full length FN (Figure 4A–C). These data demonstrated that DDR2 activity in ovarian tumor cells promotes efficient cleavage of FN, and as such, may contribute to the enhanced mesothelial clearance by DDR2 expressing cells.

MMP cleavage of FN has been shown to enhance peritoneal adhesion of ovarian cancer cells[20]. Additionally, while DDR2 itself does not bind FN, its presence has been shown to promote adhesion and spreading of cells via integrin activation[16]. We therefore sought to determine if depletion of DDR2 in ovarian cancer cells led to differential adhesion and spreading of these cells on FN. Polyacrylamide hydrogels were functionalized with FN. When plated on these FN coated hydrogels, ES2 shSCRM control cells appeared maximally spread at 3 hours, while DDR2 depleted ES2 cells showed a dramatic reduction in cell spreading (Figure 4D and E). These results indicate that DDR2 promotes the ability of ovarian cancer cells to adhere and spread on FN.

## DDR2 is critical for ovarian tumor metastasis in vivo

To determine whether DDR2 influenced ovarian cancer cell metastasis *in vivo*, we compared the ability of control scrambled shRNA (shSCRM) and shDDR2 (ES2 and A2780) (Figure 5A and Supplementary figure 4A) to form metastasis in a peritoneal xenograft model of ovarian cancer. Mice were sacrificed and the tumor burden determined by measuring tumor weight and/or counting number of tumor nodules. For both cell lines, DDR2-depleted tumor-bearing mice showed less total tumor weight (Figure 5B and Supplementary Figure S4B and S4C). Consistent with human disease, much of the peritoneal tumor burden in these mice consisted of lesions attached to the mesentery and omentum (Figure 5C and D, Supplementary Fig. S4D). These results indicated that the action of DDR2 in ovarian tumor cells is critical for the establishment of ovarian tumor metastasis *in vivo*.

## DDR2 is expressed in human ovarian carcinomas and is an indicator of poor prognosis

To determine if DDR2 expression in human ovarian tumors is associated with clinical outcome, we first analyzed gene-expression data from a previously described cohort of ovarian cancer patients with advanced stage, high-grade serous cancer[38]. Univariate survival analysis showed that stratification of patients into “DDR2 high” and “DDR2 low” groups based on an optimum threshold revealed that high DDR2 mRNA expression levels were associated with shorter overall survival ($p=0.02$) (Figure 6A). Next, we determined DDR2 expression, by IHC, in ovarian surface epithelium taken from patients with benign ovarian disease, primary tumors, and metastatic lesions. Although some benign ovarian disease exhibited low level DDR2 expression, 0% (0/9) of the specimens exhibited high DDR2 expression (3+) (Figure 6B and C). High level of DDR2 expression appeared to correlate with stage of the patient, as 44% (79/179) of early stage and 74% (28/38), $p<0.0001$, of advanced stage specimens show high levels of DDR2 expression (Figure 6B and C). Notably, 100% (12/12) of the metastatic tumor samples showed high levels of DDR2 expression (Figure 6B and C). These findings are consistent with a previous report[15] and demonstrated that DDR2 expression was induced in aggressive ovarian tumors.

## DDR2 expression level is correlated with increased invasion in patient derived primary ovarian cancer cells

The mesenchymal gene program correlates with increased metastatic behavior in the primary tumors of ovarian cancer patients[10]. We therefore assayed a number of primary ovarian cancer (POV) cells derived from the abdominal ascites of patients with advanced stage, high grade ovarian cancer for expression of mesenchymal proteins, including DDR2.

All POV cells expressed at least one of the mesenchymal markers assayed (Figure 7A). Additionally, although variable in level, all POV cells exhibited some degree of DDR2 expression. To determine whether correlations between invasiveness and DDR2 expression could be observed in POV cells, we utilized an *ex vivo* assay in which the Matrigel invasion capacity was examined. A subset of the POV cells (POV1, 9, 10, 12) with similar proliferation rates (Supplemental Figure 5), but with varying expression profiles of mesenchymal proteins, were subjected to the assay (Figure 7B and C). Notably, POV9, which displayed the lowest expression of DDR2 among the cells assayed, was least invasive. These data are consistent with results from the established ovarian cell lines, and further implicate DDR2 action as critical for the invasive capacity of ovarian cancer cells, and its potential utility as a therapeutic in the ovarian cancer setting.

## Discussion

In summary, these studies have shown that the collagen receptor DDR2 contributes to the mesothelial clearance, invasion, and metastasis of ovarian cancer cells. Our working model (Figure 7D) posits that upon EMT induction by tumor environmental signals, EMT transcription factors, including TWIST1, are expressed. During the transition to the mesenchymal state, TWIST1 induces DDR2 expression. DDR2 then modulates the expression and activity of matrix remodeling enzymes, including MMPs and LOXL2. Additionally, DDR2 enhances the ability of ovarian tumor cells to cleave fibronectin, and adhere to and spread on fibronectin substrate. This could contribute to increased mesothelial cell clearance and invasion by ovarian tumor cells. Upon reaching the collagen I-rich stromal matrix DDR2 activity maintains SNAIL1 levels, thereby serving a central role in maintaining the mesenchymal phenotype of metastatic ovarian tumor cells.

DDR2 stabilization of SNAIL1 has previously been shown to depend on the kinase function of DDR2[51]. Indeed, in our studies DDR2 is activated under the conditions that promote SNAIL1 stabilization. Notable, however, is that in the absence of ligand there are differences in the mRNA level of matrix remodeling enzymes (Figure 3C and D), as well as the ability of cells to adhere and spread on non-ligand substrate (Figure 4D and E). This raises intriguing possibility of DDR2 playing roles outside of its enzymatic activity to modulate tumor cell behavior. An accumulating body of evidence suggests the ability of RTKs carry out non-catalytic functions within the cell[31], and it will be of interest to deterimine if, and how, DDR2 is carrying out these roles in the absene of ligand.

DDR2 expression led to increased adhesion to fibronectin, yet DDR2 does not directly bind fibronectin[40]. We have shown that DDR2 expressing cells are able to more efficiently cleave FN. Cleavage of FN is necessary to expose high affinity sites for α5β1 and αvβ3 integrin binding, leading to enhanced adhesion of ovarian cancer cells[20, 29]. DDR2 has been shown to influence adhesion and spreading of cells by collagen binding α1β1 and α2β1 integrins[16]. This raises that possibility that DDR2, as a signaling receptor, could also influence the adhesive activity of integrins. As it was not determined in that study, how exactly DDR2 influences collagen and/or fibronectin, integrin adhesion remains of interest.

Whereas other epithelial tumors, including ovarian cancer, have been shown to use hematogenous or lymphatogenous routes to metastasize, ovarian cancer is unique in that cells within the ascites fluid reach distant sites within the peritoneal cavity[30, 50]. This leads to the requirement of ovarian tumor cells to clear and invade the mesothelial layer that covers the peritoneal organs in order for metastatic implants to arise[3]. Our findings demonstrate that DDR2 expression is associated with worse prognosis in high-grade serous cancers (Figure 4). Additionally, molecular profiling of the ES2 cell line used for many of our cell-based assays by The Cancer Cell Line Encyclopedia has shown the line to have a TP53 mutation. Therefore, although the ES2 cell line is classically thought of as a clear cell tumor derivative, it more likely high-grade serous[12]. The A2780 cell line used in our assays does not have a TP53 mutation and is unlikely of high-grade serous histology with PIK3CA, PTEN, and ARID1A mutations. However the results from this cell line adds to the potential generalizability of our findings. We have shown that DDR2 plays a critical role in mediating mesothelial cell clearance. While this could be due, simply, to the increased ability of DDR2 expressing cells to adhere and spread, there are other intriguing possibilities that warrant further study. Recently, DDR1, the other member of the discoidin domain family, was demonstrated to mediate myosin-dependent collagen contraction via association with non-muscle myosin IIA (NM-IIA)[7]. It has been demonstrated that ovarian cancer spheroids exert force on the mesothelial cell-associated ECM protein FN via α5β1 integrin in a NM-IIA dependent manner to clear the mesothelium[19]. As there is a rich network of collagen fibers just below the mesothelium in sites of ovarian cancer metastasis[21], it would interesting to determine if a DDR2-NM-IIA mechanism of force generation exists, and if so, whether it contributes to enhanced clearance and invasion by ovarian cancer cells.

Our findings also suggest that stabilization of SNAIL1 and regulation of MMPs may be an important component of DDR2 signaling contributing to ovarian tumor metastasis. Other studies have shown that TWIST1 and SNAIL1 promote mesothelial clearance by ovarian cancer cells, and suggest that inhibiting the pathways that drive mesenchymal programs may suppress tumor cell invasion[10]. As transcription factors such as TWIST1 and SNAIL1 can be difficult to target therapeutically[49], modulating the activity of proteins whose expression they regulate (e.g., DDR2) or upstream regulators that stimulate or maintain their expression (e.g., DDR2) could be a more practical approach. In the case of ovarian cancer metastasis, targeting DDR2 could allow for intervention at both such levels. In human samples, advanced and metastatic ovarian tumor cells have increased DDR2 expression, and this high expression is associated with poor prognosis of ovarian cancer patients. While other authors have recently shown this association[15], here we provide functional data that DDR2 expression leads to mesothelial cell clearance, and increased invasion and migration by ovarian tumor cells. Furthermore, we have demonstrated that these enhanced cellular functions translate to DDR2 promoting ovarian cancer metastasis *in vivo*. Since DDR2 is upregulated in ovarian cancer cells, but relatively undetectable in surrounding normal tissues, DDR2 could be a highly specific therapeutic target with minimal toxicity to normal cells.

In conclusion, in metastatic ovarian tumor cells DDR2 expression is a product of the mesenchymal program induced by TWIST1, and serves to maintain the mesenchymal phenotype through SNAIL1 stabilization. Our *in vitro, in vivo*, and *ex vivo* results confirm

that DDR2 is one of the critical factors contributing to the steps of ovarian cancer metastasis. Therapeutic modulation of DDR2 could provide a means of improving treatment for patients with advanced ovarian cancer.

## Materials and Methods

### Antibodies

The antibodies and sources were as follows: DDR2 (for IHC, R&D Systems MAB2538), DDR2 (for Western Blot and immunoprecipitation, Cell Signaling Technologies 12133), MT1-MMP (Millipore AB6004), pTYR 4G10 (Millipore 05321), Snail1 (Cell Signaling Technologies C15D3), Twist1 (AbCam ab50887), β-Actin (Sigma a5316), β -Tubulin (Sigma T4026), N-cadherin (BD 610920), E-Cadherin (BD 610181), a-SMA (Sigma a5228), Zeb1 (Santa Cruz sc25388). Secondary anti-mouse and anti-rabbit HRP conjugated antibodies were from Cell Signaling Techologies.

### Cell culture

Established ovarian cancer cell lines A2780 (purchased from ATCC), SKOV3.ip1 (gift from Dr. Gordon Mills, M.D. Anderson Cancer Center, Houston, TX), OVCAR3 (purchased from ATCC), OVCAR4 (purchased from National Cancer Institute-Frederick DCTD tumor cell line repository), and OVCAR5 (National Cancer Institute-Frederick DCTD tumor cell line repository) were maintained in RPMI Medium (GIBCO) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin and streptomycin. Ovarian ES2 cells were maintained in McCoy's 5A (modified) medium (Life Technologies) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin and streptomycin. Cell lines were maintained at 37°C in a 5% $CO_2$ incubator. We used IDEXX Bioresearch o authenticate our cell lines, which performs short tandem repeat (STR) profile and interspecies contamination testing. Mycoplasma testing was also performed using MycoAlert Mycoplasma Detection Kit prior to performing experiments (Lonza).

For TGF-B induction of EMT, OVCAR3 cells were treated with 2ng/mL TGFB1 (Sigma) for the indicated time points. During treatment, media was replenished every 48 hours. For collagen I stimulation of DDR2, 2mg/mL collagen I (BD) gels were prepared by dilution of concentrated collagen I into media, and titration with NaOH followed by plating for 1hr at 37°C. Cells that had been serum starved overnight, were then plated onto collagen gels for indicated time points.

### Primary Ovarian Cancer Cell Culture

Ascites from patients with ovarian cancer (POV) was obtained and plated in a 1:1 ratio in RPMI 20% FBS, 1% pen-strep. After 7–14 days, attached and proliferating cells were passaged and used for experiments. POV 1, 9, 10, and 12 were all obtained from patients with advanced stage, high-grade serous ovarian or fallopian tube cancer. Immunohistochemistry was performed with CK8 to confirm epithelial origin of these cells. All the patients who participated in this study provided written informed consent for the collection and research use of their materials, and the use of these samples was approved by the Washington University Insitutional Review Board (IRB 201309050).

### Plasmids and shRNA Constructs

TWIST1 shRNA, 5'-CCTGAGCAACAGCGAGGAAGA-3' in the pLKO vector (Sigma) was used. Two previously validated oligos for DDR2 shRNA and shSCRM control sequence were used[51]. The oligos for human DDR2 shRNA, 5'- GCCAGATTTGTCCGGTTCATT-3' and 5'-GCCAAGTGATTCTAGCATGTT-3', and scramble control, 5'-CCTAAGGTTAAGTCGCCCTCGCTC-3', were cloned into the pLKO vector and infected cells were selected in puromycin (Sigma). For all hairpins, polyclonal populations were tested for decreased DDR2 expression levels by western blot analysis.

### Western blot analysis

Cultured cell lysates were prepared using a 9M Urea, 0.075 M Tris buffer (pH 7.6), and the Bradford Assay was used to quantify protein lysates. For Western blot anaylsis, 50–100μg of protein as determined by Bradford analysis (BioRad), was subjected to reducing SDS/PAGE using standard methods. For immunoprecipitation, five hundred micrograms of whole-cell lysate was incubated with DDR2 antibody, and captured using protein A-sepharose 4B beads (Sigma). Blots were probed with indicated antibodies, followed by corresponding horseradish peroxidase-conjugated secondary antibodies. Detection was preformed using SuperSignal Chemiluminescent substrate (Thermo Scientific) on a ChemiDoc XRS+ (BioRad). Integrated relative densities of individual bands were quantified using ImageLab software. All quantification was prefomed under conditions of linear signal response.

### Gelatin Zymography

$3\times10^6$ cells were plated on plastic or 2mg/mL collagen in serum-free media. Supernatant was collected and run on a 10% gelatin gel under non-denaturing conditions at 24 hrs. Gel was developed, stained with coomassie, and destained until bands were visible. After harvesting supernatant, cells were trypsinized and counted to ensure there were no proliferation differences among the groups tested. Experiment was performed 3 times and active/total MMP2 was quantified using ImageLab software after imaging the gels using ChemiDoc XRS+ (BioRad).

### Immunohistochemical analysis using human tissue microarrays

Ovarian human tissue microarrays were obtained from US Biomax (208, 2084t) which contained a majority of early stage and a smaller number of advanced stage ovarian cancers. Slides were deparaffinized with xylene, rehydrated and unmasked following standard immunohistochemical methods. The DDR2 primary antibody (R&D Systems, MAB2538) was used at a 1:500 dilution. Negative controls for all samples were done using the secondary antibody alone. Antigen-antibody complexes were visualized using the VECTASTAIN ABC system (Vector Laboratories) and DAB Substrate Kit for Peroxidase (Vector Laboratories) following the manufacturer's protocol. Slides were counterstained in hematoxylin. DDR2 staining on the membrane of tumor cells was scored microscopically according to the percentage of cells positive for DDR2 expression both by intensity and percentage of cells with expression (0 for absent, 1 for 1%–40%, 2 for 40–60%, and 3 for > 60%).

### Invasion and Migration Assays

Matrigel invasion assays were performed according to the manufacturer's protocol (Corning). Briefly, cells were serum starved for 24 hours, and 25,000 cells were plated in media containing 1% FBS onto Matrigel coated boyden (8um pore size) chambers. In the lower chamber, media containing 10% FBS and 5ug/mL fibronectin was placed as chemoattractant. Invasion assays were stained and analyzed after 48 hours. For migration assays, these were performed in a similar fashion in uncoated Boyden chambers. Invaded cells were quantified by counting the number of invading cells per high powered field, 4 high powered fields per insert, and 3 inserts per condition were quantified. Applying sample calculation for unequal variance t-test, the same size needed was 3 per group to achieve the statistical power as 0.9. The experiment was replicated 3 times.

Collagen I invasion assays were performed in which cells were plated in 48 well format as 5000 cell per well and cells were mixed with 2mg/mL collagen. After polymerization, media was added, and for 7 days, cells were monitored for invasive branching. For scoring, 5 fields of view were examined for each well. The cells in each field were scored as either non-invasive (rounded appearance) or invasive (having ≥1 invasive protrusion). Quantfication reported as number of cells with invasive protrusions as a percent of total cells scored. The experiment was replicated 3 times.

### Proliferation Assay

Cells were plated at densities of 4,000 cells per well into four 96-well plates in medium as described previously. Proliferation was measured at 24, 48, 72, and 96 hours using an 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT)-based assay (Roche Molecular Biochemicals) as previously described[18].

### Real-time PCR with reverse transcription

Real-time PCR reactions were done using the SYBR Green PCR Master Mix (Applied Biosystems) in the ABI detection system (Applied Biosystems). The thermal cycling conditions were composed of 50 °C for 2 min followed by an initial denaturation step at 95 °C for 20 s, 40 cycles at 95 °C for 3 s, and 60 °C for 30 s. The experiments were carried out in triplicate for each data point. Using the $2^{-\Delta\Delta Ct}$ method as described previously[23], the relative quantification in gene expression was determined. Primers used for PCR with reverse transcription and real-time PCR with reverse transcription are listed in Supplemental table. The experiment was repeated 3 independent times.

### Fibronectin Cleavage Assay

ES2 or A2780 shRNAi-depleted of DDR2 or transduced with scrambled control (SCRM) were cultured for 6hours under serum free conditions on 2mg/mL of collagen I, and cell free, conditioned media was collected and incubated with 4ug human Fibronectin (BD). Extent of cleavage was measured at time intervals of 0.25, 1, and 16 hours by SDS Page followed by coomassie staining. Experiment was repeated 3 independent times. Amount of intact FN (~220 kDa) quantified by densitometry, and results of independent experiments plotted as mean +/− SEM. Control FN was incubated for 16hrs with non-conditioned media.

### Cell Spreading Assay

Polyacrylamide hydrogels were produced as previously described[52]. Briefly, acrylamide solution (Bio-Rad) was mixed with N-N'- methylene-bis-acrylamide solutions (BioRad). A glutaraldehyde-activated glass surface and hydrophobic coverslip was then used for polymerization. Polymerized substrates were then activated for protein conjugation with the heterobifunctional crosslinker Sulfo-SANPAH at 0.5 mg/mL (Pierce Chemical Co.) under UV exposure for 15 min. The substances were then washed with HEPES buffer, and then functionalized with fibronectin. Using atomic force microscopy, gels were determined to have elastic moduli of ~120 kPa. Functionalized gels were washed with PBS, and ES2 shSCRM or shDDR2 were plated. Live cells were imaged on a Nikon Ti-E microscope with a temperature and humidity controlled incubation chamber (LiveCell). Data were collected by 3×3 tiling of 10× regions of view. Time-lapse imaging was performed with 20-minute frame intervals for a 6 hour duration. Image analysis was performed using Matlab (The Mathworks, Natick MA) and Image J. Cell area was computed for each cell in each field of view using custom software to detect cell boundaries. 3 independent experiments conducted, and pooled results quantified (n>200 for each condition). Data was plotted using R graphing library[36].

### Spheroid-induced mesothelial clearance assay

Mesothelial cells were cultured on 6-well plastic plates (Techno Plastic Products) and used in the clearance assay once a confluent monolayer was formed (approximately 72 hours). Mesothelial cells were labeled 18 hours prior with CMFDA-green (Molecular Probes), washed with PBS and incubated with fresh culture medium until use. Spheroids were prepared 18 hours in advance. Cells were labeled with CMTPX-red (Molecular Probes), washed with PBS, dissociated by trypsinization and resuspended in culture medium. Labeled cells were then counted and plated at 200 cells/well in ultra-low attachment multiwell plates (Corning) with 10ug/mL of soluble bovine fibronectin for increased cohesion. Spheroids were placed onto the monolayer of mesothelial cells at the microscope and images captured at 0, 1, and 7 hours. Experiment replicated 3 independent times. Normalized clearance value is determined by measuring the total mesothelial cell area cleared by the spheroid in ImageJ, and normalizing to the size of the spheroid at time 0.

### Survival analysis

For the survival analysis, the Tothill database was accessed[38]. Raw expression mRNA values for DDR2 and overall survival was used as an endpoint. Samples were dichotomized into two groups after determining the optimal cutoff level for gene expression. Survival curves were calculated using the Kaplan–Meier method, and statistical significance was assessed using the log-rank test to determine if there was a statistically significant association between DDR2 expression and overall survival.

### Orthotopic Model of Ovarian Cancer

All procedures involving animals and their care were performed in accordance with the guidelines of the American Association for Accreditation for Laboratory Animal Care and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals. All

animal studies were also approved and supervised by the Washington University Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals and NIH guidelines (Protocol 20140178). No randomization was used as mice were injected with either shSCRM or shDDR2 cells. Sample size calculation for equal variance t-test showed the sample size needed was 7 per group to achieve the statistical power of 0.9.

For genetic studies, A2780 shSCRM or shDDR2 were injected intraperitoneally (i.p.) with $7.5\times10^6$ cells in 0.5 ml of PBS into female 6- to 8- week old ($n$=8 per group) Balb/c nude (Taconic). ES2 shSCRM or shDDR2 cells were injected intraperitoneally (i.p.) with $10\times10^6$ cells in 0.5 ml of PBS into female 6- to 8- week old ($n$=8 per group) NU/NU nude (Charles River) mice. Mice were monitored for adverse events and sacrificed with CO2 exposure and cervical dislocation at 14 days (A2780 model) and 28 days (ES2 model) after i.p. injection. Necropsy with tumor burden assessment was performed by a blinded investigator. At the completion of each experiment, aggregate tumor weight, location, and number of tumor nodules were recorded for each group.

### Statistical Analysis

Prism software (GraphPad) was used for statistical analysis. Shapiro-Wilk test was performed for the normality of samples and found to indicate no departure from normality. Thus, two-tailed unpaired Student's t test was performed to analyze statistical differences between the groups. $P<0.05$ was considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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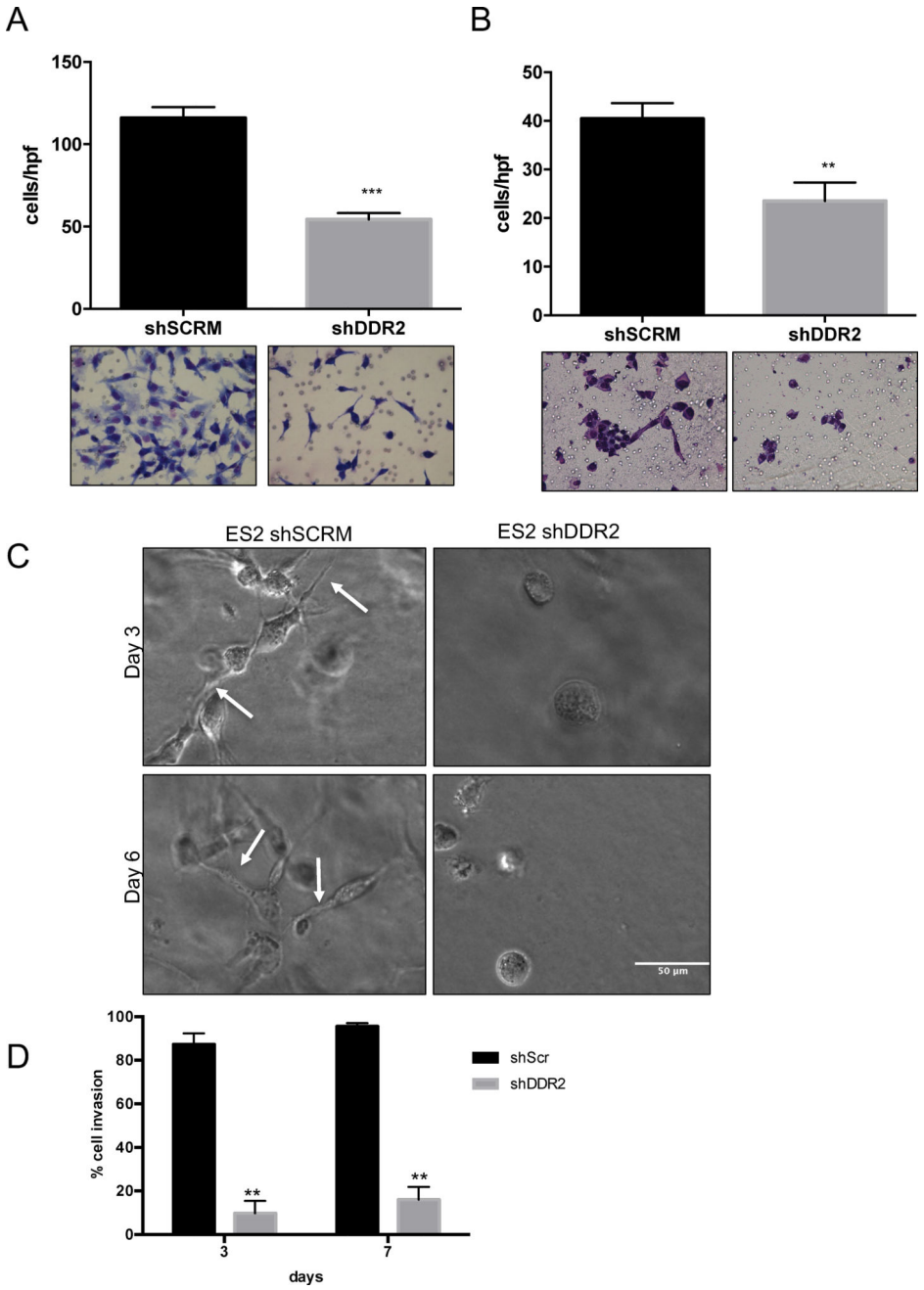



**Figure 1. TWIST1 induces DDR2 expression in ovarian cancer cells**

**A.** Western blot analysis of DDR2 and Twist1 protein expression in established ovarian cancer cell lines

**B.** A2780 or ES2 ovarian cancer cell lines were infected with lentivirus expressing the indicated shRNAi. Western blotting preformed using the indicated antibodies.

**C.** Western blot of indicated proteins from whole cell lysates of OVCAR3 cells treated with 2ng/mL TGF-B to biochemically induce EMT for indicated times.

**D.** ES2 ovarian cancer spheroids expressing the indicated shRNAi labeled with CMTCX dye (red) were added to a confluent monolayer of primary mesothelial cells that were labeled

with CMFDA dye (green) and monitored over 7 hours. Images show representative mesothelial clearance at 0 and 7 hours.

**E.** Quantification of experiment in D. >5 spheroids averaged per condition. Error bars denote SEM. **p<0.01, Student's t test.

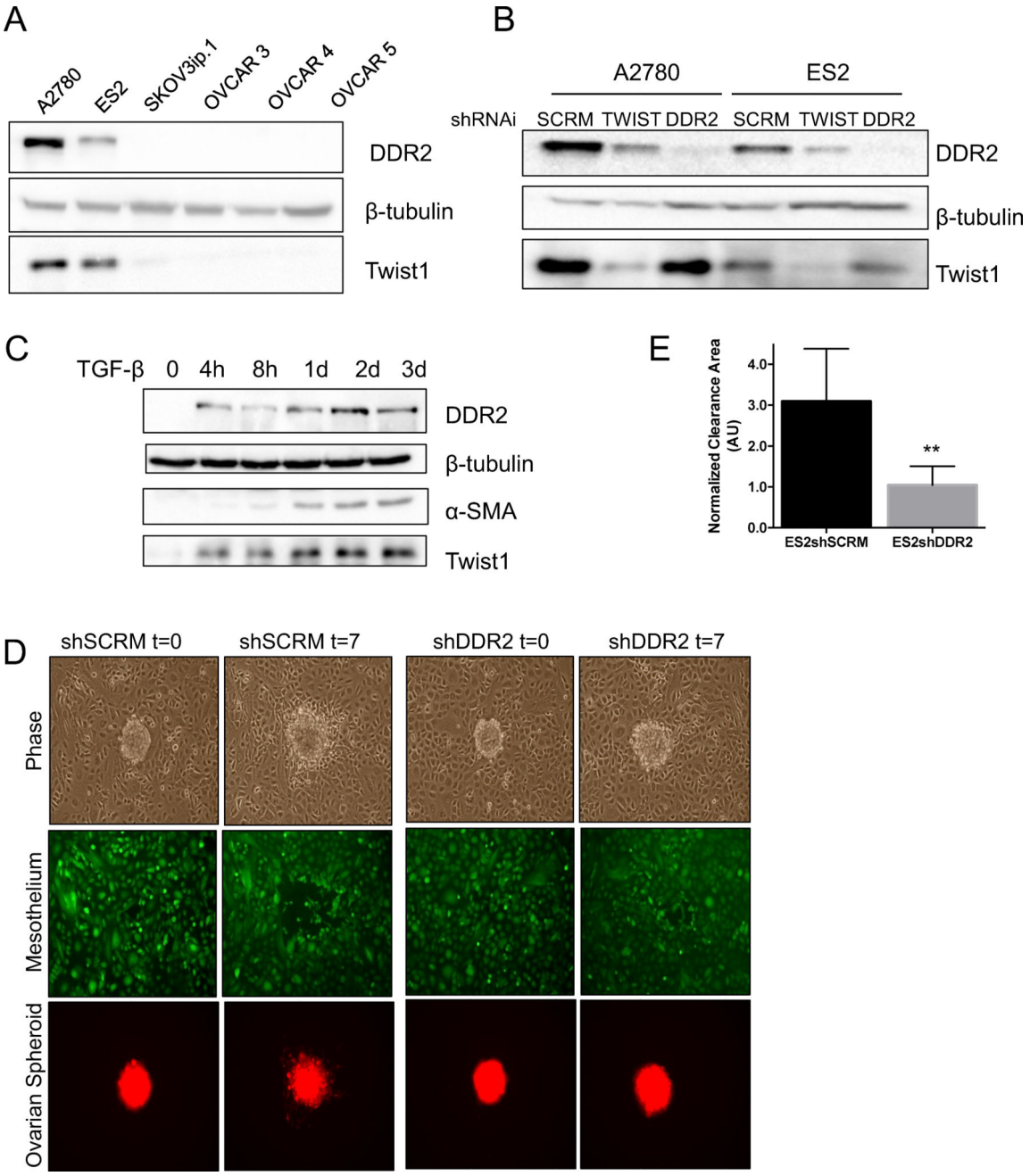



**Figure 2. DDR2 promotes invasion by ovarian cancer cells**

**A, B**. Matrigel invasion assays. **A.** ES2 and **B.** A2780 cells were depleted of DDR2 or control (SCRM) and added to Matrigel coated Boyden chambers (8um pore size).Representative images and quantification (Means and S.D. for 3 individual chambers per group plotted as number of cells invading through to lower surface 4 per high powered field (hpf)). Repeated with statistical significance in 3 independent experiments. Unpaired t-test, **p<0.01, ***p<0.001.

**C.** Collagen I invasion assays of ES2 shDDR2 or control (shSCRM) cells. Representative photographs taken at day 3 and day 6.

**D.** Quantification of collagen invasion. 20 cells per well, 3 wells per condition quantified. Mean and s.d. are shown, unpaired t-test **p<0.01

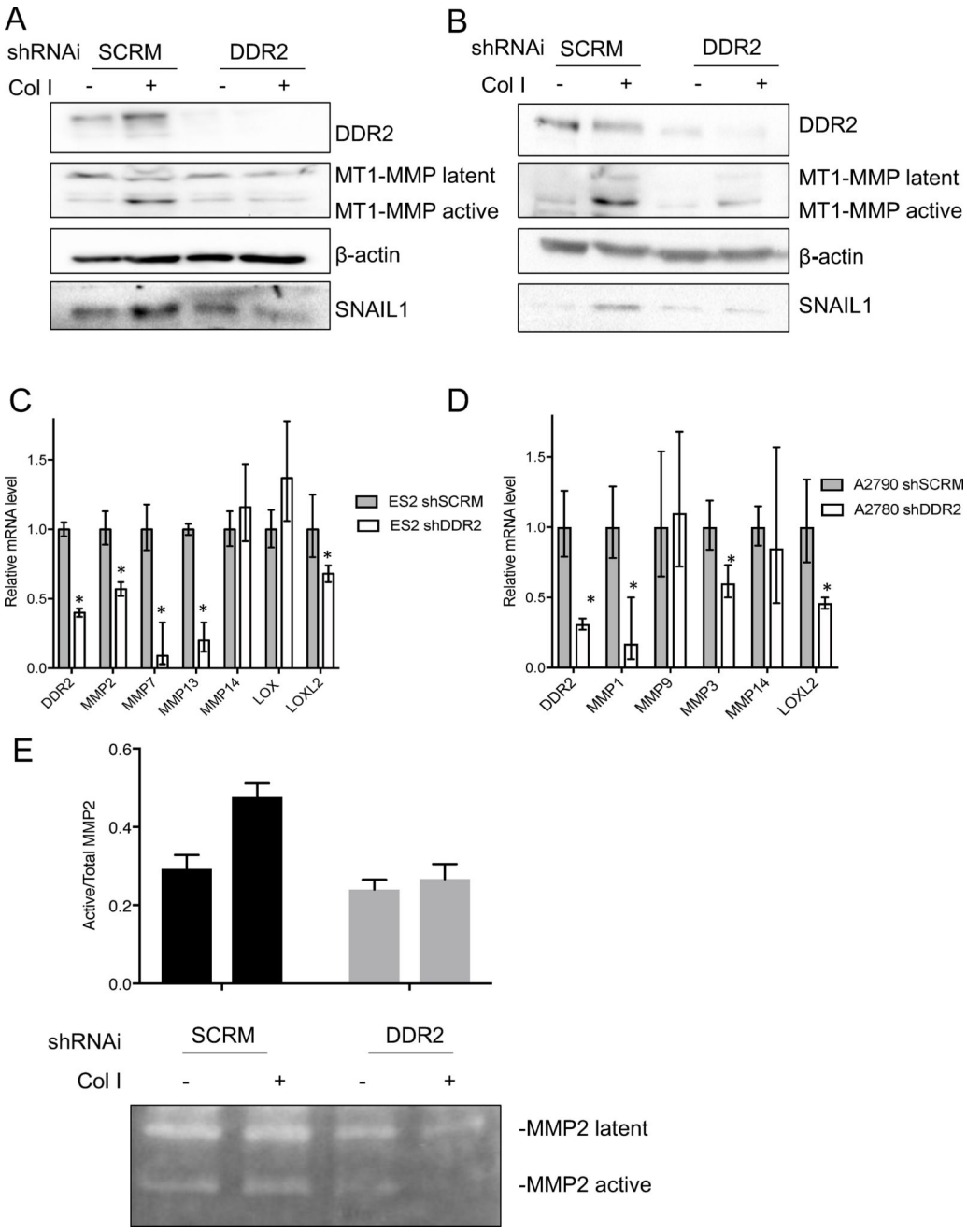



**Figure 3. DDR2 influences matrix remodeling enzyme expression and activity in ovarian cancer cell lines**

**A.** ES2 or **B.** A2780 cells depleted of DDR2 or control (SCRM) were added to collagen I coated (+) or uncoated (−) plates for 6 hours. Western blotting with the indicated antibodies was performed on cell extract. Data representative of 3 independent experiments.

**C, D.** Q-PCR analysis of mRNA isolated from C. ES2 or D. A2780 shRNA-depleted of DDR2 or transduced with scrambled control (SCRM). Three replicates of each gene were done for each experiment. Data are representative of three independent experiments. *p<0.05 student's t test

**E.** Gelatin zymography was conducted on the supernatants from ES2 cells depleted of DDR2 or control (SCRM) that had been plated on collagen I coated (+) or uncoated (−) plates for 24 hours. Representative zymogram and quantitation of 3 independent experiments shown.

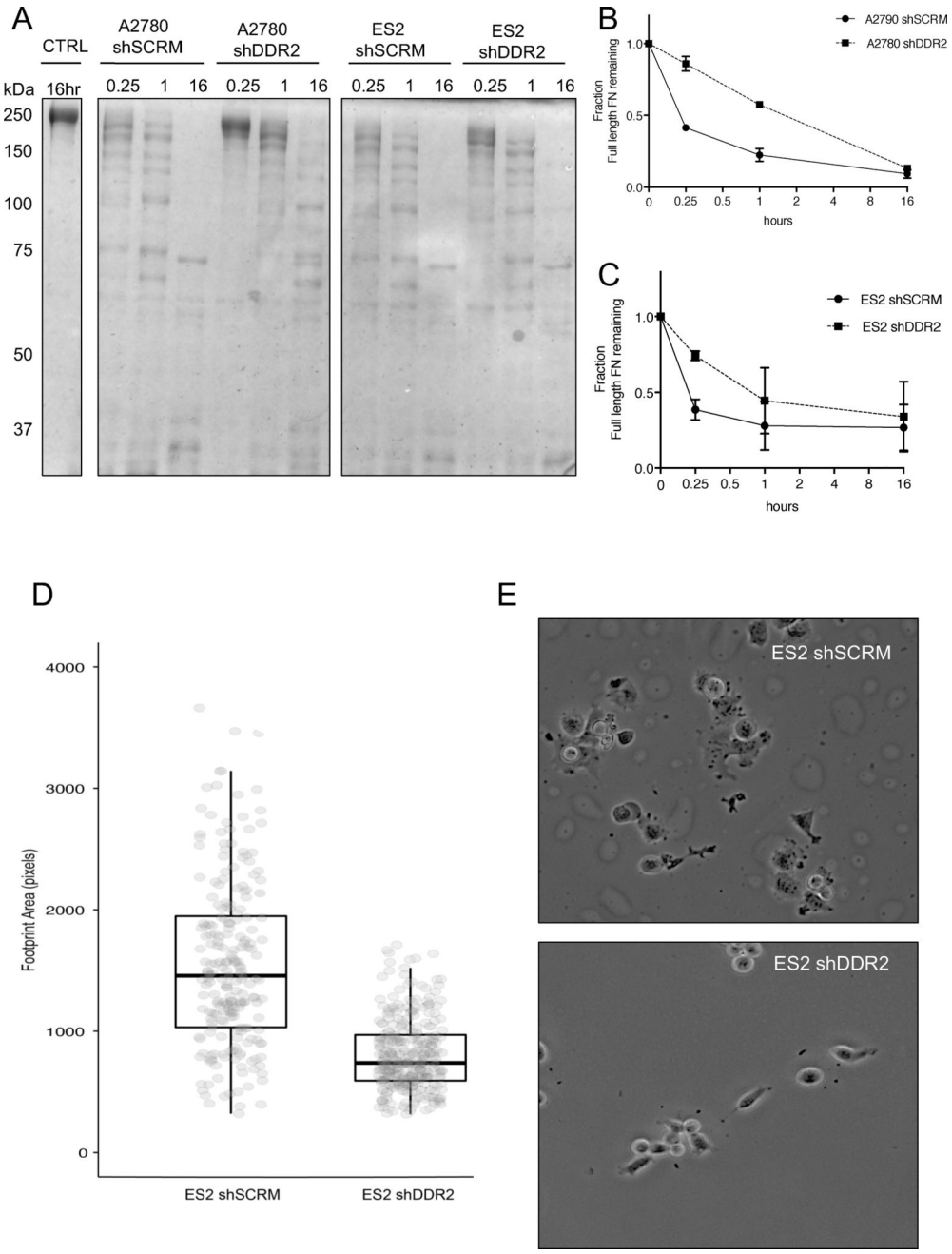



**Figure 4. DDR2 influences fibronectin cleavage and spreading by ovarian cancer cells**

**A.** ES2 or A2780 shRNAi-depleted of DDR2 or transduced with scrambled control (SCRM) were cultured for 6 hours under serum free conditions on 2mg/mL of collagen I, and cell free media was incubated with recombinant human fibronectin (FN). Extent of cleavage was measured at time intervals of 0.25, 1, and 16 hours by SDS Page followed by coomassie staining.

**B, C.** Quantification of cleavage of intact FN by B. A2780 or C. ES2 plotted as a fraction of remaining intact FN as compared to control. Amount of remaining intact FN (~220 kDa) quantified by densitometry. Data from 3 independent experiments are plotted.

**D.** Quantification of cell area when plated on fibronectin coated hydrogels for 3 hrs. Dots represent individual cell areas, with box plot overlay depicting the median and interquartile ranges of area spread. n>200 cells quantified for each group.

**E.** Representative images of ES2 shSCRM or shDDR2 cells plated on fibronectin coated hydrogels.

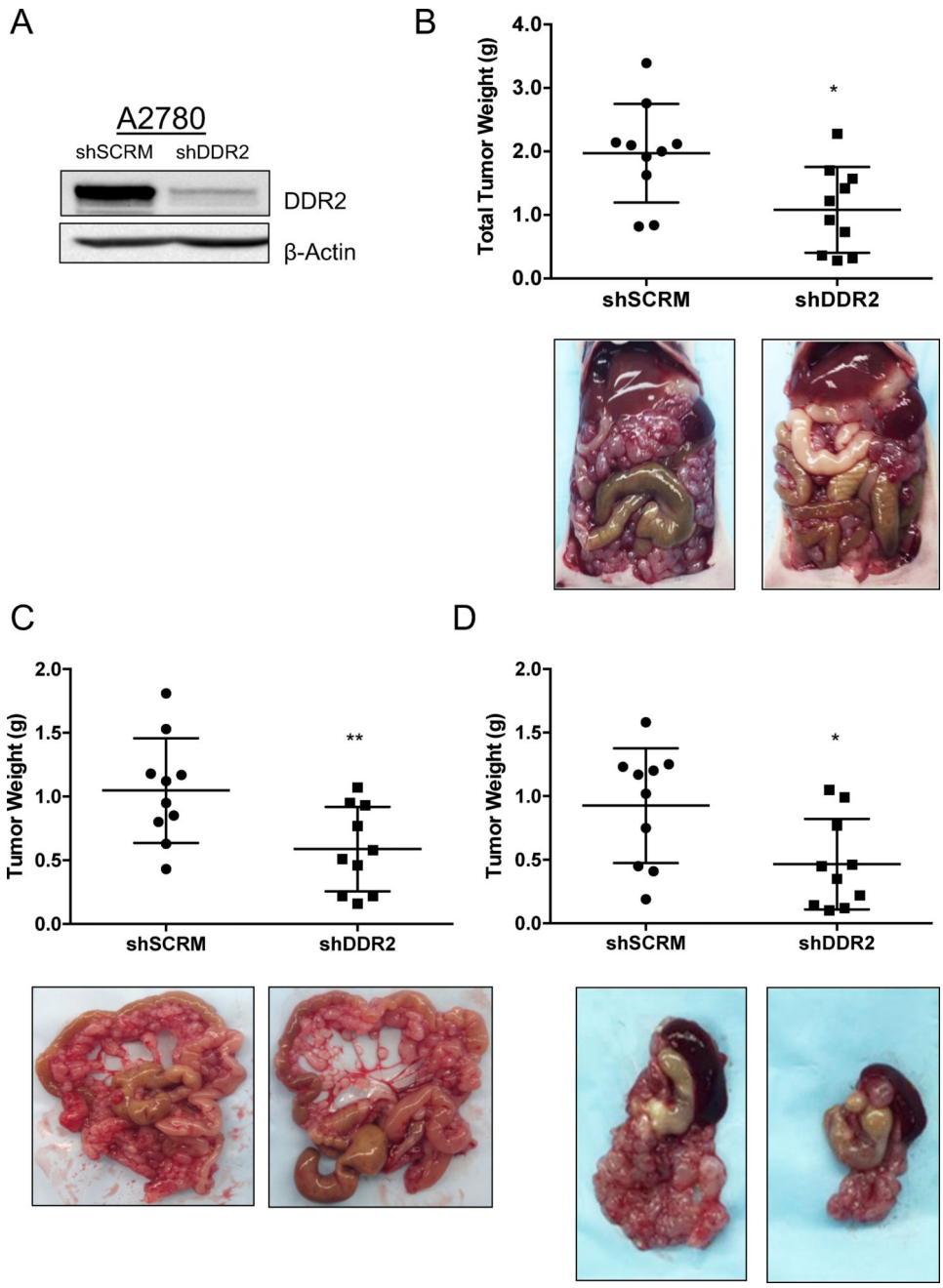



**Figure 5. DDR2 influences ovarian cancer cell metastasis in vivo**

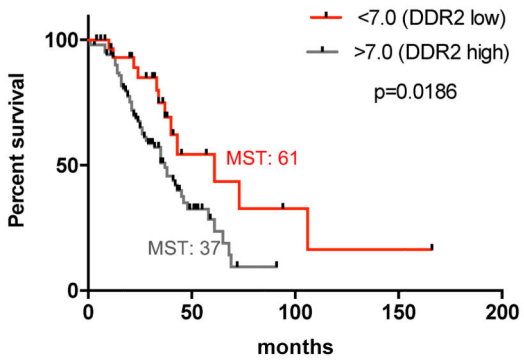
**A.** A2780 cells stably expressing shRNA targeting sequence for scramble control (shSCRM) or DDR2 (shDDR2). Beta-Actin was used as a protein loading control.

**B–D.** Representative images and quantification of Balb/c Nu mice injected IP with 7.5×106 A2780 shSCRM or shDDR2 cells. Tumor burden was assessed at 14 days post injection in the B entire peritoneal cavity

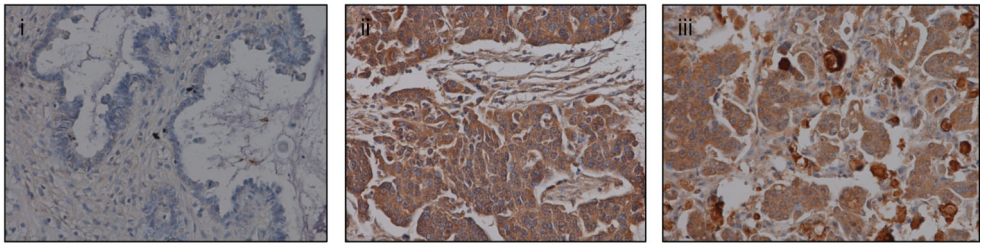
**C.** Mesentary only

**D.** Omentum only. N=10 mice per group. Means and s.d. Unpaired t-test, *P<0.05, **P≤0.01

A




B




C

| Score | 0 | 1-2 | 3+ | Total |
|---|---|---|---|---|
| Stage | | | | |
| Benign | 3 (33%) | 6 (67%) | 0 | 9 |
| Early (1/2) | 44 (25%) | 56 (31%) | 79 (44%) | 179 |
| Advanced (3/4) | 1 (2%) | 9 (24%) | 28 (74%) | 38 |
| Grade | | | | |
| Benign | 3 (33%) | 6 (67%) | 0 | 9 |
| Low Grade | 14 (29%) | 18 (38%) | 16 (33%) | 48 |
| High Grade | 29 (18%) | 45 (28%) | 87 (54%) | 161 |
| Site | | | | |
| Primary Tumor | 17 (23%) | 24 (33%) | 32 (44%) | 73 |
| Metastatic Implant | 0 | 0 | 12 (100%) | 12 |

**Figure 6. DDR2 is highly expressed in advanced ovarian tumors and metastatic implants**

**A.** Overall survival of advanced-stage III, IV serous ovarian cancers by Kaplan-Meier methods and log rank test. MST: Median survival time.

**B.** Representative images of DDR2 immunohistochemical staining in i) early stage IA primary tumor ii) advanced stage IV primary tumor iii)mesenteric metastatic implants

**C.** Classification of immunohistochemical analysis of DDR2 staining in normal (benign) ovary, human primary ovarian tumors, and metastatic implants.

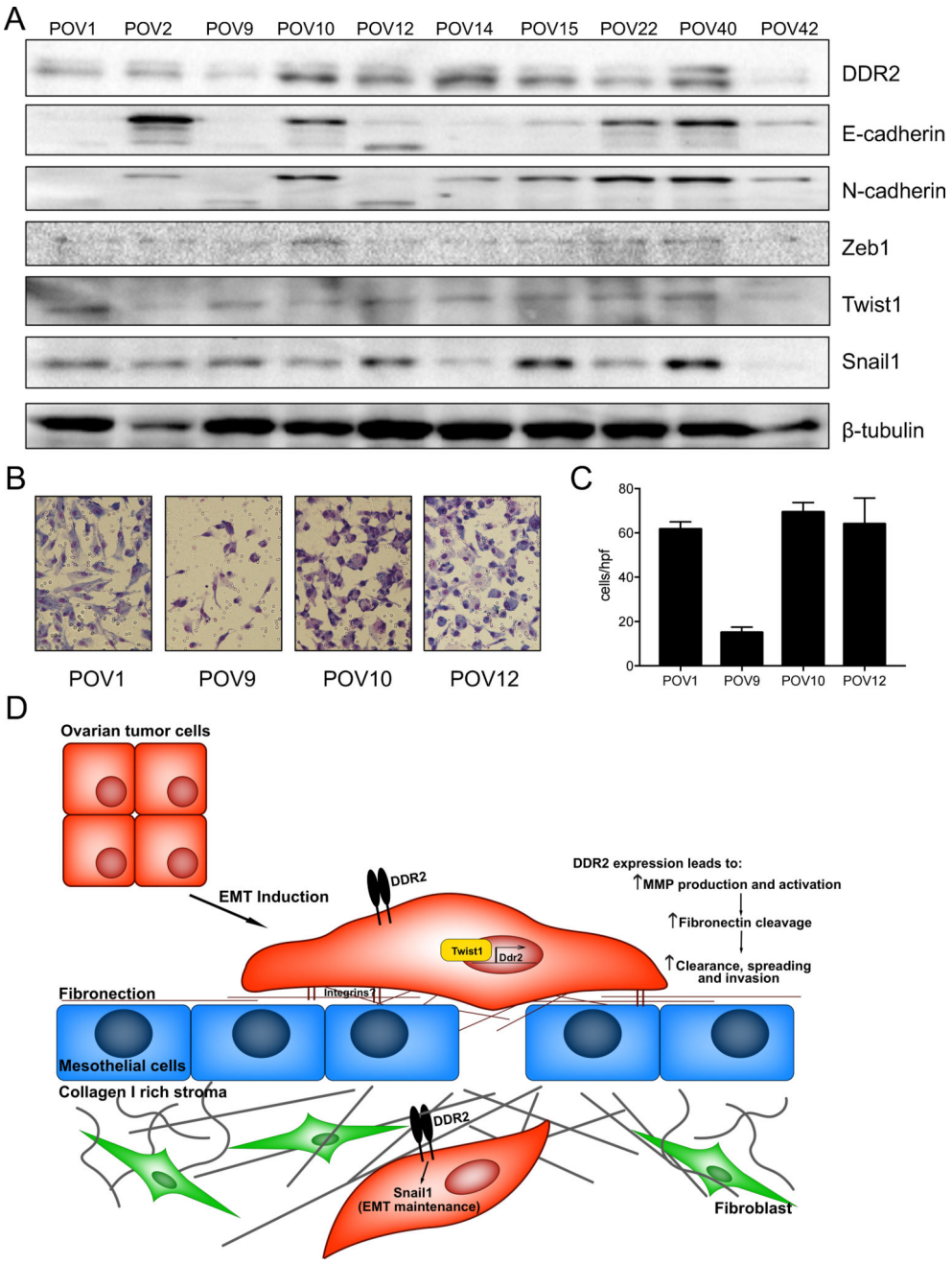



**Figure 7. DDR2 expression correlates with increased invasion of patient-derived ovarian cancer cells *ex vivo***

**A.** Western blot of patient derived primary ovarian tumor (POV) cells for expression of indicated proteins.

**B, C**. A subset of POV cells were added to Matrigel coated Boyden chambers to assay invasion. **B.** Representative images of inserts and **C.** quantification plotted as number of cells invading through to lower surface per high powered field (hpf). Means and s.d. are shown for 3 individual chambers per group, 4 hpf per chamber.

**D.** Proposed role of DDR2 in ovarian cancer metastasis