Surgical adhesions in mice derived from hypoxia-responsive mesothelial cells can be targeted with anti-mesothelial antibodies

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Overline: Peritoneal Adhesions

Single Sentence Summary:

Surgical adhesions derived from mesothelial cells can be targeted by antibodies against the mesothelial marker mesothelin in mice.

Editor's Summary:

Peritoneal adhesions are ectopic fibrotic tissues induced by surgical perturbations that result in post-operative morbidities such as small bowel obstruction. The cellular origin of adhesions remains unclear. Now, Tsai et al. show that mesothelial cells overlying organs and the abdominal wall give rise to adhesions after surgery in mice. The injured mesothelium upregulated mesothelial-specific genes that were known to be highly expressed during fetal development. Targeting adhesions with antibodies against the mesothelial marker mesothelin eliminated adhesions that had formed after surgery. Injured mesothelium responded to hypoxia under a HIF1A program. Blocking the HIF1α pathway with small molecule inhibitors prevented adhesion formation in mice after surgery.

Abstract

Peritoneal adhesions are fibrous tissues that tether organs to one another or to the peritoneal wall and are a significant cause of post-surgical and infectious morbidity. The primary molecular chain of events leading to the initiation of adhesions has been elusive, chiefly due to the lack of an identifiable cell of origin. Using clonal analysis and lineage tracing, we have identified injured surface mesothelium expressing podoplanin (PDPN) and mesothelin (MSLN) as a primary instigator of peritoneal adhesions after surgery in mice. We demonstrate that an anti-MSLN antibody diminished adhesion formation in a mouse model where adhesions were induced by surgical ligation to form ischemic buttons and subsequent surgical abrasion of the peritoneum. RNA sequencing and bioinformatics analyses of mouse mesothelial cells from injured mesothelium revealed aspects of the pathological mechanism of adhesion development and yielded several potential regulators of this process. Specifically, we show that $PDPN^{+}MSLN^{+}$ mesothelium responded to hypoxia by early upregulation of hypoxia inducible factor 1 alpha (HIF1α) that preceded adhesion development. Inhibition of HIF1α with small molecules ameliorated the injury program in damaged mesothelium and was sufficient to diminish adhesion severity in a mouse model. Analyses of human adhesion tissue suggested that similar surface markers and signaling pathways may contribute to surgical adhesions in human patients.

Introduction

Adhesions are fibrous tissues that develop after trauma to serosal membranes and surrounding cavities, which are likely to share a common pathology. Adhesions arise in response to instigations such as surgery, infection or dialysis. Their primary sequelae include small bowel obstruction, chronic pain, female infertility, poor quality of life, and death (*1–4*). The National Institutes of Health estimates that ~93% of abdominal surgeries lead to adhesions (*5*), with a ~20% rate of re-hospitalization for adhesion-related complications. The annual cost of surgery-related adhesions is over $1 billion in the United States (*1*, *3*, *5–8*). Although adhesions are a significant healthcare burden, few definitive strategies exist to prevent or treat adhesion formation.

Adhesions occur through an insult resulting in hypoxia and reactive oxygen species damage leading to inflammation and activation of the coagulation cascade, which results in the formation of a fibrin bridge between adjacent surfaces (*4*). Much is known about later stages of adhesion formation involving fibrin deposition and fibrinolysis (*9*), however the cellular and molecular details of the initial stages require further elucidation. It has been proposed that the frictionless surface of the mesothelium, the epithelial monolayer lining the peritoneal cavity and visceral organs (*9–12*), plays a protective role against adhesion formation (*13*), suggesting adhesion formation requires removal of the mesothelium. Exposed basement membrane is suspected to be the substrate for fibrin attachments between denuded surfaces, followed by fibroblast accumulation from sub-parenchymal sources (*14*, *15*). Other studies propose possible sources of adhesions are myofibroblast metaplasia (*16*), mesothelial-to-mesenchymal transition (MMT) (*17*), or extracellular matrix deposition by sub-peritoneal fibroblasts (*11*, *18–22*). As these

observations are largely dependent on histology, the early mechanisms continue to be debated. A cell-of-origin has not yet been definitively identified through lineage-tracing analyses. A few transcriptomic studies have characterized the gene expression changes leading to adhesion initiation (*23*, *24*), although it is unknown which cells sense the initial insults to initiate downstream pathology. Consequently, few effective treatments exist.

Here, we demonstrate by lineage tracing that an activated subset of mesothelial cells could be a cell-of-origin for adhesions. We identify mesothelin (MSLN) as a specific surface marker that is upregulated by mesothelial cells participating in adhesion formation and show that these cells are a necessary component of adhesion tissue in mouse and human. We found that administration of anti-MSLN antibodies diminished adhesions in a mouse model, and that this treatment was boosted by co-administration of antibodies against CD47 (*25*, *26*). We isolated and purified injured peritoneal mesothelium from mice using fluorescence activated cell sorting and performed RNA sequencing to identify molecular candidates for adhesion initiation. We show that peritoneal injury in a mouse model using surgical ligation and abrasion induced upregulation of the HIF1α signaling pathway and that this pathway activated reprogramming of the mesothelium. Disruption of the HIF1α pathway using small molecule inhibitors was sufficient to curtail adhesion formation *in vivo*.

Results

Mesothelial Cell Proliferation After Peritoneal Injury in Mice

To identify and prospectively isolate cells that could be responsible for adhesion development, we analyzed various established rodent injury models for reproducibility in adhesion severity and location. These animal models included those where adhesions were induced by intraperitoneal (ip) injections of chemicals, rough abrasion of the cecum and peritoneum (*27*, *28*), cauterization of the peritoneum and abdominal organs (*12*, *29*), and placement of ischemic buttons in the peritoneal wall (*17*, *30*–*33*). We found that the use of chemicals, cauterization, general or even localized abrasion were unpredictable in the reproducibility of adhesion formation. Instead, adhesion formation was systemic in these models, likely due to generalized inflammation.

We found that inducing formation of a small, single ischemic "button" in the peritoneal wall of mice by pulling up the peritoneal wall with forceps and ligating its base, resulted in adhesions between the button and adjacent tissue, with little morbidity (**Fig 1A**). This method was reproducible: adhesions sites were consistently in the same place, making it easier to pinpoint the locations of cells likely to give rise to adhesions. This method was physiologically relevant as the button simulated micro-pockets of ischemia that often form in response to surgical ties. We found that an optional, gentle abrasion of the ischemic button and intestinal surfaces could controllably increase adhesion severity without increasing morbidity in the mice.

Following surgery, mice were allowed to recover and analyzed at 0.5, 1, 2, 4, 24, 72 hours, and 7 days after induction of the ischemic button. Buttons were removed and stained with hematoxylin and eosin (H&E) at 30 minutes, 1 hour, 2 hours, and 4 hours

post-adhesion induction (**Fig 1B**). To confirm mesothelial cells were present following button induction, buttons were stained with antibodies against the mesothelial-specific markers podoplanin (PDPN) and keratin 19 (K19). Although other studies have reported PDPN expression on injured fibroblasts (*34*, *35*), we only observed PDPN expression in cells co-labeled with K19 on the surface monolayer of the buttons at 0, 6, 12, and 24 hours following button induction (**Fig 1C, Fig S1**) and on adjacent tissues (**Fig S2**) suggesting that $PDPN^+$ cells were mesothelial. Confocal microscopy of peritoneal wall tissue confirmed that PDPN expression remained specific to mesothelium during injury, as compared to smooth muscle actin (SMA) the expression of which was characteristic of fibroblasts (**Fig S3, A, B**).

We found that mesothelin (MSLN), a marker highly expressed on fetal peritoneal mesothelium, with low expression in adult mesothelium (*23*), was highly upregulated by the mesothelium at all time points after injury in the mice (**Fig S3, C, D, S4**). Intact cellular layers were visible on ischemic buttons with and without abrasion at post-operative time-points (**Fig 1C**), indicating that the mesothelium was neither denuded, nor had retracted following this injury. At some time points e.g. at 12 hours post injury, mesothelial cells took on an "activated" phenotype, characterized by loosening of intercellular connections (**Fig S2**). The mesothelium proliferated at four hours after injury as evidenced by its transition from a single cell layer to a multicellular layer (**Fig S5A).** By 24 hours after injury, the mesothelium showed further signs of thickening (**Fig 1C, S5B**) forming full adhesions after 7 days (**Fig S5C**). Trichrome staining and additional immunohistochemistry using antibodies against PDPN and MSLN were performed and

showed that adhesions contained cells that stained with either PDPN or MSLN (**Fig S6**, **Fig 1D**), suggesting that they may be locally derived from peritoneal mesothelium.

To investigate whether mouse adhesion tissue was derived from local surface mesothelium or circulating cells, we subjected C57BL/6J wildtype mice and C57BL/Ka Rosa26^{mRFP1} mice (*36*) that constitutively expressed red fluorescent protein in all cells to parabiosis where both mouse circulatory systems were connected (*37*, *38*). By 14 days the blood of both mice was chimeric (**Fig S7**). Adhesions induced in the non-fluorescent C57BL/6J wildtype mouse that underwent parabiosis showed little evidence of contributions from circulating cells. The majority of the PDPN^{+} cells in the adhesions were not fluorescent, although infiltrating blood or tissue myelomonocytic cells were observed (**Fig S8**) as evidenced by their co-expression of F4/80 stain.

Mice were injected with 0.025mg of 5-ethynyl-2'-deoxyuridine (EdU) intraperitoneally after adhesion induction and allowed to recover for 7 days; then their adhesions were stained for EdU, PDPN, and MSLN. Mesothelial cells (PDPN^{+} or MSLN^{+}) were counted in multiple (n=20) high power fields (0.75mm x 1mm) in control mice, and in mice following adhesion induction (n=16). Normal undamaged mesothelium showed rare mesothelial cell proliferation. However, there were marked foci of EdU^{+} MSLN^{+} mesothelial cells in areas of the mouse peritoneum where adhesions were present, and around regions of thickening (**Fig S9**). This suggested that mesothelial cell proliferation contributed to adhesion development in mice receiving ischemic buttons (**Fig 1E, 1F**).

To study the ultrastructural changes in the mesothelial cells after button induction, we performed conventional and large block-face scanning electron microscopy on

uninjured peritoneum and peritoneum on ischemic buttons 30 minutes, 2, 4, 6, 12, and 24 hours post button induction (**Fig S10-12**). These images confirmed the presence of mesothelial cells in the adhesion tissue and the basement membrane underneath the mesothelium, and suggested that within one hour after injury some mesothelial cells detached from their basement membranes, separated from neighboring cells and extended ciliary protrusions into the peritoneal space.

In Vivo and *In Vitro* Lineage Tracing of Activated Mesothelial Cells from Mouse Adhesions

We developed a lineage-tracing assay in which a cell-permeable dye, 5(6)-Carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE), was injected directly onto the surgically induced buttons to label mesothelial cells in mice. Buttons from mice treated with 10uM CFSE became fluorescent within 30 minutes (**Fig 2A**). Staining for PDPN and K19 confirmed that no CFSE labeling occurred in underlying tissue (**Fig 2B, Fig S13**), indicating that the CFSE dye specifically labeled surface mesothelial cells and did not cross the mesothelial basement membrane. Mice undergoing ischemic button placement and treated with CFSE were allowed to recover for 4-7 days. Fluorescence imaging confirmed CFSE staining within adhesions (**Fig 2C-E**) that co-localized with PDPN staining (**Fig 2F,G; Fig S14**), indicating that labeled mesothelial cells contributed to the adhesions formed after button induction.

We then crossed $Actin^{CreER}$ mice with $R26^{VT2/GK3}$ Rainbow mice (*37*, *39*, *40*) carrying a multicolored fluorescent reporter. Following low dose tamoxifen administration and Cre induction, the Rainbow reporter was activated in a very low number of cells, with each cell randomly and permanently expressing one color. We and others have found that tracing many cells simultaneously makes it difficult to determine clonality and morphological outcomes (*39*, *40*). Therefore, low dose tamoxifen studies were chosen to elucidate lineage outcomes of single cells. Adhesion induction was performed on $Actin^{CreER}$; $R26^{VT2/GK3}$ offspring and lineage-tracing was conducted on adhesions *in vivo* for seven days. Clusters (clones) of $PDPN^{+}$ cells of the same color were found within adhesion sites (**Fig 2H**) suggesting adjacent cells were derived from single

precursor cells. Some $PDPN^+$ mesothelial cells exhibited spindle-like morphology, suggesting a potential transition to a fibroblast phenotype (**Fig 2I**, **S15**).

Mesothelial explants from the kidney capsule and intestines were excised from uninjured control mice and cultured for 14 days. Cells expanded outward from the explant and stained positive for PDPN and K19 (**Fig S16**) and many $PDPN^+K19^+$ cells showed spindle-like phenotypes that were different from their original cobblestone appearance. These *in vitro* experiments suggested that adhesions may result from a transition or differentiation of epithelial-like mesothelial cells into spindle-like fibrocytic cells (**Fig S16**).

Targeting Mesothelin in Peritoneal Adhesions in Mice

To determine whether $PDPN^{+}MSLN^{+}$ mesothelial cells were a necessary component of adhesions, we depleted these cells from pre-formed adhesions in mice. Mouse monoclonal anti-MSLN antibodies were injected intraperitoneally immediately after adhesion induction (**Fig 3A**) and were found to bind only to damaged areas. Light sheet microscopy of injured mouse peritoneum revealed emerging foci of MSLN expression surrounding injury sites (**Fig 3A, 3B**, **Fig S17, Videos S1, S2**). MSLN expression was specific to injured areas surrounding the ischemic button, leaving adjacent areas unlabeled (**Fig 3B, Fig S17,** controls in **Fig S18**); antibody binding was absent in deeper tissues.

Damaged mesothelium also expressed CD47 (**Fig 3C**), a surface molecule highly upregulated on tumors that blocks macrophage-mediated phagocytosis (*26*). Blocking CD47 with anti-CD47 antibody has been shown to eliminate many human tumors in immunodeficient mice by enhancing phagocytosis of tumor cells (*25*, *26*, *41*, *42*). A human mesothelial cell line (MeT5A) was treated with anti-CD47 antibody *in vitro* and showed increased phagocytosis by macrophages (**Fig 3D, E**) compared to the MeT5A human mesothelial cell line treated with control IgG antibody.

In order to interrogate the potential therapeutic effect of antibody treatments *in vivo*, we required a method to evaluate adhesion severity. Previous studies characterized adhesions by focusing on strength (mechanical difficulty in separating adhered tissue) or the number of organs to which they adhered in order to score adhesion severity (*29, 43, 44*). We developed a method to evaluate adhesion severity based on histological criteria and adhered surface area (**Fig S19**).

Anti-CD47 antibody (200ug) alone, anti-MSLN antibody (200ug) alone, or a combined injection of both antibodies was administered 7, 10, and 13 days following adhesion induction in mice. A decrease in adhesion burden was observed using anti-MSLN antibody alone and a greater reduction was observed using a combination of anti-MSLN antibody and anti-CD47 antibody (**Fig 3G, H**) compared to vehicle controls (**Fig 3F, H**). Immunohistological analysis of the ischemic buttons resected from the antibody-treated mice confirmed the absence of $MSLN^+$ cells (**Fig 3I)** suggesting that mesothelial cells had been removed. This $MSLN^+$ subset may be a necessary component of adhesion tissue and potentially could contribute to adhesion formation. Collagen and fibronectin were still detected on the resected buttons suggesting that $MSLN^+$ cell removal was sufficient to diminish adhesion burden, despite residual extracellular matrix components. Interestingly, immunofluorescence staining for K19 and PDPN (**Fig S20**) and H&E staining (**Fig S21**) showed the persistence of mesothelium on the buttons.

RNA Sequencing of Mouse Mesothelial Cells in the Early Stages of Adhesion Formation

To determine changes in gene expression in $PDPN^+MSLN^+$ cells during adhesion formation, we isolated $PDPN^+MSLN^+$ cells using FACS from sites of injury at 6, 12, and 24 hours following adhesion formation after button induction. Our lineage gating scheme included mesothelial markers and excluded blood cells, endothelial cells, and lymphatic cells. The cells selected by FACS were $PDPN^+LYVE1^-CD31^-CD45^-$ (**Fig 4A**), and we performed RNA sequencing on this population. Our purification protocol included ischemic buttons to enrich for activated mesothelium. Peritoneal mesothelial cells were similarly isolated from mice that did not undergo surgery (t=0). Differentially expressed genes were clustered based on expression patterns across the 24 hour time course and then were analyzed for geneset activity (*45–47*) to elucidate early transcript changes occurring in the first 24 hours after adhesion formation (**Fig 4B-E).**

Differentially expressed genes broadly clustered into six expression patterns, peaking or dipping at 6, 12, and 24 hours after button induction (**Fig 4B**). Fold changes in transcript expression after 6, 12, and 24 hours were plotted against total gene expression (**Fig 4C**). Differentially expressed genes were calculated based on a $q < 0.05$ threshold and ordered by fold change. At 24 hours post injury, ~8000 genes were significantly ($q < 0.05$) differentially expressed compared to control mice that did not undergo surgery **(Fig 4C)**.

We compared the number of differentially expressed genes in mesothelial cells between injured and control mice 24 hours after button induction to genes expressed during hematopoietic stem cell (HSC) differentiation. Transcriptional changes in

activated mesothelial cells were greater than those found among HSCs, macrophages and B cells. Transcriptional changes in activated mesothelial cells were greater than those found in HSCs undergoing differentiation to multipotent progenitors, and in activated T cells compared to resting $CD69^-$ T cells. Transcriptional changes in activated mesothelial cells were greater than those found in acute myeloid leukemia (AML) cells compared to AML blast cells (**Fig 4D**). Altogether this suggested extensive reprogramming in the mesothelium after button induction and injury.

Geneset activity analysis (**Fig 4E**) showed early upregulation of genes involved in angiogenesis and hypoxia, followed by expression of genes involved in the inflammatory response encoding chemokines, chemotactic factors, cytokines, and NF-kappa-B pathway components (**Fig 4E**). Genes encoding proliferation factors were upregulated 24 hours after button induction, corroborating data from our pulse-chase experiments. Genesets associated with extracellular matrix (ECM) formation were downregulated within 24 hours after injury, including fibronectin 1 (FN1), and collagens (COL1A1, COL1A2, COL3A1) (**Fig S22**). The *HIF1A* gene was highly upregulated within 6 hours of adhesion formation, indicating that sensing of hypoxia in the mesothelium may contribute to adhesion initiation. The tumor growth factor (TGF) signaling pathway, which is thought to contribute to adhesion formation, was downregulated in the mesothelium 24 hours after surgery (**Fig 4E**).

We observed expression of the mesothelial specific markers PDPN and K19 at all time points after adhesion initiation in mice (**Fig 5A**). Many markers, which are highly expressed by peritoneal mesothelium during mouse fetal development with low expression in the uninjured adult, were upregulated after injury. These included MSLN,

which was upregulated 80-fold, and uroplakin1B (UPK1B) and Wilm's Tumor antigen 1 (WT1), which peaked at 24 hours after adhesion initiation (**Fig 5A**). Genes associated with fibroblasts, specifically S100A4 (FSP1), E-cadherin (CDH1), and smooth muscle actin (ACTA2) were not expressed in uninjured mesothelium (t = 0), but were upregulated after injury (**Fig 5A**). Adhesion molecules also were upregulated, indicating cell-to-cell contact and cellular migration were important to adhesion initiation (**Fig S22**). These RNA sequencing data show that injured mesothelial cells undergo a massive genomewide transcriptional rearrangement involving a mesothelial program with an increased fibrogenic composition.

We validated our RNA sequencing screen using immunohistochemical staining of proteins encoded by gene targets in mouse adhesions (**Fig 5B, S23B**). There was strong protein staining detected by immunofluorescence and RNA expression detected by *in situ* hybridization for the target genes, demonstrating that the RNA sequencing data were recapitulated *in vivo*. Adhesions did not stain positive for *HIF1A* expression at later stages after injury initiation; this transcription factor was upregulated within 6 hours of injury and downregulated soon thereafter. Injured mesothelium broadly expressed S100A4, MSLN, and K19, whereas WT1 was restricted to a subset of injured mesothelium, suggesting heterogeneity within adhesions.

Effect of HIF1α Blockade on Adhesion Formation in Mice

Hypoxia has been shown to be an instigator of adhesion formation (*15*) although which cells sense hypoxia and their contribution to adhesion formation has not been well characterized. We found that mesothelial *HIF1A* gene expression was upregulated early during adhesion formation before decreasing shortly thereafter (**Fig 5A**). It is established that the HIF1α transcription factor is regulated through stabilization and post-translational modification (*48*). We investigated protein partners of HIF1α known to increase HIF1α activity and found them to be upregulated at similar time points during adhesion development (**Fig S24**).

We analyzed the potential effects that HIF1α could exert on surface mesothelium *in vitro.* Primary mouse mesothelial cells that were cultured under low oxygen conditions (5% O_2 or with 100uM $CoCl_2$) for three days showed little morphological difference from mesothelial cells cultured under normoxic conditions (**Fig S25**). Mesothelium co-cultured with macrophages, which are constitutively present in the peritoneum, developed dense fibrotic foci that stained positive for PDPN and HIF1α (**Fig 6A, B**) suggesting that macrophages may contribute to mesothelial transformation.

In vivo analysis of the HIF1α signaling pathway was performed using small molecule inhibitors of HIF1α, specifically cryptotanshinone (inhibits HIF1α activation) (*49*), FM19G11 (represses alpha-subunit targets) (*50*)), echinomycin (blocks DNA binding of HIF1α) (*51*), and PX-12 (blocks transcriptional activity of HIF1α) (*52*). Mice underwent adhesion induction and were treated with cryptotanshinone (200mg/kg), FM19G11 (2mg/kg), echinomycin (10ug/kg or 20ug/kg), or PX12 (25mg/kg) immediately after injury, 4 hours after injury, and every 24 hours for 7 days. Mice were

euthanized after 7 days and adhesions were imaged and scored. H&E staining showed an intact layer of $MSLN^+$ mesothelium (**Fig 6C**). Adhesion burden was significantly ($p < 0.0001$) reduced (**Fig 6D**) in mice treated with PX12 (n = 7) or echinomycin (n=17), with many mice showing no observable adhesions (score 0) suggesting that HIF1α blockade was sufficient to prevent adhesion formation. Treatment with HIF1α inhibitors did not affect wound healing; all mice appeared healthy and abdominal wall closure was complete after 7 days similar to the situation observed for untreated control animals that underwent injury.

We injected EdU as previously described into mice that had undergone button induction and adhesion initiation and had been treated with echinomycin (20ug/kg) (n = 5). Mice were analyzed 7 days after EdU injection and were found to have reduced EdU incorporation into $PDPN^+MSLN^+$ cells (**Fig 6E**) compared to untreated injured control animals pulsed with EdU, suggesting that HIF1α inhibition decreased mesothelial cell proliferation. Mesothelial cells of echinomycin-treated mice exhibited both activated (**Fig S26A**) and normal uninjured phenotypes (**Fig S26B**), suggesting that HIF1α reduced cell proliferation but not cell activation and adhesion formation .

Transcriptional Changes in Mesothelial Cells Treated with HIF1α Inhibitors

To determine the transcriptional changes downstream of HIF1α, 10 mice were treated with echinomycin (20ug/kg) prior to and immediately following induction of four ischemic buttons in the peritoneum of each mouse. Damaged mesothelium at 24 hours post button induction was isolated from the buttons and RNA extracted for sequencing. Known HIF1α targets VEGFβ and transferrin (TRF) were downregulated after echinomycin treatment (**Fig S27**). Expression of PCNA and the ORC family of genes was analyzed (**Fig S28A, B**) (*53–55*) to confirm that echinomycin did not inhibit DNA replication in our purified mesothelial cell subset.

RNA sequencing analysis revealed that ~200 genes upregulated 24 hours post injury showed decreased expression after HIF1α inhibition. More than 600 genes with decreased expression 24 hours after injury were upregulated in response to HIF1α inhibition (**Fig 6F**). Some genes associated with adhesion formation were unaffected (ITGAM, ITGB1, and ITGB2) (**Fig S26**). Expression of PDPN, MSLN, and S100A4 increased after treatment with echinomycin and injury, whereas expression of UPK1B and WT1 decreased (**Fig 6F**) suggesting that WT1 and UPK1B may be downstream targets of HIF1α and could play a role in adhesion formation.

Upregulation of Mesothelin in Human Peritoneal Adhesions

To determine whether the potential mechanism of adhesion formation and target gene expression observed in mice was similar in human tissue, human adhesion samples were obtained from patients requiring abdominal surgeries (n=6). Samples were fixed, sectioned, and stained with H&E and trichrome. The human adhesions were filled with cells and showed collagen deposition (**Fig 7A**). Human adhesion samples stained positive for expression of MSLN, PDPN, CD47, and S100A4 by immunofluorescence and UPK1b expression by *in situ* hybridization (**Fig 7B**), demonstrating that many target genes expressed in mouse adhesions were similarly expressed in human adhesions.

Discussion

The cellular origins of peritoneal adhesions and early molecular events are unclear, largely because the cell-of-origin has not been identified. Studies have suggested that adhesions originate from stromal, sub-mesothelial, or mesothelial cells through a mesothelial-to-mesenchymal transition (*17*, *56*). Here, we employ multiple lineage-tracing approaches and propose that the mesothelium contributes to and is incorporated into adhesions due to its proliferation outwards into the peritoneal cavity. We show that preventative or post-adhesion treatment targeting these mesothelial cells results in reduction of adhesion severity in mice.

Notably, despite our finding that the mesothelium is a contributor to peritoneal adhesions, it is likely that other cell types play a role. Although carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) stained only surface mesothelial cells, there was marked heterogeneity within this surface cell population that was difficult to elucidate using only immunostaining. Future studies will need to focus on specifically labeled subsets of these cell populations within the surface mesothelium, to carefully trace individual cellular contributions to adhesions. At the same time, the scope of our study was limited only to the mesothelium. Our lineage tracing and subsequent FACS based RNA sequencing analyses were designed to gate out all other cell populations that may play a role in adhesion formation. Therefore, our study cannot exclude that other cells such as sub-mesothelial fibroblasts also contribute to adhesions. Further studies need to be done to specifically label these cells genetically or chemically and then to trace these cells after adhesion induction surgery in order to document their contributions to adhesion formation.

We propose a model of early adhesion formation (**Fig S29**) where after injury the peritoneal mesothelium responds by induction of genes responsible for cell proliferation and differentiation. This enables separation of the mesothelium from its basement membrane, likely through the downregulation of collagens and other ECM proteins, and enables the mesothelium to move into the peritoneal space. For simplicity, our model omits interactions between mesothelial cells and other cell types (fibroblasts, hematopoietic cells) that likely also contribute to adhesions. Specifically, many fibroblasts often share similar mesothelial markers when activated (*34*, *35*). It is also likely that activated mesothelium recruits leukocytes, as evidenced by an increase in chemokines and cytokines (**Fig 4E**), and initiates coagulation events. The interplay between inflammatory and mesothelial cells will be important to elucidate in future studies.

The RNA sequencing studies presented here reveal marked early gene expression changes occurring within the first 24 hours after adhesion formation. Injured mesothelium upregulated expression of fibroblast genes S100A4, CDH2, and ACTA2, which together with *in vivo and in vitro* staining suggest that the mesothelial cells become fibroblast-like cells as previously described (*17*) but migrate into the peritoneal cavity rather than into the abdominal organs (*23*). Surprisingly, our results show *TGFB* downregulation in PDPN+MSLN+ cells suggesting that TGFβ plays a role later in adhesion formation or is expressed by other cell types.

The $MSLN^+$ cells in the post-injury surface mesothelium involved in adhesion formation were heterogeneous by several gene expression criteria. It is plausible that there was a further adhesion-prone subset of cells within the $MSLN^+PDPN^+$

mesothelium. Whether different mesothelial cell subsets share common embryonic origins or represent separate mesothelial cell types as recently revealed in dermal mesenchyme (*24*) remains to be studied.

The mesothelium likely senses hypoxia and responds through HIF1α upregulation. Disruption of the HIF1α pathway by small molecule inhibitors was sufficient to induce a decrease in adhesion formation in mice, implicating a role for HIF1α in the pathogenesis of adhesion formation. Our findings implicate WT1 as a downstream target of HIF1α and a likely candidate in further regulating adhesion formation.

Many mesothelial genes were highly upregulated during peritoneal injury in mice and had low or no expression in most other tissues, making them potential targets for treatments to reverse adhesions. We identified adhesion-specific targets and then showed that an antibody against these targets could be used to treat adhesions in a mouse model. Furthermore, many malignancies, such as mesotheliomas, pancreatic ductal adenocarcinoma, ovarian cancer, and some lung cancers, express MSLN (*57–60*). Our finding that targeting MSLN enhanced phagocytosis of MSLN positive-mesothelium further suggests that certain cancers may be susceptible to a combined anti-MSLN/anti-CD47 immunotherapy although this remains to be tested.

Materials and Methods

Study Design

The objective of this study was to determine if the mesothelium contributed to the formation of adhesion tissue in a mouse model. Further objectives assessed whether small molecule inhibitors of HIF1α could prevent adhesion formation and if antibodies against two markers expressed by mesothelial cells, MSLN and CD47, could treat pre-formed adhesions in mice. In all treatment studies, each experiment used 5 mice that received treatment and 5 untreated control mice. All *in vivo* experiments were repeated at least twice; many were repeated up to six times to ensure statistical power and adequate sample sizes (see individual experiments for n). Mice were excluded only due to morbidity within 5 days of adhesion induction. Blinding was not performed. Early endpoints were taken if mice were found to be morbid. All outliers were reported and included in statistical analyses.

Adhesion Induction

All animal experiments were carried out in strict accordance with the guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Stanford University's Administrative Panel on Laboratory Animal Care (APLAC), (Protocol number #10266) and in the United States, or the European Animal Welfare Act, Directive 2010/63/EU. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Government of Bavaria, Germany, and received the permission No: 55.2-1-54-2532-150-2015. All surgery was

performed under anesthesia, and all efforts were made to minimize suffering. Adhesion induction surgeries were done on wild type B6 (C57BL/6J (The Jackson Laboratory) mice aged 6-10 weeks. Mice were anesthetized by inhaled isoflurane until determined unconscious confirmed by toe-pinch test. The abdomen was disinfected with betadine and phosphate buffered saline (PBS). A left mid-clavicular incision was made in the skin running down the length of the mouse. A similar left-mid clavicular incision was made in the peritoneum running down the length of the peritoneum. The peritoneum was gently folded to the right and held down by a hemostat. A single, ischemic button was placed on the right half of the peritoneal wall by clamping a small (~5mm diameter) piece of peritoneum with a hemostat and ligating the base with a 4-0 silk suture (Ethicon, 683G) twice before the clamp was released. Light abrasion on the button (20 times) and on the adjacent liver, cecum, and small and large bowels (7 times) was optionally performed (depending on the desired adhesion severity) with a surgical brush. Light brushing with fewer repetitions was performed to avoid pinpoint bleeding. The peritoneum was closed using 4-0 silk sutures and the skin was stapled closed (EZ Clips, 9mm, Braintree Scientific Inc). Mice were allowed to recover on a heating pad and injected with 0.05-0.1 mg/kg of buprenorphine. Mice were followed closely and monitored daily for signs of morbidity for 7 days until euthanasia. Adhesed tissues were dissected, scored, and fixed in 2% paraformaldehyde overnight at 4 degrees.

Adhesion Scoring

We assigned a single score for an adhesion, taking into consideration both surface area contact and molecular makeup as described in the text. In our scheme, an adhesion with a

519 score of 0 has no adhesion between two areas, with limited mesothelial thickening on the
520 button (**Fig S18A**). The area stains positive for MSLN and fibronectin (**Fig S18A**). Mice
521 with an adhesion score of 0 had no signs of morbidity and survived the 7 day recovery
522 period (n=25).

523 An adhesion score of 1 indicated a "string" adhesion, connecting the two adhesed
524 areas with a light fibrous bridge (**Fig S18B**). The string and surrounding areas were
525 immunopositive for MSLN and fibronectin (**Fig S18B**). Most mice with an adhesion
526 score of 1 had no signs of morbidity and survived the 7 day recovery period (n=11).

527 All adhesions with scores of 2 or above involve a single direct contact between
528 two tissues. The adhesion contact itself was light, and usually involved contact between
529 the peritoneum and an abdominal organ (**Fig S18C**). The adhesion in between the two
530 adhesed areas stained positive for MSLN, fibronectin, and F4/80, suggesting some
531 macrophage infiltration has occurred (**Fig S18C**). Most mice with an adhesion score of 2
532 had no signs of morbidity and survived the 7 day recovery period (n=12).

533 An adhesion score of 3 was characterized by direct, continuous contact of three
534 areas, usually between the peritoneum and two abdominal organs or two distinct, non-
535 continuous areas of a single organ (**Fig S18D**). The adhesed area stained positive for
536 MSLN, fibronectin, F4/80, pan collagen, and CD31 (**Fig S18D**). Most mice with an
537 adhesion score of 3 had little signs of morbidity and survived the 7 day recovery period
538 (n=9).

539 An adhesion score of 4 included direct and continuous contact of four or more
540 areas, usually between the peritoneum and three abdominal organs, or multiple separate
541 areas of one or two organs (**Fig S18E**). The adhesed area stained positive for MSLN,

fibronectin, F4/80, pan collagen, and CD31 (**Fig S18E**). Most mice with an adhesion score of 4 showed little signs of morbidity and survived the 7-day recovery period (n=14).

An adhesion score of 5 was characterized as full compaction/encapsulation of the abdominal organs. Most organs were adhesed to the peritoneum as well as to each other as a single, rigid mass (**Fig S18F)**. The adhesed areas stained positive for MSLN, fibronectin, F4/80, collagen, and CD31 (**Fig S18F**). Most mice with an adhesion score of 5 were visibly morbid with low survival rates at 7 day post injury (n=5).

It should be noted that the amount of surface area contact does not always dictate molecular phenotype and vice versa, as the severity of the adhesion likely exists on a continuum represented by our discrete criteria described above. We have observed adhesion areas with high surface area contact (a score of 3 or 4) with little or no collagen or macrophage involvement ($F4/80^-$). Conversely, we have also observed adhesion areas with low surface area contact (a score of 2) with high collagen and macrophage infiltration. In these cases we scored adhesions based on surface area contact or number of organs involved, as we predict this is a more significant indicator of clinical outcome.

Parabiosis

Parabiosis surgeries were done on age matched (4-6 week old) female wild type B6 (C57BL/6J (The Jackson Laboratory) and C57BL/Ka Rosa26 mRFP1 mice. Mice to undergo parabiosis were housed together in a single cage for 10 days prior to surgery. Mice were anesthetized by inhaled isoflurane until determined unconscious confirmed by toe-pinch test. The sides of the mice were shaved and cleaned with 70% ethanol and

betadine. Mice were laid next to each other and incisions from elbow to knee were made on adjacent sides. The elbow and knee joints were ligated using 4-0 sutures (Ethicon) and the loose skin from adjacent mice was stapled together. Mice were allowed to recover on a heating pad and injected with 0.05-0.1 mg/kg of buprenorphine. Mice were followed closely and monitored daily for signs of morbidity for 14 days. Staples were removed following 14 days and mice were bled retro-orbitally to assay for chimerism.

Histology

Tissues were fixed in 2% paraformaldehyde overnight at four degree and were embedded and frozen in optimal cutting temperature compound O.C.T (Sakura) or embedded in paraffin. Frozen sections were cut at 10-12um throughout the adhesed organs and saved for immunofluorescence. Paraffin sections were cut at 5um and hematoxylin/eosin and Masson's trichome stains were performed via standard protocols.

Immunofluorescence

Immunofluorescence studies were performed on frozen sections. Frozen sections were thawed at room temperature for ten minutes and washed in PBS twice. Slides were blocked in 5% serum for 30 minutes at room temperature. Sections were subsequently stained with primary antibodies against PDPN (1:100, hamster monoclonal, Abcam), MSLN (1:200, rabbit monoclonal, ABBIOTEC), fibronectin (1:100, rabbit monoclonal, Abcam), F4/80 (1:100, rat monoclonal, Abcam), CD31 (1:100, rabbit monoclonal, Abcam), pan-collagen (1:100, rabbit polyclonal, Abcam), WT1 (1:100, rabbit polyclonal, Abcam), HIF1α (1:100, mouse monoclonal, Abcam), S100A4 (1:100, rabbit polyclonal,

Abcam), and CK19 (1:100, rabbit monoclonal, Abcam, EP1580Y and 1:100, rabbit polyclonal, Abcam). overnight at 4 degrees, and washed three times in PBS. Slides were stained were incubated with secondary antibodies conjugated to Alex Fluor 488, 594, or 647 for one to two hours at room temperature. Stains were washed once with PBST and three times with PBS before nuclear staining with Hoechst 33342 (Life Technologies), for two minutes and mounted with Fluoromount G (Southern Biotech).

In Situ Hybridization

Tissues were fixed in 10% (vol/vol) neutral buffered formalin at room temperature for 24h-32h, dehydrated, and embedded in paraffin. Tissue sections cut at 5um thickness were processed for RNA in situ detection using the RNAscope 2.5 HD Assay-RED according to the manufacturer's instructions (Advanced Cell Diagnostics, Reference#). RNAscope probes used were Mm-Upk1b (NM_178924.4, 46-966) and Hs-UPK1B (NM_006952.3).

EdU Pulse-Chase

Following adhesion induction, mice were immediately injected subcutaneously with 0.025mg of 5-ethynyl-2'-deoxyuridine (Life Technologies) in 90% PBS and 10% ethanol. Mice were traced for 7 days and euthanized. Adhesed tissues were dissected and fixed with 2% paraformaldehyde overnight, frozen in O.C.T (Sakura), and sectioned at 12um. EdU positive cells were visualized with Click-iT EdU Imaging Kit (Life Technologies).

Lineage Tracing

Adhesion induction surgeries were performed on wild type mice as previously described. 600ul – 1000ul of CFSE (ThermoScientific) was pipetted on top of the button and into the peritoneal cavity. Mice were closed and allowed to recover for 4-7 days.

Clonal Analysis

Adhesion induction surgeries were performed on $Actin^{CreER}$; $R26^{VT2/GK3}$ mice as previously described(*36*). 1mg/kg of 4-hydroxy tamoxifen was added on top of the button. Mice were closed and allowed to recover for 7 days. *In vitro* clonal analyses were performed by explanting mesothelium from the renal capsule of $Actin^{CreER}$; $R26^{VT2/GK3}$ mice and culturing them as previously described(*23*). Explants were treated with 8mM 4-hydroxyl tamoxifen for 14 days and imaged for clonality.

Flow Cytometry

A modified adhesion induction surgery was done according to previously described procedures on wild type C57BL/6J (The Jackson Laboratory). To obtain adequate amounts of mesothelial cells, four buttons were established per mouse (two on each side of the peritoneal wall). Twenty buttons were taken for a single triplicate (n=5 mice per triplicate), and three cohorts of littermate mice were sacrificed at 6, 12, or 24 hours post injury. No abrasion of the button or abdominal organs was done. Mice were allowed to recover for 6, 12, or 24 hours and euthanized. Ischemic buttons were obtained by cutting the base and placed in dissociation media (DMEM (Life Technologies, 10565-042), 50mg/ml collagenase IV (Worthington Biochemical), 20uM $CaCl_2$) and dissected further using a single edge razor, and incubated in dissociation media for 30 minutes at 37C. The

subsequent cell suspension was filtered through a 100um filter and spun and washed with 2% fetal bovine serum (FBS) in PBS. Cells were treated with 1ml of ACK lysis buffer (Life Technologies) for 5 minutes at 4C and spun and washed. Cells were blocked with 1% goat serum (Life Technologies) for 10 minutes and stained with anti-PDPN (BioLegend, 8.1.1, 1:100), anti-LYVE-1 (eBioscience, ALY-7, 1:100), anti-CD31 (eBioscience, 390, 1:100), and anti-CD45 (BioLegend, 30-F11, 1:100) for 30 minutes at 4C. Cells were spun down, filtered, and resuspended in 200ul of 2% FBS in PBS. Cells were sorted using a FACSAria (BD Bioscences); $PDPN^{+}LYVE1^{-}CD31^{-}CD45^{-}$ cells were sorted directly into 750ul of Trizol LS (Life Technologies).

RNA Sequencing and Analysis

Total RNA from sorted mesothelial population was isolated using Trizol (ThermoFisher) as per manufacturer's recommendation and was facilitated by addition of linear polyacrylamide (Sigma) as a carrier during RNA precipitation. Purified total RNA was treated with 4 units of RQ1 RNase-free DNase (Promega) at 37 °C for 1 hour to remove trace amounts of genomic DNA. The DNase-treated total RNA was cleaned-up using RNeasy micro kit (QIAGEN). 10-50 ng of total RNA was used as input for cDNA preparation and amplification using Ovation RNA-Seq System V2 (NuGEN). Amplified cDNA was sheared using Covaris S2 (Covaris) using the following settings: total volume 120 ml, duty cycle 10%, intensity 5, cycle/burst 100, total time 2 min. The sheared cDNA was cleaned up using Agencourt Ampure XP (Beckman Coulter) to obtain cDNA fragments >= 400 base pairs (bp). 500 ng of sheared and size-selected cDNA were used as input for library preparation using NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs) as per manufacturer's recommendations. Resulting libraries

(fragment distribution: 300-700 bp; peak 500-550 bp) were sequenced using HiSeq 4000 (Illumina) to obtain 2x150 base pair paired-end reads. The reads obtained were trimmed for base call quality and the presence of adapter sequences using Skewer(*61*). High quality reads thus obtained were aligned to mouse genome using OLego(*62*) and the quantity of expressed mRNAs were estimated using cuffdiff2(*63*) and represented as fragments per kilo-base per million mapped reads (FPKM).

Gene expression intensities of each data point as well as results of pair-wise comparison by Cuffdiff are further analyzed and visualized by Gene Expression Commons (https://gexc.riken.jp)(*45*). To find similar expression pattern genes, gene expression intensities in FPKM at four time points are first standardized into z-values for each gene, then correlations to a gene of interest (e.g., Hif1a) were computed against entire genes by Pearson product-moment correlation coefficient. Genes with p-value less than 0.05 are listed as similar expression pattern genes.

Differentially regulated genes by Cuffdiff pair-wise comparison were subjected to Geneset Activity Analysis. For each gene set defined by Gene Ontology(*46*), differentially regulated genes were divided into up-regulated genes and down-regulated genes on the one hand, and those that are in the gene set and not in the gene set on the other hand, then Fisher's exact test performed. Next, q-values (estimated FDR) were computed using qvalue R package provided by Storey lab(*47*). Geneset Activity is presented by color scale based on odds ratio and p-value. In pair-wise setting, gene set with odds ratio more than 1 is considered as "activated".

Antibody Treatments

Adhesions were induced in wild type B6 (C57BL/6J (The Jackson Laboratory) and allowed to recover for 7 days. 200ug of monoclonal anti-MSLN (B35) antibody were administered via intraperitoneal injections at 7, 10, and 13 days post surgery. 200ug of monoclonal anti-CD47 (MIAP301) (BioXCell) were co-administered via intraperitoneal injections at the same frequency. Mice were euthanized 17 days after initial surgery and scored for adhesion severity. B35 anti-MSLN antibody was a gift from A. Miyajima.

Whole-Mount Immunostaining and Tissue Clearing

Whole-Mount samples were stained and cleared with a modified 3DISCO protocol(*64*, *65*). In short, samples stored in PBS-GT (DPBS (Gibco, 14190094), 0.2% Gelatin solution (Sigma, G1393), 0.5% Triton X-100 (Sigma, X100), 0.01% Thimerosal (Sigma, T8784)) were incubated with primary antibodies (anti-PDPN (Abcam, ab11936), anti-MSLN (Abbiotec, 250519), anti-α-SMA (Abcam, ab5694)) in PBS-GT with shaking, for 36 hours at room temperature. Excessive antibody was removed by thorough washing in PBS-GT for 6-12 hours and refreshing the solution every 1-2 hours. Incubation with fluorophore-coupled secondary antibodies (Molecular Probes, A-21451 & A-21207) in PBS-GT for 36 hours was followed by thorough washing in PBS-GT as described above. Samples were then dehydrated in an ascending series of tetrahydrofuran (Sigma, 186562) (50%, 70%, 3x 100%; 60 minutes each), and subsequently cleared in dichloromethane (Sigma, 270997) for 30 min and eventually immersed in benzyl ether (Sigma, 108014). Cleared samples were imaged in 35 mm glass bottom dishes (Ibidi, 81218) using a laser scanning confocal microscope (Zeiss LSM710).

Multi-Photon microscopy

Immunostained and non-cleared samples were imaged using a Leica SP8 MP (Leica Microsystems) at the Imaging Core Facility of the Biomedical Center (LMU, Martinsried). In brief, samples we were embedded in 2% agarose (Lonza, 859081) in a 35mm-dish (Corning, 351008) and submersed in PBS (Gibco, 14190094). A 25x water dipping objective (HC IRAPO L 25x/1.00 W) was used in combination with a tunable laser (Spectra Physics, InSight DS+ Single), set to 1080nm and 1225nm excitation, respectively. Data from confocal and multi-photon microscopy was analysed using Imaris 9 (Bitplane, UK). Brightness and contrast was adjusted for better visualization.

Ultramicroscopy

Peritoneum was imaged with a LaVision BioTech UltraMicroscope (LaVision BioTech GmbH, Germany) equipped with a 2x objective lens and additional zoom optics (0.63x – 6.3x). Antibodies against MSLN (B35) were labeled with the IRDye 800CW fluorophore (Li-cor, Lincoln, NE) according to the manufacturer's instructions. 1mg of the antibody-conjugate in phosphate buffered saline (PBS) was injected intraperitoneally immediately after surgery and left for 30 minutes. Fluorescence signal was collected at 800 nm using 750 nm excitation and auto-fluorescence emission was collected at 690 nm using 650 nm excitation**.** The sample was scanned in 4 µm steps in z-direction. Fluorescence signal was collected at 800 nm using 750 nm excitation and autofluorescence emission was collected at 690 nm using 650 nm excitation. Data was further processed using Imaris 8.2.1 (Bitplane, UK). 3D rendering was done using the maximum intensity projection (MIP) or blend mode. 2D representations are maximum intensity projections of 36 µm thick sections from the 3D model.

In Vitro Mesothelial Culture

Wild type B6 (C57BL/6J (The Jackson Laboratory) were euthanized and the mesothelium was excised from the renal capsule and intestine. Excised mesothelium was cut into smaller fractions and placed into culture dishes pretreated with EmbryoMax 0.1% Gelatin Solution (EmdMillipore) for 30 minutes and cultured in Dulbecco's Modified Eagle Medium (Life Technologies) with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids at 37 degrees for 7 days. Mesothelial cells were co-cultured with macrophages (added to confluency) in normal conditions or in hypoxic conditions (5% O_2 incubator or 100uM $CoCl_2$).

Preparation of Primary Mouse Bone-marrow Derived Macrophages (BMDMs)

To prepare primary BMDMs, BALB/c mice were humanely euthanized, and disinfected with 70% ethanol. An incision was made along the legs, and the muscle removed from the bones. The femur and tibia were removed from the body, and rinsed in PBS. The bones were flushed with a 6 mL syringe and 23 gauge needle, and the marrow resuspended in 10 mL RPMI. The suspension was centrifuged 5 minutes, 1200 rpm, and the pellet resuspended in 5 mL ACK lysis buffer (Invitrogen) for 5 minutes to remove blood cells. The suspension was filtered over a 70 μm Falcon cell strainer, and centrifuged again. The pellet was resuspended in 40 mL of macrophage media (RPMI+10% FBS+10% penicillin/streptomycin+10 ng/mL MCSF), and plated on four 10 cm petri dishes. On day 4, the macrophage media was replaced. On day 7, the macrophages were lifted from the dish and used.

Preparation of Human Macrophages

Leukocyte reduction system (LRS) chambers were obtained from the Stanford Blood Center from anonymous donors, and peripheral blood mononuclear cells were enriched by density gradient centrifugation over Ficoll-Paque Premium (GE Healthcare). Four hundred million PBMC's were plated per 15cm dish (Corning), washed vigorously three times after a 30 minute period of adherence and then left to differentiate into macrophages by culture for 7-10 days in IMDM+GlutaMax (Invitrogen) supplemented with 10% AB-Human Serum (Gemini) and 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

Phagocytosis Assay

Phagocytosis assays were performed by co-culture of 50,000 macrophages with 100,000 human mesothelial cells (MeT5A) (ATCC) labeled with Calcein AM (ThermoScientific) and antibodies for two hours in serum-free medium, then analyzed using a Cytoflex cell analyzer with high-throughput sampler (Beckman Coulter). Antibodies used for treatment included: human IgG4 isotype control (Eureka Therapeutics) and anti-CD47 clone 5F9. Primary human macrophages were identified by flow cytometry using anti-CD45 antibodies (BioLegend). Dead cells were excluded from the analysis by staining with DAPI (Sigma). Phagocytosis was evaluated as the percentage of green macrophages using FlowJo v9.4.10 (Tree Star) and was normalized over the isotype control. Statistical significance was determined by two-tailed paired students t-test.

Small Molecule Inhibitor Treatments

Adhesion induction surgeries were performed on 4-8 week old wild type B6 (C57BL/6J (The Jackson Laboratory) as previously described. 200mg/kg of cryptotanshinone (Sigma Aldrich), 2mg/kg of FM19G11 (Sigma Aldrich), 10ug/kg or 20ug/kg of echinomycin (Sigma Aldrich), or 25mg/kg of PX12 (Sigma Aldrich) immediately after injury, 4 hours after injury, and every 24 hours subsequently for 7 days.

Scanning Electron Microscopy (SEM)

Conventional SEM: Tissue specimens (mesothelium 'buttons') were fixed overnight in 4% PFA with 2% Glutaraldehyde in 0.1M NaCacodylate Buffer (pH 7.3), postfixed in 1% OsO_4 (1hr) and dehydrated in a graded ethanol series (50, 70, 90, 100%, 10' each) before critical point drying with liquid CO2 in a Tousimis Autosamdri-815B apparatus (Tousimis, Rockville, MD), mounting on Aluminum SEM stubs (Electron Microscopy Sciences, Hatfield, PA), and sputter-coating with 50Å of gold-palladium using a Denton Desk II Sputter Coater (Denton Vacuum, Moorestown, NJ). Specimens were visualized with a Zeiss Sigma FESEM (Zeiss Microscopy, Thornwood, NY) operated at 2-3kV using InLens Secondary Electron (SE) and SE2 detection. Images were captured in TIFF using store resolution 2048 x 1536 pixels and a line averaging noise algorithm

Large Block-Face (Thin Section) SEM to visualize internal ultrastructure: Tissue specimens (mesothelium 'buttons') were similarly fixed in 4% PFA with 2% Glutaraldehyde in 0.1M NaCacodylate Buffer (pH 7.3) before *en Bloc* staining with a series of heavy metals as described by Tapia et al (2012) and Deerinck et al (2010)(*66*, *67*). Typically, specimens were treated consecutively with ferrocyanide-reduced OsO_4 (1% OsO_4 with 1.5% tetrapotassium ferricyanide) (1hr), freshly prepared and syringe-

filtered, 1% Thiocarbohydrazide (TCH) (40min), 2% OsO_4 (1hr) and 1% Uranyl Acetate (overnight), before dehydration in a graded ethanol series (50, 70, 90, 100%, 10' each), followed by 2x in 100% Acetonitrile (10' each). Repeated washing with H_2O (3x5mins) was included between steps of staining and before dehydration. Tissue was then infiltrated with 25%, 50% and 75% EMBed 812 embedding resin (Electron Microscopy Sciences, Hatfield, PA) in Acetonitrile, followed by 100% resin (2x3hrs) and finally embedding in pure resin with polymerization for 48hr at 60ºC. Ultrathin sections (200nm each) were collected on conductive silicon wafer substrates, and visualized with a Zeiss Sigma FESEM (Zeiss Microscopy, Thornwood, NY) operated at 5-7kV using Backscattered Electron (BSE) detection. Signal inversion resulted in micrographs with grey levels similar to Transmission EM images(*68*). Images were captured in TIFF using store resolution 2048 x 1536 pixels and a line averaging noise algorithm.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6. All results were expressed as means ± SEM. Unpaired t test was used to compare two groups. $P < 0.05$ was considered significant.

List of Supplemental Figures

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Author Contributions: Y.R outlined and supervised the research study, and Y.R and J.M.T. designed experiments with suggestions from I.L.W. J.M.T. developed and performed surgical techniques, developed adhesion severity scores, and performed *in vivo* studies, *in vitro* studies, RNA sequencing analysis, immunohistochemistry, lineage tracing, clonal analyses, and antibody experiments. R.S. performed RNA sequencing and analysis. J.S. provided the bioinformatics analysis platforms. N.F. helped sort mesothelial cells. J.H.S performed, imaged and analyzed the in vitro clonal studies. L. J. prepared samples for and performed electron microscopy studies. S.C. and T.K. generated conjugated antibodies for ultramicroscopy images, performed adhesion surgeries, performed ultramicroscopy imaging, confocal imaging and multi-photon imaging of injured and non-injured peritoneal wall tissue, and analyzed the 2D and 3D datasets. K.M.M performed phagocytosis assays. S.R.G. provided macrophages for *in vitro* experiments. G.K. and J.N. provided human adhesion samples. M.M helped to plan antibody experiments. M.S. performed antibody experiments. N.P., G.W helped to perform immunohistological stains. L.X. performed all *in situ* hybridizations. Y.R and J.M.T wrote the manuscript with suggestions from I.L.W.

Competing Interests: I.L.W is a cofounder of Forty Seven, Inc. J.M.T., I.L.W, R.S, N.F and Y.R have applied for and filed a patent covering the mesothelial origin of adhesions and therapies thereof (# WO 2017/190148). J.M.T. consults for Forty Seven, Inc.

Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. The B35 anti-MSLN antibody may be obtained from A. Miyajima under a materials transfer agreement.

Figure 1. Mouse surface mesothelium proliferates in response to the induction of adhesions

(A) Adhesions were induced in mice by surgical placement of ischemic buttons in the mouse peritoneum at t=0 days. Ischemic buttons were analyzed for mesothelial proliferation 3 days after button induction. Adhesions were analyzed 7 days after button induction. (**B)** Images show haematoxylin and eosin (H&E) staining of ischemic buttons at 30 minutes, 1 hour, 2 hours and 4 hours after button induction followed by abrasion in the peritoneum (black arrow) (n=2 mice per time point). (**C)** Shown is immunofluorescence staining of ischemic buttons for podoplanin (PDPN) and cytokeratin 19 (K19) at 0, 6 hours, 12 hours, and 24 hours after button induction and abrasion of the mouse peritoneum (n=2 mice per time point). (**D)** Shown is immunofluorescence staining of string adhesions between the liver (L) and peritoneum (P) for MSLN and podoplanin (PDPN) 7 days after adhesion induction in mice. **(E)** Numbers of mesothelial cells ($MSLN^+$) (top) and double-positive EdU^+MSLN^+ mesothelial cells (bottom) were counted in n=20 high powered fields (0.75mm x 1mm) in normal mouse peritoneal tissue versus peritoneal tissue with adhesions. (**F)** Shown is immunofluorescence staining for MSLN and EdU in normal mouse peritoneum (P) (top) and adhesions (ADH) between the peritoneum and large intestine (I). All scale bars are 100um. Values are presented as mean ± SEM; *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001; NS not significant. Analyses were done with unpaired t test.

Figure 2. Lineage tracing and clonal analysis of mesothelial cells involved in adhesion formation in mice.

(A) Whole mount imaging of an ischemic button imaged *in vivo* (B) 30 minutes after adhesion induction and treatment with the stain carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE). (**B)** Shown is immunofluorescence staining of PDPN, K19 and CFSE on the surface mesothelium surrounding a single ischemic button. (Single staining for PDPN, K19, CFSE is shown in Fig S13). (**C-E)** Whole mount imaging of an ischemic button (IB) 4 days after adhesion induction and treatment with CFSE stain. Adhesions (ADH) are indicated by white boxes and white arrows (n=3). (**F,G)** Shown is immunofluorescence staining of adhesions for PDPN and CFSE 7 days after adhesion induction (n=5). (**H)** Shown is immunofluorescence staining of adhesion sites for PDPN and $Actin^{CreER}$; $R26^{VT2/GK3}$ 7 days after adhesion induction and CFSE injection. White arrows indicate cells originating from the same precursor. (**I**) Shown is immunofluorescence staining for PDPN and $Actin^{CreER}$; $R26^{VT2/GK3}$ at adhesion sites (ADH) 7 days after adhesion formation (n=3). White arrows indicate a spindle like phenotype. All scale bars are 100 um unless otherwise indicated.

Figure 3. Mesothelium is a necessary component of adhesionsin mice.

(A) Light-sheet microscopy images after injection of anti-MSLN antibodies into the mouse peritoneum following adhesion induction *in vivo*. Autofluorescence (green) indicates the suture (S) and muscle fibers of the peritoneum (P). (**B)** Further analysis by light sheet microscopy shows longitudinal expansion of $MSLN^+$ staining that is parallel to the muscle fibers. Volume rendering of 2D images (a virtual z-stack) as a 3D model was done using the maximum intensity projection or blend mode. 2D representations are maximum intensity projections of 36 μm thick sections from the 3D model. (**C)** CD47 expression at 6, 12, and 24 hours after adhesion formation in response to button induction in mice (**D)** Phagocytosis of mesothelial cells treated with anti-CD47 antibody by macrophages. **(E)** Immunofluorescence staining of macrophages (red) and mesothelial cells (green) showing phagocytosis of mesothelial cells by macrophages. **(F)** Appearance of adhesions two weeks after button induction in mice. (**G)** Appearance of adhesions after treatment with anti-MSLN antibodies injected at 7, 10 or 13 days following adhesion formation. (**H)** Adhesion score after treatment of formed adhesions in mice with anti-CD47 antibody alone (n=8), anti-MSLN antibody alone (n=3), or a combination of anti-CD47 and anti-MSLN antibodies (n=5) compared to control untreated mice injected with PBS (n=10). Antibodies were injected at 7, 10, and 13 days following induction of adhesions. Values are presented as mean ± SEM; *$p<0.05$, **$p<0.005$; NS not significant. Statistical analyses were done with an unpaired t test. (**I)** Ischemic buttons (IB) from mice after antibody treatment showing immunofluorescence staining for collagen, fibronectin, CD31, F4/80, and MSLN.

Figure 4. Mesothelial cells show a distinct transcriptional profile after adhesion induction in mice.

(A) Surface mesothelium was isolated from ischemic buttons and cells with a $PDPN^{+}LYVE1^{-}CD31^{-}CD45^{-}$ surface phenotype were obtained by flow cytometry. (**B)** Heatmap of RNA expression in purified surface mesothelium immediately after button induction in mouse peritoneum (t=0) and 6, 12, and 24 hours after button induction. Representative genes are shown above gene clusters. (**C)** Log-fold changes in transcript expression were calculated 24 hours after induction of adhesions compared to controls and were plotted against gene identity. (**D)** Number of upregulated and downregulated genes from uninjured and injured mesothelium at 24 hours post button induction is shown versus the number of upregulated or downregulated genes in hematopoietic stem cells (HSCs) and their progeny after differentiation. Differentiated hematopoietic cells included multi-potent progenitors (MPPs), common myeloid progenitors (CMPs), monocytes, natural killer (NK) cells, CD4+ and CD8+ T cells +/- CD69 expression, acute myeloid leukemia stem cell (LSCs), acute myeloid leukemia progenitor cells (LPCs), and acute myeloid leukemia blasts. (**E)** Heatmap of gene sets upregulated or downregulated in mouse surface mesothelium 6, 12, and 24 hours after adhesion formation.

Figure 5. Mouse mesothelial gene expression compared to fibroblast gene expression **(A)** Expression of genes (indicated above graphs) in mouse mesothelial cells by RNA sequencing (FPKM, Fragments Per Kilobase of transcript per Million mapped reads) over 24 hours (**B)** Immunofluorescence staining of adhesions 7 days post button induction for PDPN, MSLN, CD44, S100A4 and K19. . All scale bars are 100um. ADH, adhesions; L, liver; P, peritoneal wall; I, intestine.

Figure 6. Inhibition of HIF1α is sufficient to prevent adhesion formation in mice.

(A) Mesothelial macrophage co-cultures in vitro under normal oxygen conditions (normoxia) immunostained for PDPN or HIF1A expression. (**B)** Mesothelial macrophage co-cultures in vitro under hypoxia conditions (5% O_2 incubator) stained for PDPN or HIF1A expression. Scale bars are 250um. (**C)** Gross anatomy, immunofluorescence staining, and H&E staining of mouse adhesions 7 days after button induction and treatment with 20ug/kg echinomycin daily for 7 days. (**D)** Adhesion score after treatment of mouse adhesions with echinomycin or PX12, small molecule inhibitors of HIF1α. (**E**) Numbers of double-positive mesothelial cells ($MSLN^+PDPN^+$) and triple-positive EdU^+MSLN^+ $PDPN^+$ mesothelial cells were counted per high powered field (0.75mm x 1mm) after treatment of mice with the HIF1α inhibitor echinomycin (n=16 fields) and were compared to untreated control mice (n=5 fields). (**F)** Expression measured by RNA sequencing (FPKM) of selected target genes during a 24 hour time course after adhesion induction and treatment of mice with the HIF1α inhibitor echinomycin (n=2 mice). Heatmap showing RNA sequencing of surface mesothelium immediately after echinomycin treatment and button induction and 6, 12, and 24 hours after echinomycin treatment and button induction. Scale bars are 100um unless otherwise noted. Values are presented as mean ± SEM; *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001; NS not significant. Statistical analyses were done by an unpaired t test.

Figure 7. Gene expression in human peritoneal adhesions.

(A) H&E staining of representative human adhesion tissue from n=6 patients undergoing surgery showing mesothelial cells present within the adhesion. (**B)** Trichrome staining of representative human adhesion tissue from n=6 patients undergoing surgery showing mesothelial cells within areas of fibrosis. (**C)** Immunofluorescence staining and in situ hybridization for PDPN, MSLN, S100A4, WT1, CD47, and UPK1B in human adhesion tissue isolated from patients undergoing surgery (n=6). Scale bars are all 100um unless otherwise noted.

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