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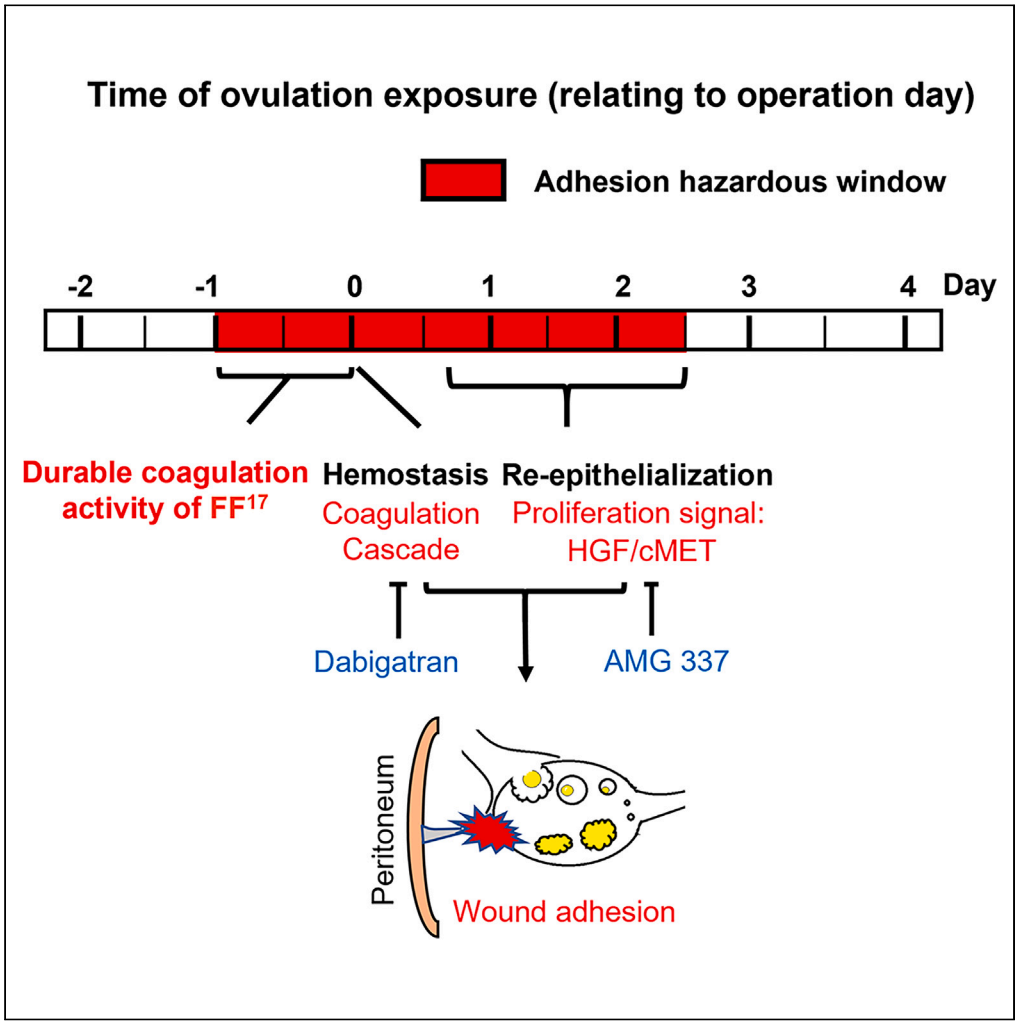

Vaishnavi Seenan, Che-Fang Hsu, Kanchana Subramani, Pao-Chu Chen, Dah-Ching Ding, Tang-Yuan Chu

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Highlights

Incidence and severity of postoperative adhesions highly depend on exposure of wound to ovulatory follicular fluid (FF)

FF exposure promotes wound adhesions in a hazardous time frame of 1 day before to 2.5 days after surgery

Coagulation cascade and HGF signals are responsible for early- and late-exposure risk, respectively

Ovulation-caused wound adhesions can be prevented by avoiding surgery at the ovulation time, or by giving a cMET inhibitor

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Ovulation provides excessive coagulation and hepatocyte growth factor signals to cause postoperative intraabdominal adhesions

Vaishnavi Seenan,[1,2] Che-Fang Hsu,[1] Kanchana Subramani,[1,2] Pao-Chu Chen,[3] Dah-Ching Ding,[2,3] and Tang-Yuan Chu[1,2,3,4,5,*]

SUMMARY

Postoperative adhesions show a higher occurrence in females aged 16–60, especially after pelvic surgeries. This study explores the role of ovulation in adhesion formation in mice. Ovarian surgery in mice with normal- or super-ovulation led to pronounced adhesions, whereas ovulation-defective *Pgr*-KO mice showed minimal adhesions. Specifically, exposure to ovulatory follicular fluid (FF) markedly increased the adhesion. The hazardous exposure time window was one day before to 2.5 days after the surgery. Mechanistically, early FF exposure triggered adhesions via the blood coagulation cascade, while later exposure relied on the HGF/cMET signaling pathway. Prophylactic administration of a thrombin inhibitor pre-operatively or a cMET inhibitor postoperatively effectively mitigated FF-induced adhesions, while COX inhibitor treatment exhibited no discernible effect. These findings underscore ovulation as a pivotal factor in the development of pelvic wound adhesions and advocate for targeted preventive strategies such as c-MET inhibition, scheduling surgeries outside the ovulatory period, or employing oral contraceptive measures.

INTRODUCTION

Intra-abdominal adhesions are one of the most important complications of open pelvic or abdominal surgeries, which may develop in up to 90% of patients.[1–3] The consequences of adhesions include infertility, pelvic pain, small bowel obstruction, and repetitive invasive surgeries. In the USA, the total cost of hospitalization for adhesiolysis was estimated at $1.33 billion in 1994.[4] Despite therapeutic advances, such as improved surgical techniques, interventions using anti-inflammatory drugs,[5,6] and installation or placement of biomaterials as barrier agents,[7,8] the incidence of adhesion development has not diminished substantially.

Gender is an important contributing factor to adhesion development in intra-abdominal surgery.[3,9] Female patients undergoing appendectomy have an estimated 4-fold higher risk of adhesive small bowel obstruction (ASBO) as compared to men receiving the same procedure.[10] Reproductive age is another risk factor. A longitudinal study based on the Scottish National Health Service (SNHS) medical records, the Surgical and Clinical Adhesions Research (SCAR), has shown that patients aged between 16 and 60 years old have a higher risk of readmission directly related to adhesions after colorectal surgery or appendectomy.[11] An updated analysis of SCAR confirmed that sex and age are associated with adhesion-related readmissions.[12] A prospective multicenter trial in France also found a higher rate of recurrence in younger patients (age <40 years) who underwent surgery for ASBO.[13] Together, these studies support that woman of reproductive age are at an increased risk for postoperative intraperitoneal adhesions.

Studies on the prevalence of adhesions among different surgical sites have indicated that ovarian surgery is the procedure most associated with adhesion development. A meta-analysis of the incidence of ASBO following 446,331 abdominal surgeries revealed that gynecologic surgery had a relatively high incidence of 11.1%, and open adnexal surgery had the highest incidence of 23.9%.[14] Other studies also showed that ovarian surgery has the highest rate of readmissions related to adhesions.[9,15]

The aforementioned risk factors for postoperative adhesions prompted us to hypothesize that the exposure of a wound to ovulation fluid or follicular fluid (FF) may promote adhesion formation after a surgery. Based on previous studies on the constituents of ovulatory FF and their functions in tissue repair after ovulation wounding,[16,17] we presumed this to be a likely mechanism behind the ovulation-induced wound adhesion.

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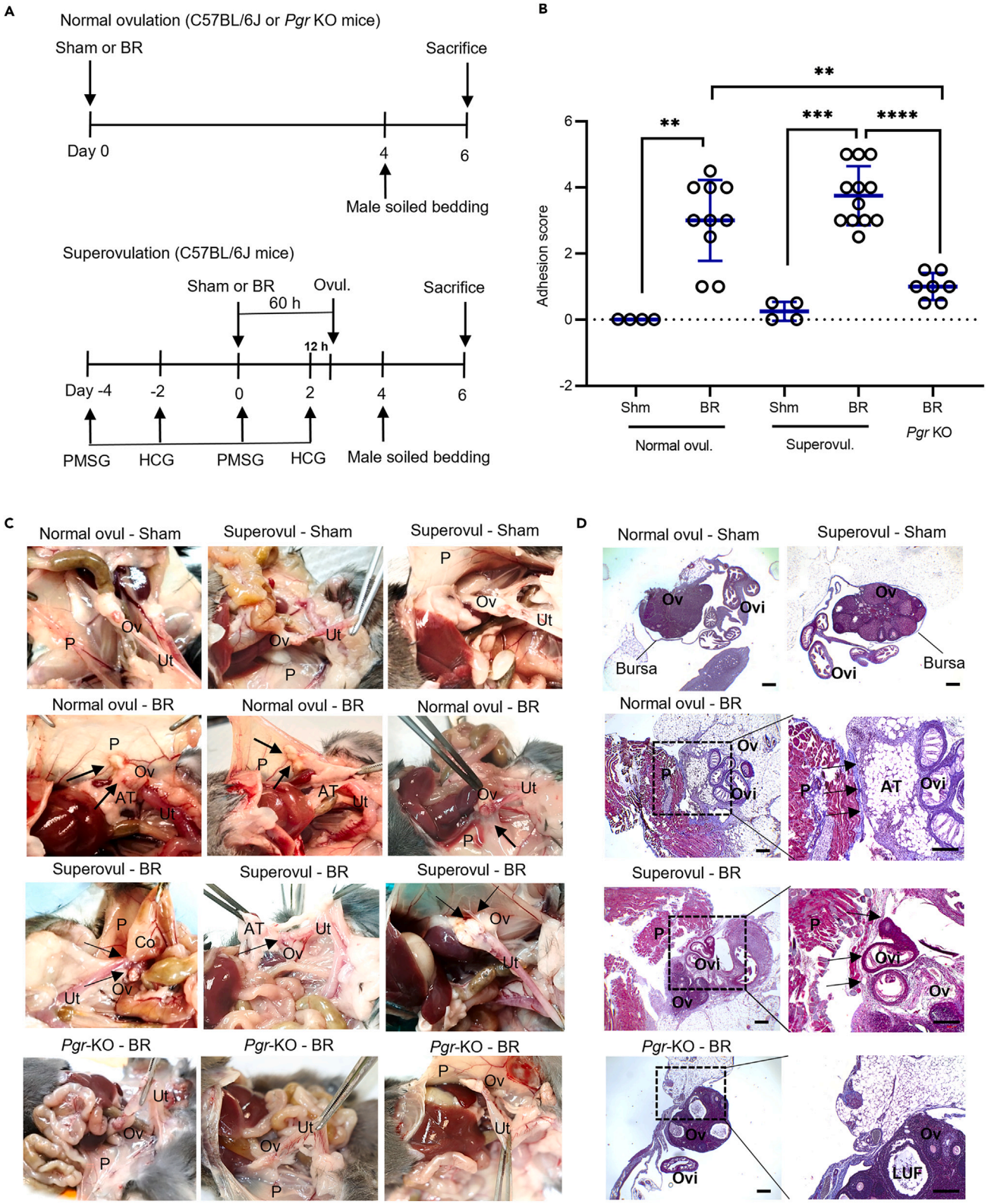
A
Normal ovulation (C57BL/6J or Pgr KO mice)
Sham or BR
Sacrifice
Day 0
4
6
Male soiled bedding
Superovulation (C57BL/6J mice)
Sham or BR
Ovul.
60 h
Sacrifice
12 h
Day -4
-2
0
2
4
6
PMSG
HCG
PMSG
HCG
Male soiled bedding
B
Adhesion score
6
4
2
0
-2
**
**
***
****
Shm
BR
Shm
BR
BR
Pgr KO
Normal ovul.
Superovul.
C
Normal ovul - Sham
Superovul - Sham
Superovul - Sham
Normal ovul - BR
Normal ovul - BR
Normal ovul - BR
Superovul - BR
Superovul - BR
Superovul - BR
Pgr-KO - BR
Pgr-KO - BR
Pgr-KO - BR
P
Ov
Ut
AT
Co
D
Normal ovul - Sham
Superovul - Sham
Normal ovul - BR
Superovul - BR
Pgr-KO - BR
Ov
Ovi
Bursa
P
AT
LUF

Figure 1. Ovulation promoted periovarian adhesions following bursectomy

(A) Schematic experimental timelines of bursa removal (BR) or sham surgery with or without ovulation induction. Ovulation was induced via PMSG/hCG injections such that ovulation occurred at 60 h after the surgery. Estrus cycle was synchronized in the mouse group by exposure to male-soiled bedding before sacrifice on day 6.

(B) Adhesion scores of different mouse groups with normal ovulation, superovulation, and luteinized non-ovulation (*Pgr*-KO) after sham or bursectomy (BR) surgery are shown and the average adhesion score of left and right adhesions are plotted as mean ± SD.

(C) Representative images of wound adhesions in three biological replicates of bursectomy mice in contrast to sham-operation wild-type mice and *Pgr*-KO mice after bursectomy.

(D) Representative trichrome stain histology of adhesions in the five groups. Adhesions of the wounding site at the ovary (Ov)/oviduct (Ovi) to the adjacent adipose tissue (AT), peritoneum (P), and colon (Co) are shown in the gross and microscopic views. Ut: Uterus, LUF: Luteinized unruptured follicle, black arrows: adhesion sites. Scale bar: 100 μm. Normal Ovul. Sham (N = 4) BR (N = 10); Superovul. Sham (N = 4) BR (N = 12); *Pgr* KO (N = 7). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, two-tailed, unpaired Student's t test.

Wound healing consists of four overlapping phases: hemostasis, inflammation, proliferation, and remodeling.[18] Ovulatory FF is rich in coagulation cascade proteins, inflammatory cytokines,[19] and growth factors, such as HGF, IGF2, and PDGF.[17,20,21] These molecules are essential for the quick healing of ovulation wounds. They may also promote adhesions when encountering a surgical wound.

Using mouse ovarian bursectomy and ovariectomy surgical models, this study investigated the effect of ovulation and specifically exposure of surgical wounds to FF on the incidence of intra-abdominal adhesions. We discovered a potent pro-adhesive effect of ovulation, identified multiple phase-specific mechanisms, and demonstrated targets for pharmaceutical prevention. We also provide the time window for risk avoidance, and a new surgical concept for adhesion prevention.

RESULTS

Ovulation enhanced adhesion development following minor ovarian surgery

To determine whether ovulation has a causal relationship with post-surgical adhesion development, we manipulated ovulation in mice and performed a minor surgery at the periphery of the ovary. As shown in Figure 1A, ovarian bursectomy or sham operation was performed, and the severity of wound adhesions was scored 6 days later (Table 1). In the super-ovulation group, ovulation was controlled for 60 h after the operation. To examine the effect of ovulatory follicle rupture on wound adhesion more precisely, we used progesterone receptor (*Pgr*) knockout mice as the ovulation-defective model. *Pgr*-KO mice maintain a relatively normal estrus cycle with normal follicle and oocyte maturation and luteinization; however, the follicles are not ruptured.[22] As expected, ovarian surgery resulted in significantly higher adhesion scores than the sham surgery in both the normal ovulation (3 ± 1.2 vs. 0, $p = 0.0005$) (mean ± SD) and superovulation (3.75 ± 0.9 vs. 0.25 ± 0.3, $p < 0.0001$) groups. Compared to the normal ovulation mice, the superovulation mice showed a non-significant increase in adhesion score (3.75 ± 0.9 vs. 3 ± 1.2, $p = 0.1$). However, in ovulation-defective *Pgr*-KO mice, there was a significant decrease in adhesion score (1 ± 0.4, $p = 0.0009$) (Figure 1B). Adhesions were confirmed histologically using trichrome staining. Adhesions of the ovarian adipose tissue to the peritoneum were seen in the normal ovulation mice and adhesions of the ovary and oviduct to the peritoneal adipose tissue and colon were seen in the superovulation mice. In contrast, in *Pgr*-KO mice, there were only loose filmy adhesions, which were difficult to visualize in tissue sections (Figures 1C and 1D).

Ovulatory follicular fluid exposure promoted adhesion growth with a hazardous time frame peaking at 16 h to 2.5 days post-operation

As adhesion development was diminished by ovulation suppression, we assumed that wound exposure to ovulatory FF was responsible for adhesion growth. We tested this hypothesis by using the intraperitoneal (i.p.) injection of human FF into mice that had undergone ovariectomy (OVX) and investigated the time window where this treatment increased the risk of adhesion growth. As shown in Figure 2A, 10% FF or saline was intraperitoneally injected at different times in relation to OVX surgery, beginning 2 days before to 4 days after the surgeries. At all six time points

Table 1. Adhesion severity grading score

Score	Adhesion type	Description (Bursectomy)	Description (Ovariectomy)
0	No adhesion	No adhesion observed	No adhesion observed
1	Thin filmy	Ovarian fat to the peritoneum	Uterine ligament to the peritoneum
2	Thin filmy	Ovary to the peritoneum (easy to separate)	Uterus or oviduct to the peritoneum (easy to separate)
3	Moderate	Ovary to the peritoneum (blunt dissection)	Uterus or oviduct to the peritoneum (blunt dissection)
4	Severe	Ovary to the peritoneum (sharp dissection)	Uterus or oviduct to the peritoneum (sharp dissection)
5	More severe	Ovary deeply embedded into the peritoneum or to other neighboring organs	Uterus or oviduct deeply embedded to the peritoneum or to other neighboring organs

For morphological illustrations, see Figure S1.

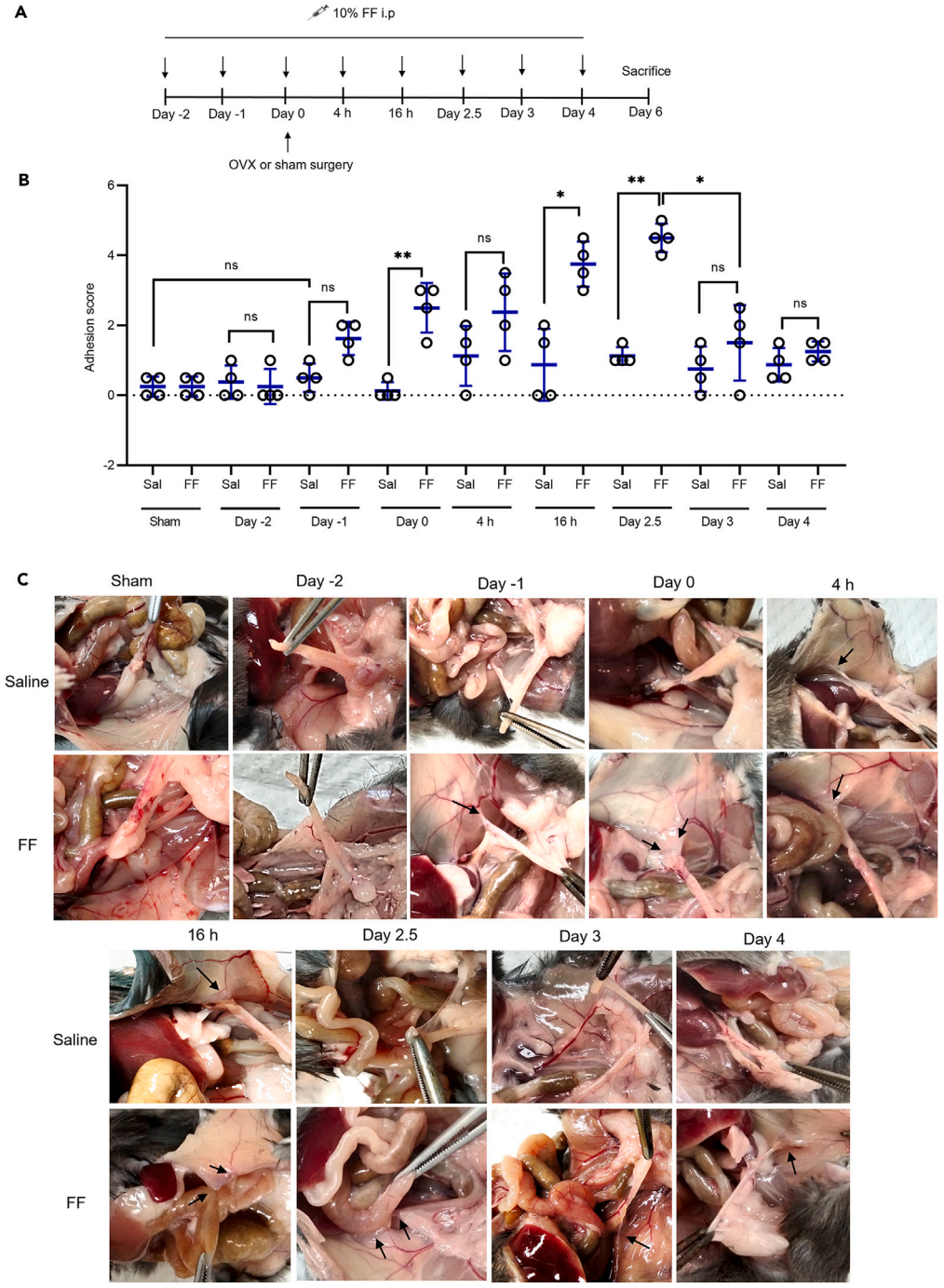

Figure 2. FF exposure at different time points pre/post-surgery shows varied ovarian wound induced adhesion development that maximizes with exposure 2.5 days after surgery

(A) Schematic experimental timeline showing the intraperitoneal injection of follicular fluid (FF) or saline at different times in relation to ovariectomy (OVX) or sham surgery.

(B) Adhesion scores in the operated mice with FF- or saline-exposure at different times are shown and the average adhesion score of left and right adhesions are plotted as mean ± SD.

(C) Representative images showing the adhesion site. Black arrows indicated adhesions of the oviduct or uterus to the peritoneum, and the uterus to the colon. $N = 4$ mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-tailed, unpaired Student's t test. ns: nonsignificant.

between day −1 to day 2.5, FF injection resulted in an increase in adhesion score compared to saline injection and the FF-injected sham surgery controls (Figure 2B). The maximum increase was seen in mice exposed between 16 h and 2.5 days after surgery, with scores of 3.75 ± 0.6 and 4.5 ± 0.4, respectively. For those exposed after day 2.5, the scores reduced to an insignificant level of 1.5 ± 1.1 on day 3 and 1.25 ± 0.3 on day 4 (Figure 2B). The adhesion pattern in the OVX + i.p. FF injection mice was similar to that observed in bursectomy + endogenous ovulation mice. There were either no adhesions or very thin, filmy adhesions in the day 0 + sham and day −2 + FF or saline exposure mice, whereas moderate adhesions of the oviduct to the peritoneum were observed in the day −1, 0 h, and 4 h exposure groups, and severe adhesions of the oviducts to the colon were seen in the 16 h and day 2.5 groups. In the groups exposed after day 2.5, there were either thin and filmy or no adhesions (Figure 2C). Together, these results show that FF exposure immediately before and early after wound healing results in enhanced adhesion development. Thus, performing surgery near the time of ovulation, both before and shortly after, increases the risk for developing postoperative adhesions. The hazardous time window was between 2.5 days before and one day after ovulation, and the worst time was 2.5 days before.

In a model mimicking immediate ovulatory exposure, the pro-adhesion activity of follicular fluid during the early phase of wound healing has been demonstrated

Since FF quickly diffuses into the peritoneal fluid after ovulation, we designed an extracorporeal FF exposure model to investigate the pro-adhesion activity of pure FF and focused on the exposures at the earliest (within 45 min) phase of wound healing. The adnexa of each uterine horn was surgically exposed and each was treated with 100% FF or saline for 3 min. The exposure was set at 0 min, 5 min, 15 min, 30 min, and 45 min after OVX in the different mouse groups (Figure 3A). Instead of the 10% FF solution used in the i.p. injection model, which simulates FF exposure after dilution with the peritoneal fluid, short exposure with 100% FF was conducted to mimic immediate exposure after ovulation. The adhesion pattern in the pure FF exposure at 0 h was more severe than that in the i.p. 10% FF exposure model at the same time point. At different exposure time points, adhesion scores on the FF-treated side (ranging from 4 to 4.75) were significantly higher than those on the contralateral saline-treated side (ranging from 1 to 3) (Figure 3B). Severe adhesions were observed between the oviduct and peritoneum or colon in the FF-exposed wounds and moderate to no adhesions on the saline-exposed wounds (Figure 3C). The results demonstrated that undiluted FF has a more potent pro-adhesion development activity, causing more severe adhesions than the 10% FF.

Meanwhile, the adhesion scores in the saline-treated group increased progressively as the time of saline treatment was delayed (thus resulting in a longer duration of air-drying) after OVX. The scores increased from 1 to 0.75 when the wound was rinsed with saline at 0 min and 5 min, and to 1.25, 2.0, and 3.0 when saline was given at 15 min, 30 min, and 45 min, respectively. This result is consistent with the previously observed results demonstrating the adhesion-promoting effect of air-drying.[23]

Coagulation cascade components in follicular fluid are responsible for adhesions resulting from early follicular fluid exposure

Coagulation is responsible for the earliest phase of wound healing and promotes hemostasis by activating coagulation factors.[24] Our previous study revealed the abundant presence of coagulation cascade proteins in FF, which readily formed a clot after treatment with tissue factor.[17] We proposed that this coagulation machinery was contributing to adhesion formation upon FF exposure during the early phases of wound healing. We tested this hypothesis by disrupting the coagulation cascade through the depletion of coagulation factors with tissue factor or treatment with the thrombin inhibitor dabigatran (Figure 4A). Indeed, OVX wounds exposed to coagulation factor-depleted FF (CD FF) had significantly lower adhesion scores than the contralateral FF-exposed wound (0.8 ± 0.9 vs. 4.0 ± 0.8, $p = 0.03$). When the mice were administered oral dabigatran 24 h before the operation, FF exposure no longer resulted in wound adhesions. The adhesion score was lowered to 1.0 in all four tested mice, compared to the average score of 4.25 ± 0.95 in vehicle-treated mice with the same FF exposure ($p = 0.0005$) (Figures 4C and 4D).

Hepatocyte growth factor-cMET signaling is responsible for adhesion development following later follicular fluid exposure

Hepatocyte growth factor (HGF) is a well-known growth factor responsible for tissue repair and regeneration.[17,25] Previously, we showed that HGF and its activator HGFA in FF are continuously activated once the coagulation protease cascade is activated after ovulation.[17] Through the activation of thrombin via the extrinsic coagulation pathway, HGFA cleaves pro-HGF to activate HGF. Thus, ovulation releases the HGF activation machinery to be activated at the injured tissues expressing the cMET receptor.[17] We tested whether coagulation-induced HGF/cMET signaling is responsible for the pro-adhesion activity of late FF exposure. Using the i.p. FF injection model, a treatment protocol was designed where FF was i.p. administered 0 h, 16 h, and 2.5 days where OVX and cMET inhibitor (AMG-337) was administered 30 min before the FF injection (Figure 5A). A complete reduction of adhesions to that of non-FF treated levels was achieved by AMG-337 treatment when the wound was exposed to FF at +16 h (average adhesion score of 0.37 ± 0.25) and +2.5 days (score of 1.25 ± 0.43). Meanwhile, cMET inhibition did not suppress the adhesion caused by immediate FF exposure at 0 h (Figure 5A). In addition, we performed the same treatment in the bursectomy model, choosing the most severe protocol (exposure at 2.5 days after surgery). As shown in Figure 5B, AMG 337 pretreatment effectively prevented the adhesions with adhesion scores significantly lower than the saline-treat control (3.87 ± 0.75 vs. 1.5 ± 0.4, $p = 0.0014$). Furthermore, we discovered cMET expression in the adhesion tissues (Figure 5C). The function of cMET signaling in the mesothelial cells was confirmed by showing that Met-5A cells highly expressed cMET which was readily phosphorylated 30 m after 10% FF treatment and was inhibited by AMG 337 (Figure 5D). These results indicate that ovulation-sourced HGF/cMET signaling is responsible for adhesions due to exposure at 16 h to 2.5 days after surgery.

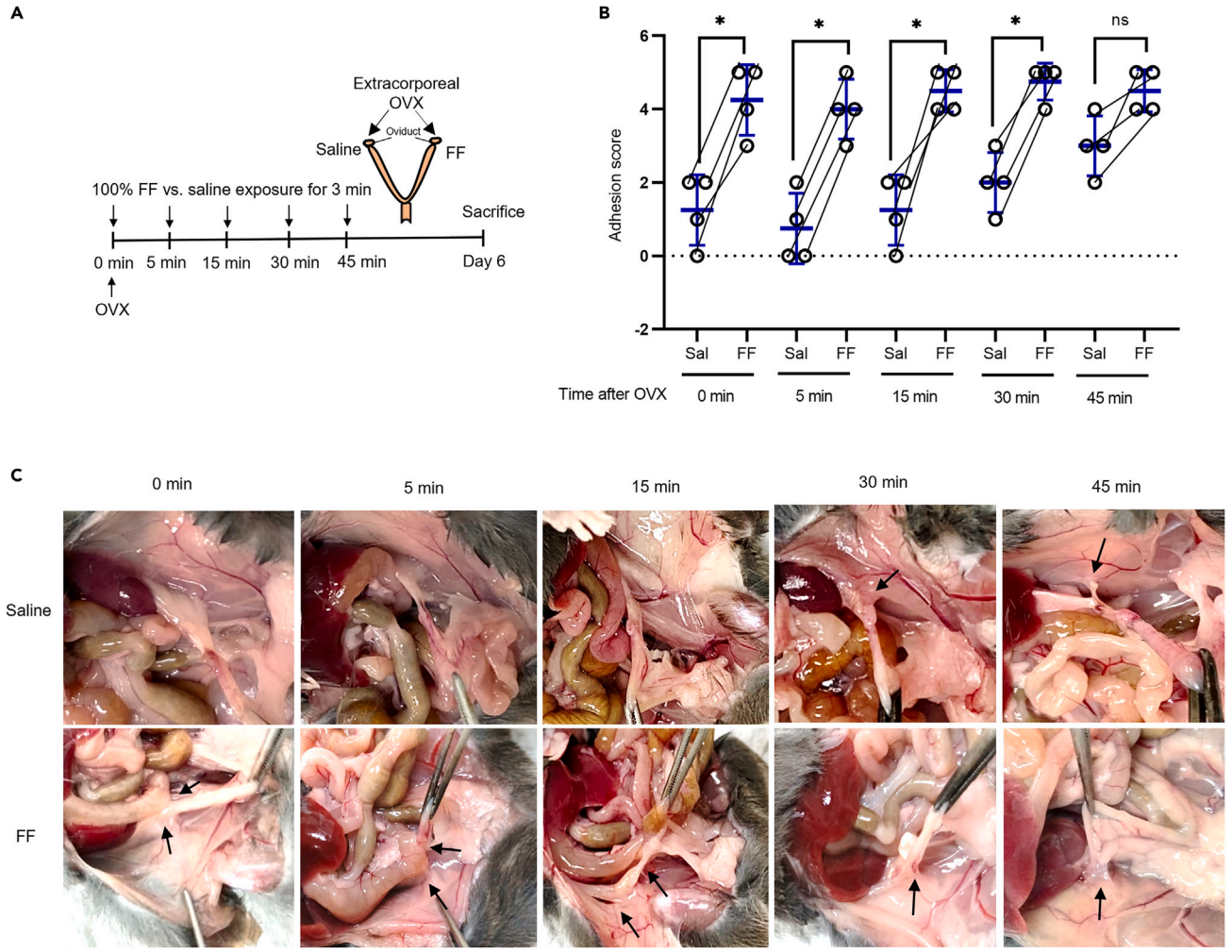

Figure 3. Extracorporeal short exposure demonstrated the pro-adhesion activity of FF in the early phase of wound healing

(A) Schematic timeline showing transient (3 min) exposure of bilateral ovariectomy (OVX) wounds each with 100% FF or saline extracorporeally. In different groups, the exposures were performed at 0 min, 5 min, 15 min, 30 min, or 45 min after wounding, and mice were sacrificed on day 6.

(B) Pairwise comparisons of adhesions were scored for each of the OVX wound sides. Adhesion scores of the saline- and FF-exposed sides were compared by two-tailed, paired Student's t test.

(C) Representative images showing adhesions on the side with FF exposure (arrows). $N = 4$ mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Ovulation-induced adhesion development is independent of COX-mediated inflammation

In different intra-abdominal surgery models, a non-selective COX inhibitor, indomethacin, was found to be effective in inhibiting intraperitoneal adhesions post-operation.[26,27] Although ovulation is a consequence of acute inflammation, FF does not contain the COX enzyme. We investigated whether treating animals with COX inhibitors would reduce FF-induced wound adhesion scores. We performed extracorporeal FF exposure in which bilateral OVX wounds were immediately exposed to FF or saline for 3 min. Mice were administered indomethacin or vehicle daily from −1 to 3 days after the operation and sacrificed on day 6 (Figure 6A). We found the same severity of adhesions (average scores of 4 and 4.25) in both the FF-treated groups with and without indomethacin (Figures 6B and 6C). These results suggest that adhesions caused by ovulatory FF are independent of COX-mediated inflammation.

DISCUSSION

In this study, we discovered that ovulation is the main cause of postoperative adhesions in adnexal surgeries. Gynecological surgeries, especially tubo-ovarian procedures, are highly vulnerable to postoperative adhesion growth of unknown etiology. This study has elucidated crucial elements of the pro-adhesion mechanisms harbored in the FF of ovulation. Among them, coagulation cascade proteins act in the early phase, and HGF-cMET signaling acts in the late phase of wound healing or adhesion development.

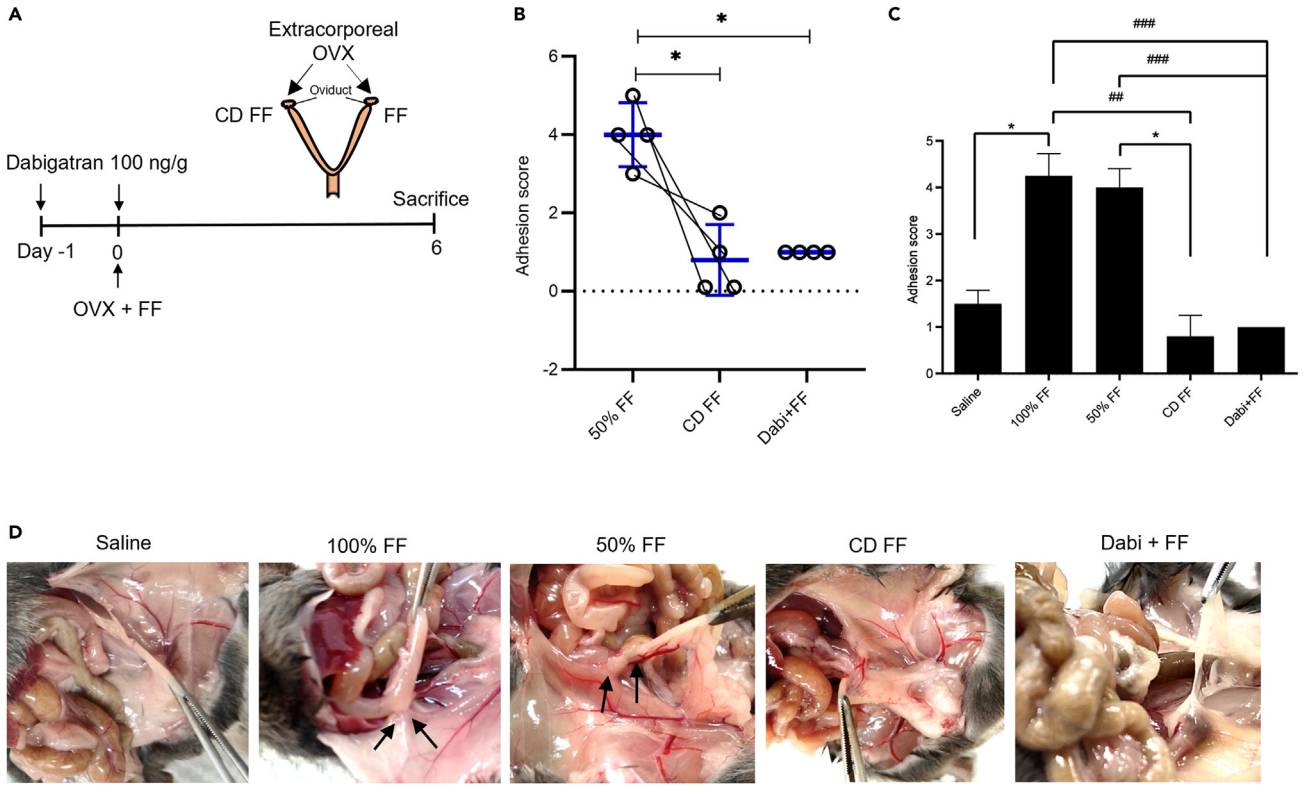

Figure 4. The pro-adhesion activity of FF in early wound healing results from coagulation factors and thrombin

(A) In the bilateral extracorporeal transient wound exposure model, bilateral OVX wounds were exposed with 50% FF vs. clotting factor-depleted FF (CD FF) at 0 min. In another set of studies, mice were fed with 100 ng/g dabigatran one day before OVX surgery with immediate FF exposure.

(B) Paired comparison of the FF- vs. CD FF-exposed wound sides and comparison with the dabigatran treated mice.

(C) A summarized comparison of adhesion scores in extracorporeal wound exposure to 100% or 50% FF, CD FF, or saline with or without dabigatran pretreatment.

(D) Representative images showing the adhesion sites (Black arrows). $N = 4$ mice per group. *$p < 0.05$ compared between left and right side of the mouse by two-tailed, paired Student's t test; ##$p < 0.01$, ###$p < 0.001$ compared between different mice by two-tailed, unpaired Student's t test.

By administering FF at different time points before and after surgery, we defined the hazardous time window for ovulation as one day before to 2.5 days after surgery. Interestingly, within this time window, the later exposures resulted in more severe adhesions, with the most severe adhesions arising from exposure 2.5 days after surgery. Exposure later than 2.5 days after surgery no longer resulted in an increase in adhesion number or severity (Figure 2B). This sharp decrease in vulnerability to adhesion development is consistent with the time required for complete healing of mucosal wounds.[28,29]

Although the time of risk was derived from mouse surgery. According to previous studies including genome-wide comparisons of molecular changes during wound healing, the time scales of different stages (hemostasis, inflammation, proliferation, and remodeling) of wound healing after either surgical or burn injury was very similar in mouse and human.[30] Therefore, the time window to avoid ovulation to protect from adhesions should be similar in both mouse and human.

The mesothelium lines the organs and walls of the peritoneal cavity to provide a non-adhesive surface. Adhesions occur when the mesothelial layer is denuded or damaged.[23,31] In rodents, the ovary and distal oviduct are surrounded by a mesothelial structure called the ovarian bursa to ensure oocyte pickup. We chose bursectomy as a model for minimal ovarian surgery to observe the effect of ovulation on adhesion formation. Few adhesions were noted in the ovulation-disabled *Pgr*-KO mice, in contrast to the overt adhesions in normal- and super-ovulated mice. Importantly, follicles in *Pgr*-KO mice are fully proficient in luteinization and maturation but fail to rupture,[22] further indicating that exposure to ovulation-released FF is to blame for the adhesion formation.

Interestingly, we observed only a small, non-significant increase in adhesion score in super-ovulated versus normal-ovulated mice. Under normal ovulatory FF exposure, wound healing signaling is likely affected to a maximum capacity, such that stimulation from further ovulation could no longer have any effect. We observed the same result in an i.p. xenograft tumorigenesis mouse experiment where, compared to the pro-tumorigenic effect of normal ovulation, superovulation did not increase the severity of the intraperitoneal seeding of high-grade serous carcinoma cells.[32] The same study also showed that the bursa-removed ovary ovulated normally and readily released FF to enhance tumor growth, thus clarifying whether bursectomy would compromise ovulation function.[32] Meanwhile, we also, chose ovariectomy surgery in the

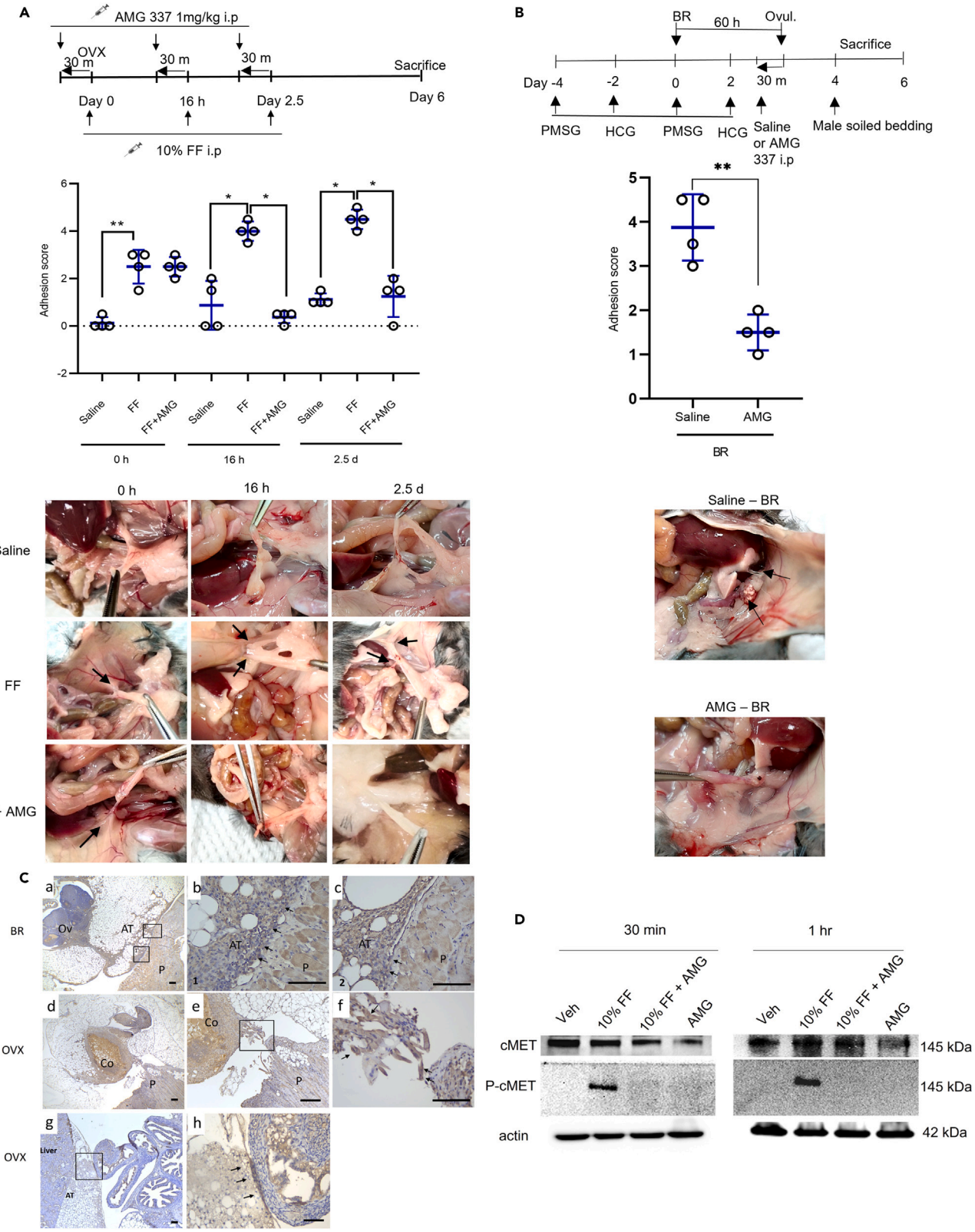
A
AMG 337 1mg/kg i.p
OVX
30 m
30 m
30 m
Sacrifice
Day 0
16 h
Day 2.5
Day 6
10% FF i.p
Adhesion score
Saline
FF
FF+AMG
0 h
16 h
2.5 d
B
BR
60 h
Ovul.
Sacrifice
Day -4
-2
0
2
30 m
4
6
PMSG
HCG
PMSG
HCG
Saline or AMG 337 i.p
Male soiled bedding
Adhesion score
Saline
AMG
BR
Saline – BR
AMG – BR
C
BR
OVX
OVX
Ov
AT
P
Co
Liver
D
30 min
1 hr
Veh
10% FF
10% FF + AMG
AMG
cMET
P-cMET
actin
145 kDa
145 kDa
42 kDa

Figure 5. Inhibition of cMET prevents adhesions from FF exposure in late stage wound healing at 16 h and 2.5 days

(A) Schematic timeline showing exposure of OVX wound to i.p. FF at 0 h, 16 h or 2.5 days after surgery, with or without pretreatment (30 min before surgery) with cMET inhibitor AMG-337 (upper). Comparisons of adhesion scores of mice in different treatment groups are shown and the average adhesion score of left and right adhesions are plotted as mean ± SD (middle). Representative images showing adhesion sites (lower).

(B) Schematic experimental timeline showing BR with ovulation induction. Ovulation was induced via PMSG/hCG injections such that ovulation occurred at 60 h after the surgery with pretreatment 30 m before ovulation (upper). Adhesion scores of saline and AMG groups with surgery are shown and the average adhesion score of left and right adhesions are plotted as mean ± SD (middle). Representative images showing adhesion sites (lower).

(C) IHC stain showing cMET expression in the adhesion tissue.

(D) Expression of phosphorylated and non-phosphorylated forms of cMET 30 m and 1 h after treatment with or without 10% FF or pretreated 30 m with 10μM cMET inhibitor (AMG 337) or AMG 337 only. Scale bar: 100 μm. Black arrows: adhesion sites. $N = 4$ mice per group. ***$p < 0.001$, ****$p < 0.0001$ by two-tailed, unpaired Student's t test.

i.p. FF injection mouse model to avoid the disturbance of endogenous ovulation and specifically examine the effect of exogenous FF exposure on wound healing.

Immediately after tissue injury, wound healing starts with the activation of the coagulation system to form a fibrin clot.[24] In addition to blood, ovulatory FF also harbors highly abundant coagulation cascade proteins[33] which readily form clots upon interaction with tissue factor.[17] The functions of FF-coagulants were first identified in our previous study. We found that FF has a long-lasting mitogenic and transforming activity that can be attributed to a high reserve of coagulation cascade proteins as well as components of the thrombin/HGFA/HGF cascade.[17] The sustained effects of the coagulation protein reserve explain why FF exposure before surgery also results in adhesion development (Figure 2B) and why exposure to bloodless wounds would result in remarkable adhesions and why cMET inhibitor works effectively in preventing the adhesions.

HGF, a key growth factor, mediates tissue regeneration in multiple organs.[34] In various cell culture models mimicking wound healing, HGF promotes the motility and growth of epithelial, dermal stromal, and endothelial cells.[35–37] There is also *in vivo* evidence showing improved skin healing when the wound is locally injected with an HGF-expressing plasmid.[35] Several phase one and two clinical trials are using the same *HGF* gene delivery strategy to treat critical limb ischemia.[38–40] The present study is an opposite approach to HGF/cMET signaling, showing that blocking signaling with cMET inhibitors can prevent excessive healing or adhesions from ovulation exposure. Previously, we found that the average concentration of HGF in FF is 48 times higher than that in serum, and the activation of HGF through the coagulation protease cascade in FF is maintained throughout the menstrual cycle.[17] Consistent with the ultra-high level of HGF, in FF and ultra-high level of cMET mRNA in the mesothelium (Human Protein Atlas) ovulation wounds demonstrate accelerated total healing rates, often within hours. This study further discovered that HGF/cMET-mediated adhesion formation happened at 16 h and 2.5 days after wounding or at the reepithelization and remodeling stage. Disturbance at this stage results in more severe adhesion formation than that in the early stage and cMET inhibition worked only during this period. Whether the same cMET inhibitor also prevents adhesions not related to ovulation deserves further investigation.

The results of this study may be translated clinically to prevent ovulation-derived intra-abdominal wound adhesions. In scheduling operations, the hazardous window, spanning three days before to one day after ovulation, should be avoided. Oral contraceptives can be administered to patients whose ovulation time cannot be determined by Naegale's rules of the menstrual cycle from the last menstrual period (LMP) to the previous menstrual period (PMP). Contents within the preovulatory follicle should be treated as contaminants. The concept of "dangerous follicles" should be considered during abdominal surgery. Practice based on this principle may include the following: (i) Routine examination of the follicle status of ovaries before any operative procedure. When there are signs of recent ovulation or in the case of follicle rupture during operation, the FF leak should be aspirated, and the exposed surgical areas should be thoroughly washed. (ii) Do not disturb growing follicles on the ovaries. (iii) To prevent subsequent ovulatory FF exposure during wound healing, the FF content in the Graafian follicle should be aspirated and cleared. Alternatively, medications, such as NSAIDs[41] or RU486[42] can be administered postoperatively to prevent or deter ovulation.

In this study, the oral administration of dabigatran, a prescription drug used for thromboembolic diseases,[43,44] inhibited FF-induced adhesions when administered in the early phase of wound healing. Dabigatran is an oral anticoagulant commonly used to inhibit thrombin. It inhibits fibrinogen to fibrin formation and thus prevents clot. However, the clinical use of this and other anticoagulants is contraindicated in fear of hemorrhage during surgery. Under the harshest FF exposure scenario, a single dose of AMG 337 administered 30 min before the operation completely prevented subsequent adhesion growth. Over a dozen cMET/HGF inhibitors are currently being tested for cancer therapy, and at least two have been approved by the FDA for multiple indications.[45]

The study also found that treatment with indomethacin failed to prevent adhesions from ovulatory FF exposure. Anti-inflammatory drugs, such as indomethacin or ibuprofen, have been found to reduce adhesions from inflammation-prone procedures, such as cecum abrasion, foreign body placement, or salpingostomy.[26] In these adhesion scenarios, the COX inhibitor prevents adhesions by blocking the synthesis of prostaglandins, ceases the early inflammation of the wound, and reduces adhesions. However, in the case of ovulatory FF exposure to the sterile miniscule wounds, inflammation or prostaglandin synthesis seems to play a minor role. For ovulatory FF, prostaglandin is synthesized, and acute inflammation occurs at the time of luteinization. At the time of FF exposure, COX is no longer active.[46]

In summary, exposure of surgical wound to ovulation contents causes adhesions in a three-day time window where coagulation cascade acts in the immediate-early phase and the thrombin/HGF cascade acts at the reepithelization and remodeling phases of wound healing. Upon

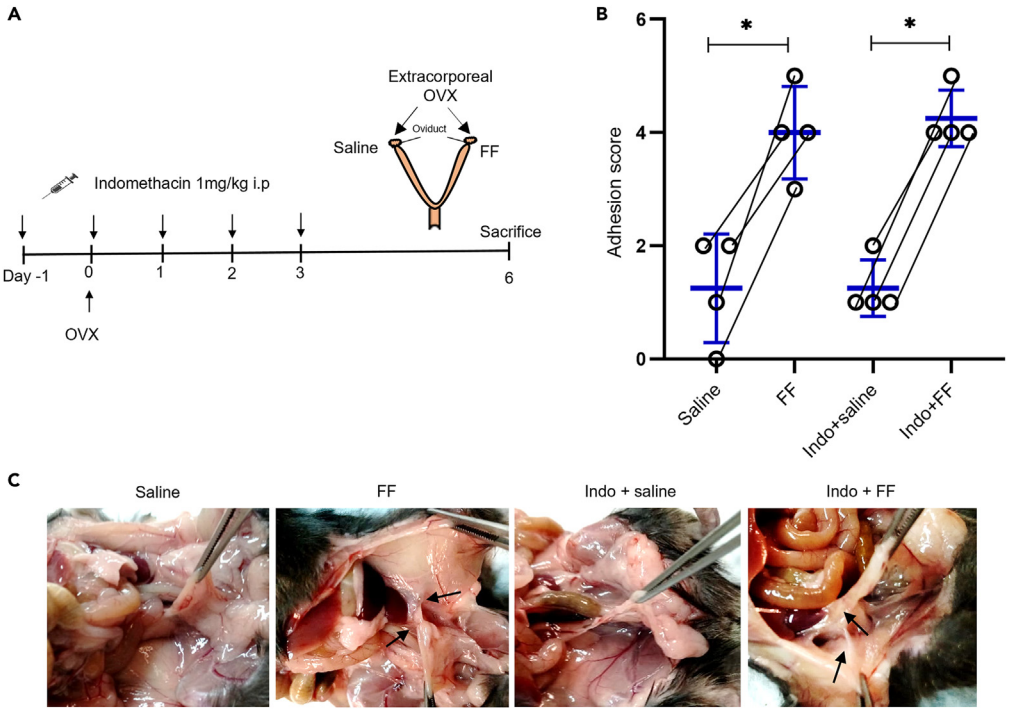

Figure 6. FF induced adhesions are independent of COX-mediated inflammation

(A) Schematic timeline showing the i.p. injection of indomethacin (Indo) or saline at different times relative to OVX surgery on bilateral adnexa each exposed with 100% FF vs. saline for 3 min. Mice were sacrificed 6 days post-surgery.

(B) Adhesion scores of the paired adnexal sides with different treatments. Significance determined using two-tailed, paired Student's t test. *$p < 0.05$, **$p < 0.01$

(C) Representative images showing the adhesion sites (Black arrows). $N = 4$ mice per group.

clinical translation, the results may lead directly to prevention measures of "ovulation-free" surgical scheduling and a concept of "dangerous follicle" during operation.

Limitations of the study

This study used different genetic and surgical models to investigate the effect of ovulation on wound healing, allowing the characterizing of both the effect and the mechanism and confirming the key findings. The limitation of this study is obviously from the preclinical setup of the study. Mouse differs from humans in histological structure and molecular profile in the epithelium,[47] although the difference in visceral organs may be less than the epidermis.[48] Mouse also carries a short ovulation cycle and may confound the observations. We tried to overcome it by performing OVX and exposing the wound to exogenous FF from IVF women, a cross-species and a non-natural source. Thus, clinical studies are needed for validation.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109788.

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AUTHOR CONTRIBUTIONS

TYC proposed the hypotheses, conceived and supervised the study, and provided resources. VS, CFH, and KS designed and performed experiments. TYC, VS, and CFH interpreted data. PCC and DCD provided essential patient samples and interpreted data. VS and TYC wrote and revised the article. All authors read and approved the final article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-MET	Bioss	Cat#BS-0668R; RRID:AB_10856924
anti-p-MET	Cell Signaling	Cat#3077; RRID:AB_2143884
anti-actin	Cell Signaling	Cat#4970; RRID:AB_2223172
Chemicals, peptides, and recombinant proteins		
Dabigatran	boehringer ingelheim	Pradaxa
AMG 337	Cayman Chemical	Cat#21333
PMSG	ProSpec	Cat#HOR-272
hCG	Sigma-Aldrich	Cat#C1063
Dade® Innovin®	SIEMENS	Cat#B4212-41
Critical commercial assays		
Trichrome Stain	ScyTek Laboratories	TGB-IFU
Experimental models: Cell lines		
MeT-5A	BCRC	#60352; RRID:CVCL_3749
Experimental models: Organisms/strains		
C57BL6/J mice	NLAC	Cat#RMRC1105
C57BL/6JNarl-Pgr^{tm}1Tyc/Tmc	NLAC	N/A
Software and algorithms		
GraphPad Prism (ver. 8.0)	GraphPad Software, Inc.	RRID:SCR_002798

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tang-Yuan Chu (hidrchu@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell culture

The human mesothelial cells, MeT-5A (BCRC #60352) was maintained in M199 media supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and 100 IU/mL penicillin, and 100 μg/mL streptomycin (P/S, Corning Inc., Corning, NY, USA).

Follicular fluid specimen

FF samples were collected from patients undergoing oocyte retrieval in an IVF program as previously described.[17,20,49,50] Aliquots from 50 cases were pooled, filtered, frozen at −80°C, and thawed before each experiment (Approval ID: IRB110-238-A).

Animals

Female wild-type C57BL6/J and progesterone receptor (*Pgr*) knockout C57BL/6JNarl-Pgr^{tm}1Tyc/Tmc mice, between 8 and 14 weeks of age, were obtained from the National Laboratory Animal Center (NLAC), Taiwan. Animals were housed at the Laboratory of Animal Center, Tzu Chi University for one week before the experiment and maintained on a 12-h dark/light cycle with a standard diet and water. This study was reported in line with the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) statements.[51] Each experiment was conducted in two rounds with at least two mice per round. The protocols were approved by the Institutional Animal Care and Use Committee of Tzu-Chi University (Approval ID:111-39).

Study design

Bursectomy or ovariectomy was performed in mice at day 0 followed by superovulation or normal ovulation or treatment with FF or inhibitors (AMG 337 or dabigatran) at appropriate time points as mentioned in the timeline of each figure. The animals were sacrificed on day 6 using CO_2 inhalation and examined for adhesions and scored.

METHOD DETAILS

Surgical procedure for bursectomy

Mice were anesthetized, and a lower dorsal side incision was made to enter the peritoneum, where the ovarian fat pad was pulled out using blunt forceps. Using a surgical microscope, the bursa was carefully peeled off around the ovary. After the bleeding check, the ovary was placed back into the peritoneum, and the wound was closed using 9 mm surgical wound clips. The same procedure was repeated on the opposite side. For sham surgery, the ovaries were exposed to the environment for 10 min and returned. The animals were placed on a heating pad until recovery from anesthesia.

Surgical procedure for ovariectomy

The ovary was exposed with a 1 cm dorsolateral incision, clamped between the oviduct and uterus using hemostat scissors, and held for a few seconds. The ovary was removed by cutting below the hemostat, the uterus was placed back to the peritoneum, and the wound was closed.

All the surgical procedures were performed in an aseptic environment. None of the animals died during the experiments. After the appropriate treatment, the animals were sacrificed on day 6 using CO_2 inhalation.

Adhesion score

The adhesions were scored by visualizing the gross morphology of the adhesions on each side of the adnexa in a group-blinded manner. Scores were given based on the severity and location as described in Table 1 and illustrated in Figure S1. 0 – no adhesion, 1 – thin filmy adhesion of the injured organ to adjacent fat, 2 – thin filmy adhesions of the injured organ to the adjacent peritoneum, 3–3.moderate adhesions of the injured organ to the peritoneum, which could be dissected with surgical forceps; 4 – severe adhesions of the injured organ to the peritoneum, which could be dissected only with scissors; 5 – injured organ deeply embedded into the peritoneum and/or to the colon, adipose tissue of the liver or spleen in such an extent that sharp dissection would injure and impair the function of the adhering organs.

Superovulation induction

Superovulation was induced by i.p. injection of PMSG 5 IU followed by HCG 5 IU after 48 h, and ovulation was expected after 12 h.[17]

Estrous cycle synchronization and observation of vaginal cytology

The estrous cycle of the mice was synchronized by the modified Whitten effect, as described in a previous study.[52] The experimental cages were exposed to soiled male mouse bedding for three days to attain the estrus phase on the third day. In the presurgical FF injection groups, we used the same method as previously described to prevent ovulation before surgery (48). Vaginal lavage fluid was collected twice (before FF injection and before OVX) by pipetting 100 μL of saline back and forth three times using a sterile tip. The collected fluid was smeared under a microscope, air-dried, and stained with crystal violet. The slides were then checked for the stages of estrous cycle.

Coagulation factor depleted FF

The coagulation factor-depleted FF (CD FF) was prepared as described in our previous study.[17] Briefly, an equal volume of FF was added to recombinant tissue factor solution (1 ×; Innovin, Dade Behring, Deerfield, IL) or ddH_2O and incubated overnight at 37°C to form a fibrin clot. The supernatant obtained was used for extracorporeal exposure and labelled CD FF and 50% FF.

Immunohistochemistry

To evaluate cMET expression levels in adhesion tissues, samples were randomly selected and paraffin-embedded. Sections (4 μm) were subjected to IHC staining using an UltraVision™ Quanto Detection System (Thermo Fisher, Waltham, MA). The sections were deparaffinized and hybridized with anti-Met primary antibody (BS-0668R, Bioss, Woburn, MA) at a 1:100 dilution, incubated at 4°C overnight and stained with

DAB. The stained sections were imaged under 4x, 10x, and 20x magnification using an inverted microscope (Axio Vert A1, Zeiss, Oberkochen, Germany.).

Masson's trichrome staining

To visualize tissue adhesion, trichrome staining was performed using a modified Masson's trichrome stain kit (ScyTek Laboratories, Logan, UT). In brief, after deparaffinization, the slides were incubated for 60 min in preheated Bouin's solution and washed with ddH_2O. Samples were then immersed in Weigert's iron hematoxylin solution for 10 min, followed by incubation with Briebrich Scarlet-Acid Fuchsin solution for 4 min. Samples were washed with ddH_2O after each step and then incubated with phosphotungstic/phosphomolybdic acid solution for 5 min, without rinsing. Samples were then incubated with aniline blue solution for 5 min, rinsed in 1% acetic acid solution for 3 min, further washed with ddH_2O, and then dehydrated with an ethanol gradient. After mounting, the sections were imaged under a light microscope.

Western blotting

The phosphorylated and non-phosphorylated forms of cMET expression were seen by western blotting. 30 μg of protein was separated on a 10% SDS-PAGE and transferred to the PVDF (Polyvinylidene fluoride) membrane. The membranes were then blocked with 5% non-fat milk powder in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for one hour followed by incubation with primary antibodies such as anti-MET (BS-0668R, Bioss), anti-p-MET (#3077, Cell Signaling), and anti-actin (#4970, Cell Signaling). After TBST wash, the blots were incubated with appropriate secondary antibodies, developed using ECL (EMD Millipore, MA, USA) and detected under a high-sensitivity biomedical imaging system UVP Chemstudio PLUS.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using GraphPad Prism version 8.0 and Microsoft Excel 2021 and the adhesion scores were presented as mean ± standard deviation. The comparison of adhesion scores between the left and right side of each mouse was performed by a two-tailed, paired Student's *t*-test and the comparison between groups was performed by a two-tailed, unpaired Student's *t*-test. $p < 0.05$ were considered statistically significant.