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Full-length Article

Perioperative escape from dormancy of spontaneous micro-metastases: A role for malignant secretion of IL-6, IL-8, and VEGF, through adrenergic and prostaglandin signaling

Rita Haldar [a], Lee Shaashua Berger [a,b], Ella rossenne [a], Arielle Radin [c], Anabel Eckerling [a], Elad Sandbank [a], Erica K. Sloan [d], Steve W. Cole [c], Shamgar Ben-Eliyahu [a,b,*]

[a] School of Psychological Sciences, Tel-Aviv University, Tel-Aviv, Israel
[b] Sagol School of Neuroscience, Tel-Aviv University, Tel-Aviv, Israel
[c] Departments of Medicine and Psychiatry and Bio-behavioral Sciences, David Geffen School of Medicine, UCLA, Los Angeles, CA, USA
[d] Monash Institute of Pharmaceutical Sciences, Monash University, Parkville VIC 3052, Australia

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ABSTRACT

We recently showed that a minimally-invasive removal of MDA-MB-231^{HM} primary tumors (PTs) and elimination of their secreted factors (including IL-6, IL-8, VEGF, EGF, PDGF-aa, MIF, SerpinE1, and M−CSF), caused regression of spontaneous micro-metastases into a non-growing dormant state. To explore the underlying mechanisms and potential clinical ramifications of this phenomenon, we herein used the MDA-MB-231^{HM} human breast cancer cell-line, *in-vitro,* and *in vivo* following orthotopic implantation in immune-deficient BALB/C nu/nu mice. Employing bioluminescence imaging, we found that adding laparotomy to minimally-invasive removal of the PT caused an outbreak of micro-metastases. However, perioperative β-adrenergic and COX-2 inhibition, using propranolol + etodolac, maintained metastatic dormancy following laparotomy. *In-vitro*, β-adrenergic agonists (epinephrine or metaproterenol) and prostaglandin-E2 markedly increased MDA-MB-231^{HM} secretion of the pro-metastatic factors IL-6, IL-8, and VEGF, whereas cortisol reduced their secretion, effects that were maintained even 12 h after the washout of these agonists. *In-vivo*, laparotomy elevated IL-6 and IL-8 levels in both plasma and *ex-vivo* PT spontaneous secretion, whereas perioperative propranolol + etodolac administration blocked these effects. Similar trends were evident for EGF and MIF. Promoter-based bioinformatics analyses of excised PT transcriptomes implicated elevated NF-kB activity and reduced IRF1 activity in the gene regulatory effects of laparotomy, and these effects were inhibited by pre-surgical propranolol + etodolac. Taken together, our findings suggest a novel mechanism of post-operative metastatic outbreak, where surgery-induced adrenergic and prostanoid signaling increase the secretion of pro-metastatic factors, including IL-6, IL-8, and VEGF, from PT and possibly residual malignant tissue, and thereby prevent residual disease from entering dormancy.

1. Introduction:

Dormant tumors and dormant metastases are usually small sized, avascular, and asymptomatic, and can remain in a state of growth arrest for years (Sargent et al., 2007; Saphner et al., 1996; Tsao et al., 1997; Crowley and Seigler, 1992). Several non-exclusive mechanisms for cancer dormancy have been suggested, including (i) cell quiescence, (ii) balance between proliferation and apoptosis, (iii) incomplete immune control over malignant tissue, and (iv) lack of vascularization, limiting oxygen and nutrients supply (reviewed in Aguirre-Ghiso, 2007; Sosa et al., 2014). Through yet unknown mechanisms, dormant micro-metastases can transform into a fast-growing state, resulting in disease recurrence. Therefore, understanding mechanisms that contribute to dormancy and/or escape from dormancy bears clinical significance, especially given the prevalence of dormant primary tumors (PTs) in the general population (Black and Welch, 1993; Harach et al., 1985), and dormant micro-metastases following PT excision (Sauer et al., 2021; Cackowski and Heath, 2022).

During the last fifteen years, progress has been made in developing animal models of PT dormancy based on (i) syngeneic cancers in mice (e.

* Corresponding author at: Sagol School of Neuroscience and School of Psychological Sciences, Tel Aviv University, Tel Aviv 69978, Israel.
E-mail address: Shamgar@tauex.tau.ac.il (S. Ben-Eliyahu).

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g., hepatocellular (Shachaf et al., 2004) and breast (Gattelli et al., 2004), and (ii) human cancer xenografts, including breast adenocarcinoma, osteosarcoma, and glioblastoma (Naumov et al., 2006). Unlike in the above models, in the clinical setting, following PT excision, dormancy mainly occurs in minimal residual disease, including metastases (Recasens and Munoz, 2019). We recently developed a model of spontaneous post-operative dormant metastases, also used in the current study, based on a human MDA-MB-231^{HM} breast cancer xenograft orthotopic transplantation in immune-deficient mice (Shaashua et al., 2020). Following PT removal with a minimal surgical procedure, spontaneously-growing micro-metastases in the lungs/lymph-nodes regress in size and transform into a latent, non-progressing state for at least 50 days (Shaashua et al., 2020). We further found that the PT secretes pro-metastatic factors that promote progression of its spontaneous early-stage metastases (but not larger metastases), and that combined *in-vivo* blockade of four of these factors (PDGF-AA, Serpin-E1, IL-8, and MIF) can arrest the growth of micro-metastases in the presence of the PT (Shaashua et al., 2020).

The excision of a PT is a lifesaving procedure in most types of solid cancers. However, surgery and other forms of stress have been shown to facilitate the growth of preexisting micro-metastases (dormant or growing) (Auer et al., 2011; Wang et al., 2022; Hiller et al., 2018; Onuma et al., 2020; Tsuchiya et al., 2003; Xu et al., 2018), through various mechanisms (Neeman et al., 2012; Horowitz et al., 2015; Rolls et al., 2015), many of which are activated through catecholamines (CAs) and prostaglandins (PGs) signaling (Neeman et al., 2012; Horowitz et al., 2015; McGregor and Antoni, 2009; Cole et al., 2015). For example, these signaling pathways were shown to (i) regulate the secretion of pro- and anti-inflammatory soluble factors (e.g. IL-6, CRP, TNFα and IL-10) (Hinson et al., 1996; Baumann and Gauldie, 1994; Elenkov et al., 2005; Madden et al., 2011), locally and systemically, and to (ii) suppress NK and *T*-cell cytotoxicity (Ben-Eliyahu et al., 2000; Andersen et al., 1998; Rosenne et al., 2014; Muthuswamy et al., 2017; Pockaj et al., 2004; Hellstrand and Hermodsson, 1989), thus promoting cancer metastasis. More recently, CAs and PGs were shown to directly impact tumor cells by promoting their growth (Coelho et al., 2015; Lee et al., 2009), motility (Masur et al., 2001), invasion capacity (Sood et al., 2006), resistance to cell death (apoptosis and anoikis) (Sood et al., 2010; Liao et al., 2010; Zhang et al., 2009), epithelial-to-mesenchymal transition (EMT) (Neil et al., 2008), and secretion of pro-angiogenic factors (Lee et al., 2009; Singh et al., 2006; Yang and Beattie, 2008; Le et al., 2016). Thus, the perioperative blockade of these pathways can be expected to restrict cancer progression through multiple mechanisms.

Indeed, *in-vitro* and pre-clinical *in-vivo* studies have indicated that β-adrenergic blockade and/or PGs synthesis inhibition can reduce the immune-suppressive and pro-metastatic effects of stress and surgery in several tumor cell-lines and models (Ben-Eliyahu et al., 2000; Lee et al., 2009; Masur et al., 2001; Sood et al., 2006; Le et al., 2016; Nagaraja et al., 2016; Yakar et al., 2003; Melamed et al., 2005; Inbar et al., 2011; Fujita et al., 2011; Goldfarb et al., 2011; Kim-Fuchs et al., 2014; Sloan et al., 2010; Shaashua, 2017). Specifically, in mice and rats, the perioperative use of the β-blocker, propranolol, and the COX-2 inhibitor, etodolac, was proven safe in the perioperative setting (Benjamin et al., 2010; Hazut et al., 2011), and reduced post-operative metastases and mortality rates (Goldfarb et al., 2011; Benish et al., 2008; Glasner et al., 2010; Sorski et al., 2016). Importantly, in some studies only the combined use of the two drugs was effective (Benish et al., 2008; Glasner et al., 2010; Sorski et al., 2016), specifically when using models of spontaneous metastasis following surgical removal of the PT. As CAs and PGs are both abundantly released perioperatively, and as each can promote metastasis through activating the cAMP-PKA pathway in immune or malignant cells (Masera et al., 1989; Whalen and Bankhurst, 1990; Torgersen et al., 1997); blocking only one of these pathways may not suffice to improve long-term cancer outcomes (Ben-Eliyahu, 2020; Ricon et al., 2019; Eckerling et al., 2021). In the MDA-MB-231^{HM} cell-line used herein, *in-vivo* knockdown of β_2-adrenoceptors significantly reduced the impact of chronic stress on metastatic progression, but not the impact of surgery (Chang et al., 2016). Overall, the above findings suggest the need of simultaneous blockade of both CAs and PGs in the context of tissue injury.

Given that dormancy of metastases may occur following removal of the PT (Sauer et al., 2021; Shaashua et al., 2020), and that stress and surgery are known to promote cancer metastasis, it is possible that surgical stress may prevent post-operative fast-growing-to-dormant transformation, and/or may facilitate escape from dormancy in established dormant foci (e.g. Krall et al., 2018; Zappalà et al., 2013). We hypothesize that one mechanism that may mediate such deleterious effects of surgery, and of excess CAs and PGs release, is over-secretion of pro-metastatic factors from the PT and/or residual disease. In the current study, employing the MDA-MB-231^{HM} human cell-line in immune deficient mice, we study this hypothesis using *in-vivo, ex-vivo*, and *in-vitro* approaches.

2. Materials and Methods:

2.1. Animals

Eight-week old female BALB/c nu/nu mice (Envigo, Israel) were housed under SPF conditions on a 12-h dark/ light cycle, in compliance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Animals were randomly assigned to the experimental conditions, and the experimenters were blind to group allocation when assessing outcome parameters. All procedures were approved by Tel-Aviv University Animal Ethics Committee.

2.2. Cell-line:

A highly metastatic variant of the triple-negative human breast adenocarcinoma cell-line, MDA-MB-231^{HM}, was transduced with a codon-optimized firefly luciferase-mCherry vector as previously described (Kaminskas et al., 2013). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10 % fetal bovine serum (FBS) unless specified otherwise, 1 % GlutaMAX (Thermo Fisher Scientific), 4.5 g/l D-glucose, and 110 mg/l sodium pyruvate. Cells were maintained at 37 °C and 5 % CO^2 as described elsewhere (Shaashua et al., 2020).

2.3. Breast MDA-MB-231^{HM} xenograft model of regression of spontaneous metastases

This model was recently described elsewhere by us (Shaashua et al., 2020). Briefly, MDA-MB-231^{HM} cells (2×10^5 cells in 20 μl PBS), labeled with luciferase-mCherry vector were injected into the 4th mammary fat-pad of eight-week old female BALB/c nu/nu mice under 2 % isoflurane anesthesia for PT formation. The growth of the PT and of spontaneous metastases were frequently monitored by bioluminescence imaging (BLI) using an IVIS100 spectrum apparatus (Perkin Elmer) 10 min following i.p. injection of 150 mg/kg D-luciferin sodium salt (Regis Technologies). PT removal was conducted under 2 % isoflurane anesthesia through a minimal incision to the skin without injuring the underling muscle and without administration of additional analgesic agents. The skin was then sutured, and complete removal was verified by BLI. Further metastatic development in lungs and axillary lymph-nodes was monitored thereafter through BLI in a similar manner (Fig. 1).Fig. 2.

2.4. Metastatic progression, dormancy, and the effects of surgery:

Once metastatic foci reached a total count of 10^6 photons/s (~3–4 weeks post-injection), the PT was removed, with or without additional laparotomy (as detailed in Glasner et al., 2010). Propranolol was injected s.c., 4 times, in 8 h intervals, and etodolac was injected s.c.

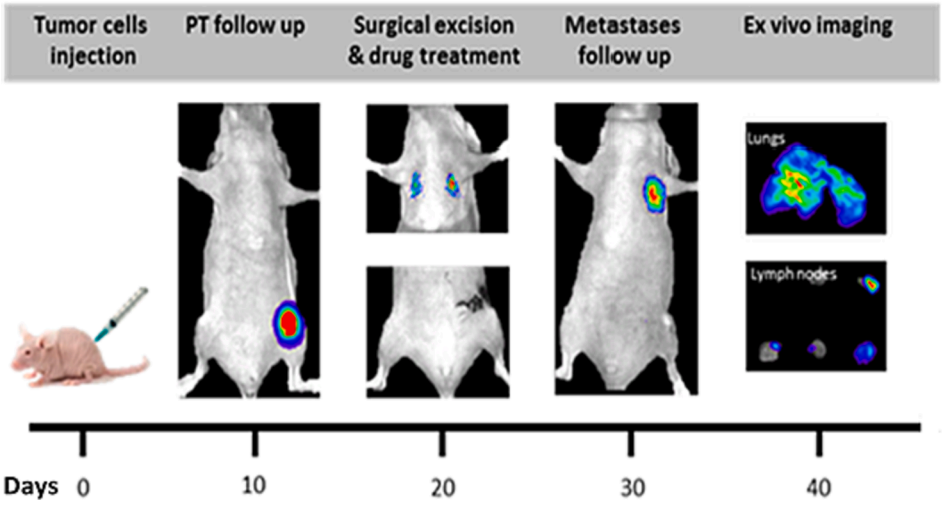

Fig. 1. Breast MDA-MB-231^{HM} xenograft model of regression of spontaneous metastases: MDA-MB-231^{HM} cells (2×10^5 cells in 20 μl PBS), labeled with luciferase-mCherry vector were injected into the 4th mammary fat-pad of female BALB/c nu/nu mice for PT formation. The growth of the PT and of spontaneous metastases were frequently monitored by bioluminescence imaging (BLI). Once metastatic foci reached a total count of 10^6 photons/s (~3–4 weeks post-injection), the PT was removed, with or without additional laparotomy, and with propranolol + etodolac or vehicle. PT removal was conducted under 2 % isoflurane anesthesia through a minimal incision to the skin without injuring the underling muscle. The skin was then sutured, and complete removal was verified by BLI. Long-term follow-up of metastatic progression was conducted thereafter through daily BLI. At the end of this experiment, animals were euthanized 10 min following luciferin injection, and lungs and lymph nodes were harvested for high-sensitivity *ex-vivo* BLI.

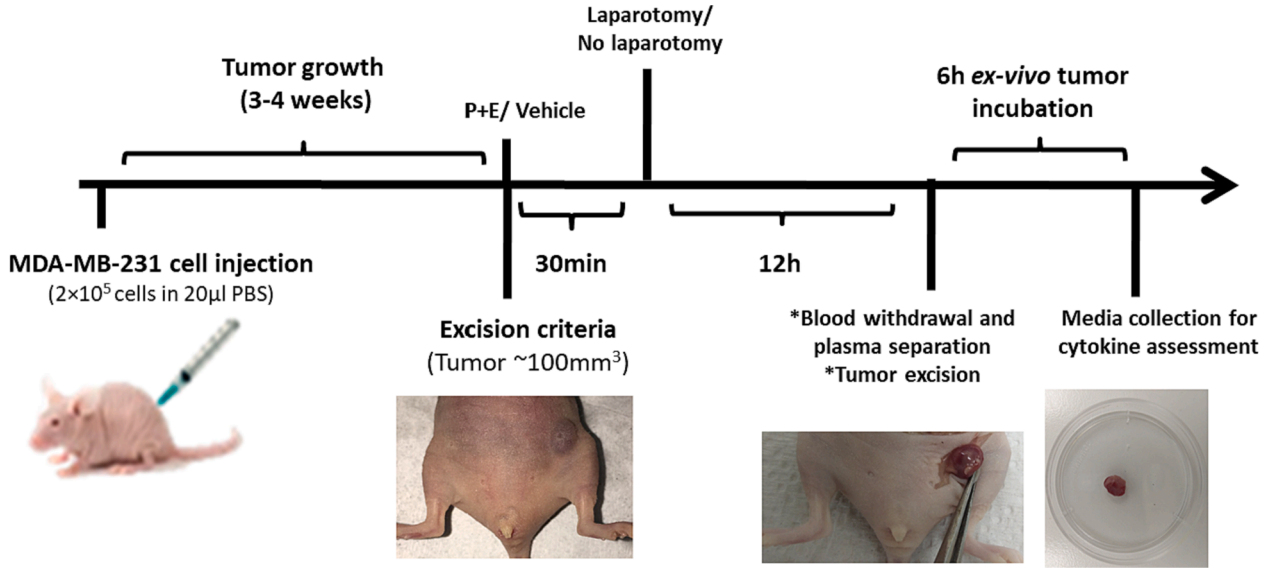

Fig. 2. *Schematic presentation of ex-vivo approach; Effects of surgical stress and its blockade on factors secreted by malignant tissue:* MDA-MB-231^{HM} cells (2×10^5 cells in 20 μl PBS), labeled with luciferase-mCherry vector were injected into the 4th mammary fat-pad of female BALB/c nu/nu mice for PT formation. Once the PT reached ~ 100 mm^3 (20–40 days following cell injection), animals were injected with propranolol + etodolac or vehicle, 30 min prior to laparotomy or no laparotomy. Twelve h after laparotomy/no laparotomy, animals were euthanized, and blood was withdrawn via cardiac puncture, and plasma was tested for human cytokines using human ELISA kits. In addition, PTs were removed for evaluation of their ex-vivo secretion. PTs were placed in media without FBS, and following 6 h incubation period, media was collected for assessment of human cytokines.

twice, in 16 h intervals, both starting 1 h prior to laparotomy. Control animals received vehicle injections. Long-term follow-up of metastatic progression was conducted thereafter through bi-weekly BLI. At the end of this experiment, animals were euthanized 10 min following luciferin injection, and lungs and lymph nodes were harvested for high-sensitivity *ex-vivo* BLI (Fig. 1).

2.5. *Effects of surgical stress and its blockade on factors secreted by malignant tissue:*

Primary tumors were measured by a caliper, and volume was calculated by the formula: (length × width^2) × 0.5. Once the PT reached ~ 100 mm^3 (20–40 days following cell injection), animals were injected with propranolol + etodolac or vehicle, 30–60 min prior to laparotomy (as detailed elsewhere[60]) or no laparotomy. Group assignment of each animal along tumors development was counterbalanced, and the mean duration from cell injection to PT removal, volume of removed tumors, and tumor weights, were equivalent between groups (tumor volume, mean = 104.43 mm^3, SE = 1.16; tumor weight, mean = 88 μg, SE = 2.97). Twelve hours after laparotomy/no laparotomy, animals were euthanized, and PTs were removed for evaluation of their *ex-vivo* secretion and mRNA gene expression. Specifically, MDA-MB-231^{HM} tumors were excised without interrupting the tumor capsule, weighted, and cut to quarters using a scalpel. One quarter of each sample was snap-frozen in liquid nitrogen and kept in −80 °C for whole-genome RNA sequencing analysis, and each of the other 3 quarters were placed in 24-well plates with 800ul of serum-free media (DMEM (Thermo Fisher Scientific) supplemented with 1 % GlutaMAX (Thermo Fisher Scientific), 4.5 g/l D-glucose, and 110 mg/l sodium pyruvate). Following 6 h incubation period, media was collected, centrifuged (4 °C, 5 min, 754 x

g), and kept in −80 °C until assayed using human ELISA kits. Secretions from the 3 quarters were summed, and divided by their combined weight (weight of 3 quarters of tumor, mean = 68.34 μg, SE = 2.1). In addition, at the time of tumor removal, blood was withdrawn via cardiac puncture using EDTA (1.8 mg/ml), centrifuged (4 °C, 20 min, 1126 x g), and plasma was kept in −80 °C for ELISA cytokine analyses. We employed human ELISA kits for assessment of cytokine levels, thus identifying PT secreted factors, rather than host-derived cytokines (see below).

We removed the PT 12 h after laparotomy to simulate and study important aspects of the clinical setting, specifically the impact of stress-inflammatory responses that start days before tumor removal (Hanalis-Miller et al., 2022); and surgical stress which starts several hours before the removal of the PT (e.g. in colorectal cancer (Huang et al., 2020)). Recent clinical trials reported that blocking CAs (Hiller et al., 2020) or blocking CAs and PGs (Shaashua et al., 2017; Haldar et al., 2018; Haldar et al., 2020; Ricon-Becker et al., 2022) signaling, during these days and hours that precede PT removal, affect pro-metastatic characteristics of the PT. Thus, herein we deliberately chose this 12 h lag, to enable the impact of surgery-induced stress-inflammatory responses on the malignant tissue.

2.6. *In vitro approach*

500,000 MDA-MB-231^{HM} cells/well were seeded in 6-well plates and cultured in 1 ml/well of growth media (DMEM media containing 10 % FBS, 1 % GlutaMax, 4.5 g/l D-glucose and 110 mg/l sodium pyruvate) for 24 h. Then, cells were washed 3 times with serum-free media (DMEM (Thermo Fisher Scientific) supplemented with 1 % GlutaMAX (Thermo Fisher Scientific), 4.5 g/l D-glucose, and 110 mg/l sodium pyruvate) and re-suspended in 1.2 ml/well of serum-free media. Stress/inflammatory agonists (30 μl/well) or vehicle were added for 12 h of incubations. Agonists included epinephrine (Sigma), metaproterenol (Sigma), PGE2 (Sigma), and cortisol in a 10-fold concentration gradient from 10^{-9} to 10^{-5}M. Conditioned media was collected, centrifuged (10 min, 393 x g, 4 °C) and kept in −80 °C for ELISA assessment of cytokine levels. All conditions were tested in triplicates. The same approach was used to study the effects of specific blockers (i.e. propranolol (Sigma-Aldrich), ONO-AE3-208 (Tocris), or RU-486 (Sigma), 30 μl/well at selected concentrations), which were added 30 min prior to their respective stress agonists. Removal of stress agonists was conducted by washing cells 3 times with serum-free media, and re-supplementation of wells with 1.2 ml/well of serum-free media for a second 12 h incubation period. Several studies were conducted twice, yielding very similar effects.

2.7. *Enzyme-linked immunosorbent assay*

Human ELISA kits (R&D Systems) were used to assess levels of IL-6, IL-8, VEGF, PDGF-aa, MIF, Serpin E1, EGF, and M−CSF, according to the manufacturer's instructions. As the human ELISA kits show no cross-reactivity to mice cytokines (except M−CSF that showed low cross-reactivity of 0.11 %, according to manufacturer), detectable levels in plasma and *ex-vivo* PT secretion can be ascribed to secretion by the human PT cells and/or its established metastases (i.e., by MDA-MB-231^{HM} cells).

3. Drugs and their administration

3.1. ***In vivo***: *Propranolol*

A nonselective β-adrenergic antagonist injected s.c. in slow-release emulsion (SRE); Propranolol (Sigma-Aldrich) was dissolved in a slow-release emulsion that consists of a mixture of PBS, mineral oil and mannide monooleate, in a 4:3:1 ratio, respectively. In experiments 1 and 2, studying the effects of laparotomy (experiment 1) and of drug treatment (experiment 2) on metastatic regression and outbreak, propranolol was injected s.c., 4 times, in 8 h intervals, starting one h prior to PT excision. The first injection was given in a dose of 5 mg/kg, while the second-fourth injections were given in a dose of 2.5 mg/kg. In the 3rd *in-vivo* experiment, studying the effects of surgery and of the drug treatment on plasma *ex-vivo* secretion of selected cytokines, propranolol was administered once, 30 min prior to surgery at a dose of 5 mg/kg. All injections were given in a total volume of 100 μl SRE. Control animals received equivalent schedule of SRE.

Etodolac

A semi-selective COX-2 inhibitor (Taro, Israel) was injected s.c. dissolved in corn oil. In experiments 1 and 2, etodolac was injected s.c. twice, in 16 hr interval, starting one hr prior to PT excision. The first injection was given in a dose of 50 mg/kg, while the second injection was given in a dose of 25 mg/kg. In experiment 3 etodolac was injected s.c once 30 min prior to surgery at a dose of 50 mg/kg. All injections were given in a total volume of 80 μl corn oil. Control animals received equivalent schedule of corn oil.

3.2. *In-vitro*

Agonists used include epinephrine, metaproterenol, PGE2, and cortisol (hydrocortisone – water soluble, Sigma-Aldrich) in concentration of 10^{-9} to 10^{-5}M. The antagonists used include propranolol, ONO-AE3-208, or RU-486 in concentration of 10^{-8} to 10^{-6}M. The range of concentrations used herein was chosen to correspond with *in vivo* systemic concentration of endogenous hormones. Specifically, systemic corticosterone and cortisol levels range from 10^{-8} to 10^{-6} M (Gotlieb et al., 2015), and the chosen *in-vitro* concentrations are 10^{-7}, $3x10^{-7}$, and 10^{-6} M. Epinephrine and prostaglandins are secreted *in vivo* both systemically and locally, ranging from 10^{-10} to 10^{-5} M or lower (Gotlieb et al., 2015). The chosen *in vitro* concentrations are 10^{-7} to 10^{-5} M for prostaglandin, and 10^{-8} to 10^{-6} M for epinephrine. Importantly, systemic levels of such hormones cannot be fully translated to the *in vitro* setting, given many biological differences (for more detailed information see Gotlieb et al., 2015).

3.3. *Tumor RNA profiling*

Tumor RNA profiling was conducted as previously described (Hiller et al., 2020). Briefly, total RNA was extracted from approximately 20 μg of frozen tumor tissue, was tested for suitable mass (RiboGreen) and integrity (Agilent TapeStation), reverse transcribed to complementary DNA (Lexogen QuantSeq 3′ FWD), and sequenced on an Illumina NovaSeq instrument (Lexogen Services, GmbH), following the manufacturers' standard protocols. Sequencing targeted > 5 million 89-nt single stranded reads per sample (achieved mean 9.1 million/sample), which were mapped to the GRCh38 human transcriptome and quantified as transcripts per million mapped reads using the STAR aligner. Transcript abundance values were $\log_2$-transformed for statistical analysis using standard linear statistical models to quantify the magnitude of differential expression across groups in a 2 (Surgery: minimally invasive vs extensive/laparotomy) × 2 (Treatment: vehicle control vs propranolol + etodolac) factorial design controlling for differences in tumor weight as a covariate. *A priori* hypotheses regarding activity of key pro-inflammatory transcription factors (NF-κB, AP-1, Sp1, OCT1, and STAT3) and anti-inflammatory transcription factors (IRF, ISRE, GR) were assessed using TELiS promoter-based bioinformatics analysis (Cole et al., 2020; Cole et al., 2005) of core promoter sequences from all genes that showed ≥ 1.5-fold difference in response to laparotomy (vs minimally invasive surgery) in drug-treated tumors vs placebo-treated tumors, using TRANSFAC position-specific weight matrices as described previously (Cole et al., 2020).

3.4. *Statistical analyses*

Factorial analysis of variance (ANOVA), with a pre-determined

significance level of 0.05, was conducted. Provided significant group differences were found, Fisher's protected least significant difference (Fisher's PLSD) contrasts were performed to test pair-wise post hoc comparisons. Student's *t* test was performed for comparing two experimental conditions (following F-test of equality of variance). All statistical tests were two-sided. Mauchly's Test of Sphericity was used, and in any case of sphericity violation the Huynd-Feldt Correction was used. All *in-vivo* experiments contained 7–8 animals per group, based on prior experiments and expected effect size. *In-vitro* studies were conducted in triplicates or more. In *ex-vivo* incubation experiments, two samples from each group were suspected for contamination, and removed from further analyses.

4. Results:

1 The impact of surgery on escape from dormancy of metastatic foci following PT excision.

Animals bearing both PTs and verified metastases (10^6 photons/s in bioluminescent imaging (BLI)), were subjected to a minimally-invasive or an extensive surgery (laparotomy) to remove the PT, after which metastatic progression was monitored by BLI up to 25 days post-surgery. Laparotomy, in addition to the minimal surgery necessary for PT removal, induced the outbreak of early-stage micro-metastases, as evident 25 days post PT removal (Fig. 3, $p < .05$). On day 1 post-operatively, group differences did not reach statistical significance, but we hypothesize that the long-term impact is ascribed to processes initiated immediately post-operatively.

2 The impact of treatment with the β-blocker, propranolol, and the COX-2 inhibitor, etodolac, on metastatic outbreak.

A pre-operative administration of propranolol + etodolac (vs vehicle) was conducted prior to laparotomy/no laparotomy, and metastatic progression was monitored thereafter. As in the previous experiment, laparotomy induced metastatic outbreak ($p < .05$), which was prevented by the combined drug treatment ($p < .05$, Fig. 4), as was evident by BLI as early as day 14 following PT excision.

3 *In-vitro* baseline secretion of cytokines.

We recently established[15], using this model, that factors secreted from the PT have a crucial role in promoting early metastatic growth. Out of 359 secreted proteins identified in tumor secretome, 8 were investigated based on their *in-vivo* and/or *in-vitro* secretion by the PT[15, 82] and literature reports of their pro-metastatic properties (detailed in Discussion): IL-6, IL-8, VEGF, PDGF-aa, EGF, M−CSF, Serpin-E1 and MIF. In the current study, we tested the release of these 8 cytokines from MDA-MB-231 tumor cells *in-vitro*, and found that supernatant levels of

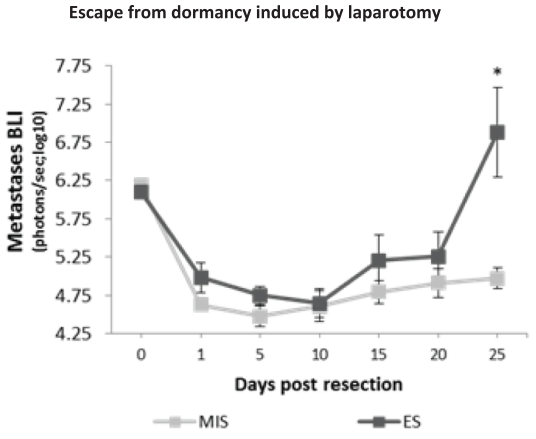

Fig. 3. *The impact of extensive surgery on metastatic foci escape from dormancy post PT excision*: *In-vivo* quantification of metastatic progression over time by BLI following extensive surgery (ES – laparotomy) or minimal surgery (MIS), (n = 5–6 per group). All data represent mean ± SEM. * $p < 0.05$.

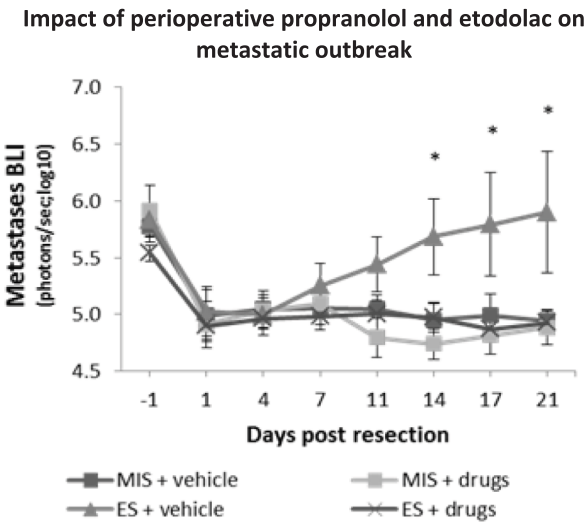

Fig. 4. *Propranolol and etodolac attenuated metastatic outbreak of* MDA-MB-231^{HM} spontaneous metastases following extensive surgery: tumor-bearing mice were repeatedly monitored for metastatic progression using BLI. Once metastatic foci reached a total count of 10^6 photons/s (~3–4 weeks post-injection), the PT was removed, with additional laparotomy (i.e. extensive surgery, ES) or without it (i.e. minimal invasive surgery, MIS). In addition, animals were injected with propranolol and etodolac (i.e. drugs) or vehicle. (n = 5–6 per group). All data represent mean ± SEM. * $p < 0.05$.

EGF and M−CSF were below detection, whereas IL-6, IL-8, VEGF, PDGF-aa, Serpin-E1 and MIF were within detection levels, (Fig. 5).

4 Adrenergic and prostanoid signaling, but not cortisol, facilitate *in-vitro* secretion of IL-6, IL-8, and VEGF: Dose dependent effects and their blockade.

Using an *in-vitro* approach to study how adrenergic and prostanoid signaling modulate the secretion of the above 8 cytokines, we employed epinephrine and metaproterenol (a β-adrenergic agonist), PGE2, cortisol, and their respective antagonists (propranolol, ONO-AE-208, and RU-486). EGF and M−CSF were below detection levels in this experiment as well. Epinephrine, metaproterenol, and PGE2 caused dose-dependent elevations in IL-6 (Fig. 6A), IL-8, and VEGF levels (Supplementary S1, $p < .001$ for all). These effects were blocked by the respective antagonists, propranolol and ONO-AE-208, ($p < .01$ for all, Fig. 6B and Supplementary S1). No effects of the agonists or the antagonists were evident in PDGF-aa, MIF, or SerpinE1 ($p < .4$ for all, Supplementary S1,). Cortisol caused a reduction in IL-6 and IL-8 levels

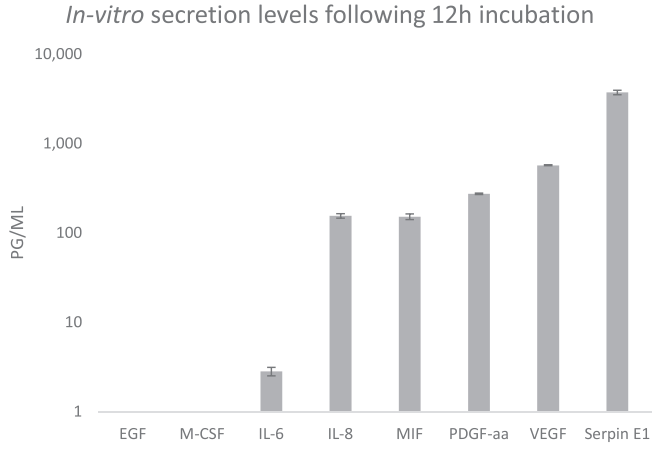

Fig. 5. *In-vitro secretion levels of 500,000 MDA-MB-*231^{HM} *cells following 12 h incubation.* Data presented in mean pg/ml ± SEM. n = 3 biological replicates for each cytokine. The experiment was repeated twice.

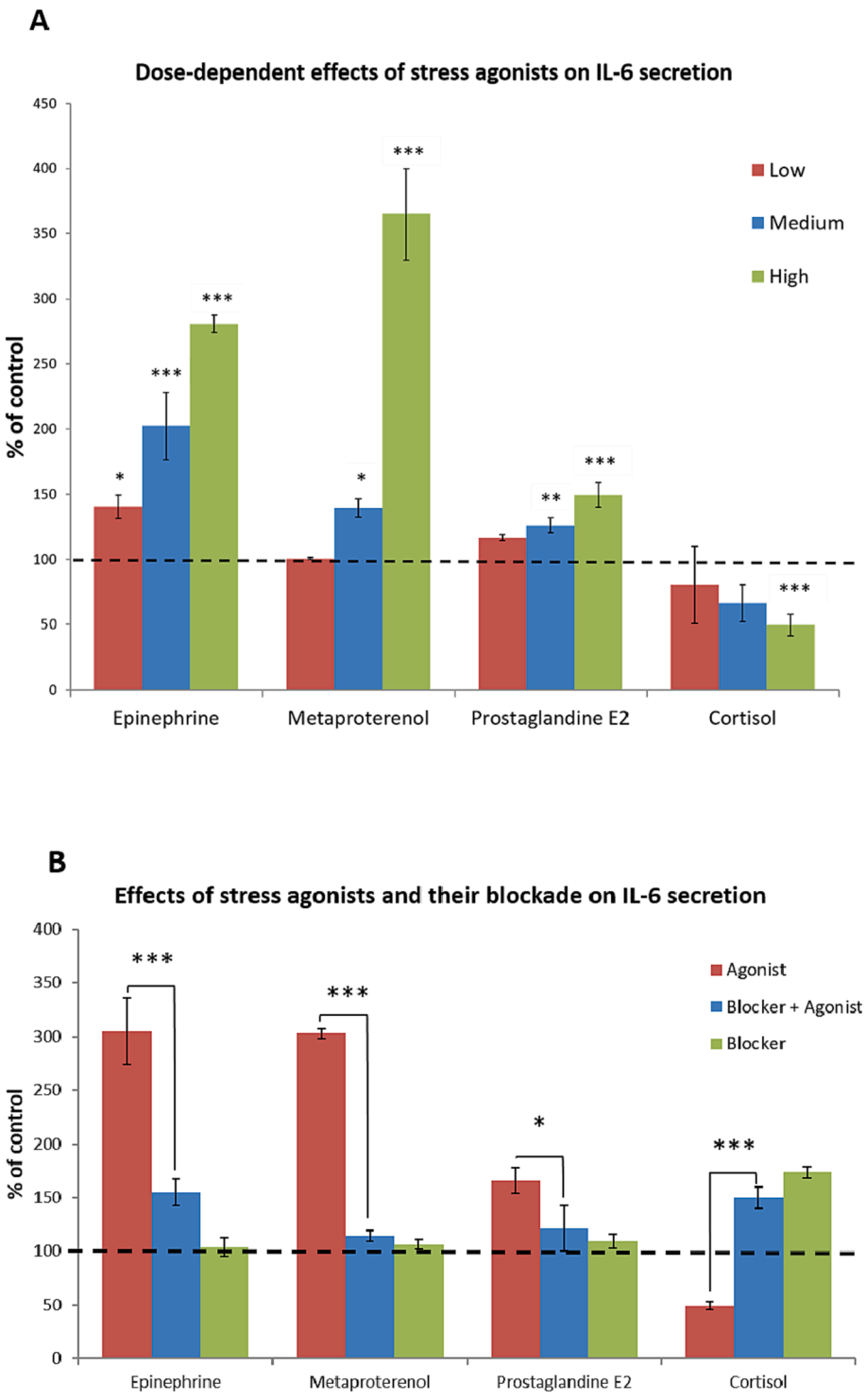

Fig. 6. *In-vitro effects of stress agonists and their blockade on MDA-MB-231^{HM} cells secretome.* (A) *Dose dependent effects of stress hormones on IL-6 secretion.* Epinephrine doses – 10^{-8}M (low), 10^{-7}M (medium), and 10^{-6}M (high). MP doses – 10^{-9}M (low), 10^{-8}M (medium), and 10^{-7}M (high). PGE2 doses – 10^{-7}M (low), 10^{-6}M (medium), and 10^{-5}M (high). Cortisol doses – 10^{-7}M (low), $3*10^{-7}$M (medium), and 10^{-6}M (high). (B) *Effects of stress agonists and their blockade on IL-6 secretion.* Epinephrine and metaproterenol were blocked with propranolol at a dose of 10^{-8}M, PGE2 was blocked with ONO-AE3-208 at a dose of 10^{-6}M, and cortisol was blocked by RU-486 at a dose of 10^{-6}M. Data presented as % of control ± SEM. Group differences are indicated by * ($p < .05$), ** ($p < .01$), or *** ($p < .001$). n = 3 biological replicates per experimental group. The experiment was repeated twice.

($p < .0001$ and $p = .0035$), which was blocked by the blocker RU-486 ($p < .0001$, Fig. 6A-B and Supplementary S1).

5 Long-lasting effects on the secretion of IL-6, IL-8, and VEGF following the removal of stress-inflammatory agonists.

Here we tested whether the effects of adrenergic, prostanoid, and glucocorticoid factors are long-lasting and maintain their impact on *in-vitro* secretion of IL-6, IL-8, and VEGF following their washout. Results indicated that following 12 h of incubation with epinephrine, metaproterenol, PGE2, and cortisol, and their consequent complete removal (see Methods), *MDA-MB-231^{HM}* cells maintained their elevated/decreased secretion of these 3 cytokines, with similar effect sizes to those evident in the presence of the agonists ($p < .0069$ for all, Fig. 7A-C). The increase in secretion levels of VEGF was even greater following the removal of epinephrine and metaproterenol ($p < .0001$ for both, Fig. 7C).

6 Plasma levels and *ex-vivo* secretion of cytokines that potentially mediate the effects of surgery on the outbreak of dormant micrometastases.

To study *in-vivo* whether surgical stress elevates the secretion of the eight cytokines, MDA-MB-231^{HM}- PT bearing mice were monitored for PT growth. Once PTs reached removal criteria (volume of 100 mm^3), animals were treated with propranolol + etodolac or with vehicle, and

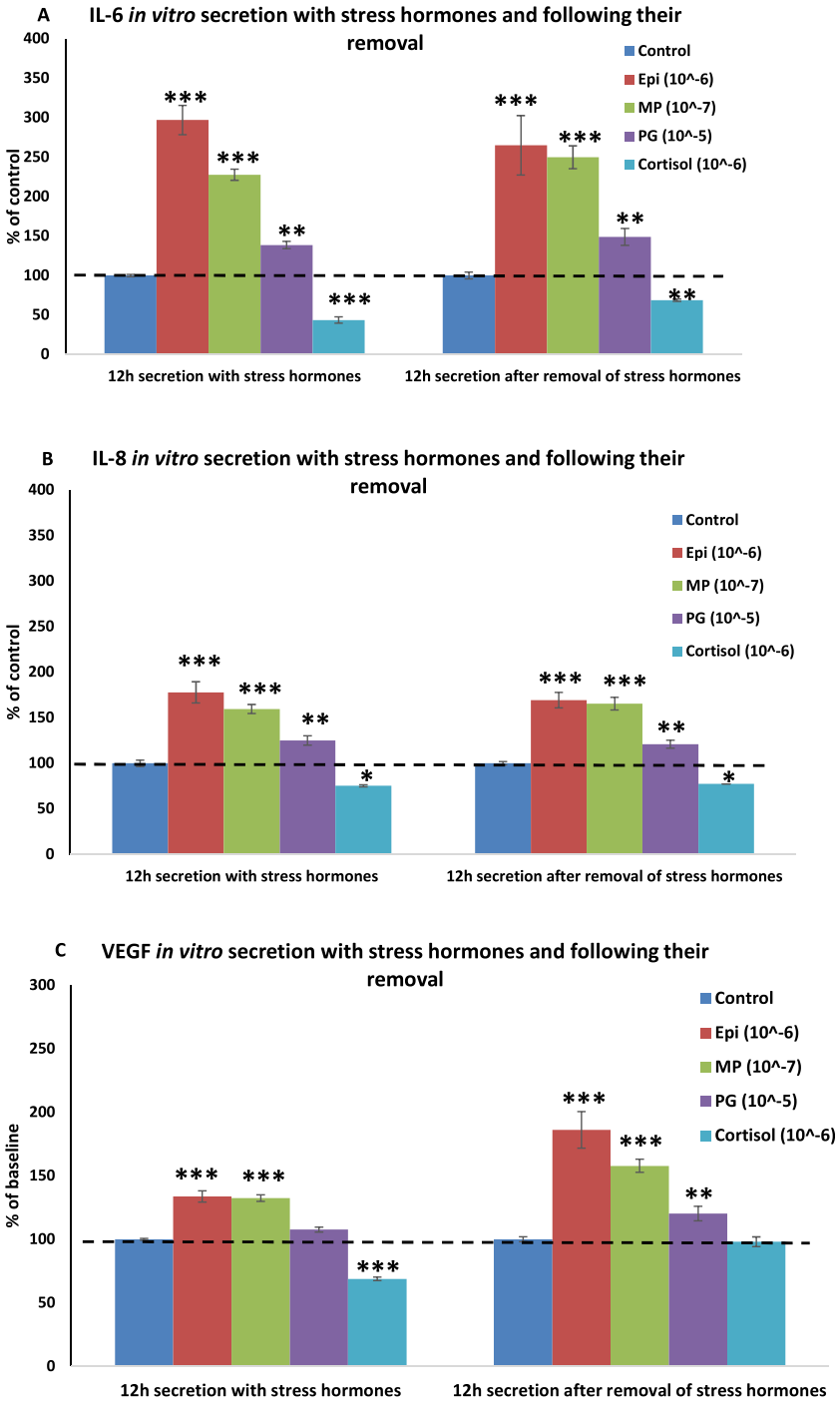

Fig. 7. *Secretion of pro-metastatic cytokines by MDA-MB-231^{HM} cells following the removal of stress agonists; long-lasting effects:* Tumor cells maintained their elevated secretion of (A) IL-6, (B) IL-8, and (C) VEGF (cortisol maintained its inhibitory impact), with similar effect sizes to those evident in the presence of the agonists ($p < .0069$ for all). Doses: Epinephrine 10^{-6}M, MP 10^{-7}M, PGE2 10^{-5}M, Cortisol 10^{-6}M. Data presented as % of control ± SEM. Differences from control group are indicated by * ($p < .05$). n = 3 biological replicates per experimental condition. The experiment was repeated twice.

30 min later underwent laparotomy/no laparotomy. Twelve hours later, animals were sacrificed, blood was drawn, and the PTs were removed and incubated *ex-vivo* in serum-free medium to assess their post-operative secretion of cytokines. Given the *in vitro* findings, we assessed plasma levels of IL-6, IL-8, and VEGF, and chose to study *ex-vivo* secretion of all 8 cytokines.

Analyses of plasma levels indicated an interaction between laparotomy and propranolol + etodolac treatment in IL-6 and IL-8 ($p = .02$ and $p = .07$ respectively, Fig. 8A-B). Post-hoc analyses indicated that within vehicle control groups, laparotomy elevated IL-6 levels ($p = .04$, Fig. 8A), and within laparotomy groups, propranolol + etodolac treatment reduced it ($p = .03$, Fig. 8A). Similar trends appeared for IL-8, but failed to reach significance (Fig. 8B). No conclusive results were available for plasma VEGF due to low detection levels in some samples and limited amount of plasma. *Ex-vivo,* 6 h incubation of excised PTs in serum-free media indicated an interaction between laparotomy and propranolol + etodolac treatment in the secretion of IL-6 and IL-8 ($p = .035$, $p = .054$ respectively, Fig. 8C-D). Post-hoc analyses of IL-6 levels within vehicle groups, laparotomy elevated IL-6 levels ($p = .049$, Fig. 8C), and within laparotomy groups, propranolol + etodolac treatment reduced it ($p = .02$, Fig. 8C). Similar trends appeared for IL-8 (as well as EGF and MIF; interaction $p = .095$, $p = .07$ respectively), but failed to reach significance (Fig. 8E-F). Laparotomy elevated SerpinE1 levels (Supplementary S2, $p = .075$), no effects were evident in VEGF and PDGF-aa (Supplementary S2, $p > .46$ for both), and M−CSF was below detection.

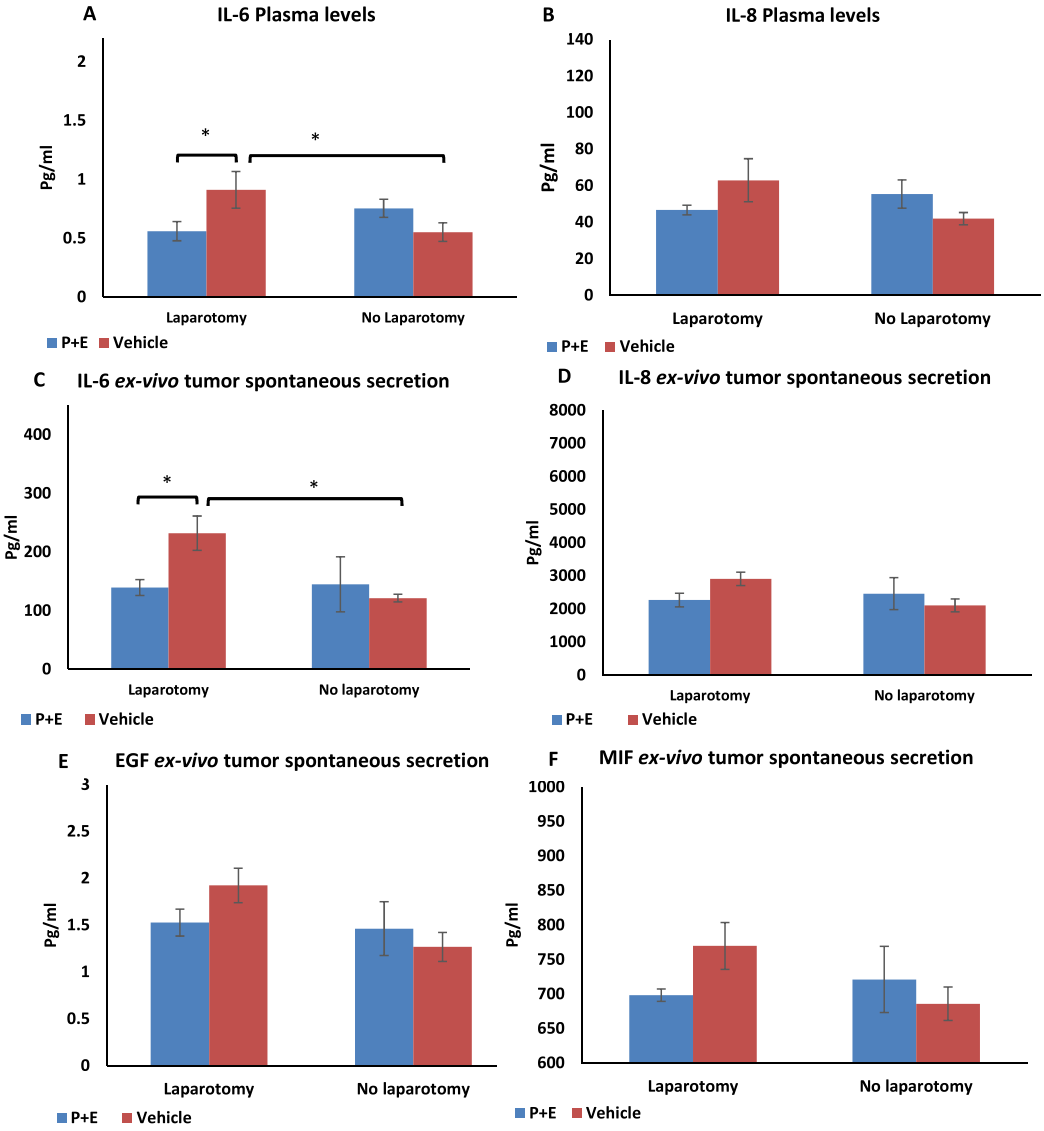

Fig. 8. Ex-vivo secretion of pro-metastatic cytokines by MDA-MB-231^{HM} primary tumors following laparotomy and drug treatment: PT bearing mice were treated with propranolol + etodolac or with vehicle, and 30 min later underwent laparotomy/no laparotomy. Twelve hours later, animals were sacrificed, blood was drawn for plasma analyses, and the PTs were removed and incubated *ex-vivo* for 6 h in serum-free medium. Plasma cytokine levels were assessed for(A) IL-6 and (B) IL-8. *Ex-vivo* cytokine PT secretion levels of(C), IL-6, (D) IL-8, (E) EGF and (F) MIF were also evaluated. In (A-B), n = 7–8 per experimental group. In (C-F) n = 5–6 per experimental group. Data is presented as % of control ± SEM. Group differences are indicated by * ($p < .05$).

7 Transcriptional regulation of pro-metastatic cytokine responses, inflammatory responses, and cortisol:

To identify specific transcriptional pathways that may mediate the effects of surgical stress on PT cytokine production, excised PT from the experiment described in section 6 underwent whole-genome RNA sequencing, following by promoter-based bioinformatics analyses. All 246 gene transcripts showing > 1.5-fold transcriptional response to laparotomy (164 up-regulated and 82 down-regulated) were considered in the analysis, focusing on transcription factors known to drive pro-metastatic cytokine responses (NF-κB, AP-1, Sp1, and C/EBP), immune responses (IRF1 and OCT1), and cortisol (GRE). Results (see Table 1 and Fig. 9) implicated increased NF-κB activity in structuring the transcriptome-wide alterations in tumor cell gene expression response to laparotomy (75 %±SE 17 % greater prevalence of NF-κB response elements in up- vs down-regulated promoters, p =.0006; Fig. 9, black bars), and this effect was substantially inhibited by pre-surgical propranolol + etodolac treatment (38 % ± SE 8 % decrease, p =.0024; Fig. 9, gray bars). Results also linked laparotomy to a marginal reduction in IRF1 activity (-18 % ± SE 11 %, p =.066), and pre-surgical propranolol + etodolac to elevated IRF1 activity (39 % ± 15 % increase, p <.0001). In addition, while laparotomy did not affect Sp1 activity (2 % ± SE 14 %, p =.91), pre-surgical propranolol + etodolac caused a significant reduction in Sp1 activity (29 % ± SE 12 % decrease, p =.0001). Last, laparotomy did not affect OCT1 (17 % ± SE 14 %, p =.22), while pre-surgical propranolol + etodolac caused a significant elevation in OCT1 (52 % ± SE 9 % increase, p <.0001). No effects of surgery or drug treatment were evident regarding AP1, STAT3, and GRE (see Table 1 and Fig. 9, all p >.16). All these effects are consistent with elevation of metastatic progression by surgery, and/or their attenuation by the drug treatment, as is elaborated in the discussion.

5. Disscussion and Conclusions:

Metastatic recurrence can occur even decades following successful tumor removal (Sauer et al., 2021), and dormant cancer foci have been suggested to underlie metastatic relapse (Recasens and Munoz, 2019),

Table 1

Transcriptional activity associated with pro-metastatic cytokine response, inflammatory responses, and cortisol: Whole-genome RNA sequencing was performed on excised tumors from propranolol and etodolac or vehicle treated animals that underwent laparotomy or not. TELiS promoter-based bioinformatics analysis of core promoter sequences from all genes that showed ≥ 1.5-fold difference was performed. Table shows full name of transcription factor (TF) and effect sizes of laparotomy (Lap) (n = 7) relative to control (n = 7), and laparotomy + drugs (n = 8) relative to laparotomy + vehicle (n = 7). Data presented as effect size ± SE. Drugs – propranolol and etodolac.

TF Name	TFBM	Effects of Lap vS control		Effects of Lap + Drugs vS Lap	
		Effect size ± SE	p-value	Effect size ± SE	P-value
NF-kB; Nuclear Factor kappa B	V$CREL_01	75 % ±17	**0.0006**	37 % ±8	**0.0024**
AP-1; Activator Protein 1	V $AP1_Q6_01	−11 % ±11	0.2848	−3% ±6	0.1607
STAT3 - Signal Transducer and Activator of Transcription 3	V $STAT3_01	0 % ±53	1	29 % ±36	0.4069
Sp1; Specificity protein 1	V$SP1_01	−2% ±14	0.9054	−31 % ±9	**0.0001**
IRF1; Interferon Regulatory Factor 1	V$IRF1_Q6	−18 % ±11	0.0666	21 % ±8	**0.0000**
OCT1; Octamer Transcription Factor 1	V$OCT1_03	17 % ±14	0.2224	69 % ±9	**0.0000**
GRE; Glucocorticoid Response Elements	V$GRE_C	4 % ±27	0.8853	45 % ±21	0.0779

emphasizing the importance of understanding mechanisms mediating escape from dormancy (Zappalà et al., 2013). Herein, we have established that following PT removal, if conducted by a minimal surgical procedure, spontaneous metastases may regress and remain dormant for prolonged periods, whereas a major surgery (laparotomy) can induce an outbreak of these metastases. Additionally, surgery, or *in-vitro* adrenergic and prostaglandin signaling, elevated the secretion of pro-metastatic cytokines from malignant tissue/cells, including IL-6, IL-8, and VEGF, and this elevated secretion was evident even hours following termination of exposure to adrenergic and prostaglandin signaling (*in-vivo* or *in-vitro*). Corresponding effects were evident in transcriptional activity of the excised tumors. Taken together, this study indicates that surgery can promote post-operative transition of MRD to a progressive state, potentially through elevating secretion of pro-metastatic factors from the PT and MRD, and thereby promoting cancer recurrence or progression.

We recently reported that MDA-MB-231^{HM} cells secrete IL-6, IL-8, VEGF, EGF, PDGF-aa, MIF, serpinE1 and M−CSF, cytokines which can promote metastatic outbreak (Shaashua et al., 2020; Haldar et al., 2019). Herein, levels of IL-6, IL-8, and VEGF were markedly elevated in response to surgery (*in-vivo*) and/or *in-vitro* pharmacological adrenergic and PG signaling. EGF and MIF showed the same pattern of effects, not reaching statistical significance, and the other three cyokines were not affected or were below ELISA detection levels. Thus, based on known pro-metastatic impacts of these 8 candidates (Shaashua et al., 2020; Jones and Jenkins, 2018; Ellis and Hicklin, 2008; Laoui et al., 2014; Sasaki et al., 2013), their blockade may reduce metastatic progression in general. The current work suggests that IL-6, IL-8, and VEGF (and potentially also MIF and EGF) are key candidates for mediating the metastasis-promoting effects of surgery and their attenuation by β-adrenergic and COX-2 inhibition. The involvement of IL-6, IL-8, and VEGF in cancer progression was suggested in several cancer types and in cancer patients. For example, in colorectal cancer patients, serum levels of IL-6 and IL-8 were significantly higher than in healthy volunteers (Malicki et al., 2009), and tumor tissue expressed higher level of IL-8-mRNA than in normal mucosa (Malicki et al., 2009). Serum IL-8 and VEGF levels were suggested to be prognostic markers (Bunger et al., 2011), and high tumor VEGF expression was associated with reduced survival (Bruhn et al., 2014). Interestingly, vascularization of colorectal micro-metastases is significantly increased 3 months following PT excision (Peeters et al., 2004; Peeters et al., 2006), presumably as a result of a drop in anti-angiogenic (Peeters et al., 2005) and an increase in pro-angiogenic (Kong et al., 2010) factors following PT removal. In patients with melanoma, lung, hepatocellular, or renal cell carcinomas, serum IL-8 levels were associated with tumor burden, stage, survival,

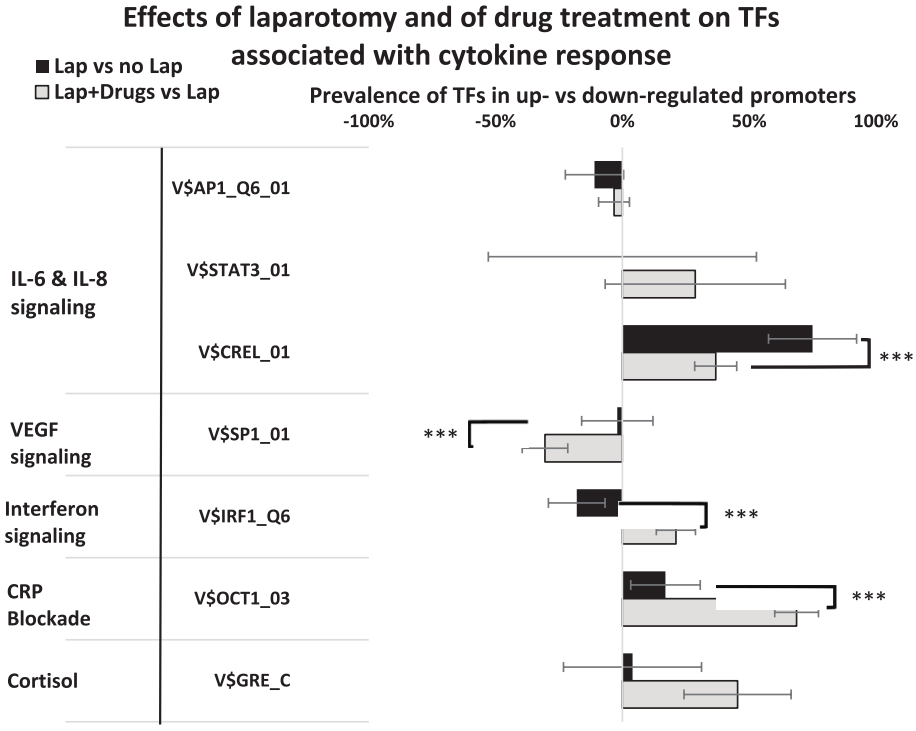

Fig. 9. *Effects of laparotomy and of propranolol and etodolac treatment on TFs associated with cytokine response:* Whole-genome RNA sequencing was performed on excised tumors from propranolol and etodolac or vehicle treated animals that underwent laparotomy or not. *A priori* hypotheses regarding activity of key pro-inflammatory transcription factors (NF-κB, AP-1, Sp1, OCT1, and STAT3) and anti-inflammatory transcription factors (IRF, ISRE, GRE) were assessed using TELiS promoter-based bioinformatics analysis of core promoter sequences from all genes that showed ≥ 1.5-fold difference in response to laparotomy vs control. Data is presented as relative prevalence of TFs in up- vs down-regulated gene promotors, in % ± SEM. n = 7–8 per experimental group. Group differences are indicated by *** (p <.001). TF – transcription factors. Lap – laparotomy. Drugs – propranolol and etodolac.

and objective responses to therapy (Sanmamed et al., 2014). In lung cancer patients, VEGF levels increased within the first 24 h after pulmonary surgery, and in a mouse model using Lewis lung carcinoma, VEGF was suggested to play a role in the rapid growth of dormant micrometastases (Maniwa et al., 1998). Last, in breast cancer patients, higher serum levels of IL-6 (Zhang and Adachi, 1999), IL-8 (Benoy et al., 2004), and VEGF (Banys-Paluchowski et al., 2018) were associated with poorer prognosis. Given that IL-6, IL-8, and VEGF are broadly associated with cancer progression, the current study suggests new mechanisms underlying post-operative outbreak of metastasis, through surgery-induced excess secretion of these cytokines from malignant tissue, and the prevention of malignant dormancy.

Herein, laparotomy increased *in-vivo* and *ex-vivo* secretion levels of IL-6 and IL-8 (and potentially of EGF and MIF) from the PT, and previous studies linked the extent of surgery with the secretion levels of these cytokines. Specifically, in an animal model of colon cancer, laparotomy increased serum IL-6 levels more than laparoscopy (Shiromizu et al., 2000). In a non-cancerous animal model, the incision length of laparotomy correlated with serum IL-6 levels (Ishibashi et al., 2006). In colorectal cancer patients (in a RCT), serum levels of IL-6 and IL-8 were increased 1–6 h post-operatively in patients undergoing open surgery, but not in patients undergoing laparoscopic surgery (Siekmann et al., 2017). In patients undergoing surgery for benign adnexal masses, IL-6 serum levels (but not IL-8) were increased 4 h, 24 h, and 48 h following surgery, and to a significantly higher levels in patients undergoing laparotomy than laparoscopy (Torres et al., 2007). Similarly, in patients undergoing abdominal non-cancer surgery, *ex-vivo* secretion levels of IL-6 (but not IL-8) increased in peritoneum samples taken one hr following open surgery compared to samples taken immediately on surgery initiation, but no such increase occurred if laparoscopy was conducted (Yahara et al., 2002). Overall, it seems that the extent of surgery influences the secretion of the pro-metastatic cytokines IL-6 and IL-8, from cancer cells and from other cell types, with higher secretions evident in more extensive surgeries. In the current study, increased plasma levels of these cytokines are attributed to PT secretions, which are human cytokines specifically assessed by human ELISA kits, rather than to a systemic response to the surgery from non-cancerous host tissue.

In the current study, perioperative use of the β-blocker propranolol and the COX-2 inhibitor etodolac attenuated surgery-induced *ex-vivo* and *in-vivo* PT secretion of IL-6 and IL-8 (and possibly also EGF, and MIF), and arrested the surgery-induced escape from dormancy of MRD evident herein. Thus, this study indicates that *in-vivo* neuroendocrine signaling can stimulate pro-inflammatory and pro-metastatic factors from tumors (mostly shown in cell lines Madden et al., 2011; Lutgendorf et al., 2003; Yang et al., 2009; Hu et al., 2017; Clark et al., 2018), and extends those findings by suggesting a key *in-vivo* role for this pathway in supporting the survival and growth of MRD.

Recently, in breast (Shaashua et al., 2017; Haldar et al., 2018) and colorectal (Haldar et al., 2020) cancer patients, we showed that propranolol and etodolac improve several biomarkers associated with cancer recurrence, including serum IL-6 and CRP levels, as well as tumor EMT status and GATA1, GATA3, STAT3, and GRE activity (Shaashua et al., 2017). In addition, IL-6, IL-8, and VEGF were recently shown to be reduced post-operatively by propranolol in patients with ovarian, fallopian tube, or primary peritoneal cancers (Thaker et al., 2017). As higher serum levels of IL-6 (Zhang and Adachi, 1999), IL-8 (Benoy et al., 2004), and VEGF (Banys-Paluchowski et al., 2018) were associated with poorer prognosis in breast cancer patients, their perioperative attenuation by propranolol and etodolac may have significant clinical ramifications.

Looking for potential mechanisms underlying the elevated PT secretion of IL-6, IL-8, and VEGF by adrenergic and inflammatory signaling, we studied *in-vivo* the activity of several transcription factors in the excised PT following surgery and pre-operative treatment with propranolol + etodolac. Laparotomy elevated NF-κB activity in excized tumors, whereas pre-surgical propranolol + etodoloac treatment reduced NF-κB and Sp1 activity. NF-κB is a major pro-inflammatory driver suggested to have a tumor-promoting impact in most cancer types (Ben-Neriah and Karin, 2011). In breast cancer, NF-κB pathway regulates gene expression of IL-6 and IL-8 (Choi et al., 2019), and in MDA-MB-231 cells, high NF-κB activity was associated with the expression of VEGF mRNA, while its inhibition resulted in reduced VEGF expression (Shibata et al., 2002). Sp1 is a prominent transcription factor involved in carcinogenesis via numerous mechanisms (Vizcaíno et al., 2015), incuding induction of VEGF (Pore et al., 2004); and *in-vivo* experiments support its key role in controling VEGF-related tumor growth and microvessel formation (Su et al., 2017). Interestingly, aspirin, a widely-used NSAID, was shown to arrest tumor growth in a colon cancer mouse xenograft model, and was accompanied by downregulation of Sp1 proteins and decreased expression of VEGF and NF-κB (Pathi et al., 2012), similar to the herein results using propranolol + etodolac. Taken together, the reduction of NF-κB and Sp1 by propranolol and etodolac can be suggested to bear clinical advantages.

Also, laparotomy reduced IRF1 activity, whereas pre-surgical propranolol + etodolac treatment significantly elevated IRF1, ISRE, and OCT1 activity. OCT-1 acts as a transcriptional repressor of CRP expression, a protein whose concentrations increase under conditions of inflammation, stress, or cellular injury (Voleti et al., 2012), and predicts poor cancer prognosis (Haldar et al., 2018). Thus, high levels of OCT1 are expected to reduce CRP levels and metastasis. Indeed, in a clinical trial in breast cancer patients, we found that perioperative treatment with propranolol and etodolac elevated OCT1 and reduced systemic levels of CRP, as well as molecular tumor biomarkers of cancer progression (Shiromizu et al., 2000; Ishibashi et al., 2006). However, OCT1 is associated with poor cancer prognosis in breast cancer patients (Ogura et al., 2021), and it is thus not clear whether its high expression levels are causally related to prometastatic processes, or merely associated with other factors driving poor prognosis. IRF1 is a transcription factor in the IFNγ signaling pathway known for its tumor supressor activity (Nicolini et al., 2006), which binds to ISRE and promotes gene transcription (Mancino and Natoli, 2016). In invasive breast carcinoma, higher IRF1 expression was negatively correlated with tumor grade (Connett et al., 2005). Similarly, high IRF1 tumor expression was associated with improved DFS and OS in ovarian cancer patients (Zeimet et al., 2009).

We suggest that simultaneous neutralization of IL-6, IL-8, and VEGF during the critical perioperative period should be studied as a novel anti-metastatic treatment. Attempts to neutralize specific cytokines (e.g. anti-VEGF (Aalders et al., 2017; Choi et al., 2015) or anti-IL-6 (Rossi et al., 2015) treatments) have been previously made with limited effectiveness in cancer patients, and such systemic treatments bear adverse effects (Pichler and Campi, 2007). Nevertheless, we believe that a focus on the critical perioperative period (Horowitz et al., 2015; Ben-Eliyahu, 2020), on simultaneous neutralization of several secreted factors, and on employing malignant-targeted therapies (e.g. nanoparticles (Awasthi et al., 2018)), may have greater impact on long-term cancer outcomes. Moreover, we have shown herein that a pre-operative treatment with propranolol and etodolac attenuated the secretion of these pro-metastatic cytokines from the malignant tissue, as well as arrested metastatic outbreak. Clinically, propranolol and etodolac treatment have already been tested in clinical trials and found safe and effective (e.g. Eckerling et al., 2021; Haldar et al., 2020), but approximately 50 % of patients are excluded due to contraindications to these medications, and thus targeted blockade of tumor secretion of pro-metastatic cytokines could be considered as an alternative approach in the perioperative context.

Our *in-vitro* approach to study the effects of stress agonists on the secretion of pro-metastatic cytokines has several limitations, including the absence of the tumor microenvironment, which was repeatedly shown to interact with tumor cells and affect tumor secretome (Paltridge et al., 2013). Also, *in-vitro* doses used may markedly differ from relevant

in-vivo doses, which may dramatically differ between systemic and local levels. Thus, as our *in-vitro* studies are inherently limited (also see Gottlieb et al., 2015), we have conducted *ex-vivo* and *in-vivo* studies that yielded parallel findings with greater ecological and clinical relevance. Another limitation is the use of immunodeficient mice, which is necessary for employing this xenograft model, but does not enable a more comprehensive and ecological settings. On the other hand, this model has several advantages, including our ability to detect in the plasma of mice cytokines that are secreted only from the human PT, as well as the study of a human cancer line. Last, pre-clinical assessment of the separate and combined blockade of IL-6, IL-8, and VEGF in the perioperative context should be conducted in future studies, in this model and in immune competent models.

Taken together, in the current study we suggest a novel mechanism of post-operative escape from metastatic dormancy, meditated through surgery-induced excess cytokine secretion. Specifically, surgery through CAs and PGs signaling, triggers PTs, and potentially residual disease, to secrete higher levels of factors that support the growth of micro-metastases, including IL-6, IL-8, and VEGF, preventing post-operative transformation of MRD to a dormant state, and/or causing metastatic escape from dormancy. Perioperative treatment with propranolol and etodolac was shown effective in attenuating such pro-metastatic processes in both pre-clinical and clinical studies, but clinically this treatment is currently contraindicated in approximately 50 % of the patients (Shaashua et al., 2017). Thus, directly targeting an array of specific malignant-tissue-secreted factors in the perioperative context, through targeted therapy, may constitute a novel and potentially more effective clinical approach to overcome post-operative cancer recurrence. Tumor-related IL-6, IL-8, VEGF and other pro-metastatic factors may be identified even before surgery through proteomic/transcriptomic analyses of biopsies secretome, enabling personalized treatments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.01.005.

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