Tissue Factor signals airway epithelial basal cell survival via coagulation and PAR1/2

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ONLINE DATA SUPPLEMENT

Methods:

Cellular immunofluorescence analysis: Cells grown on glass coverslips in 6-well plates or cytospun onto glass slides were fixed in 4% paraformaldehyde (PFA) for 10 minutes, rinsed in TBS, and permeabilized with 0.4% Triton-X-100 in 10 mM sodium citrate for 20 minutes. After blocking in 5% donkey serum for 20 minutes, cells were incubated with non-specific IgG or individual primary antibodies specific to: anti-human annexin V (Abcam, Cambridge, MA), Muc5AC (Affinity Bioreagents, Golden, CO.), MUC5B (Santa Cruz Biotechnology, Santa Cruz CA), keratin 5 (Covance, Emeryville, CA), or gamma-tubulin (Sigma Chemical Co., St. Louis MO) for 1 hour. Secondary antibodies were conjugated with Alexa-488 or Alexa-594 (Invitrogen, Grand Island, NY) and incubated for 60 minutes. Cells were mounted on slides with Prolong Gold-DAPI and allowed to dry overnight. Slides were viewed using a Zeiss Axiovert 200M fluorescent microscope and digital images recorded using Axiovision software (Carl Zeiss, Inc, Thornwood, NY).

TF activity assay: TF activity was assayed as Factor VII (FVII)-dependent activation of Factor X (FX) as described previously (1). Primary epithelial cultures were plated on 96-well plates at a density ranging from 0 – 400 cells/well and allowed to grow for 24 hr. Wells were rinsed with HBSA (137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, 10 mM HEPES, 0.1% BSA) and then incubated at 37°C with 100 μl of HBSA containing a 1:50 dilution of isotype-matched control antibody or anti-TF antibody. For TF activity inhibition studies, cultures were treated with a buffer containing 300 mM L-arginine in 20 mM sodium citrate pH 5.5 (vehicle) or 5 μg/ml TFPI (Novartis, Basel Switzerland). After a 30-minute incubation, the wells were rinsed and incubated with 105 μl of an HBSA solution containing 20 nM FVII, 300 nM FX, and 12 mM $CaCl_2$. After

30 minutes, 100 µl of the solution was transferred to a new 96-well plate containing 25 µl of 25 mM EDTA. Twenty-five µl of S-2765 FXa substrate (6 mM; Chromogenix; Bedford, MA) was added to each well and absorbance was measured after 2 min incubation at 37 °C. TF activity was expressed relative to the activity of standards produced by serial dilution of Innovin recombinant human thromboplastin reagent (Dade, Miami FL). The activity of the undiluted standard (1x, by manufacturer's specifications) was designated as 1,000 units of relative TF activity.

RNA abundance probes: Housekeeping controls included 18S (Hs99999901_s1) and beta-actin (Hs99999903_m1). Specific mRNA probes: tissue factor (TF, Hs01076032_m1); tissue factor pathway inhibitor (TFPI, Hs00196731_m1); thrombin (Hs01011988_m1); antithrombin (Hs00166654_m1); coagulation factor V (Hs00914120_m1); and coagulation factor VII (Hs01551992_m1); coagulation factor IX (Hs01592597_m1); coagulation factor X (Hs00173450_m1); coagulation factor XIII (Hs00173388_m1); protease activated receptor 1 (PAR1, Hs00169258_m1); and protease activated receptor 2 (PAR2, Hs00173741_m1).

Cycle conditions were: 95°C for 12 minutes, [95°C for 15 seconds, 60°C for 1 minute] x 40 cycles. Differential gene expression was determined using a Step One Plus Sequence Detection System and relative expression calculated by the ΔCT method (2).

Scanning electron microscopy (SEM): Human airway epithelial basal cells were cultured at ~10,000 cells/cm^2 on 14 mm coverslips. On the next day of plating treatments were performed and cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 4 h, rinsed four times and stored in 0.1 M sodium cacodylate buffer (pH 7.4). Samples were dehydrated in graded ethanol solutions (50%, 70%, 80%, 90%, 100%, 100%) for 5 min each, then chemically

dried once in 50% and then twice in 100% hexamethyldisilazane for 5 min each. Samples were air dried overnight at room temperature in a desiccator. A thin film (~10 nm) of gold was sputtered on samples prior to imaging. Images were taken with an accelerating voltage of 5 KV and at a 6 mm working distance on a JEOL 7000 FE scanning electron microscope. Each experimental condition was imaged in ten positions at magnifications of 2,000x and 25,000x. Brightness and contrast were adjusted on SEM images to optimize viewing. No other image manipulation was performed.

Western blot: Cell lysates were prepared in lysis buffer (20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM Na_3VO_4, protease inhibitor cocktail (Calbiochem, La Jolla, CA), 1 mM PMSF and 1% NP-40. Protein concentration was determined using the BioRad DC (detergent compatible) protein assay kit (Bio-Rad, Hercules, Ca) in a 96-well plate with bovine serum albumin as a standard. Western blots were performed as previously described (3) and membranes were probed with rabbit polyclonal antibodies against TF (Affinity Bioreagents, Golden, CO) at 1:1,000 dilution, overnight at 4°C. Blots were washed with TBS-T and incubated with secondary peroxidase-conjugated IgG (Bio-Rad, Hercules, CA) at 1:2,000 dilution for 1 h at room temperature. Immunoreactive bands were detected using an ECL detection kit (Pierce, Rockford, IL) followed by exposure to Hyperfilm (Amersham Pharmacia Biotech Inc. UK).

Determination of cell survival and death: The MTT assay was performed as described (4). Caspase 3/7 activity was measured by using Caspase-Glo^R 3/7-assay kit (Promega Corporation, Madison, WI). Annexin binding was assessed by using Vybrant apotosis assay kit (Molecular Probes, Eugene, OR) (5). In this method, apoptotic cells bearing phosphatidylserine on the

plasma membrane outer leaflet were identified as those binding Alexa Fluor 488-labeled annexin V and necrotic cells were identified as those that incorporate DNA intercalating Sytox Green dye.

Determination of cellular proliferation: HAEC were seeded on 24-well plates at a density of 1.0×10^4 cells per well in BEGM. The next day, cells were transduced with lentiviral TF shRNA. 1.0 μCi of [*methyl*-^{3}H] thymidine (NEN Life Science Products, Boston, MA) was added after 24 h, and cells were incubated for 48 h. Incorporated radioactivity in total cell lysates was determined using a Beckman LS6500 counter as described previously (6).

Determination of PAR activity: PAR-induced intracellular calcium mobilization was measured by using a fluorescence technique as described previously (7, 8). Cells cultured at 30, 000 cells/cm^2 in collagen-coated 96-well microtiter plates were loaded with 5 μM Fluo-3-AM (Molecular Probes) for 90 min. Plates were washed five times to remove unincorporated dye. Subsequent steps were performed with a Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices). Test compounds were added, and cells were monitored for 20 min to detect agonist activity. Net peak calcium, expressed in arbitrary fluorescence units, was measured.

Legends to Supplemental Figures

Figure E1. A. Cultured human airway epithelial cells express TF. To determine if cultured human tracheobronchial airway epithelial cells (HAEC) express tissue factor (TF), passage 1 cells were allowed to adhere to collagen-coated culture dishes for 24 hours and immunostained for TF. Panel A shows differential interference contrast (DIC) image of staining of HAECs and panel B shows immunofluorescence staining (green) for TF. **B-D**. ***Basal cell-associated tissue factor activity requires added Factor X (FX) and Factor VII (FVII).*** Basal cells were allowed to adhere to collagen-coated plates for 24 hours. TF activity was determined by the S-2765 FXa substrate assay in the presence of $CaCl_2$. Data are presented as the mean ± SEM (n=6). * $p<0.05$ for medium vs. cultures. NS – not significant. B. TF activity as a function of cell number. C. TF activity in the presence of added Factor X and in the absence of Factor VII. D. TF activity in the presence of added Factor VII and in the absence of Factor X.

Figure E2. Effect of collagen on TF activity. To determine if collagen (coated (1.0 μg/cm^2) on assay plates, A or soluble (4.0 μg/ml) in assay, B) affects TF activity, TF activity assay was performed as described in legend to Figure E1 and in the Methods Section. Symbol (-■-) represents time course of TF activity in absence of collagen and (-□-) represents time course of TF activity in presence of collagen. Symbols (-●-) (-▲-)(-▼-) indicate negative controls where TF, FX or FVII respectively, were omitted from the TF assay in presence of collagen. Data are presented as the mean ± SEM (n=8).

Figure E3. TF inhibition causes basal cell apoptosis. A. Basal cells were allowed to adhere to collagen-coated plates for 24 hours and treated with diluent (Dil) or TFPI for 18 hours. Cell death was measured post treatment.

B. Caspase 3/7 activity was measured in culture medium and using the Caspase-Glo^{R} 3/7 assay. Caspase 3/7 release indicates the luminescence (RLU) with the background signal subtracted. Data are presented as the mean ± SEM (n=6). *$p<0.05$ for diluent vs. TFPI treatment.

C. Annexin V staining of diluent (Dil) and TFPI treated cultures. Annexin V (red), DAPI (blue). The images are representative of three independent experiments performed with cells isolated from airways of three different donors.

D. PE-labeled Annexin stained cells were evaluated by flow cytometry and the mean fluorescence (MFI) quantified. * $p<0.05$ for Dil vs. TFPI.

Figure E4. Effect of TFPI on Caspase 8 and TF phosphorylation. (A) Basal cells were allowed to adhere to collagen-coated plates for 24 hours and treated with diluent or TFPI for 6 or 18 hours. Caspase 8 was measured post treatment as described in the Methods. Tumor necrosis factor, TNF (10 μg/ml for 6 h) was used as a positive control. Data are presented as the mean ± SEM (n=6). *$p<0.05$ for diluent vs. treatment (TFPI or TNF).

(B) To assess TF phosphorylation by TFPI, basal cells were cultured and treated as described above and cell lysates were prepared. Treatment with PAR1 and PAR2 agonists were included as positive controls. Western was performed using anti-human phospho-TF antibody or anti-human TF antibody. The figure shows a representative blot.

(C) Quantitative analysis of TFPI-dependent TF phosphorylation analysis. Image includes Western blots for pTF and TF of three different donor cells treated with TFPI. Data are presented as the mean ± SEM (n=3).

Figure E5. Basal cells express mRNA of clotting cascade components. Basal cells were cultured on collagen-coated plates for 24 hours. A subset of cultures was mechanically injured (scratch stimulus to assess potential induction) using a pipette tip, incubated for 24 hours, followed by RNA preparation (A). Expression of various clotting cascade mRNA was determined by quantitative RT PCR. Individual mRNAs were normalized to 18S rRNA, and fold change relative to human liver RNA was calculated. (B) Tissue factor mRNA abundance. (C) Tissue factor pathway inhibitor (TFPI) mRNA abundance. (D) Thrombin mRNA abundance. (E) Anti-thrombin mRNA abundance. (F) Factor V mRNA abundance. (G) Factor X mRNA abundance. (H) Fibrinogen gamma chain mRNA abundance. Cells isolated from airways of three different donors were evaluated. Data are presented as mean ± SEM (n=9). * $p<0.05$ for control vs. scratch.

Figure E6. Scanning electron microscopic (SEM) analysis of fibrin networks on the surface of basal cells. Basal cells were cultured on collagen-coated glass coverslips for 24 hours. One set of cultures were then treated with diluent, peptide Gly-Pro-Arg-Pro (GPRP) or TFPI for 30 min. Another set of cultures were grown on collagen-coated plastic, tranduced with non-target (NT) or TF-specific shRNA for 24 hours. The cultures were then treated with $CaCl_2$ in HBSA and various additives: 1) no factors (control); 2) fibrinogen (Fbn); 3) FBN, prothrombin (prothr), Factor VII (FVII), Factor X (FX). Cells were then fixed and processed for SEM.

A. SEM analysis. Low magnification (mag) images of basal cells treated with the agent(s) indicated at the top of each column. 2000x, scale bar = 10 μm. Arrows: white, tight network fibrin fibers on the cell; filled white arrowheads, smooth cell surface; open arrowheads, fibrin networks of fibrin formed from exogenously added fibrinogen; open arrows, larger fibrin aggregates.

B. SEM image to show fibrin network formation on the basal cell surface. (11,000X). White arrowhead indicates the ruffled cell surface that is due to fibrin polymers. Scale bar = 10 μm.

Figure E7. tPA dose response of basal cells. Basal cells were allowed to adhere for 24 hours, and then treated for 18 hours with diluent or different concentrations of tissue plasminogen activator (tPA). Apoptosis and cell number were assayed. (A) Caspase 3/7 activity was measured in culture medium and using Caspase-Glo^{R} 3/7 assay. Caspase 3/7 release indicates luminescence (RLU) with the background signal subtracted. (B) Cell number was determined using the MTT assay. Each data set represents three independent experiments performed with cells isolated from three different donors. Data are presented as mean ± SEM (n=8). *$p<0.05$ for diluent vs. tPA.

Figure E8. PAR activation protects against TF expression inhibition-induced apoptosis. Basal cells were allowed to adhere for 24 hours and pretreated with PAR agonists as described in the legend to Figure 6. Cells were transduced with NT- or TF-shRNA. Fresh media containing agonist was added 24 h and Caspase 3/7 release measured 48 h post transduction. Data are presented as mean ± SEM (n=6). * $p<0.05$ vs. NT-shRNA and #$p<0.05$ vs. TF-shRNA.

Figure E9. TF-dependent fibrin network formation and PAR activation cooperate to promote basal cell survival. Basal cells were allowed to adhere for 24 hours and pre-treated with a PAR1 antagonist (3-mercaptopropionyl-F-Cha-Cha-RKNDK-amide) or a PAR2 (FSLLRY-Amide) antagonist. Two hours later, fibrin network formation was evaluated in the presence of FITC-labeled fibrinogen (Fbn) and $CaCl_2$ or Fbn + Prothrombin (Prothro) + factor VII (FVII) + Factor X (FX). A merge of the DIC (transmitted light) and the green fluorescence image is shown.

References:

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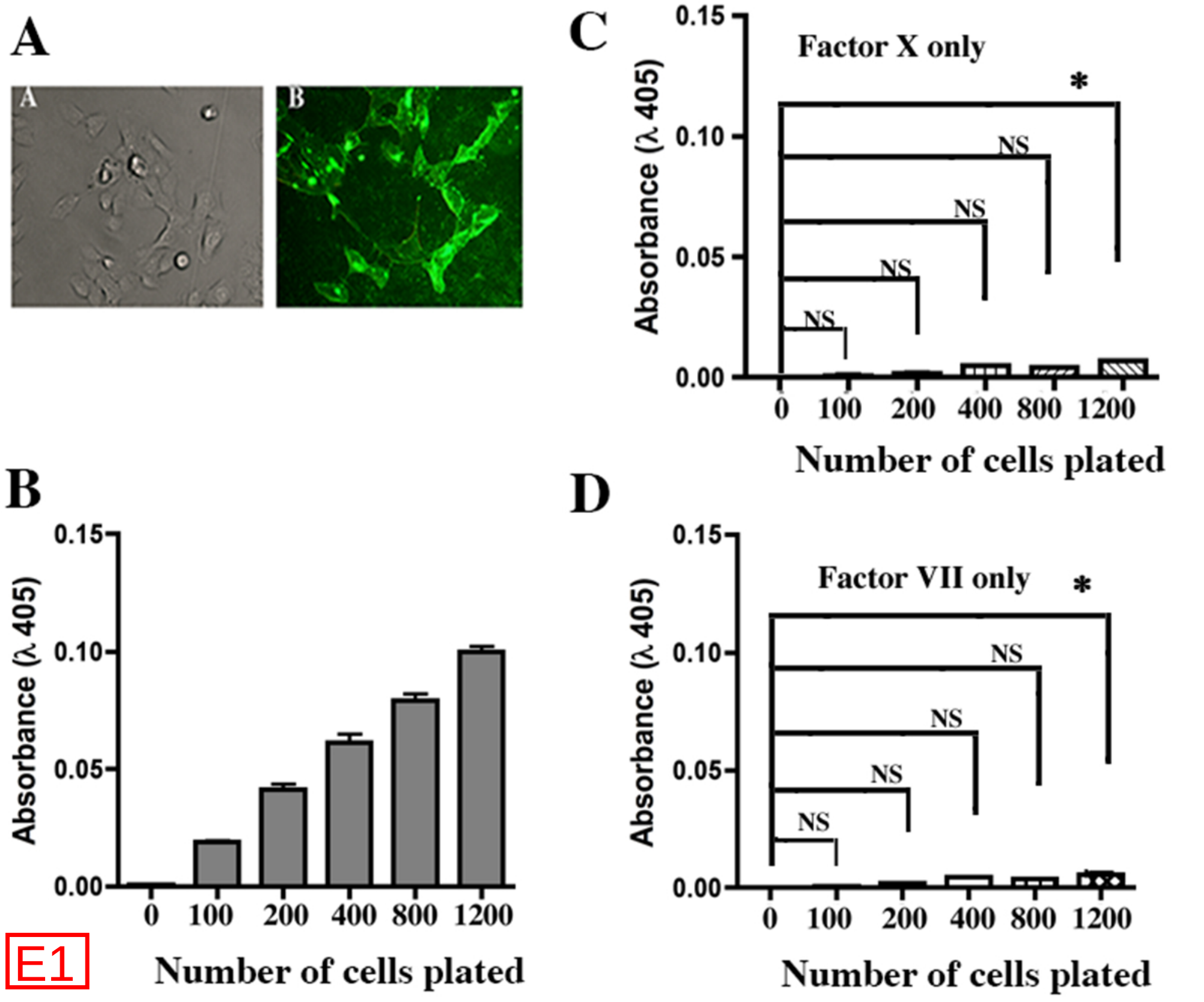
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B
B
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0.10
0.05
0.00
0
100
200
400
800
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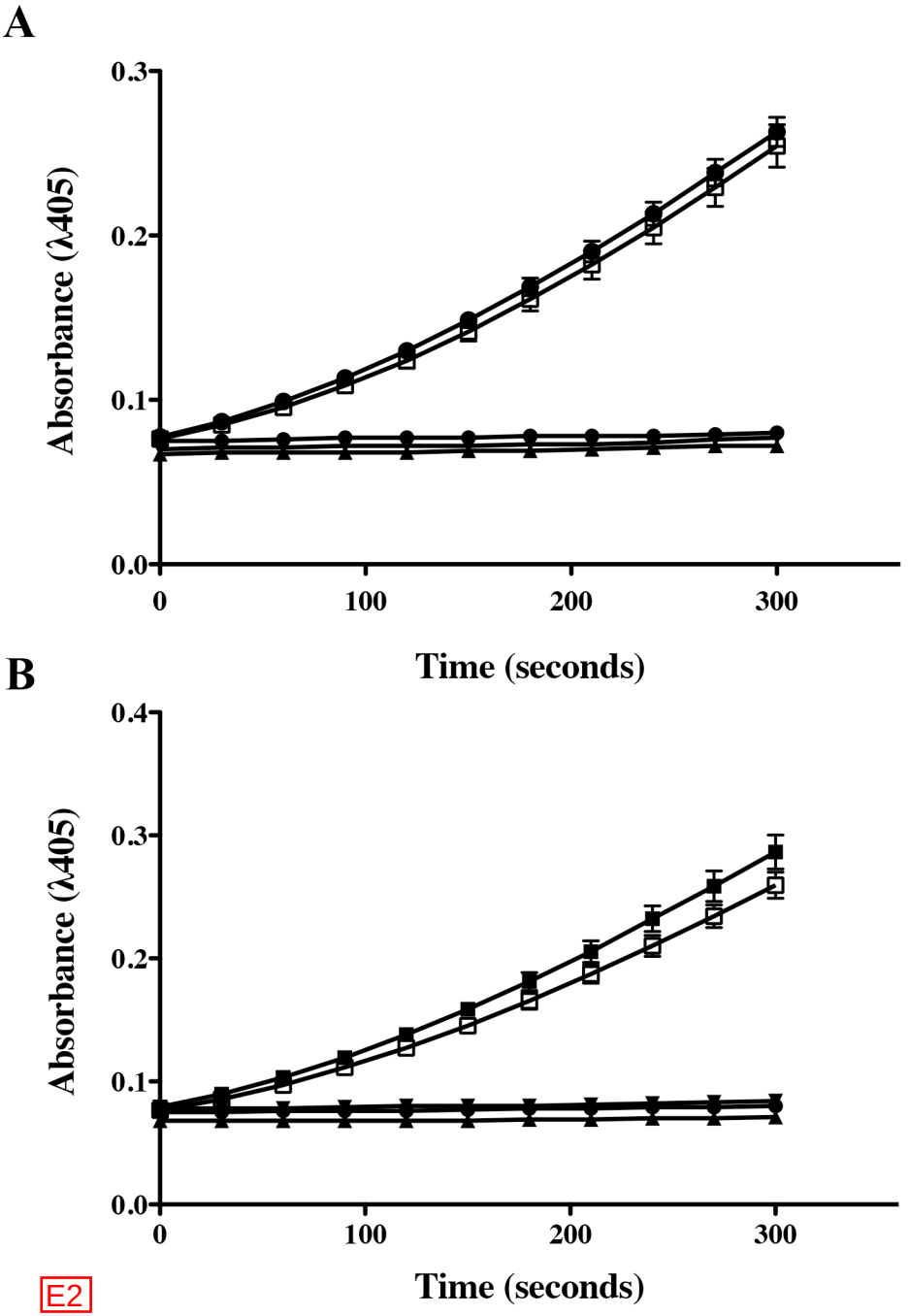
NS
NS
E1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200
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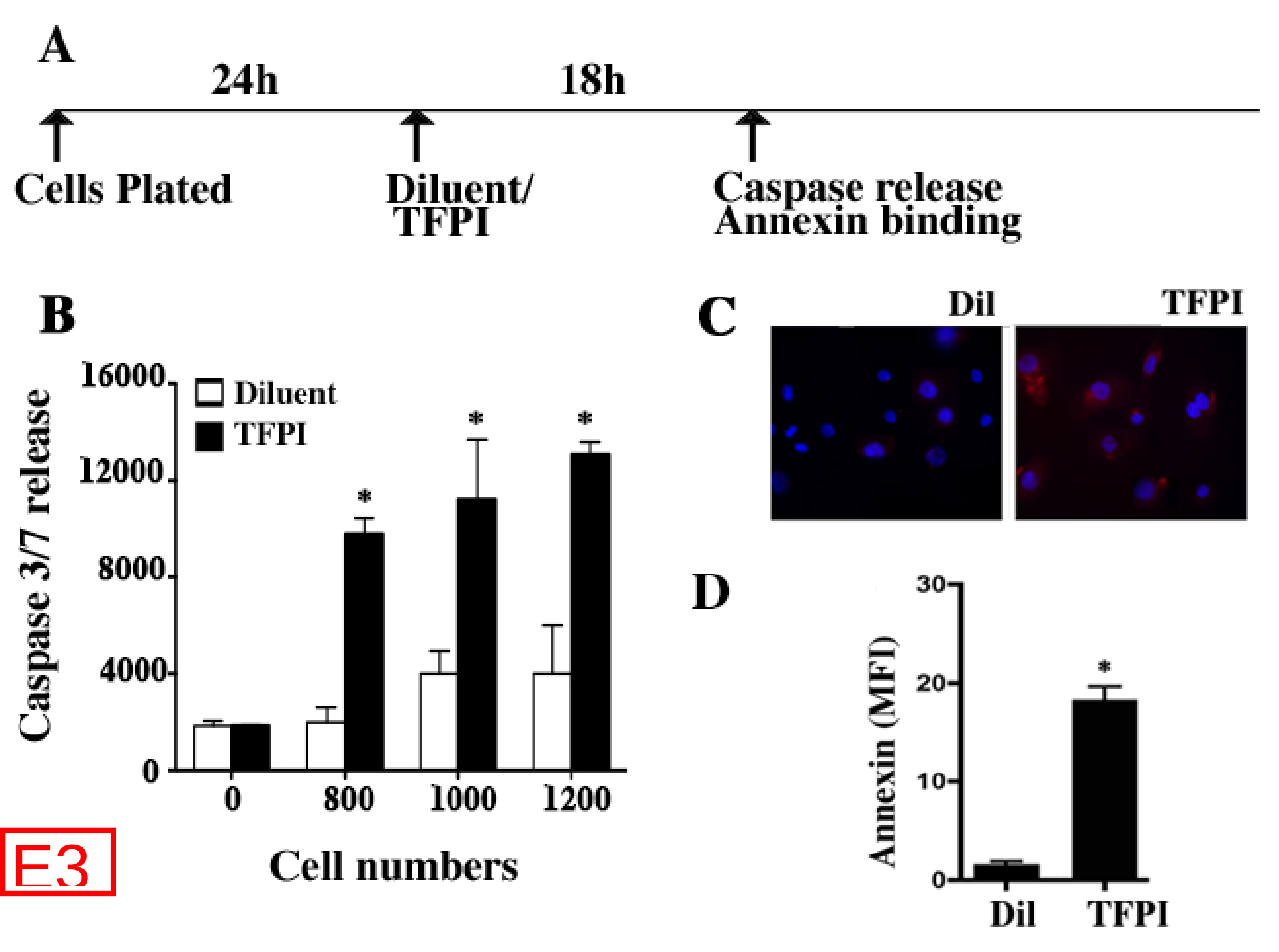
0
100
200
300
Time (seconds)

E2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24h
18h
Cells Plated
Diluent/
TFPI
Caspase release
Annexin binding
B
Caspase 3/7 release
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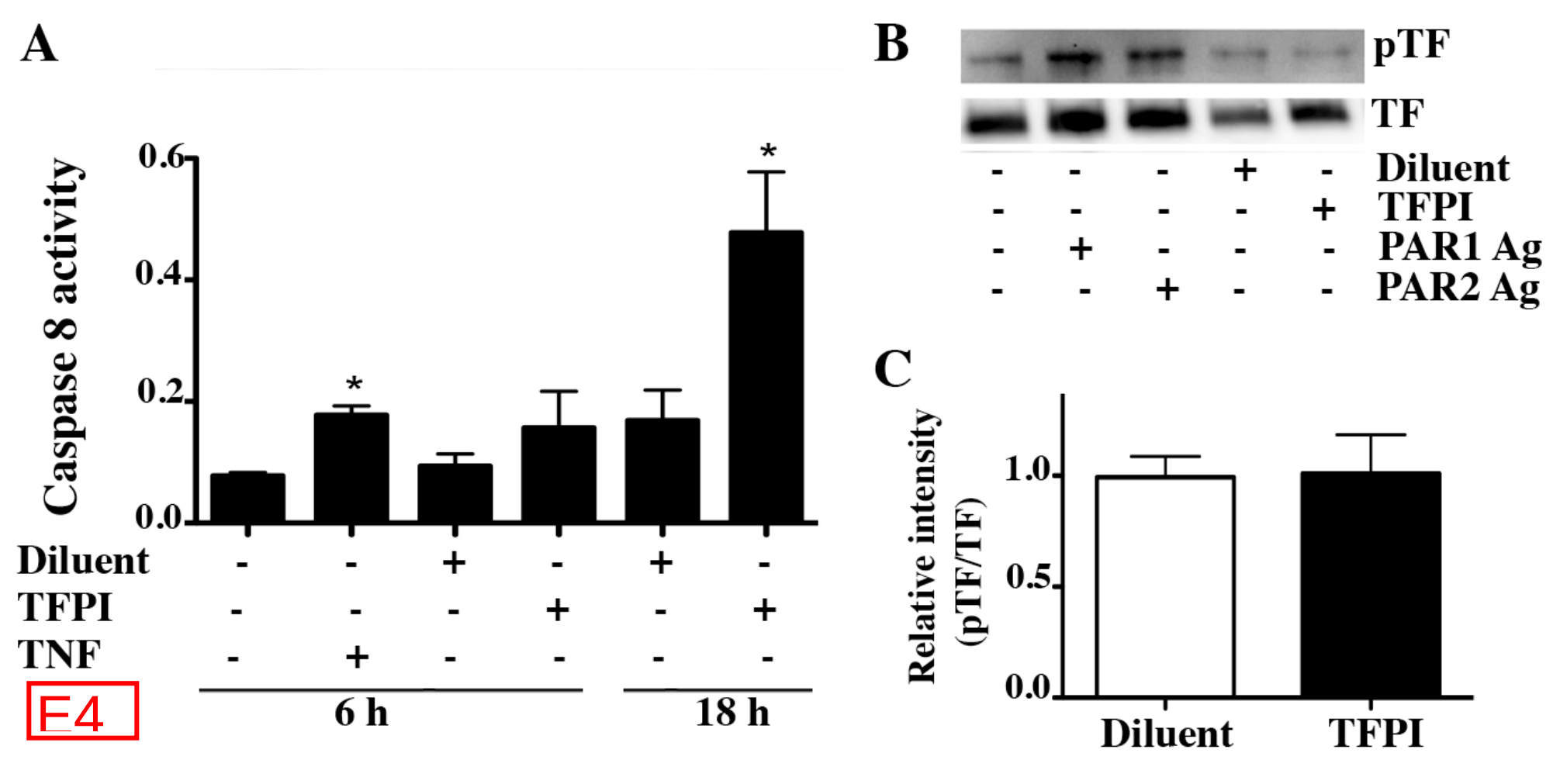
12000
8000
4000
0
Diluent
TFPI
*
*
*
0
800
1000
1200
Cell numbers
C
Dil
TFPI
D
Annexin (MFI)
30
20
10
0
*
Dil
TFPI
E3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Caspase 8 activity
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0.4
0.2
0.0
*
*
Diluent - - + - + -
TFPI - - - + - +
TNF - + - - - -
6 h
18 h
E4
B
pTF
TF
- - - + - Diluent
- - - - + TFPI
- + - - - PAR1 Ag
- - + - - PAR2 Ag
C
Relative intensity (pTF/TF)
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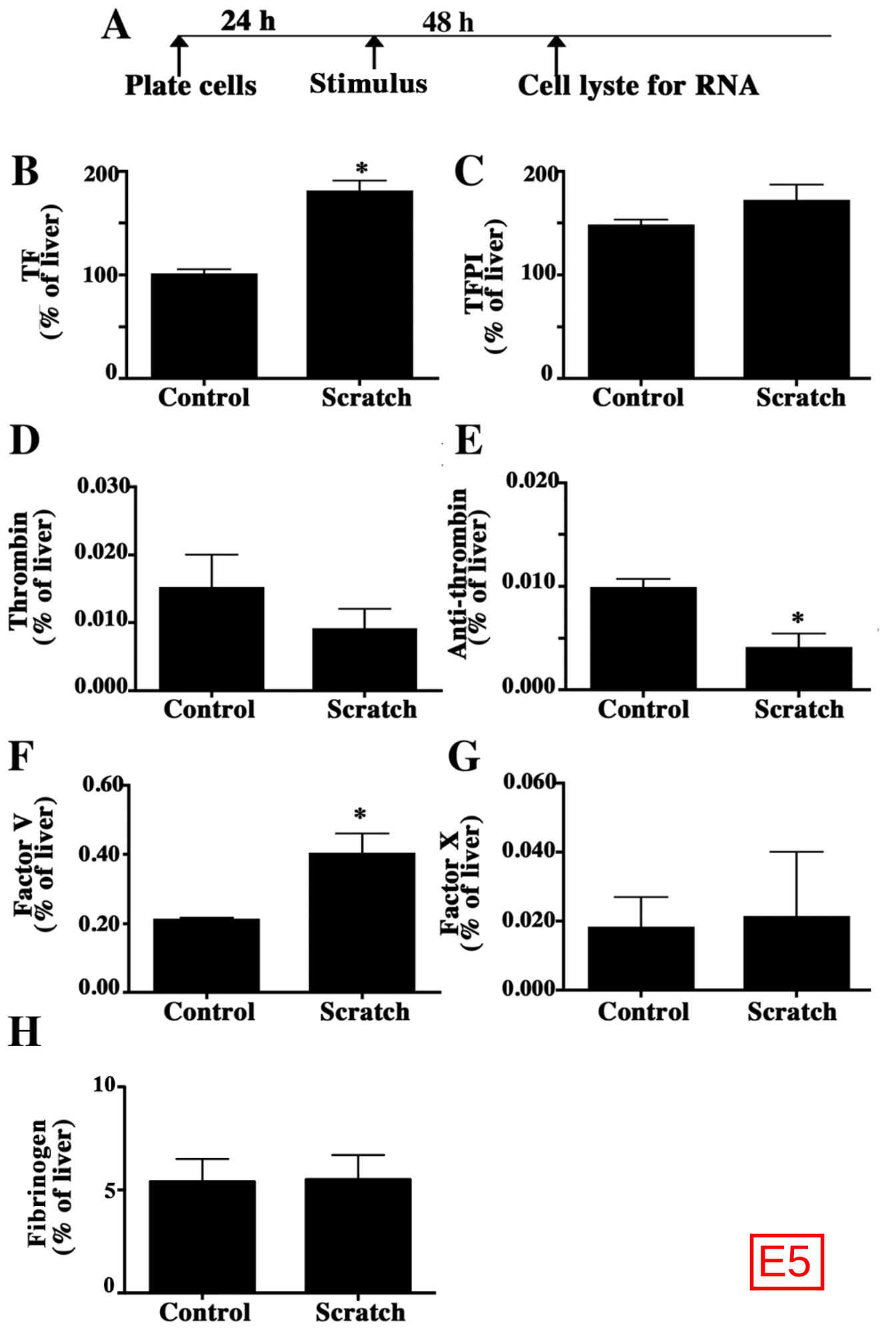
0.5
0.0
Diluent
TFPI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24 h
48 h
Plate cells
Stimulus
Cell lyste for RNA
B
TF
(% of liver)
200
100
0
*
Control
Scratch
C
TFPI
(% of liver)
200
100
0
Control
Scratch
D
Thrombin
(% of liver)
0.030
0.020
0.010
0.000
Control
Scratch
E
Anti-thrombin
(% of liver)
0.020
0.010
0.000
*
Control
Scratch
F
Factor V
(% of liver)
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0.40
0.20
0.00
*
Control
Scratch
G
Factor X
(% of liver)
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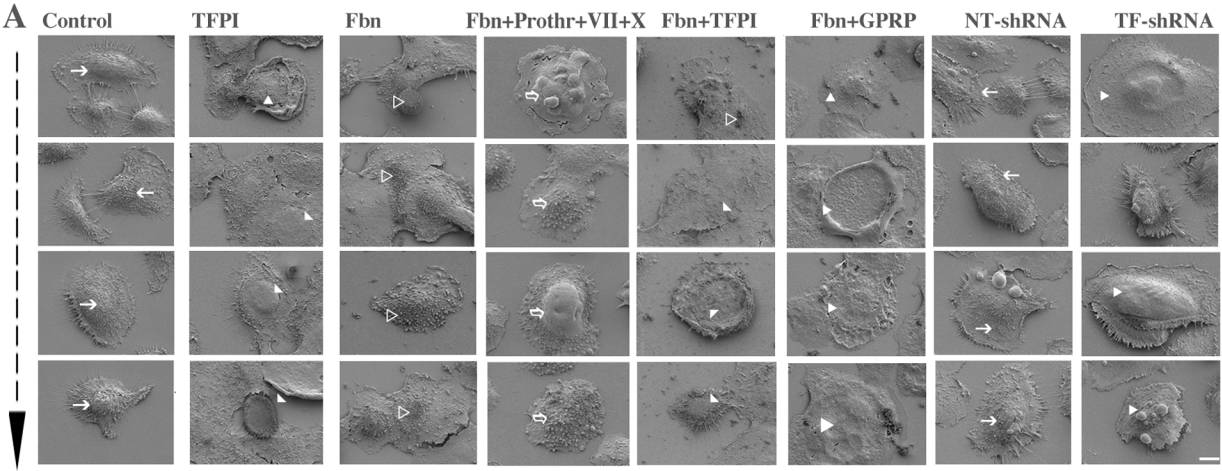
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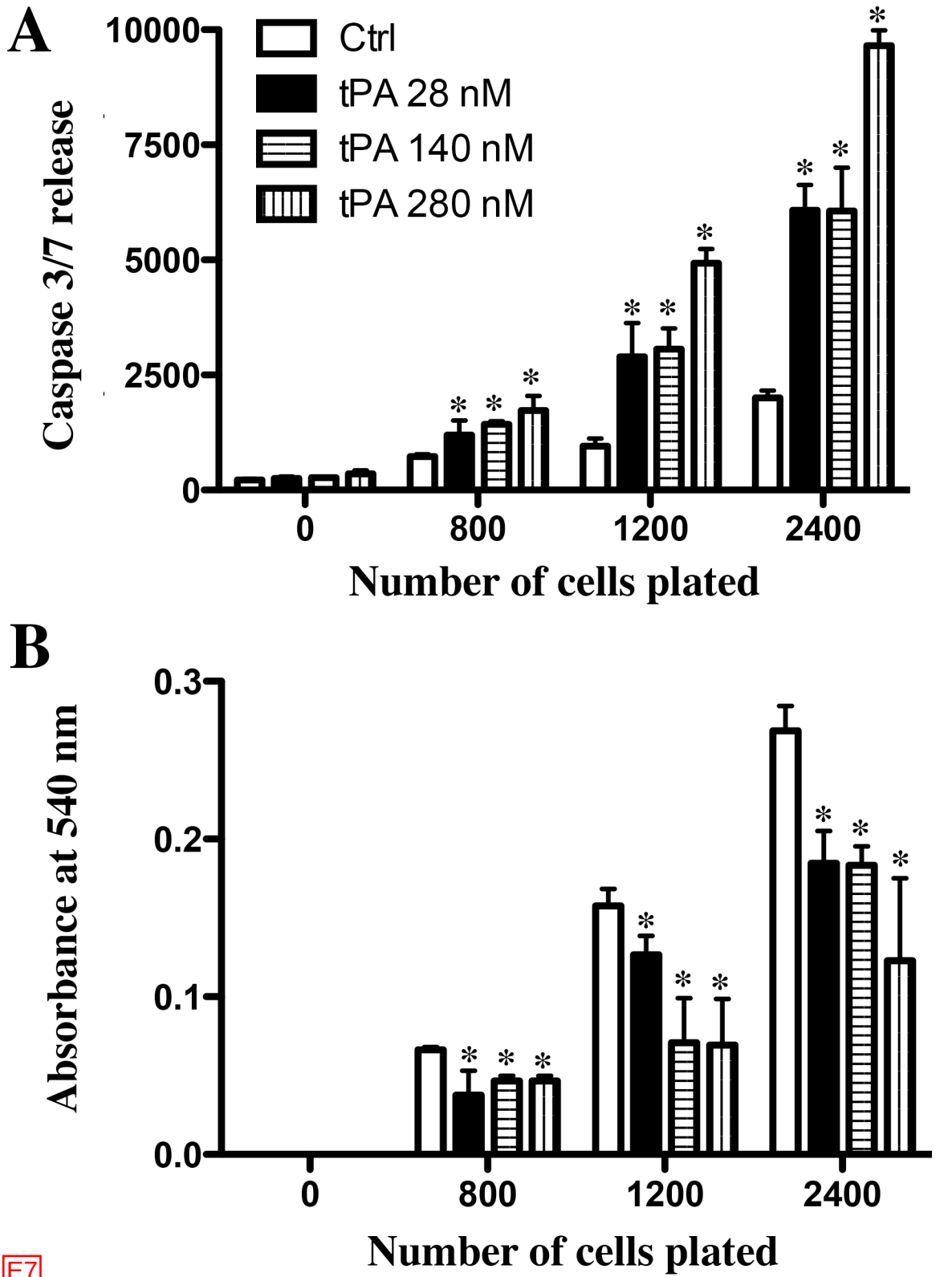
E6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2500
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tPA 140 nM
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1200
2400
Number of cells plated
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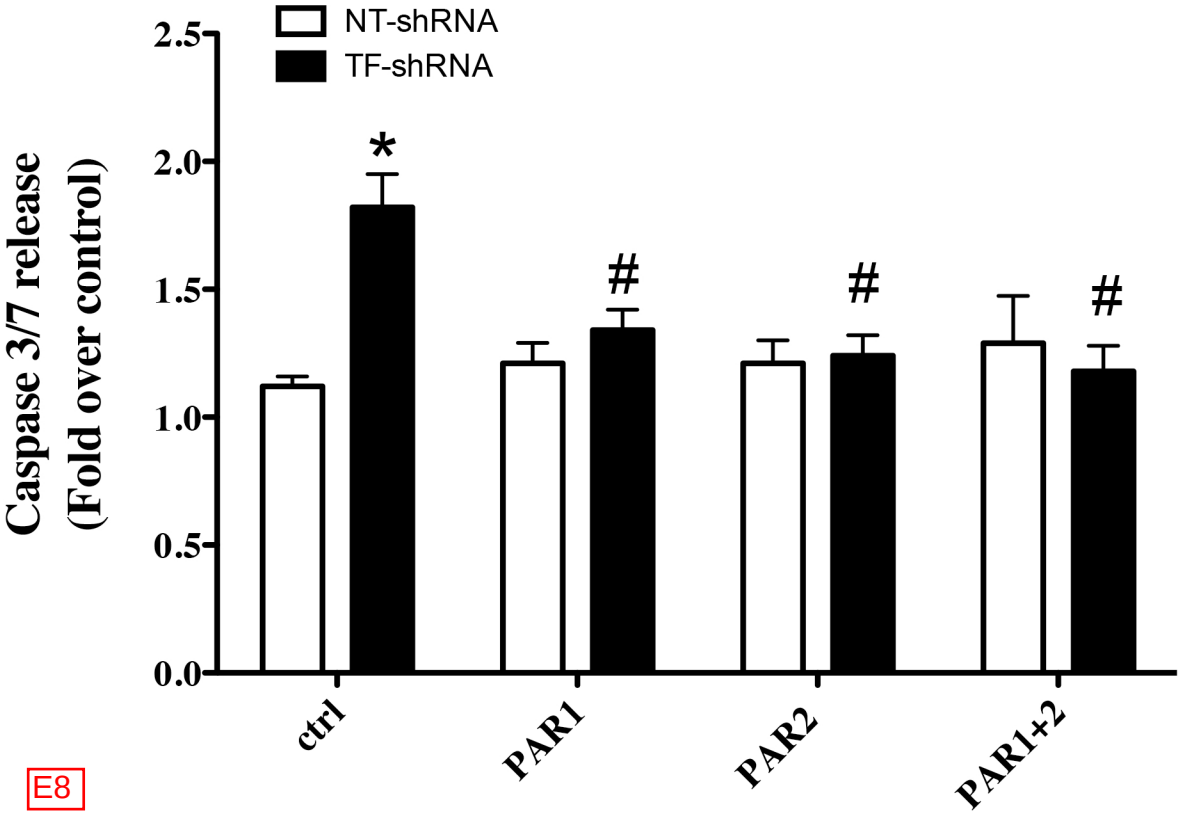
0.2
0.1
0.0
0
800
1200
2400
Number of cells plated

E7


NT-shRNA
TF-shRNA
Caspase 3/7 release
(Fold over control)
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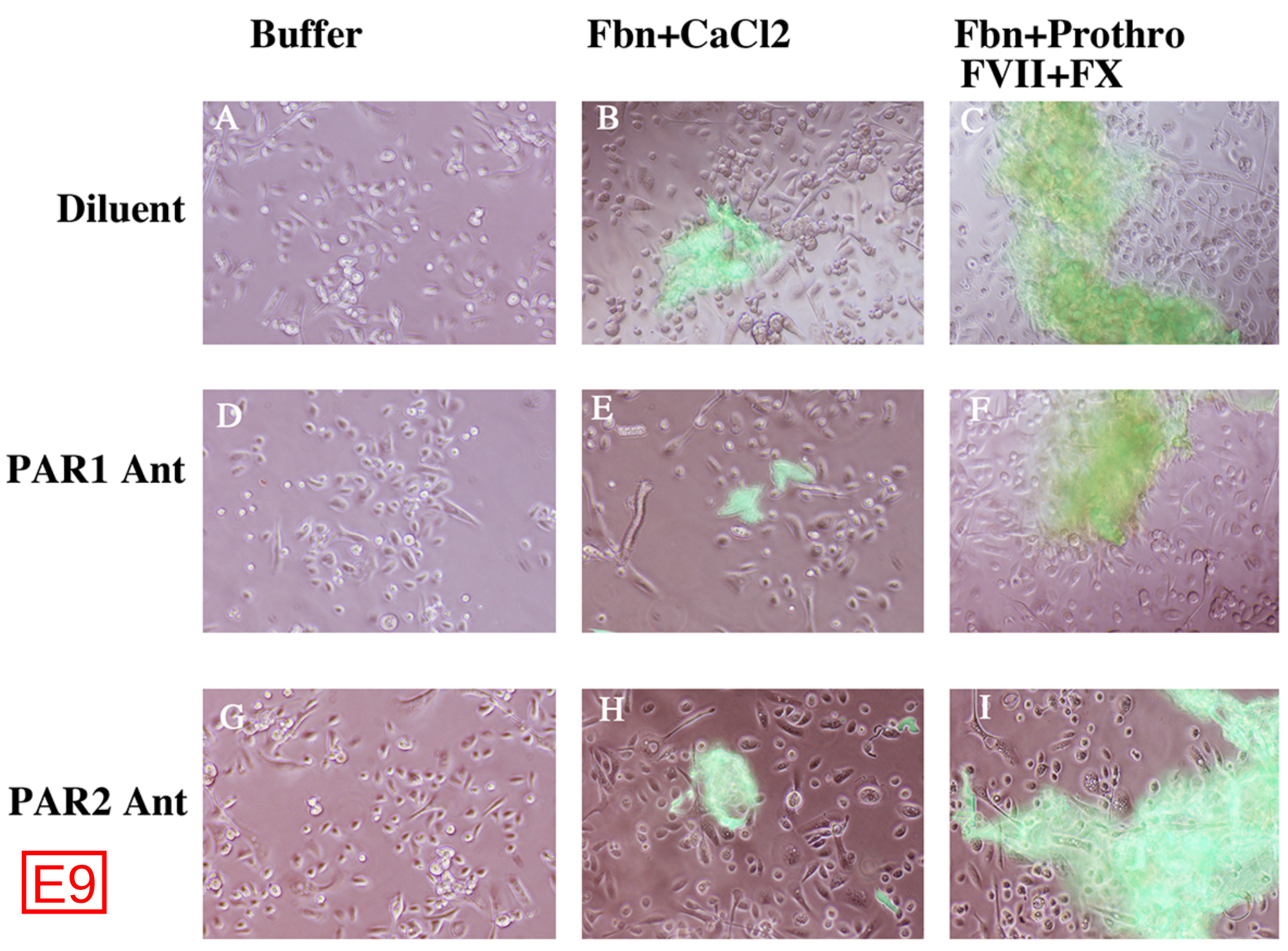
*
#
#
#
ctrl
PAR1
PAR2
PAR1+2

E8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FVII+FX
Diluent
PAR1 Ant
PAR2 Ant
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B
C
D
E
F
G
H
I
E9