Primary tumors secrete factors that alter the microenvironment of distant organs, rendering those organs as fertile soil for subsequent metastatic cancer cell colonization. Although the lungs are exposed to these factors ubiquitously, lung metastases usually develop as a series of discrete lesions. The underlying molecular mechanisms of the formation of these discrete lesions are not understood. Here we show that primary tumors induce formation of discrete foci of vascular hyperpermeability in premetastatic lungs. This is mediated by endothelial cell-focal adhesion kinase (FAK), which up-regulates E-selectin, leading to preferential homing of metastatic cancer cells to these foci. Suppression of endothelial-FAK or E-selectin activity attenuates the number of cancer cells homing to these foci. Thus, localized activation of endothelial FAK and E-selectin in the lung vasculature mediates the initial homing of metastatic cancer cells to specific foci in the lungs.

Results

Distant Tumors Induce Focal Hyperpermeability in the “Premetastatic” Lungs, and This Effect Can Be Reproduced by Infusion of Tumor-Seeded Factors. To investigate the effects of primary tumors on the normal lungs, we grew tumors endowed with a well-characterized metastatic potential (E0771 mammary carcinoma and LLC lung carcinoma) in syngeneic C57BL/6 mice. Once tumors reached ≈6 mm in diameter, we infused Evans Blue (EB) i.v. and then excised and examined the lungs 3 h later. We found increased EB leakage in the lungs of tumor-bearing mice, with distinct, focal regions of macroscopically detectable hyperpermeability (Fig. L4). To rule out the possibility that focal EB leakage was a consequence of metastatic cancer cell seeding to the lungs but rather is a direct effect of secreted factors from primary tumors, we repeated the experiments using a previously established experimental model (6, 10). We infused i.v. tumor-conditioned medium (TCM) from E0771 (ETCM) or LLC (LTCM) in non–tumor-bearing mice and examined the lungs immediately after the injection. Both ETCM and LTCM induced pulmonary vascular hyperpermeability (Fig. 1B) with discrete areas of EB leakage. Using ELISA, we found that LTCM/ETCM contained high concentrations of VEGF and placental growth factor (PIGF) in contrast to non-tumor cell conditioned media (NTCM). These factors are known to increase vascular permeability through direct and indirect mechanisms, respectively (20–22). Infusion of recombinant (r) VEGF or rPlGF also induced—albeit to a lesser extent—discrete foci of hyperpermeability with focal EB leakage in the lungs (Fig. S1A). The increase in EB leakage peaked at 3 h after stimulation and was specific to the lungs after either i.v. or intracardiac injection of the stimulus (Fig. 1 C and D). Treatment of tumor-bearing mice with an anti-VEGF blocking antibody partially inhibited lung vascular hyperpermeability (Fig. S1B), confirming that VEGF modulates this effect.

Metastatic Cancer Cells Preferentially Home to Hyperpermeable Foci in Lungs. To study metastatic cancer cell colonization, we developed a three-step experimental assay system (Fig. 2A). First, we induced focal lung hyperpermeability by tumor implantation or i.v. infusion of TCM, rVEGF, or rPlGF in non–tumor-bearing mice. Second, we infused EB or PEG-coated microbeads systemically to detect the sites of focal lung vessel leakage. Finally, we infused i.v. fluorescence-labeled metastatic cancer cells and measured the number of cells that homed to the lungs 5 or 24 h later. In tumor-bearing mice, we found that metastatic cancer cells preferentially homed to areas of high macroscopic EB leakage compared with areas of low EB leakage (after separating them by tissue microdissection; Fig. 2B). Prior TCM infusion
also significantly increased the total number of fluorescent cancer cells homing to the lungs of healthy mice after both 5 and 24 h, and the cells localized preferentially in hyperpermeable areas (Fig. S2 A–C). Finally, prior anti-VEGF antibody treatment significantly reduced the number of cancer cells homing to the lungs (Fig. S2D).

To investigate the association of cancer cells with the permeable vessels, we injected PEG-coated microbeads (instead of EB) after the induction of focal hyperpermeability by TCM or rVEGF infusion and followed by the infusion of metastatic cancer cells. We found a higher density of extravasated PEG microbeads in microscopic fields containing metastatic cancer cells (Fig. 2C). Of interest, microbead density was uniformly increased over a distance of 200 μm from cancer cells, before decreasing abruptly (Fig. S2E). This suggested that cell homing to the normal lung occurred over a region of hyperpermeability rather than a specific site of leakiness (i.e., a preexisting gap between endothelial cells within a vessel). Of note, infiltrated cancer cells in the TCM-stimulated lungs appeared larger and had a more irregular shape compared with those in healthy lungs (Fig. S2 F and G). In this model, metastatic cancer cells homed selectively to the lungs, and homing to other organs was negligible regardless of whether the cells were infused via the tail or portal vein (Fig. S2H).

**Endothelial Focal Adhesion Kinase (FAK) Mediates Focal Lung Vessel Hyperpermeability and Metastatic Cancer Cell Homing.** Because VEGF can activate the Src–FAK complex in lung endothelial cells, we next determined whether FAK activity is instigated before the arrival of cancer cells in the lungs. We found increased levels of phosphorylated FAK protein (pFAK) in areas of TCM-induced hyperpermeability (Fig. 3A). The expression of pFAK in lung tissue was largely colocalized with MECA-32 expression, an endothelial cell marker (Fig. S3 A and B). To determine whether endothelial cell FAK mediates the formation of hyperpermeable foci and subsequent cancer cell homing, we developed a transgenic mouse model conditionally overexpressing FRNK—the dominant negative form of FAK (23)—in endothelial cells under the control of a tetracycline response element (tTA-FRNK mouse; Materials and Methods). FRNK expression level was higher than endogenous FAK expression in the lung tissue (Fig. S3C) and was reduced in a time-dependent fashion after administration of doxycycline (Dox) (Fig. S3D and E). Endothelial-specific FRNK overexpression significantly reduced the formation of hyperpermeable foci in the lungs after ETCM infusion and LTCM infusion compared with control non-transgenic mice (Fig. 3 B and C). We confirmed this finding by comparing EB leakage in tTA-FRNK mice at time points 0, 24, and 48 h after i.p. administration of Dox (Fig. S3F). Moreover, endothelial cell FRNK overexpression resulted in a significant decrease in the homing of infused metastatic cancer cells to the lungs, both of healthy mice stimulated by TCM (Fig. 3D and Fig.
E-selectin in Lung Endothelial Cells Mediates Metastatic Cell Homing to Hyperpermeable Foci. We next performed in vivo assays to determine the role of E-selectin in metastatic cancer cell homing to hyperpermeable foci. Immunohistochemistry revealed colocalization of pFAK and E-selectin in endothelial cells adjacent to metastatic cancer cells in the lungs of wild-type mice after TCM and cancer cell injection (Fig. S4B). Tumor cell homing to lungs after TCM stimulation was significantly reduced by treatment with anti–E-selectin blocking antibody in wild-type mice as well as in E-selectin−/− mice (Fig. 4C and Fig. S4C). Consistent with the observed reduction of E-selectin expression and tumor cell homing after knockdown of endothelial cell FAK function, no significant further change was seen in tTA-FRNK mice by E-selectin knockdown (Fig. 4C). To further dissect the relative contribution of FAK and E-selectin in the induction of hyperpermeable foci and tumor cell homing, we examined hyperpermeable lesions and cancer cell homing in the lungs of E-selectin−/− mice (24) after TCM stimulation and EB injection. Although macroscopically detectable regions of vascular hyperpermeability persisted despite the E-selectin deficiency, we found a reduction in metastatic cancer cell homing to the lungs of E-selectin−/− mice at both 5 and 24 h after metastatic cancer cell injection (Fig. 4D and E). This reduction was specific for areas of hyperpermeability (Fig. 4D). Importantly, a reduction in cancer cell homing to the lungs was also observed in E-selectin−/− mice bearing primary tumors (Fig. 4F).

FAK Activation Directly Induces E-selectin Expression in Lung Endothelial Cells. To confirm that endothelial cell FAK directly regulates E-selectin expression (i.e., not via induction of local plasma leakage and inflammation), we isolated primary lung endothelial cells from wild-type and tTA-FRNK mice and exposed them to rVEGF in vitro. As expected, VEGF stimulation significantly increased E-selectin expression in wild-type endothelial cells (25) but not in tTA-FRNK endothelial cells (Fig. 5A and B). Thus, E-selectin expression in lung endothelial cells can be induced by VEGF via FAK activation. Next, we evaluated the role of E-selectin induction by FAK in metastatic cancer cell–endothelial cell adhesion in an in vitro cancer cell adhesion assay (26). rVEGF stimulation increased the number of fluorescently labeled metastatic cancer cells attached to lung endothelial cells (Fig. 5C and D). Addition of neutralizing anti-mouse E-selectin antibodies abolished the tumor cell adhesion to the endothelial cells (Fig. 5D). Conversely, rVEGF failed to induce cancer cell adhesion to the lung endothelial cells from tTA-FRNK mice, and anti–E-selectin blocking antibody had no effect in this setting. This indicates that FAK-induced E-selectin mediates cancer cell adhesion to lung endothelium (Fig. 5D).

Discussion

Lung tissue can be “activated” before the arrival of metastatic tumor cells by activation of the resident endothelial cells and macrophages by distant primary tumors in the “pre-metastatic phase” (5). Several reports have converged toward the idea of a “preparation” of the metastatic soil. Despite these reports, the precise role of various molecular and cellular factors as facilitators of nascent metastases remains controversial (12, 13). Furthermore, the presence of the candidate molecules throughout

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S3G) and in primary tumor-bearing mice, as measured 48 h after metastatic cancer cell infusion (Fig. 3E). Importantly, the total macroscopic metastatic burden in the lungs was also significantly reduced 3 wk after i.v. TCM and metastatic cancer cell infusion in tTA-FRNK mice (Fig. 3F). Collectively, these data show that blocking lung endothelial cell-FAK activity reduces not only formation of discrete foci of vascular permeability but also metastatic tumor cell homing.

Lung Endothelial Cells in Hyperpermeable Foci Overexpress E-selectin. We next evaluated the microenvironmental changes within the hyperpermeable foci that might mediate the increase in metastatic cancer cell homing. Quantification of the number of myeloid (CD11b+) cells in areas of high vs. low EB leakage showed no significant difference in inflammatory cell infiltration at 3.5 h after TCM injection (Fig. S4A). We next performed PCR array analysis to assess whether the adhesive properties of the local endothelium were altered in areas of hyperpermeability. To this end, we extracted RNA from microdissected tissue from leaky vs. nonleaky areas—induced by TCM injection in healthy wild-type mice—and measured gene expression of a panel of endothelial adhesion molecules. We found only modest changes (e.g., E-selectin gene showed a 1.7-fold increase after rVEGF and ≈1.4-fold after TCM stimulation). However, measurement of E-selectin protein in lung endothelial cells by immunofluorescence microscopy showed a significant increase in E-selectin expression in the hyperpermeable areas (Fig. 4A). Moreover, E-selectin expression after TCM stimulation was significantly reduced in the lung tissue of tTA-FRNK mice compared with wild-type mice (Fig. 4B).

Collectively, these data suggest that endothelial cell-FAK activity mediates E-selectin expression in the hyperpermeable area.
The phenomenon of focal response of lung vasculature to stimuli (such as increased permeability after exposure to toxins) has been reported for decades (18, 19). However, the exact molecular mechanisms responsible for this heterogeneity remain elusive. Multiple secreted factors—often overexpressed in tumors (e.g., VEGF, TGF-β, TNF-α, and angiopoietin-2)—can promote vascular permeability in the lungs (5–11). VEGF released from lung metastasizing cancer cells can activate the Src-FAK complex in lung endothelial cells and promote vascular hyperpermeability, up-regulation of endothelial adhesion molecules, and cancer cell homing (27, 28). Here we demonstrate that metastatic primary tumors and soluble factors released by them can induce distinct macroscopic regions of FAK-dependent vascular hyperpermeability in the lungs.

Endothelial cell E-selectin is traditionally associated with the homing of leukocytes through rolling and tethering (29–32), and its expression is rapidly induced in response to inflammatory stimuli, such as TNF-α (peaking at 2–6 h) (33). It has previously been noted that VEGF overexpression can lead to an E-selectin-dependent increase in leukocyte rolling (34), that exposure of cultured endothelial cells to tumor-secreted factors increases E-selectin expression (25), and that VEGF directly induces E-selectin expression in endothelial cells (35). Finally, tumor cell engagement with the lung endothelium is mediated in part by E-selectin (33, 36). Here we show that lung regions that serve as discrete, fertile fields of premetastatic “soil” demonstrate an increased tumor cell homing facilitated by E-selectin up-regulation in endothelial cells via FAK.

In summary, we demonstrate that VEGF and other factors derived from primary tumors can set in motion molecular and physiological changes in distant organs before the homing of metastatic cancer cells. We show that circulating metastatic cancer cells localize preferentially in regions of vascular hyperpermeability via endothelial FAK/E-selectin mediated homing. These findings and further understanding of the cascade of pathological changes in the “pre-metastatic” stroma and the molecular determinants of the metastatic cell colonization may impact strategies for preventing or controlling lung metastasis.

Materials and Methods

Reagents. Human rVEGF was supplied by NCI Research Resources. Mouse PGF1α was purchased from R&D Systems. Dox, tetracycline hydrochloride, and EB were from Sigma-Aldrich and fluorescein-labeled tomato lectin was from Vector Laboratories.

Metastatic Cancer Cell Lines and TCM. The C57BL/6 mouse-syngeneic LLC1 cell line was purchased from ATCC. The E0771 breast cancer cell line was originally established by Dr. Sirotnak (Memorial Sloan-Kettering Cancer Center, New York) and kindly provided by Dr. Mihich (Roswell Park Memorial Institute, Buffalo, NY) (37). To obtain TCM, cell lines were incubated overnight in serum-free media (DMEM, ATCC). We measured mouse VEGF and PlGF levels using the ELISA (SI Materials and Methods).

Animals and Tumor Models. All animal procedures were performed following the guidelines of Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.
Syngeneic tumor grafts were generated by s.c. implantation of 5 × 10^6 LLC1 or E0771 cells in 8- to 12-wk-old C57BL/6 mice (Jackson Labs). For tumor cell homing assays, 1–4 × 10^6 rhodamine-labeled metastatic cells were infused via tail vein. We used inducible overexpression of FRNK because, whereas endothelial-specific FAK−/− mice are embryonically lethal, in inducible endothelial-specific FAK−/− mice there is normal embryogenesis and modulation of vascular permeability in response to VEGF after FAK inhibition (38–40). For transgenic mouse experiments, we used 7- to 8-wk old ITA-VECadherin promoter-driven FRNK and their wild-type littermates. E-selectin−/− mice (#008236, B6.129S4-Seletm1Dmil/J) were purchased from Jackson Labs. Age- and sex-matched C57BL/6 mice were used as controls, because in mice with no tumors, labeled cells were infused i.v. 6 h after cancer cell infusion. Thirty randomly selected lung tissue fragments (3 mm in diameter) were used as a control. In mice with no tumors, labeled cells were infused i.v. 5 min later. Gene and protein expression levels in EB-high and EB-low areas (after i.v. LTCM infusion, as previously described (41). In brief, the pulmonary microvasculature was first flushed with PBS under physiologic pressure. The lungs were then excised, rinsed in PBS, and snap-frozen in liquid nitrogen. Frozen tissue was later homogenized in PBS and incubated in formamide at 60 °C for 16 h. To determine EB concentrations, we recorded supernatant absorbance at 620 (A_{620}) and 740 nm (A_{740}). Tissue EB content (μg EB/g lung) was calculated by correcting A_{620} for the presence of heme pigments:

\[ A_{620} \text{ (corrected)} = A_{620} \times (1.426 \times A_{740} + 0.030) \]

and comparing this value to a standard curve of EB in formamide/PBS.

Cancer Cell Labeling and in Vivo Homing Assays. E0771 and LLC cells were fluorescently labeled with the PKH26 staining kit (Zynaxis), and then 2 × 10^6 labeled cells were infused i.v. In mice with primary tumors, the labeled cells were infused when the tumor reached 6 mm in diameter. In mice with no tumors, labeled cells were infused i.v. 5 min later. Gene and protein expression levels in EB-high and EB-low areas (after i.v. LTCM infusion) were measured using PCR array and Western blotting (SI Materials and Methods).

Quantitative Analysis of Extravasated Microbeads. Analysis of immunostained images was performed using in-house algorithms in Matlab software (Mathworks). Bead density was quantified by digitizing the beads using user-defined thresholds and measuring the fraction of lung tissue area covered by beads (pixels). Spatial analysis of the location of PEG microbeads relative to metastatic cancer cells was performed by measuring the bead density at specific distances from the closest cancer cell (using a 2D map).

Construction of Plasmids and Generation of Transgenic Mouse. The mouse FRNK expression vector (23), a 1.1-kb FRNK coding region corresponding to amino acids 691–1053 of mouse FAK, was kindly provided by Dr. R. Lee (Harvard Medical School, Boston). We modified the −3 upstream ATG nucleotide of FRNK from C to A and inserted it into a pTRE-Tight vector (Clontech). Transgene DNA for pronuclear injection was excised as an ApaLI fragment. The linearized construct was injected into fertilized oocytes of DBA2 × C57BL6 (DBF) mice, and the eggs were implanted into pseudo-pregnant foster mothers. The offspring (F2) were tested for chromosomal
integration of transgene by PCR. For genotyping, the PCR primers used were as follows (5' to 3'): AAGGCTCAGAATGCTTGGCG (forward) and AAATGCAGACGGTGGCC (reverse). Then these mice were mated with VE-Cadherin-TTA mice (42). Our TTA-FRNK mice did not show embryonic lethality. To rapidly regulate the FRNK expression in vivo, 2 mg of Dox was administered via i.p. injection (43). Littermates were used as controls for all experiments using TTA-FRNK mice.

### Immunofluorescence

For immunostaining of lung frozen tissue sections or dissociated lung cells suspensions, we used rat anti-mouse E-selectin (BD Pharmingen), rabbit anti-mouse FAK (y397), and mouse anti-pF2 (Ty397, Mab 1144; Upstate), rat anti-mouse MECA32 (BD Pharmingen), and rat anti-mouse Mac1/CD11b (Serotec) antibodies. Cy3- or Cy5-conjugated secondary antibodies were used for the detection of signals by confocal microscopy. Slides were counterstained with DAPI nuclear staining.

### E-selectin Antibody Blockade in Vivo

Blocking monoclonal antibodies against E-selectin (10E9.6) or control rat IgG2ax antibodies (both from BD Pharmingen) were infused i.v. at a dose of 4 mg/kg 30 min before metastatic cancer cell infusion.

### Anti-VEGF Antibody Experiments

Neutralizing anti-VEGF antibodies (R&D Systems) was incubated with TCM for 30 min before systemic infusion. In experiments using tumor-bearing mice, 10 μg of anti-VEGF antibody was infused 30 min before cancer cell infusion.

### Metastatic Cancer Cell–Endothelial Cell Adhesion Assay

Lung endothelial cells were obtained using enzymatic digestion and magnetic immuno-separation as follows (57).

Supporting Information
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Materials and Methods

PCR Array. Total RNA samples were isolated from frozen tissues using TRIzol (Invitrogen). Gene expression levels in EB-high and EB-low areas [after i.v. LLC tumor-conditioned medium (LTCM) and recombinant VEGF infusion] were measured using Mouse Endothelial Cell Biology PCR array plates (SABiosciences).

Immunoprecipitation and Western Blot Analysis. Lung tissues were lysed with lysis buffer [50 mM Hepes (pH 7.4), 1% Triton X-100, 150 mM sodium chloride, and 1 mM EGTA and 5 mM EDTA] with protease inhibitor mixture, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, and 1 mM sodium orthovanadate. For FRNK detection, we used the rabbit antibody to the carboxyl terminal FAK (C-20; Santa Cruz Biotechnology).

Isolation of Endothelial Cells. Minced mouse lungs were digested with 2 mg/mL of collagenase type 1A, 2 mg/mL of hyaluronidase, and DNase at 37 °C for 30 min, and then filtered through a 70-μm cell strainer (all from Sigma). The cellular filtrate was washed, and a layer containing endothelial cells was separated using Histopaque (Sigma). Cells from this layer were incubated with magnetic Dynabeads (Dynal) coated with anti-rat IgG antibody, to which rat anti-mouse CD31 and MECA32 antibodies were attached. Cells were separated using a magnetic separator and then cultured on a collagen-coated plate in VEGF-free EBM2 medium (Lonza) overnight.

Soluble Protein Measurement in Cell Culture Supernatant. We measured mouse VEGF and mouse placental growth factor levels using the ELISA plate from R&D Systems, according to the manufacturer’s instructions.

Fig. S1. (A) Upper: Distinct macroscopic regions of Evans Blue (EB) leakage in the lungs of healthy mice after stimulation by recombinant (r)VEGF or recombinant placental growth factor (rPlGF). Lower: Total EB leakage into lungs of healthy mice 3 h after stimulation with rVEGF or rPIGF. *P < 0.05, n = 6 and n = 4, respectively. (B) Total EB leakage into the lungs of mice bearing LLC primary tumors measured 3.5 h after EB injection, with or without treatment with an anti-VEGF antibody. n = 4, ± SEM.
Fig. S2. (Continued)
Fig. S2. (A) Number of i.v. injected metastatic cancer cells homed to areas of high vs. low Evans Blue (EB) leakage in tumor-bearing mice measured 5 h after injection; n = 4, *P < 0.05, ± SEM. (B) Tumor cell homing to lungs stimulated by E0771 tumor-conditioned medium (ETCM) or LLC TCM (LTMC) 5 h after tumor cell injection. *P < 0.05, n = 6. (C) Tumor cell (LLC) homing to the lungs of mice stimulated by LTMC, measured 24 h after tumor cell injection. *P < 0.05, n = 6. NTCM, non-tumor-conditioned medium. (D) Number of E0771 metastatic cancer cells homed to the lungs of ETCM-stimulated mice measured 5 h after tumor cell injection, with or without anti-VEGF antibody treatment; n = 4, *P < 0.05. (E) Density of PEG microbeads (y-axis) measured against distance from tumor cell (x-axis) in the lungs of mice stimulated by NTCM, TCM, VEGF, and PBS (y-axis units are in multiples of 10⁻³). (F) Representative images of tumor cells in the lungs of wild-type mice after stimulation with NTCM or TCM (24 h after stimulation). (G) Representative image of an extravasated LLC cell 24 h after i.v. injection (red, tumor cell; yellow, PEG microbeads; green, lectin-stained vessels). (H) LLC tumor cell homing to lungs, liver, kidney, and brain after stimulation with i.v. PBS (left bars) or TCM (right bars). *P < 0.05; n = 6 mice per group.
Fig. S3. (A) Representative images showing immunofluorescent staining for phosphorylated FAK (Y397) in the Evans Blue (EB)-low vs. EB-high areas of the lung after stimulation by E0771 tumor-conditioned medium (ETCM). Staining is by DAPI (blue), anti-MECA32 (endothelial cell marker, green), and anti pFAK (red). Center: Merged image. (B) Quantification of pFAK and mECA32 colocalization by double immunostaining. (C) Western Blot showing expression of FAK and FRNK proteins in tTA (control), FRNK (control), and tTA-FRNK mice (in the absence of doxycycline). (D) Kinetics of FRNK protein suppression after i.p. doxycycline injection in tTA-FRNK mice. (E) FRNK expression as a ratio of baseline FAK expression at different time points after i.p. doxycycline. (F) EB leakage in ETCM-stimulated lungs from wild-type and tTA-FRNK mice at 0, 24, and 48 h after i.p. injection of doxycycline. *P < 0.05, n = 4. (G) Number of E0771 cells homing in ETCM-stimulated lungs from tTA-FRNK mice 0, 24, and 48 h after i.p. injection of doxycycline. *P < 0.05, n = 4.
Fig. S4.  (A) Immunohistochemical quantification of CD11b myeloid cells in the tumor-conditioned medium (TCM)-stimulated lungs of wild-type mice (3.5 h after i.v. TCM injection); \( n = 4 \). (B) Representative immunohistochemical image depicting colocalization of phosphorylated FAK expression (green), E-selectin expression (blue), and tumor cell homing (red). (C) Metastatic burden in mice after i.v. infusion of 200 \( \mu \)L of LLC TCM followed by i.v. infusion of 50,000 LLC cells after 3.5 h: E-selectin deficiency reduces the number of lung metastases formed after 20 d. \( *P < 0.05, n = 6 \).