Pancreatic secretory trypsin inhibitor causes autocrine-mediated migration and invasion in bladder cancer and phosphorylates the EGF receptor, Akt2 and Akt3, and ERK1 and ERK2

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PSTI/TATI on cellular actions and signaling pathways relevant to tumor growth and metastases.

METHODS

All chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated.

Cell lines. 253J cells are derived from a metastatic transitional cell carcinoma originating in the bladder isolated from a retroperitoneal lymph node from a 53-yr-old Caucasian male (6). HT1376 cells are derived from a urinary bladder grade 3 carcinoma from a 58-yr-old Caucasian female (26). RT4 cells are derived from an urinary bladder transitional cell papilloma from a 63-yr-old Caucasian male (27). 253J and HT1376 cells were chosen as examples of high-grade bladder carcinomas, whereas RT4 cells are examples of a lower-grade bladder transitional cell papilloma.

Production of recombinant human PSTI/TATIs. “Normal” sequence recombinant PSTI/TATI was prepared as previously described (18). In addition, two mutant PSTI/TATI-expressing clones were produced from this construct using a Stratagene Quickchange II site-directed mutagenesis kit with specific primers. One is a naturally occurring variant found in some patients with familial pancreatitis where Asp34 is replaced by a serine residue (N34S), and one is a non-naturally occurring variant in which Lys18-Ile19 residues were changed to Arg18-Val19 (R18/V19). The three forms of PSTI/TATI (PSTI/TATI, R18/V19 PSTI/TATI, and N34S PSTI/TATI) were assessed for relative trypsin inhibitor activity using a standard Na-benzoyl-DL-arginine-p-nitroanilide “BAPNA” trypsin inhibition assay as previously described (17). Results from these experiments showed, as expected, an approximately equimolar inhibitory effect of normal sequence PSTI/TATI against trypsin, with the N34S mutant showing a similar trypsin inhibitory activity to normal sequence PSTI/TATI. In contrast, the N34S mutant showing a similar trypsin inhibitory activity to normal sequence PSTI/TATI. In contrast, the
mutant with the active site mutated (R18/V19) showed no significant trypsin inhibitor activity even when added at four times the molar concentration of trypsin.

Quantitation of PSTI/TATI mRNA and protein. Protein expression analyses used a commercial ELISA kit using a PSTI/TATI matched antibody pair (H0000690-AP41, Abnova) as per the manufacturer’s instructions. Results are expressed as means ± SE of triplicate wells.

For PSTI/TATI mRNA analyses, cDNA was synthesized from 2 μg of total RNA using a high-capacity RNA-to-cDNA kit (Applied Biosciences). Quantitative real-time PCR was then performed using Taqman Gene expression assays for PSTI/TATI (Hs00162154_m1) and β-actin (Hs9999903_m1), and reactions were carried out in triplicate. Data are expressed as PSTI/TATI mRNA levels normalized with β-actin expression as an internal control.

PSTI/TATI RNA interference knockdown of 253J and HT1376 cells. To examine the influence of endogenously produced PSTI/TATI on cell functions, PSTI/TATI RNA interference knockdown techniques were used. 253J and HT1376 cells were stably transfected with a PSTI/TATI short hairpin (sh)plasmid (sc-45801-SH) or control shRNA plasmid sh-A (sc-108060), a negative control plasmid encoding a scrambled shRNA sequence that does not result in the specific degradation of any known cellular mRNA. The PSTI/TATI shRNA (sc-45801-SH) plasmid is a pool of three target-specific lentiviral plasmids, each encoding 19–25 nt (plus hairpin) shRNAs designed to knockdown PSTI/TATI gene expression and also encoding a puromycin resistance gene for the selection of cells stably expressing shRNA. Both plasmid constructs and the shRNA transfection reagent (sc-108061) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell migration assays. Cell migration assays were performed using our previously published methods (25). Briefly, cells were grown as a monolayer, and a standard wound was inflicted. Serial photomicrographs were taken at various time points. All results are expressed as means ± SE of three separate wells.

Collagen cell invasion assays. Collagen gel motility/invasion assays were performed using a Cultrex 96-well Cultrex 1 Cell Invasion Assay Kit (R&D Systems) as per the manufacturer’s instructions. Each condition was tested in triplicate, and data are expressed as invasive cells as a percentage of the control (cells plated in wells without collagen added).

Cell proliferation assays. All three cell lines were studied using Alamar blue assays (Invitrogen, Paisley, UK), which we have previously described (Refs. 19 and 20). This assay incorporates a specially selected oxidation-reduction indicator that undergoes colorimetric change in response to cellular metabolic reduction. The assay was performed as per the manufacturer’s instructions measuring changes in absorbance at 570 nm. For each condition, the effects were measured in six separate wells.

Cell signaling assays. To examine the signaling pathways involved in PSTI/TATI-mediated effects, two related methods were used. First, the effect of addition of PSTI/TATI or EGF on the degree and speed of phosphorylation of the EGFR receptor (EGFR) was determined using standard ELISA techniques (R&D Systems) as per the manufacturer’s instructions. Cells were treated with either EGF or PSTI/TATI (15 nM) for various times at 37°C, and results are expressed as means ± SEM of triplicate wells. Second, downstream signaling pathways stimulated by PSTI/TATI or EGF were analyzed using a human phospho-MAPK proteome profiler antibody array kit (R&D Systems). This array allows the parallel determination of relative levels of phosphorylation of a range of MAPKs and other serine/threonine kinases. The protocol was performed as per the manufacturer’s instructions.

Statistical analysis. ANOVA was used for all experiments. Experiments of the effect of PSTI/TATI on migration and invasion used treatment and dose as factors. For the EGFR time-course experiment, treatment and time were used as factors; for the signaling pathway experiments, treatment and pathway were used as factors. All values are expressed as means ± SE. Where a significant effect was seen (P < 0.05), individual comparisons were performed using t-tests based on the group means, residual, and degrees of freedom obtained from the ANOVA, a method equivalent to repeated-measures analyses.

RESULTS

Cell migration assays. PSTI/TATI stimulated cell migration in a dose-dependent manner in 253J, HT1376, and RT4 bladder cancer cells (Fig. 1A). Maximal restitution was seen at a PSTI/TATI concentration of 1.5 μM. Concentrations above this showed no additional stimulation (data not shown).

Both the N34S PSTI/TATI variant peptide and the trypsin inhibitor site-inactivated (R18/V19) PSTI/TATI mutant peptide stimulated migration in a dose-dependent manner that was approximately equipotent to the normal sequence recombinant PSTI/TATI peptide (Fig. 1B). In contrast, the addition of soybean trypsin inhibitor had no stimulatory effect on cell migration (Fig. 1C).

Blocking experiments showed that the promigratory effects of normal sequence PSTI/TATI were virtually completely prevented by the copresence of the PSTI/TATI-neutralizing antibody, the EGFR-blocking antibody (100 μg/ml, Cetuximab, Bristol-Myers Squibb, Princeton), or the EGFR tyrosine kinase inhibitor tyrphostin (AG1478, 100 nM; Fig. 2A). In contrast, the copresence of a transforming growth factor-β-neutralizing antibody (100 μg/ml, British Biotechnology, Avingdon, UK) resulted in only a minor reduction of the migratory activity of PSTI/TATI (~20% reduction; Fig. 2A).

PSTI and EGF are both present in human urine (5, 28, 29). We therefore performed experiments to examine potential synergistic effects. These experiments showed that when given alone, incubation of damaged monolayers with either 10 nM EGF or 10 nM PSTI/TATI did not significantly affect the rate of migration compared with baseline (Fig. 2B). However, when given together, 10 nM EGF and PSTI/TATI showed a synergistic effect, resulting in a significantly greater amount of migration compared with use of 20 nM of either peptide alone (P < 0.01 vs. either peptide alone at 20 nM).

Collagen cell invasion assays. PSTI/TATI induced 253J and HT1376 cell lines to migrate and invade vertically through the gel to the basal surface in a dose-dependent manner (Fig. 3A). When normal sequence PSTI/TATI was added at 10 μM, the numbers of cells invading were approximately threefold compared with the negative control (Fig. 3B). Treatment with the N34S PSTI/TATI mutant also caused increased cell invasion, although its potency was only ~70% of that caused by adding an equivalent concentration of normal sequence PSTI/TATI (P = 0.039 compared with normal sequence PSTI/TATI). In contrast, mutation of the active site inactivated R18/V19 PSTI/TATI mutant did not result in any increase in cell invasion above baseline (Fig. 3B).

As seen in the migration assays, the proinvasive effects of exogenously administered PSTI/TATI could be blocked by coincubation with the PSTI/TATI-neutralizing antibody, the EGFR-blocking antibody, or the tyrosine kinase inhibitor tyrphostin (Fig. 3C).

Addition of EGF alone also caused proinvasive activity (Fig. 3D). This could be blocked by coincubation with the EGFR-blocking antibody or the tyrosine kinase inhibitor tyrphostin.
significantly greater percentage of invading cells compared with use of 20 nM of either peptide alone ($P < 0.01$ vs. either peptide alone at 20 nM).

Cell proliferation assays. For all three cell lines, incubation in medium containing either 10% FCS or EGF (1.5 μM), both positive controls, resulted in an approximate doubling in proliferation compared with medium alone (Fig. 4). PSTI/TATI did not increase cell proliferation above baseline levels (Fig. 4).

Fig. 1. Effect of exogenous pancreatic secretory trypsin inhibitor (PSTI)/tumor-associated trypsin inhibitor (TATI) administration on cell migration in 253J cells. A: addition of PSTI/TATI to wounded monolayers of the human bladder cancer 253J cell line stimulated a dose-dependent increase in the rate of migration of the cells. Shown are the negative control (RPMI alone), or 1.5 μM PSTI/TATI concentrations above 1.5 μM did not increase the rate of migration above the maximal response (data not shown). RPMI + 10% FCS was used as a positive control. $P < 0.01$ vs. the negative control for all time points after 4 h for 1.1 and 1.5 μM PSTI/TATI. B: addition of normal sequence recombinant PSTI/TATI (1.5 μM), the naturally occurring N34S PSTI/TATI variant peptide (23 μM; 1.5 μM), or the trypsin inhibitor site-inactivated variant R18/V19 PSTI/TATI (1.5 μM) all caused similar increases in migration compared with the control (C). For each peptide, $P < 0.01$ vs. the negative control for all time points after 6 h. C: addition of soybean trypsin inhibitor (structurally unrelated to PSTI/TATI) at various doses (0.23 μM; 0.46 μM; and 2.3 μM) did not stimulate cell migration above the negative control (C). For comparison, the effect of PSTI/TATI (1.5 μM) is also shown. Each of the experiments (A–C) were also performed using RT4 and HT1376 cells and gave similar results (data not shown).

However, addition of PSTI/TATI-neutralizing antibody had no effect on EGF-induced invasion (Fig. 3D).

Experiments examining the potential additive/synergistic effects of PSTI/TATI and EGF showed when given alone, treatment of cells with either 10 nM EGF or 10 nM PSTI/TATI did not significantly increase cell invasion compared with baseline (Fig. 3E). However, when given together, 10 nM EGF and PSTI/TATI showed a synergistic effect, resulting in a
Influence of endogenous (autocrine) PSTI/TATI production on migration and invasion. Western blot analysis (data not shown), immunoassay of cell lysates (Fig. 5A), and PSTI/TATI mRNA analyses (Fig. 5B) demonstrated endogenous production of PSTI/TATI.

PSTI/TATI RNA interference knockdown resulted in both PSTI/TATI protein (Fig. 5A) and RNA (Fig. 5B) expression being reduced by 75–80% (all \( P < 0.02 \) vs. nontransfected or scrambled controls) in the relevant cells.

Transfection with scrambled control plasmid did not influence the baseline rate of migration (Fig. 5C) or invasion (Fig. 5D) compared with nontransfected cells. In contrast, cells that had PSTI/TATI expression silenced by transfection with PSTI/TATI shRNA had significantly reduced baseline rates of migration (23% compared with the scrambled control; Fig. 5C) and reduced baseline invasion rates (32%, both \( P < 0.01 \); Fig. 5D). Transfected cells were still able to respond to the prostimulatory effects of the addition of FCS, EGF, or PSTI/TATI (data not shown).

Additional evidence for an autocrine effect of PSTI/TATI in these cell lines was suggested by the fact that the addition of PSTI/TATI-neutralizing antibody or the EGFR-neutralizing antibody had no effect on EGF-induced invasion. **\( P < 0.05 \) and ***\( P < 0.01 \) vs. the negative control (RPMI + 2% FCS). D: neutralizing antibodies and EGF. EGF-induced invasion was blocked by the addition of an EGF-blocking antibody or the tyrosine kinase inhibitor tyrphostin (AG-1478). However, the addition of PSTI/TATI-neutralizing antibody had no effect on EGF-induced invasion. **\( P < 0.05 \) and ***\( P < 0.01 \) vs. the negative control (RPMI + 2% FCS). E: synergistic effect of EGF and PSTI/TATI on cell invasion. The effects of various doses of PSTI/TATI and EGF, given alone or in combination, on cell invasion are shown. **\( P < 0.01 \) vs. the negative control (RPMI + 2% FCS); **\( P < 0.01 \) vs. RPMI alone or in combination, on cell invasion are shown.
increased cell migration and invasion into collagen gel but did not promote growth, and proliferation. Exogenous administration of PSTI/TATI upregulation, seen in most bladder tumours, on various.

**DISCUSSION**

The effect of PSTI/TATI on the EGFR and downstream pathways. In experiments comparing the effect of PSTI/TATI or EGF on the degree and rate of phosphorylation of the EGFR, similar results were seen in both 253J and HT1376 cell lines. The addition of EGF caused a rapid and intense phosphorylation of EGFR, with this effect beginning to diminish within 3 min (Fig. 6A). In contrast, the addition of PSTI/TATI resulted in a more gradual but more prolonged phosphorylation of EGFR, reaching a lower level than that caused by the addition of EGF (Fig. 6B).

Experiments examining downstream signaling pathways using the phospho-MAPK proteome profiler showed that, as expected from a study of non-urological cell lines (e.g., Ref. 16), incubation with EGF resulted in the phosphorylation of many of the MAPK pathways and other serine/threonine kinases (Fig. 7A). The highest increases in phosphorylation were seen in ERK1 and ERK2 and in Akt1, Akt2, and Akt3.

In contrast to the profile obtained by the addition of EGF, incubation with PSTI/TATI resulted in a less widespread phosphorylation profile (Fig. 7B). Large changes in the phosphorylation signal were seen in ERK1, ERK2, and Akt2 and, to a lesser extent, Akt3. In addition, small but significant changes were seen in JNK1, MKK3, and ribosomal protein S6 kinase (RSK)1.

The profile obtained by the addition of mutant R18/V19 PSTI/TATI resulted in a similar phosphorylation profile as incubation with normal sequence PSTI/TATI, with the exception of a much smaller increase in Akt2 phosphorylation (compare Fig. 7, B and C).

**Effect of PSTI/TATI on the EGFR and downstream pathways.** In the experiments comparing the effect of PSTI/TATI or EGF addition on the degree and rate of phosphorylation of the EGFR, similar results were seen in both 253J and HT1376 cell lines. The addition of EGF caused a rapid and intense phosphorylation of EGFR, with this effect beginning to diminish within 3 min (Fig. 6A). In contrast, the addition of PSTI/TATI resulted in a more gradual but more prolonged phosphorylation of EGFR, reaching a lower level than that caused by the addition of EGF (Fig. 6B).

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**DISCUSSION**

Using a variety of models, we examined the effect of PSTI/TATI upregulation, seen in most bladder tumours, on various pathophysiological actions relating to urothelial migration, invasion, and proliferation. Exogenous administration of PSTI/TATI increased cell migration and invasion into collagen gel but did not increase proliferation. Site-directed mutagenesis showed that an active trypsin inhibitor site within the PSTI/TATI sequence was not required to stimulate migration but was necessary to increase invasion. 253J and HT1376 cell lines both demonstrated PSTI/TATI-mediated autocrine stimulation of migration and invasion. At least some of these actions were dependent on the presence of functional EGFR, although postreceptor signaling experiments and the lack of proliferative activity of PSTI/TATI demonstrated marked disparity between PSTI/TATI and EGF.

PSTI/TATI is a 56-amino acid Kazal-type serine protease inhibitor that was initially identified in the pancreas. The subsequent realization of a wider distribution, such as in the colonic mucosa, suggests, however, that it may play additional roles in mucosal defence and immune modulation (16–18) and also led to two additional alternative names: serine protease inhibitor Kazal-type 1 (SPINK1; usually used in relation to studies on familial pancreatitis) and TATI.

The present series of experiments focused on its potential relevance in the malignant urothelium. Three cell lines were examined: 253J and HT1376 cells as examples of a high-grade invasive phenotype and RT4 cells as an example of a low-grade, noninvasive (3) phenotype. All three cell lines are in common use for growth factor activation and chemotherapy studies.

Two related well-established models of motility were used, one analyzing the effect of PSTI/TATI on the rate of migration of cells across wounded monolayers and the other examining changes in the invasion of cells through extracellular matrices, which has particular relevance to malignant spread and progression (16, 25).

PSTI/TATI stimulated cell migration in all cell lines in a dose-dependent manner. The two variant sequence PSTI/TATI peptides were found to possess similar promigratory activity. The N34S variant is found in some cases of familial pancreatitis, although the reason behind its association with this condition is unclear as our present study and other groups have found it retains its trypsin inhibitor activity (2). The R18/V19 variant is not found naturally and was produced to examine the importance of PSTI/TATI’s trypsin inhibitor active site in any effects seen. Despite having no trypsin inhibitor activity, the R18/V19 variant maintained similar promigratory activity to normal sequence PSTI/TATI, whereas addition of the structurally distinct soybean trypsin inhibitor did not induce migration. These findings suggest the generic trypsin inhibitor activity of PSTI/TATI was not relevant to its promigratory activity.

Experiments examining collagen invasion showed that proinvasive activity was induced by the addition of normal PSTI/TATI and the naturally occurring N34S variant, although the efficacy of the N34S variant was slightly reduced. However, in contrast to the migration experiments, the R18/V19 variant (trypsin inhibitor inactive) did not stimulate invasion, suggesting that serine protease inhibition was important in this effect. The mechanism underlying this difference is probably due to our finding that normal sequence PSTI/TATI stimulates upregulation of Akt2, whereas the R18/V19 variant caused only modest increase in phosphorylation. Akt activation stimulates cell invasion via increased motility and metalloproteinase production, and knockdown of Akt2, but not Akt1 or Akt3, has been shown to prevent the proinvasive activity of EGF in human breast cancer lines (1, 13).

The identity of the PSTI/TATI receptor(s) involved in stimulating migration and invasion is unclear, although Niinobu and coworkers (22) have reported 125I-labeled PSTI-binding.
sites on a variety of cell lines, including human skin fibroblasts (BUD-8), pancreatic cancer (A-431) cells, and colon cancer (HCT-15) cells. These binding sites were able to be saturated and displaced by excess noniodinated PSTI but not by EGF (22). In a subsequent study (23) using mouse fibroblasts, Niinobu et al. reported the presence of high-affinity plasma membrane receptors for human PSTI/TATI. These putative receptors were not cell surface proteinases, and the receptor ligand complex had a molecular weight of \(140\,\text{kDa} (23)\). Our present study showed truncation of PSTI/TATI-mediated effects in the presence of an EGFR-neutralizing antibody and that phosphorylation of EGFR occurred in response to the addition of PSTI/TATI. These findings are in keeping with our previous report (18) showing that the deletion of EGFR prevents the promigratory activity of EGF and PSTI/TATI in human colon cancer cells. Taken together, these results support the idea that at least some of the actions of PSTI/TATI are mediated through EGFR, although the relationship between PSTI/TATI and EGFR is probably not that of a direct receptor ligand. PSTI/TATI and EGFR share a similar three Cys-Cys disulfide bridge structure and have moderate sequence homology (50%). However, most radiolabeled EGFR PSTI/TATI/EGF displacement studies have reported that PSTI/TATI is not a direct EGFR ligand (7, 22, 23). Further evidence demonstrating that PSTI/TATI does not act as a standard EGFR ligand comes from our present finding that PSTI/TATI did not stimulate the proliferation of 253J, RT4, or HT1376 cells despite inducing phosphorylation of EGFR, whereas, using the same cell lines, EGF stimulated proliferation as well as inducing phosphorylation of EGFR. This finding is also supported by the lack of proliferative effects of PSTI/TATI reported by our and other groups using a variety of nonurothelial cells (18, 22, 23), although a notable exception is the proproliferative activity of PSTI/TATI when tested on the pancreatic cell line AR4-2J (6). It, therefore, seems more likely that PSTI/TATI is inducing cross-phosphorylation of EGFR and/or influencing its downstream pathways.

To further investigate downstream signaling pathways, we used a commercial human phospho-MAPK proteome profiler antibody array kit, which allows parallel determination of relative levels of phosphorylation of a wide range of MAPKs and other serine/threonine kinases. As expected, the addition of EGF gave a strong and widespread increase in phosphorylation. In contrast, a much more highly focused response was seen when normal sequence PSTI/TATI was added. The two major families undergoing phosphorylation in response to PSTI/TATI were Akt and ERK. Smaller increases in phosphorylation were seen in JNK1, whose functions include influencing apoptosis and the inflammatory response, and RSK1, which is involved in transcriptional regulation by phosphorylating c-Fos and CREB (24).
The Akt kinase family is composed of three highly homologous isoforms: Akt1 (PKB-α), Akt2 (PKB-β), and Akt3 (PKB-γ). Although there is an overlap in their functions, gene knockout and other models suggest that Akt1 plays an important role in cellular growth and angiogenesis, whereas Akt2 is probably important in cell migration and invasion (5). Our finding that EGF phosphorylates Akt1 and Akt2, whereas PSTI/TATI only phosphorylates Akt2, may be relevant in explaining the promigratory and invasive activity of both EGF and PSTI/TATI but discordance in regard to proliferation.

Akt2 phosphorylation is also important in glucose homeostasis and insulin signaling (9, 10). Our finding that PSTI/TATI stimulated Akt2 and MKK3 phosphorylation, which are both involved in glucose homeostasis and glucose transporter expression, may also have particular relevance to its production in the human pancreas (9, 10).

ERK1 and ERK2 (also known as MAPK3 and MAPK1) are part of the Ras-Raf-ERK signal transduction cascade often found downstream of growth factor receptor activation. Phosphorylation of ERK1 and ERK2 causes multiple effects on transcription factors and scaffolding proteins (15, 30).

Caution must always be shown in extrapolating from the in vitro to the in vivo situation. Nevertheless, the concentrations of PSTI/TATI and EGF used in the present study, which showed biological effects, especially in regard to synergistic responses (10–20nM), are similar to those found in humans in vivo.

Fig. 6. Time course of phosphorylation of EGFR in 253J cells. The addition of EGF caused a rapid and intense phosphorylation of EGFR, which began to diminish within 10 min (A). In contrast, the addition of PSTI/TATI caused a more progressive gradual increase in the phosphorylation of EGFR, although peak levels were somewhat lower than those seen using EGF (B). A450, absorbance at a wavelength of 450 nm. Data are expressed as means ± SE. All data points are P < 0.01 vs. time 0. Experiments using HT1376 cells gave similar results (data not shown).

Fig. 7. Effect of EGF (A), PSTI/TATI (B), and R18/V19 PSTI/TATI (C) on the phosphorylation of MAPKs and other serine/threonine kinases in 253J cells. GSK, glycogen synthase kinase; HSP, heat shock protein; RSK, ribosomal protein S6 kinase; TOR, target of rapamycin. Data are expressed as means ± SE. Experiments using HT1376 cells gave similar results (data not shown).
mean concentration of PSTI/TATI in urine from normal subjects is ~1.5 nM, rising to ~3.1 nM in patients with bladder cancer (4, 29). Similarly, the concentration of EGF present in the urine of healthy subjects is ~2.2 nM, with similar values seen in the urine of patients with bladder cancer (28). Furthermore, our study showed that endogenously produced PSTI/TATI was at least partially responsible for the migratory and invasive activity, acting in an autocrine fashion. Taken together, these findings suggest that PSTI/TATI expression in bladder carcinoma, possibly acting in concert with urinary EGF, may have relevance for tumor invasion and dissemination, opening the possibility of influencing clinical progression through interference of this system.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.M. and R.J.P. conception and design of research; T.M. and A.M. performed experiments; T.M., A.M., and R.J.P. analyzed data; T.M., A.M., and R.J.P. interpreted results of experiments; T.M. prepared figures; T.M. and R.J.P. drafted manuscript; T.M. and R.J.P. edited and revised manuscript; T.M., A.M., and R.J.P. approved final version of manuscript.

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