

ORIGINAL ARTICLE

Platelet polyphosphate induces fibroblast chemotaxis and myofibroblast differentiation

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Funding information

National Heart, Lung, and Blood Institute, Grant/Award Number: R35 HL135823, T32 HL007622 and UM1 HL120877; University of Michigan, Grant/Award Number: Mcubed

Abstract

Background: Platelets secrete many pro-wound healing molecules such as growth factors and cytokines. We found that releasates from activated human platelets induced the differentiation of cultured murine and human fibroblasts into a myofibroblast phenotype. Surprisingly, most of this differentiation-inducing activity was heat-stable, suggesting it was not due to the protein component of the releasates. Inorganic polyphosphate is a major constituent of platelet-dense granules and promotes blood coagulation and inflammation.

Objectives: We aim to investigate the contribution of polyphosphate on myofibroblast differentiating activity of platelet releasates.

Methods: Using NIH-3T3 cells and primary human fibroblasts, we examined the effect of human platelet releasates and chemically synthesized polyphosphate on fibroblast differentiation and migration.

Results: We found that the myofibroblast-inducing activity of platelet releasates was severely attenuated after incubation with a polyphosphate-degrading enzyme, and that fibroblasts responded to platelet-sized polyphosphate by increased levels of α -smooth muscle actin, stress fibers, and collagen. Furthermore, fibroblasts were chemotactic toward polyphosphate.

Conclusions: These findings indicate that platelet-derived polyphosphate acts as a cell signaling molecule by inducing murine and human fibroblasts to differentiate into myofibroblasts, a cell type known to drive both wound healing and fibrosing diseases. Polyphosphate therefore not only promotes early wound responses through enhancing fibrin clot formation, but also may play roles in the later stages of wound healing, and, potentially, progression of fibrotic diseases, by recruiting fibroblasts and inducing their differentiation into myofibroblasts.

KEYWORDS

blood platelets, chemotaxis, fibroblasts, myofibroblasts, platelet activation

1 | INTRODUCTION

Polyphosphate is a simple inorganic molecule, consisting of linear polymers of phosphate groups that range in length from a few phosphates to more than a thousand phosphates long.¹ Polyphosphate has been identified in all kingdoms of life, making it a remarkably conserved molecule.² The majority of polyphosphate studies has been performed in prokaryotes, where polyphosphate has roles in stress endurance, quorum sensing, virulence, phosphate storage, biofilm formation, and heavy metal resistance.³⁻⁹ In unicellular eukaryotes, polyphosphate has roles in germination, development, migration, nutrient homeostasis, proliferation, and differentiation.¹⁰⁻¹⁵

Although the roles and modes of action of polyphosphate in mammalian cells are less understood, extracellular polyphosphate is an emerging regulatory molecule with recently identified functions including blood clotting, complement, biomineralization, inflammation, differentiation, and cell migration.¹⁶⁻²⁵ Polyphosphate is contained in the dense granules of platelets, and upon platelet activation it is released into the microenvironment where it modulates coagulation and fibrinolysis by enhancing fibrin clot structure, acting as cofactor for the activation of factor XI by thrombin, accelerating factor V activation by factor XIa, and acting as cofactor for inactivation of alpha tissue factor pathway inhibitor by factor XIa.²⁶⁻³¹ Platelet-derived polyphosphate acts as a pro-inflammatory and pro-coagulant molecule for endothelial cells by increasing adhesion molecule expression and von Willebrand factor secretion.^{17,18,32}

Polyphosphate released from activated platelets induces differentiation of monocytes into fibrocytes, a circulating cell type that can promote scar tissue.²⁴ These findings raise the possibility that polyphosphate secreted by activated platelets may act as a cell signaling molecule in wound healing for other cell types. Tissue resident fibroblasts are known to be recruited to wound or injury sites and to differentiate into myofibroblasts, which generate contractile force and secrete tissue matrix proteins to aid in wound closure and scar formation.³³ Myofibroblasts are also the primary effector cells driving fibrotic disease, in which the wound healing process becomes dysregulated in otherwise healthy organs or tissues, which in turn can lead to cellular dysfunction, organ failure, and death.^{34,35} There is currently no cure for fibrosis, which contributes to up to 45% of all deaths in the United States.³⁶ We now report that platelet polyphosphate is chemotactic for mouse and human fibroblasts, and that it potently induces their differentiation into myofibroblasts.

2 | METHODS

2.1 | Materials

α -Smooth muscle actin (α -SMA) antibody (14-9760-82), goat anti-mouse IgG2a secondary antibody (A-21133), Dulbecco's Modified Eagle Medium (DMEM), SYBR Green I, polycarbonate cell culture inserts, ProLong Gold Antifade Mountant, and CyQUANT Cell

Essentials

- Polyphosphate is released from activated platelets.
- Platelet releasates induce myofibroblast differentiation.
- Platelet-sized polyphosphate promotes migration and myofibroblast differentiation.
- Platelet polyphosphate acts as a pro-wound healing and chemotactic molecule for fibroblasts.

Proliferation Assay were from Thermo Fisher Scientific. Bovine calf serum, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Tween 20, and Triton X-100 were from Fisher Scientific. Collagen 1 (84336) and vimentin (5741) antibodies were from Cell Signaling Technology. The PAR-1 selective activating peptide, TRAP (S1820), prostaglandin E1, theophylline, sodium pyrophosphate, and sodium tripolyphosphate were from Sigma-Aldrich. Phalloidin-iFluor 647 (ab176759) was from Abcam. Goat anti-rabbit IgG was from Jackson ImmunoResearch Labs. Poly-L-Lysine was from ScienCell Research Laboratories. L-glutamine, penicillin/streptomycin, and insulin-transferrin-selenium were from Corning. The P2Y₁ receptor competitive antagonist MRS 2279 was from Tocris.

Narrowly sized fractionated polyphosphate was produced from chemically synthesized polyphosphate by preparative PAGE as previously described.³⁷ Approximately platelet-sized polyphosphate, with a modal polymer length of 75 phosphates and a range of 20 to 280 phosphates, was prepared using differential acetone precipitation of P70 (a more heterogeneous, chemically synthesized polyphosphate preparation that was a kind gift from Dr Thomas Staffel, BK Giulini GmbH). Briefly, to a stirred solution of 500 mmol/L P70 in 100 mmol/L NaCl, acetone was gradually added to a final concentration of 12.2% v/v. The precipitated polyphosphate was collected by centrifugation for 20 minutes at 13 000g, dried under nitrogen gas, and dissolved in a solution of 20 mmol/L HEPES-NaOH buffer (pH 7.4). Polyphosphate concentrations were quantified as inorganic phosphate after acid hydrolysis as previously described.³⁸ In this paper, polyphosphate concentrations are given in terms of the concentration of phosphate monomer.

2.2 | Cell culture

The murine embryonic fibroblastic cell line, NIH-3T3 (ATCC CRL-1658), and primary human dermal fibroblasts, GM1381 (Coriell Institute), were routinely cultured in DMEM supplemented with 10 mmol/L HEPES, penicillin, streptomycin, 4 mmol/L L-glutamine, and 10% bovine calf serum, in a 5% CO₂ environment at 37°C. Cells were maintained at <90% confluence and were cultured for no more than 2 weeks at a time. Experiments performed in serum-free media were cultured in DMEM supplemented as detailed previously, without serum but with the addition of insulin-transferrin-selenium.

2.3 | Platelet releasates

Recently expired platelet units were kindly provided by the University of Michigan Blood Bank. Platelet releasates were prepared as described.²⁹ Briefly, platelets were washed in Tyrode's buffer and suspended in the same buffer at 2.0×10^9 platelets/mL, then incubated with 5 $\mu\text{mol/L}$ TRAP to activate the platelets. After 20 minutes, platelets were removed by centrifugation, and the protein content was estimated using a NanoDrop 1000 spectrophotometer (assuming an A_{280} of 1 equals 1 mg/mL protein). Protein concentrations in such releasates averaged 415 ± 86 $\mu\text{g/mL}$ (mean \pm standard deviation). Releasates were diluted to 300 $\mu\text{g/mL}$ protein in Tyrode's buffer containing 10 mmol/L EDTA (to inhibit phosphatases), then filter-sterilized and stored at -80°C until use. In some cases, releasates were heated to 95°C for 30 minutes to denature proteins, then clarified by centrifugation for 10 minutes at 13 000g. (Polyphosphate is stable during this heat treatment.²⁹) In other cases, polyphosphate was degraded by incubating samples with recombinant yeast exopolyphosphatase (ScPPX1), as previously described.²⁹ Effects of platelet releasates on cell differentiation were tested by incubating adherent cells for 48 hours in medium with 1.5% serum plus 20-fold-diluted releasate (yielding a final releasate protein concentration of 15 $\mu\text{g/mL}$).

2.4 | Immunofluorescence

For immunofluorescence studies, cells were seeded on glass coverslips at a density of 3.75×10^3 cells/cm² in DMEM containing 2.5% serum and allowed to adhere overnight. The next day, cells were treated in fresh medium (with 2.5% serum) containing the desired stimulant for varying times, after which cells were washed once with Tris-buffered saline (TBS; 50 mmol/L Tris-Cl pH 7.5, 150 mmol/L NaCl) and fixed for 10 minutes at room temperature using 4% paraformaldehyde in TBS. Cells were washed three times with TBS, permeabilized in TBS containing 0.2% Triton X-100 for 5 minutes, then washed again three times with TBS. Cells were then blocked in TBS plus 0.05% Tween-20 and 5% bovine serum albumin for 1 hour at room temperature, after which they were incubated with primary antibody for 1 hour at room temperature in TBS plus 0.05% Tween-20 and 1% bovine serum albumin, and then washed three times with TBS. Cells were then incubated with secondary antibody plus SYBR green I (to stain DNA, 1:250 000 dilution) in TBS plus 0.05% Tween-20 and 1% bovine serum albumin for 1 hour at room temperature in the dark. Cells were washed three times in TBS, briefly rinsed with water, and mounted onto glass microscope slides using Antifade Mountant. Samples were imaged the next day or stored at 4°C in the dark. Primary antibodies were used at the following dilutions: α -SMA at 1:1000, COL1A at 1:500, and vimentin at 1:100. Secondary antibodies were typically diluted 1:500, whereas 647-conjugated phalloidin was diluted 1:1000. Images were obtained on a Leica SP5 confocal microscope (Leica Microsystems). A minimum of 25 individual cells from a minimum

of five representative images were analyzed for each experiment. Integrated density (defined as product of cell area and mean fluorescence intensity) was determined using ImageJ and the average integrated density for each experiment was determined.³⁹ Cell body area was quantified using ImageJ.

2.5 | Migration assay

A total of 5×10^4 cells in DMEM were seeded in the top chamber of a NUNC polycarbonate 24-well transwell (Thermo Fisher Scientific) migration assay in which the top chamber insert contained an 8-micron porous membrane growth surface. Media with or without polyphosphate was added into the bottom chamber of the well to generate a polyphosphate gradient. Plates were incubated in a 5% CO₂ environment at 37°C for 20 hours, after which cells in the bottom chamber were collected, subjected to one freeze/thaw cycle, and resuspended in RIPA lysis buffer. DNA was quantified in lysates using the CyQUANT proliferation assay using a SpectraMax i3x fluorescent microplate reader (Molecular Devices).

3 | RESULTS

3.1 | Ability of platelet releasates to induce myofibroblast differentiation in NIH 3T3 cells is largely dependent on its polyphosphate content

To characterize platelet-secreted factors that promote wound healing, we examined the ability of platelet releasates to induce differentiation of cultured fibroblastic cells into myofibroblasts, as depicted by increased α -SMA levels. Figure 1A reports a significant increase in α -SMA levels in NIH-3T3 cells 48 hours after incubating the cells with platelet releasates in 1.5% serum, relative to cells cultured in the same medium without releasates. Releasates that had been heated to 95°C for 30 minutes exhibited only a slightly diminished ability to induce an increase α -SMA levels in NIH-3T3 cells, suggesting that the activity was not due to a protein. The inorganic polymer, polyphosphate, is an emerging signaling molecule that is not a protein or peptide, and is released from dense granules of activated platelets.²⁶ Furthermore, polyphosphate is relatively heat-stable under conditions that denature most proteins,¹⁷ so it seemed a good candidate for the differentiation-induce activity in platelet releasate. When platelet releasates were incubated with ScPPX1 to degrade polyphosphate,⁴⁰ they lost most of their ability to increase α -SMA levels in NIH-3T3 cells. Finally, the combination of heat-treating the releasates followed by digestion with ScPPX1 completely destroyed their ability to induce an increase in α -SMA levels in NIH-3T3 cells ($P = 0.23$). These data support the idea that the polyphosphate content of platelet releasates is essential for most of their ability to induce myofibroblast differentiation.

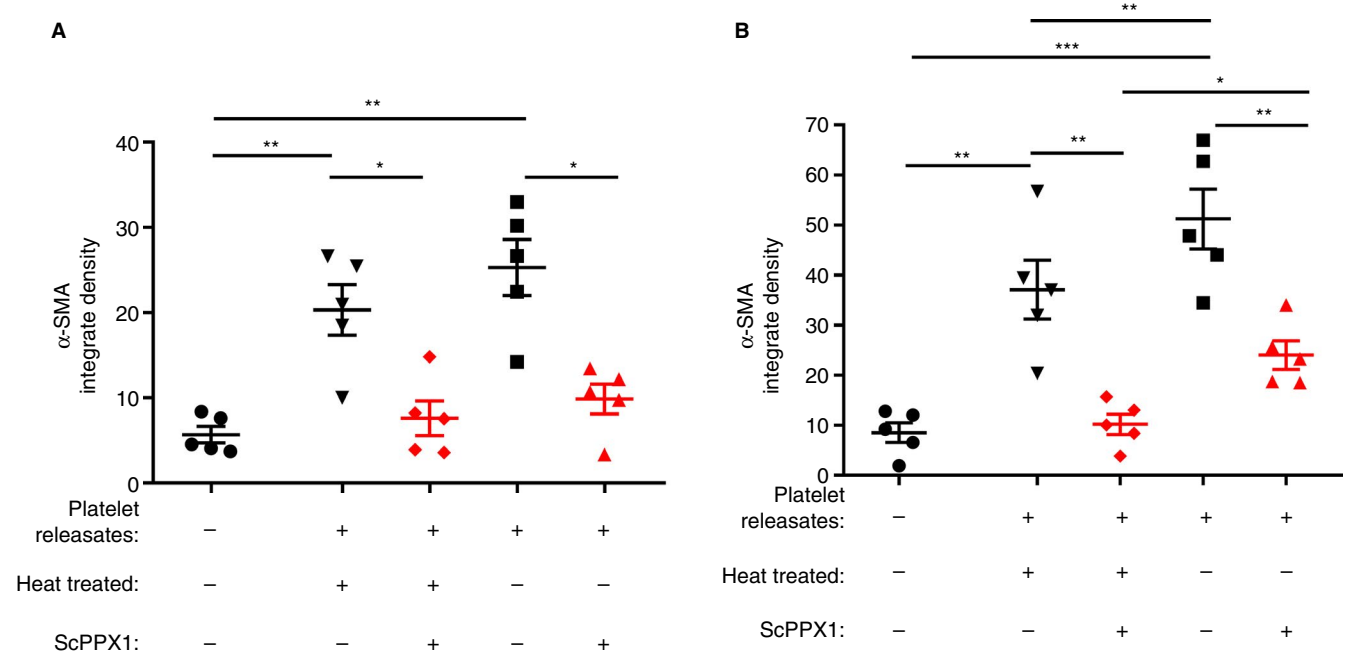


FIGURE 1 Platelet releasates induce myofibroblast differentiation, as evidenced by increased α -SMA levels. A, NIH-3T3 cells or (B) GM1381 cells were incubated in 1.5% serum in the presence or absence of releasates from activated human platelets (diluted to 15 μ g/mL protein). As indicated, some releasates were previously heat-treated to denature proteins, or digested with ScPPX1 to destroy polyphosphate (red indicates treatment with ScPPX1). After 48 h, antibody staining for α -SMA was quantified by confocal microscopy as a marker of myofibroblast differentiation. All values are mean \pm SEM ($n = 5$). Asterisks highlight statistical significance of the indicated comparisons: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (paired t-tests)

3.2 | Platelet releasates also induce myofibroblast differentiation of primary human skin fibroblasts

NIH-3T3 cells are an established cell line derived from mouse embryonic fibroblasts. To examine whether polyphosphate could induce myofibroblast differentiation in primary human cells, we cultured normal human dermal fibroblast cells (GM1381)⁴¹ under the same conditions we used for NIH-3T3 cells. As with NIH-3T3 cells, incubating GM1381 cells with releasates from human platelets induced significant accumulation of α -SMA, whereas this activity was substantially reduced when the releasates were digested with ScPPX1 (Figure 1B). As with NIH-3T3 cells, heat-treating the releasates resulted in only a modest decrease in accumulation of α -SMA by GM1381 cells. GM1381 cells incubated with platelet releasates that had been both heat-treated and digested with ScPPX1 showed no significant increase in α -SMA over control cells. Taken together, these findings show that primary human dermal fibroblasts respond to platelet-derived polyP in a manner that is very similar to those of NIH-3T3 cells regarding induction of markers of myofibroblast differentiation.

3.3 | Platelet-sized polyphosphate induces hallmarks of myofibroblast differentiation

To assess directly if polyphosphate can act as a signaling molecule for fibroblasts, we incubated NIH 3T3 cells with chemically

synthesized polyphosphate (of approximately the size range released by activated platelets) in low-serum medium for 48 hours. As can be seen in Figure 2A, 5 μ mol/L platelet-sized polyphosphate induced a robust increase in α -SMA levels which localized along stress fibers, both of which are hallmarks of differentiation into myofibroblasts.^{33,35} We further found that concentrations of platelet-size polyphosphate ranging from 0.1 to 25 μ mol/L resulted in significantly increased α -SMA levels and stress fiber formation (Figure 2B and C). These polyphosphate concentrations are well within those expected in the presence of activated human platelets.²⁷ In addition to increased α -SMA levels and stress fiber formation, fibroblasts increase in cell body size upon myofibroblast differentiation.⁴² Cells treated for 48 hours with 1 μ mol/L platelet-sized polyphosphate increased in cell body area (Figure 2D).

3.4 | Polyphosphate of a variety of polymer lengths induces myofibroblast differentiation

The biological effects of polyphosphate can depend strongly on polymer length.³⁷ To evaluate the polymer length dependence of polyphosphate's ability to induce myofibroblast differentiation, we incubated NIH-3T3 cells for 48 hours with a series of narrowly sized fractionated polyphosphate preparations (all tested at 1 μ mol/L) and evaluated α -SMA levels and stress fiber localization. Polyphosphate preparations ranging from

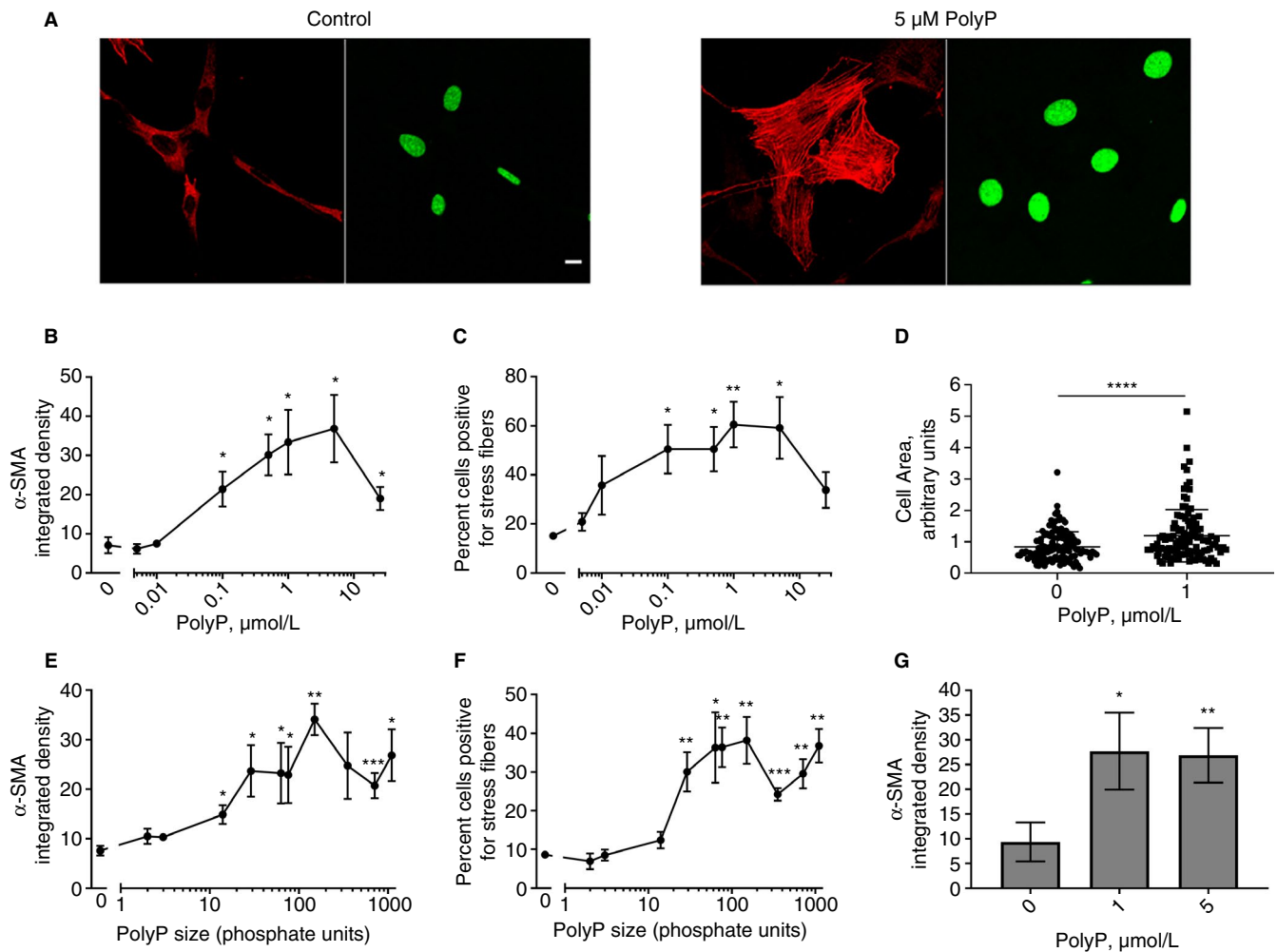


FIGURE 2 Polyphosphate induces myofibroblast differentiation, as evidenced by increased $\alpha\text{-SMA}$ levels and stress fiber formation. A and C, Platelet-size polyphosphate (polyP) increases $\alpha\text{-SMA}$ levels in NIH-3T3 cells. A, Representative images in which NIH-3T3 cells were cultured in 1.5% serum in the absence (control) or presence of 5 $\mu\text{mol/L}$ platelet-sized polyphosphate. After 48 h, cells were stained for $\alpha\text{-SMA}$ (red) or DNA (green) and imaged by confocal microscopy (bar = 10 μm). B and C, Dose-response of NIH-3T3 cells to platelet-size polyphosphate. In 5 experiments, cells were treated for 48 h in 1.5% serum plus the indicated concentrations of platelet-size polyphosphate, after which the following were quantified: (B) integrated intensity of $\alpha\text{-SMA}$ staining per cell, or (C) percent cells positive for $\alpha\text{-SMA}$ staining localized along fibrin tendrils (stress fibers). D, Platelet-size polyphosphate increases cell body area. NIH-3T3 cells were cultured in 1.5% serum in the absence or presence of 1 $\mu\text{mol/L}$ polyphosphate for 48 h and imaged by confocal microscopy. Cell body area of individual cells was quantified. E and F, Polymer length-dependence of polyphosphate's ability to induce myofibroblast differentiation in NIH 3T3 cells. Cells were incubated in 1.5% serum for 48 h in the presence or absence of 1 $\mu\text{mol/L}$ polyphosphate of the indicated polymer lengths (in phosphate units): (E) integrated intensity of $\alpha\text{-SMA}$ staining per cell, or (F) percent cells positive for $\alpha\text{-SMA}$ staining localized along fibrin tendrils (stress fibers). G, Dose-response of GM1381 cells to platelet-size polyphosphate. Cells were incubated in 1.5% serum with varying concentrations of platelet-size polyphosphate for 48 h, after which the integrated intensity of $\alpha\text{-SMA}$ staining was quantified. Values in panels B-C and E-G are mean \pm SEM ($n = 5$). Asterisks highlight statistical significance relative to the no-polyphosphate controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (paired t tests). Values in panel D are mean \pm SEM of individual cells across five experiments. **** $P < 0.0001$ (unpaired t test)

14 to 1100 phosphates long showed significant ability to induce $\alpha\text{-SMA}$ accumulation (Figure 2E), whereas polyphosphates ranging from 29 to 1100 phosphates in length supported increased stress fiber formation (Figure 2F). These polymer lengths encompass those secreted by activated platelets, mast cells, and basophils, which are ~ 60 to 100 phosphates long, as well as those found in microbes, which can range up to more than 1000 phosphates long.

3.5 | Platelet-sized polyphosphate induces myofibroblast differentiation of primary human fibroblasts

We next examined whether polyphosphate could induce myofibroblast differentiation in primary human fibroblasts. GM1381 cells were incubated for 48 hours with 1 or 5 $\mu\text{mol/L}$ platelet-sized polyphosphate. Polyphosphate induced significant increases in the

levels of α -SMA, in a manner very similar to that observed with NIH-3T3 cells (Figure 2G).

3.6 | Polyphosphate increases polymerized actin levels

To determine if platelet-sized polyphosphate induced an increase in F-actin levels, we stained polyphosphate-treated NIH-3T3 cells with phalloidin. Polyphosphate increased F-actin levels within 24 hours, and by 48 hours showed clear fibril localization (Figure 3A and B). This further suggests that polyphosphate promotes the formation of actin stress fibers in fibroblastic cells.

3.7 | Polyphosphate increases collagen synthesis and vimentin levels

We next assessed if platelet-sized polyphosphate induced increases in other markers of myofibroblast differentiation. Myofibroblasts produce high levels of extracellular matrix proteins to propagate scar-tissue formation and promote reepithelialization.³⁵ Polyphosphate nanoparticles have previously been shown to increase collagen levels in the osteoblastic cell line MC3T3-E1, as well as in vivo when topically applied to skin lesions of mice.⁴³ To determine if polyphosphate induces increased collagen synthesis in fibroblasts, NIH-3T3 cells were stained for collagen 1 alpha after incubation for 48 hours with platelet-sized polyphosphate. Polyphosphate

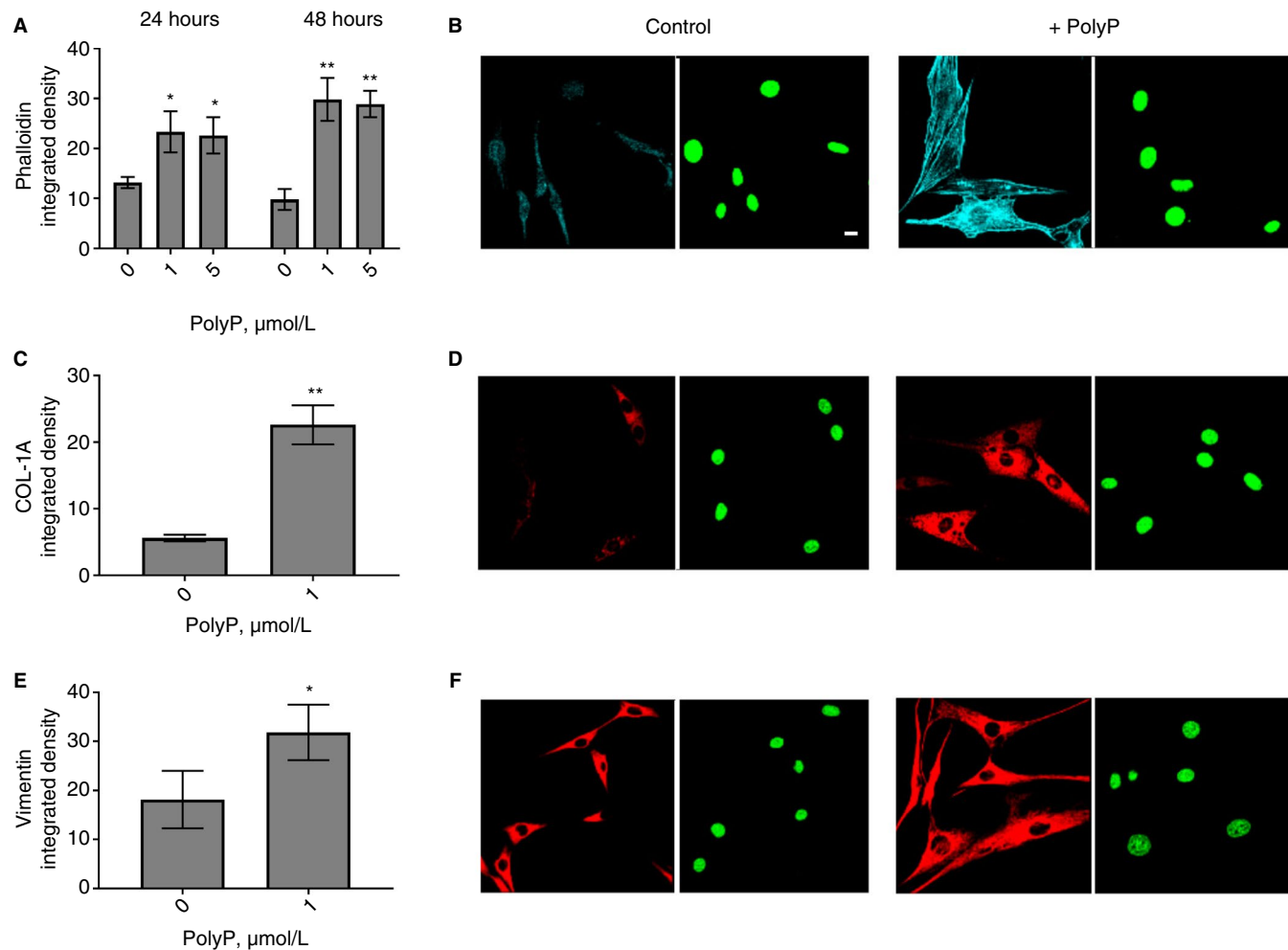


FIGURE 3 Polyphosphate increases other myofibroblast markers. A and B, phalloidin levels. NIH-3T3 cells were incubated in 1.5% serum for 24 or 48 h in the presence of the indicated concentrations of platelet-sized polyphosphate, after which phalloidin staining levels from confocal images were assessed. Images at 48 h are shown in panel B (representative of 5 experiments; bar = 10 μm). C and D, collagen type 1 alpha (COL-1A) levels. NIH-3T3 cells were incubated in 1.5% serum for 48 h in the presence or absence of 1 $\mu\text{mol/L}$ platelet-sized polyphosphate, after which COL-1A staining levels from confocal images were assessed. D, representative images from 5 experiments. E and F, vimentin levels. NIH-3T3 cells were incubated in 1.5% serum for 48 h in the presence or absence of 1 $\mu\text{mol/L}$ platelet-sized polyphosphate, after which vimentin staining levels from confocal images were assessed. F, representative images from 5 experiments. Values in panels A, C, and E are mean \pm SEM (n = 5). Asterisks highlight statistical significance compared to the no-polyphosphate controls: * $P < 0.05$, ** $P < 0.01$ (paired t tests)

induced an increase in collagen production (Figure 3C and D). The intermediate filament vimentin is a mesenchymal marker that is important in wound healing, and increases upon myfibroblast differentiation.^{44,45} Polyphosphate caused an increase in vimentin staining (Figure 3E and F). These data suggest that polyphosphate increases the synthesis of collagen and vimentin, both of which are markers of myfibroblasts.

3.8 | Polyphosphate is a chemoattractant for NIH-3T3 cells

Tissue-resident fibroblasts are recruited to wound sites preceding myfibroblast differentiation.^{33,35} Polyphosphate has been

implicated in endothelial cell migration during bone and cartilage formation,⁴⁶ modulates leukocyte migration,⁴⁷ and acts as a chemoattractant for human neutrophils.²⁴ We therefore examined whether polyphosphate was a chemoattractant for NIH-3T3 cells by culturing NIH-3T3 cells in transwell migration plates with a gradient of polyphosphate. Polyphosphate at 1 or 5 $\mu\text{mol/L}$ enhanced NIH-3T3 migration toward the bottom chamber (Figure 4A). To test whether polyphosphate was a chemoattractant rather than simply enhancing motility, polyphosphate was incubated in the top, bottom, or both chambers of transwell migration plates. Cells incubated with polyphosphate in the top chamber remained in the upper chamber in higher numbers compared with untreated cells (Figure 4B). Polyphosphate incubated in both the top and bottom chambers did not induce directional cell migration, whereas

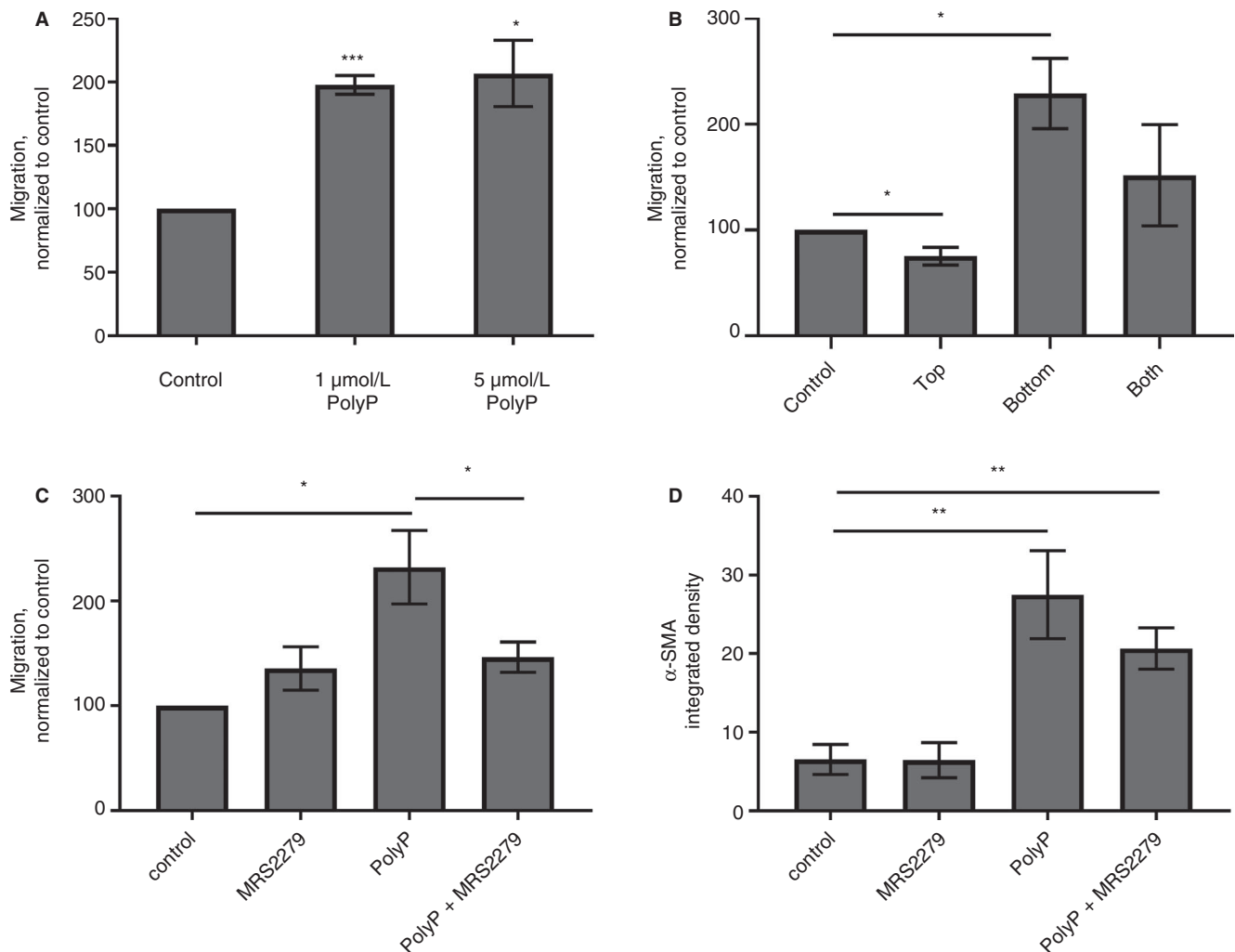


FIGURE 4 Polyphosphate is a chemoattractant. A, NIH-3T3 cells were incubated in 1% serum for 20 h in the upper chamber of migration chambers, with the indicated concentrations of platelet-sized polyphosphate in the lower chamber. B, NIH-3T3 cells were incubated in 1% serum for 20 h with 5 $\mu\text{mol/L}$ platelet-sized polyphosphate in the indicated chamber(s), or without polyphosphate (control). C, NIH-3T3 cells were incubated in 1% serum for 20 h in the upper chamber of migration chambers, with the indicated treatments of 3 $\mu\text{mol/L}$ MRS2279 or 5 $\mu\text{mol/L}$ polyphosphate in the lower chamber. Control chambers received no treatment. After collecting and lysing the cells in the lower chamber, CyQUANT signals were expressed as relative fluorescence units (RFU) normalized to the no-polyphosphate control (set to 100 RFU). D, NIH-3T3 cells were cultured in 1.5% serum in the absence (control) or presence of 5 $\mu\text{mol/L}$ platelet-sized polyphosphate or 3 $\mu\text{mol/L}$ MRS2279. After 48 h, the integrated intensity of α -SMA staining was quantified. All values are mean \pm SEM (n = 5). Asterisks highlight statistical significance compared to the no-polyphosphate controls: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (paired t tests)

polyphosphate in just the bottom chamber resulted in directional cell migration (Figure 4B). The purinergic receptor P2Y₁ mediates polyphosphate signaling in endothelial cells and astrocytes.^{32,48} Blocking P2Y₁ signaling with the high-affinity competitive antagonist MRS 2279 effectively inhibited polyphosphate-induced migration (Figure 4C). Thus, in addition to inducing fibroblast differentiation, polyphosphate may also play a role in fibroblast recruitment, mediated by the P2Y₁ receptor. In contrast, we found that MRS 2279 did not significantly diminish the ability of polyphosphate to induce α -SMA accumulation by NIH-3T3 cells (Figure 4D). This suggests that the differentiation-inducing ability of polyphosphate may act via different receptors than its chemotaxis-inducing ability.

4 | DISCUSSION

Here, we show that inorganic polyphosphate promotes myofibroblast differentiation in both the embryonic fibroblast-like cell line, NIH-3T3, and primary human dermal fibroblasts. Platelet-sized polyphosphate induced increased α -SMA expression, which localized along F-actin stress fibers, the two hallmarks of myofibroblast differentiation. Furthermore, polyphosphate promoted increased F-actin levels, collagen synthesis, and vimentin levels. These data suggest that polyphosphate activates fibroblasts to differentiate into a tissue-remodeling phenotype that promotes contractile force generation to aid in wound closure, and increases tissue matrix protein synthesis to generate scar tissue, both essential to wound resolution.

Polyphosphates over a wide range of sizes promoted myofibroblast differentiation, with 29 phosphate groups being the minimum length necessary, and polymer lengths of up to 1100 phosphates showing activity. Eukaryotic cells such as *Dictyostelium discoideum*, mast cells, basophils, and platelets secrete relatively small chains of polyphosphate containing less than 100 phosphate groups.^{13,25,26} Prokaryotic organisms, on the other hand, contain polyphosphates with more heterogeneous size distributions, often well over 1000 phosphate units in length.³⁷ It is therefore possible that microbial polyphosphate could also promote chemotaxis of fibroblasts and differentiation into myofibroblasts. Although severe bacterial infection may be detrimental, low levels of bacterial colonization have actually been shown to be beneficial to wound healing.⁴⁹

Polyphosphate is an emerging pro-migratory molecule, enhancing migration of the social amoeba *Dictyostelium discoideum*,¹⁵ human endothelial cells,⁴⁶ and human leukocytes.^{24,47} Our finding that platelet-sized polyphosphate promoted chemotaxis of NIH-3T3 cells suggests that in addition to inducing differentiation into a pro-wound healing and pro-fibrotic phenotype, polyphosphate may act to recruit fibroblasts to sites of tissue injury. The purinergic receptor P2Y₁ mediates polyphosphate signaling in endothelial cells and astrocytes.^{32,48} Migration of NIH-3T3 cells induced by polyphosphate was also mediated by P2Y₁, adding further evidence that P2Y₁ may be a conserved receptor for polyphosphate in mammalian cells. In contrast, inhibiting P2Y₁

receptors did not appreciably diminish 3T3 cell differentiation into a myofibroblast phenotype, suggesting that this aspect of polyphosphate signaling uses different receptors.

Polyphosphate nanoparticles enhance wound healing in vivo, increasing collagen and α -SMA levels in the osteoblast-like cell line, MC3T3.⁴³ Here, we show that polyphosphate synthesized and released by human platelets recapitulated the effects of chemically synthesized polyphosphate on myofibroblast differentiation. This was demonstrated in experiments in which releasates from activated human platelets induced fibroblast differentiation, but this activity was largely eliminated following treatment of releasates with the polyphosphate-degrading enzyme, exopolyphosphatase, suggesting that polyphosphate is the major source of activity in platelet releasates. Polyphosphate released from activated platelets and other cell types at sites of tissue damage may therefore act as a pro-wound healing, cell signaling molecule for fibroblasts. It also suggests that polyphosphate may be an appealing therapeutic for treatment of chronic wounds.

In this study, platelet polyphosphate promoted myofibroblast differentiation of both murine fibroblastic cells and human primary dermal fibroblasts. Myofibroblasts drive fibrosis progression in tissues and organs, raising the intriguing possibility that polyphosphate, which also promotes the formation of the pro-fibrotic fibrocyte cell,²⁴ may be a novel target for the suppression of fibrotic diseases, which account for 45% of all deaths in the United States.^{24,36} Patients with idiopathic pulmonary fibrosis show increased platelet reactivity and activation, which is due to a currently unknown plasma factor.^{50,51} Primary myelofibrosis, in which the normal spongy marrow of the bone is replaced by dense scar tissue, is thought to be driven in large part by megakaryocytes, which are responsible for platelet production.^{52,53} Megakaryocytes contain pro-fibrotic molecules such as transforming growth factor-beta and platelet-derived growth factor.⁵³ The role of platelet-derived and potentially megakaryocyte-derived polyphosphate in these fibrotic disorders has not been investigated, but might provide insight into the progression of these fibrotic pathologies in the lung and bone marrow.

Mycobacterium tuberculosis and *Trypanosoma cruzi* are microorganisms that accumulate polyphosphate.^{54,55} In *M tuberculosis*, polyphosphate plays roles in infection persistence, survival, and virulence, while in *T cruzi*, polyphosphate is important for environmental stress adaptation.^{54,56-58} Tuberculosis infection can lead to the development of pulmonary fibrosis, whereas *T cruzi* infection can lead to the development of cardiac fibrosis.⁵⁹⁻⁶¹ Microbial polyphosphate may therefore be a source of this fibrosis-inducing activity, which warrants further investigation.

ACKNOWLEDGMENTS

This work was supported by Mcubed funding from the University of Michigan and by grants R35 HL135823, UM1 HL120877, and T32 HL007622 from the National Heart Lung & Blood Institute of National Institutes of Health (NIH). The content is solely the responsibility of the authors and does not necessarily represent the views of the NIH.

CONFLICT OF INTEREST

Dr. Suess, Dr. Smith, and Dr. Morrissey are inventors on patents and patent applications for medical uses of polyphosphate and polyphosphate inhibitors.

AUTHOR CONTRIBUTIONS

Patrick M. Suess designed and performed the research, collected data, analyzed, and interpreted the data, and wrote the paper. Stephanie A. Smith designed the protocol and generated platelet-sized polyphosphate and revised the paper. James H. Morrissey coordinated and conceived the study, acquired funding, interpreted the data, and revised the paper.

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REFERENCES

- Brown MR, Kornberg A. Inorganic polyphosphate in the origin and survival of species. *Proc Natl Acad Sci USA*. 2004;101:16085-16087.
- Rao NN, Gómez-García MR, Kornberg A. Inorganic polyphosphate: essential for growth and survival. *Annu Rev Biochem*. 2009;78:605-647.
- Rao NN, Kornberg A. Inorganic polyphosphate supports resistance and survival of stationary-phase *Escherichia coli*. *J Bacteriol*. 1996;178:1394-1400.
- Nikel PI, Chavarría M, Martínez-García E, Taylor AC, de Lorenzo V. Accumulation of inorganic polyphosphate enables stress endurance and catalytic vigour in *Pseudomonas putida* KT2440. *Microb Cell Fact*. 2013;12:50.
- Rashid MH, Rumbaugh K, Passador L, et al. Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA*. 2000;97:9636-9641.
- Srisanga K, Suthapot P, Permsirivisarn P, Govitrapong P, Tungpradabkul S, Wongtrakongate P. Polyphosphate kinase 1 of *Burkholderia pseudomallei* controls quorum sensing, RpoS and host cell invasion. *J Proteomics*. 2019;194:14-24.
- Tocheva EI, Dekas AE, McGlynn SE, Morris D, Orphan VJ, Jensen GJ. Polyphosphate storage during sporulation in the gram-negative bacterium *Acetonebacterium longum*. *J Bacteriol*. 2013;195:3940-3946.
- Kulaev I, Kulakovskaya T. Polyphosphate and phosphate pump. *Annu Rev Microbiol*. 2000;54:709-734.
- Kulakovskaya T. Inorganic polyphosphates and heavy metal resistance in microorganisms. *World J Microbiol Biotechnol*. 2018;34:139.
- Zhang H, Gómez-García MR, Brown MR, Kornberg A. Inorganic polyphosphate in *Dictyostelium discoideum*: influence on development, sporulation, and predation. *Proc Natl Acad Sci USA*. 2005;102:2731-2735.
- Livermore TM, Chubb JR, Saiardi A. Developmental accumulation of inorganic polyphosphate affects germination and energetic metabolism in *Dictyostelium discoideum*. *Proc Natl Acad Sci USA*. 2016;113:996-1001.
- Bru S, Martínez-Láinez JM, Hernández-Ortega S, et al. Polyphosphate is involved in cell cycle progression and genomic stability in *Saccharomyces cerevisiae*. *Mol Microbiol*. 2016;101:367-380.
- Suess PM, Gomer RH. Extracellular polyphosphate inhibits proliferation in an autocrine negative feedback loop in *Dictyostelium discoideum*. *J Biol Chem*. 2016;291:20260-20269.
- Suess PM, Watson J, Chen W, Gomer RH. Extracellular polyphosphate signals through Ras and Akt to prime *Dictyostelium discoideum* cells for development. *J Cell Sci*. 2017;130:2394-2404.
- Suess PM, Tang Y, Gomer RH. The putative G protein-coupled receptor Gr1D mediates extracellular polyphosphate sensing in *Dictyostelium discoideum*. *Mol Biol Cell*. 2019;30:1118-1128.
- Usui Y, Uematsu T, Uchihashi T, et al. Inorganic polyphosphate induces osteoblastic differentiation. *J Dent Res*. 2010;89:504-509.
- Biswas I, Panicker SR, Cai X, Mehta-D'souza P, Rezaie AR. Inorganic polyphosphate amplifies high mobility group Box 1-mediated von Willebrand factor release and platelet string formation on endothelial cells. *Arterioscler Thromb Vasc Biol*. 2018;38:1868-1877.
- Hassanian SM, Dinarvand P, Smith SA, Rezaie AR. Inorganic polyphosphate elicits proinflammatory responses through activation of the mammalian target of rapamycin complexes 1 and 2 in vascular endothelial cells. *J Thromb Haemost*. 2015;13(5):860-871.
- Morrissey JH, Choi SH, Smith SA. Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation. *Blood*. 2012;119:5972-5979.
- Morrissey JH, Smith SA. Polyphosphate as modulator of hemostasis, thrombosis, and inflammation. *J Thromb Haemost*. 2015;13(Suppl 1):S92-S97.
- Wat JM, Foley JH, Krisinger MJ, et al. Polyphosphate suppresses complement via the terminal pathway. *Blood*. 2014;123:768-776.
- Gajszewicz JM, Smith SA, Morrissey JH. Polyphosphate and RNA differentially modulate the contact pathway of blood clotting. *J Biol Chem*. 2017;292:1808-1814.
- Azevedo C, Livermore T, Saiardi A. Protein polyphosphorylation of lysine residues by inorganic polyphosphate. *Mol Cell*. 2015;58:71-82.
- Suess PM, Chinae LE, Pilling D, Gomer RH. Extracellular polyphosphate promotes macrophage and fibrocyte differentiation, inhibits leukocyte proliferation, and acts as a chemotactic agent for neutrophils. *J Immunol*. 2019;203:493-499.
- Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA, Docampo R. Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. *J Biol Chem*. 2012;287:28435-28444.
- Ruiz FA, Lea CR, Oldfield E, Docampo R. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. *J Biol Chem*. 2004;279:44250-44257.
- Smith SA, Mutch NJ, Baskar D, Rohloff P, Docampo R, Morrissey JH. Polyphosphate modulates blood coagulation and fibrinolysis. *Proc Natl Acad Sci USA*. 2006;103:903-908.
- Smith SA, Morrissey JH. Polyphosphate enhances fibrin clot structure. *Blood*. 2008;112:2810-2816.
- Choi SH, Smith SA, Morrissey JH. Polyphosphate is a cofactor for the activation of factor XI by thrombin. *Blood*. 2011;118:6963-6970.
- Choi SH, Smith SA, Morrissey JH. Polyphosphate accelerates factor V activation by factor XIa. *Thromb Haemost*. 2015;113:599-604.
- Puy C, Tucker EI, Ivanov IS, et al. Platelet-derived short-chain polyphosphates enhance the inactivation of tissue factor pathway inhibitor by activated coagulation factor XI. *PLoS One*. 2016;11:e0165172.
- Dinarvand P, Hassanian SM, Qureshi SH, et al. Polyphosphate amplifies proinflammatory responses of nuclear proteins through interaction with receptor for advanced glycation end products and P2Y₁ purinergic receptor. *Blood*. 2014;123:935-945.
- Hinz B. Myofibroblasts. *Exp Eye Res*. 2016;142:56-70.
- Bochaton-Piallat ML, Gabbiani G, Hinz B. The myofibroblast in wound healing and fibrosis: answered and unanswered questions. *F1000Res*. 2016;5:752.
- Darby IA, Zakuan N, Billet F, Desmouliere A. The myofibroblast, a key cell in normal and pathological tissue repair. *Cell Mol Life Sci*. 2016;73:1145-1157.
- Pilling D, Gomer RH. The development of serum amyloid P as a possible therapeutic. *Front Immunol*. 2018;9:2328.
- Smith SA, Choi SH, Davis-Harrison R, et al. Polyphosphate exerts differential effects on blood clotting, depending on polymer size. *Blood*. 2010;116:4353-4359.

38. Smith SA, Morrissey JH. Sensitive fluorescence detection of polyphosphate in polyacrylamide gels using 4',6'-diamidino-2-phenylindol. *Electrophoresis*. 2007;28:3461-3465.
39. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9:671-675.
40. Wurst H, Shiba T, Kornberg A. The gene for a major exopolyphosphatase of *Saccharomyces cerevisiae*. *J Bacteriol*. 1995;177:898-906.
41. Karlsson C, Paulsson Y. Age related induction of platelet-derived growth factor A-chain mRNA in normal human fibroblasts. *J Cell Physiol*. 1994;158:256-262.
42. Baum J, Duffy HS. Fibroblasts and myofibroblasts: what are we talking about? *J Cardiovasc Pharmacol*. 2011;57:376-379.
43. Müller WEG, Relkovic D, Ackermann M, et al. Enhancement of wound healing in normal and diabetic mice by topical application of amorphous polyphosphate. Superior effect of a host-guest composite material composed of collagen (host) and polyphosphate (guest). *Polymers*. 2017;9(12):300.
44. Tarbit E, Singh I, Peart JN, Rose-Meyer RB. Biomarkers for the identification of cardiac fibroblast and myofibroblast cells. *Heart Fail Rev*. 2019;24:1-15.
45. Eckes B, Colucci-Guyon E, Smola H, et al. Impaired wound healing in embryonic and adult mice lacking vimentin. *J Cell Sci*. 2000;113(Pt 13):2455-2462.
46. Müller WEG, Ackermann M, Wang S, et al. Inorganic polyphosphate induces accelerated tube formation of HUVEC endothelial cells. *Cell Mol Life Sci*. 2018;75:21-32.
47. Bae JS, Lee W, Rezaie AR. Polyphosphate elicits pro-inflammatory responses that are counteracted by activated protein C in both cellular and animal models. *J Thromb Haemost*. 2012;10:1145-1151.
48. Holmström KM, Marina N, Baev AY, Wood NW, Gourine AV, Abramov AY. Signalling properties of inorganic polyphosphate in the mammalian brain. *Nat Commun*. 2013;4:1362.
49. Edwards R, Harding KG. Bacteria and wound healing. *Curr Opin Infect Dis*. 2004;17:91-96.
50. Crooks MG, Fahim A, Naseem KM, Morice AH, Hart SP. Increased platelet reactivity in idiopathic pulmonary fibrosis is mediated by a plasma factor. *PLoS One*. 2014;9:e111347.
51. Fahim A, Crooks MG, Morice AH, Hart SP. Increased platelet binding to circulating monocytes in idiopathic pulmonary fibrosis. *Lung*. 2014;192:277-284.
52. Papadantonakis N, Matsuura S, Ravid K. Megakaryocyte pathology and bone marrow fibrosis: the lysyl oxidase connection. *Blood*. 2012;120:1774-1781.
53. Malara A, Abbonante V, Zingariello M, Migliaccio A, Balduini A. Megakaryocyte contribution to bone marrow fibrosis: many arrows in the quiver. *Mediterr J Hematol Infect Dis*. 2018;10:e2018068.
54. Singh R, Singh M, Arora G, Kumar S, Tiwari P, Kidwai S. Polyphosphate deficiency in *Mycobacterium tuberculosis* is associated with enhanced drug susceptibility and impaired growth in guinea pigs. *J Bacteriol*. 2013;195:2839-2851.
55. Docampo R, Huang G. Acidocalcisomes of eukaryotes. *Curr Opin Cell Biol*. 2016;41:66-72.
56. Singh M, Tiwari P, Arora G, Agarwal S, Kidwai S, Singh R. Establishing virulence associated polyphosphate kinase 2 as a drug target for *Mycobacterium tuberculosis*. *Sci Rep*. 2016;6:26900.
57. Tiwari P, Gosain TP, Singh M, et al. Inorganic polyphosphate accumulation suppresses the dormancy response and virulence in *Mycobacterium tuberculosis*. *J Biol Chem*. 2019;294:10819-10832.
58. Ruiz FA, Rodrigues CO, Docampo R. Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation, and environmental stress in *Trypanosoma cruzi*. *J Biol Chem*. 2001;276:26114-26121.
59. Dheda K, Booth H, Huggett JF, Johnson MA, Zumla A, Rook GA. Lung remodeling in pulmonary tuberculosis. *J Infect Dis*. 2005;192:1201-1209.
60. Garzoni LR, Adesse D, Soares MJ, Rossi MI, Borojevic R, de Nazareth Leal de Meirelles M. Fibrosis and hypertrophy induced by *Trypanosoma cruzi* in a three-dimensional cardiomyocyte-culture system. *J Infect Dis*. 2008;197:906-915.
61. Coelho LL, Pereira IR, Pereira MCS, et al. *Trypanosoma cruzi* activates mouse cardiac fibroblasts in vitro leading to fibroblast-myofibroblast transition and increase in expression of extracellular matrix proteins. *Parasit Vectors*. 2018;11:72.

How to cite this article: Suess PM, Smith SA, Morrissey JH. Platelet polyphosphate induces fibroblast chemotaxis and myofibroblast differentiation. *J Thromb Haemost*. 2020;18:3043-3052. <https://doi.org/10.1111/jth.15066>