Chapter

Inorganic Polyphosphates Are Important for Cell Survival and Motility of Human Skin Keratinocytes and Play a Role in Wound Healing

Cynthia M. Simbulan-Rosenthal, Bonnie C. Carney, Anirudh Gaur, Manish Moghe, Elliott Crooke, Lauren T. Moffatt, Jeffrey W. Shupp and Dean S. Rosenthal

Abstract

Inorganic polyphosphate (polyP) is a simple ancient polymer of linear chains of orthophosphate residues linked by high energy phospho-anhydride bonds ubiquitously found in all organisms. Despite its structural simplicity, it plays diverse functional roles. polyP is involved in myriad of processes including serving as microbial phosphagens, buffer against alkalis, Ca^{2+} storage, metal-chelating agents, pathogen virulence, cell viability and proliferation, structural component and chemical chaperones, and in the microbial stress response. In mammalian cells, polyP has been implicated in blood coagulation, inflammation, bone differentiation, cell bioenergetics, signal transduction, Ca^{2+}-signaling, neuronal excitability, as a protein-stabilizing scaffold, and in wound healing, among others. This chapter will discuss (1) polyP metabolism and roles of polyP in prokaryotic and eukaryotic cells, (2) the contribution of polyP to survival, cell proliferation, and motility involved in wound healing in human skin keratinocytes, (3) the use of polyP-containing platelet-rich plasma (PRP) to promote wound healing in acute and chronic wounds, including burns, and (4) the use of polyP-containing PRP in excisional wound models to promote faster healing. While polyP shows promise as a therapeutic agent to accelerate healing for acute and chronic wounds, the molecular mechanisms as a potent modulator of the wound healing process remain to be elucidated.

Keywords: inorganic polyphosphate, wound healing, keratinocytes, platelet-rich plasma

1. Introduction

PolyP is a simple prebiotic molecule that varies in chain length between three and several thousand inorganic phosphates linked by phosphoanhydride bonds (**Figure 1A**). It is continuously synthesized from ATP or GTP and degraded by cellular enzymes in bacteria [1–5] and eukaryotes, yet its pleiotropic functions remain

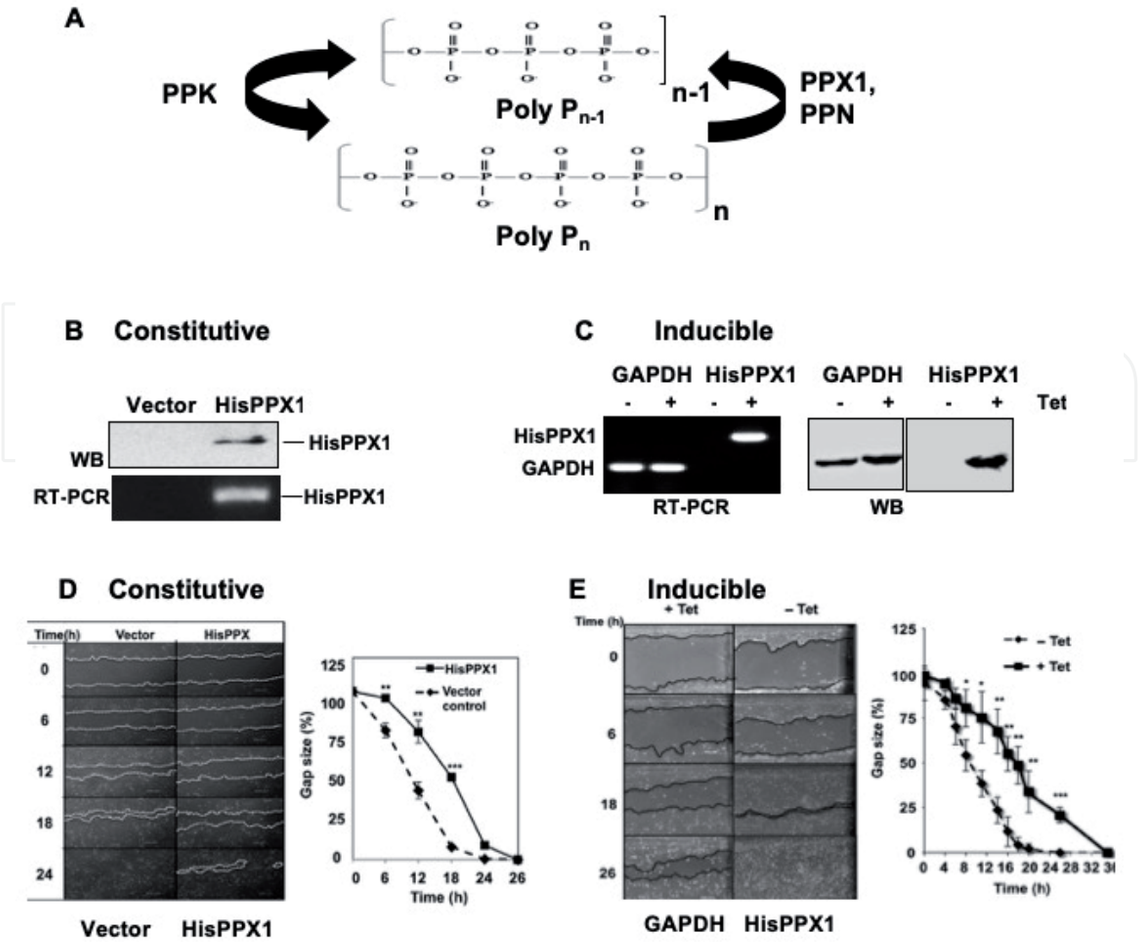

Figure 1.
(A) Schematic of linear structure of polyP, and its synthesis by polyphosphate kinase (PPK) and degradation by exopolyphosphatases (PPX1) or endopolyphosphatases (PPN). Constitutive (B and D) or Tet-inducible PPX1 (C and E) expression slows wound healing in cultured human keratinocytes (modified with permission from [33]).

to be clarified. In bacteria, but not mammalian cells, the enzymes that catalyze these activities have been identified [6–10]. polyP is synthesized following osmotic, oxidative, UVB, or other cellular stresses, and augments bacterial survival [11–16]; four roles have been proposed: an energy source, chelation of metal ions, storage of phosphate and response to cellular stresses. Necessary for bacterial survival, polyP is implicated in essential biological processes in prokaryotes including stress response, motility, biofilm formation [17–22], virulence, sporulation, and quorum-sensing [21–23]. Enzymes involved in polyP synthesis and degradation have been isolated and characterized in bacteria and other lower eukaryotes, and maintain tight control of polyP levels, as might be expected for a polymer controlling vital biological processes.

1.1 PolyP in prokaryotes

Drawing from the cellular ATP pool, bacterial polyphosphate kinase (PPK1) catalyzes the reversible transfer of the terminal γ-phosphate of ATP to polyP [24], whereas PPK2, expressed in other prokaryotes, transfers the terminal inorganic phosphate (Pi) from polyP to GDP to form GTP [4, 25, 26]. In contrast, the exopolyphosphatase PPX1 hydrolyzes polyP into phosphate monomers, thus maintaining phosphate homeostasis [5]. PPK levels and activity are tightly regulated, maintaining steady-state polyP concentrations in the bacterial cytosol at low micromolar levels, even in mutant strains deficient in PPX [27]. PolyP synthesis is

upregulated during nutrient deprivation [28, 29], or during osmotic [28], acidic pH [30], oxidative [31], or heat [32] stresses, potentially depleting cellular ATP pools by converting millimolar levels of ATP to long polyP chains (>1000 Pi) [31]. polyP levels are measured using enzyme-based assays that employ ppk to generate ATP from ADP, using luciferase as a reporter [33]. In another assay, cells are labeled with $^{32}P_i$; polyP is isolated and hydrolyzed with PPX1, and thin-layer chromatography or phosphoimage analysis is performed [17]. Toluidine blue binding assays are effective for different chain-lengths but are relatively insensitive. Recently, a rapid and simple method has been described [34, 35]. ^{31}P-NMR Spectroscopy is an effective and accurate method for measuring polyP in intact cells [36]. Electron ionization mass spectrometry [37], cryoelectron tomography and spectroscopy imaging [38] have also been employed. Protein affinity labeling *in vivo* uses the affinity of a recombinant polyP-binding domain of *E. coli* PPX1 (PPXbd) [39], which we and other investigators have used to specifically inhibit the function of polyP.

Not surprisingly, *ppk* mutants are extremely sensitive to environmental stresses [13, 31, 32, 40, 41], and exhibit reduced motility, virulence, and biofilm production [42]. *ppk* gene expression is regulated by σ38, a transcriptional regulator for late stationary phase genes [43] and polyP, in turn, amplifies its own synthesis by inducing transcription of the gene encoding σ38 (RpoS) [29, 41, 42]. In response to oxidative or heat stress, polyP synthesis is regulated at a transcriptional and/or post-translational level, as PPK synthesis and levels are altered by antisense RNA that target *ppk* mRNA transcripts [44] as well as transient inactivation of PPX by stress-sensitive regulators that allow polyP levels to remain high until normal conditions are restored [45].

1.2 PolyP in eukaryotes

While polyP is found in eukaryotes from protists to mammalian cells [11], the mechanism of polyP synthesis remains largely unknown for most eukaryotic organisms [1, 45], except for *S. cerevisiae*, where the vacuolar transporter chaperone 4 (VTC4) synthesizes polyP from ATP and then transports the polymer into vacuoles [46, 47]. Vacuolar polyP maintains phosphate homeostasis by appropriating phosphate during growth in phosphate-rich conditions [46], and releases phosphate during the cell cycle to provide precursors for DNA replication [48].

There is no sequence or structural homology between the polyP-synthesizing enzymes PPK1, PPK2, or VTC4, and homologues have yet to be found in higher eukaryotes [1]. While phylogenetic analysis of the prokaryotic branch reveals no clear homologues of *E. coli* PPK in a large number of polyP-synthesizing species [49], a few enzymes have been identified that use polyP as phosphate donor in reactions that can be reversed in the presence of excess substrate *in vitro* [49, 50]. In the absence of a polyP-synthesizing enzyme, polyP may be synthesized by the mitochondrial proton-motive force [51], in a complex process involving intact mitochondrial membranes [52]. Decreased polyP production resulting from depolarization of the mitochondrial membrane [52, 53] suggests that this may be a spontaneous process that does not need catalysis. Alternatively, inositol phosphates have also been implicated in polyP metabolism, since polyP levels are diminished in cells lacking the enzyme that synthesizes highly phosphorylated inositols [54–57]. Similar to polyP synthesis, polyP-degrading enzymes, such as yeast PPX1, have been found in lower eukaryotes, while mammalian polyP-specific degradation enzymes are mostly uncharacterized. However, h-prune, which regulates cell migration, also acts as a exopolyphosphatase for short-chain polyP *in vitro* [58, 59].

Subcellular fractionation, immunofluorescent staining, and biochemical quantification reveal that polyP is localized to the nucleus, cell membrane, cytoplasm, and

intracellular organelles in mammalian cells. It is specifically enriched in nucleoli, acidocalcisomes (organelles rich in protons, calcium (Ca^{2+}), and phosphorus), and mitochondria [11, 60–63]. In the brain, astrocytes secrete polyP, which is taken up by neurons, indicating both intra- and extracellular localization [64, 65]. Similar to vesicular packaging of ATP, astrocytes release polyP *via* exocytosis from vesicular nucleotide transporter (VNUT)-containing vesicles [65]. A putative G protein-coupled receptor in *D. discoideum* mediates cell surface binding of extracellular polyP, which as a signaling molecule, elicits differential effects on cell-substratum adhesion and cytoskeletal F-actin levels [66].

Eukaryotic polyP levels are in the 20–100 μM range (expressed as Pi concentration), with chain lengths ranging from 50 to 800 Pi residues (rat tissues) [67], averaging ~80 Pi residues in human platelets [68] to 200 Pi residues in yeast [67], compared to bacterial polyP, which can range up to thousands of Pi units long [53]; however, up to 130 mm medium-sized polyP chains are stored in dense granules in thrombocytes and mast cells [62, 68, 69]. Brain tissue exhibits among the highest polyP levels (~100 μm), which drop with age and neurodegenerative disease [11, 70–72], consistent with the role of polyP in stabilizing protein unfolding intermediates as amyloid-like precursors [32]. High levels of polyphosphate are also found in osteoblast matrix vesicles, the initial sites of bone mineral formation [73]. PolyP concentrations and chain lengths are dynamic, and depend on growth conditions of cells; for example in *Plasmodia*, polyP has an average chain length of 100 Pi, which is degraded to 10 Pi during sporulation [67].

Studies on the myriad roles of polyP in higher eukaryotes have recently gained momentum. PolyP is directly or indirectly involved in diverse cell processes, including control of cell bioenergetics, signal transduction, activation of the mitochondrial permeability transition pore (mPTP), Ca^{2+}-signaling [74, 75], and maintenance of the mitochondrial membrane potential [74]. Associated with mPTP [74] and voltage-gated channels, polyP regulates neuronal excitability [76] and astroglial signaling [64]. About 39% of intracellular polyP pools in astrocytes are in mitochondria [77], playing a role in bioenergetics [52, 77] and Ca^{2+}-handling [74, 78, 79]. As a signaling molecule, polyP released from astrocytes can mediate the physiological response to brain hypoxia [65].

In addition to its role as a gliotransmitter in the autonomic nervous system [64], the polymer also interacts with a variety of proteins, such as mammalian target of rapamycin (mTOR), fibroblast growth factor (FGF)-2, TRPM8, integrin β1, and glycosomal and ribosomal proteins and enzymes, consequently modulating cell survival and cell growth [80–86]. A fascinating finding of several recent studies is that polyP can covalently and non-enzymatically modify a small number of specific proteins in yeast [81] and humans containing lysine residues located in poly-acidic, serine, and lysine-rich (PASK) motifs, some of which are involved in ribosome biogenesis [87].

PolyP is involved in mTOR signaling, cell proliferation, and apoptosis [74, 80], and stimulates the mTOR pathway [80] at concentrations normally found in mammalian cells (0.15–1.5 mM); [11], suggesting a role for the polymer in mammalian cell proliferation. By promoting release of translation initiation factor eIF4E, mTOR stimulates initiation of translation, particularly proteins involved in cell growth and proliferation. PolyP also enhances the mitogenic activity of FGF-2 by promoting its binding to cell surface receptors [83]. PolyP appears to regulate apoptosis by inducing activation of caspase-3 in human plasma cells [88]. This polyanion also chelates metals, such as manganese and cadmium, blocking metal-induced cell damage [89, 90].

Other roles for polyP in mammalian cells include coagulation *via* activation of blood clotting factor XII [69], inflammation [91] as well as Ca^{2+} chelation for bone

mineralization and osteogenic differentiation [92]. The polymer also contributes to pro-inflammatory responses upon release from mast cells [62]. Finally, serving as a stabilizing scaffold for protein-folding intermediates, polyP was recently shown to work as a protein-like chaperone protecting cells against stress-induced protein aggregation [93].

2. The contribution of polyP to cell survival, proliferation, and motility involved in wound healing in skin keratinocytes

Inorganic polyP shows promise in different phases of wound healing, including hemostasis and re-epithelialization, as polyP is a normal component of different cells that play a role in this process, including platelets, dermal fibroblasts, and keratinocytes. The use of polyP as a therapeutic for acute and chronic wounding has begun to garner interest, in part because of experiments elucidating its role in wound healing [33], as well as in hemostasis [69, 94–99]. We have recently shown a role for polyP in the response to UV survival, cell motility, and wound healing [33]. Addition of exogenous polyP increased the rate of wound healing in standard scratch wound assays *in vitro* [33].

Whereas candidates for mammalian polyP metabolism have been shown to exhibit additional enzymatic activities [59], more specific polyphosphatases have been identified in lower eukaryotes including yeast, trypanosomes, and *Dictyostelium*. We therefore used exopolyphosphatase derived from *S. cerevisiae* (ScPPX1) to target intracellular polyP in human skin keratinocytes, an obvious choice for UV resistance, motility, and wound healing. The functions of polyP in the response of keratinocytes to UVB or wounding was studied by expressing ScPPX1, which selectively breaks down endogenous inorganic polyP, and not phosphoproteins, DNA, RNA, or nucleotide mono-, di-, or triphosphates [6]. Cells depleted of intracellular polyP by ScPPX1 expression exhibited increased sensitivity to UVB *via* enhanced apoptosis, and impaired wound healing [33]. Human keratinocytes stably expressing constitutive HisPPX1 or tetracycline (Tet)-inducible HisPPX1 were used to deplete cells of endogenous polyP, and study its role in wound healing assays performed on confluent monolayers, mimicking cell re-epithelialization during wound healing *in vivo*. RT-PCR and immunoblot analysis confirmed PPX1 expression in stable HisPPX1-expressing cells or in the presence of Tet (**Figure 1B** and **C**). Scratch gaps demonstrate marked attenuation of wound healing following constitutive HisPPX1 or Tet-induced expression (**Figure 1D** and **E**).

Since keratinocyte proliferation and migration are crucial to re-epithelialization during wound healing, the contribution of polyP to cell growth and motility involved in wound healing was next determined. Vector control cells exhibited significantly higher rates of cell growth as well as BrdU incorporation into newly synthesized DNA in cells at the wound edge, compared with polyP-depleted HisPPX1-expressing cells (**Figure 2**). Further, real time monitoring and measurement of cell motility performed in an xCelligence impedance-based system revealed significant decreases in cell motility in polyP-depleted keratinocytes (**Figure 3**). These results demonstrate that polyP depletion by either constitutive or inducible expression of PPX1 retards the rate of wound healing in human skin keratinocytes, by decreasing cell proliferation and motility. To determine if the loss of endogenous polyP can be supplemented with exogenous extracellular polyP, ScPPX1-expressing cells were grown in the presence of different concentrations of polyP, or with polyP-rich platelet lysate (next section). Exogenously added polyP was found to accelerate wound healing in human keratinocytes in polyP dose-response experiments on confluent monolayers of keratinocytes subjected to scratch wound healing assays

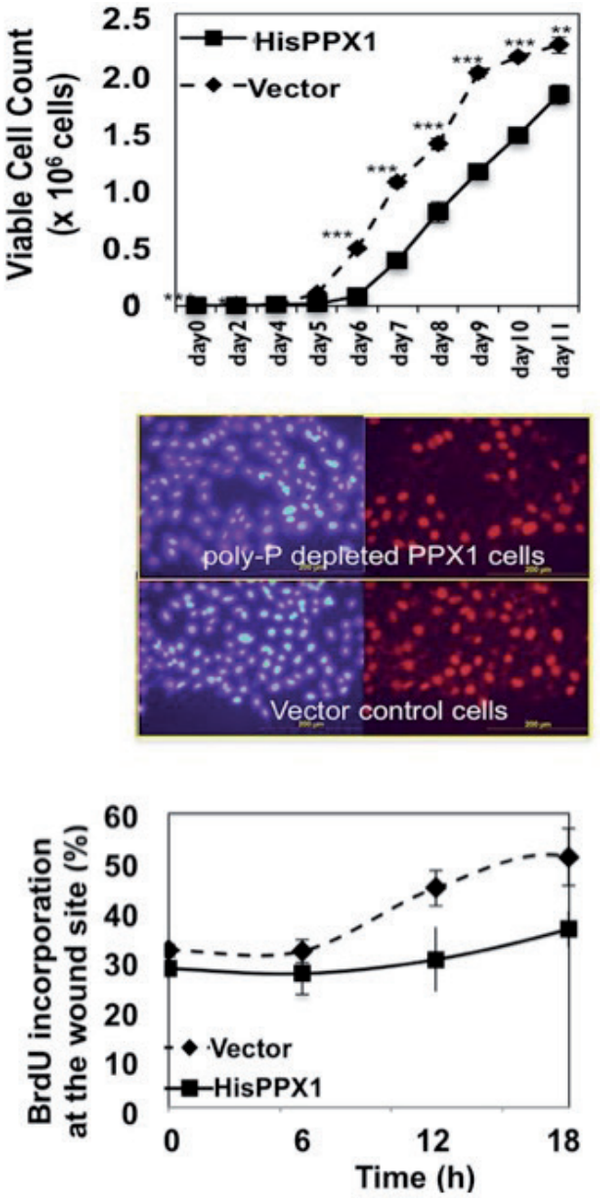

Figure 2.
Constitutive PPX1 *expression decreases growth rate of keratinocytes. Viable cell counts were performed over 11 days, and growth curves plotted for HisPPX1-expressing cells compared to vector cells (top). HisPPX1 and vector controls were subjected to scratch assays and proliferation was measured by in situ BrdU incorporation in cells at the wound edge (middle and bottom; modified with permission from [33]).*

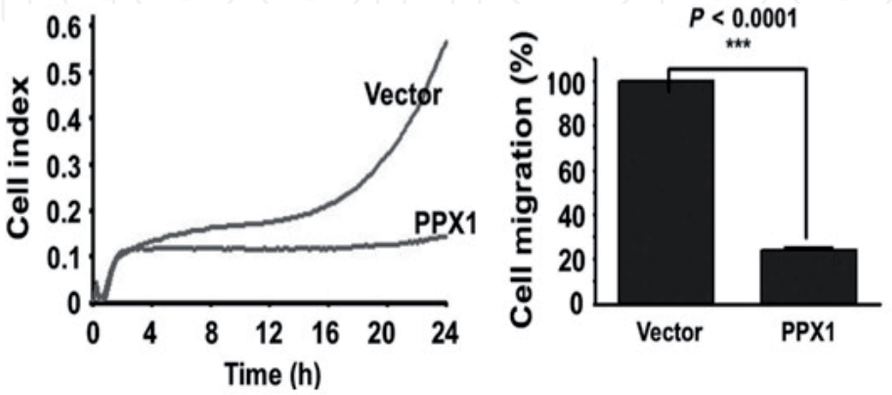

Figure 3.
Real-time monitoring and measurement of cell motility was performed in an xCelligence impedance-based system. Total number of cells attached to the bottom chamber were measured every 15 min over 24 hours, and are shown as technical duplicates, with assays repeated twice (left). Percentage of cell migration at the 24-hour time-point based on 100,000 cells plated at the top chamber (right; modified with permission from [33]).

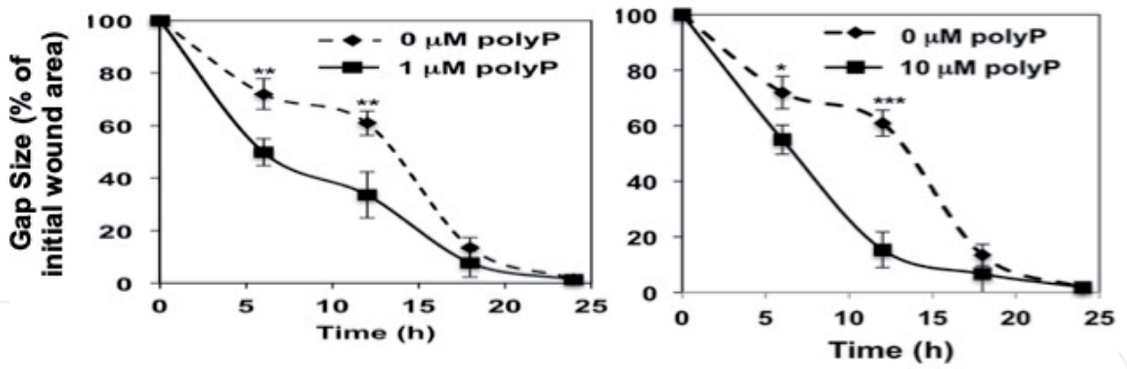

Figure 4.
PolyP dose-dependently accelerates wound healing in cultured human keratinocyte, using polyP at 1 μM (left) or 10 μM (right; modified with permission from [33]).

(**Figure 4**). Interestingly, both intracellular and extracellular polyP dose-dependently increased the rate of wound healing *in vitro*.

3. The use of polyP-containing PRP to promote wound healing in acute and chronic wounds, including burns

Wound healing is a highly coordinated process involving biochemical and physiological interplay of keratinocytes and fibroblasts to restore skin integrity. Platelets are components of blood responsible for blood clotting and wound healing. Although the importance of platelets in wound healing has been extensively studied, the bioactive substance playing a major role in skin re-epithelialization during wound healing is still unclear. PRP has been shown to support the survival and proliferation of human keratinocytes [100], and is currently used as therapeutic for both acute and chronic wounds (for review see [101]). Discovery of the release of growth factors triggered an interest in using PRP for wound healing, and platelet lysates have been examined as a replacement for using fetal bovine serum in cell cultures, which may contain contaminants such as prions, or elicit an unwanted immune response in patients. Platelet-rich lysates derived from platelets are a by-product of blood preparation, and are thus inexpensive. Most studies focused on lysates for mesenchymal stromal cell culture for cell therapy, in which platelets are activated by thrombin and $CaCl_2$, or by freeze-thaw.

In recent years, PRP has gained traction in many different specialties including in dermatology where it is used to treat acne [102], scarring [103], and alopecia [104, 105], in regenerative medicine where it is used to treat acute and chronic injuries to bone and cartilage [106, 107], in orthopedics and sports medicine where it is used to treat rotator cuff tears, osteoarthritis of the knee, hamstring injuries, and Achilles tendinopathy [108–110], in dentistry where it is used during tooth extractions, periodontal surgery, and dental implant surgery [111, 112], and more recently in wound healing to promote enhanced healing [113–118]. PRP is an autologous blood product generated from multiple rounds of centrifugation that serve to concentrate the number of platelets in plasma. PRP contains high concentrations of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor beta (TGFβ) compared to plasma and whole blood [119, 120]. It also contains higher levels of pro- and anti-inflammatory cytokines that promote enhanced healing. Lastly, PRP is known to contain inorganic polyP which is continually synthesized from ATP or GTP, and is degraded by cellular enzymes in bacteria and eukaryotes.

The role of polyP secreted by platelets and present in PRP on cell proliferation and wound healing was investigated in human HaCaT keratinocytes co-transduced

with either ScPPX1 or vector control, along with DsRed or GFP, respectively, as fluorescent markers in order to visualize and track cells that have reduced or normal levels of polyP. Cells stably expressing fluorescent-tagged DsRed-PPX1 or GFP-empty vector were incubated with platelet lysate (4%) supplemented with or without exogenous pure polyP (1 μM). In both vector-GFP control and polyP-depleted PPX1-DsRed cells treated with polyP, platelet lysate, or platelet lysate + polyP, cell growth curves revealed a significant increase in cell proliferation compared to untreated controls (data to be published elsewhere). PolyP quantification in platelet lysates using a micromolar polyP assay kit showed that a 4% platelet lysate contains ~8 μM polyP, which was within the range used for exogenously added polyP. This assay measures increase in fluorescence intensity (emission 550 nm, excitation 415 nm) of a PPD dye upon binding to polyP.

Cell migration/scratch assays were performed on PPX1-DsRed or vector-GFP control keratinocytes to assess the effects on wound healing and cell motility. Fluorescent pictures were taken at 10 min intervals for 36 hours using an EVOS FL time-lapse imaging system, and gap closure was quantified by Image J. In both GFP-vector cells and PPX1-expressing cells, the rate of wound closure in the scratch assays were significantly increased when cells were incubated either with platelet lysate alone, polyP alone, or both (data to be published elsewhere). These results together indicate that exogenous polyP, delivered either purified or from platelet-enriched plasma, can accelerate wound healing.

To assess whether the increased rate of wound healing is attributable to polyP in platelet lysates, specific polyP inhibitors (polyP-binding protein PPXbd or UHRA-9, a kind gift from Dr. James Morrissey) were utilized in wound healing assays. PPXbd, a recombinant polyP-binding domain of *E. coli* exopolyphosphatase, binds to platelet-derived polyP and blocks FXI activation, thrombin and fibrin generation, and consequently, inhibiting polyP procoagulant activity [99]. Interestingly, the enhanced rates of wound healing in vector control or polyP-depleted ScPPX-expressing cells induced by supplementation with exogenous extracellular polyP from pure polyP or in platelet lysates, was completely reversed by addition of the polyP inhibitors PPXbd or UHRA-9 (data to be published elsewhere). PolyP secreted by platelets and present in platelet lysate or PRP may therefore play an essential role in re-epithelialization during wound healing.

3.1 Chronic wounds

The acceleration of wound healing is of paramount importance in the setting of acute and chronic wounds, as well as burn wounds. Open chronic wounds are a significant cause of additional morbidity in patient populations that already have a plethora of comorbidities [121]. Significant improvements in complete healing were reported in chronic wounds treated with PRP compared to no topical treatments in a 2011 systematic review and meta-analysis on the use of PRP in acute and chronic wounds [101]. Another review of PubMed and Cochrane databases found significant benefit of PRP for diabetic chronic wounds, specifically in wounds unresponsive to standard of care treatment options [113]. A third systematic review of nine randomized controlled clinical trials (RCT) suggested that well-designed high-powered RCTs are needed to demonstrate increased wound healing with PRP treatment [122].

Treatment of 56 patients with diabetic foot ulcers with twice weekly applications of PRP resulted in complete healing in 86% of patients in the treated groups vs. only 68% in the control group [123]. Animal models using exosomes derived from PRP for full thickness skin wounds in a diabetic rat model also showed increased healing, as well as increased fibroblast proliferation and migration [124]. Platelet-rich fibrin also improved diabetic animal skin wound healing [125]. Overall, while there is no

consensus on this treatment modality in chronic wounds, it is becoming widely used, and many trials seek to understand its potential beneficial effects. Improvements in open wound area have been shown in a number of animal and clinical studies.

3.2 Acute wounds

Meta-analysis of rodent and non-rodent studies using a systematic review conducted under preferred reported items for systematic review of interventions (PRISMA) guidelines indicated that the treatment of wounds with PRP resulted in reduction of open wound area [126]. In addition to its role in wound healing, PRP reduced complications such as wound infection, exudate (mass of cells and fluid that seeps out of a wound), drainage, and hematoma formation [101]. PRP and PRP with keratinocyte and fibroblast cells were shown to increase re-epithelialization at 7–14 days post-injury in mouse models, compared to non-treated controls [127]. In full thickness porcine wounds treated with the secreted proteins of PRP, wound re-epithelialization and collagen deposition were significantly increased in treated animals vs. saline controls [128]. Thus, PRP may improve wound healing in acute surgical wounds by secreting growth factors that support local microenvironments that promotes healing [129]. PRP's effectiveness has also been shown in bone grafting, cartilage regeneration, and non-cutaneous surgical procedures. The impact of PRP on normal and damaged (derived from chronic ulcers or irradiated) fibroblasts have been described [130]. In addition, despite the lack of reproducibility of platelet concentrations due to differences in manufacturer-specific protocols for PRP preparation and differences in treatment methodologies, PRP has been shown to affect fibroblast proliferation and migration in a number of *in vitro* studies. As with chronic wounds, it is unclear why some studies, but not others, show a beneficial effect of PRP treatment.

3.3 Burns

PRP has been used as a topical treatment to accelerate wound healing in burn wounds, however, like in chronic and acute wounds, its use is still debated due to conflicting results [114]. Some papers recommend its use [115, 117, 118, 131–135], while others have shown non-significant changes in outcomes after treatment with PRP, and advise caution in using it in a wide-spread manner [114, 136–138]. A review of PRP for burns concluded that PRP may be useful in regeneration of dermal structures, increasing graft-take, and increasing re-epithelialization, but recommended further research on characterization of the mechanisms by which PRP can improve burn wound healing, donor site healing, and scar outcomes [139].

The use of side-by-side treatment of a split thickness skin graft (STSG) donor site with standard treatment or with PRP showed complete re-epithelialization in the PRP-treated side at day 11 *vs.* day 13 for the control. Histological samples taken from these healing wounds, and by H and E staining revealed increased epidermal thickness in PRP-treated wounds, as well as a significant increase in the number of blood vessels. After platelet concentrate in conjunction with STSG was used for deep burns, monitoring of viscoelastic properties of the resultant scars over 12 months revealed that the skin's return to normal viscoelastic properties was accelerated in burns treated with PRP compared to controls [133]. Compared to historic institutional standard of care controls, treatment of deep partial thickness (DPT) burns using PRP applied with the autograft during skin grafting, pain scores, inflammation, pruritis (itchiness), cosmesis of the scar, and perfusion all showed improved outcomes [135].

Animal models of burn injury in rats treated with topical PRP or control showed that PRP treatment resulted in increased hydroxyproline, decreased inflammatory

cells infiltration, but no difference in fibroblast collagen production or angiogenesis [134]. In a rat animal model of DPT burns, PRP was effective in increasing % wound closure, but showed little effectiveness in the full thickness injury group. PRP treatment resulted in increased neo-epidermal thickness at day 21, as well as decreases in CD31, 68, and 163, TGFβ1, MMP2, and MPO+ cells indicating an increased resolution of inflammation [118]. PRP injection in burn wound scars in a rat animal model of burn injury also showed pain-associated markers to be decreased with treatment [132]. While positive healing was observed in most models, in a recent study using a swine model of burn, tangential excision, and grafting with or without PRP, PRP showed similar effect on re-epithelialization and scarring in full thickness wounds compared to control wounds [136].

In a randomized clinical trial from 2018, 27 patients with DPT burns that did not get autografted were treated with lyophilized PRP powder, and showed a significant increased percent wound closure at 3 weeks in the treated group compared to control. Additionally, the infection rate in the PRP group was 26%, while 33% of control patients had postoperative infections [115]. PRP's concentrated secretion of growth factors may include basic fibroblast growth factor, epidermal growth factor, platelet-derived growth factor (PDGF), insulin-like growth factor, transforming growth factor β (TGFβ1), and vascular endothelial growth factor (VEGF) as probable mechanisms by which it can accelerate healing [139]. However, quantification of growth factors TGFβ1, PDGF-AA, and VEGF in a cohort of five burn patients compared to five healthy volunteers showed comparable levels of growth factors in the PRP from burn patients and health volunteers. Thus, there may be an additional factor in PRP that is possibly altered in burn patients (or patients with other pathologies such as diabetes or other conditions that would lead them to have surgical procedures yielding acute wounds) that may contribute to its success in treating some wounds and failure in others. Due to the effect burns have on the pathophysiology of blood coagulopathy [140–142] and capillary endotheliopathy [143, 144] after injury, it is reasonable to assume that platelets from burn patients may have differing levels of polyP. Associating this data with what is known about polyP, its ubiquitous presence in all prokaryotic and eukaryotic organisms, and its role in the response to cellular stress, it was hypothesized that polyP may contribute to wound healing.

4. PolyP and polyP-containing platelet rich plasma accelerates re-epithelialization *in vitro* and *in vivo*

A HaCaT keratinocyte polyP-depleted cell line and vector control was used in growth curves and scratch assays to evaluate polyP, platelet lysate, or combined treatment to accelerate wound healing *in vitro*. PolyP-containing PRP was also evaluated as a treatment in a splinted model of excisional wounding *in vivo*. Exogenous polyP was also spiked into PRP to assess its role. Treatment with the polyP-containing treatments increased cell growth and attenuated open wound area *in vitro* ($p < 0.001$). Addition of a polyP inhibitor abrogated these effects ($p < 0.0001$). PRP-treated wounds re-epithelialized faster compared to untreated wounds when analyzed at Days 3 and 5 ($n = 6$ wounds, $p < 0.05$). Re-epithelialization was further enhanced by exogenous polyP addition to PRP as evidenced by elongated epithelial tongues (**Figure 5**) in the low and high dose PRP + polyP treatment groups compared to PRP alone ($n = 8$ wounds, $p < 0.05$; data to be published elsewhere). Due to its autologous nature, PRP serves as a safe and efficacious option for accelerating wound healing, and may be enhanced by the exogenous addition of polyP.

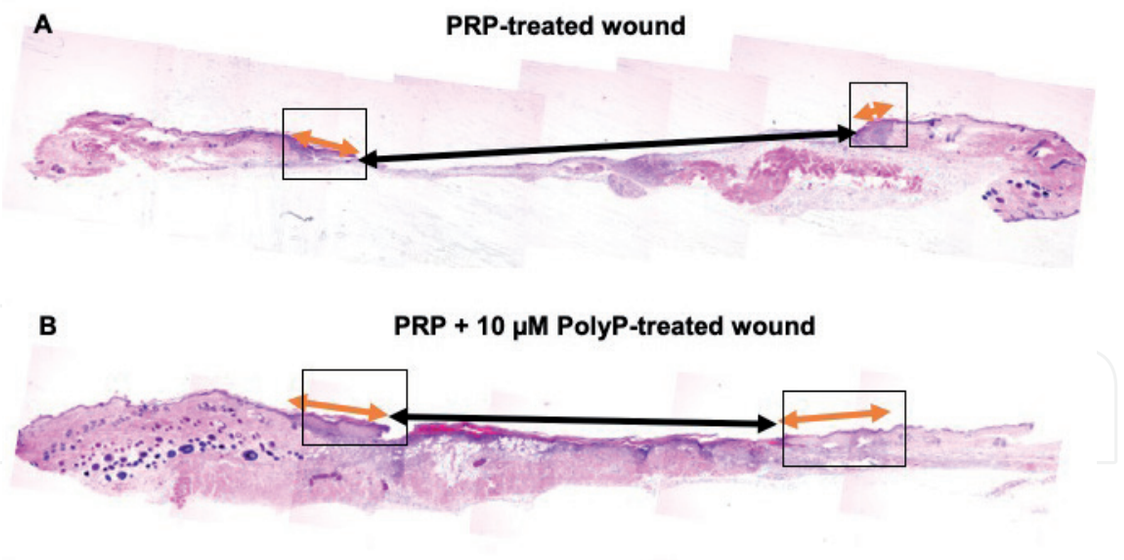

Figure 5.
Untreated or treated wounds were excised on day 5 and fixed in formalin, paraffin embedded, sectioned, and stained with H&E. Sections were imaged, and composited to create an image with areas of normal skin on both sides with the epithelial tongue (orange arrow) protruding from each side of normal skin. The epithelial gap is demarcated by the black arrow where no epithelium is present. Scale bar = 200 μm. Representative histology of untreated (A), PRP only treated (B), PRP + 10 μM polyP-treated wounds are shown. Epithelial tongue length was measured with Image J and quantified.

4.1 PRP-treated wounds heal faster than controls *in vivo*

PRP was generated based on a previously published protocol [145]. Briefly, whole blood was collected, and centrifuged to create platelet-poor plasma (PPP) and a pellet of platelets. PPP was then removed and the platelets were resuspended and activated with thrombin and calcium chloride ($CaCl_2$) to form a "biobandage"-like gel. Whole blood, packed RBCS, and PRP were stained with Wright and Giemsa stains to confirm PRP platelet concentration. A murine model of full thickness excisional wound healing was used where 6 mm punch biopsies were created on dorsal flanks of C57BL/6 mice [146, 147]. Wounds were splinted to encourage healing by re-epithelialization as opposed to contracture. They were subsequently treated with PRP, or no treatment was applied. Tegaderm dressing was applied and kept in place for 3 days. Pictures were taken at days 3, 4, 5, 6, or 7. By day 7, wounds were mostly closed.

In a second experiment, wounded mice were divided into four treatment groups: untreated, PRP only, PRP + 10 μM polyP (low dose) and PRP + 100 μM polyP (high dose). Doses of polyP were calculated by examining historical data from the literature on platelet levels, as well as polyP concentrations in platelets. The mean platelet count for C57BL/6 mice is 9.85 ± 1.40 × 10^{11} platelets/L, however, because there are no reliable reports of polyP concentrations per platelet in mice, human platelet counts and polyP concentrations were extrapolated for this experiment [148]. Human platelet concentrations range from 1.5 to 4.5 × 10^{11} platelets/L [149]. It is also known that platelets contain 0.74 ± 0.08 μmol polyP/1 × 10^{11} platelets [150]. Therefore, whole blood should contain between 1 and 3 μM polyP, and PRP, which contains at least 3-fold higher levels of platelets compared to whole blood, should contain 3–9 μM of polyP. We added ~10 μM in the low dose group, and a 10× concentration compared to the low dose group for the high dose group (100 μM). Lyophilized polyP was reconstituted in dH_2O to 1 M. PolyP took up 10% of the total treatment volume of PRP.

Treatments were applied on Day 0, and *Tegaderm*™ dressings stayed in place through day 3. On day 3 and 4, dressings were removed, and pictures were taken. On day 5, dressings were removed, pictures were taken, and wounds were excised

with underlying fascia and were sewn into histological cassettes to retain wound orientation. These samples were then paraffin embedded and H&E stained. Sections were imaged, and epithelial tongue length was measured using Image J. Epithelial tongues were defined as new epithelium if there was no uninjured dermis underneath the epithelium.

Compared to whole blood, PRP contained a higher concentration of platelets (data to be published elsewhere). The splinted wound model was used to shift the healing towards re-epithelialization instead of the normal contraction observed in mice. PRP application was easily applied as a "bio-bandage" gel-like liquid (data to be published elsewhere). The 6 mm punch biopsies allowed for the creation of similar wound size between animal groups at day 0 ($0.33\ cm^2 \pm 0.13$ vs. $0.39 \pm 0.15\ cm^2$, p = n.s.; data to be published elsewhere). At days 3 (0.09 ± 0.06 vs. 0.23 ± 0.13) and 5 (0.12 ± 0.07 vs. 0.25 ± 0.12) PRP-treated wounds had significantly smaller open wound areas compared to control animals ($n = 6$, $p < 0.05$). At days 6 and 7, this difference leveled off.

4.2 Exogenous spiking of PRP with polyP further accelerates healing *in vivo*

To further investigate the potential role of polyP in PRP, wounds were treated with PRP or with PRP with low or high dose-spiked polyP. Untreated wounds were largely open by day 5, while PRP treated wounds were smaller and contained newly formed epithelium (data to be published elsewhere). Spiking with low or high dose polyP further stimulated epithelialization, and wounds were smaller with increasing doses. By histomorphometric analysis, epithelial tongues can be seen by H&E staining. In untreated wounds, these tongues are small and shallow. PRP treatment results in a more proliferative epithelium that is thicker and longer than untreated samples. Spiking with low and high dose polyP creates longer epithelial tongues). Epithelial tongue measurement by Image J shows a significant decrease in tongue length in untreated and PRP only treated vs. PRP + high dose polyP (737.38 ± 121.21 and 925.55 ± 214.17 vs. $1186.91 \pm 255.06\ \mu M$, $n = 8$, $p < 0.0001$, $p < 0.05$). PRP + high dose polyP-treated wounds also had significantly longer epithelial tongues compared to PRP + low dose polyP ($n = 8$, $p < 0.05$). PRP contains polyP at a concentration near $5\ \mu M$. The exogenous addition of polyP to the PRP promoted keratinocyte growth and proliferation, as is evidence by the increased epithelial tongue length with increasing doses of polyP administration.

5. Conclusion

PolyP plays key roles in essential biological processes in bacteria, and its increasing importance in eukaryotes is becoming apparent, including its participation in blood coagulation and wound healing. Recent advances in measurement and localization of polyP, along with our growing understanding of polyP metabolism and its interaction with specific proteins allows us to begin to analyze mechanisms responsible for cell-specific roles of polyP. Our ability to regulate polyP in eukaryotic cells opens possibilities for therapeutic intervention. Future work related to wound healing should be aimed at investigating the specific roles of intra- and extracellular polyP in keratinocytes, as well as the potential importance of polyP in other skin cells, including dermal fibroblasts, as these cells make up the majority of the skin. As polyP is also secreted by activated platelets and is important for normal blood clotting, the application of polyP-containing PRP as a biologic dressing may positively contribute to wound healing. We have completed two clinical trials using platelet-rich plasma for wound healing, a phenomenon that may be explained by

the presence of polyP. PolyP levels and chain lengths should also be quantified in healthy and pathologic conditions in order to assess appropriate levels when treating acute or chronic wounds in the future.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author details

Cynthia M. Simbulan-Rosenthal[1], Bonnie C. Carney[1,2], Anirudh Gaur[1], Manish Moghe[1], Elliott Crooke[1], Lauren T. Moffatt[1,2], Jeffrey W. Shupp[1,2,3,4] and Dean S. Rosenthal[1*]

1 Department of Biochemistry and Molecular and Cellular Biology, Georgetown University School of Medicine, Washington, DC, USA

2 Firefighters' Burn and Surgical Research Laboratory, MedStar Health Research Institute, Washington, DC, USA

3 The Burn Center, Department of Surgery, MedStar Washington Hospital Center, Washington, DC, USA

4 Department of Surgery, Georgetown University School of Medicine, Washington, DC, USA

*Address all correspondence to: rosenthd@georgetown.edu

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