



## Original Article

Inorganic polyphosphate in platelet rich plasma accelerates re-epithelialization *in vitro* and *in vivo*

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## ARTICLE INFO

## Article history:

Received 8 April 2020

Received in revised form

25 June 2020

Accepted 16 July 2020

## Keywords:

Inorganic polyphosphate

PPX1 exopolyphosphatase

Wound healing

Keratinocytes

## ABSTRACT

Wound healing requires well-coordinated events including hemostasis, inflammation, proliferation, and remodeling. Delays in any of these stages leads to chronic wounds, infections, and hypertrophic scarring. Burn wounds are particularly problematic, and may require intervention to ensure timely progression to reduce morbidity and mortality. To accelerate burn wound healing, Platelet-Rich Plasma (PRP)<sup>1</sup> can be of value, since platelets release growth factor proteins and inorganic polyphosphates (polyP) that may be integral to wound healing. We used polyP-depleted keratinocyte (HaCaT) and fibroblast cell culture models to determine cell proliferation and scratch-wound repair to determine if polyP, platelet lysate, or combined treatment could accelerate wound healing. While polyP and PRP significantly reduced the open scratch-wound area in fibroblasts and keratinocytes, polyP had no effect on keratinocyte or fibroblast proliferation. PRP was also evaluated as a treatment in a murine model of full thickness wound healing *in vivo*, including a treatment in which PRP was supplemented with purified polyP. PRP induced significantly more rapid re-epithelialization by Day 3. Pure polyP enhanced the effects of PRP on epithelial tongues, which were significantly elongated in the PRP + high-dose polyP treatment groups compared to PRP alone. Thus, PRP and polyP may serve as an effective therapeutic combination for treating wounds.

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## 1. Introduction

In recent years, platelet rich plasma (PRP) has garnered attention in diverse disciplines of medicine. For example, it has been

*Abbreviations:* endopolyphosphatase, PPN; epidermal growth factor, EGF; exopolyphosphatase, PPX; human foreskin fibroblasts, HFF; mammalian target of rapamycin, mTOR; platelet-derived growth factor, PDGF; platelet-poor plasma, PPP; platelet-rich plasma, PRP; polyP kinase, PPK; polyphosphates, polyP; reactive oxygen species, ROS; total body surface area, TBSA; transforming growth factor beta, TGFβ; vacuolar transporter chaperone 4, VTC4.

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

<https://doi.org/10.1016/j.reth.2020.07.004>

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used in the treatment of alopecia, acne and scarring (dermatology); cartilage and bone injury (regenerative medicine); rotator cuff tears, osteoarthritis of the knee, hamstring injuries, and Achilles tendinopathy (orthopedics); tooth extractions, periodontal and implant surgery (dentistry); and in cutaneous wound repair, as we and others have tested its efficacy to promote enhanced healing [1–5]. PRP is derived from the patient's blood using centrifugation to concentrate plasma platelets, and its derived growth factors and cytokines including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGFβ) [6] that enhance wound healing.

The timely and proper sequence of events is of utmost importance in the setting of chronic and acute wounds, including burn wounds. Improper healing of chronic wounds are a significant factor contributing to morbidity in patient populations that already

have a plethora of comorbidities [7]. Burn patients with large total body surface area (TBSA) involvement have restricted donor site availability. As such, the healing of these large TBSA wounds is a significant clinical challenge that can lead to wound infection, sepsis, multi-organ failure, and death if not treated appropriately. Agents that can act to heal donor sites for quicker re-harvest and autografting, or to accelerate the healing of deep partial thickness injuries with, or in conjunction to, autografting are rare and are in need.

PRP has been used to promote acute and chronic wound healing, including burn wounds; however, its use is still debated, as many papers report conflicting results [1]. A review of the use of PRP in burns in 41 primary manuscripts was reported in 2010 [8]. PRP can be helpful in stimulating regeneration of dermal structures, increasing successful rates of skin graft assimilation, and promoting re-epithelialization, although additional research is recommended. Since 2010, many studies have been published to try to define the pathways by which PRP can promote burn wound healing and improve scar outcomes. Some studies support its use [2–4], while others do not show substantial changes after PRP treatment [1]. Many investigators have commented on the rich reservoir of growth factors present in PRP as a probable mechanism by which it can accelerate healing [8]. As such, a recent paper quantified growth factors TGF $\beta$ 1, PDGF-AA, and VEGF in a cohort of 5 burn patients compared to 5 healthy volunteers in an effort to compare the levels of these factors from individual to individual as a possible explanation for the variable findings associated with autologous PRP treatment. Surprisingly, they showed similar levels of growth factors in PRP generated from burn patients and healthy volunteers.

With this information in mind, it was hypothesized that there may be an additional factor(s) altered in burn patients (or those with other pathologies) responsible for its success in autologous treatment for some burn patients and failure in others. Due to the impact of burns on the pathophysiology of blood coagulopathy [9] and endotheliopathy [10], it is not unreasonable to infer that thrombocytes from burn patients have varying levels of inorganic polyphosphates (polyP). Coupled with polyP's omnipresence in a wide range of organisms, and its function in cellular stress, we hypothesized that polyP is vital to wound healing.

Many clinicians and research scientists have become interested in inorganic polyP, which is synthesized from polyP kinases 1 and/ or 2 (PPK1/2) and degraded by both exo- (PPXs) and endo- (PPNs/ epps) polyphosphatases. PPX cleaves polyP into phosphate monomers, thereby preserving phosphate homeostasis [11]. In general, polyP synthesis is induced by environmental stress from nutrient deprivation, osmotic changes, acidic pH, reactive oxygen species (ROS), heat, or UV irradiation, which deplete ATP reserves by converting ATP to polyP (>1000 Pi residues in prokaryotes vs. 50 to 800 Pi chain lengths in eukaryotes) [12,13]. The mechanism for synthesis is largely unknown for most eukaryotes [14], although the Ca<sup>2+</sup>-ATPase of the human erythrocyte membrane have been shown to display polyP-synthesizing activity in humans [15]. Poly P may also be generated by a complex process in intact mitochondrial membranes [16] that generate a proton-motive force. To maintain phosphate homeostasis, phosphate is sequestered as polyP in phosphate-rich conditions [17], and released to provide precursors for DNA replication.

PolyP is localized to the nucleus, cell membrane, cytoplasm, and intracellular organelles, particularly those rich in H<sup>+</sup>, Ca<sup>2+</sup> and phosphorus, such as acidocalcisomes and mitochondria [18,19]. Platelet-dense granules are similar to acidocalcisomes, and have very high concentrations of polyP, as do dense granules of mast cells (130 mM) [19,20]. PolyP is efficiently secreted after platelet activation [20], and intra- and extracellular localization of polyP is of central importance in the regulation of the clotting cascade.

Patients with platelet-dense granule defects and bleeding demonstrated approximately 10 times lower platelet polyP levels than normal [21]. We have also demonstrated a role for intracellular and extracellular polyP in human keratinocytes [22].

PolyP is implicated in bioenergetics, signal transduction, Ca<sup>2+</sup>-signaling, and regulation of the mitochondrial membrane potential [23]. In *Dictyostelium discoideum* a G protein-coupled receptor mediates cell-surface binding of extracellular polyP, which alters cell adhesion and cytoskeletal F-actin levels [24]. PolyP can interact with a number of other proteins, including mammalian target of rapamycin (mTOR), to which it binds and stimulates at physiological levels in mammalian cells (0.15–1.5 mM) [18], and thus appears to play a role in mTOR-induced autophagy [25], as well as cell proliferation, and apoptosis [23]. Ribosome biogenesis has been shown to be the major metabolic effort in proliferating cells [26], and thus important in wound healing. PolyP also promotes binding of FGF-2 to its receptors, and thus enhances its mitogenicity [27]. PolyP plays other key roles in mammalian cells including inflammation [28] after its release from mast cells [19], and Ca<sup>2+</sup> metabolism during osteogenic differentiation and bone mineralization [29].

Many of these processes, especially blood clotting, inflammation, oxidative stress, motility, proliferation, and differentiation are intimately involved in wound healing, including burn wound healing. However, a definitive role for polyP in mammalian cell wound healing has only been tested in a few cell culture models [22]. It was our hypothesis that polyP from PRP or platelet lysate promotes wound healing, and we tested this hypothesis using wound healing models in cultured cells and *in vivo*. Previous work by our group was used as the foundation for this study [22]. An immortalized keratinocyte cell line that is depleted of polyP was derived (HaCaT PPX1), and it was shown that polyP depletion had a negative effect on cell growth, motility, proliferation, and wound healing in scratch assays *in vitro*. The goal of the current work was to expand and confirm this finding through the use of the HaCaT PPX1 cell line and evaluate wound healing *in vitro* and *in vivo* with the secreted products of platelets, focusing on polyP.

## 2. Methods

### 2.1. Reagents

#### 2.1.1. PolyP and human platelet lysate

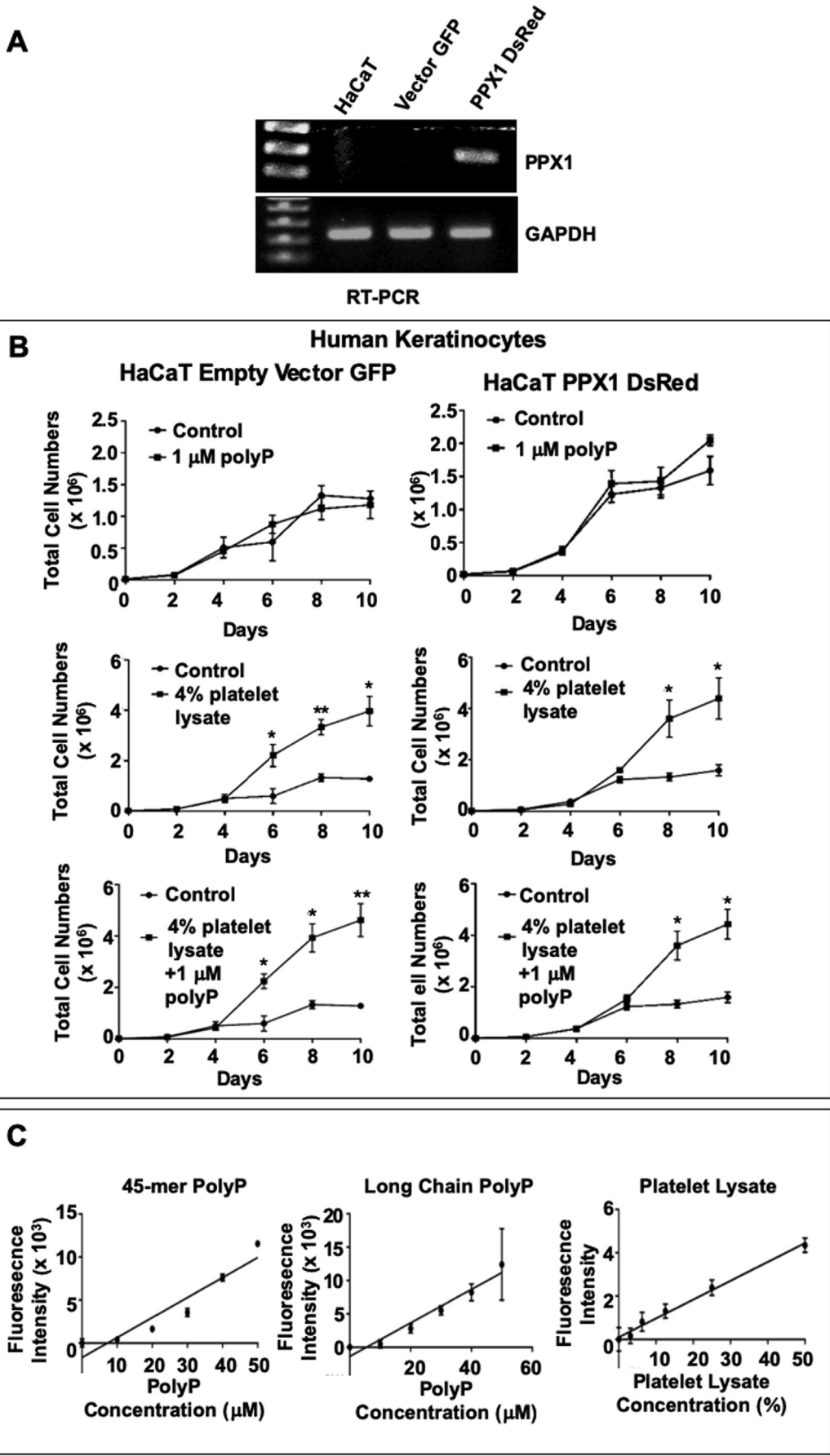
Medium chain polyP (75 residues) and human platelet lysate were purchased from Kerfast Inc., Boston, MA and MilliporeSigma, St. Louis, MO, respectively.

#### 2.1.2. PolyP assay

PolyP concentrations were quantified using a polyP assay according to manufacturer's instructions (ProFoldin, Hudson, MA), which measures fluorescence of a PPD dye upon binding to polyP, using a 45-mer sodium polyphosphate for the creation of a standard curve (emission 550 nm, excitation 415 nm).

#### 2.1.3. Platelet-Rich Plasma preparation

PRP was generated as described previously [30]. Multiple mouse donor blood was pooled, collected in sodium citrate, and centrifuged at 200×g (20 min at room temperature) to separate plasma. A small number of RBCs containing platelets and the full volume of plasma was then further centrifuged at 400×g (15 min at room temperature) to generate platelet-poor plasma (PPP) as well as a platelet pellet. PPP was removed and platelets re-suspended in a small volume of PPP. PRP was activated with thrombin and CaCl<sub>2</sub> at a ratio of 10:1 (Recothrom®, Mallinckrodt Pharmaceuticals, Surrey, United Kingdom) to form a gel, which makes a “bio-bandage”-like



substance that is convenient to apply to wounds. Recothrom was not dissolved in the saline supplied by the manufacturer, but instead with 10% CaCl<sub>2</sub>. Smears of Whole blood, packed RBCs, and PRP were stained with Wright Giemsa stain (American Mastertech Scientific, Lodi, CA) to confirm high concentration of platelets in the PRP. Whole blood and PRP were diluted in HEPES buffered saline 1:100 for counting by hemocytometer.

## 2.2. Cell culture

HaCaT immortalized human keratinocytes [31] or primary human foreskin fibroblasts (HFF), derived as described previously [32,33], were grown in DMEM with 10% FBS and 1% penicillin/streptomycin in a cell culture incubator (37 °C, 5% CO<sub>2</sub>). HaCaT cells were transfected with vector pCMV (Clontech Labs, Inc) or with pCMV-PPX1 using Lipofectamine-LTX; clones were selected using G418, and screened for PPX1 expression by RT-PCR and immunoblot analysis [22]. Packaging ØNX-cells were transfected with pLHCX vector with either GFP or DsRed to track cells fluorescently; HaCaT-vector or HaCaT-PPX1 cells were then transduced with the viral supernatants from ØNX-cells; clones were selected in hygromycin.

## 2.3. Cell growth curves

HaCaT empty vector GFP, HaCaT PPX1 DsRed cells, or HFF were seeded into 6-well plates using 10,000 cells/well, allowed to attach for 24 h, and further grown in media containing (1) media only (control), (2) 1 µM polyP, (3) 4% platelet lysate and (4) 4% platelet lysate + 1 µM polyP. Cells from triplicate wells were collected every two days for 10 days, and subjected to total and viable cell counts with an Eve™ Automated Cell Counter (NanoEnTek Inc, Waltham, MA) after trypan blue staining. All cells used were above 90% cell viability, indicating cells were healthy. Cell growth curves were plotted over time.

## 2.4. Scratch-wound assays

500,000 cells/well were plated in 12-well plates for 24 h, followed by addition of media containing 4% platelet lysate with or without 1 µM polyP. Three scratches/well with consistent widths were made on confluent monolayers of cells using sterile pipette tips. The scratch wounds were analyzed using an EVOS fluorescence time-lapse auto-imager (Life Technologies, Carlsbad, CA) with an onstage incubator. Fluorescent images were taken at 10-min intervals for a period of 36 h using time-lapse imaging. Average wound gap areas were quantified with Image J software based on the photomicrographs, and gap areas were calculated and converted to percent gap area relative to the original wound gap. Multiple scratch assays were performed where HaCaT PPX1 DsRed, HaCaT empty vector GFP cells were treated with 1 µM polyP, 4% platelet lysate, or combination treatment and compared to control cells that received no treatment. HFF were treated as above after exposure to either 1 µM polyP or 4% platelet lysate. Results are representative of 3 independent experiments with reproducible results.

## 2.5. Murine excisional wounding model

The protocol and procedures employed were reviewed and approved by the MedStar Health Research Institute's Institutional Animal Care and Use Committee. For *in vivo* experiments, a murine full thickness excisional wound healing model was used in which 6 mm punch biopsy injuries were generated on the dorsal flanks of C57BL/6 mice [34]. To induce healing by reepithelialization, punch wounds were then splinted to minimize contracture. PRP was then applied to wounds, followed by Tegaderm dressing for 3 days. Images were captured at days 3–7, after which wounds were healed.

In another experiment, mice were wounded as above, then divided into 4 treatment groups: 1) untreated, 2) PRP 3) PRP + 10 µM polyP (low dose) and 4) PRP + 100 µM polyP (high dose). C57BL/6 mice contain  $9.85 \pm 1.40 \times 10^{11}$  platelets/L [35], and platelets contain  $0.74 \pm 0.08$  µmol polyP/ $10^{11}$  platelets [36]. Therefore, whole mouse blood contains 7.3 µM polyP, while PRP should contain 3–5X that much, or >22 µM polyP. We therefore used 10 µM polyP in the “low dose” group, and 100 µM for the “high dose” treatment.

After treatment, Tegaderm® dressings remained in place for three days, after which dressings were taken off and images captured at days 3 and 4. On the fifth day, dressings were removed, images were captured, and wounds excised and sewn into histological cassettes to maintain correct orientation of sections. Tissues were embedded in paraffin and stained with H&E, and epithelial tongue lengths measured by Image J. Criteria for “new epithelium” was determined by whether only injured dermis appeared directly below keratinocytes migrating into the wound.

## 2.6. Statistical analysis

Data from three experiments were compared using two-tailed Student's t-test to determine significance. When multiple comparisons were made, a Bonferroni correction was used after the t-test.

## 3. Result

### 3.1. Inorganic polyP and platelet lysate contribute to increased wound closure in cultured cells alone and in combination

To examine the effects of exogenous polyP and platelet lysate on cell proliferation of HaCaT keratinocytes, platelet lysate supplemented with or without 1 µM polyP was incubated with fluorescently-tagged DsRed-PPX1 or GFP-empty vector-containing cells. RT-PCR analysis revealed that HaCaT-PPX1- DsRed cells express high levels of PPX1 RNA, whereas vector-GFP control and parental HaCaT cells did not (Fig. 1A). The presence of functional scPPX1 was verified by quantifying degradation of <sup>32</sup>P-labeled long chain polyphosphates with increasing protein concentrations in cell lysates. As expected, scPPX1 activity was detected and increased linearly with protein levels in HaCaT PPX1 cells, but not in the vector control cells; further, intracellular polyP levels were reduced by 48% in ScPPX1-expressing cells [22].

In both empty vector-GFP control (Fig. 1D, left column) and polyP-depleted PPX1-DsRed cells (Fig. 1D, right column) treated

**Fig. 1.** Ectopic expression of ScPPX1 enzyme in HaCaT cells and effects on proliferation upon treatment with 4% platelet lysate either with or without 1 µM polyP. A) RNA isolated from HaCaT cells stably transfected with vector pCMV, or with pCMV-ScPPX1, was subjected to RT-PCR using primers specific for ScPPX1. B) Growth curves of HaCaT empty vector (left) or polyP-depleted PPX1 cells (right) reveal significant increases in cell proliferation upon treatment with 4% platelet lysate either with or without 1 µM polyP treatment. C) polyP standard curves generated using a 45-mer polyP control (left) and long-chain polyP (200–1300-mer; middle), and of different concentrations of platelet lysate (right). For all experiments \*, \*\*, or \*\*\* represent  $p < 0.0167$ ,  $p < 0.0033$ , or  $p < 0.00033$  (Bonferroni correction for 3 comparisons) compared to controls; results are shown as the means ± SEM of three replicates of a representative experiment; essentially the same results were obtained in three independent experiments.

**Table 1**  
Levels of polyP in platelet lysate as quantified using two standard curves.

Platelet Lysate (% of pure)	45-mer PolyP standard curve	Long Chain PolyP standard curve
50%	25.96 $\mu$ M	22.80 $\mu$ M
25%	17.50 $\mu$ M	14.98 $\mu$ M
12.5%	12.86 $\mu$ M	10.68 $\mu$ M
6.25%	10.75 $\mu$ M	8.73 $\mu$ M
3.125%	7.92 $\mu$ M	6.12 $\mu$ M
<b>4%</b>	<b>9.18 <math>\mu</math>M</b>	<b>7.28 <math>\mu</math>M</b>

The bold value represents the % of pure platelet lysate used in cell cultures.

with 4% platelet lysate (Fig. 1D, middle row), or 4% platelet lysate + 1  $\mu$ M polyP (Fig. 1D, bottom row), cell proliferation was significantly enhanced, but to the same extent in the presence or absence of added purified polyP; there is no significant difference between 4% platelet lysate with or without 1  $\mu$ M polyP. polyP quantification showed that 4% platelet lysate harbors approximately 8  $\mu$ M polyP (Fig. 1B, Table 1); thus adding 1  $\mu$ M exogenous polyP does not enhance proliferation *in vitro*.

To determine changes in keratinocyte migration and wound healing, scratch wound assays were performed. Images were captured every 10 min for 36 h (EVOS FL time-lapse; Fig. 2A), and analyzed by Image J (Fig. 2B). Closure in scratch wounds was accelerated with 1  $\mu$ M polyP (Fig. 2B, top row), 4% platelet lysate alone (Fig. 2B, middle row), or a combination of the two (Fig. 2B, bottom row;). Cells treated with the combined treatment had a smaller open wound area compared to the individual treatments. The scratch gaps completely closed by 16 h, 14 h, and 12 h in polyP-treated cells, 4% platelet lysate-treated cells, and with the combined treatment, respectively. PolyP, platelet lysate, or the combination may therefore enhance re-epithelialization during wound healing.

We next determined the effects of platelet lysate and polyP on HFF primary epidermal fibroblasts. Cells were derived from neonatal foreskin (Materials and Methods), and scratch wound assay closures performed in the presence or absence of platelet lysate (Fig. 3A) or polyP (Fig. 3B) as described above for keratinocytes. Fig. 3A and B shows that in the presence of platelet lysate or purified polyP, scratch wounds close faster than control fibroblasts. To determine whether proliferation was contributing to fibroblast wound closure, growth assays were performed. Platelet lysate, but not purified polyP, increased the proliferation of HFF (Fig. 3C), suggesting that purified polyP contributes to fibroblast migration into the wound, but unlike its effect on keratinocytes, polyP does not promote fibroblast proliferation.

### 3.2. PRP accelerates wound healing *in vivo*

As expected, PRP was determined to contain more platelets than equivalent amounts of whole blood (Fig. 4A). To prevent the dramatic contraction that occurs in murine wound healing compared to humans, a splinted model variation of our previously used skin grafting technique [32,33] was used to study the process of re-epithelialization. PRP was introduced on the wound as a “bio-bandage” (Fig. 4C and 4D). Punch biopsies were utilized to generate wounds of similar dimensions at day 0. At the third and fifth days, PRP significantly reduced the sizes of wounded areas compared to controls (Fig. 4E), after which differences between the groups was not significant.

### 3.3. Addition of purified of polyP to PRP further increase rate of healing *in vivo*

To determine the potential role of polyP as a therapeutic in PRP-induced wound healing, PRP alone, or with 10  $\mu$ M (“low dose”) or

100  $\mu$ M (“high dose”) additional purified polyP was used. Control wounds were still not healed by day 3, while those treated with PRP were noticeably reduced with freshly generated epithelium (Fig. 5A). Addition of high levels of purified polyP to the PRP appeared to promote epithelialization. Image J quantification of open wound area revealed that treatment with PRP and/or PRP + low or high-dose polyP resulted in lower % open wound area normalized to Day 0 at Day 3 (Untreated =  $85.34 \pm 2.49\%$  vs. PRP =  $68.89 \pm 3.08\%$  ( $p < 0.0059$ ) vs. PRP + low dose polyP =  $69.91 \pm 2.95\%$  ( $p = 0.0106$ ) vs. PRP + high dose polyP =  $65.17 \pm 3.12\%$  ( $p = 0.0007$ ). This quantification did not reveal significant differences with the addition of polyP, and hence, epithelial tongue length was further quantified to elucidate any difference.

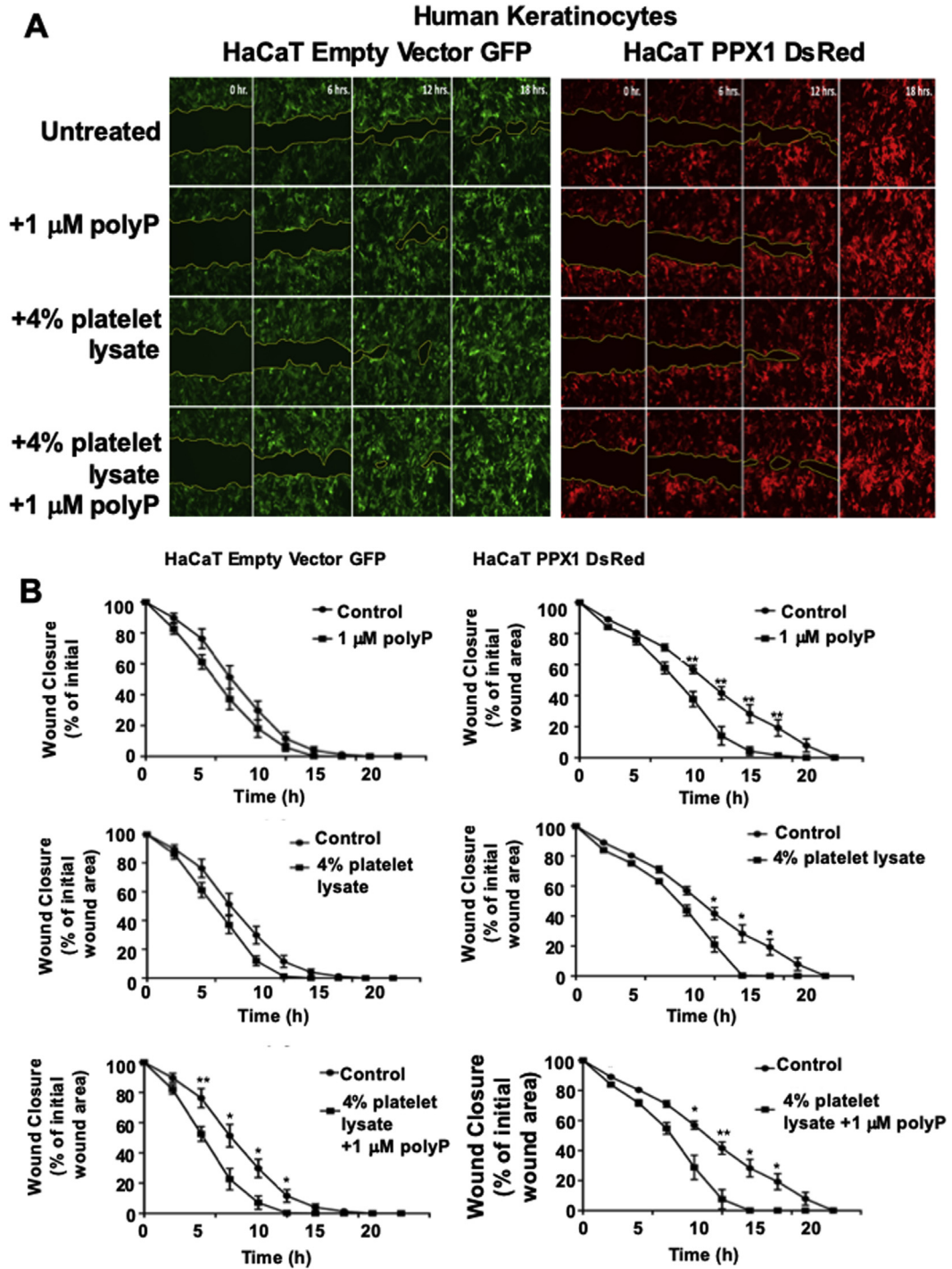
### 3.4. Addition of purified of polyP to PRP increases epithelial tongue length

Upon cutaneous wounding, platelets release clotting factors that prevent bleeding and invasion by bacteria, which are further eliminated by inflammatory cells. Fibroblasts also enter the wound bed and secrete ECM containing collagen. Finally, keratinocytes migrate over this granulation tissue until two epithelial “tongues” meet in the center of the wound, as part of the re-epithelialization process [37]. Following H&E staining, these epithelial tongues can be measured by quantitative analysis. In controls, epithelial tongues were reduced in size in all dimensions. Addition of PRP generated an epithelium that contained more layers and was longer than controls (Fig. 5C). Addition of 10  $\mu$ M purified polyP to PRP generated epithelial tongues of greater length (Fig. 5D). A polyP dose-dependency was also observed with significantly longer tongues observed with 100  $\mu$ M polyP (Fig. 5E). Image J analysis revealed a significant increase in tongue length in wounds exposed to PRP with 100  $\mu$ M purified polyP, compared to controls and wounds exposed to PRP alone (Fig. 5F).

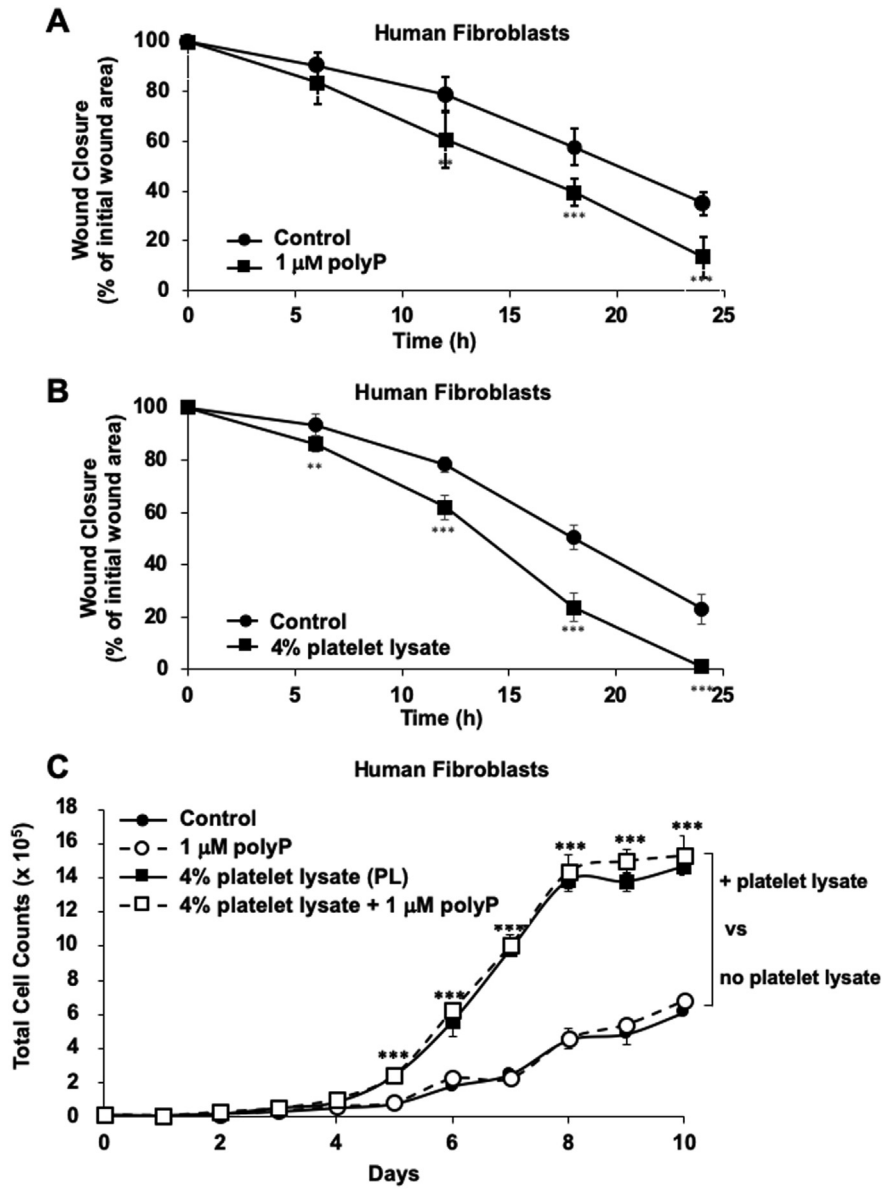
## 4. Discussion

PolyP has been shown to play potential roles in different stages of normal wound healing, including hemostasis, dermal wound healing, and re-epithelialization. We have shown in cell culture models that both intracellular and extracellular polyP enhances scratch-wound closure of HaCaT skin keratinocytes [22]. Keratinocytes, the cells that make up the majority of the epithelium, were shown to have increased cell growth in the presence of 4% platelet lysate. Moreover, addition of polyP attenuated open wound area in scratch assays of HaCaT keratinocyte cells in cells depleted of polyP (PPX1 DsRed), as well as empty vector controls.

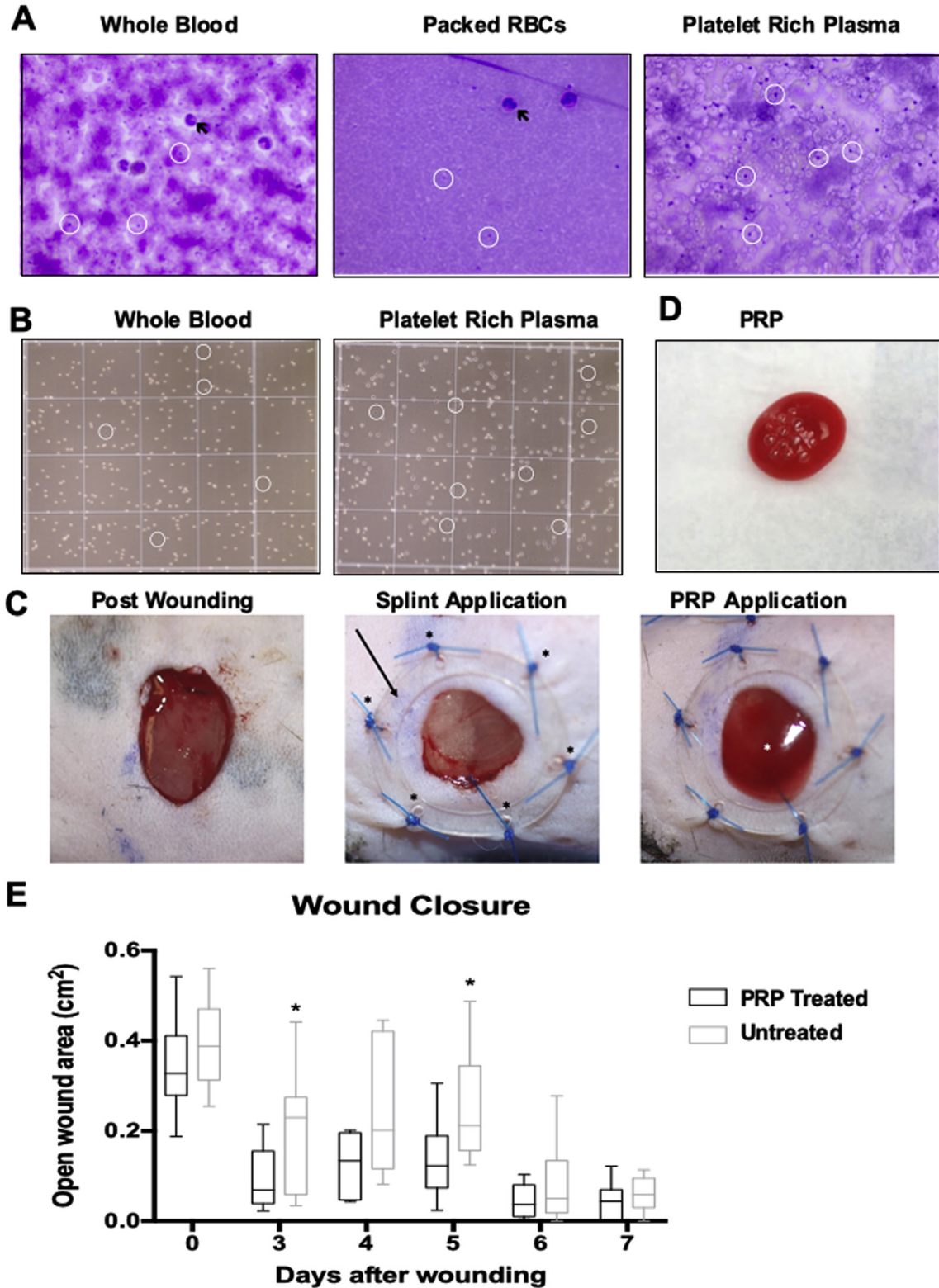
Reepithelialization of wounds depends on the processes of cell proliferation and cell motility. Effects on cell growth were evaluated to elucidate whether treatment with polyP, platelet lysate, or the combination of the two, can enhance the rate of wound healing in HaCaT cells by affecting cell proliferation, cell motility, or both.



**Fig. 2.** A) Representative fluorescent images of wound healing scratch assays at indicated time points for HaCaT empty vector GFP (left panel) or polyP-depleted PPX1 Ds-Red cells (right panel) that are untreated (top, control), treated with 1  $\mu$ M polyP (upper middle), 4% platelet lysate (lower middle) and 4% platelet lysate + 1  $\mu$ M polyP (bottom). Images were taken at 10 $\times$  magnification, with the margin of wound shown as a yellow line. B) Wound gap closure of HaCaT empty vector (left) or polyP-depleted PPX1 cells (right) reveal significant increases in rates of wound healing in cells treated with 1  $\mu$ M polyP (top), 4% platelet lysate (middle), or 4% platelet lysate + 1  $\mu$ M polyP (bottom) compared to untreated control cells. Results are the means  $\pm$  SEM of three replicates of a representative experiment; essentially the same results were obtained in three independent experiments.

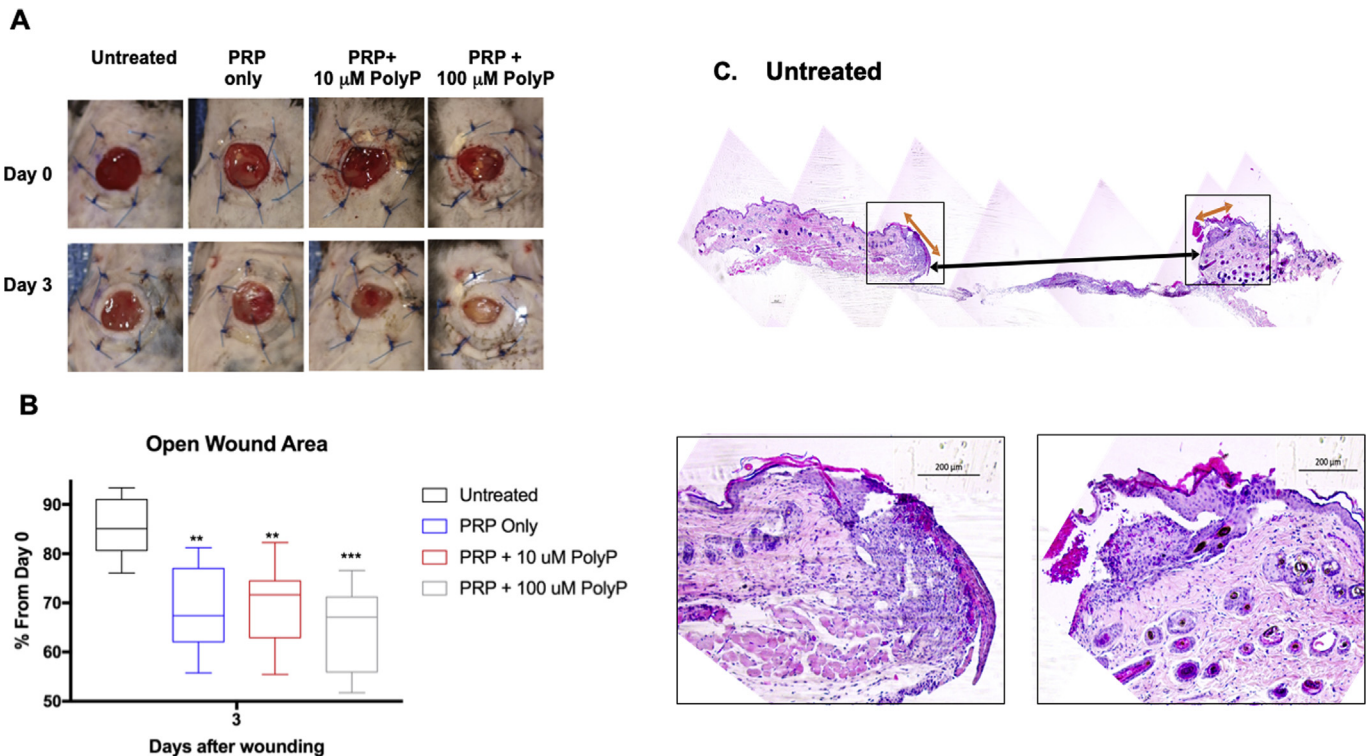


**Fig. 3.** Fibroblast scratch wound closure is enhanced by PRP and purified polyP. Scratch wounds were made in fibroblast cultures, and measurements were made from representative fluorescent images at indicated time points for HFF that are untreated or treated with 1 μM polyP (A), 4% platelet lysate (B). C) HFF Proliferation is enhanced by PRP but not polyP. HFF were plated in the presence or absence of purified polyP, and cell counts were performed each day for 10 days.



**Fig. 4.** A) PRP was created by multiple rounds of centrifugation. Blood smears were stained with Wright Giemsa. B) Whole blood and PRP were diluted and loaded onto a hemocytometer to determine platelet concentrations. White blood cells are indicated with black arrowheads. Platelets are indicated in white circles. C) The splinted excisional wound mouse model was used to evaluate treatment of wounds with PRP and polyP. 6 mm punch biopsies were used to create full thickness wounds (left), splints were applied to prevent contraction (middle), and treatment was applied as a gel (right, D). Black arrow = splint; black asterisk = suture; white asterisk = PRP. E) Wounds were splinted to prevent contraction, and were left untreated, or were treated with PRP. On Days 0 and 3–7, pictures were taken and open wound areas were quantified by Image J to assess wound healing. Box and whiskers plot of wound areas (N ≥ 6), showing average ± SD of the mean, and range. \*p < 0.05, \*\*p < 0.01 Results are the means ± SEM of three replicates of a representative experiment; essentially the same results were obtained in three independent experiments.





**Fig. 5.** The splinted mouse model was used, and representative pictures of wounds at days 0 and 3 are shown (5A). B) Image J quantification of open wound area revealed that treatment with PRP and/or PRP + low or high-dose polyP resulted in lower % open wound area normalized to Day 0 at Day 3 (Untreated =  $85.34 \pm 2.49\%$  vs. PRP =  $68.89 \pm 3.08\%$  ( $p < 0.0059$ ) vs. PRP + low dose polyP =  $69.91 \pm 2.95\%$  ( $p = 0.0106$ ) vs. PRP + high dose polyP =  $65.17 \pm 3.12\%$  ( $p = 0.0007$ ). This quantification did not reveal significant differences with the addition of polyP, and hence, epithelial tongue length was further quantified to elucidate any difference. Wounds were removed after exposure to test compounds for five days, then formalin fixed, paraffin embedded, sectioned, and examined histologically with H&E. Wounds were left untreated (5C), treated with PRP only (5D), PRP + 10 μM polyP (low dose; 5E) or PRP + 100 μM polyP (high dose; 5F). For 5C–5F, images were photo stitched to observe unwounded skin as well as the epithelial tongues entering the wound bed (orange arrows). Black arrows denote areas lacking epithelium. Image J analysis was performed and revealed a significant increase in tongue length in wounds exposed to PRP with 100 μM purified polyP, compared to controls and wounds exposed to PRP alone (5G).

We have previously shown significantly higher rates of cell growth in vector control cells compared to PPX1-expressing cells, indicating that intracellular polyP depletion by PPX1 decreases the rate of cell growth [22]. As a measure of individual cell proliferation, an *in situ* BrdU incorporation assay was also conducted at indicated times on the cells at the wound edge. Fluorescent images of cells at wound-edge were taken, and % BrdU incorporation into their nascent DNA was quantified and plotted. Consistent with the cell growth curves, BrdU incorporation was significantly lower for the PPX1 cells compared to vector control cells. Depletion of intracellular polyP by PPX1 may therefore impair the ability of HaCaT skin keratinocytes to close wounds, in part by interfering with their ability to proliferate [22].

Interestingly, while intracellular polyP may play an essential role in cell proliferation, our current results show that addition of exogenous polyP did not have any significant effect on cell growth of HaCaT cells, although polyP-rich platelet lysate did. The significant increase in the rate of wound healing in cells treated with extracellular polyP, platelet lysate or the combination, can therefore be attributed to increased cell motility rather than cell proliferation. Consistently, results of real time monitoring and measurement of cell motility using an xCelligence impedance-based system revealed a marked attenuation of cell motility in polyP-depleted PPX1-expressing HaCaT keratinocytes [22]. PPX1 cells exhibit only one-fifth the motility of the vector control cells, suggesting that polyP depletion leads to significantly impaired cellular

migration. Taken together, our results demonstrate that constitutive expression of PPX1, which degrades intracellular polyP, retards the rate of wound healing in human skin keratinocytes, a response that, at least in part, may be attributable to a decrease in cell proliferation as well as cell motility.

The human h-Prune cancer metastasis regulator protein exhibits inorganic polyphosphatase activity ( $K_m = 2.2 \mu\text{M}$  for polyP<sub>3</sub> [38]). Interestingly, h-Prune has been shown to increase cell motility [39,40] as a mechanism to promote cancer metastasis [38,41,42]. Normal wound healing *in vivo*, however, is a coordinated series of events, including angiogenesis, collagen deposition, granulation tissue formation, re-epithelialization, and wound contraction. Consistent with our results, corneal epithelial cell migration during wound healing is also stimulated by diadenosine polyphosphates presumably due to activation of MAPK pathways [43]. The role(s) of polyP in wound healing may also be mediated *via* mTOR, a serine threonine kinase stimulated by inorganic polyphosphate [44]. mTOR is involved in cell proliferation and survival, and is upregulated during wound healing [45].

These promising *in vitro* results prompted an investigation into the effect polyP may have *in vivo*. PRP, which contains polyP at a concentration near 8 μM polyP, was shown to accelerate wound healing in a splinted excisional wound mouse model. The addition of purified polyP to PRP accelerated keratinocyte proliferation, as shown by augmented epithelial tongue length in a polyP dose-dependent fashion. Thus, extracellular polyP appears to function

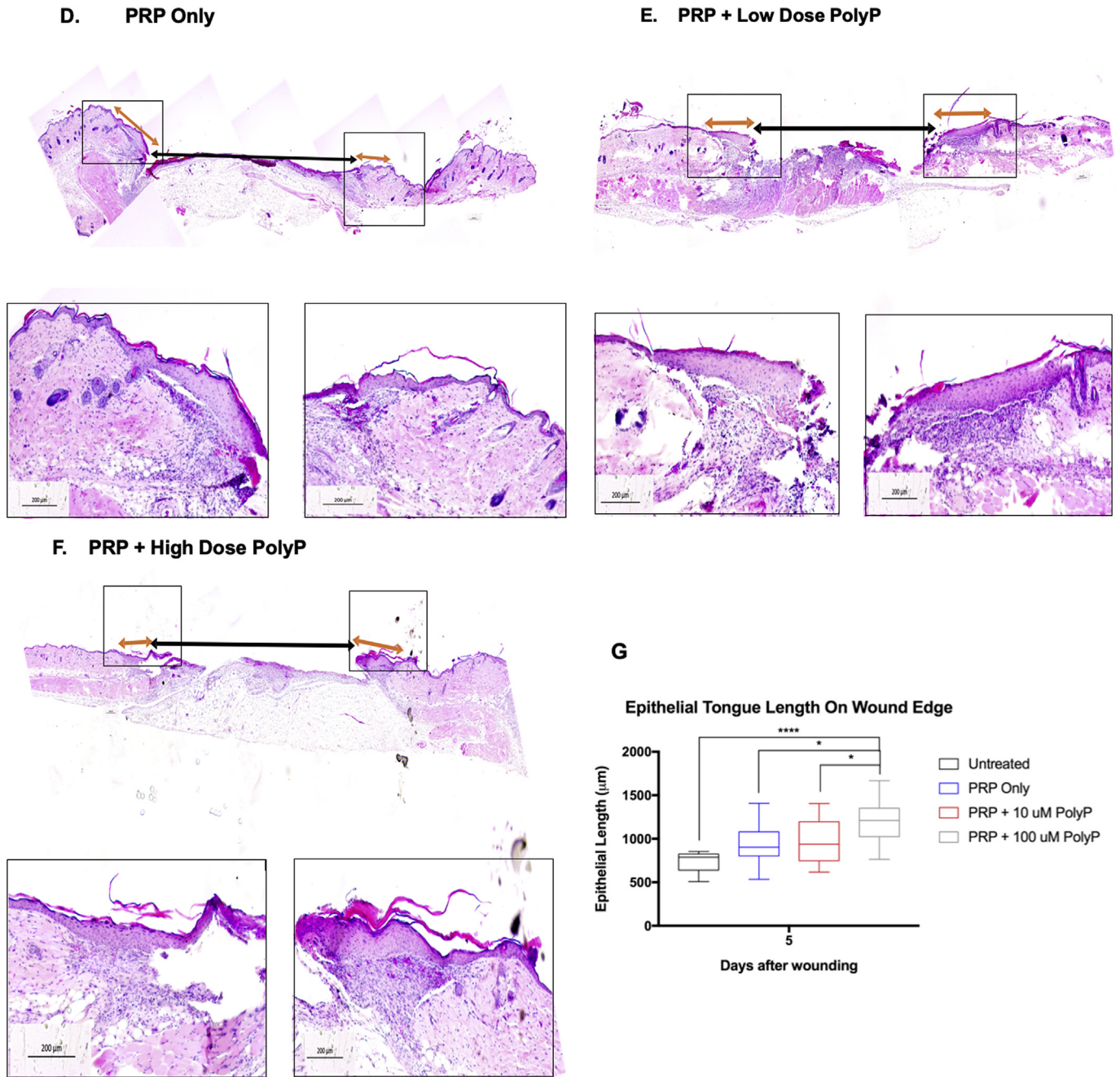


Fig. 5. (continued).

as an inducer of migration, but not proliferation in keratinocytes as well as dermal fibroblasts, which comprise the bulk of the skin. PolyP levels need to be compared in healthy and pathologic conditions allowing us to supplement PRP with appropriate amounts of polyP when treating acute burn wounds or chronic wounds in the future.

**5. Conclusions**

In conclusion, we have shown that in HaCaT skin keratinocytes, treatment with polyP, platelet lysate, and their combination increases rates of wound scratch closure. Additionally, in a splinted excisional wound mouse model, PRP-treated wounds had smaller open areas compared to controls as quantified by gross pictures.

Additionally, when purified polyP is added to PRP, acceleration of healing is increased. PRP is a safe option for the treatment of wounds due to its autologous nature. It can be used to treat wounds or partial thickness burns, and further studies should further examine the role of polyP modulation to accelerate wound healing.

**Declaration of competing interest**

The authors state no conflict of interest.

**Acknowledgements**

Authors acknowledge technical aid of Sixian Song, Ahmed Dang, Takashi Kitani, and Jozef Schmidt for the scratch wound and

proliferation assays, and polyP measurements. The work was funded in part by NIH (2R42ES026908-02; DSR).

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