Optimising platelet secretomes to deliver robust tissue specific regeneration

Article

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Optimising platelet secretomes to deliver robust tissue-specific regeneration

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Authors response

We are indebted to the anonymous reviewers for the constructive criticism they provided, that enabled us to carefully revise and improve our work.

Referee(s)' Comments to Author:

Reviewing: 1

Major issues include:

1. The platelet releasates in this study were prepared from single donor platelets. It has been known that the amount of growth factors or other alpha-granule components in platelets can be highly variable among different individuals. (Journal of Translational Medicine, 2017, 15:107, the concentration range for TGF-b1 among donors is 50-68000 ng/ml). In Fig 1., they compared the concentration of 37 molecules in PR and SR from 2 donors, and found that unlike many other molecules, the concentration of TGFb decreased in SR and concluded that "the formation of platelet releasate is a regulated process and not one that relies on mass action. The releasates produced under physiological and supra-physiological conditions differ both quantitatively and qualitatiavely." (page 11, line 41 to 50). However, in Fig 1B, SR1 contains similar level of TGFb to PR1. Only SR2 shows lower level of TGFb compared to PR2. The other two molecules in the same cluster, Osteopontin and RANTES, show same problem. The difference they observed could be simply caused by the variation between donors. The data is not convincing. The "N" number needs to be increased to draw a conclusion.

Response: We thank the reviewer for this comment. As requested by the reviewer, we have conducted new multiplex analysis on two more donors using Thrombin as an agonist which uses not only the PAR1 pathway but also PAR4 in order to give a deeper insight into the variations indicated by the reviewer. This data in the revised manuscript backs up the overall reduction seen with TGFβ in PAR1 from the original manuscript and makes a robust point about qualitative and quantitative releasate composition under physiological vs. supraphysiological conditions (see revised Figure 1 and Suppl. Figure 1).

We also believe that by focusing only on one or two particular releasate analytes one misses the full picture on the composition of physiological and supra-physiological releasates. We are aware of inter-individual variability on releasate composition, but the point this study makes is about the higher potential of supra-physiological releasate for tissue regeneration. To this aim, we employed PCA analysis on these data which clustered quite well for the majority of analytes, both between donors as well as between platelet concentrations. Therefore, we do not encourage the over-simplification and interpretation of these datasets on isolated analytes. Wer are aware that, TGFβ detection is also a complicated one, it cannot be performed with other analytes (Single-plex), and requires citrate treatment of the sample that would interfere with the detection of other analytes. As stated above, in order to circumvent potential technical problems with TGFB detection, we provided new data on thrombin-activated physiological and supraphysiological releasates, which are in line with the conclusions drawn previously.

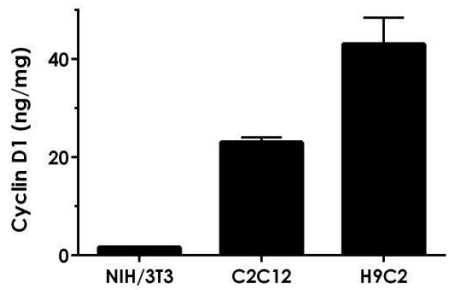
2. In Fig 1C, they showed color-coded symbols for 10 growth factors, however, there are only 9 molecules shown in the graph. (VEGF-D is missing). In Fig 1E, they showed color-coded symbols for 7 high concentration analytes, however, there are only 6 molecules shown in the graph. (CD40L is missing.)

Response: Thank you for pointing this out. The values for the above two analytes were much smaller in relation to others and difficult to see. In the revised manuscript, we have added 2 new conditions (Thrombin as described in Q1). We have also expanded these smaller samples x10 and x5 respectively so as that they are now visible in the revised manuscript. See revised Figure 1.

3. In Fig 2B, C2C12 cells and H9C2 cells responded to FBS(GM) very differently. The C2C12 cell number (DAPI positive cells) increased significantly after GM /FBS treatment compared with SF condition. However, the majority of these cells are negative for cyclin D1. How to explain it? Do the cells exit cell cycles faster and became cyclin D1 negative? Then Cyclin D1 expression is not a good measure for cell proliferation.

Response: The C2C12 and H9C2 cell lines respond differently in culture because they are fast and slow proliferating cells respectively (Senyo et al. *Stem Cell Research* (2014) 13, 532–541). We have also added to the manuscript Suppl. Figure 5 to show the cumulative population growth rate for both H9C2 and C2C12 cells to show this discrepancy in growth rates. The majority of cells are negative for Cyclin D1 simply because Cyclin D1 staining is a "snapshot in time" of cell proliferation. We are aware of the limitations of this marker which does not encapsulate the full story. This is why we have backed up our findings on H9C2 cells by other markers for cell proliferation such as Ki-67 staining and most importantly EdU live cellular staining, which are already present in the original submission. Therefore, an emphasis on Cyclin D1 data in isolation is not representative of the message this study delivers about the role of platelet releasate concentration on proliferation of these cell types.

Moreover, the C2C12 cells are proliferating faster than the H9C2 cells and Cyclin D1 protein concentration is higher in H9C2 cells compared to C2C12 cells as reported by a commercial supplier:



https://www.abcam.com/human-cyclin-d1-elisa-kit-ab214571.html#description_images_6 , accessed 12/07/2019.

4. In Fig.1B- D, Physiological releasate alone can stimulate C2C12 and HDF proliferation, but not H9C2 and HaCaT. How about GM+R? Will PR induce stronger H9C2 and HaCaT proliferation in the presence of FBS?

Response: To address the reviewers' point we have conducted new experiments in H9C2 and HaCaT cell lines cultured with growth medium versus growth medium and releasate. These data add value to this manuscript by showing that growth medium plus PR does not increase the proliferation rate above GM alone in H9C2 cells. Importantly, HaCaT cultured in GM+PR are significantly less proliferative as compared to GM alone which suggests that there is an impaired proliferation rate using PR in culture even in serum-rich conditions. These data are illustrated as a new Suppl. Figure 6.

5. Are Fig. 2E and S.Figure1 show the same experiment? The figure legends for both of them are confusing. Which condition is treated with IL-1b? How are the cell numbers quantitated in Fig. 2E? Will PR alone stimulate chondrocytes proliferation without GM/FBS?

Response: We are thankful for this comment. We have revised the manuscript as recommended to improve clarity. S. Figure 2 (Suppl. Figure 1 in the previous submission) refers to the same experiment as shown in Fig. 2E and depicts the single channel images of this experiment. The figure legends were amended to provide clarity and consistency. Quantification was performed with the ZEN 2.3 Zeiss software. Chondrocytes under serum free conditions are not viable and the effect of PR on this cell type proliferation can be hardly assessed.

6. In Figure Legend for Fig. 3B, the C2C12 cells were treated with 10-30% releasate (SR). However, in Result section, it says 10% SR was used (page 12, bottom line). Which concentration is correct?

Response: We thank the reviewer for pointing out this discrepancy. We used 10% PR or SR in all data sets of the manuscript except figure 4B, where a dose response including 10-30% SR was studied on H9C2 cells. Additionally, we have used 30% SR in the qRT-PCR for H9C2 cells.

7. To show if stronger proliferation induced by SR compared with PR in different cell types is just because SR is a more concentrated product, higher concentration of PR (30-40%) should be added to culture to compare with 10% SR in different cell types (Fig. 3D and 4D). If adding more PR could not induce similar level of response to 10%SR, then the effect of SR is unique, not just because it is a more concentrated product.

Response: This is a very valid point. To address the reviewers point we conducted new experiments on C2C12 cells. These new data have been added to the revised manuscript and a supplementary figure and the text were revised accordingly, see Suppl. Figure 7.

8. In Fig. 4C, they found SR alone promoted HaCaT proliferation compared with serum free condition. (also as described in Page 13, line 50-54: "In contrast, supra-physiological releasate did not stimulate HaCaT proliferation in serum-rich conditions, however there was a significant increase in serum-free conditions as compared to serum-free alone".) In Fig. 2D, PR alone could not promote HaCaT proliferation. However, in Fig. 4D they found PR and SR had similar effects on HaCaT

proliferation. These data are conflicting. Whether PR or SR alone can promote HaCaT proliferation is not conclusive.

Response: We show here that the physiological platelet releasate does not induce proliferation when compared to serum free conditions (Figure 2D). The absence of significant effects of PR on HaCaT cell proliferation in our study is in line with published data (see S. Table 2 in this manuscript and particularly Bayer et al. *Mediators of Inflammation Volume* 2017, Article ID 5671615, 12 pages) and (Bayer et al. *Annals of Anatomy 215* (2018) 1–7). We also show that PR and SR have similar effects on HaCaT cell proliferation (Fig. 4D). However, when normalised to SF conditions the SR treatment reached the level of statistical significance. To strengthen these findings, we performed a new experiment on HaCaT cells under serum free conditions with or without SR and found that SR consistently increases HaCaT cell proliferation (Suppl. Figure 9). The take home message from our study is that SR drives late keratinocyte cell differentiation which is a novel finding with implications for regenerative medicine.

9. In Suppl. Figure 2A, GM alone did not induce Scrib expression in C2C12 cells, which conflicts with what they had shown in the previous publication. (Figure 8C in their 2018 publication in Acta Physioligica, e13207 showed GM alone induced Scrib expression significantly in C2C12 cells.) The authors need to be more careful about their statistics. In this paper, their statement "We here report that platelet releasate does not show the same Scrib expression as observed in the C2C12 cells as compared to the serum-free and growth medium group (Suppl. Figure 2)." (on page 14, line 9-14) is hard to understand.

Response: We would like to thank the reviewer for this valid point. There is a significant difference that was overlooked in our initial submission of the manuscript that has now been rectified. The statement on page 14 has now been altered to read more clearly.

10. Suppl. Figure 3 is very confusing. What caused the formation of high density and low density areas? Is this simply a consequence of uneven seeding of the cells in culture dishes? Does it mean uneven seeding of the cells can significantly affect the result of EdU live staining?

Response: High density areas of the HaCaT cells is a normal process of cell lineage progression not observed in any other cell type we have cultured and does not represent an unequal seeding issue. Typical keratinocyte differentiation involves an increase in cell density (Buerger et al. *PlosOne*, July 10, 2017, p1-20). This point and reference has now been added to the discussion for the reader's clarity.

11. Why 30% SR (instead of 10%) were used in Fig. 5A? The sudden change of the concentration makes it hard to compare and explain data. In Fig. 5A, SR alone, but not GM alone, induced VEGF and VEGFR expression in H9C2, suggesting GM and SR work through different pathways in H9C2 cells. In Fig. 5B, SF alone condition should be included as control. The effect of SR should be compare with SF condition, not just GM condition. VEGFR inhibitor should be used to show if the cell proliferation is driven by VEGFR activation.

Response: 30% SR was used for the qRT-PCR as according to our dose response in Figure 4B, 30% SR resulted in the most potent proliferation rate of the H9C2 cells. The point of adding the serum-free condition alone was one we thought of previously and have this data at hand. The condition

has now been re-introduced into the manuscript (see Figure 5B). Adding a VEGFR inhibitor is a valid point; we have conducted a new experiment on H9C2 cells with either GM or SR in the presence or absence of a VEGFR inhibitor (AAL-993; 1.30µM, Merck).

12. In Fig. 6A, SR alone need to be added to show if the effect of SR is GM-dependent or the effects of SR and GM are additive. There is no data support their conclusion:"Unlike the C2C12 cells, platelet releasate did not seem to play a significant role in increasing total H9C2 myotube number. (on page 14, line 49-51) ".

Response: Suppl. Figure 8 was added to the revised manuscript in which the above-mentioned experiment (Figure 6A) was repeated on C2C12 cells with the SF+SR condition added. The sentence in question has been rephrased to make the point more clear that we are addressing the SR+SF condition when stating that there was no increase in myotube number.

13. In Fig. 7A, what are the control conditions for ACTA2 and CDH1 expression? Are they same with the control condition for Gel Weight experiment on the right side? Or do both control and SR conditions for ACTA2 and CDH1 contain TGFb1? Can SR alone (without TGFb1) stimulate HDF differentiation since platelet releasate contains significant amount of TGFb? Will PR work stronger since PR contains more TGFb compared with SR?

Response: The control conditions for *ACTA2* and *CDH11* expression are media only (DMEM with 2% FBS) without TGFβ1 or releasate. The control for the gel weight is the same as the control for the *ACTA2* and *CDH11* expression. The increased *CDH11* expression following SR treatment indicates that SR alone (without TGFβ1) is able to stimulate HDF differentiation. We have not assessed PR in these experiments. PRP at physiological levels has previously been shown to not induce differentiation of fibroblasts (Chellini et al. *Cells* 2018, 7, 142). The results section and fibre legends have been changed for clarity.

14. In Fig. 7B and C, SR only condition (without Ca) should be added. Ca alone did not induce IVL expression, indicating SR and Ca may work through different mechanisms. Ca may not be required for SR function.

Response: The reviewer's point is valid and for this reason the *KRT1* and *IVL* expression was conducted again on fresh NHEK cells to include the missing SR without Ca condition. Please see the revised Figure 7.

15. On page 19, line3-14:" Additionally, when H9C2 cells are treated for a longer period of time in differentiation media; the additive effect of platelet releasate with 10% FBS was more pronounced resulting in greater Myogenin expression, higher cell numbers, a greater myofusion index and myotube number. One can speculate that this additive effect is due to a higher total cell number in the proliferation period, leading to more cells to undergo differentiation as a whole." This is hard to understand since the differentiation index was normalized with cell numbers (# of DAPI).

Response: We are thankful for this observation. We think that cell confluence is crucial for cell differentiation which we have backed up with a reference (Tanaka et al. 2011 on page 20), stating that the denser cells are, the more they differentiate. We hope that this clarifies the above point.

Minor issues include:

Response: We have fully addressed all the minor points mentioned below.

1. Page11, line 35: “Similarly, growth factors driving such as EGF…” driving should be deleted.

2. Fig. 4A: Platelet concentration should be 10x108, not 2.5x108.

3. Fig. 6B: “SR+SR” in quantitated graphs should be “SF+SR”.

4. Page 27, line 39-48: “Outcome measures include the nuclei number, number of Myogenin+ve nuclei, myotubes (n=2 nuclei/ myotube). The myotube fusion index was calculated by Myogenin+ve nuclei in myotubes/ DAPI as a percentage. Outcome measures include the nuclei number, number of Myogenin+ve nuclei, myotubes (n=2 nuclei/ myotube).” Repeated twice.

Reviewing: 2

Comments to the Author

A very interesting and topical manuscript from a highly reputable group in this emerging field. The manuscript builds on their recent output in this area, offering insights into the relationship between platelet releasate supplementation concentrations and cellular proliferation rates. Following an initial basic characterisation of physiological vs. supra-physiological platelet releasate composition (Fig. 1), the authors describe various proliferative responses (and lack thereof in cases) to the aforementioned on: (i) rat cardiomyocytes (H9C2), (ii) primary human skin fibroblasts (HDF), (iii) immortalised human skin keratinocytes (HaCaT), (iv) primary human epidermal keratinocytes (NHEK), (v) primary murine chondrocytes (C57BL/6), and (vi) murine skeletal myoblasts (C2C12) as a positive control in vitro. Notable findings include (a) the disproportionate elevation and reduction of various components in physiological vs. supra-physiological platelet releasate, (b) increased HaCaT differentiation in response to platelet releasate exposure but not proliferation, and (c) elevated HDF, H9C2, and C2C12 cell proliferation following supra-physiological releasate supplementation. Plausible explanations for these findings are presented, revolving around additional qRT-PCR data. In closing, these data are of value and support the authors’ conclusions. I believe this paper will be of great interest to the Journal of Tissue Engineering and Regenerative Medicine readership.

Response: We are thankful to reviewer 2 for the careful proof reading and the positive feedback. We have fully addressed all the raised concerns as follow:

General Comments

1. Individual page lines are neither correctly numbered nor continuous. In future, the authors are advised to amend this. Herein, I refer to both pages and approximate line numbers (e.g. P1L11 as page 1 line 11).

Response: Apologies for the inconvenience caused by the page line formatting from the automated submission system.

2. Given the high number of independent and dependent variables, coupled with the sparse provision of experimental replicates in the main body of the manuscript, I believe a brief schematic diagram outlining the experimental designs would greatly benefit the reader. This would also negate the effective repetition in Figs. 1A, 2A, 3A, 4A, and 6A. However, the authors may choose not to do this if it markedly increases manuscript length, without prejudice to the contribution of the paper.

Response: Since the experimental set up is not the same among all figures, we find it hard to make a one-fits-all diagram and we think that more than one schematic diagrams support the data flow.

3. Referencing is inconsistent. For example Ranzato, Martinotti, Volante, Mazzucco, & Burlando (2011) on P4L38 vs. Ranzato et al. (2011) on P4L56. Please correct all references in line with the journal policy throughout.

Response: We have used to correct referencing format (i.e. Publication Manual of the American Psychological Association (6th edition)) throughout, which gives the exact format as you have quoted above, i.e. Ranzanto et al. (2011) in the text and as you correctly stated; authors followed by year in the bibliography.

4. Were physiological and supra-physiological platelet releasate experiments conducted in parallel? i.e. Did the authors control for potential donor-to-donor platelet variation? This should be stated in the manuscript.

Response: This is a valid point; we thank the reviewer for directing our attention to this. The methods section has been updated on page 6 to include this information.

5. Please consider sharpening the title along the lines of “Modified platelet releasate alters cellular proliferation and differentiation”

Response: The title has been altered to fit the reviewer’s recommendation as follows: “Optimising platelet secretomes to deliver robust tissue-specific regeneration”.

6. I believe the manuscript would benefit from elaborating upon the “future work” section (P20L27). For example, testing the impact of physiological vs. supra-physiological platelet releasate from mice on murine chondrocyte parameters.

Response: We have added to the details of our future work section and have incorporated current work being conducted in our lab.

7. The English, in parts, requires improvement. Please see below.

Response: We have fully addressed all the minor points mentioned below.

Specific Comments

8. P2L13 — Replace “differentiation for specific” with “differentiation of specific”

9. P2L14 — Replace “profiled for” with “profiled from”

10. P2L23 — Delete “the”

11. P2L31 — Rephrase to read “induced H9C3, C2C12, HDF, and keratinocyte differentiation”

12. P3L11 — Replace “controversial evidence” with “controversial data surrounding”

13. P3L51 — Replace “as fibrinogen as previous”: with “as fibrinogen; previous”

14. P4L27 — Replace “is” with “has”

15. P4L35 — Delete “have previously been reported to”

16. P4L39 — Delete “have recently been shown to”

17. P4L48 — Rephrase to read “seem to inhibit keratinocyte proliferation”

18. P4L60 — Consider replacing “a possible outcome” with “possible”

19. P5L14 — Replace “We here” with “Here we”

20. P5L16 — Replace “insights to” with “insights into”

21. P6L21 — Replace “theatres” with “operating theatres”

22. P6L41 — Delete “labelled as” here and elsewhere 23. P6L49 — Please state how releasate supernatant aliquots were stored.

24. P7L20 — Please provide a citation elaborating upon the myofusion index methodology.

25. P7L33 — Replace “on Dispase” with “in Dispase”

26. P7L42 — Please provide the surface area of the wells used here and elsewhere.

27. P20L41 — Replace “would” with “could”

28. P20L49 — Consider replacing “is not due” with “may not be due”

Original research paper

Optimising platelet secretomes to deliver robust tissue-specific regeneration

Running title; Insights from the use of customised platelet releasate in cell lines

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Summary

Promoting cell proliferation is the cornerstone of most tissue regeneration therapies. As platelet-based applications promote cell division and can be customised for tissue-specific efficacy, this makes them strong candidates for developing novel regenerative therapies. Therefore, the aim of this study was to determine if platelet releasate could be optimised to promote cellular proliferation and differentiation of specific tissues. Growth factors in platelet releasate were profiled for physiological and supra-physiological platelet concentrations. We analysed the effect of physiological and supra-physiological releasate on C2C12 skeletal myoblasts, H9C2 rat cardiomyocytes, human dermal fibroblasts (HDF), HaCaT keratinocytes and chondrocytes. Cellular proliferation and differentiation were assessed through proliferation assays, mRNA and protein expression. We show that supra-physiological releasate is not simply a concentrated version of physiological releasate. Physiological releasate promoted C2C12, HDF and chondrocyte proliferation with no effect on H9C2 or HaCaT cells. Supra-physiological releasate induced stronger proliferation in C2C12 and HDF cells compared to physiological releasate. Importantly, supra-physiological releasate induced proliferation of H9C2 cells. The proliferative effects of skeletal and cardiac muscle cells were in part driven by VEGFα. Furthermore, supra-physiological releasate induced differentiation of H9C2 and C2C12, HDF and keratinocyte differentiation. This study provides insights into the ability of releasate to promote muscle, heart, skin and cartilage cell proliferation and differentiation and highlights the importance of optimising releasate composition for tissue-specific regeneration.

Key words: biomaterial, cardiomyocyte, chondrocyte, fibroblast, injury, keratinocyte, platelet releasate, regeneration

Introduction

Platelet-based applications have been studied in many tissue types *in vitro*, *ex vivo*, *in vivo* and clinically. Although outcomes have been largely successful in terms of improving tissue regenerative capacity (reviewed by (Scully, Naseem, & Matsakas, 2018)), there is controversial data surrounding the translational aspect of platelet-based applications from laboratory to clinic (Mosca & Rodeo, 2015). One potential aspect that may be overlooked in the preparation of platelet-based therapies is the concentration of platelets used; however, this has yet to be established. Importantly, in slow proliferating cell types such as cardiomyocytes and chondrocytes, optimised platelet releasate may be used to enhance regeneration (Hargrave, Varghese, Barabutis, Catravas, & Zemlin, 2016; Ishibashi, Hikita, Fujihara, Takato, & Hoshi, 2017; Senyo, Lee, & Kuhn, 2014). Previously, we have found a strong correlation between the platelet concentration used to make platelet releasate and proliferation of skeletal myoblasts (Scully, Sfyri, et al., 2018).

Exercise-induced silent myocardial ischemia increases in prevalence with aging with an estimated 3 million people having asymptomatic ischemia in the United States of America (Stern, 2005). Platelet-rich plasma (PRP) has been studied as an attractive biomaterial for the treatment of myocardial ischemia and cardiac tissue regeneration as it is autologous, inexpensive and easily obtained (Gallo et al., 2013; Hargrave & Li, 2012; Hargrave et al., 2016; X. H. Li et al., 2008; Morschbacher et al., 2016; Patel, Selzman, Kumpati, McKellar, & Bull, 2016; Spartalis et al., 2015; Tang et al., 2017). However, concerns have been raised over the efficacy of PRP as an effective method of stimulating cardiac restoration (Morschbacher et al., 2016). One issue of using PRP may be the clotting factors contained in blood plasma such as fibrinogen; previous studies have described that adding clotting factors to the heart may cause serious negative side-effects (Hargrave & Li, 2012). Additionally, the cardiomyocyte self-renewal rate is known to be low (Senyo et al., 2014), which may offer a plausible explanation for the limited success of platelet-based applications to-date. Removing plasma, cellular debris and clotting factors from activated platelet-rich

plasma (i.e. a cocktail of growth factors and cytokines described previously as platelet releasate) may offer an alternative, more effective regenerative platelet-based application for cardiac regeneration (Scully, Naseem, et al., 2018).

Similarly, cartilage shows poor regenerative capacity due to its relatively avascular nature and chondrocyte heterogeneity, where various sub-populations differ in proliferation rate (Ishibashi et al., 2017). Platelet releasate has recently been shown to promote chondrocyte anabolic gene expression, relieve inflammatory stress and ameliorate cartilage degeneration *in vivo* (Yang et al., 2018). By optimising the preparation of platelet releasate for this poorly vascularised tissue, growth factors, hormones and nutrients can be delivered where they normally would not have access during typical healing. Furthermore, delivery of these growth factors may increase proliferation in slow dividing sub-populations of chondrocytes and enhance regeneration during injury, but this has yet to be determined.

In contrast to the cardiomyocytes and cartilage, the epidermis and dermis of the skin as well as skeletal muscle have a high capacity for regeneration and repair. Both the heart and skin respond positively to platelet-based applications for regenerative and wound healing purposes (Hargrave et al., 2016; Ranzato, Martinotti, Volante, Mazzucco, & Burlando, 2011). Such approaches effectively upregulate human dermal fibroblast proliferation, differentiation and migration (Cho et al., 2018; Kushida, Kakudo, Suzuki, & Kusumoto, 2013). However, contrary to fibroblasts and cardiomyocytes, platelet-based applications seem to inhibit keratinocyte proliferation of and induce terminal-differentiation (Bayer et al., 2018). Interestingly, keratinocytes are slow proliferating cells, however upregulate their proliferation rate in response to injury (Freedberg, Tomic-Canic, Komine, & Blumenberg, 2001). Taken together, platelet-based applications promote keratinocyte migration and regulate fibroblast matrix deposition (Ranzato et al., 2011). By optimising the preparation of platelet releasate for these cell types, faster, more complete regenerative wound healing with less scar tissue accumulation may be a potential outcome.

In light of this, the aim of this study was to determine if the method of platelet releasate production could be further optimised for increasing cellular proliferation and differentiation and aiding the regenerative capacity of different cell types. We hypothesised that supra-physiological platelet concentrations would yield more concentrated platelet secretomes by increasing the levels of all components and induce better proliferation in various cell types. Here, we focus on cells from tissues with high- (i.e. skeletal muscle and skin) and lower- (i.e. heart and cartilage) regenerative capacities. This article provides methodological insights into optimising the composition of the platelet secretome for cell-type specific applications. This may provide an effective intervention for treating injury and trauma such as sports injuries, wound healing and joint disorders.

Materials and Methods

Ethical standards. The study was approved by the local Ethics Committee of the University. Primary chondrocytes were obtained from mice bred in house after Schedule 1 euthanasia. Animals were maintained under a project license from the United Kingdom Home Office in agreement with the revised Animals (Scientific Procedures) Act 1986 and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985). Blood sampling from healthy human volunteers was performed with their written informed consent and was approved by the University's Ethics Committee. Human skin was collected from operating theatres at Castle Hill hospital (Cottingham, UK) under full UREC (FEC_47_2017) and LREC (17/SC/0220) approval conforming with the Declaration of Helsinki.

Preparation of platelet releasate. Human platelet releasate was prepared as described previously from male donors between the ages of 26 and 29 (Scully, Sfyri, et al., 2018). Briefly, acid citrate dextrose to whole blood at a ratio of 1:5 was centrifuged at 190g for 15 minutes followed by PRP collection and inactivation of platelets using prostaglandin I_2. The PRP was then centrifuged at 800g for 12 minutes and the platelet-poor plasma supernatant was then removed. Modified tyrode's buffer (NaCl, HEPES, NaH_2PO_4, $NaHCO_3$, KCl, $MgCl_2$ and D-Glucose) was used to re-suspend the platelet pellet to a concentration of $2.5x10^8$ platelets mL^{-1} (Physiological Releasate) or $10x10^8$ platelets mL^{-1} (Supra-physiological Releasate). The platelet preparation was activated using a PAR-1 (Protease-activated receptor-1) agonist (TRAP6; 20μM; AnaSpec; cat. AS-60679) or a Thrombin agonist (0.05-0.1 NIH Units mL-1; Sigma Aldrich; cat.9002-04-4; **see Figure 1**) until reaching at least 70% aggregation. Platelets were centrifuged at 9500g for 10 minutes, the releasate supernatant was aliquoted from the cellular debris and stored for up to 24 hours at -80°C.

Cell cultures and treatments. Murine C2C12 skeletal myoblasts (American Type Culture Collection, USA) and rat H9C2 cardiomyocytes (LGC-PromoChem, Teddington, UK) were cultured in growth medium (GM) at 37°C in a humidified atmosphere of 5% CO_2. GM

consisted of high glucose Dulbecco's Modified Eagle's Medium (DMEM; HyClone with (H9C2s) or without (C2C12s) sodium pyruvate) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin/streptomycin (PS; Sigma-Aldrich) and 0.1% amphotericin B (AB; Sigma-Aldrich). All releasate treatments were cultured in serum-free (SF) conditions when not indicated to be in a GM group. To induce differentiation, C2C12 cells were cultured in GM ± releasate until reaching 80% confluence (4 days) before switching to differentiation media (DM), containing DMEM plus 2% horse serum (HS; Gibco) 1% PS and 0.1% AB for a further 4 days. H9C2 cells were cultured in GM ± releasate until reaching confluence (4 days) before switching to DM containing DMEM with sodium pyruvate, 1% FBS, 1% PS and 0.1% AB for a further 4 - 7 days. The myofusion index was calculated as myogenin-stained cells per myotube (n=2 nuclei/myotube) divided by DAPI (4',6-diamidino-2-phenylindol-(Dako)-stained cells as a percentage (Scully, Sfyri, et al., 2018). For proliferation in all experimental groups, unless otherwise stated, C2C12 and H9C2 cells were cultured in SF conditions (DMEM, 1% PS and 0.1% AB). For VEGFR inhibition, VEGFR Inhibitor (AAL-993; 1.30μM, Merck) was used on H9C2 cells (see **Figure 5C**).

Skin was collected in DMEM plus 2% antibiotic-antimycotic solution (Thermo Fisher Scientific, Paisley, UK) and kept on ice during transport. Skin was placed in 2% Dispase II (Thermo Fisher Scientific) overnight at 4°C to separate the epidermis and dermis. Human dermal fibroblasts (HDFs) were isolated from the dermal tissue as described previously (Wilkinson et al., 2019). HDFs were cultured in phenol red-free DMEM with 10% FBS and 1% PS. For qRT-PCR differentiation experiments, HDFs were seeded into wells at a density of $1x10^5$ cells/ml for 24 hours with 2% FBS. For gel contraction analysis, HDFs were treated with differentiation media (DMEM plus 10ng/ml transforming growth factor-beta1; TGFβ1) alone or in combination with 10% supra-physiological releasate for 72 hours.

HaCaT keratinocytes (an aneuploid immortal keratinocyte cell line from adult human skin) were grown in calcium free DMEM with 10% FBS, 1% PS solution and 1mM calcium chloride (Sigma-Aldrich, Dorset, UK). For differentiation experiments, HaCaTs were seeded at a

density of $1x10^5$ cells/ml in 6-well plates. After 24 hours, media was replaced with DMEM containing 2.5mM calcium chloride and 10% supra-physiological releasate where appropriate. HaCaTs were left to differentiate for 24 hours and collected for RNA. HaCaTs were also seeded into 24-well plates, treated as above, and collected in crystal violet for bright-field imaging.

Primary normal human epidermal keratinocyte (NHEK) isolation and culture, human skin was de-fatted and washed in Hank's balanced salt solution and DPBS (Dulbecco's phosphate buffered saline). Skin was cut into strips and placed epidermis-side-up in 0.2% Dispase II (in DPBS) overnight at 4°C. The epidermis was then removed from the dermis, placed in 0.25% Trypsin and cut into small pieces before being neutralised with FBS and passed through a 70µm cell strainer and pelleted. NHEKs were re-suspended in Epilife medium (containing 1% growth supplement, GS) in coated 12-well plates at $2x10^5$ cells/ml. Plates were coated with coating matrix (Gibco, UK). For NHEK differentiation; NHEK media was replaced with Epilife containing 0.5% GS. Calcium chloride (1mM) was added to differentiation treatment groups, and 10% supra-physiological releasate (SR) was added where appropriate. NHEKs were differentiated for 24 hours and collected for RNA isolation.

Primary chondrocytes were obtained from 2-day old C57BL/6 mice as described previously (Wang et al., 2017). In brief, the articular cartilage derived from the terminal of the tibia and femur was digested with 0.2% type II collagenase, then expanded in medium containing F12 (Life Technologies, Cat. #88215) with 10% FBS, and 1% PS. 24 hours after cell seeding, media was changed with or without 2 ng/mL Interleukin 1 beta (IL-1β) as per experimental design. Forty-eight hours later, the cells were collected for analysis. All cell cultures were conducted in standard 24-well or 6-well plates (Corning Costar, UK).

Cell proliferation analysis. Cell proliferation was evaluated by the pyrimidine analogue EdU incorporation assay using the fluorescent Click-iT® EdU Cell Proliferation Assay (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Proliferating cells were measured as EdU divided by DAPI-stained nuclei as a

percentage. This was confirmed with monoclonal anti-Ki-67 (ThermoFisher Scientific. Cat. 14-5698-80) and with anti-Cyclin D1 (1:200 Santa Cruz; cat. sc-450) immunohistochemistry.

Immunohistochemistry. Cells were seeded on coverslips in 1mL of media in 24-well plates (Corning Costar TC-Treated 24-Well Plates). Media was removed at the end of experiments with 4% paraformaldehyde added for 15 minutes, followed by two washes in phosphate-buffered saline. Permeabilisation buffer was then added for 20 minutes followed by two washes in wash buffer before applying onto optical slides. Primary antibodies for anti-Myogenin (Santa Cruz; cat. sc-52903), Scrib (Santa Cruz; cat. sc-374139), mouse monoclonal anti-VEGF (Santa Cruz; cat. sc-7269), mouse anti-Col II antibody (EMD Millipore, cat. #MAB8887) and rabbit anti-ADAMTS5 (Abcam, cat. #ab41037) were added (1: 200 in wash buffer) overnight. Primary antibodies were removed with 3 washes in wash buffer, followed by the addition of secondary antibodies (Alexa fluor 488 Goat-anti-mouse; Life Technologies; cat. A11029 of 1: 200 in wash buffer. Cells were measured by the intensity of fluorescence per cell divided by DAPI-stained nuclei as a percentage (ZEN 2.3 blue edition © Carl Zeiss Microscopy GmbH, 2011, UK).

Luminex Multiplex assay. All releasate samples (PAR-1- and Thrombin- activated) were analysed by multiplex immunoassay based on Luminex 200 technology (Luminex Corporation, USA). We used the ProcartaPlex Human kits (Invitrogen) which test a panel of 37 molecules, including growth factors, cytokines, chemokines and immune stress markers. The multiplex assay was performed following the manufacturer's instructions and the plates were read using the xPONENT software (Luminex Corporation, USA). The specific factors analysed were: Caspase-3, CD40L, EGF, FGF-2, FGF-23, G-CSF (CSF-3),GM-CSF, GITRL, Granzyme B, GRO alpha (KC/CXCL1), HGF, ICAM-1, IFN gamma, IL- 1a, IL-1b, IL-2, IL-6, IL-7, IL-8 (CXCL8), IL-10, MIP-1a (CCL3), MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MIP-1b (CCL4), Osteopontin, PDGF-BB, PECAM-1, P-Selectin, RANTES (CCL5), SDF-1α, Thrombopoietin (TPO), TGFβ, TNFα, VCAM, VEGF-A and VEGF-D.

RNA extraction and real-time PCR analysis. Quantitative PCR was performed as described previously (Matsakas, Yadav, Lorca, Evans, & Narkar, 2012; Scully, Sfyri, et al., 2018). In brief, $3x10^4$ H9C2s were seeded per well of a 6-well plate in serum-free or growth medium (10% FBS) with or without 30% platelet releasate. After 24 hours (proliferation phase) cells were harvested in TRIzol (AMRESCO RiboZol™ RNA Extraction Reagent) for RNA isolation and qPCR. Details of primers are given in **Suppl. Table 1**. Relative expression was calculated using the ΔΔCt method with normalisation to Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), hypoxanthine-guanine phosphoribosyl-transferase (*Hprt*) and 14-3-3 protein zeta/delta (*YWHAZ)*. mRNA levels of *Vegfa165, Vegfr1, Igf1*, *Cyclind1*, *ACTA2, CDH11, KRT1 and IVL* were measured.

Contraction Assay. A contraction assay was performed as in (Hardman, Emmerson, Campbell, & Ashcroft, 2008) to assess the contractile ability of HDFs *in vitro*. A 10x DMEM and $NaHCO_3$ (sodium bicarbonate) solution was prepared (Thermo Fisher Scientific, UK) and mixed with rat tail collagen type 1 (Corning, Flintshire, UK) in a 1:4 ratio. HDFs were seeded at a density of $1.5x10^5$ cells/ml collagen solution in 24-well plates. HDF media with 2% FBS was then added to each well, with TGFβ1 (10ng/ml) and 10% supra-physiological releasate where appropriate. HDFs were differentiated for 48 hours, and then the gels were detached from tissue culture plates for a further 24 hours. After this time, gel areas and weights were recorded.

Statistical analysis. Data normal distribution was checked by the D'Agostino-Pearson omnibus test. Data are reported as mean±SD. Statistical differences among experimental groups were determined by one-way ANOVA followed by the Tukey post-hoc test. Differences between two groups were detected by using Student's *t*-test. Statistical differences were considered as significant for $p < 0.05$. Statistical analysis was performed using SPSS software (IBM SPSS Statistics version 24).

Results

Growth factor composition of physiological and supra-physiological releasate. We have previously shown a positive dose response for C2C12 skeletal myoblast proliferation when treated with platelet releasate made with increasing concentrations of platelets (Scully, Sfyri, et al., 2018). Therefore, we aimed to profile human physiological and supra-physiological releasate for their growth factor and cytokine constituents, with the hypothesis that more concentrated platelet releasate would yield better proliferation in various cell types by increasing the levels of all components. In this study, we use PAR-1- and thrombin-activated platelet releasate from physiological and supra-physiological platelet concentrations (**Figure 1A**). Using multiplex technology, the concentration of 37 molecules, including growth factors, cytokines, chemokines and immune stress markers were assessed in both concentrations of releasate (**Figure 1A-B, Suppl. Figure 1**). Differences in concentration of growth factors, high concentration analytes and cytokines between physiological and supra-physiological releasate are illustrated in **Figure 1C-E**. Of note, PDGFβ and VEGFα were increased in supra-physiological releasate 1.56- and 4.42-fold respectively. VCAM-1 was upregulated in supra-physiological releasate 3.8-fold over physiological releasate. Similarly, growth factors such as EGF and FGF-2 were upregulated 1.92- and 3.43-fold respectively. However not all factors were found at higher levels in the supra-physiological releasate compared to the physiological releasate; TGFβ, a growth factor that can induce fibrosis in muscle regeneration, and inhibits keratinocyte proliferation was reduced by 0.5-fold in the supra-physiological releasate. These results imply that the formation of platelet releasate is a regulated process and not one that relies on mass action. The releasates produced under physiological and supra-physiological conditions differ both quantitatively and qualitatively.

Physiological releasate is beneficial for myoblast, fibroblast and chondrocyte proliferation but not cardiomyocyte and keratinocyte proliferation. Physiological concentrations of platelets used to make platelet releasate are a powerful inducer of skeletal

muscle stem cell proliferation (Scully, Sfyri, et al., 2018). Therefore, we aimed to determine the effect of physiological releasate (i.e. $2.5x10^8$ platelets/mL) on cardiomyocyte, fibroblast, keratinocyte and chondrocyte proliferation. To this end, H9C2 cardiomyocytes and C2C12 murine skeletal myoblasts were cultured in either serum-free, growth medium (GM; 10% FBS) or 10% platelet releasate (**Figure 2A**). H9C2s and C2C12s were stained for the cell-cycle marker Cyclin D1 (immunocytochemistry), Ki67 (immunocytochemistry) and EdU (live staining). In the present study, platelet releasate promoted the proliferation of C2C12 cells in a reproducible manner as reported previously (Scully, Sfyri, et al., 2018). In contrast, physiological levels of releasate failed to impact proliferation of H9C2 cells for any parameter examined. (**Figure 2B-C**). Human dermal fibroblasts (HDF) cells showed an increase in proliferation as compared to both growth medium and serum-free conditions; however keratinocyte (HaCaT) cells did not proliferate with platelet releasate as compared to serum-free conditions (**Figure 2D)**. This is in line with previous studies on both of these cell types in response to platelet-based applications (see **Suppl. Table 2**). Furthermore, we have shown that physiological releasate was sufficient in stimulating chondrocyte proliferation as compared to growth medium (**Figure 2E**). In addition, we were able to show that the expression of ADAMTS5 an inflammation associated marker induced by IL-1b was reduced in the presence of platelet releasate (**Suppl. Figure 2**).

Supra-physiological releasate induces a stronger proliferative response in skeletal myoblasts and fibroblasts. Given the quantitative and qualitative differences of physiological and supra-physiological releasate reported in this study, we next hypothesised that supra-physiological releasate would exhibit a stronger proliferative effect on cell types that responded positively with physiological releasate. For this reason, we next aimed to analyse a supra-physiological platelet concentration ($10x10^8$ platelets/mL) on C2C12 myoblasts and HDF cells (**Figure 3A**). We have shown in a previous study that a higher concentration of platelets used in making releasate correlates strongly with higher proliferation of C2C12 myoblasts in serum-free conditions (Scully, Sfyri, et al., 2018). We

here report that 10% supra-physiological releasate significantly increased C2C12 myoblast and HDF cell proliferation under both serum-free and growth medium conditions, based on a 3-hour live staining with EdU (**Figure 3B-C**). Of note, the pronounced effect of supra-physiological releasate on C2C12 cell proliferation was evident independent of the presence of growth medium (**Figure 3B**). This finding indicates that supra-physiological releasate can substitute the serum used in growth medium. Compared to standard culture conditions with growth medium (GM), supra-physiological releasate induced a 1.54- and 2.89-fold increase in proliferation for C2C12 and HDF cells respectively. Most importantly, supra-physiological releasate induced a stronger proliferative response as compared to physiological releasate by 1.39- and 1.32-fold in skeletal myoblasts (C2C12) and fibroblasts (HDF) respectively (**Figure 3D**).

Supra-physiological releasate induces a proliferative response in cardiomyocytes. We next aimed to analyse the effect of supra-physiological releasate on cells that did not show increased proliferation with physiological releasate. For this reason, 10, 20 and 30% (v/v) supra-physiological platelet (i.e. $10x10^8$ platelets/mL) releasate was applied in serum-free conditions on the H9C2 cells (**Figure 4A**). We found that 10-30% (v/v) supra-physiological releasate induced significantly higher proliferation compared to serum-free (SF), showing a (v/v) dose response. Further to this, both 10% supra-physiological releasate plus growth media (GM+SR) and releasate at 30% v/v (30% SR) were the highest proliferative groups for the cardiomyocytes based on a 3-hour live staining with EdU. Supra-physiological releasate on growth medium (GM+SR) had a 1.61-fold increase in H9C2 cell proliferation compared to the GM condition. Of note, 30% supra-physiological releasate in serum-free conditions induced significantly higher H9C2 cell proliferation compared to standard culture conditions with growth medium (**Figure 4B**). In contrast, supra-physiological releasate did not stimulate HaCaT proliferation in serum-rich conditions, however there was a significant increase (i.e. 1.95-fold) in serum-free conditions as compared to serum-free alone (**Figure 4C**). Most

importantly, supra-physiological releasate induced a strong proliferative response by 3.1-fold in H9C2 cardiomyocytes as compared to physiological releasate (**Figure 4D**).

We have previously shown that skeletal myoblast cell progression is heavily driven by platelet releasate through Scrib expression (Scully, Sfyri, et al., 2018). Of note, Scrib has been shown to be a crucial factor involved in cardiomyocyte development and progression (Boczonadi et al., 2014). We here report that H9C2 cells cultured with platelet releasate differ in terms of Scrib expression levels when compared to C2C12 cells; such that was a significant increase in Scrib expression in SR versus GM for the C2C12 cells while Scrib expression was decreased with the H9C2 cells in SR versus GM (**Suppl. Figure 3**). HaCaT cells in 20% physiological releasate and growth medium alone exhibited equal differentiation morphology in high- and low-density regions and equal proliferation only in low-density regions (**Suppl. Figure 4**).

Supra-physiological releasate drives cardiomyocyte proliferation at the gene and protein levels. We have previously reported a critical role for the vascular endothelial growth factor (VEGF) in the platelet releasate for skeletal muscle stem cell proliferation and differentiation (Scully, Sfyri, et al., 2018). VEGF has also been shown to be beneficial in H9C2 cells and may potentially drive proliferation (H. Li et al., 2016). Having shown that 30% platelet releasate causes significantly increased proliferation of H9C2 cells, we here show that 30% releasate drives *Vegfa165, Vegfr1 and Cyclind1* expression of H9C2 cells, independent of FBS. *Igf1* expression was markedly reduced in all proliferating conditions (**Figure 5A**). Interestingly, VEGF protein synthesis was increased by supra-physiological releasate in serum-free conditions over growth media for both C2C12s and H9C2 cells (**Figure 5B**).

Supra-physiological releasate increases myoblast and cardiomyocyte differentiation. We next wanted to establish the direct effect of supra-physiological releasate on C2C12 myoblast and H9C2 cardiomyocyte differentiation. Cells were grown in serum-free or growth medium conditions with or without 10% supra-physiological releasate before switching to

differentiation medium for 4 days. Unlike the C2C12 cells, platelet releasate in serum-free conditions did not seem to play a significant role in increasing total H9C2 myotube number. However, the trends between cardiomyocytes and skeletal muscle myoblasts remain similar both in terms of increased total cell number, increased Myogenin^{+ve} cell number and no significant increase in both of their myofusion indexes between GM and GM+SR (**Figure 6A-B**). We next sought to determine the effects of platelet releasate in a later stage of differentiation. The same experimental setup was conducted; however the cells were cultured in differentiation medium for 7 days. Interestingly, with longer incubation in differentiation media, there is a significant increase between GM and GM+SR in cell number, myogenin^{+ve} cells, myofusion index and myotube number ($p<0.05$) when supplementing H9C2s with releasate in the proliferative phase with the standard growth medium culture (**Figure 6C**).

Supra-physiological releasate stimulates fibroblast and keratinocyte differentiation. We next wanted to analyse the effect of supra-physiological releasate on both fibroblast and keratinocyte differentiation. For fibroblast differentiation, qRT-PCR showed that alpha-actin-2 (*ACTA2*) was not significantly affected with SR treatment, however, *CDH11;* a fibroblast differentiation marker was upregulated with SR treatment. Of note, negative controls were used (media only) to show that TGF-β1 causes differentiation/contraction. SR treatment caused a significantly increased percentage area contraction and significantly reduced gel weights than the TGF-β1 only group (**Figure 7A**). HaCaT cells differentiated more readily in the presence of SR as shown morphometrically both *via* crystal violet staining and cellular density, with withdrawal from the cell cycle (**Figure 7B, Suppl. Figure 4**). Additionally, the schematic of the stratum layers of keratinocyte differentiation outlines cellular markers along this gradient to provide clarity of where keratin 1 (*KRT1)* and Involucrin (*IVL*) are primarily expressed (**Figure 7C**) qRT-PCR data of normal human epidermal keratinocytes (NHEKs) shows reduced expression of the early differentiation marker, *KRT1* and a dramatic

upregulation of *IVL* (**Figure 7D**). Taken together, this data shows that HDF, HaCaT and NHEK cells differentiate in response to supra-physiological releasate.

Discussion

To date, platelet-based applications have gained a lot of attention for regenerative purposes in a variety of applications such as musculoskeletal injuries, skin, bone, nerve, liver conditions (Scully & Matsakas, 2019; Scully, Naseem, et al., 2018). However, with regards to the use of autologous platelets for the regeneration of skeletal and heart muscle; the literature has been more stringent in accepting its potential benefits. This may be due to clinical trials reporting no advantageous outcomes over conventional treatments (Mosca & Rodeo, 2015). Previously, we have shown robust myoblast proliferation was stimulated by optimising the platelet-preparation method, using platelet releasate devoid of plasma and cellular debris made with the TRAP-6 platelet agonist (Scully, Sfyri, et al., 2018). For this reason, we aimed to apply this method of preparing platelet releasate to additional cell types to test if it was commonly beneficial for skeletal myoblasts, cardiomyocytes, human dermal fibroblasts, primary chondrocytes and normal human epidermal keratinocytes. To achieve this, we prepared releasates from Thrombin- (PAR1 and PAR4 receptors) and TRAP6- (PAR1 receptor) activated platelets from physiological and supra-physiological platelet concentrations.

Supra-physiological releasate had a higher concentration of growth factors, analytes in higher abundance and cytokines compared to physiological releasate. PECAM-1 and P-selectin were the most abundant components of the releasate which have been shown to exhibit anti-apoptotic, pro-angiogenic and regenerative functions in different cell types (see **Suppl. Table 3**). Our data on the composition of platelet releasate revealed that known key growth factors driving myoblast and/or chondrocyte proliferation such as PDGFβ and VEGFα were increased several-fold in supra-physiological releasate (Kieswetter, Schwartz, Alderete, Dean, & Boyan, 1997; Scully, Sfyri, et al., 2018). VCAM-1 was previously shown to increase cardiomyocyte proliferation and skeletal myoblast differentiation and was 3.8-fold more abundant in supra-physiological releasate compared to physiological releasate (Choo, Canner, Vest, Thompson, & Pavlath, 2017; Iwamiya, Matsuura, Masuda, Shimizu, & Okano,

2016). Similarly, growth factors driving fibroblast proliferation such as EGF and FGF-2 were increased 1.92- and 3.43-fold respectively (Yu, Matsuda, Takeda, Uchinuma, & Kuroyanagi, 2012). Importantly, TGFβ, a growth factor that can induce fibrosis in muscle regeneration, and inhibits keratinocyte proliferation was reduced by 50% in supra-physiological releasate (H. Li et al., 2016).

We determined the effect of physiological releasate on the proliferation of various cell types. To this end, we used C2C12 cells, with a relatively fast proliferative rate, as a positive control as previously described, where proliferation was elevated with the application of releasate (Scully, Sfyri, et al., 2018). However, in a slower proliferating cell line (H9C2 cardiomyocytes) Cyclin D1, Ki-67 and live EdU staining for proliferation were all markedly lower than the growth media group, showing similar levels to serum-free conditions. Indeed, this reinforces the notion that cardiomyocytes are a slow-proliferating cell line and difficult to stimulate into proliferation (**Suppl. Figure 5, Suppl. Figure 6**). Cardiac conditions such as exercise-induced silent myocardial ischemia has proven difficult to both diagnose and treat (Stern, 2005). However major studies have been conducted to combat cardiac conditions, such as the application of platelet-based applications delivering deliver growth factors to damaged heart tissue (Gallo et al., 2013; Hargrave & Li, 2012; Hargrave et al., 2016; X. H. Li et al., 2008; Morschbacher et al., 2016; Patel et al., 2016; Spartalis et al., 2015; Tang et al., 2017). Promising *in vitro* and *in vivo* data was generated by Hargrave et al., regarding PRP on the ischemic heart; however they held concerns about clotting factors in the platelet preparation (Hargrave & Li, 2012; Hargrave et al., 2016). Therefore, we speculated that using platelet releasate, without the clotting factors associated with plasma, was an ideal candidate to target the slowly proliferating cardiomyocytes in circumstances such as myocardial ischemia. Previous experimental evidence for the effect of these cytokines and growth factors analysed in human platelet releasate has been studied on skeletal and cardiac muscle as outlined in **Suppl. Table 3**. Interestingly, here we have shown that human dermal fibroblasts, a relatively fast proliferating cell-type, showed similar proliferative effects

following platelet releasate to the C2C12 skeletal myoblasts. In contrast, HaCaT keratinocytes demonstrated no additional proliferation, but increased differentiation in response to platelet releasate treatment. Concurrently, previous authors have demonstrated decreased proliferation in HaCaTs subjected to platelet-based applications (Bayer et al., 2018). Published experimental evidence of platelet-based applications on keratinocytes, fibroblasts and chondrocytes are summarised in **Suppl. Table 2**.

As physiological releasate failed to stimulate H9C2 or HaCaT proliferation, we opted to use supra-physiological releasate to stimulate proliferation. This decision was based on pervious work with skeletal myoblasts; where increased platelet concentration in the releasate preparation correlated strongly with enhanced proliferation (Scully, Sfyri, et al., 2018). Here we show for the first time, evidence that supra-physiological releasate indeed enhanced H9C2 proliferation to the same levels as growth medium. An increase in proliferative capacity was also observed in HDFs and C2C12s treated with supra-physiological releasate as compared to physiological releasate. In addition, supra-physiological releasate has the advantage over physiological releasate such that 40% PR caused a fibrin clot in culture (**Suppl. Figure 7**). For this reason, we conducted qRT-PCR to show the transcription levels of *Vegf* and translation into protein through immunohistochemistry in cardiomyocytes after application of platelet releasate. Supra-physiological releasate increased *Vegfα165*, *Vegfr* and *Cyclind1* mRNA expression in both serum-free and serum-rich expression indicating an increase in H9C2s cellular proliferation. Additionally, there was increased VEGF protein expression in both C2C12 and H9C2 cells. Notably, *Igf1* mRNA was reduced in all groups versus serum-free, indicating a cell cycle progression and a subsequent differentiation suppression at the proliferative stage of growth (Smith, Klaasmeyer, Woods, & Jones, 1999). Therefore, we surmise that cardiomyocyte proliferation may be driven, at least in part, through the VEGF pathway.

We have previously shown that platelet releasate does not increase the differentiation of C2C12 myoblasts at human-physiological levels (Scully, Sfyri, et al., 2018). However, in the

current study, we have shown a beneficial effect of supra-physiological releasate on C2C12 differentiation (total cell number, Myogenin^{+ve} cells and the total myotube number, **Suppl. Figure 8**). To our knowledge, this is the first evidence of H9C2 cells treated with supra-physiological releasate resulting in increased total cell numbers and myogenin^{+ve} cells (**Suppl. Figure 9**). Additionally, when H9C2 cells are treated for a longer period of time in differentiation media; the additive effect of platelet releasate with 10% FBS was more pronounced resulting in greater Myogenin expression, higher cell numbers, a greater myofusion index and myotube number. One can speculate that this additive effect is due to a higher total cell number in the proliferation period, leading to more cells to undergo differentiation as a whole (Tanaka et al., 2011).

It is known that skeletal muscle satellite cells upregulate the Notch signalling pathway during quiescence, with a critical role in proliferation, where they switch to the Wnt signalling pathway upon differentiation (Brack, Conboy, Conboy, Shen, & Rando, 2008). In contrast to this, keratinocytes have been shown to upregulate Notch1 and Notch2 signalling during differentiation, directly upregulating Involucrin; a terminal stage differentiation marker (Nakamura et al., 2014; Rangarajan et al., 2001). Here we speculate that there may be a possible connection between the Notch signalling pathway and platelet releasate's opposing effects on the proliferation and differentiation of both myoblasts and keratinocytes; however this has yet to be established. PRP has been shown previously to downregulate keratin-1 and upregulate Involucrin, indicating faster progression of the keratinocyte differentiation pathway (Denecker, Ovaere, Vandenabeele, & Declercq, 2008; Sandilands, Sutherland, Irvine, & McLean, 2009). To our knowledge, we are the first to replicate these results with platelet releasate on normal human epidermal keratinocytes (NHEKs) and HaCaTs. Typical keratinocyte differentiation involves an increase in cell density (Buerger et al., 2017). Further to this, the observed morphological differences between platelet releasate and growth media on the inhibition of proliferation in high density keratinocyte populations supports the lineage

progression results, exiting the cell cycle faster and upregulating terminal differentiation genes greater than normal culture conditions.

Platelet-rich plasma has previously been shown to stimulate both fibroblast proliferation and human fibroblast-populated collagen gel contraction (Caceres, Martinez, Martinez, & Smith, 2012). Moreover, platelet releasate has been shown to increase proliferation and differentiation, where TGF-β1 has been speculated to be the key factor in platelet releasate affecting fibroblastic differentiation (Rothan et al., 2014). To our knowledge, this is the first study to show more pronounced effects of supra-physiological releasate on HDF's proliferation and differentiation.

We show that physiological levels of platelet releasate had two possible significant clinically valuable outcomes on chondrocytes; it promoted their proliferation and decreased the levels of markers associated with inflammation. Both of these factors could be beneficial in the treatment of osteoarthritis, a common disease found across the globe that impacts negatively on patients quality of life. Osteoarthritis can be caused by genetic factors, age as well as life-style factors such as performing sports as well as diet. Our work shows the potential of platelet releasate to promote cartilage regeneration (chondrocyte proliferation) and decrease the inflammatory response (ADAMTS5 levels). Our future work will investigate this exciting potential directly by injecting platelet releasate into the knee joint of surgically induced rodent model of osteoarthritis. Further work will be conducted on the proteomics of the platelet releasate content of physiological versus supra-physiological platelet releasate and analysing the effects of both concentrations *in vivo*.

It is worth contemplating the mechanism of action that may underpin the ability of supra-physiological releasate, but not the physiological releasate, to support a biological process e.g. the proliferation of H9C2 cardiomyocytes. At the simplest level, it could be that proliferation inducing molecules are below a threshold level in the physiological releasate but the level is breached in the supra-physiological releasate. This certainly could be the case

for many of the common constituents found in the two different preparations. However, it is worth emphasising that a number of components were present at lower levels in the supra-physiological releasate than in the physiological releasate. Therefore, it is worthy to contemplate that proliferation induced by supra-physiological releasate may not be due wholly to the increased presence of molecules but also due to the decreased levels of inhibitory species.

Conclusion

Aiding the regenerative potential of different tissues is particularly challenging, due in part to the unique proliferative capacity of their cells. As platelet-based applications can be customised for tissue-specific efficacy, this makes them strong candidates for developing innovative regenerative therapies. This study provides novel insights into the role of platelet releasate on C2C12s, H9C2s, HaCaTs, NHEKs, HDFs and chondrocytes and suggests an optimised preparation method to maximise the proliferative/differentiative response for potential regeneration. This study highlights the benefit of concentrating the composition of the platelet secretome for optimal cell-type targeted applications in regenerative medicine. Here, we show for the first time that supra-physiological releasate either significantly improves proliferation and/ or differentiation of various cell types. In summary, our data show that standardising the concentration of platelets for therapeutic use may be a key factor determining varied results surrounding clinical success.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

D.S. and A.M. performed conceptualization. D.S., P.S., S.V., H.N.W., A.A-H. and A.P. carried out methodology. D.S., M.C.M-T., K.P. and A.M. carried out formal analysis. D.S., L.G., M.J.H. and A.M. performed investigation. D.S., K.P., M.J.H., L.G. and A.M. carried out writing. A.M. carried out supervision.

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Figure legends

Figure 1. Composition of physiological and supra-physiological platelet releasate. Human platelet suspensions were aggregated using TRAP6 (a PAR1 agonist; P1/2) or Thrombin (T1/2). Concentrations of specific analytes contained in (ProcartaPlex Human kits) were measured in platelet releasate. (**A**) A schematic depicting the methodology behind the experimental setup. (**B**) Hierarchical clustering representing the average expression intensity of each analyte (PR; physiological platelet releasate ($2.5x10^8$ platelets/mL), SR; supra-physiological releasate ($10x10^8$ platelets/mL)). (**C**) Concentration of growth factors, (**D**) cytokines and (**E**) analytes in high abundance detected in physiological (PR) and supra-physiological (SR) platelet releasates averaged from both PAR1 and Thrombin-stimulated platelets.

Figure 2. Physiological releasate does not induce cardiomyocyte or keratinocyte proliferation. (**A**) A schematic depicting the methodology behind the experimental setup; application of human-physiological releasate to various cell types for 24 hours. (**B**) Representative images and quantitative data for H9C2 cardiomyocyte and C2C12 skeletal myoblast cellular proliferation, conducted in serum-free (SF), growth medium (GM; 10% FBS) or 10% physiological releasate (R) staining for Cyclin D1 (Orange) and DAPI. (**C**) Representative images and quantitative data for H9C2 cardiomyocyte Ki-67 expression and EdU proliferative live-staining (Green) co-stained with DAPI (Blue). Both cell lines were treated with SF, GM, and R. (**D**) Representative images and quantitative data for Human dermal fibroblasts (HDF) and keratinocytes (HaCaT) live staining with EdU for 3 hours after a 21-hour culture. (x5 magnification, scale bar 200 μm). (**E**) Chondrocytes were stained with Collagen II, ADAMTS5 and DAPI and treated with GM, IL-1β+GM and IL-1β+GM+R (scale bar 60 μm). All releasate was made with $2.5x10^8$ platelets/mL for Figure 2. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test or Student's *t*-test as appropriate. Differences are *p<0.05, **p<0.01, and ***p<0.001, #P<0.05 vs. every other group.

Figure 3. Supra-physiological releasate induces a stronger proliferative response in skeletal myoblasts and fibroblasts. (**A**) EdU proliferative live-staining was conducted on C2C12 skeletal myoblasts and human dermal fibroblasts (HDF) in serum-free (SF), growth medium (GM; 10% FBS) and/or 10% supra-physiological releasate. (**B**) C2C12 skeletal myoblasts cells for 3-hours after 21 hour incubation for serum-free, growth medium and 10% releasate (SR; $10x10^8$ platelets/ml) conditions. (**C**) Representative images and quantitative data for HDF live staining with EdU (x5 magnification, scale bar 200 μm). (**D**) C2C12 and HDF cell differences between physiological releasate ($2.5x10^8$ platelets/mL) and SR as measured by 3-hour proliferation (EdU)/DAPI as a percentage. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test or Student's *t*-test as appropriate. Differences are *p<0.05, **p<0.01, and ***p<0.001, #P<0.05 vs. every other group.

Figure 4. Supra-physiological releasate induces a strong proliferative response in cardiomyocytes (**A**) EdU proliferative live-staining was conducted on H9C2 cardiomyocytes and HaCaT keratinocytes in serum-free (SF), growth medium (GM; 10% FBS) and/or 10% (HaCaTs)-30% (H9C2s) releasate (SR; $10x10^8$ platelets/ml) conditions. (**B**) Representative images and quantitative data for H9C2 cells (**C**) Representative images and quantitative data for HaCaT cells. (x5 magnification, scale bar 200 μm).). (**D**) H9C2 and HaCaT cell differences between physiological releasate ($2.5x10^8$ platelets/mL) and SR as measured by 3-hour proliferation (EdU)/DAPI as a percentage. Statistical analysis was performed by one-way ANOVA followed by Tukey's post-hoc test or Student's *t*-test as appropriate. Differences are *p<0.05, **p<0.01, and ***p<0.001, #P<0.05 vs. every other group.

Figure 5. Supra-physiological releasate drives cardiomyocyte gene expression and protein synthesis for proliferation markers. (**A**) Gene expression for *Vegfa165, Vegfr1, Cyclind1* and *Igf1* were measured for H9C2 cardiomyocytes in serum-free and growth media (GM: 10% FBS) conditions with or without 30% platelet releasate. (**B**) C2C12 and H9C2

immunohistochemical staining for VEGF expression during proliferation in GM or 10% platelet releasate (SR; $10x10^8$ platelets/mL) in serum-free conditions (x10 magnification, scale bar 200 μm). (**C**) Cell count and VEGF expression/DAPI for H9C2 cells treated with or without GM or 10% SR with or without a VEGFR inhibitor (V⊣). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test or Student's *t*-test as appropriate. Differences are *p<0.05, **p<0.01 and ***p<0.001.

Figure 6. Supra-physiological releasate increases myoblast and cardiomyocyte fusion. (**A-B**) C2C12 myoblasts and H9C2 cardiomyocytes were proliferated in serum-free, growth medium conditions with or without 10% platelet releasate (SR; supra-physiological concentrations $10x10^8$ platelets/mL). Differentiation was measured after 4 days in differentiation medium (2% horse serum). Representative images for Myogenin and DAPI (x10 magnification, scale bar 200μm). (**C**) Differentiation was measured after 7 days in differentiation medium (2% horse serum). Representative images for Myogenin and DAPI (x10 magnification, scale bar 200μm). Outcome measures include the nuclei number, number of Myogenin^{+ve} nuclei, myotubes (n=2 nuclei/ myotube). The myotube fusion index was calculated by Myogenin^{+ve} nuclei in myotubes/ DAPI as a percentage. Data are mean±SD (n=3/group, 3 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Differences are *p<0.05, **p<0.01, ***p<0.001 and #P<0.05 vs. every other group.

Figure 7. Supra-physiological releasate stimulates fibroblast and keratinocyte differentiation. (**A**) Primary Human dermal fibroblasts (HDF) were differentiated over a period of 4 days in 2% FBS with 10% platelet releasate made using supra-physiological levels of platelets (SR; $10x10^8$/mL) with RNA collected for qPCR analysis of *ACTA2* and *CDH11* differentiation markers. Contraction assays were performed for HDFs in the presence or absence of TGF-β1 to induce differentiation with or without supra-physiological levels of platelet releasate (SR) (Scale bar 2mm). (**B**) HaCaT keratinocytes were

differentiated using 2.5mM Calcium (Ca) in the presence or absence SR for Crystal Violet staining. Representative images x10 magnification, scale bar 200μm. (**C**) Schematic of the general structure of the epidermis and epidermal differentiation of keratinocyte layers; adapted from both (Denecker et al., 2008; Sandilands et al., 2009). (**D**) Normal human epidermal keratinocyte (NHEK) differentiation markers were assessed in the presence or absence of SR, with and without calcium, for *KRT1* (early differentiation) and *IVL* (late differentiation). Data are mean±SD (n=3/group, 3 independent experiments). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test or Student's *t*-test as appropriate. Differences are *p<0.05, **p<0.01, ***p<0.001.

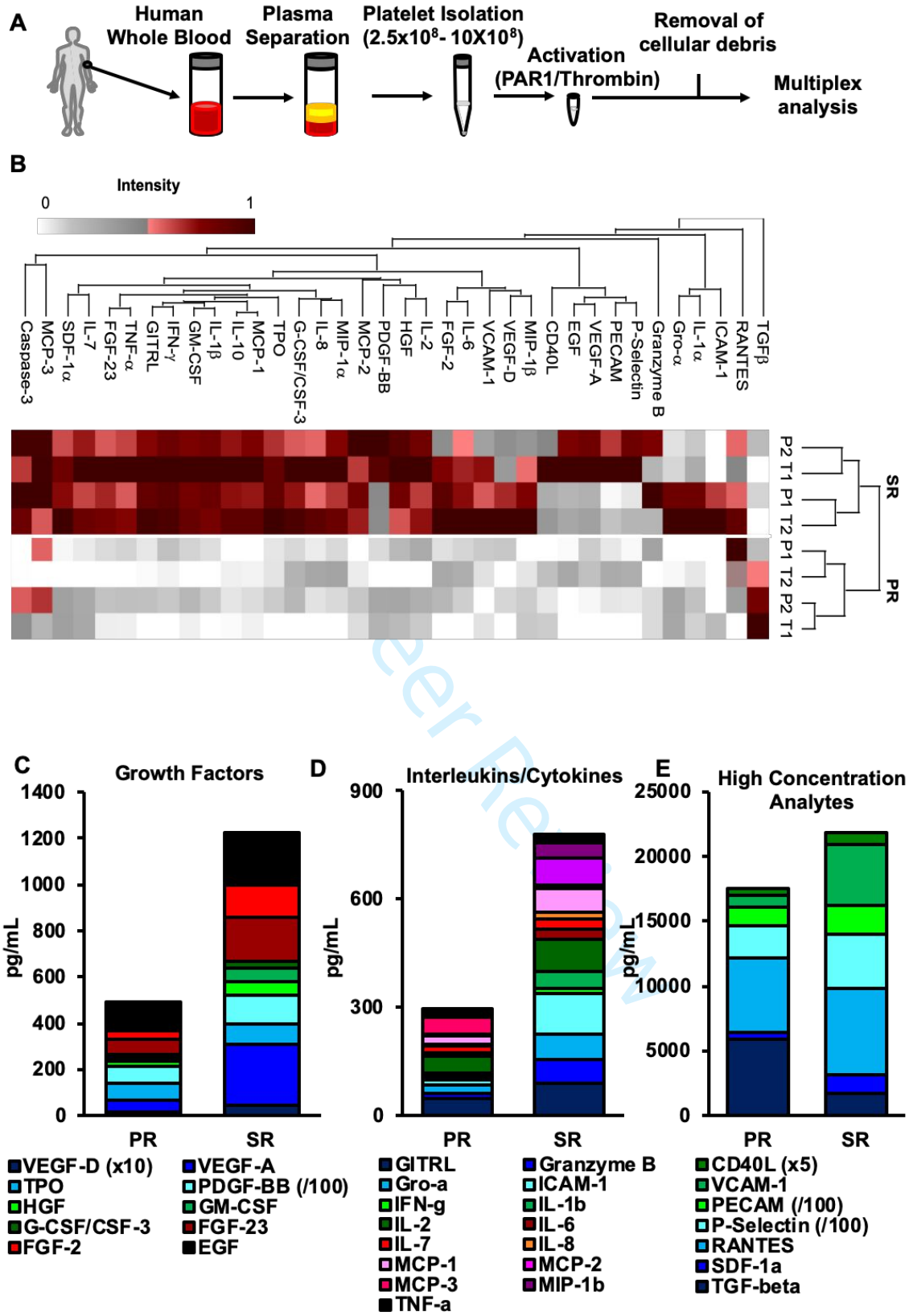
A
Human Whole Blood
Plasma Separation
Platelet Isolation (2.5x10⁸- 10X10⁸)
Activation (PAR1/Thrombin)
Removal of cellular debris
Multiplex analysis
B
Intensity
0
1
Caspase-3
MCP-3
SDF-1α
IL-7
FGF-23
TNF-α
GITRL
IFN-γ
GM-CSF
IL-1β
IL-10
MCP-1
TPO
G-CSF/CSF-3
IL-8
MIP-1α
MCP-2
PDGF-BB
HGF
IL-2
FGF-2
IL-6
VCAM-1
VEGF-D
MIP-1β
CD40L
EGF
VEGF-A
PECAM
P-Selectin
Granzyme B
Gro-α
IL-1α
ICAM-1
RANTES
TGFβ
P2 T1 P1 T2 P1 T2 P2 T1
SR
PR
C
Growth Factors
pg/mL
1400
1200
1000
800
600
400
200
0
PR
SR
VEGF-D (x10)
TPO
HGF
G-CSF/CSF-3
FGF-2
VEGF-A
PDGF-BB (/100)
GM-CSF
FGF-23
EGF
D
Interleukins/Cytokines
pg/mL
900
600
300
0
PR
SR
GITRL
Gro-a
IFN-g
IL-2
IL-7
MCP-1
MCP-3
TNF-a
Granzyme B
ICAM-1
IL-1b
IL-6
IL-8
MCP-2
MIP-1b
E
High Concentration Analytes
pg/mL
25000
20000
15000
10000
5000
0
PR
SR
CD40L (x5)
VCAM-1
PECAM (/100)
P-Selectin (/100)
RANTES
SDF-1a
TGF-beta

Figure 1

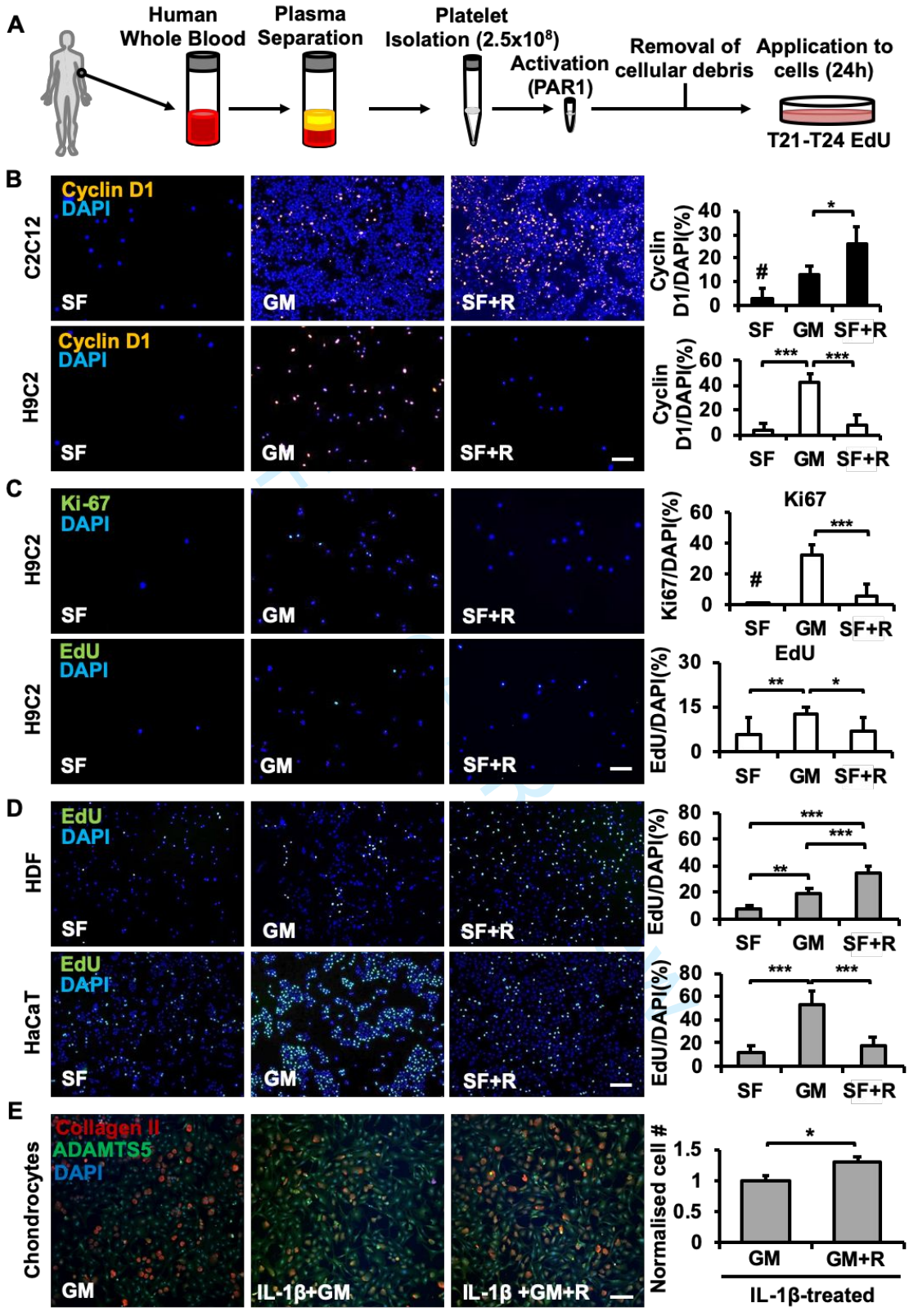
A
Human Whole Blood
Plasma Separation
Platelet Isolation (2.5x10^8)
Activation (PAR1)
Removal of cellular debris
Application to cells (24h)
T21-T24 EdU
B
C2C12
Cyclin D1
DAPI
SF
GM
SF+R
Cyclin D1/DAPI(%)
H9C2
C
Ki-67
Ki67/DAPI(%)
EdU
EdU/DAPI(%)
D
HDF
HaCaT
E
Chondrocytes
Collagen II
ADAMTS5
IL-1β+GM
IL-1β +GM+R
Normalised cell #
GM
GM+R
IL-1β-treated

Figure 2

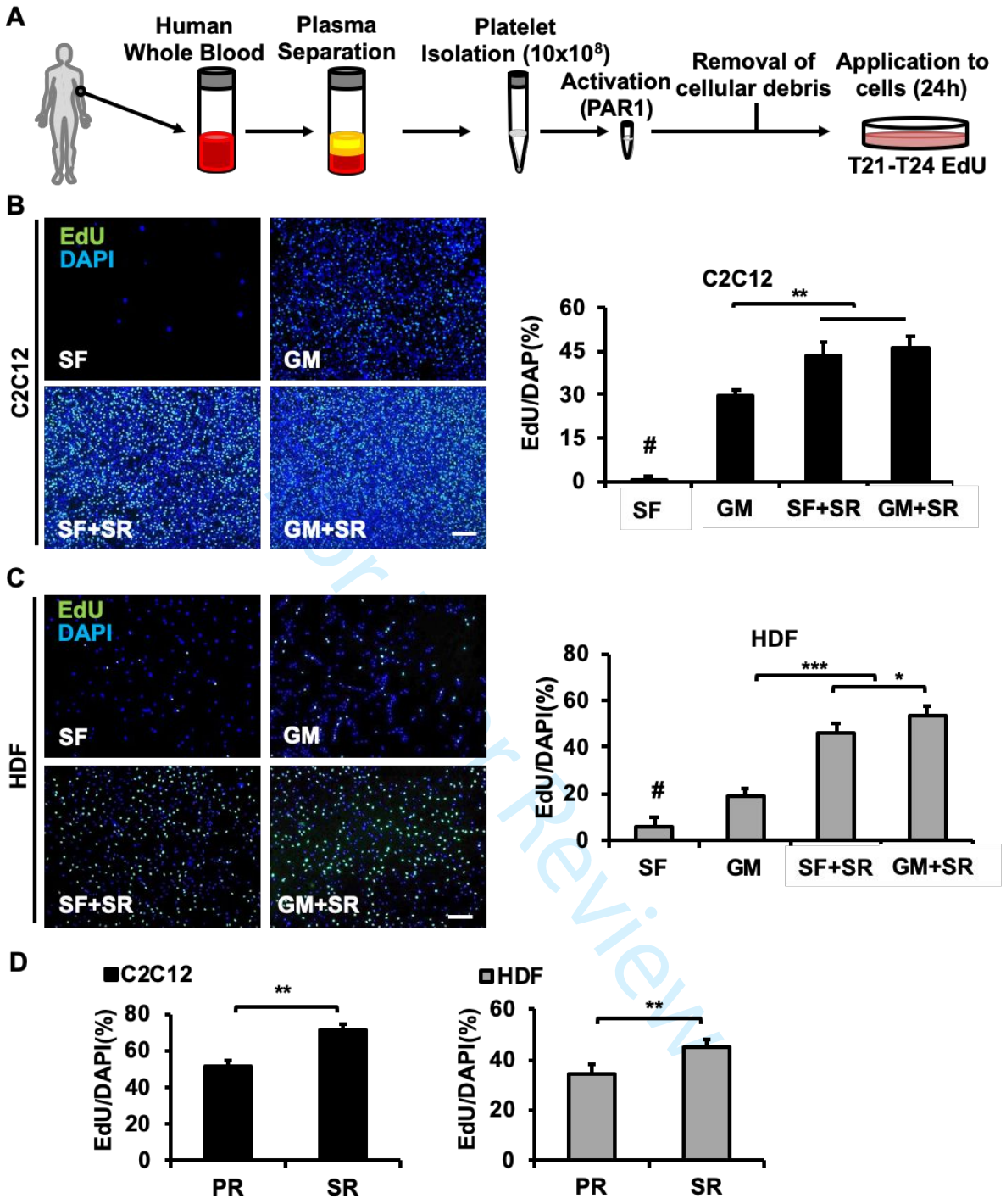
A
Human Whole Blood
Plasma Separation
Platelet Isolation (10x10⁸)
Activation (PAR1)
Removal of cellular debris
Application to cells (24h)
T21-T24 EdU
B
C2C12
EdU
DAPI
SF
GM
SF+SR
GM+SR
C2C12
EdU/DAP(%)
60
45
30
15
0
#
**
SF
GM
SF+SR
GM+SR
C
HDF
EdU
DAPI
SF
GM
SF+SR
GM+SR
HDF
EdU/DAPI(%)
80
60
40
20
0
#
***
*
SF
GM
SF+SR
GM+SR
D
C2C12
EdU/DAPI(%)
80
60
40
20
0
**
PR
SR
HDF
EdU/DAPI(%)
60
40
20
0
**
PR
SR

Figure 3

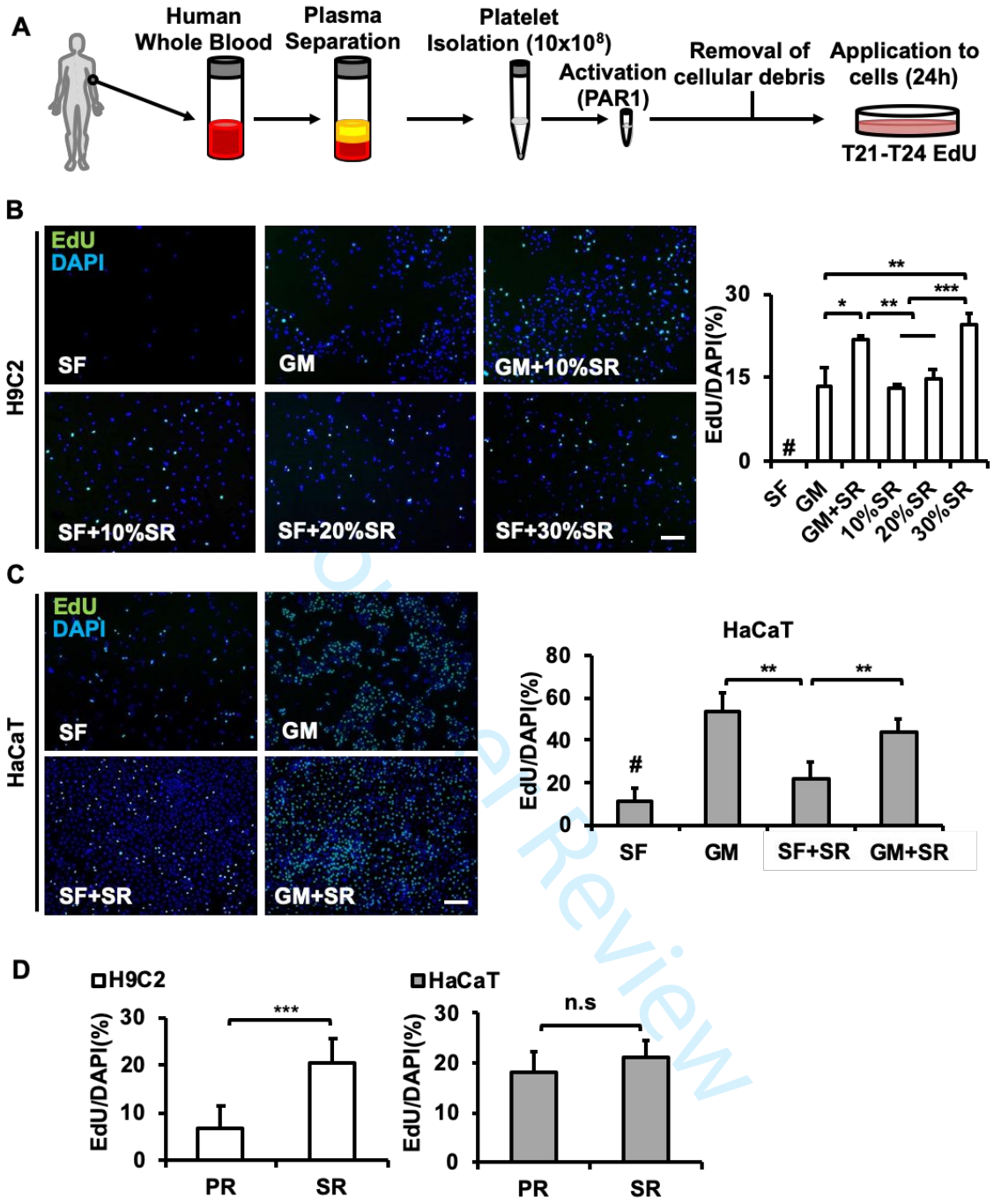
A
Human Whole Blood
Plasma Separation
Platelet Isolation (10x10⁸)
Activation (PAR1)
Removal of cellular debris
Application to cells (24h)
T21-T24 EdU
B
H9C2
EdU
DAPI
SF
GM
GM+10%SR
SF+10%SR
SF+20%SR
SF+30%SR
EdU/DAPI(%)
SF
GM
GM+SR
10%SR
20%SR
30%SR
C
HaCaT
SF
GM
SF+SR
GM+SR
HaCaT
EdU/DAPI(%)
D
H9C2
HaCaT
n.s
PR
SR

Figure 4

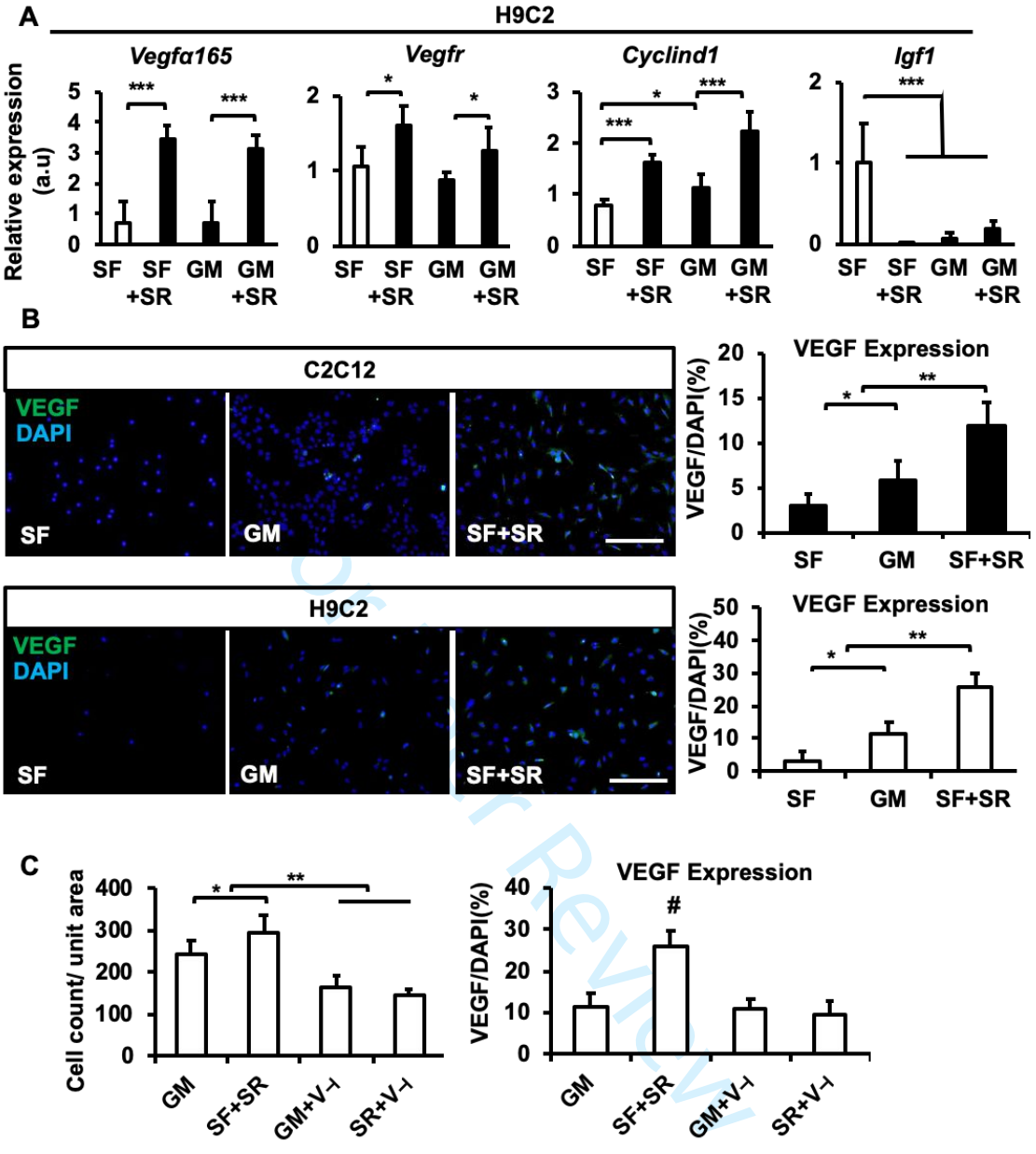
A
H9C2
Vegfa165
Vegfr
Cyclind1
Igf1
Relative expression (a.u)
SF
SF +SR
GM
GM +SR
B
C2C12
VEGF
DAPI
SF
GM
SF+SR
VEGF Expression
VEGF/DAPI(%)
H9C2
C
Cell count/ unit area
GM
SF+SR
GM+V-I
SR+V-I

Figure 5

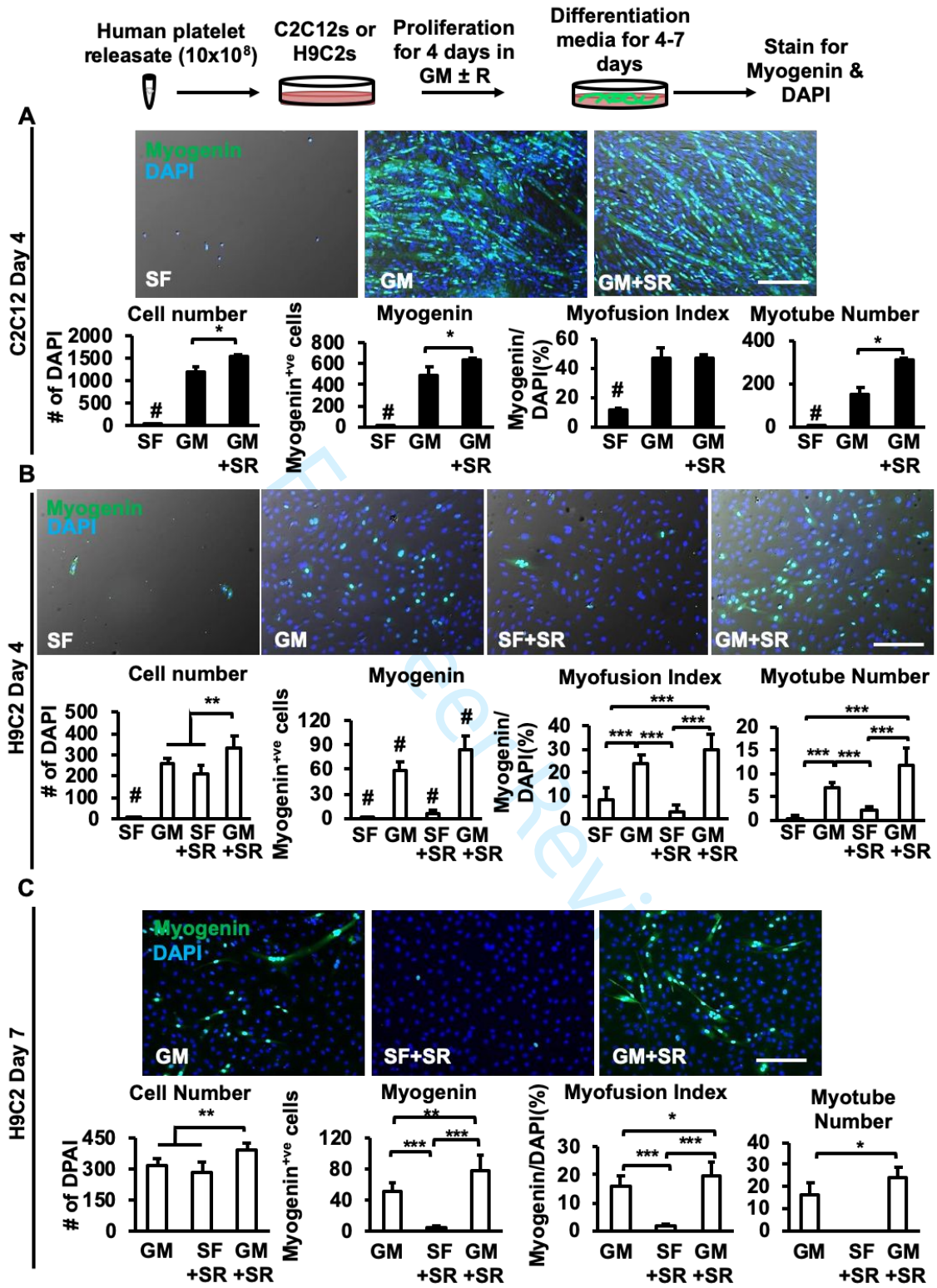
Human platelet releasate (10x10⁸)
C2C12s or H9C2s
Proliferation for 4 days in GM ± R
Differentiation media for 4-7 days
Stain for Myogenin & DAPI
A
C2C12 Day 4
Myogenin
DAPI
SF
GM
GM+SR
Cell number
# of DAPI
Myogenin
Myogenin+ve cells
Myofusion Index
Myogenin/ DAPI(%)
Myotube Number
SF GM GM +SR
B
H9C2 Day 4
SF+SR
Cell number
# of DAPI
Myogenin
Myogenin+ve cells
Myofusion Index
Myogenin/ DAPI(%)
Myotube Number
SF GM SF GM +SR +SR
C
H9C2 Day 7
Cell Number
# of DPAI
Myogenin
Myogenin+ve cells
Myofusion Index
Myogenin/DAPI(%)
Myotube Number
GM SF GM +SR +SR

Figure 6

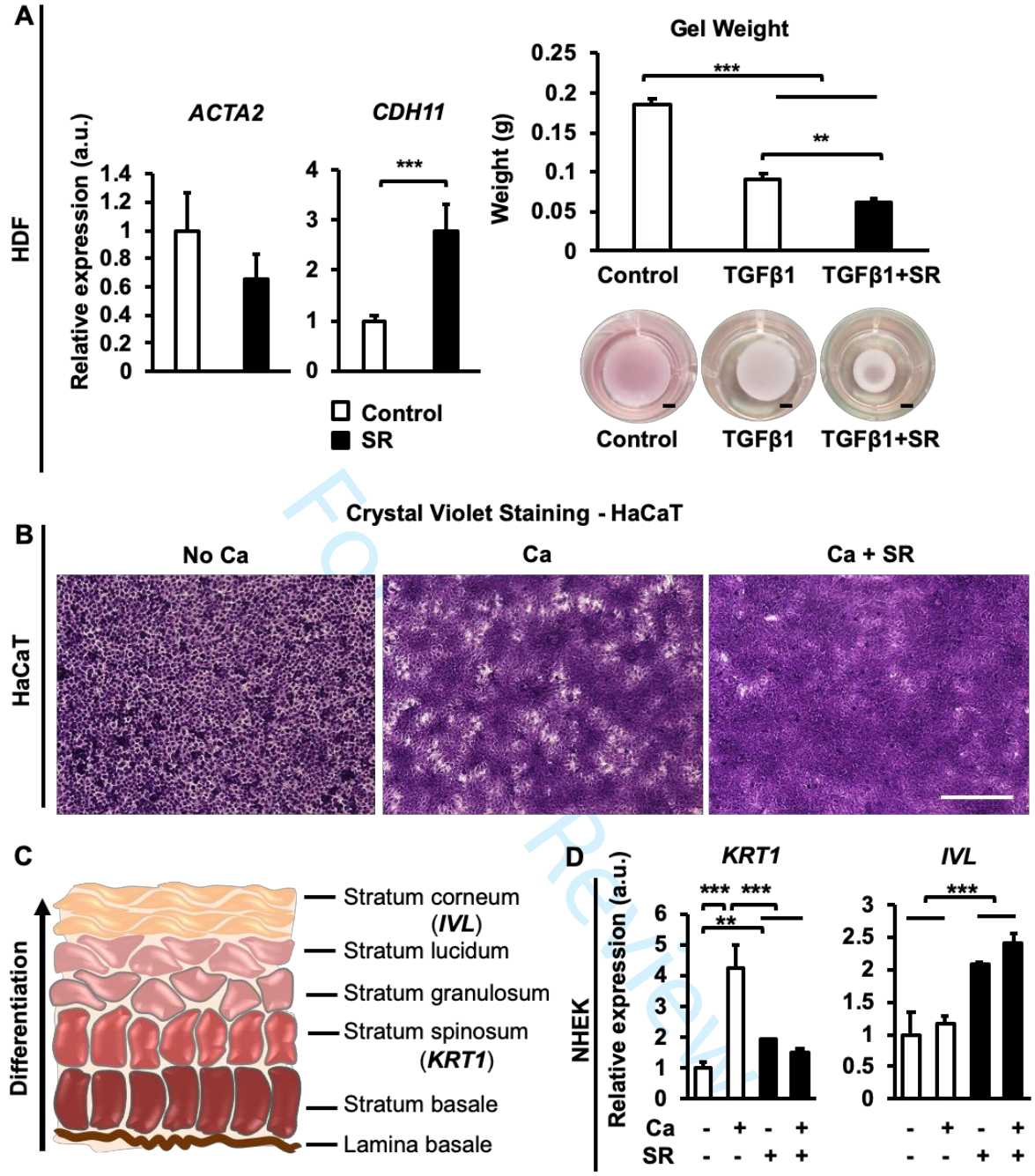
A
HDF
ACTA2
CDH11
Relative expression (a.u.)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
4
3
2
1
0
***
Control
SR
Gel Weight
Weight (g)
0.25
0.2
0.15
0.1
0.05
0
***
**
Control
TGFβ1
TGFβ1+SR
Control
TGFβ1
TGFβ1+SR
B
HaCaT
Crystal Violet Staining - HaCaT
No Ca
Ca
Ca + SR
C
Differentiation
Stratum corneum
(IVL)
Stratum lucidum
Stratum granulosum
Stratum spinosum
(KRT1)
Stratum basale
Lamina basale
D
NHEK
Relative expression (a.u.)
KRT1
IVL
***
***
**
***
6
5
4
3
2
1
0
3
2.5
2
1.5
1
0.5
0
Ca - + - +
SR - - + +
- + - +
- - + +

Figure 7