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Signal Transduction:

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Shockwave treatment enhances cell proliferation and improves wound healing by ATP release coupled extracellular signal-regulated kinase (ERK) activation*

Anna M. Weihs[1,2#], Christiane Fuchs[1,2#§], Andreas H. Teuschl[1,2], Joachim Hartinger[2,3], Paul Slezak[2,3], Rainer Mittermayr[2,3], Heinz Redl[2,3], Wolfgang G. Junger[3,4], Harald H. Sitte[5], and Dominik Rünzler[1,2]

[1]University of Applied Sciences Technikum Wien, Department of Biochemical Engineering, Vienna, Austria
[2]The Austrian Cluster for Tissue Regeneration, Vienna, Austria
[3]Ludwig Boltzmann Institute for Experimental and Clinical Traumatology/AUVA Research Center, Vienna, Austria
[4]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, United States of America
[5]Institute of Pharmacology, Center for Physiology and Pharmacology, Medical University of Vienna, Austria

[#]both authors contributed equally to this work

[*]Running title: Signaling underlying shockwave enhanced cell proliferation

[§]To whom correspondence should be addressed: Christiane Fuchs, University of Applied Sciences Technikum Wien, Department of Biochemical Engineering and The Austrian Cluster for Tissue Regeneration, Vienna, Austria; Tel.: +43 1 333 40 77 -989; Fax: +43 1 333 40 77 -99 989; E-mail: fuchsc@technikum-wien.at

Keywords: shockwave treatment; ATP; extracellular signal-regulated kinase (ERK); cell proliferation; wound healing; signal transduction

Background: Signaling pathways underlying beneficial effects of extracorporeal shockwave treatment (ESWT) remain to be completely elucidated.
Results: ESWT enhances cell proliferation *in vitro* and wound healing *in vivo*.
Conclusion: ESWT-induced ATP release and subsequent extracellular signal-regulated kinase (ERK) activation are prerequisites for enhanced cell proliferation and wound healing.
Significance: Deciphering the involved signaling cascades provides the basis for ESWT as clinical wound healing treatment.

ABSTRACT

Shockwave treatment accelerates impaired wound healing in diverse clinical situations. However, the mechanisms underlying the beneficial effects of shockwaves have not yet been fully revealed. Since cell proliferation is a major requirement in the wound healing cascade, we used *in vitro* studies and an *in vivo* wound healing model to study whether shockwave treatment influences proliferation by altering major extracellular factors and signaling pathways involved in cell proliferation. We identified extracellular ATP, released in an energy- and pulse number-dependent manner, as a trigger of the biological effects of shockwave treatment. Shockwave treatment induced ATP release, increased Erk1/2 and p38 MAPK activation, and enhanced proliferation in three different cell types (C3H10T1/2 murine mesenchymal progenitor cells, primary human adipose derived stem cells, human Jurkat T cell line) *in vitro*. Purinergic signaling-induced Erk1/2 activation was found to be essential for this proliferative effect, which was further confirmed by *in vivo* studies in a rat wound healing model where shockwave treatment induced proliferation and increased wound healing in an Erk1/2 dependent fashion. In summary, this report demonstrates that shockwave treatment triggers release of cellular ATP, which subsequently activates purinergic

receptors and finally enhances proliferation *in vitro* and *in vivo* via downstream Erk1/2 signaling. In conclusion, our findings shed further light on the molecular mechanisms by which shockwave treatment exerts its beneficial effects. These findings could help to improve the clinical use of shockwave treatment for wound healing.

The application of extracorporeal shockwave treatment (ESWT) is a novel and effective therapeutic strategy to treat musculoskeletal disorders as well as chronic soft tissue wounds including bedsores, burn wounds, and diabetic and vascular ulcers (1,2). In addition to the accelerated healing rates in patients treated with ESWT, this therapeutic approach is non-invasive and cost-effective. Despite the growing evidence of the beneficial clinical effects of ESWT in treating musculoskeletal disorders and soft tissue wounds in animal models and clinical studies (3-10), the underlying mechanisms of how ESWT exerts these beneficial effects have remained unclear.

The process of wound healing consists of four major phases, namely hemostasis, inflammation, proliferation and tissue remodeling that involve complex cellular processes such as chemotaxis, phagocytosis, angiogenesis, collagen synthesis, and epithelialization (11). In recent years various research groups have focused on the angiogenic impact of shockwaves (4,5,12,13). They have shown that ESWT upregulates angiogenesis via mediators such as vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), hypoxia inducible factor 1a (HIF1a), and CD31 (4,5,14,15). ESWT was shown to augment the expression of proliferating cell nuclear antigen (PCNA), the recruitment of fibroblasts, and to downregulate production of pro-inflammatory cytokines (16). Other studies have shown that the expression of certain cytokines, chemokines, and matrix metalloproteinases with pro-angiogenic roles is enhanced during ESWT promoted wound healing (12).

Shockwaves generate an impulse that elicits mechanical stimulation via pressure changes that elicit a biological response such as cell differentiation and proliferation (17,18). Shockwaves are capable of initiating such cellular responses by triggering various intracellular signaling events that involve integrins, calcium channels, phospholipase C (PLC), and mitogen-activated protein kinases (MAPKs). For example in osteoblasts, one of the earliest intracellular signaling responses caused by mechanical stimulation is the phosphorylation of extracellular signal-regulated kinase 1/2 (Erk1/2) (18). In a similar fashion ESWT has been shown to induce Erk1/2 or p38 MAPK activation in mesenchymal stem cells (MSCs), immune cells, and osteoblasts resulting in distinct cellular effects including osteogenic differentiation of mesenchymal stem cells, angiogenesis, and T-cell proliferation (19-24). MAPK signaling pathways are known to play a central role in cell proliferation, differentiation, apoptosis, inflammation, and development (25-28). Moreover, Erk1/2 can induce HIF1a activation and angiogenesis via VEGFA expression (20).

Recent evidence suggests that shockwaves trigger adenosine triphosphate (ATP) release by activating purinergic signaling and p38 MAPK activation (22,23). The crucial role of purinergic signaling in the regulation of numerous physiological processes has only recently been appreciated (29,30). In addition to the regulation of neurotransmission and –modulation it is involved in differentiation, motility, apoptosis, regeneration and proliferation. ATP that is released from stimulated cells can bind to P2X and P2Y purinergic receptors that are ion channels and G-protein coupled receptors, respectively (29,31). Purinergic signaling also contributes to wound healing (29). Signaling in wound repair is to some extent dependent on the local release of ATP from the wound, which subsequently causes the activation of Erk1/2 effector pathways to induce wound healing (32).

In our current study, we show that shockwave treatment triggers the release of cellular ATP, which in turn elicits Erk1/2 signaling pathway activation and increases cell proliferation *in vitro* and wound healing *in vivo*.

EXPERIMENTAL PROCEDURES

Reagents – Unless otherwise stated, all reagents were purchased from Sigma-Aldrich. Antibodies for phospho-p38 MAPK (Thr180/Tyr182), total p38 MAPK, phospho-Akt (Ser473), total Akt, phospho-p44/42 MAPK (Thr202/Tyr204) (phospho-Erk1/2), total p44/42 MAPK (total Erk1/2), phospho-Mek1/2 (Ser217/221), total Mek1/2, phospho-S6 ribosomal

protein (Ser240/244), total-S6 ribosomal protein were obtained from Cell Signaling Technology. The secondary Antibodies IRDye® 680LT donkey anti-rabbit IgG, IRDye® 800CW goat anti-rabbit IgG or IRDye® 800CW goat anti-mouse IgG were obtained from LI-COR Biosciences.

Cell culture – C3H10T1/2 is a stable multipotent mesenchymal cell line derived from mouse embryos with a finite lifetime (33). Cells were grown in BME medium supplemented with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin. Cells used in experiments were between passages 7 and 11. Adipose derived stem cells (ASCs) were cultured in EGM-2 medium (Lonza, Walkersville, MD, USA) supplemented with 5% FBS. Cells were used between passage 2 and 7. For proliferation experiments ASCs were grown in DMEM/F12 medium. Human Jurkat T-cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin. To assess cell cycle progression and proliferation, cells were first deprived of serum for 24 hours in medium containing 0.2% FBS (starvation medium) to obtain synchronization in G0/G1. Cells were then stimulated to re-enter the cell cycle by adding 10% FBS. The shockwave group additionally received shockwave treatment at this time point.

Shockwave treatment – In this study shockwave treatment was performed with the electrohydraulic device DermaGold®100 and an OP155 applicator (Tissue Regeneration Technologies, LLC, manufactured by MTS Europe GmbH).

To allow the unhampered physical propagation and reproducible application of shockwaves to the sample *in vitro*, shockwave treatment was performed using a water bath set-up (34), where 8 x 10^5 cells suspended in 400 µl medium in a 15 ml PP tube are exposed to shockwaves under uniform and reproducible treatment conditions in terms of temperature and distance to the shockwave applicator.

In vitro, 10 to 300 pulses of shockwaves using energy flux densities between 0.03 and 0.19 mJ/mm^2 at 3 Hz were applied. According to previous experiments and *in vivo* studies (35) 100 pulses at 0.13 mJ/mm^2 and 3 Hz were used for the shockwave treatment in a rodent ischemic excision wound healing model. Control animals were treated alike while receiving no shockwave treatment.

Metabolic activity – To exclude possible adverse effects of shockwave treatment on the metabolic activity of cells the effect of 100 shockwave pulses at 0.07 and 0.19 mJ/mm^2 was analyzed using a MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]) bromide assay.

After shockwave treatment, cells were seeded to 96 well plates and incubated for indicated timeframes. MTT reagent was added at a final concentration of 650 µg/ml and cells incubated for 1 hour at 37°C in a 5% CO_2 environment. Medium was discarded, precipitated formazan was dissolved in DMSO by mechanical shaking in the dark for 20 minutes and absorbance was measured immediately at 540 nm.

Cell proliferation – Propidium iodide DNA staining was used to specifically determine the amount of cells undergoing S-phase. Cells were harvested by trypsinization and fixed by rapid submersion in ice-cold 85% EtOH. Samples were stored at -20°C for at least overnight or longer. For cell cycle analysis DNA was stained with 0.25 mg/ml propidium iodide, 0.05 mg/ml RNAse A and 0.01% Triton X-100 in citrate buffer, pH 7.8. Cells were analyzed on a BD FACSCanto II using BD FACSDiva software and data were further processed using FlowJo software.

BrdU (5-bromo-2-deoxyuridine) incorporation into newly synthesized DNA of cells treated with/without shockwaves was used as an indicator for actively proliferating cells. The BrdU enzyme-linked immunosorbent assay (Roche Molecular Biochemicals) was performed according to manufacturer's instructions. In brief, cells were deprived of serum for growth arrest and restimulated by serum addition combined with/without shockwave treatment. Cells were then seeded into 96-well plates and incubated with media containing 100 µM BrdU for 3 hours at indicated timepoints. FixDenat® solution was added for 30 min followed by incubation with anti-BrdU POD (peroxidase) antibody for 1 hour at room temperature. After 3 washing steps with PBS, tetramethyl benzidine was added as a substrate for 30 minutes. By adding 1 M H_2SO_4 the reaction was terminated and absorbance measured at 450 nm.

ATP release – The amount of ATP release of C3H10T1/2, Jurkat T cells and adipose derived stem cells into the supernatant was determined with the CellTiter-Glo assay (Promega). Cells were adjusted to 8 x 10^5/400 µl and allowed to rest

for 1 hour at 37°C in a humidified incubator before shockwave treatment was applied. Afterwards cells were centrifuged at 1000 g for 5 minutes at 4°C and 100 µl of supernatant was transferred to a 96-well plate. After an equal amount of CellTiter-Glo reagent was added, the plate was horizontally shaken for 2 minutes and after incubation for 10 minutes at room temperature the luminescence was measured. The calibration of measured luminescence to ATP concentrations was performed by using ATP standard solutions of known concentrations.

Immunoblotting – Total protein of cells was extracted by repeated freeze and thaw cycles. In brief, cells were harvested by trypsinization, cell pellets were washed 3 times with PBS and lysed in NP-40 buffer containing 40 mM HEPES pH 7.9, 120 mM NaCl, 1 mM EDTA pH 8.0, 10 mM 2-glycerolphosphate, 50 mM NaF, 0.5 mM Na_3VSO_4, 1% NP-40 nonidet substitute and 1 mM PMSF supplemented with 2 µg/ml aprotinin, 2 µg/ml leupeptin, 0.3 µg/ml benzamidinechloride and 10 µg/ml trypsin inhibitor. Samples were incubated on ice for 20 minutes and then centrifuged at 15,000 rpm for 20 minutes at 4°C. Supernatants were collected and protein concentrations were determined (Protein Assay Kit II, Bio-Rad).

Equal amounts of protein (up to 20 µg per lane) were resolved by SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk in TBS buffer with 0.1% Tween (TBS/T) and primary antibodies in 5% BSA in TBS/T were incubated at 4°C overnight. Membranes were incubated in 5% milk-TBS/T containing secondary antibody and signals were detected using the OdysseyFC Infrared Imaging System (LI-COR Biosciences). After membranes were incubated in 1x Stripping Buffer (LI-COR Biosciences) on a shaker at room temperature for 5 minutes and washed 3 times in PBS, membranes were re-probed with total antibodies. Ratios of phosphorylated/total proteins were analyzed using densitometry (Image Studio Lite, LI-COR Biosciences).

Erk1/2 inhibition - The Mek1/2 inhibitor U0126 (Cell Signaling Technology) was solubilized in DMSO and used *in vitro* to block Erk1/2 activation. Cells were serum deprived using starvation medium for 24 hours with 10 µM U0126 being present for the last 2.5 hours. Cells were detached and adjusted to 8 x 10^5/400 µl and allowed to rest for 1 hour, followed by shockwave treatment or the addition of ATP to the supernatant of untreated cells at indicated concentrations for 10 minutes, respectively. Cells were then either seeded on 96-well plates for cell proliferation assays or washed with PBS and processed for total protein isolation.

Purinergic Signaling – Cells were adjusted to 8 x 10^5/400 µl and allowed to rest at 37°C in a humidified incubator for 30 minutes. Purinergic receptor antagonists suramin, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) and theophylline were added at a concentration of 100 µM and cells were incubated for further 30 minutes. Cells received either shockwave treatment at indicated energies or ATP at indicated concentrations was added for 10 min. Directly after treatment protein was isolated for immunoblotting. Purinergic receptor agonists adenosine, UTP, ATP, 2-MeSATP were added at indicated concentrations (5, 50 or 100 µM) after cells were allowed to rest at 37°C in a humidified incubator for 1 hour. Cells were incubated with agonists at 37°C for 10 minutes and immediately processed for protein isolation. For hydrolysis of released ATP in the supernatant of shockwave treated cells 20 U/ml apyrase was added prior to treatment.

Erk1/2 inhibition in vivo – Sprague-Dawley rats were randomly assigned to one of the following four groups: control group, control group receiving inhibitor, ESWT group and ESWT group receiving inhibitor. Rats in the inhibitor groups were treated with GSK1120212 (AbMole BioScience), a potent inhibitor of Mek1/2 activity (36). GSK1120212 was dissolved in DMSO, diluted in hydroxyethylcellulose (1% w/v) and was administered orally once a day. Treatment with the inhibitor started 4 days before surgery with a singular dose of 0.3 mg/kg followed by 0.1 mg/kg of GSK1120212 until day 10 after surgery. Control animals received vehicle (DMSO) in hydroxyethylcellulose via the same administration route.

Rodent ischemic excision wound healing model – Fourty male Sprague-Dawley rats, weighing between 350 and 450 g, were used in this study. A standard rodent ischemic epigastric flap model was modified to study the effect of shockwave treatment on ischemia impaired wound healing (37) (Hoffmann A. et al., submitted). In

brief, rats were anesthetized in an inhalation box using isoflurane (2.5 Vol.-%). Anesthesia was maintained by intraperitoneal (i.p.) injection of a mixture of ketamin (110 mg/kg; Pharmacia & Upjohn, Germany) and xylazin (12 mg/kg; Bayer, Leverkusen, Germany).

The abdomen of the rats was shaved and depilated, and an epigastric adipocutaneous flap was created, as described in detail elsewhere (35). Ligation of an unilateral inferior epigastric neurovascular bundle induces ischemia in the corresponding abdominal area whereas the contralateral side remains adequately perfused by the intact neurovascular bundle. After the flap was sutured back the ischemic side of the flap was treated with 100 shockwave pulses at 0.13 mJ/mm^2. Thereafter, circular excision wounds with a diameter of 1.3 cm were created on both the ischemic and normal perfused side, leaving the fascia intact. Thus, the wound healing progress in an ischemia disturbed excision wound can be compared to the regular perfused internal counterpart. Control animals were treated identically without receiving shock wave treatment.

To determine the rate of wound healing, wounds were traced on a transparent acrylic foil which was captured by digital imaging and further analyzed using a planimetric software program. Wound size obtained immediately after surgery represents baseline values and wound size recordings from days 1, 5 and 10 after surgery were referred to this baseline. Wounds from the ischemic side reflect impaired healing and were compared to wound healing in the contralateral normal perfused area.

Animals were euthanized on day 10 and tissue biopsies from all wounds with adjacent unaffected tissue were harvested for histological and immunohistochemical analysis.

Histology and immunohistochemistry – Full-thickness biopsies from the excision wounds were processed according to standard procedures. In brief, samples were fixed in formalin for 24 hours, dehydrated in a gradient series of alcohol and embedded in paraffin. Deparaffinized sections were rehydrated in graded alcohols and stained with hematoxylin and eosin (HE) for standard histology. Immunohistochemical staining for proliferation and activation of Erk1/2 was performed on formalin-fixed, paraffin-embedded sections using a Ki-67 (RM-9106, Thermo Scientific) and phospho-Erk1/2 antibody, respectively. Quantification of positively stained cells in the area of the wound was performed in a double-blinded fashion with the HistoQuest analysis software (TissueGnostics GmbH, Vienna, Austria).

Statistical analysis – All data is presented as mean+standard deviation (SD) except indicated otherwise. Normal distribution of data was tested with the Kolmogorov-Smirnov-test. Comparisons between groups were calculated using either Student's *t*-test (unpaired, two-tailed), One-way ANOVA with Tukey's multiple comparison test or Kruskal-Wallis test with Dunn's multiple comparison test, and *P*-values ≤ 0.05 were considered as statistically significant. All calculations were performed using GraphPad Software (GraphPad Software, Inc., SanDiego, CA, USA).

Study approval – The *in vivo* study was conducted in accordance with protocols approved by the local Committee on Animal Experiments of Vienna, Austria. All experiments were performed according to the policies, procedures and responsibilities for the care and use of laboratory animals at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria. For human ASCs, liposuction material of male and female patients between 31 and 40 years of age was used. The collection of human adipose tissue was approved by the local ethical review board and informed, written consent was obtained from each patient prior to the use of the collected material for this research project.

RESULTS

Shockwave treatment increases in vitro proliferation of mesenchymal progenitor cells in a dose- and time-dependent fashion – To date, no routine standard protocol exists for the application of *in vitro* shockwave treatment. Therefore we began our studies by assessing the influence of shockwave treatment on the metabolic activity of C3H10T1/2 mesenchymal progenitor cells *in vitro* (Figure **1A**). Cells were treated with 100 shockwave pulses at an energy level of 0.07 or 0.19 mJ/mm^2 and metabolic activity was measured with an MTT assay after 15, 24, and 36 h. Shockwave treatment significantly increased metabolic activity 15 h after treatment but not at later time points. These findings indicate that shockwaves can enhance the metabolic activity of C3H10T1/2 cells and that energy levels of 0.07 or

0.19 mJ/mm^2 do not adversely affect cell viability under our experimental conditions.

Next, we studied whether shockwave treatment affects cell proliferation. C3H10T1/2 cells were subjected to shockwave treatment using 100 pulses at 0.03, 0.07, or 0.19 mJ/mm^2. Along with respective controls that did not receive shockwave treatment, cells were harvested and fixed at indicated timepoints, stained with propidium iodide, and cell cycle characteristics were assessed using flow cytometric analysis (Figure **1B**). The cell cycle profiles revealed more cells in the S-phase in the samples treated with shockwaves compared to control cells without shockwaves. This effect increased with energy and was particularly clear 21 h after treatment with 100 pulses at 0.19 mJ/mm^2, revealing a significant increase in proliferating cells in the S-phase (Figure **1D**). However, shockwave treatment with 100 pulses using 0.19 mJ/mm^2 increased the cell numbers in S-phase by up to 48 % compared to untreated control already 15 h after treatment (Figure **1C**). These results indicate a dose- and time-dependent pro-proliferative effect of shockwave treatment *in vitro*.

Shockwave treatment triggers ATP release and activates MAPK signaling pathways – Shockwave treatment exerts a mechanical stimulus on cells, which can release cellular ATP into the extracellular space (23). We exposed C3H10T1/2 cells to shockwave treatment and measured ATP release immediately, Erk1/2, its upstream effector Mek1/2 as well as p38 MAPK activation 10 minutes after shock wave treatment. To assess the dose-dependent effects of shockwaves, the cells were treated at a constant energy level (0.19 mJ/mm^2) with different numbers of shockwave pulses (0 to 300; Figure **2A, B and E**) or with 100 shockwave pulses at different energy levels (0 to 0.19 mJ/mm^2; Figure **2C, D and F**).

The application of 50, 100, or 300 shockwave pulses at 0.19 mJ/mm^2 caused a significant release of ATP into the supernatant that increased with the number of shockwave pulses administered (Figure **2E**). Western blot analysis 10 min after shockwave treatment revealed significant and dose-dependent activation of Erk1/2, Mek1/2 and p38 MAPK signaling reaching levels up to 10-fold higher than in unstimulated controls (Figure **2B**). Activation of Akt and ribosomal S6 protein, a downstream target of the mammalian target of rapamycin (mTOR), did not follow this dose dependent pattern, which can be appreciated in the representative Western blots (Figure **2A**).

Shockwave treatment elicited ATP release in an energy level-dependent fashion, reaching levels significantly higher than in control samples at $\geq$ 0.07 mJ/mm^2 (Figure **2F**). Similarly, Erk1/2, Mek1/2 and p38 MAPK activation was increased by up to 8-fold in response to shockwaves at 0.19 mJ/mm^2 while the phosphorylation of Akt and ribosomal S6 remained unchanged (Figure **2C** and **D**). Thus, shockwave treatment results in pulse- and energy-dependent increase in ATP release and Erk1/2 and p38 MAPK activation.

Shockwave treatment promotes proliferation and MAPK signaling pathway activation via purinergic signaling – Shockwave treatment has been shown to have pro-proliferative effects on T cells, where ATP release plays an essential role (22). BrdU incorporation assays showed that shockwave treatment increases proliferation of C3H10T1/2 cells (Figure **3A**). Shockwave treatment with energy levels of 0.07 mJ/mm^2 or 0.19 mJ/mm^2 significantly increased proliferation by up to 1.5 times the value of untreated control cells. To test the hypothesis that ATP release contributes to the observed pro-proliferative effect of shockwave treatment, we treated cells with shockwaves in the presence of apyrase (20 U/ml) to rapidly hydrolyze extracellular ATP. Scavenging ATP from the supernatant of shockwave-treated cells abolished the enhancing effect of shockwaves on proliferation (Figure **3A**). Analysis of Erk1/2 phosphorylation showed that apyrase (20 U/ml) and the non-specific P2 receptor antagonist suramin (100 μM) reversed the stimulatory effects of shockwave treatment on Erk1/2 activation (Figure **3B** and **C**). Apyrase abolished p38 MAPK phosphorylation in control cells and cells treated with ESWT, while suramin did not reduce p38 MAPK phosphorylation, suggesting the involvement of suramin-insensitive mechanisms in the p38 MAPK signaling of C3H10T1/2 cells (Figure **3C**). These results indicate that purinergic signaling is responsible for the enhancement of Erk1/2 activation and proliferation of shockwave-treated C3H10T1/2 cells.

Erk1/2 signaling is crucial for the proliferative effects of shockwave treatment – To further test the notion that shockwave treatment accelerates proliferation by Erk1/2 activation, we used the Mek1/2 inhibitor U0126. C3H10T1/2

cells were treated with U0126 (10 μM) for 2.5 h before shockwave treatment at 0.19 mJ/mm^2 and Western blot analysis was performed to assess Erk1/2 activation. In some cases, exogenous ATP (500 nM) was added for 10 min prior to analysis of Erk1/2 activation. The significant increase in Erk1/2 phosphorylation induced by shockwave treatment was reduced 6-fold by inhibition of Mek1/2, the upstream kinases of Erk1/2 (Figure **4A**). Addition of extracellular ATP had no significant effect on Erk1/2 phosphorylation compared to control cells without shockwave treatment (Figure **4A**). Under control conditions shockwave treatment with 100 pulses at 0.07 and 0.19 mJ/mm^2 increased proliferation by up to 1.6-fold as measured by BrdU incorporation 21 h after treatment (Figure **4B**). Pretreatment with U0126 reduced the enhancing effect of shockwave treatment on proliferation (Figure **4B**). These findings support the conclusion that Erk1/2 signaling is essential for the proliferative effects of shockwave treatment.

Shockwave treatment activated purinergic signaling stimulates Erk1/2 phosphorylation – Extracellular ATP is rapidly broken down by ectonucleotidases that are found on the cell surfaces of many cell types. This results in the formation of adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine and the potential stimulation of P2X, P2Y, as well as P1 (adenosine) receptors (38). To determine whether these different purinergic receptor classes contribute to Erk1/2 activation in response to shockwave treatment, we used agonists and antagonists of these different receptors. P2X purinergic receptors are solely activated by ATP, whereas ATP and uridine triphosphate (UTP) are both agonists for P2Y receptors (39). We found that extracellular ATP and UTP increased Erk1/2 activation (Figure **5A**). Treatment with 2-methylthio-ATP (2-MeSATP), an agonist of P2X receptors and adenosine, a P1 agonist (39), did not significantly increase Erk1/2 activation. Taken together, these findings suggest that P2Y receptors are primarily responsible for the stimulatory effect of purinergic signaling on C3H10T1/2 cells.

In order to investigate the roles of P2 and P1 receptors in Erk1/2 activation by shockwave treatment, we used PPADS, an antagonist of P2X receptors (40), suramin which is a nonselective antagonist of P2X and P2Y receptors (30), and theophylline, which is an antagonist of P1 receptors (30). Cells were treated with these antagonists for 30 min before shockwave treatment (Figure **5B**) or addition of extracellular ATP (Figure **5C**) and Erk1/2 activation was assessed. We found that suramin but neither PPADS nor theophylline reduced Erk1/2 activation by shockwave or ATP treatment (Figure **5B** and **C**). These findings suggest that P2Y receptors seem to be predominantly responsible for Erk1/2 activation in response to shockwave treatment.

Shockwave treatment enhances proliferation by inducing ATP release and subsequent Erk1/2 activation in human cells – Shockwave treatment has been previously shown to beneficially influence wound healing in pre-clinical and clinical trials. Therefore, we study the effect of shockwave treatment on human cell types that may be involved in wound healing, namely human stem cells and T lymphocytes. Primary, adherent adipose derived stem cells were obtained from 3 different donors and Jurkat T lymphocytes were cultured *in vitro*. First we tested whether shockwave treatment has an effect on the proliferation of these cells similar to that described above for C3H10T1/2 cells. Cell cycle time course experiments using flow cytometry suggested the accumulation of both cell types in the S-phase after shockwave treatment. ASCs and Jurkat T cells showed significantly higher percentages of cells in S-phase at 21 h and 18 h after shockwave treatment, respectively, (Figures **6A** and **7A**). Similar to C3H10T1/2 cells, both human cell types showed dose-dependent increases in ATP release and Erk1/2, Mek1/2 and p38 MAPK activation in response to shockwave treatment (Figures **6B, C, F, G, H, J** and **7B, C, F, G, H, J**). In order to test whether the activation of Erk1/2 and p38 MAPKs is dependent upon ATP released in response to shockwave treatment, ASCs and Jurkat T cells were either treated with apyrase or suramin, which hydrolyze ATP or block purinergic P2 receptors, respectively. As with C3H10T1/2 cells, both, apyrase and suramin treatment abolished the activation of Erk1/2 and p38 MAPK in both human cell types (Figures **6D-E** and **7D-E**). Finally, we found that shockwave treatment induced proliferation in both ASCs and Jurkat T cells, as assessed by BrdU incorporation. This proliferative effect was reduced by apyrase and U0126, indicating that proliferation in response to shockwave treatment depends on ATP release and

activation of Erk1/2 signaling (Figures **6I** and Figure **7I**).

Taken together with the results in C3H10T1/2 cells shown above, these findings indicate that ATP release, P2 receptor activation, and Erk1/2 signaling are universal mechanisms by which shockwave treatment influences the activation and function of different cell types.

Shockwave treatment enhances wound healing in a rat model by promoting proliferation via Erk1/2 signaling – Shockwave treatment of ischemic skin flaps has been shown to improve wound healing in a standard rodent ischemic epigastric flap model (35). We used this model and the Mek1/2 inhibitor GSK1120212 to test the hypothesis that Erk1/2 signaling is crucial for improved wound healing in response to shockwave treatment. Planimetric analysis of the wound size area on the ischemic side of the epigastric flap was performed immediately after surgery (day 0) and on days 1, 5, and 10 after surgery (Figure **8C** and **D**) Shockwave treatment of the ischemic side of the epigastric flap (100 pulses at 0.13 mJ/mm^2) significantly decreased the wound size compared to control animals (Figure **8A**)**.** In animals receiving the Mek1/2 inhibitor GSK1120212 (0.1 mg/kg daily), wound sizes in the control and shockwave groups did not differ (Figure **8B**). These results suggest that shockwave treatment exerts its beneficial effects on wound healing by induction of Erk1/2 signaling.

Quantitative immunohistochemical analysis of the effects of shockwave treatment on proliferation and Erk1/2 phosphorylation in ischemic wounds was performed by staining with the proliferation marker Ki-67 and a phospho-Erk1/2 antibody, respectively. Regions of interest (ROI) in the granulation tissue area close to the remaining wound were analyzed under the microscope and representative pictures of each study group showing sections of the ischemic wound harvested 10 days after surgery are depicted in Figure **9A**. Wounds of animals treated with 100 shockwave pulses at 0.13 mJ/mm^2 exhibited a significant increase in Ki-67 and phospho-Erk1/2 positive cells in the granulation tissue that was 6-fold and ~5-fold higher than in untreated controls, respectively. In animals receiving GSK1120212, shockwave treatment did not significantly change the percentage of Ki-67 or phospho-Erk1/2 positive cells (Figure **9B** and **C**). These data support our findings that ischemic wound healing in shockwave treated animals is significantly enhanced by shockwave treatment and is dependent on Erk1/2 pathways.

DISCUSSION

Despite the clinical evidence that ESWT can benefit patients with musculoskeletal disorders, non-union bone fractures, and chronic soft tissue wounds (2,8,9), the molecular mechanisms underlying these beneficial effects have remained unclear. Here, we sought to fill this gap in knowledge by exploring these mechanisms using several cell types that contribute to wound healing. Our results indicate that shockwave treatment affects target cells and tissues by eliciting ATP release, which in turn promotes P2 receptor-mediated Erk1/2 signaling and thereby enhances cell proliferation (Figure **10**). Through this mechanism, ESWT improved ischemic wound healing in a rodent model that recapitulates one of the clinically most remarkable features of ESWT, namely its ability to induce the healing of chronic ulcers and non-healing wounds (1-5).

Shockwave treatment has been shown to influence the function of a number of different cells including MSCs (24,41), osteoblasts (20), tenocytes (42), and T cells (22). In mouse mesenchymal progenitor cells we could show that the proliferative effects of shockwave treatment are mediated by the release of cellular ATP in an energy and dose-dependent manner and through P2 receptors. P2 receptor stimulation has been shown to induce proliferation of many different cell types, suggesting that purinergic signaling plays an important, perhaps universal role in processes involved in wound healing (29,32,43-45). We found that ATP is released from shockwave treated cells. However, the mechanisms of release are unclear. ATP can be released by damaged and dying cells or in response to physiological stimuli and mechanical stretch (30). Mechanical stimulation has been shown to mediate the release of cellular ATP of various cells, such as astrocytes, osteoblasts, chondrocytes or epithelial cells (46-49). Studies of Sun et al. and Yu et al. support our findings that ESWT at our chosen settings releases ATP without negatively affecting cell viability, as they showed that subjection of hMSCs to <200 shockwave pulses at 0.18 mJ/mm^2 resulted in >95% viability directly or 24h after ESWT (22,23).

We found that the released ATP activated P2 receptors and increased Erk1/2 and p38 MAPK signaling via mechanisms similar to those reported in osteoblasts, MSCs, and T cells (20,22-24). In addition to Erk1/2 and p38 MAPK, the downstream signaling mechanisms triggered by shockwave-induced P2 receptor activation may also involve additional signaling intermediates such as Ras and HIF1a transactivation (20,23). A study dealing with stretch-induced injury reported the activation of Erk1/2 to be dependent on the rate and amplitude of mechanical impact, involving released ATP and activated P2 receptors (46). AKT and mTOR signaling pathways are known as essential regulators of cell proliferation (50-52). However, we did not observe a change in the phosphorylation of AKT and ribosomal S6 protein in our study, suggesting these pathways do not play a major role in the pro-proliferative effects of shockwave treatment of C3H10T1/2 cells.

Once released into the extracellular space, ATP cannot only stimulate P2 receptors, but it can also be degraded by ectonucleotidases found on cell surfaces to ADP, AMP, and adenosine, which in turn can activate different sets of P2X, P2Y and P1 (adenosine) receptors (30,53). We found that exogenous ATP and UTP could both replicate the stimulatory effect of shockwave treatment on Erk1/2 signaling in C3H10T1/2 cells, implying the involvement of P2Y receptors in this responses (39,47,54). Moreover, in epithelial cells the P2Y receptor antagonist reactive blue 2 inhibited wound healing *in vitro* (49) and released ATP after injury induced Erk1/2 phosphorylation through activation of P2Y receptors (55).

We could demonstrate that shockwave treatment caused Erk1/2 activation in the mouse mesenchymal progenitor cell line (C3H10T1/2) as well as in two different human cell types that may be involved in wound healing, namely primary adherent adipose derived stem cells and the Jurkat T cell line. ESWT has been shown to trigger inflammatory processes by activating immune cells including T cells (3,22,34,56). Our findings with these different cell types suggest that ESWT may aid tissue repair by influencing the inflammatory and regenerative aspects of wound healing. The effects of shockwaves on primary human stem cells suggest that clinical ESWT may promote the growth and perhaps the differentiation of stem or progenitor cells during tissue regeneration (1,57,58). In all three cell types we tested *in vitro*, we found a uniform scheme of shockwave-induced ATP release, subsequent stimulation of purinergic receptors, and the activation of Erk1/2 signaling, finally resulting in enhanced cell proliferation. We therefore conclude that this mechanism plays a central role in the response of mammalian cells to shockwave treatment. Our *in vivo* experiments demonstrated that the biological responses triggered by ESWT improved tissue repair. It remains to be seen to what extent the here proposed mechanism induced by ESWT also influences other aspects of wound healing, such as neovascularization, angiogenesis, stem cell differentiation, and the inflammatory response involved in wound repair, as shockwave treatment already has been reported to favor these phases of wound healing (2,10,12,19,59). Our *in vivo* studies further revealed that ESWT improves wound healing in a rat ischemic skin flap model and that Erk1/2 signaling is involved in this process. Taken together with previous reports (5,16) and with our *in vitro* and *in vivo* findings, this suggests that ESWT promotes wound healing by local ATP release and P2 receptor activation that fosters cell proliferation and tissue remodeling via Erk1/2 activation.

Patients with diabetes often suffer from chronic ulcers resulting in significant discomfort, risk of infection, and impaired quality of life. The treatment of such chronic wounds is difficult and success is uncertain. ESWT is a novel treatment approach with a remarkable success rate (1,2,9). Schaden et al. reported that a single session of ESWT can induce wound healing in patients suffering from different wound disorders (1). The clinical observation that a single ESWT session can affect outcome is echoed by our *in vitro* findings where shockwave treatment had prolonged effects on subsequent cellular functions. Furthermore, the advantage of using ESWT in an outpatient setting and its cost effectiveness (compared to other clinical procedures) underline the clinical relevance and the need for the integration of shockwave treatment into future clinical routine. Additionally, our results might help to explain the non-responsiveness to shockwave treatment in some patients suffering wound healing disorders. Besides well-known factors contributing to wound healing such as age and lifestyle, the Erk1/2 signaling pathway has been described as crucial in the wound healing

process (60,61). We therefore hypothesize that the non-responding patients might have an impaired Erk1/2 signaling pathway resistant to shockwave treatment.

In conclusion, our findings shed new light on the underlying mechanisms by which ESWT may exert its clinical effects, namely by causing the release of ATP, the subsequent stimulation of P2 receptors and activation of Erk1/2 signaling, which initiate processes involved in wound healing. While future studies are required to further define these processes, we think that our findings may help to refine the use of shockwave treatment to further improve its clinical efficacy.

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FOOTNOTES

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[§]To whom correspondence should be addressed: Christiane Fuchs, University of Applied Sciences Technikum Wien, Department of Biochemical Engineering and The Austrian Cluster for Tissue Regeneration, Vienna, Austria; Tel.: +43 1 333 40 77 -989; Fax: +43 1 333 40 77 -99 989; E-mail: fuchsc@technikum-wien.at
The abbreviations used are: ESWT, extracorporeal shock wave therapy; ATP, adenosine triphosphate; extracellular signal-regulated kinase (ERK).

FIGURE LEGENDS

FIGURE 1. Dose- and time-dependent effect of shockwave treatment on metabolic activity and proliferation of mesenchymal progenitor cells in vitro. (A) C3H10T1/2 cells were treated with 100 shockwave pulses at 0.07 mJ/mm^2 and 0.19 mJ/mm^2 and metabolic activity was assessed with an MTT assay 15, 24, and 36 h after treatment (n=4 in triplicates; mean+SD; Kruskal-Wallis test with Dunn's multiple comparison test). (B) For determination of proliferation, C3H10T1/2 cells were synchronized in G0/G1 phase using 0.2% serum for 24 h and re-stimulation using 10% serum. Then cells were treated with 100 shockwave pulses at 0.03, 0.07, or 0.19 mJ/mm^2. Untreated cells were treated alike except they did not receive shockwave treatment. Cells were harvested at indicated timepoints, stained with propidium iodide and DNA content was assessed by flow cytometry (2N, 4N: diploid and tetraploid DNA content, respectively). Characteristic cell cycle profiles of untreated cells (grey area) compared to shockwave treated cells (overlaying black line) are shown 15 h (upper panel) and 21 h (lower panel) after shockwave treatment. Corresponding percentage of proliferating cells in S-phase 15h (C) and 21h (D) after shockwave treatment (n=6; *P<0.05, **P<0.01; One-way ANOVA with Tukey's multiple comparison test).

FIGURE 2. Shockwave treatment releases cellular ATP and activates intracellular signaling in a pulse number and energy dependent fashion. Immediately after shockwave treatment with indicated pulse numbers at a constant energy of 0.19 mJ/mm^2 (A, B, E) or varying energies at a constant number of 100 shockwave pulses (C, D, F), cells were placed on ice and ATP concentrations in supernatants were determined (E, F; n=2 in triplicates; **P<0.01, ***P<0.001; Kruskal-Wallis test with Dunn's multiple comparison test). Cells were lysed and Akt, Erk1/2, Mek1/2 and p38 MAPKs, and S6 kinase activation was determined by immunoblot analysis. Representative blots of 3 different experiments are shown (A and C, asterisk denotes unspecific band) and intensity ratios depicted in corresponding bar graphs were calculated using phosphorylated and total protein expression and normalized to the untreated controls (B and D; n=3; mean+SD; *P<0.05, **P<0.01, ***P<0.001; One-way ANOVA with Tukey's multiple comparison test compared to untreated controls).

FIGURE 3. Shockwave treatment promotes proliferation and Erk1/2 and p38 MAPK pathway activation via purinergic signaling. (A) C3H10T1/2 cells were growth arrested for 24 h, re-stimulated by addition of 10% serum, and treated with 100 shockwave pulses at 0.07 or 0.19 mJ/mm^2 in the absence or presence of 20 U/ml apyrase during shockwave treatment. BrdU incorporation at 21 h post-treatment was used as a measure of cell proliferation (n=3 in triplicates; *P<0.05, **P<0.001, ***P<0.001; One-way ANOVA with Tukey's multiple comparison test compared to unstimulated controls). (B and C) Representative Western blots are shown of 3 independent experiments with C3H10T1/2 cells subjected to 100 shockwave pulses at 0.19 mJ/mm^2 in the presence or absence of apyrase (20 U/ml) or suramin (100 µM). Protein lysates were prepared immediately after shockwave treatment for determination of MAPK activation. Intensity ratios of Erk1/2 and p38 MAPK were calculated using activated and total protein expression and normalized to corresponding untreated controls (n≥3; mean+SD; two-tailed unpaired student's t-test compared to unstimulated controls; n.d.: not detectable).

FIGURE 4. Erk1/2 signaling is crucial for the proliferative effects of shockwave treatment. (A) C3H10T1/2 cells were serum deprived for 24 h and incubated with the Mek1/2 inhibitor U0126 (10 µM) for 2.5 h before re-stimulation and treatment with 100 shockwave pulses at 0.19 mJ/mm^2. Extracellular ATP (500 nM) was incubated with cells at 37°C for 10 min. Directly after treatment samples were placed on ice and cells processed for protein extraction and Western blot analysis. Representative Western blot of 3 independent experiments is shown. Ratios between activated and total Erk1/2 were calculated and data were normalized to non-treated control group. (n=3; mean+SD; *P<0.05, ***P<0.001; One-way ANOVA with Tukey's multiple comparison test). (B) BrdU incorporation was assessed 21 h after shock wave treatment of C3HT101/2 cells pretreated with or without U0126 [10 µM]. (n=3 in duplicates; *P<0.05, ***P<0.001; data are normalized to corresponding controls, One-way ANOVA with Tukey's multiple comparison test).

FIGURE 5. Shockwave treatment activated purinergic signaling stimulates Erk1/2 activation. (A) C3H10T1/2 cells were treated with the indicated concentrations of the P2 receptor agonists ATP, UTP, and MeSATP or with the P1 receptor agonist adenosine for 10 min at 37ºC. Then cells were placed on ice and protein was extracted for Western blotting. Bar graph shows fold stimulation of Erk1/2 protein level normalized to control. Representative Western blot of 4 independent experiments is shown (n=4; mean+SD; *P<0.05, **P<0.001; Kruskal-Wallis test with Dunn's multiple comparison test). (B and C) C3H10T1/2 cells were pretreated with 100 µM P2 receptor antagonists PPADS or suramin, or P1 receptor antagonist theophylline. Cells were shockwave treated using (B) 100 pulses at 0.19 mJ/mm^2 or (C) 5 µM ATP was added and incubated for 10 minutes before protein was extracted. Bar graphs depict fold change of Erk1/2 protein level normalized to untreated controls (B, n=4; C, n=5, mean+SD; *P<0.05, **P<0.001; One-way ANOVA with Tukey's multiple comparison test; lanes were run on the same gel but were noncontiguous).

FIGURE 6: Purinergic signaling and Erk1/2 activation are essential in the shockwave treatment induced proliferation in human adipose derived stem cells. (A) For assessment of cell cycle progression, ASCs were serum deprived for 24 h. Cells were then re-stimulated with 10% serum and shockwave treated with 0.19 mJ/mm² and 100 pulses. Cell were harvested and fixed at indicated time points and DNA content was assessed by flow cytometry. Corresponding percentages of cells in S-phase at various time points (0, 15, 18, 21, and 24 h) after shockwave treatment are shown (n=3 in duplicates; *P<0.05; two-tailed unpaired student's t-test for pairwise comparison at different time points). Immediately after shockwave treatment with varying energies at a constant number of 100 pulses (B, C, F) or with indicated pulse numbers at a constant energy of 0.19 mJ/mm² (G, H, J), ATP concentrations in supernatants were determined (F, J; n=3 in triplicates; *P<0.05, ***P<0.001; Kruskal-Wallis test with Dunn's multiple comparison test). Cells were lysed and Erk1/2, Mek1/2 and p38 MAPK activation was determined by Western blot analysis. Representative blots of 3 different experiments are shown (B and G, asterisk denotes unspecific band). Intensity ratios depicted in corresponding bar graphs were calculated using

phosphorylated and total protein expression and normalized to untreated controls (C and H; n=3; mean+SD; *P<0.05, **P<0.01, ***P<0.001; One-way ANOVA with Tukey's multiple comparison test). (D and E) Representative Western blots are shown of 5 independent experiments with ASCs subjected to 100 pulses at 0.19 mJ/mm² in the presence or absence of apyrase (20 U/ml) or suramin (100 µM). Intensity ratios of Erk1/2 and p38 MAPK were calculated and normalized to corresponding controls (n=5; mean+SD; two-tailed unpaired student's t-test). (I) ASCs were serum deprived for 24 h and either incubated with the Mek1/2 inhibitor U0126 (10 µM) for 2.5 h or with 20 U/ml apyrase for 30 minutes before restimulation with 10% serum and shockwave treatment with 100 pulses at 0.19 mJ/mm². BrdU incorporation was assessed 18 and 21 h after shockwave treatment as a measure of cell proliferation (n=3 in triplicates; *P<0.05; two-tailed unpaired student's t-test compared to corresponding controls).

FIGURE 7: Purinergic signaling and Erk1/2 activation are essential in the shockwave treatment induced proliferation in human Jurkat T cells. (A) For assessment of cell cycle progression, Jurkat T cells were serum deprived for 48 h. Cells were re-stimulated with 10% serum and treated with 100 shockwave pulses at 0.19 mJ/mm². Cell were harvested and fixed at indicated time points and DNA content was assessed by flow cytometry. Corresponding percentages of cells in S-phase at various time points (0, 15, 18, 21, and 24 h) after shockwave treatment are shown (n=3; *P<0.05; two-tailed unpaired student's t-test for pairwise comparison at different time points). Immediately after shockwave treatment with varying energies at a constant number of 100 pulses (B, C, F) or with indicated pulse numbers at a constant energy of 0.19 mJ/mm² (G, H, J), ATP concentrations in supernatants were determined (F, J; n=3 in triplicates; *P<0.05, ***P<0.001; Kruskal-Wallis test with Dunn's multiple comparison test). Cells were lysed and Erk1/2, Mek1/2 and p38 MAPK activation was determined by Western blot analysis. Representative blots of 3 different experiments are shown (B and G). Intensity ratios depicted in corresponding bar graphs were calculated using phosphorylated and total protein expression and normalized to untreated controls (C and H; n=4; mean+SD; *P<0.05, **P<0.01, ***P<0.001; One-way ANOVA with Tukey's multiple comparison test). (D and E) Representative Western blots are shown of 2 independent experiments with Jurkat T cells subjected to 100 pulses at 0.19 mJ/mm² in the presence or absence of apyrase (20 U/ml) or suramin (100 µM). Intensity ratios of Erk1/2 and p38 MAPK were calculated and normalized to corresponding controls (n=8; mean+SD; two-tailed unpaired student's t-test). (I) Jurkat T cells were serum deprived for 48 h and either incubated with the Mek1/2 inhibitor U0126 (10 µM) for 2 h or with 20 U/ml apyrase for 30 minutes before restimulation with 10% serum and shockwave treatment with 100 pulses at 0.19 mJ/mm². BrdU incorporation was assessed 18 and 21 h after shockwave treatment as a measure of cell proliferation (n=4 in triplicates; *P<0.05, **P<0.01; two-tailed unpaired student's t-test compared to corresponding controls).

FIGURE 8. *In vivo* shockwave treatment enhanced wound healing is dependent on Erk1/2 signaling. Sprague-Dawley rats did not receive (A and C) or received GSK1120212 (B and D) on a daily basis, starting 4 days before surgery and until day 10 post surgery. After detachment of the epigastric adipocutaneous flap the unilateral inferior epigastric neurovascular bundle was ligated according to a randomization protocol to induce an ischemic area. This area was then shockwave treated using 100 pulses at 0.13 mJ/mm^2, followed by the creation of 1.3 cm^2 diameter wounds on the ischemic (ischemic) as well as on the adequately perfused side (vital) of the flap. (A and B) Wound size on the ischemic side was monitored at days 1, 5 and 10 post surgery and is depicted as percent of postoperative wound area (day 0) for each animal (n=9; mean+SEM; *P<0.05; two-tailed unpaired student's t-test or Mann-Whitney-U test (GSK-d5) for pairwise comparison at different time points).

FIGURE 9. Erk1/2 pathway inhibition impedes the proliferative effect of shockwave treatment *in vivo*. (A) Representative IHC sections of each study group at day 10 post surgery, showing HE, Ki-67 and p-Erk1/2 staining in control group and group receiving GSK1120212. Bar graphs depict percentage of (B) Ki-67 positive or (C) p-Erk1/2 positive cells of all cells assessed in ROIs (n=7; mean+SD; **P<0.01, ***P<0.001; two-tailed unpaired student's t-test comparing shockwave treated group of control

animals/animals receiving GSK1120212 inhibitor to corresponding untreated group, scale bars: 100 µm, ns: not significant).

FIGURE 10: Shockwave treatment triggers purinergic signaling and regulates cell function. Schematic illustration of how shockwave treatment induces intracellular signaling pathways in cells and tissue finally leading to enhanced proliferation and wound healing. Shockwaves exert a mechanical stimulus onto cells and tissue possibly leading to changes in membrane integrity or even actively opening of channels to release ATP. The released ATP is then binding to purinergic receptors, in this model predominantly P2Y receptors, leading to downstream signaling events. Uptake of ATP consequently activates p38 MAPK as well as Mek1/2 – Erk1/2 signaling pathways, ultimately leading to increased cellular proliferation *in vitro* and enhanced wound healing *in vivo*. If extracellular released ATP is either degraded by apyrase or P2Y receptors are blocked by suramin, the transduction of the stimulus into the cell to activate Erk1/2 is inhibited and the proliferative effect of shockwave treatment is abolished. Inhibition of Mek1/2 by using U0126 *in vitro* or GSK1120212 *in vivo* to block the activation of Erk1/2 results in the loss of the proliferative and wound healing effect of shockwave treatment. Together these signaling events transduce the primary mechanical stimulus of shockwaves resulting in proliferation and wound healing.

Figure 1

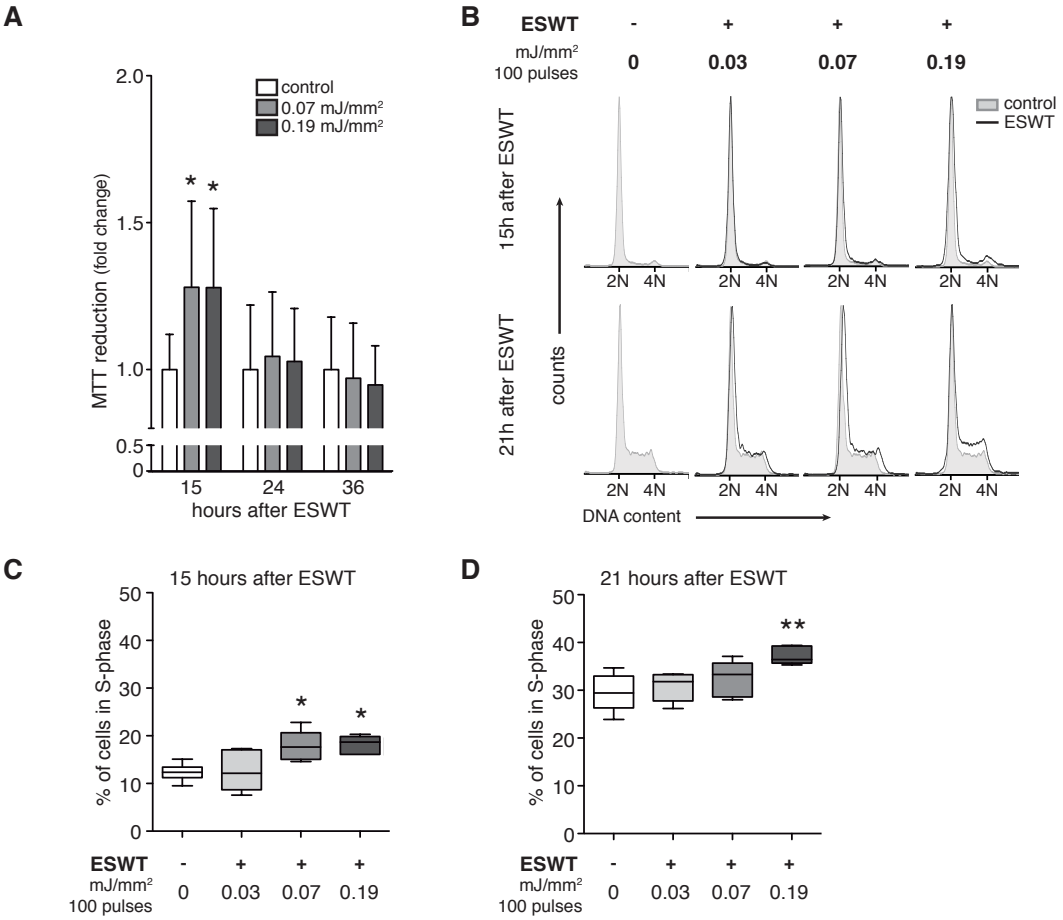
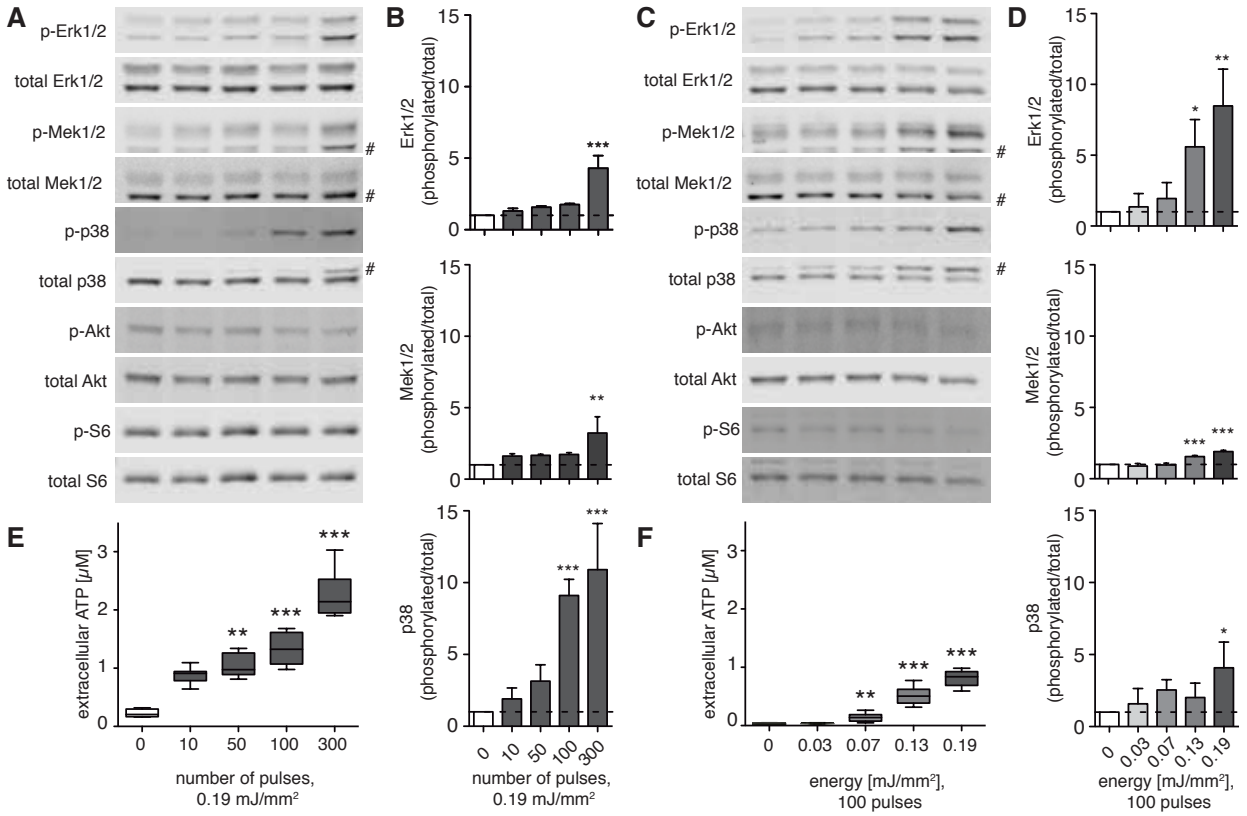


Figure 2



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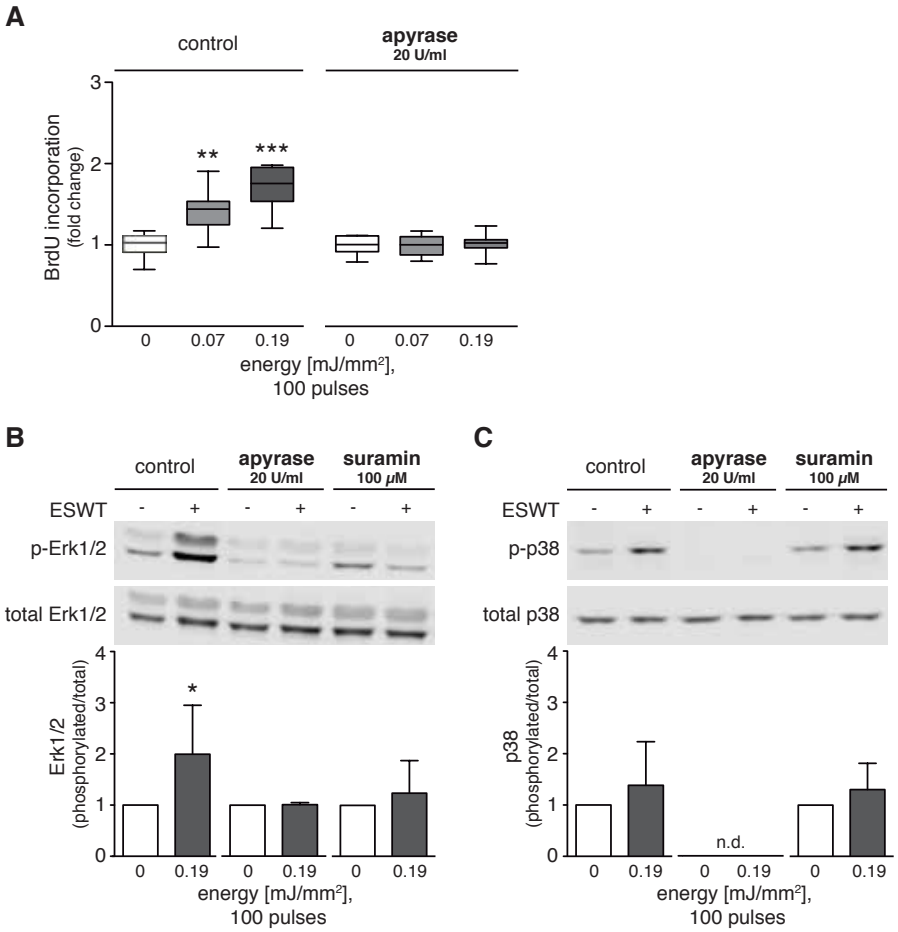

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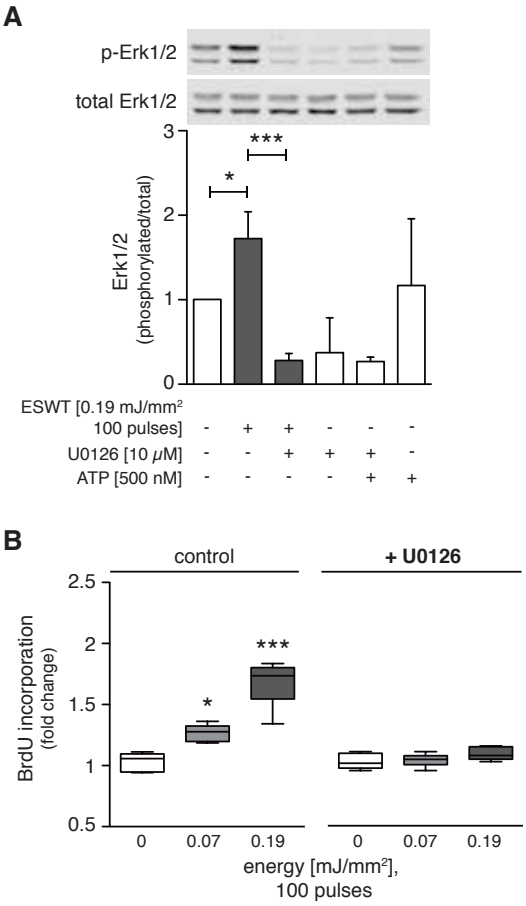

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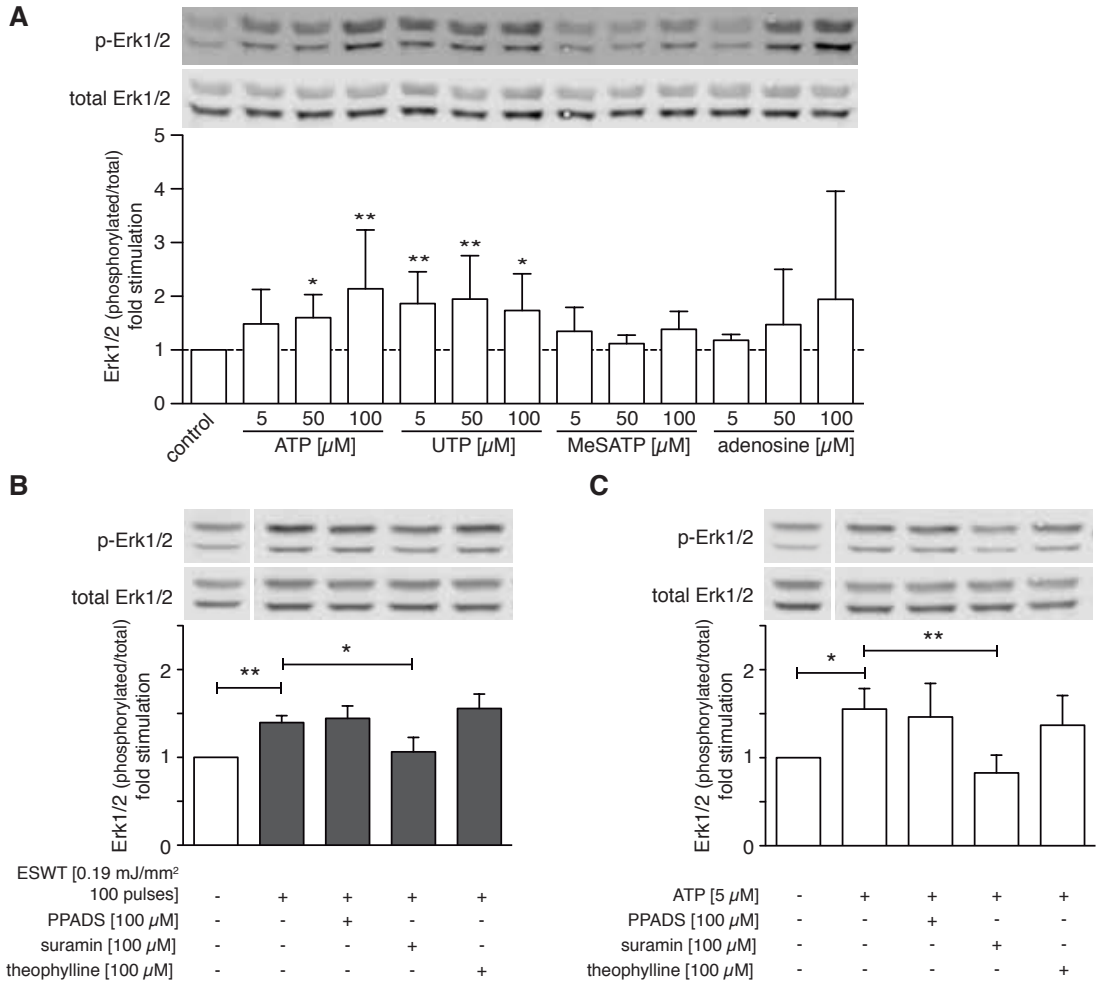

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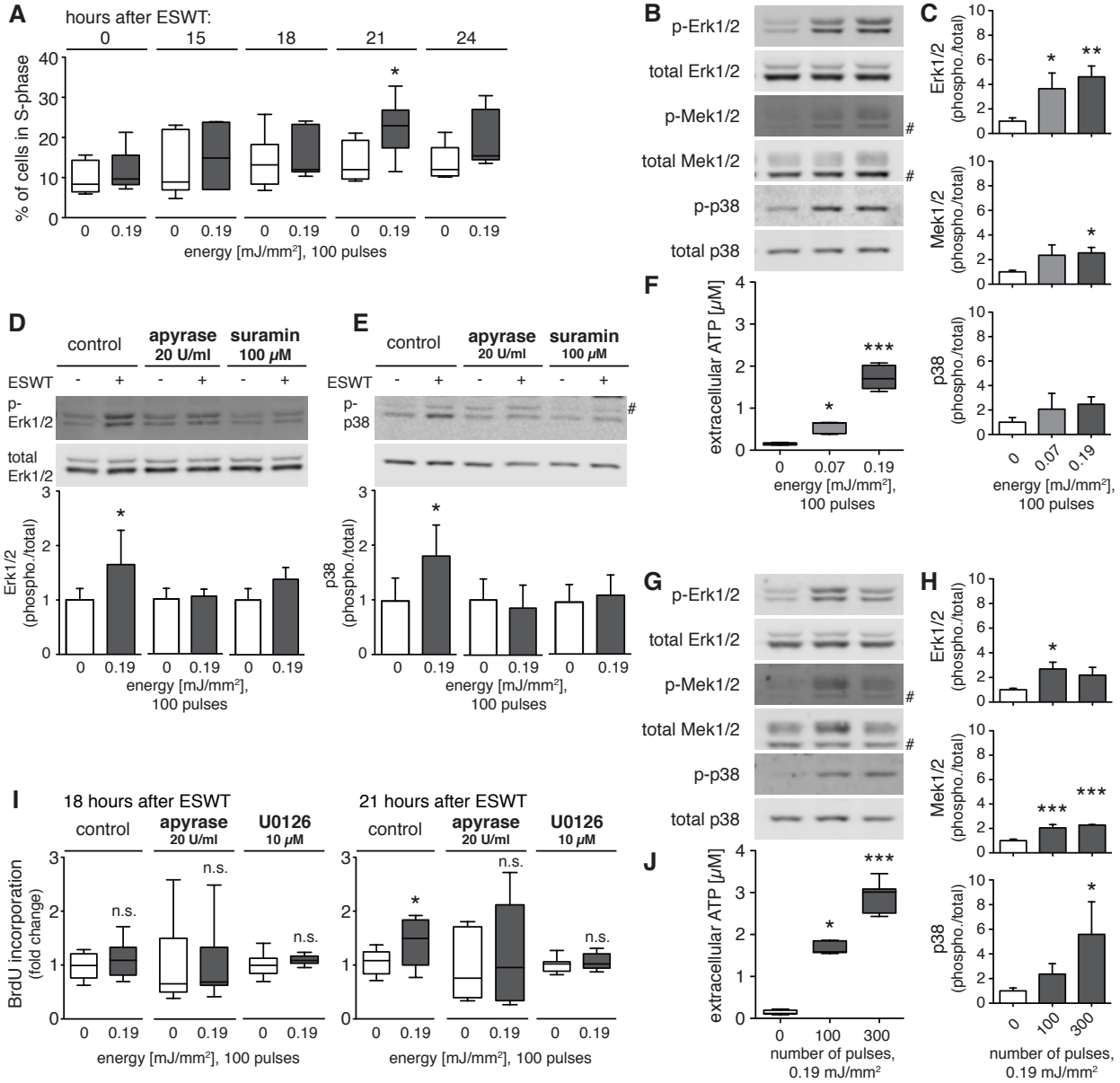

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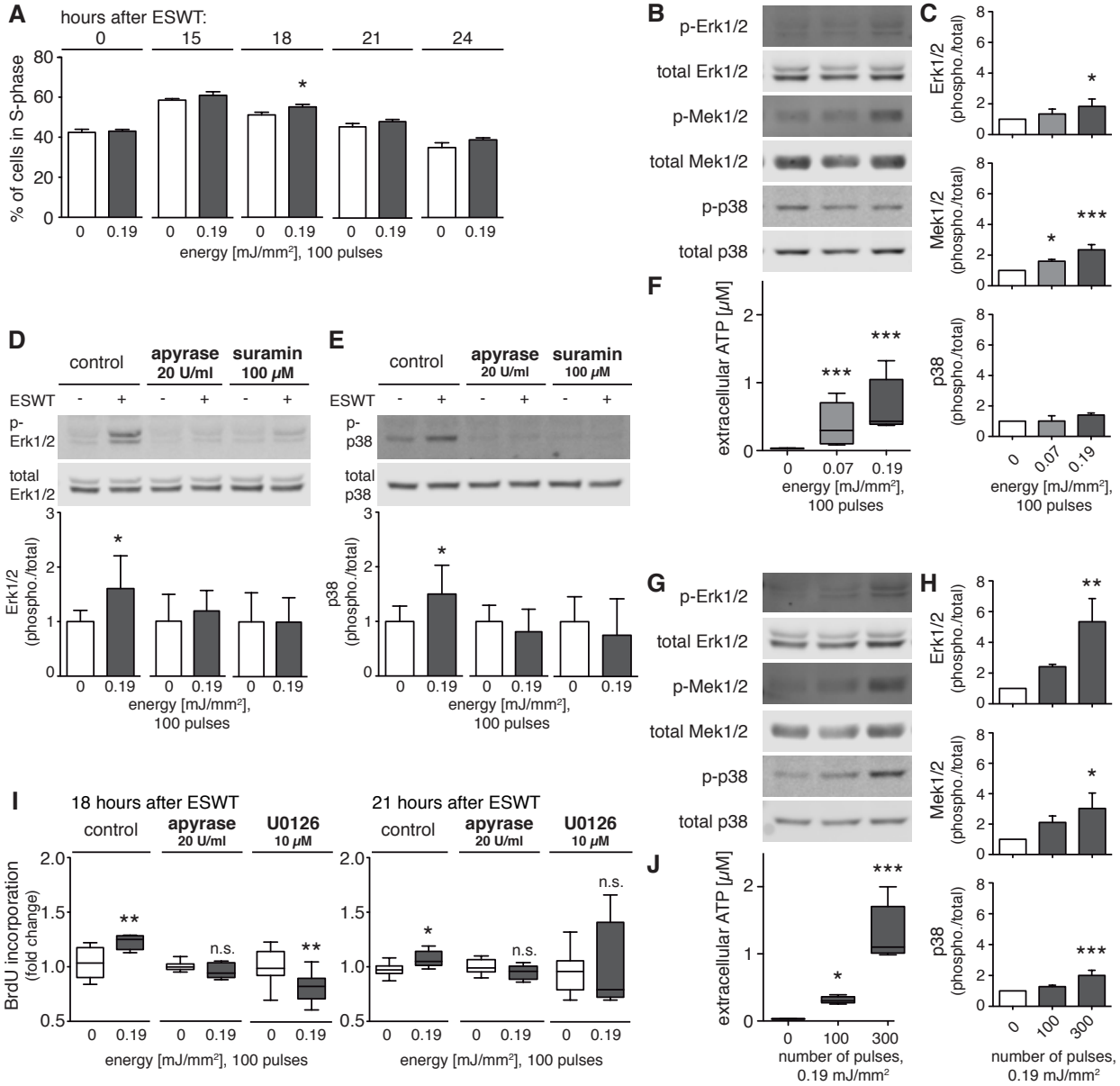

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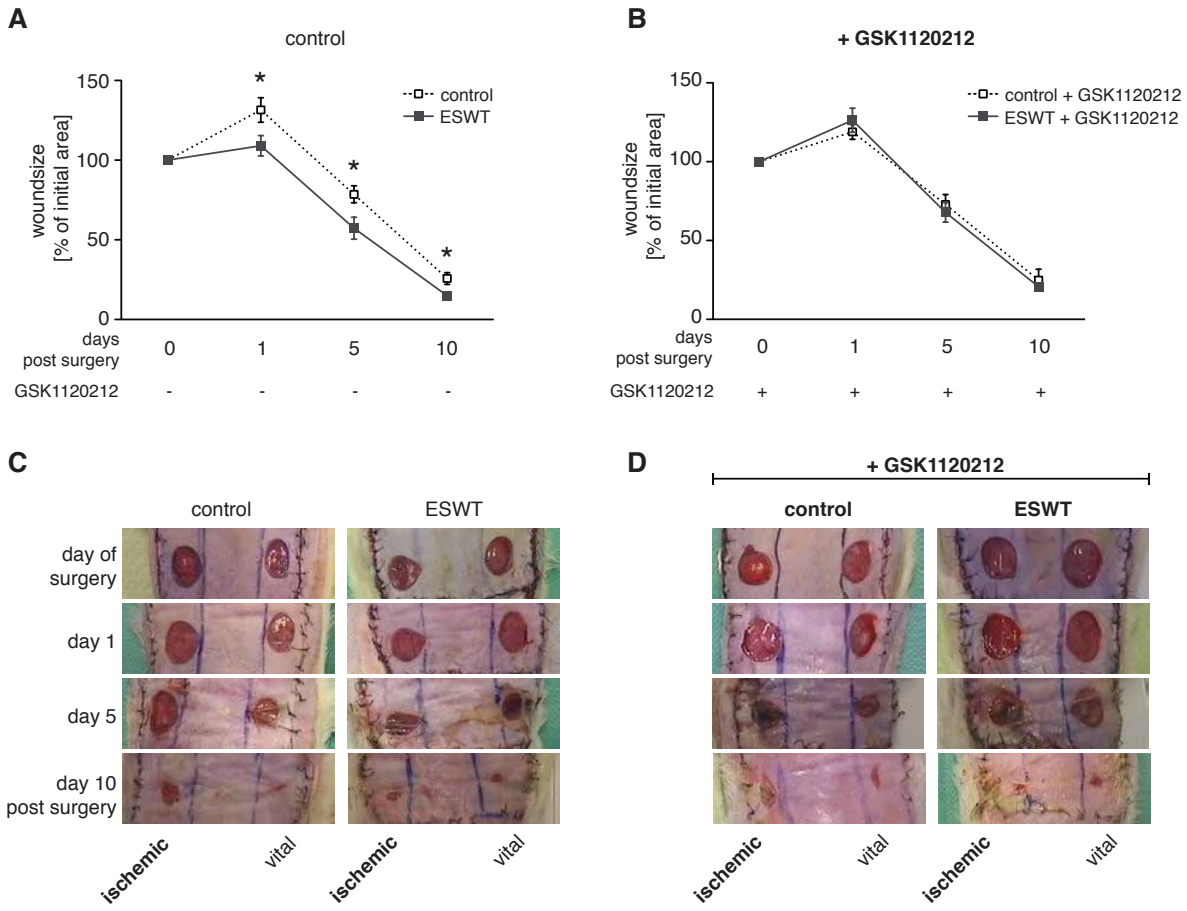

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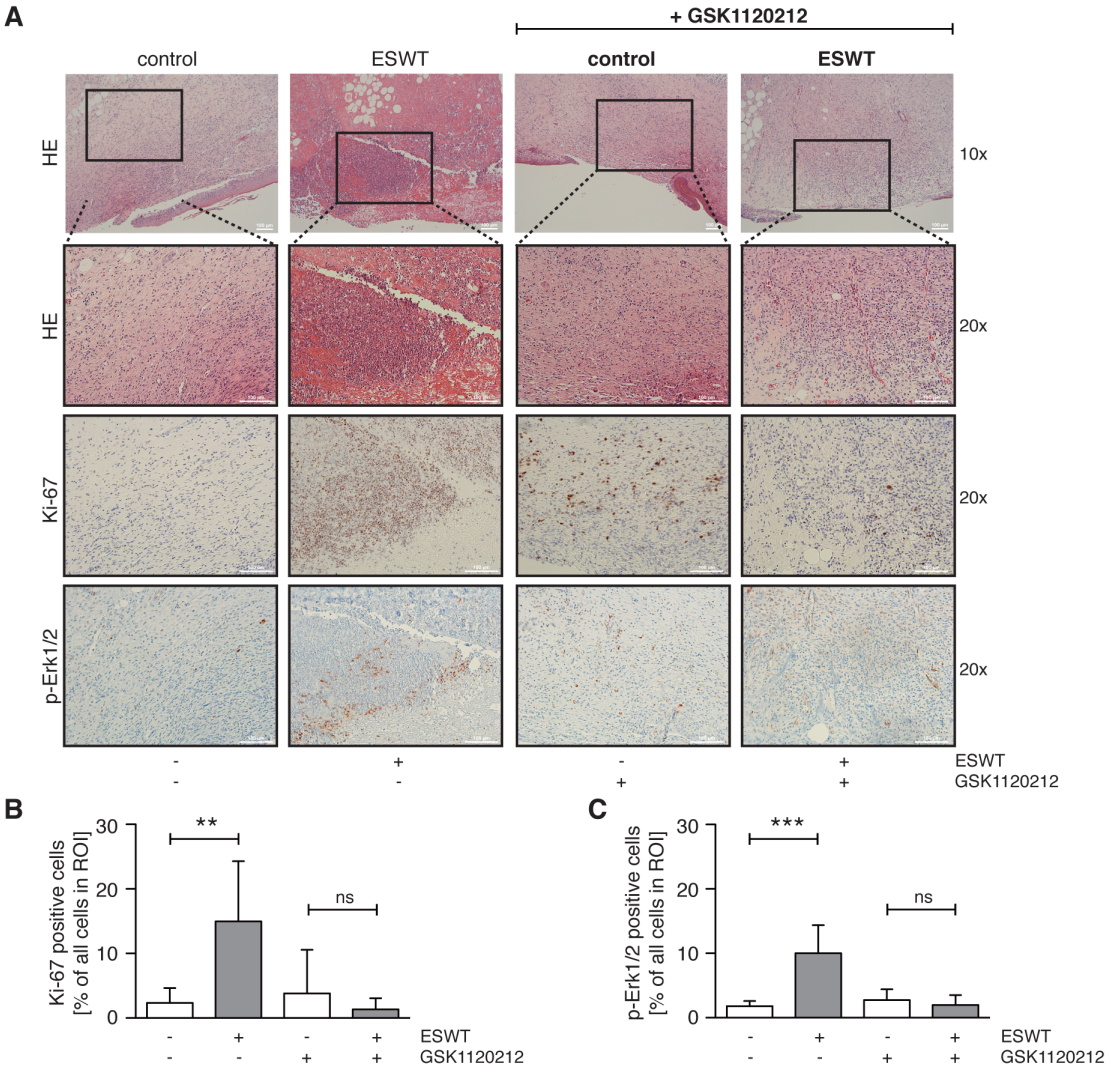

Figure 10