HGFA Is an Injury-Regulated Systemic Factor that Induces the Transition of Stem Cells into $G_{\text{Alert}}$

**Graphical Abstract**

**Highlights**

- Systemic factors control the $G_{\text{Alert}}$ transition of stem cells
- HGFA is a circulating HGF protease that is activated in response to injury
- Active HGFA is sufficient to stimulate $G_{\text{Alert}}$ in MuSCs and FAPs
- Pre-injury HGFA administration accelerates stem cell activation and tissue repair

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**In Brief**

Rodgers et al. show that HGFA is a systemic protease that is activated by tissue injury and relays a signal to stem cells in non-injured tissues that induces their transition into a primed, "$G_{\text{Alert}}$" state in which they possess an enhanced potential to activate and repair tissue damage.
HGFA Is an Injury-Regulated Systemic Factor that Induces the Transition of Stem Cells into $G_{\text{Alert}}$

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SUMMARY

The activation of quiescent stem cells into the cell cycle is a key step in initiating the process of tissue repair. We recently reported that quiescent stem cells can transition into $G_{\text{Alert}}$, a cellular state in which they have an increased functional ability to activate and participate in tissue repair. However, the precise molecular signals that induce $G_{\text{Alert}}$ in stem cells have remained elusive. Here, we show that the injury-induced regulation of hepatocyte growth factor (HGF) proteolytic processing via the systemic pro-tease, hepatocyte growth factor activator (HGFA), stimulates $G_{\text{Alert}}$ in skeletal muscle stem cells (MuSCs) and fibro-adipogenic progenitors (FAPs). We demonstrate that administering active HGFA to animals is sufficient to induce $G_{\text{Alert}}$ in stem cells throughout the body and to significantly accelerate the processes of stem cell activation and tissue repair. Our data suggest that factors that induce $G_{\text{Alert}}$ will have broad therapeutic applications for regenerative medicine and wound healing.

INTRODUCTION

Tissue damage induces the activation of quiescent stem cells, initiating a cascade in which stem cells enter the cell cycle, divide, and proliferate to generate the cells required to repair or regenerate damaged tissue (Cheung and Rando, 2013; Li and Clevers, 2010; Weissman, 2000). Stem cell activation is a limiting step in the process of tissue repair (McClain et al., 1996; Sugiuira et al., 2015). In many stem cell pools, the first cell division following activation is slow and can take many days to complete, whereas subsequent cell divisions are much more rapid (Coller, 2007; Laurenti et al., 2015; Siegel et al., 2011). Defects in stem cell activation, such as a lengthening in the time of first cell division or a failure in stem cells to activate, can result in significant impairments in the healing process (Jones and Rando, 2011). Little is known about the biologic regulation of stem cell quiescence and activation. Approaches to accelerate the rate-limiting step of stem cell activation could have broad therapeutic applications in regenerative medicine.

We previously reported an acceleration of the activation properties of quiescent stem cells in response to a prior injury, distant from the tissue in which the stem cells were residing (Rodgers et al., 2014). We described this regulation as a transitioning of stem cells from the $G_0$ to the $G_{\text{Alert}}$ state of quiescence, where $G_{\text{Alert}}$ stem cells are poised to activate quickly in response to injury and to repair tissue damage more effectively. Because of the enhanced functional properties of $G_{\text{Alert}}$ stem cells, there may be clinical applications for factors that induce the $G_{\text{Alert}}$ state. However, the endogenous signals that stimulate the $G_0$ to $G_{\text{Alert}}$ transition of stem cells in response to distant injuries have not been previously described. Here, we show that a single systemic factor, hepatocyte growth factor activator (HGFA), is sufficient to induce the transition of multiple pools of stem cells into $G_{\text{Alert}}$ and that administration of HGFA to animals, prior to an injury, improves the subsequent kinetics tissue repair.

RESULTS

Systemic Factors Mediate the Injury-Induced Transition of Stem Cells into $G_{\text{Alert}}$

The global response to injury that we observed, that injury to one part of the body resulted in the $G_{\text{Alert}}$ transition of many different types of stem cells in uninjured areas throughout the body (Rodgers et al., 2014), suggested that a systemic factor is involved. To test this hypothesis, we injected animals with serum isolated from mice that had been subjected to a muscle injury 2.5 days prior to serum isolation (we term this “injured serum”), and we compared this to serum obtained from mice that were not injured (“non-injured serum”) (Figure 1A). We sacrificed the animals 2.5 days after serum injection, FACS (fluorescence-activated cell sorting)-purified the muscle stem cells (MuSCs), and assayed the MuSCs for characteristics of stem cells in $G_{\text{Alert}}$. Using time-lapse microscopy to analyze the kinetics of cell division, we found that MuSCs isolated from animals injected with injured
serum required less time to complete the first cell division after isolation than did the MuSCs isolated from animals injected with non-injured serum (Figure 1B). Similarly, using incorporation of EdU (5-ethyl-2'-deoxyuridine) nucleotide to identify cells that had entered S-phase of the cell cycle, we found that a higher percentage of MuSCs isolated from animals injected with injured serum had entered the cell cycle 24 hr after isolation (Figure 1C). MuSCs from animals injected with injured serum were also slightly larger than MuSCs from animals injected with non-injured serum, as measured by an increase in forward scatter (Figures 1D and S1A). We also observed a similar set of functional and phenotypic changes in fibro-adipogenic progenitors (FAPs) isolated from animals injected with injured serum (Figures 1E, 1F, and S1B). Activation of mTORC1 (mechanistic target of rapamycin complex 1) signaling is the key molecular change in G0/G1 arrest of stem cells, and, using genetic models, we demonstrated that it is necessary and sufficient for the transition of MuSCs into G0/G1 arrest (Rodgers et al., 2014). Using Pax7 antibodies to identify sub-laminar quiescent MuSCs in tibialis anterior (TA) muscle sections (Seale et al., 2000), we found that a higher frequency of MuSCs were positive for phosphorylated S6 (pS6), a marker of mTORC1 signaling, in muscles from animals injected with injured serum (Figure 1G). Collectively, these data show that injurious animals with arrested serum is sufficient to induce a set of molecular, phenotypic, and functional changes in stem cells that are similar to the injury-induced G0/G1 arrest response and suggest that the factor or factors that induce the transition of stem cells into G0/G1 arrest are systemic in nature.

Our previous work identified that cMet was required for the injury-induced transition of MuSCs into G0/G1 arrest (Rodgers et al., 2014). To test whether injured serum signaled through the same pathway, we utilized the Pax7CreER driver and a cMetCreERtop mouse strain to specifically ablate cMet expression, conditional upon tamoxifen administration, in MuSCs. Using a similar experimental design as described earlier (Figure 1A), we found that MuSCs from these cMet conditional-knockout (cKO) mice displayed no phenotypic or functional response to injured serum (Figures S1C–S1E). As a control, we analyzed FAPs that were isolated in parallel with MuSCs from cMet cKO animals and found that they displayed a similar response to injured serum as that of FAPs from control mice (Figures 1E and S1F). As FAPs do not express Pax7 (Joe et al., 2010), they remain wild-type (in terms of cMet expression) in cMet cKO animals. These data suggest that signaling through cMet is required for the response of MuSCs to injured serum.

**HGFA Is an Injury-Regulated Systemic Factor**
cMet is a receptor tyrosine kinase that is activated by the binding of hepatocyte growth factor (HGF) (Trusolino et al., 2010). A plausible mechanism for injured-serum-mediated induction of G0/G1 arrest is that injured serum contains higher levels of HGF. To test this, we measured serum HGF levels by ELISA. Surprisingly, we found no differences in serum HGF levels when comparing serum samples from injured and non-injured mice (Figure S1G). Also surprising was that we were unable to induce the transition of MuSCs into G0/G1 arrest in vivo by administering exogenous, purified, active HGF to animals (data not shown). However, and importantly, both MuSCs and FAPs responded to direct HGF stimulation, ex vivo, by activating mTORC1 signaling (Figure S1H). Even though both MuSCs and FAPs can respond to HGF, we obtained no evidence that circulating HGF accounted for the in vivo induction of G0/G1 arrest by injured serum. These results suggest that a different systemic factor is responsible for activating signaling through the HGF-cMet pathway.

HGF is resident in the extracellular matrix (ECM) of most tissues in a latent, biologically inactive form (Fajardo-Puerta et al., 2016; Stoker et al., 1987). In order to possess biologic activity (i.e., bind to and activate cMet), HGF must undergo...
The proteolytic processing of a unprocessed single-chain form (pro-HGF) into a processed two-chain form (active HGF) (Gak et al., 1992). Western blot analysis revealed a clear increase in levels of processed, active HGF in muscles contralateral to an injury (Figure S1I). Thus, the induction of active HGF in muscles contralateral to injury suggests that the pathways that regulate HGF processing are induced in response to injury.

To test whether systemic factors could directly mediate the processing of HGF, we measured the ability of serum to catalyze the proteolytic cleavage of recombinant pro-HGF into active HGF in vitro. In HGF-processing assays, we found that injured serum possessed twice the HGF-processing activity as non-injured serum (Figure 1H and S2A). These results suggest a mechanism in which systemic factors regulate the processing of tissue-resident HGF and that local (now active) HGF signals through cMet-mTORC1 to induce the G₀-to-G₁ transition of MuSCs. Moreover, these results suggest that an HGF-processing enzyme is the “alerting” signal in injured serum.

HGF is the primary HGF protease; is very abundant in the blood; and, like HGF, is also regulated by proteolytic cleavage (Miyazawa, 2010). HGF is processed from an enzymatically inactive single-chain form (pro-HGF) into a two-chain form (active HGF) that possesses HGF-processing enzymatic activity (Kataoka and Kawaguchi, 2010) (Figure 1I). The site on which HGF is processed to induce enzymatic activity is a consensus thrombin cleavage site, and it is well established that both thrombin and HGF activity are induced in response to injury (Coughlin, 2000; Miyazawa, 2010; O’Reilly et al., 2008; Shimomura et al., 1993). Consistent with this, we found that injured serum contained a higher proportion of HGFA in the active form (Figures 1J and S2B). These results suggest that active HGFA is the factor in injured serum that induces the transition of stem cells into G₁.

To test whether HGFA enzymatic activity is the crucial component of injured serum that induced G₁ transition, we utilized a monoclonal antibody (Ab40) that was developed to specifically inhibit HGFA enzymatic activity (Figure S2O) (Ganesan et al., 2009). We incubated injured and non-injured serum with Ab40 or control immunoglobulin G (IgG) prior to injection of the serum samples into mice (Figure 1K). Two and a half days after injection, we analyzed HGF processing in the TA muscles of these animals and found that Ab40 blocked the injured-serum-mediated induction of active HGF (Figure S2D). Consistent with the decrease in active HGF, we found that Ab40 significantly reduced—and, in the case of FAPs, completely abolished—the injured-serum-mediated induction of EdU incorporation in MuSCs and FAPs (Figure 1L). By contrast, Ab40 did not have any significant effect on the EdU incorporation of MuSCs and FAPs when it was pre-incubated and injected with non-injured serum (Figure 1L). These results suggest that HGFA enzymatic activity is necessary for the ability of injured serum to induce the G₁ transition.

Active HGFA Is Sufficient to Induce the G₁ Transition of Stem Cells

Next, we tested if active HGFA itself is sufficient to recapitulate the effects of injection of injured serum. To do this, we obtained purified, recombinant, active HGFA (hereinafter referred to as “HGFA”) and administered it to animals (Figure 2A). Strikingly, we found that a single intravenous dose of HGFA was sufficient to induce the G₁ transition of MuSCs and FAPs. Animals injected with HGFA had a higher frequency of pS6+ MuSCs (Figure 2B). Upon isolation and culturing, MuSCs from animals administered HGFA displayed faster kinetics of cell-cycle entry and cell division (Figures 2C, 2D and S2E). MuSCs from HGFA-injected animals also displayed an increase in cell size (Figures 2E and S2F). Importantly, we observed a similar set of molecular, phenotypic, and functional changes in FAPs (Figures 2F–2H, S3A, and S3B). These data show that administering an active form of the serum HGF protease, HGFA, is sufficient to induce the G₁ transition of both MuSCs and FAPs.

Pre-injury Administration of HGFA Improves the Kinetics of Tissue Repair

A key physiological observation about the functional changes in G₁ stem cells is that they strongly correlate with enhancements in the process tissue repair (Rodgers et al., 2014). Therefore, we tested whether administration of HGFA affects tissue repair. To do this, we administered a single dose of HGFA or vehicle (PBS) to animals via the tail vein 2 days before we subjected the animals to muscle injury (Figure 3A). One metric of injury-induced muscle regeneration is the size of nascent muscle fibers, which are small immediately following injury and gradually increase in size (Figure 3B). We found that HGFA stimulated a notable enhancement in the progression of muscle fiber size (Figures 3B and 3C). These data suggest that, compared to control, the injured TA muscles from HGFA-injected mice are at an advanced stage in muscle regeneration. HGFA did not have an effect on the number of regenerating muscle fibers in injured muscle (Figure S3C). Taking this a step further, we found that animals that were injected with HGFA displayed faster recovery in their wheel-running behavior following injury to a gastrocnemius muscle (Figure 3D), suggesting an improvement in functional recovery from muscle injury (Carmichael et al., 2005; Knab et al., 2009).

The improved recovery of HGFA-injected animals from muscle injury suggests that HGFA may have a clinical application in contexts where muscle injury can be anticipated, such as surgery. Therefore, we tested whether HGFA has an effect on another common aspect of surgery, skin wound healing. We found that animals that had been administered HGFA 2 days prior to being subjected to full-thickness skin wounds healed these wounds more quickly (Figures 3E and 3D). Interestingly, at later time points following skin wounding, we observed that animals injected with HGFA had more complete regrowth of hair in the shaved area (Figure 3F). Aspects of both skin wound healing and the initiation of hair growth are dependent upon the activation of quiescent stem and progenitor cells (Arwert et al., 2012). The improvements that we observed suggest that HGFA induced the cells required for these processes into a G₁ state that is similar to that of the stem cells in the muscle compartment.

DISCUSSION

In most tissues, efficient and successful repair is dependent upon the coordinated activation of many different pools of stem and progenitor cells. We suspect that the HGFA-mediated improvements in tissue repair that we observe are due to a
coordinated GAlert transition of many cell types. Indeed, many populations of stem and progenitor cells—such as mesenchymal stem cells, epidermal stem cells, keratinocytes, and hematopoietic stem cells—have been shown to express cMet and could respond to HGFA via a similar mechanism as MuSCs (Allen et al., 1995; Chmielowiec et al., 2007; Neuss et al., 2004; Tesio et al., 2011). Another potential mechanism by which HGFA could induce a cellular response is through the proteolytic activation of the other known substrate of HGFA, macrophage-stimulating protein (MSP), and its signaling through the RON receptor tyrosine kinase (Kataoka and Kawaguchi, 2010). The data we present here, as well as our previous work, provide evidence that several different stem cell pools adopt a functionally similar GAlert state in response to a common stimulus. Combined, they suggest that HGFA is a central and single factor that is capable of “alerting” many types of stem cells throughout the body.
priming them for an accelerated functional response to injury. We propose that HGFA could have applications as a treatment administered prior to situations where tissue damage is expected, such as surgery or combat, and that this constitutes a conceptually different treatment strategy for regenerative medicine.

EXPERIMENTAL PROCEDURES

Statistics

Data are presented as mean or geometric mean ± SEM and overlaid with the individual biologic replicate measurements. In figures presented as bar graphs, the mean or geometric mean of the biologic replicates is represented as a column, and individual biologic replicates are represented as open circles. When experiments involved the isolation and analysis of primary cells, they were performed as paired measurements (i.e., control versus experimental, performed on the same day, in parallel, using the same instrumentation) and are presented as lines connecting the individual paired measurements. This is to control for day-to-day variability in the preparation of primary cells and subtle differences in FACS instrumentation, settings, and gating. Bar graphs summarizing paired measurements are presented as the geometric mean ± SEM. Time-lapse analyses are presented in two ways: as cumulative histograms, binned per hour, and displayed as the mean ± SEM of the percentage of the cells that had divided at each bin; and as bar graphs displaying the

Figure 3. Administering HGFA prior to an Injury Accelerates the Process of Tissue Repair

(A) Schematic depicting experimental design testing the effects of a single dose of HGFA or vehicle (PBS) administered 2 days prior to subjecting animals to muscle injury.

(B) HGFA accelerates muscle regeneration. Data are presented as the mean cross-sectional area (CSA) of nascent muscle fibers from TA muscles at indicated days post-injury (DPI) as measured by IF-IHC analysis. Data are presented as mean ± SEM. Replicate values are presented as open circles (HGFA) or triangles (PBS) (HGFA: 5 DPI, n = 6; 10 DPI, n = 4; 16 DPI, n = 2; 21 DPI, n = 5. PBS: 5 DPI, n = 6; 10 DPI, n = 4; 16 DPI, n = 4; 21 DPI, n = 4). Dashed lines represent the mean CSA of muscle fibers in TA muscles contralateral to injury, measured 21 days after injury. Significance was calculated by two-way ANOVA.

(C) Representative IF-IHC staining with laminin (green) and DAPI (blue) of TA muscles at 21 DPI. Scale bars, 500 μm in the top row of images and 50 μm in the bottom row.

(D) Animals injected with HGFA display improvements in running behavior following injury. Data are presented as the wheel-running distance in the 24-hr period prior to the time point; mean ± SEM (n = 7). Significance was calculated by two-way ANOVA.

(E) Animals injected with HGFA show improvements in skin-wound healing. Quantification of wound closure is presented as the mean ± SEM of the percentage of initial wound size (n = 4). Significance was calculated by two-way ANOVA.

(F) Animals administered HGFA prior to shaving and skin wounding display improvements in hair regrowth. Presented are representative images of animals 28 days post-injury.

See also Figure S3.
mean time to cell division for each replicate sample. Analysis of the forward scatter (FSC) by FACS is presented as a representative FACS histogram plot and a bar graph displaying the mean of the median FSC ± SEM of all replicate experiments. All replicate data points represent individual biologic replicates (i.e., different mice). Except when stated otherwise, statistical significance (p value) was calculated using two-tailed Student's t test, unpaired or paired where appropriate. The statistical significance of the data presented in Figure 3 was calculated using two-way ANOVA (GraphPad, Prism).

**Mice**
Pax7CreERT2 mice were provided by Dr. Charles Keller (Oregon Health & Science University; OHSU), Rosa26YFP and cmetCreER mice were obtained from Jackson Laboratories. All experiments were performed with 12- to 20-week-old male C57BL/6 mice (Jackson Laboratories), except for experiments using conditional cmet KO mice, which had a mixed background of C57BL/6 and FVB. Animals were genotyped by PCR of tail DNA; primer sequences are available upon request. Tamoxifen (Sigma) was prepared in 7% ETOH and corn oil and administered to 8- to 12-week-old mice in five doses of 5 mg per mouse every 2–3 days by intraperitoneal injection. Mice were housed and maintained in the Veterinary Medical Unit at the Veterans Affairs Palo Alto Health Care System. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) board of the VA Palo Alto and/or University of Southern California (USC).

**HGFA Administration**
Purified, recombinant-active HGFA (R&D Systems, #1200-SE) was administered via intravenous tail vein injection at a dose of 1 μg diluted into 200 μL of sterile PBS. Control injections were performed using 200 μL of sterile PBS. Experiments using HGFA were performed over the course of 2 years utilizing HGFA obtained from several different lots (HG07152A, HG0714051, HG0715011, HG0614041, and HG0613101). The specific activity of each lot was validated prior to use.

**Serum Isolation and Injection**
Serum was isolated from blood samples collected via cardiac puncture immediately after CO2 euthanasia of the animals. Blood was left to clot for 1 hr, subject to centrifugation, and serum was isolated by removing the upper clear layer of the blood sample. Serum samples were stored at −80°C until use. Serum was administered to animals via intravenous tail vein injection. Injections were performed in a total volume of 200 μL, with serum diluted 1:1 with sterile PBS. In experiments utilizing serum, each replicate measurement represents a biologic replicate serum sample (i.e., serum from a different mouse).

**Muscle Injury**
Muscle injuries were induced by intramuscular injection of BaCl2. Animals were anesthetized using isoflurane, and 30 μL of 1.2% BaCl2 (w/v H2O) was injected along the length of a TA muscle, or 70 μL was injected into the gastrocnemius muscle. Animals were then administered buprenorphine and Baytril and allowed to recover. Sham muscle injuries were performed by anesthetizing the mice, followed by administration of buprenorphine and Baytril.

**Cell Isolation, FACS Purification, and Cell Culture**
MuSCs and FAPs were purified using FACS as previously described (Liu et al., 2015). MuSCs were purified as a population of YFP+ cells when utilizing mice containing the alleles Pax7CreERT2; Rosa26YFP or as a population of CD31+;CD45−; Sca-1−; VCAM+ cells. FAPs were purified as a population of CD31+;CD45−; Sca-1+; cMet+ cells. Antibodies used for FACS isolation are listed in Figure S3E. All experiments that involve the comparison of primary cells were performed in parallel using the same FACS instrumentation (BD FACSaria II or IIIB). Cells were sorted into plating medium (Ham’s F-10 [Cellgro], 10% FBS [Invitrogen], 5 ng/mL basic fibroblast growth factor [bFGF] [Invitrogen] and 1× Pen/Strep [penicillin/streptomycin] [GIBCO]). After isolation, MuSCs and FAPs were plated and cultured on poly-D-lysine (Millipore) and ECM (Sigma E1270)-coated eight-well chamber slides (Lab-Tech II). Twelve hours after isolation, the medium was changed to culturing medium (Ham’s F-10 [Cellgro], 10% FBS [Invitrogen], 10% horse serum [Invitrogen], and 1× Pen/Strep [GIBCO]). Cells were then used for EdU incorporation assays or time-lapse microscopy.

**EdU Incorporation Assay**
Twelve hours after isolation, EdU was added to culturing medium at a final concentration of 10 μM, and cells were then fixed at either 24 or 40 hr after plating. EdU was detected using the Click-It Kit (Invitrogen) according to the manufacturer’s instructions. Data on EdU incorporation are presented as the percentage of total cells (measured by DAPI).

**Time-Lapse Microscopy**
After changing to culturing medium, chamber slides were transferred to a temperature- and CO2-controlled Zeiss Axio Observer Z1. Time-lapse acquisition was performed under 10× magnification, capturing images every 10 min, and controlled by Axiovision software. Time to division was recorded only for cells that stayed in the field of view during acquisition and was presented as the time from plating. For each biologic replicate, the time to division was manually measured for at least 40 cells.

**In Situ Analysis of Muscle Fiber Size and pS6**
Immediately after euthanasia, TA muscles were dissected, mounted onto tragacanth gum, and snap-frozen in liquid-N2-cooled isopentane. After cryosectioning, 8-μm muscle sections were fixed in 4% paraformaldehyde (PFA) for 5 min and washed with PBS with 0.3% Triton. Muscle fiber size measurements were performed on TA muscle section taken at 1/3 to 1/2 the distance from the distal end of the TA. Muscle sections were stained with laminin antibodies to outline muscle fibers, and fiber area was measured by morphometric analysis of centrally nucleated muscle fibers (ImageJ). For each biologic replicate, at least 300 muscle fibers along the lateral/anterior border of the TA muscle were analyzed.

**pS6 Analysis**
To identify MuSCs, muscle sections were stained with Pax7 antibodies using the M.O.M. Kit (Vector) according to the manufacturer’s instructions. After Pax7 staining, sections were stained with phospho-S6 and laminin antibodies overnight. FAPs were identified as PDGFR-alpha-positive cells in the interstitia between muscle fibers. Antibodies and concentrations used are listed in Figure S3E. Data on pS6 staining are presented as the percentage of sub-laminar Pax7-positive cells or PDGFR-alpha-positive cells that possessed detectable pS6 signal; at least 50 MuSCs or FAPs were analyzed per biologic replicate.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at dx.doi.org/10.1016/j.celrep.2017.03.066.

**AUTHOR CONTRIBUTIONS**

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Supplemental Information

HGFA Is an Injury-Regulated Systemic Factor that Induces the Transition of Stem Cells into $G_{Alert}$

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Figure S1

A

\[
\begin{align*}
\text{Median FSC} & \quad 150 & \quad 350 \\
\text{Non-injured serum} & \quad \square & \quad \blacklozenge \\
\text{Injured serum} & \quad \blacklozenge & \quad \square \\
\end{align*}
\]

B

\[
\begin{align*}
\text{Median FSC} & \quad 150 & \quad 350 \\
\text{Non-injured serum} & \quad \square & \quad \blacklozenge \\
\text{Injured serum} & \quad \blacklozenge & \quad \square \\
\end{align*}
\]

C

\[
\begin{align*}
\% \text{ of dividing cells} & \quad 0 & \quad 100 \\
\text{Time after plating (hrs)} & \quad 20 & \quad 100 \\
\text{Injured serum} & \quad \text{---} & \quad \text{---} \\
\text{Non-injured serum} & \quad \text{---} & \quad \text{---} \\
\end{align*}
\]

D

\[
\begin{align*}
\text{Cell} \times \text{MuSCs} & \quad 0 & \quad 6 \\
\text{Count} & \quad 0 & \quad 6 \\
\text{FSC} & \quad 150 & \quad 350 \\
\text{Non-injured serum} & \quad \square & \quad \blacklozenge \\
\text{Injured serum} & \quad \blacklozenge & \quad \square \\
\end{align*}
\]

E

\[
\begin{align*}
\text{FSC} & \quad 150 & \quad 350 \\
\text{Median FSC} & \quad 200 & \quad 300 \\
\text{Non-injured serum} & \quad \square & \quad \blacklozenge \\
\text{Injured serum} & \quad \blacklozenge & \quad \square \\
\end{align*}
\]

F

\[
\begin{align*}
\text{EdU} \times \text{FAPs} & \quad 0 & \quad 3 \\
\text{Non-injured serum} & \quad \text{---} & \quad \text{---} \\
\text{Injured serum} & \quad \text{---} & \quad \text{---} \\
\end{align*}
\]

G

\[
\begin{align*}
\text{Serum HGF (ng/ml)} & \quad 0 & \quad 10 \\
\text{Days after injury} & \quad 0 & \quad 2.5 \\
\text{Non-injured} & \quad 0 & \quad 8 \\
\end{align*}
\]

H

\[
\begin{align*}
\text{Serum Starve} & \quad \text{overnight} & \quad \text{6 hrs} & \quad +/\text{-} \text{HGF} & \quad \text{30 min} & \quad \text{Fix Cells} \\
\text{FACS purify MuSCs/FAPs} & \quad \text{and plate} & \quad \text{Serum} \\
\text{MuSCs} & \quad \text{pS6} & \quad \text{DAPI} \\
\text{FAPs} & \quad \text{pS6} & \quad \text{DAPI} \\
\end{align*}
\]

I

\[
\begin{align*}
\text{Noninjured Animal} & \quad \text{Contralateral to injury} \\
\text{HGF} & \quad \text{pro-HGF} & \quad \text{active HGF} \\
\text{WB: HGF} & \quad \text{WB: Gapdh} \\
\end{align*}
\]
Figure S1. cMet is required for the MuSC response to injured serum. Relates to Figure 1
A) MuSCs in animals injected with injured serum are larger. Data from FSC measurements of MuSCs by FACS are presented as geometric mean ± s.e.m. (n = 7).
B) FAPs in animals injected with injured serum are larger. Data from FSC measurements of FAPs by FACS are presented as geometric mean ± s.e.m. (n = 5).
C) MuSCs from cMet cKO animals display no changes in time to first division in response to injured serum. A cumulative histogram of the time to first division is displayed on the left (n = 2) and a bar graph of the mean time to division on the right; NS denotes not statistically significant.
D) Injured serum does not improve the cell cycle entry kinetics of cMet cKO MuSCs. Data from EdU incorporation assays are presented as geometric mean ± s.e.m. of the percentage of cells which were EdU+ 24 hours after isolation (n = 3).
E) Injured serum does not increase the size of cMet cKO MuSCs. Data are presented as a representative FACS histogram of the FSC parameter (left) and a bar graph of replicate experiments (right) (geometric mean ± s.e.m.; n = 3).
F) MuSC-specific cKO of cMet does not alter the response of FAPs to injured serum. A higher percentage of FAPs isolated from cMet cKO mice (cMetflx/flx; Pax7creER/+.Rosa26EYFP+/+) injected with injured serum incorporated EdU after 24 hours in culture. Data from EdU incorporation assays are presented as a bar graph of the geometric mean ± s.e.m. (n = 3).
G) Serum HGF levels do not change in response to injury. Serum samples were prepared from non-injured or injured mice at indicated days after injury and HGF levels were measured by ELISA. Bar graphs represent mean ± s.e.m. (non-injured, n = 26; 1 day, n = 5; 1.5 days, n = 4; 2.5 days, n = 15; NS denotes not statistically significant).
H) MuSCs and FAPs induce mTORC1 activity in response to ex vivo HGF stimulation. The day following isolation, serum starved MuSCs and FAPs were stimulated with indicated levels of recombinant, active HGF. Thirty minutes after stimulation, cells were fixed and mTORC1 activity was assayed by IF staining for pS6. Data are displayed as percentages of cells that are pS6+ (mean ± s.e.m., n = 3). Displayed on the right are representative images of pS6 staining of MuSCs and FAPs treated with 0 or 100 ng/ml HGF. The scale bar is 50 µm.
I) Levels of active HGF are increased in muscles contralateral to injury. Western blotting of whole TA muscle tissue extracts showed a clear increase in active HGF in muscles that were contralateral to the site of muscle injury (2.5 days after injury). Purified recombinant active human HGF (rHGF) was run as control for the size of active HGF.
**Figure S2**

**A**

Serum + rHGF-His$_6$-Ni-Agarose  
↓ React - 5 Hrs  
↓ Wash beads, SDS-PAGE

**B**

pro-HGFA

---

Theoretical MW: 70kDa  
Apparent MW: ~120kDa

active HGFA

---

L-chain ~39kDa

**C**

Reaction Rate (Δ fl. units/min)

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- IgG
- Ab40
- No Enzyme

**D**

Non-injured serum  
Injured serum  
Injured serum + IgG  
Injured serum + Ab40  
rHGF  

WB: HGF  
↓ pro-HGF  
↓ active-HGF

**E**

DAPI

EdU

PBS

HGFA

**F**

Median FSC

- PBS
- HGFA

P = 0.018
**Figure S2. The activity of serum HGFA increases following injury.** Relates to Figure 1.

A) Injured serum has greater HGF-processing activity than non-injured serum. Displayed is a schematic depiction of HGF-processing assay (left) and of pro- and active HGF proteins (middle). On the right is Western blot analysis of the HGF-processing reactions.  
B) Injured serum contains a higher proportion of HGFA in the active form. Displayed on the left is a schematic depiction of pro- and active HGFA proteins. In the middle is detection and molecular weight validation of pro- and active HGFA using purified recombinant proteins by Western blotting. On the right is Western blotting of injured and non-injured serum samples with HGFA antibodies.  
C) Ab40 inhibits HGFA enzymatic activity *in vitro*. Purified mouse recombinant HGFA was mixed with Ab40 or control IgG and HGFA enzymatic activity measured by cleavage of a fluorogenic substrate peptide. Data are displayed as mean ± s.e.m. of the reaction rate at each antibody concentration.  
D) Ab40 blocks the injured serum-mediated processing of pro-HGF into active HGF *in vivo*. Western blotting of whole TA muscle extracts shows a clear increase in the amount of active HGF compared to animals injected with non-injured serum. Animals injected with injured serum that was incubated with Ab40 show a strong decrease in the amount of active-HGF. Purified recombinant active HGF was run as a control for the molecular weight of active HGF.  
E) HGFA improves the cell cycle entry kinetics of MuSCs. Data presented are representative images of EdU incorporation assays of MuSCs isolated from PBS- and HGFA-injected mice after 24 hours in culture. The scale bar is 100 µm.  
F) MuSCs in animals injected with HGFA are larger. Data from FSC measurements of MuSCs by FACS are presented as geometric ± s.e.m. of replicate experiments (n = 7).
Figure S3

(A) Comparison of DAPI and EdU images under PBS and HGFA treatments. Scale bars indicate 100 μm.

(B) Graph showing the median FSC with different treatment groups. Graph legend: PBS and HGFA.

(C) Bar graph illustrating the comparison of eMHC fibers/mm² between PBS and HGFA treatments. PBS and HGFA are represented by different colors.

(D) Time points for wound monitoring: 2 Days, Shave and wound, Monitor wound closure. Images show wound closure progress at 0 DPI, 6 DPI, and 11 DPI.

(E) Table listing the antibodies used in the experiments, including the host, clone, company, catalog number, and notes.

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Notes:
- Pax7 and eMHC used with 1:50 with M.O.M kit.
- PDGFR-alpha and phospho-S6 used with 1:100, stained overnight.
- Laminin used with 4H8-2 Abcam 1:500.
- CD31-FITC and CD31-APC used with 102506 1.5ug per muscle preparation.
- CD45-FITC and CD45-APC used with 103108 1.5ug per muscle preparation.
- Sca-1-PacBlue used with 108120 2ug per muscle preparation.
- VCAM-Biotin used with 553331 4ug per muscle preparation.
- HGF and HGF used with 1:1000.
- CD45-APC used with 103112 1.5ug per muscle preparation.
- GPDH-HRP used with 25778 1:5000.
- Streptavidin-PE-Cy7 used with 405206 4ug per muscle preparation.
Figure S3. HGFA improves stem cell activation and tissue repair. Relates to Figure 3.
A) HGFA improves the cell cycle entry kinetics of FAPs. Data presented are representative images of EdU incorporation assays of FAPs isolated from PBS- and HGFA-injected mice after 40 hours in culture. The scale bar is 100 µm.
B) FAPs in animals injected with HGFA are larger. Data from FSC measurements of FAPs by FACS are presented geometric ± s.e.m. of replicate experiments (n = 7).
C) HGFA does not affect the density of regenerating myofibers in injured muscle. Data are presented as mean ± s.e.m. of the number of eMHC⁺ regenerating myofibers per mm² of injured muscle at 5 DPI (n = 5); NS denotes not statistically significant. Panels on the right are representative images of damaged muscles in PBS- and HGFA-injected mice. The scale bar is 50 µm.
D) Schematic depiction of skin wound healing experiments and representative images showing the HGFA-induced acceleration of skin wound closure. The scale bar is 5 mm.
E) Table detailing the antibodies used.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES (Relates to Experimental Procedures)

SDS-PAGE and Western Blotting
Serum samples were diluted 1:10 with PBS and 5 µL of diluted serum was subject to reducing SDS-PAGE. Whole muscle tissue extracts were prepared by boiling 15 10 µm TA muscle cryosections in SDS sample buffer prior to reducing SDS-PAGE.

HGF quantification
HGF levels were measured using the Mouse/Rat HGF Quantikine Elisa kit (R&D Systems). Serum samples were diluted 1:10 with PBS for ELISA analysis. Muscle HGF quantifications were performed using whole muscle tissue extracts and measurements of HGF were normalized to the muscle tissue extract protein concentration.

Ex vivo HGF stimulation
Twelve hours after isolation and maintenance in culture medium, MuSCs and FAPs were washed twice with 1X PBS (HyClone) and medium was replaced with serum starvation medium (Hams F10 (Cellgro), 0.2% FA-free BSA (Alfa Aesar J64949), and 1x Pen/Strep (Gibco)). Six hours later, the medium was aspired and replaced with serum starvation medium with or without addition of purified recombinant active HGF (Life Technologies). Cells were fixed 30 minutes after HGF stimulation.

HGF Processing Assay
Briefly, purified recombinant pro-HGF was incubated with serum samples and the amount of HGF processing was determined by SDS-PAGE and Western blotting. Purified recombinant pro-HGF was prepared by transfecting HEK 293F cells (Invitrogen) with plasmids containing the entire cDNA sequence of human pro-HGF with a C-terminal myc-His6 tag (pcDNA4c-HGF-myc-His). Media from transfected cells was collected and pro-HGF was purified using Ni-agarose beads (Sigma). Serum samples were prepared as described above. Approximately 200 ng of pro-HGF, bound to Ni-Agarose beads, was mixed with 30 µL of serum, the volume was brought to 500 µL with reaction buffer (20 mM Tris pH 8.3, 150 mM NaCl, 2.5 mM CaCl2), and samples were incubated, with agitation, for five hours at room temperature. Following the reactions, the samples were washed two times with reaction buffer, pelleting the Ni-Agarose beads between washes, and once with PBS 0.3% Triton. Samples were resolved by reducing SDS-PAGE and processing of HGF was determined by Western blotting using an anti-His antibody to detect the proportion of HGF that was in the processed form (~35 kDa) versus total HGF (active HGF plus pro-HGF). Quantification of Western blots was performed using a BioRad Versa Doc system and quantification software.

HGFA enzyme assay
Briefly, HGFA was mixed with Ab40 or control IgG and enzymatic activity was determined by measuring the cleavage of a fluorogenic substrate peptide. In a final volume of 100 µL, 100 ng of purified, recombinant, active, mouse HGFA (R&D Systems) was mixed with differing amounts of Ab40 or control IgG antibodies and HGFA substrate peptide (R&D Systems #ES002) (10 µM final concentration) in reaction buffer (described above). Reactions were monitored in a fluorescence plate reader by taking measurements every minute for 20 minutes. Reaction rates were determined by calculating the slope of the fluorescence vs. time plot.

Wheel running experiments
Wheel running studies were performed by administering HGFA or PBS to animals one day before transferring and singly housing them in computer-monitored wheel running cages (Lafayette, model 80820). One day after the being placed in wheel running cages, animals were subject to BaCl2-mediated muscle injury to the left gastrocnemius muscle and returned to wheel running cage. Data in wheel running experiments are presented as the distance the animal ran in the previous 24-hour period (i.e. day 0 represents running distance in the 24-hours prior to muscle injury).

HGFA blocking antibody
HGFA blocking antibody (Ab40) was obtained from Dr. Daniel Kirchhofer (Genentech) (Ganesan et al., 2009). Prior to injection into mice, 100 µL of non-injured or injured serum was mixed with 50 µg of Ab40 or control IgG, brought to a final volume of 200 µL with sterile PBS, and incubated for 30 minutes on ice. Serum-antibody mixtures were then administered to mice by intravenous tail vein injection.

Skin Wounding
Skin wounding was performed using a biopsy punch. Briefly, 12 week old male C56BL6 mice were anesthetized using isoflurane. The back fur was shaven and the skin was cleaned with isopropanol and Betadine. The back skin was pulled using forceps and two 5-mm full-thickness skin wounds were created along the midline using a sterile 5 mm circular biopsy punch by pressing through both layers of the skin pull. Animals were then administered buprenorphine and Baytril and allowed to recover. Skin wound healing was measured every 2-3 days by anesthetizing the animals and imaging the wounded area.