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Short Report

Pharmacological blockage of fibro/adipogenic progenitor expansion and suppression of regenerative fibrogenesis is associated with impaired skeletal muscle regeneration

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ABSTRACT

Acute skeletal muscle injury triggers an expansion of fibro/adipogenic progenitors (FAPs) and a transient stage of fibrogenesis characterized by extracellular matrix deposition. While the perpetuation of such phase can lead to permanent tissue scarring, the consequences of its suppression remain to be studied. Using a model of acute muscle damage we were able to determine that pharmacological inhibition of FAP expansion by Nilotinib, a tyrosine kinase inhibitor with potent antifibrotic activity, exerts a detrimental effect on myogenesis during regeneration. We found that Nilotinib inhibits the damage-induced expansion of satellite cells *in vivo*, but it does not affect *in vitro* proliferation, suggesting a non cell-autonomous effect. Nilotinib impairs regenerative fibrogenesis by preventing the injury-triggered expansion and differentiation of resident CD45$^-$:CD31$^-$:α7integrin$^-$:Sca1$^+$ mesenchymal FAPs. Our data support the notion that the expansion of FAPs and transient fibrogenesis observed during regeneration play an important trophic role toward tissue-specific stem cells.

1. Introduction

Acute muscle damage triggers the activation and expansion of Pax7$^+$ tissue-specific stem cells called satellite cells (myogenic progenitors, MPs). Satellite cell proliferation gives rise to a population of CD31$^-$:CD45$^-$:Sca1$^-$:α7 integrin$^+$ myoblasts, that embark into a stepwise process characterized by the sequential upregulation of myogenic regulators such as MyoD, myogenin and MRF4 to eventually lead to differentiation (Le Grand and Rudnicki, 2007). Upon differentiation, myoblasts fuse and rebuild the damaged myofibers, regenerating the architecture of the muscle.

Acute skeletal muscle damage also triggers the activation of a population of CD45$^-$:CD31$^-$:α7$^-$ integrin:Sca1$^+$ mesenchymal progenitors that reside in the interstitial space between muscle fibers. Based on their ability to originate adipocytes and fibroblasts both *in vivo* and *in vitro*, these cells have been named fibro/adipogenic progenitors (FAPs) (Joe et al., 2010; Uezumi et al., 2010; Heredia et al., 2013). FAPs proliferate early during the response to acute damage, and they transiently synthesize extracellular matrix (ECM). Such fibrogenic stage is brief and its end is marked by both a decline in the number of FAPs and clearance of the collagen deposited in the extracellular space (Joe et al., 2010; Uezumi et al., 2010; Lemos et al., 2015). Recent data indicate that besides their fibrogenic activity, FAPs support developmental (Mathew et al., 2011) and regenerative myogenesis through the release of promyogenic cytokines, including IL6 and IL10 (Joe et al., 2010; Lemos et al., 2012). In order to confirm their relevance in muscle regeneration, experiments involving genetic ablation of FAPs have been attempted, reporting somewhat detrimental effects to the regenerative process (Murphy et al., 2011). However, these studies have been somewhat limited by the inability to fully ablate FAPs in skeletal muscle. An alternative strategy is to pharmacologically inhibit FAP expansion following acute damage.

Abbreviations: FAPs, fibro/adipogenic progenitors; ECM, extracellular matrix; MP, myogenic progenitor cells; TA, tibialis anterior muscle; RTK, receptor tyrosine kinase; TKIs, tyrosine kinase inhibitors; (NTX), notexin; (MyoG), Myogenin.

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Tyrosine kinase inhibitors (TKIs) have been effectively used in the treatment of the most common and progressive forms of human fibrosis (Rosenbloom and Jiménez, 2008; Beyer and Distler, 2013). Nilotinib (Tasigna®, AMN107; Novartis), a second-generation TKI, has been rationally designed to overcome Imatinib resistance in Chronic Myeloid Leukemia (Saglio et al., 2010) and is characterized by better bioavailability, tolerability and lacks the toxic effects commonly seen with Imatinib treatment, such as fluid retention, edema, and weight gain (Kantarjian et al., 2006). Nilotinib showed a more potent antifibrotic effect than Imatinib in liver and lung fibrosis (Rhee et al., 2011; H. Liu et al., 2011; Y. Liu et al., 2011; Shaker et al., 2011). Nilotinib simultaneously targets PDGFR and TGFβ pathways, which explains its potent antifibrotic effects. These data suggest that TKI, could be effectively used as antifibrotic agents (Rokosz et al., 2008).

Taking advantage of the fact that both PDGFR –a tyrosine kinase family member receptor- and TGFβ receptor drive FAP activity *in vitro* and *in vivo* (Uezumi et al., 2011; Lemos et al., 2015), here we used Nilotinib to pharmacologically block FAPs in the context of skeletal muscle regeneration. Our results show that Nilotinib reduces FAP proliferation and expansion, dampening transient fibrogenesis during muscle regeneration, an effect that is associated with a reduction of fibrogenic gene expression and collagen deposition. We also show that this effect correlates with poor regeneration after acute muscle damage, due to non-cell autonomous reduced myoblast expansion. These results suggest that while tyrosine kinase inhibitor-based therapies could prove useful to reduce excessive fibrosis associated with degenerative pathologies, such therapies could also have a detrimental effect on the overall regenerative capacity of healthy patients.

2. Materials and method

2.1. Animals

All mice were maintained in pathogen-free facility, and all experiments were performed in accordance with University of British Columbia Animal Care Committee regulations. C57BL/6 and PDGFRα-H2B::EGFP were purchased from The Jackson Laboratory. Col1a1*3.6-eGFP mice were a gift from D.W. Rowe (Center for Regenerative Medicine and Skeletal Development, University of Connecticut Health Center). Mice were treated by intraperitoneal (i.p.) injection with vehicle (DMSO) or Nilotinib (20 mg/kg/day) in DMSO (concentration 5 mg/ml). Muscle damage was induced by intramuscular injection of 0.15 μg notexin (NTX) snake venom (Latoxan), into the tibialis anterior muscle (TA).

2.2. Skeletal muscle cells preparation

TA muscle was carefully dissected and gently torn with tissue forceps until homogeneous. Collagenase type 2 (Sigma; 250 μl of 2.5 U/ml), in 10 mM $CaCl_2$, was added to each sample, and the preparation was placed at 37 °C for 30 min. After washing, a second enzymatic digestion was performed with Collagenase D (Roche Biochemicals; 1.5 U/ml) and Dispase II (Roche Biochemicals; 2.4 U/ml), in a total volume of 250 μl per each sample, at 37 °C for 60 min. Preparations were passed through a 40-μm cell strainer (Becton Dickenson), and washed. Resulting single cells were collected by centrifugation at 1600 rpm for 5 min.

2.3. Flow cytometry/FACS

Cell preparations were incubated with primary antibodies for 30 min at 4 °C in supplemented PBS containing 2 mM EDTA and 2% FBS at ~3×10^7 cells/ml. We used the following monoclonal primary antibodies: anti-CD31 (clones MEC13.3, Becton Dickenson, and 390, Cedarlane Laboratories), anti-CD45 (clone 30-F11, Becton Dickenson), anti-Sca-1 (clone D7, eBiosciences) and anti-α7 integrin (produced in-house). Typical antibody dilutions used were: antiCD31, 1:100–400; anti-CD45, 1:200–400, 1:200–400; anti-Sca-1, 1:2000–5000; anti-α7 integrin, 1:100–400. For all antibodies we performed fluorescence minus one controls by staining with appropriate isotype antibodies. Cells were stained Hoechst 33342 (2.5 μg/ml) and resuspended at ~1×10^7 cells/ml immediately before sorting or analysis. Analysis was performed on LSRII (Becton Dickenson) equipped with three lasers. Data were collected using Facs DIVA software. Biexponential analysis was performed using FlowJo X (Treestar) software. Sorts were performed on a FACS Vantage SE (Becton Dickenson) or FACS Aria (Becton Dickenson), both equipped with three lasers. Sorting gates were strictly defined based on isotype control (fluorescence minus one) stains.

2.4. FAP cell culture

FAPs were FACS sorted from either wildtype or transgenic mice expressing EGFP under a collagen1a1 enhancer (Collagen1a1 3.6-EGFP) and grown in high–glucose Dulbecco's modified eagle medium (DMEM) (Invitrogen), supplemented with 10% FBS and 2.5 ng/ml bFGF (Invitrogen) at density of 10.000 cell/well in a 48 well-plate. For TGFβ treatment experiments, after 72 h in culture the cells were stimulated with 1 ng/ml TGFβ (eBioscience) along with different concentrations of Nilotinib (0.5, 1 and 3 mM). Cells were trypsinized and resuspended in PBS containing 2 mM EDTA, 2% FBS and Hoechst 33342 (2.5 μg/ml). Col1-GFP levels in FAPs were evaluated by FACS after 72 h of treatment.

2.5. Gene expression analysis

RNA isolation was performed using RNeasy mini kits (Qiagen) and reverse transcription was performed using the Superscript Reverse Transcriptase (Applied Biosystems). The cDNA was diluted ten times in TE buffer and 5 μl was used in a reaction mix containing Droplet Digital PCR Supermix (Bio-Rad), 1. TaqMan assay and H_2O. Droplets were generated with a QX100 droplet generator (Bio-Rad), after mixing 20 μl of reaction mix and 70 μl of droplet generator oil (Bio-Rad). The emulsified samples were loaded onto 96-well plates and endpoint PCRs were performed in C1000 Touch thermal cycler (Bio-Rad) at the following cycling conditions: 95 °C for 10 min, followed by 45 cycles at 94 °C for 30 s and 60 °C for 1 min, followed by 98 °C for 10 min. The droplets from each sample were read through the QX100 droplet reader (Bio-Rad). Resulting PCR-positive and PCR-negative droplets were counted using QuantaSoft software (Bio-Rad). Expression levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT). A similar approach was taken to assess the expression of *Tgfb1* and *TNF* in single cells. Briefly, single macrophages were sorted directly into a 96-well plates containing 5.5 μl of lysis buffer (CellsDirect Resuspension & Lysis Buffer, Life Technologies). After RNA isolation and reverse transcription (High Capacity cDNA Reverse Transcription Kit, Life Technologies), diluted cDNA was used for droplet generation, endpoint PCRs, and droplet reading as discussed above. Resulting PCR-positive and PCR-negative droplets were counted to calculate the absolute number of transcripts per cell.

2.6. Isolation, culture and immunostaining of single myofibers

Single myofibers were isolated and cultured *ex vivo* as previously described (Collins and Zammit, 2009). Briefly, whole extensor digitorum longus muscles from 6 to 8 week old C57BL6 mice were removed and digested in 0.025% collagenase I for 1 h 45 min at 37 °C. Liberated single myofibers and their associated satellite cells were then maintained in myofiber media (DMEM, 20% v/v FBS and 1% v/v chicken embryo extract, 1% v/v pren-strep) for up to 72 h. For immunostaining, single myofibers were fixed in 4% paraformaldehyde (PFA) for 10 min and then permeabilized with 0.5% v/v Triton X-100 in PBS for 6 min. Fibers were incubated in blocking buffer (10% v/v goat serum and 10% v/v

horse serum in PBS) for 1 h and then stained with primary antibodies overnight at 4 °C with gentle rocking. Primary antibodies consisted of mouse anti-MyoD (BD Bioscience, 5.8 A) and mouse anti-MyoG (DSHB, F5D). The next day, myofibers were washed 3 times in 0.025% Tween-20 in PBS and then incubated in species specific fluorescent-conjugated secondary antibodies for 1 h before being mounted with Vectashield (Vector Laboratories, USA) mounting medium. Quantification of MP numbers and percentages of $MyoG^+$ MPs was performed by manual counting of MP clusters (colonies of >3 cells positioned above the basal lamina on single myofibers) on a minimum of 20 fibers per replicate.

2.7. FAP-single fiber co-culture experiment

FAPs were FACs sorted (as above) from TAs of mice 3 days following NTX injury, seeded into 24 well plates at low (5000 cells/well), medium (20,000 cells/well) and high (50,000 cells/well) density and cultured as described above. Forty-eight hours after isolation, media was replaced with DMEM supplemented with 0.5% (v/v) FBS, 0.5% (v/v) chicken embryo extract and freshly isolated EDL myofibers added to the FAPs using a co-culture transwell system (24 well, 1 μm pore size). After 72 h of co-culture, myofibers were fixed in 4% PFA and processed for immunohistochemical analysis.

2.8. EdU (5-ethynyl-2′-deoxyuridine)-labelling studies

For *in vivo* studies, EdU was administered by i.p. injection (1 mg/mouse/day) starting the day after damage. For flow cytometric analysis, cells were stained for surface markers as indicated. After the last post-staining wash, each sample was resuspended in 1% BSA PBS, transferred to U-bottom 96-well plates. Samples were fixed in 4% PFA for 25 min in the dark, centrifuged 5 min at 1800 rpm, membranes were permeabilized in 0.2% saponin for 15 min in the dark and, after 5 min centrifugation at 1800 rpm, incubated in dark with reaction mix, prepared under kit indication Click-iT® EdU HCS Assays (Invitrogen). After washing with 1% BSA PBS, each sample was resuspended in FACS buffer.

2.9. Histology and imaging

Before tissue collection, animals were perfused transcardially with 20 ml PBS/2 mM EDTA, followed by 20 ml 4% PFA. Tissues were processed for cryosectioning or paraffin-embedding using standard methods. Sections of muscle tissues were stained with hematoxylin and eosin (H&E), and images were acquired using a bright field microscope (Axioplan2, Carl Zeiss Microimaging), equipped with a charge-coupled device camera (Retiga Ex, Axioplan2; qImaging), operated *via* OpenLab4 software (Improvision). Images were captured using the shortest possible exposure time, and manipulation of brightness and contrast, coloring adjustments and assembly into figures were performed using ImageJ, OpenLab4 (Improvision), Illustrator CS5 (Adobe) and Photoshop CS5 (Adobe).

2.10. Statistical analysis

Statistical tests, including one-way ANOVA and Student's *t*-test were performed using Prism 6 (GraphPad Software). A probability of <5% ($p \le 0.05$) or 1% ($p \le 0.01$) is considered statistically significant. Error bars in all figures represent the mean ± standard deviation (SD).

3. Results

3.1. Nilotinib inhibits acute damage-triggered FAP proliferation

Following acute damage, FAPs proliferate and the population expands for a period of 72–96 h (Joe et al., 2010). Since FAPs constitute the major cell population producing extracelluar matrix in the skeletal muscle (Uezumi et al., 2011), we decided to study the effect of Nilotinib on the fibrogenic activity of FAPs during regeneration. We first investigated the effect of Nilotinib on FAP expansion. TAs were collected at D0 (undamaged) and D4 (4 days) after damage and FAP proliferation was analyzed by incorporation of EdU -a nucleotide analog- by flow cytometry. Nilotinib delivery between D0 and D3 post-damage significantly reduced the percentage of FAPs that incorporated EdU (Fig. 1A) as well as a significantly reduced FAP numbers (Fig. 1B), providing evidence that this compound inhibits FAP proliferation following acute damage. Next we performed immunohistological analysis of muscle sections from a mouse strain in which GFP expression identifies FAPs (PDGFRa::H2B-EGFP) (Joe et al., 2010; Uezumi et al., 2010), 4 days following NTX damage. This analysis confirmed that Nilotinib blunted the accumulation of GFP^+ FAPs in skeletal muscle (Fig. 1C). Thus, Nilotinib treatment reduces the capacity of FAPs to expand following muscle injury.

3.2. Nilotinib prevents transient ECM deposition during muscle regeneration

To assess the effects of Nilotinib on damage-induced fibrogenesis we took advantage of the Col1a1-EGFP transgenic mouse, in which collagen 1a1 expression can be traced by the intracellular presence of GFP. Analysis of GFP expression after damage indicated that FAPs actively synthesize extracellular matrix throughout the regenerative process, reaching a peak on D7 post-damage (Fig. 2A). In order to test the effect of Nilotinib on the fibrogenic activity of FAPs, we assessed collagen gene expression in the TAs of mice that received Nilotinib daily between D0 and D4 post-damage. Consistent with a blockade of proliferation, Nilotinib treatment resulted in a significant reduction of both the frequency of Col1a1-expressing FAPs as well as the total number of FAPs per TA on D7 post-damage (Fig. 2B). Histological analysis confirmed that Nilotinib treatment reduced the number of Col1a1-GFP expressing cells at D7 after damage, and revealed a concomitant reduction in the deposition of COL1A1 protein, indicating that the drug reduces extracellular matrix deposition (Fig. 2C).

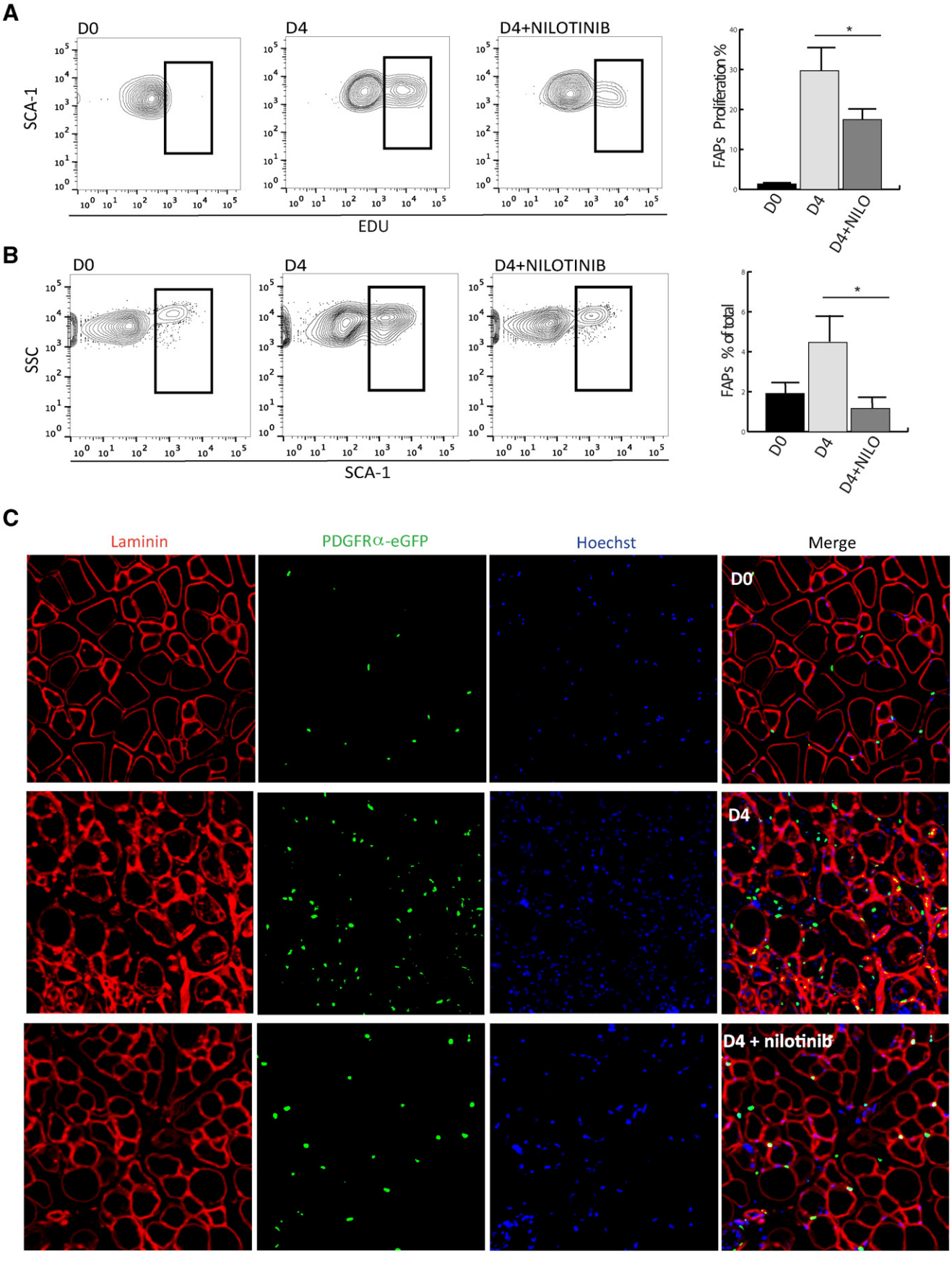
Nilotinib is known to act by inhibiting PDGFRα, while its effects on the TGFβ pathway are less characterized. To confirm that Nilotinib inhibits the pro-fibrogenic effect of TGFβ we purified GFP- FAPs from TAs (D3 post NTX) of Col1a1-EGFP mice and stimulated differentiation by addition of 1 ng/ml TGFβ. Nilotinib inhibited both Col1a1-driven GFP and fibrogenic gene expression induced by TGFβ (Fig. 2D, E). Thus Nilotinib acts directly on FAPs to inhibit their fibrogenic differentiation.

Altogether, our data indicate that Nilotinib blocks transient fibrogenesis by blocking both FAP proliferation and extracellular matrix production.

3.3. Nilotinib indirectly inhibits myoblast expansion

Next we tested the effects of pharmacological blockage of FAP expansion and fibrogenesis on muscle regeneration *in vivo*. Mice received a daily intraperitoneal injection of Nilotinib, from the day prior through D4 after damage, and muscle regeneration was assessed histologically on days 14 and 21 post-damage. Nilotinib treatment lead to a significant reduction in myofiber cross sectional area and an accumulation of small centrally nucleated myofibers after 14 and 21 days following damage – indicative of impaired/delayed muscle regeneration (Fig. 3). Myofiber regeneration begins with satellite cells becoming activated and proliferating to generate a pool of myoblasts, which goes on to differentiate and fuse to repair and replace damaged myofibers. We investigated whether Nilotinib affects MP activation/expansion during regeneration. Quantification of MP number on D4 post-NTX damage revealed a significant decrease in Nilotinib treated animals (Fig. 4A), suggesting a role for the drug in influencing MP proliferation. Such an effect could be due to a direct effect on MPs, or could be the result of dampening FAP proliferation and thus reducing the trophic support they provide to myoblasts. To

distinguish between these two possibilities we cultured isolated myofibers and their associated satellite cells for 48 h in the presence or absence of Nilotinib. In contrast with our *in vivo* results, Nilotinib had no significant effect on EdU incorporation in satellite cells, indicating that a direct effect of Nilotinib on MP proliferation is unlikely (Fig. 4B). Next we purified proliferating FAPs from TAs three days after



damage, plated them at different densities and co-cultured them with myofiber-associated satellite cells for 72 h. The results show a clear dose-dependent increase in the number of MPs (Fig. 4C) and a decrease in the percentage of satellite cells expressing Myogenin (MyoG) (Fig. 4D), suggesting that FAPs act to expand MPs. Confirming the supportive effect of FAPs, we observed a dose-dependent effect on MP clonal efficiency, measured as the frequency of myofibers containing satellite cell colonies (Fig. 4E). This result indicated a trophic effect of FAPs on MPs. Lastly, we tested whether Nilotinib could inhibit the response of satellite cells to FAP-derived trophic factors. To that end, we assessed the effect of Nilotinib on myofiber-associated satellite cells co-cultivated with FAPs. Consistent with the previous result, we found that the number of MPs increased significantly in the presence of FAPs (Fig. 4F), and importantly, Nilotinib had no effect on such increase (Fig. 4F), indicating that this compound does not affect MP proliferation by blocking their response to FAP secreted factors.

Taken together these results confirm that FAPs support the expansion of MPs, and suggest that Nilotinib does not impair muscle regeneration by acting directly on the ability of satellite cells to respond to trophic signals, but rather by reducing the trophic support provided by FAPs.

4. Discussion

Here we took advantage of a well-characterized model of skeletal muscle damage to study the effect of FAP ablation on skeletal muscle regeneration *in vivo*. Our results show that Nilotinib can effectively prevent transient ECM deposition following acute muscle damage, by inhibiting FAP activity. This was first evident in the Col1a13.6-GFP mouse, in which Nilotinib treatment resulted in a large reduction of collagen synthesis following acute damage as well as reduced FAP number. The direct cell autonomous effect of Nilotinib on TGFβ-induced synthesis of Col1a1 in FAPs was further tested *in vitro*, confirming that Nilotinib inhibits TGFβ signaling in those cells. Together these data strengthen our understanding of the cellular mechanisms by which Nilotinib and tyrosine-kinase inhibitors exert their anti fibrotic effect in fibrogenesis *in vivo* targeting the expansion and ECM deposition of fibrogenic progenitors. It is important to mention, however, that Nilotinib potently inhibits other tyrosine kinases, including the ABL kinases, BCR-ABL, KIT and DDR1 (Hantschel et al., 2008), which play important roles in mitosis, cell growth, survival and differentiation. In this study we have not assessed the effect of Nilotinib on those kinases, and therefore we cannot rule out the possibility that their inhibition contributes to the blockage of FAP activity.

In addition to their role in ECM production, FAPs have been suggested to exert a 'promyogenic' effect on myogenic progenitors (Joe et al., 2010; Mathew et al., 2011), however the precise details of this cellular relationship remain vague. Our co-culture experiments with FAPs and myofiber associated MPs –*i.e.* quiescent MPs– indicated that FAPs act to promote activation/proliferation of MPs. During this stage, we observed that MPs do not differentiate, a finding that is consistent with those cells undergoing cell cycle. These results are consistent with previous attempts to ablate a phenotypically identical population of Tcf4 + fibroblasts in skeletal muscle – which lead to premature differentiation and decreased numbers of $Pax7^{+}$ and $MyoD^{+}$ MPs following damage (Murphy et al., 2011).

The positive effect of FAPs on satellite cell proliferation seems to conflict with previous data showing that FAPs promote MP differentiation without affecting proliferation (Joe et al., 2010). The two seemingly disparate results may stem from the different experimental settings. In Joe et al. (2010) the FAPs were co-cultured with MPs isolated from post-damage day 3 TAs and expanded on plastic substrates. This means that the progenitor satellite cells had already gone through the stage of activation and proliferation, and therefore the cells used were myoblasts going into the early stages of differentiation. In that scenario, FAPs were shown to support differentiation. In the current study, we used quiescent satellite cells associated with myofibers. In this setting, we observed that FAPs favor satellite cell proliferation over differentiation. It is important to consider that in this more "intact" experimental setting, the satellite cells have not been exposed to the full plethora of damage-associated signals and therefore, may be less prone to move forward into the differentiation stages, compared to the MPs used previously (Joe et al., 2010).

Our data do not rule out the possibility that Nilotinib is influencing additional cell types, such as immune cells, or blocking satellite cell autonomous RTK signaling, induced by damage-associated signals and inflammatory cytokines *in vivo*, our *in vitro* data provide evidence for a contribution of FAPs to satellite cell proliferation. Altogether, the results indicate that a FAP-MP collaborative axis is important for effective muscle regeneration and provide further characterization of this cellular relationship in the setting of tissue regeneration.

The precise paracrine signals responsible for the trophic effects of FAPs remain unclear, although IL10 and IL6 present obvious candidates as they are both highly expressed in FAPs and have established roles in regulating satellite cells and muscle regeneration (Joe et al., 2010; Lemos et al., 2012; Zhang et al., 2013; Serrano et al., 2008; Strle et al., 2007; Deng et al., 2012; Judson et al., 2013). *In vivo*, it is likely that temporal alterations in the ECM environment coordinated by FAP matrix deposition may also play a role in influencing MP behavior. This regulation could happen at the level of modifications in substrate elasticity/stiffness for myoblasts (Gilbert et al., 2010) or changes in signaling events, through interactions between the remodeling ECM and cell adhesion molecules such as integrins (H. Liu et al., 2011; Y. Liu et al., 2011; Wilschut et al., 2011; Bröhl et al., 2012), both of which are important regulators of MPs and muscle regeneration. Functional studies on regenerating muscle based on macrophage depletion have been shown to lead to a reduction of the diameter of regenerating myofibers (Arnold et al., 2007). In conditions of mouse model of chronic muscle injury we previously demonstrated that, TGF-β-blockade by Nilotinib restores FAP apoptosis and consequently reduces excess of matrix deposition that lead to fibrosis (Lemos et al., 2015). Here, in condition of acute muscle injury, we demonstrated that Nilotinib inhibits FAP expansion, impairing muscle reparative process. Suggesting that the cell-cell communications as well as the composition of the ECM can influence the capacity of the skeletal muscle to regenerate properly.

5. Conclusion

While our *in vivo* data show that Nilotinib blocks fibrogenesis in the context of skeletal muscle regeneration, our *in vitro* model provides evidence that mesenchymal progenitor cells prompt the expansion of tissue-specific stem cells, strengthening the notion that fibrogenic cell activity following acute damage is important for effective regeneration.

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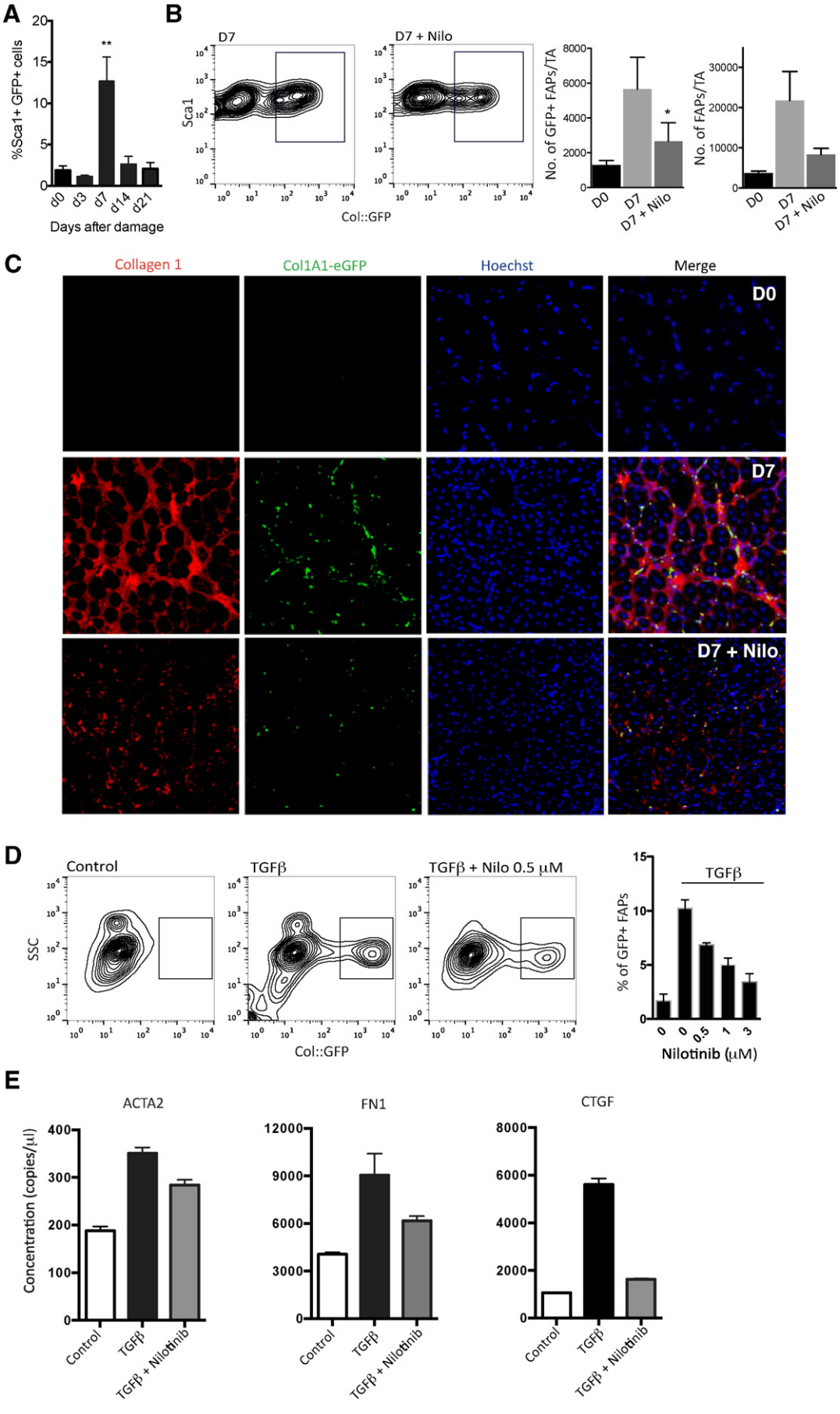
Disclosure of potential conflict of interest

The authors declare no conflict of interest.

Fig. 1. Nilotinib inhibits fibroadipogenic progenitor proliferation following acute skeletal muscle damage. A) Representative FACs plots and quantification of fibroadipogenic progenitor proliferation at 0, and 4 days following NTX injury with/without Nilotinib administration (N = 3, ANOVA: *p < 0.05, mean ± S.D.). B) Representative FACs plots and quantification of fibroadipogenic progenitor population as a percentage of the whole sample. C) Representative immunofluorescence images of TA muscles from PDGFRa::H2B-eGFP mice in which FAPs can be traced by nuclear GFP expression, at 0, and 4 days following NTX injury with/without Nilotinib administration.


A
%Sca1+ GFP+ cells
Days after damage
d0 d3 d7 d14 d21
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B
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No. of GFP+ FAPs/TA
No. of FAPs/TA
D0 D7 D7 + Nilo
C
Collagen 1
Col1A1-eGFP
Hoechst
Merge
D0
D7
D7 + Nilo
D
Control
TGFβ
TGFβ + Nilo 0.5 μM
SSC
Col::GFP
% of GFP+ FAPs
Nilotinib (μM)
E
ACTA2
FN1
CTGF
Concentration (copies/μl)
Control TGFβ TGFβ + Nilotinib

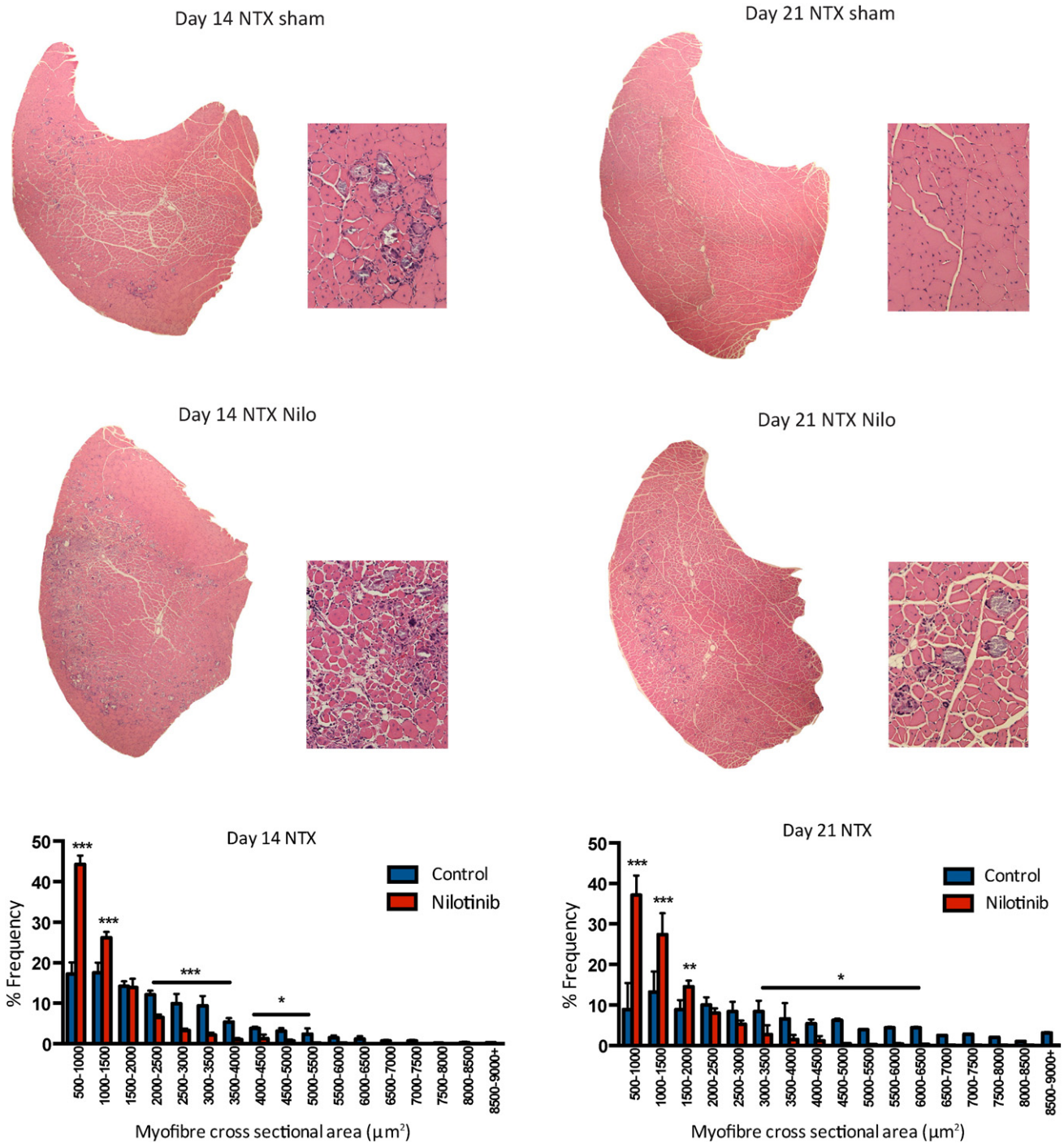

Fig. 3. Nilotinib treatment negatively affects skeletal muscle regeneration. A) Representative sections of WT TA muscles at 14, and 21 days following NTX injury with/without Nilotinib administration. B) Frequency distribution for defined ranges of fiber cross-sectional area in WT TAs at 14, and 21 days following NTX injury with/without Nilotinib administration.

Author contributions

D.F. and R.N.J. designed and carried out the experiments, analyzed the data and wrote the manuscript. M.L., carried out the experiments, analyzed the data, and wrote the manuscript; S.L., E.Z., C.H., P.X. carried out the experiments ad analyzed the data; A.L.: discussion of data and critical revision of manuscript; and F.M.V.R. conceived and designed the experiments, provided financial support and wrote the manuscript.

Fig. 2. Nilotinib inhibits transient fibrogenesis during regeneration. A) Col1 expression in FAPs after muscle damage. B) Representative FACs plots and quantification of total GFP^+ FAPs numbers at 0, and 7 days following NTX injury with/without Nilotinib administration ($N = 3$, ANOVA: $^*p < 0.05$, mean ± S.D.). C) Representative immunofluorescence images depicting Col1 deposition in the TAs of PDGFRa::H2B-eGFP mice at 0, and 7 days following NTX injury with/without Nilotinib administration. D) Representative FACs plots and quantification of the dose-response effect of Nilotinib on Col1 expression in PDGFRa::H2B-eGFP FAPs stimulated with TGFβ *in vitro* ($N = 3$, ANOVA: $^*P < 0.05$, mean ± S.D.). E) Analysis of gene expression using digital PCR.

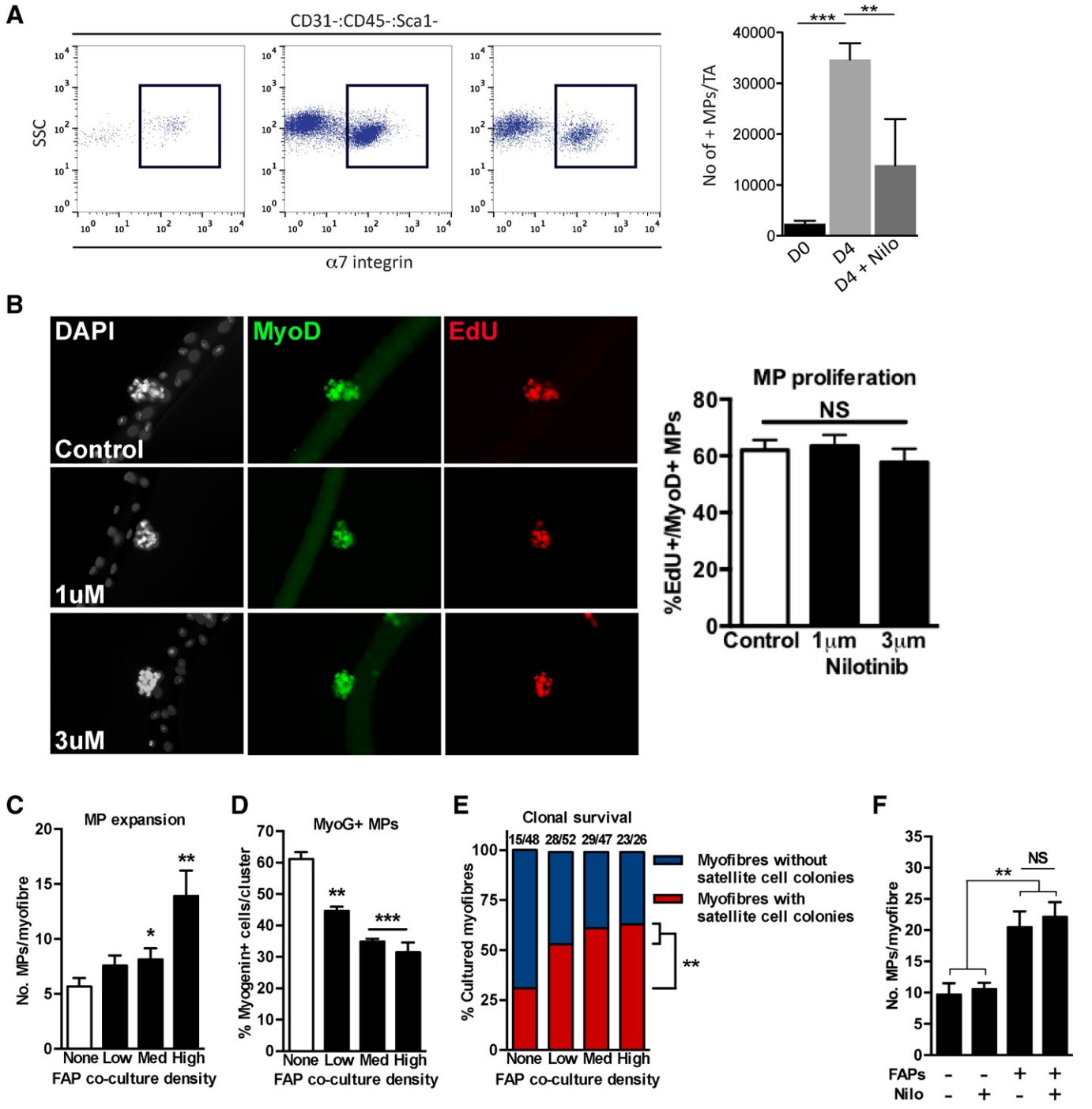

Fig. 4. Nilotinib impairs FAP-induced MP expansion. A) Representative FACs plots and quantification of total myogenic progenitor numbers at 0, and 4 days following NTX injury with/without Nilonitib administration (N = 3, ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ mean ± S.D.). B) MPs associated with EDL myofibers were cultured *ex vivo* in the presence of 0 μM (control), 1 μM and 3 μM Nilonitib. After 72 h, MPs were pulsed with EdU for 4 h before being fixed and processed for immunofluorescent co-staining with MyoD specific antibodies and EdU visualization. (N = 3, ANOVA: $^{*}p < 0.05$; mean ± S.D.). C) Quantification of total MP numbers (N = 3, ANOVA: $^{*}p < 0.05$, $^{**}p < 0.01$, mean ± S.D.). D) Quantification of % MPs (associated with single EDL myofibers) expressing myogenin (MyoG) after 72 h of co-culture with FAPs. (N = 3, ANOVA: $^{**}p < 0.01$, $^{***}p < 0.001$ mean ± S.D.). E) Quantification of % myofibers with or without MP colony growth (N = 3, ANOVA: $^{**}p < 0.01$, mean ± S.D.). F) Quantification of total MP numbers after 48 h of co-culture, in the presence or absence of Nilotinib (N = 3, ANOVA: $^{**}p < 0.01$, mean ± S.D.).

D.R.L. conceived, designed and carried out experiments, directed the project and wrote the manuscript.

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Appendix A. Supplementary data

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References

Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R.K., Chazaud, B., 2007. Inflammatory monocytes recruited after skeletal muscle injury

switch into antiinflammatory macrophages to support myogenesis. J. Exp. Med. 204 (5), 1057–1069 (May 14).
Beyer, C., Distler, J.H., 2013. Tyrosine kinase signaling in fibrotic disorders: translation of basic research to human disease. Biochim. Biophys. Acta 1832 (7), 897–904. http://dx.doi.org/10.1016/j.bbadis.2012.06.008 (Jul).
Bröhl, D., Vasyutina, E., Czajkowski, M.T., Griger, J., Rassek, C., Rahn, H.P., Purfürst, B., Wende, H., Birchmeier, C., 2012. Colonization of the satellite cell niche by skeletal muscle progenitor cells depends on Notch signals. Dev. Cell 23 (3), 469–481. http://dx.doi.org/10.1016/j.devcel.2012.07.014 (Sep, 11).
Collins, C.A., Zammit, P.S., 2009. Isolation and grafting of single muscle fibres. Methods Mol. Biol. 482, 319–330. http://dx.doi.org/10.1007/978-1-59745-060-7_20.
Deng, B., Wehling-Henricks, M., Villalta, S.A., Wang, Y., Tidball, J.G., 2012. Interleukin-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J. Immunol. 189 (7), 3669–3680 (Oct 1).
Gilbert, P.M., Havenstrite, K.L., Magnusson, K.E., Sacco, A., Leonardi, N.A., Kraft, P., Nguyen, N.K., Thrun, S., Lutolf, M.P., Blau, H.M., 2010. Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. Science 329 (5995), 1078–1081. http://dx.doi.org/10.1126/science.1191035 (Aug 27).
Hantschel, O., Rix, U., Superti-Furga, G., 2008. Target spectrum of the BCR-ABL inhibitors imatinib, nilotinib and desatinib. Leuk. Lymphoma 49, 615–619.
Heredia, J.E., Mukundan, L., Chen, F.M., Mueller, A.A., Deo, R.C., Locksley, R.M., Rando, T.A., Chawla, A., 2013. Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. Cell 153, 376–388.
Joe, A.W., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M.A., 2010. Rossi FM muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. Nat. Cell Biol. 12 (2), 153–163. http://dx.doi.org/10.1038/ncb2015 (Feb).
Judson, R.N., Zhang, R.H., Rossi, F.M., 2013. Tissue-resident mesenchymal stem/progenitor cells in skeletal muscle: collaborators or saboteurs? FEBS J. 280 (17), 4100–4108. http://dx.doi.org/10.1111/febs.12370 (Sep).
Kantarjian, H., Giles, F., Wunderle, L., Bhalla, K., O'Brien, S., Wassmann, B., Tanaka, C., Manley, P., Rae, P., Mietlowski, W., Bochinski, K., Hochhaus, A., Griffin, J.D., Hoelzer, D., Albitar, M., Dugan, M., Cortes, J., Alland, L., Ottmann, O.G., 2006. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N. Engl. J. Med. 354 (24), 2542–2551 (Jun 15).
Le Grand, F., Rudnicki, M.A., 2007. Skeletal muscle satellite cells and adult myogenesis. Curr. Opin. Cell Biol. 19, 628–633.
Lemos, D.R., Paylor, B., Chang, C., Sampaio, A., Underhill, T.M., Rossi, F.M., 2012. Functionally convergent white adipogenic progenitors of different lineages participate in a diffused system supporting tissue regeneration. Stem Cells 30 (6), 1152–1162. http://dx.doi.org/10.1002/stem.1082 (Jun).
Lemos, D.R., Babaeijandaghi, F., Low, M., Chang, C.K., Lee, S.T., Fiore, D., Zhang, R.H., Natarajan, A., Nedospasov, S.A., Rossi, F.M., 2015. Nilotinib reduces muscle fibrosis in chronic muscle injury by promoting TNF-mediated apoptosis of fibro/adipogenic progenitors. Nat. Med. 21 (7), 786–794. http://dx.doi.org/10.1038/nm.3869 (Jul).
Liu, H., Niu, A., Chen, S.E., Li, Y.P., 2011a. Beta3-integrin mediates satellite cell differentiation in regenerating mouse muscle. FASEB J. 25 (6), 1914–1921. http://dx.doi.org/10.1096/fj.10-170449 (Jun).).
Liu, Y., Wang, Z., Kwong, S.Q., Lui, E.L., Friedman, S.L., Li, F.R., Lam, R.W., Zhang, G.C., Zhang, H., Ye, T., 2011b. Inhibition of PDGF, TGF-β, and Abl signaling and reduction of liver fibrosis by the small molecule Bcr-Abl tyrosine kinase antagonist Nilotinib. J. Hepatol. 55 (3), 612–625. http://dx.doi.org/10.1016/j.jhep.2010.11.035 (Sep).
Mathew, S.J., Hansen, J.M., Merrell, A.J., Murphy, M.M., Lawson, J.A., Hutcheson, D.A., Hansen, M.S., Angus-Hill, M., Kardon, G., 2011. Connective tissue fibroblasts and Tcf4 regulate myogenesis. Development 138 (2), 371–384. http://dx.doi.org/10.1242/dev.057463 (Jan).
Murphy, M.M., Lawson, J.A., Mathew, S.J., Hutcheson, D.A., Kardon, G., 2011. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Development 138 (17), 3625–3637. http://dx.doi.org/10.1242/dev.064162 (Sep).
Rhee, C.K., Lee, S.H., Yoon, H.K., Kim, S.C., Lee, S.Y., Kwon, S.S., Kim, Y.K., Kim, K.H., Kim, T.J., Kim, J.W., 2011. Effect of nilotinib on bleomycin-induced acute lung injury and pulmonary fibrosis in mice. Respiration 82 (3), 273–287. http://dx.doi.org/10.1159/000327719.
Rokosz, L.L., Beasley, J.R., Carroll, C.D., Lin, T., Zhao, J., Appell, K.C., et al., 2008. Kinase inhibitors as drugs for chronic inflammatory and immunological diseases: progress and challenges. Expert Opin. Ther. Targets 12 (7), 883–903. http://dx.doi.org/10.1517/14728222.12.7.883 (Jul).
Rosenbloom, J., Jiménez, S.A., 2008. Molecular ablation of transforming growth factor beta signaling pathways by tyrosine kinase inhibition: the coming of a promising new era in the treatment of tissue fibrosis. Arthritis Rheum. 58 (8), 2219–2224. http://dx.doi.org/10.1002/art.23634 (Aug).
Saglio, G., Kim, D.W., Issaragrisil, S., le Coutre, P., Etienne, G., Lobo, C., Pasquini, R., Clark, R.E., Hochhaus, A., Hughes, T.P., Gallagher, N., Hoenekopp, A., Dong, M., Haque, A., Larson, R.A., Kantarjian, H.M., 2010. ENESTnd Investigators. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. N. Engl. J. Med. 362 (24), 2251–2259. http://dx.doi.org/10.1056/NEJMoa0912614 (Jun 17).
Serrano, A.L., Baeza-Raja, B., Perdiguero, E., Jardí, M., Muñoz-Cánoves, P., 2008. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab. 7 (1), 33–44. http://dx.doi.org/10.1016/j.cmet.2007.11.011 (Jan).
Shaker, M.E., Zalata, K.R., Mehal, W.Z., Shiha, G.E., Ibrahim, T.M., 2011. Comparison of imatinib, nilotinib and silymarin in the treatment of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis. Toxicol. Appl. Pharmacol. 252, 165–175.
Strle, K., McCusker, R.H., Tran, L., King, A., Johnson, R.W., Freund, G.G., Dantzer, R., Kelley, K.W., 2007. Novel activity of an anti-inflammatory cytokine: IL-10 prevents TNFalpha-induced resistance to IGF-I in myoblasts. J. Neuroimmunol. 188 (1–2), 48–55 (Aug).
Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S., Tsuchida, K., 2010. Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. Nat. Cell Biol. 12 (2), 143–152. http://dx.doi.org/10.1038/ncb2014 (Feb).
Uezumi, A., Ito, T., Morikawa, D., Shimizu, N., Yoneda, T., Segawa, M., Yamaguchi, M., Ogawa, R., Matev, M.M., Miyagoe-Suzuki, Y., Takeda, S., Tsujikawa, K., Tsuchida, K., Yamamoto, H., Fukada, S., 2011. Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. J. Cell Sci. 124 (Pt 21), 3654–3664. http://dx.doi.org/10.1242/jcs.086629 (Nov 1).
Wilschut, K.J., van Tol, H.T., Arkesteijn, G.J., Haagsman, H.P., Roelen, B.A., 2011. Alpha 6 integrin is important for myogenic stem cell differentiation. Stem Cell Res. 7 (2), 112–123. http://dx.doi.org/10.1016/j.scr.2011.05.001 (Sep).
Zhang, C., Li, Y., Wu, Y., Wang, L., Wang, X., Du, J., 2013. Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration. J. Biol. Chem. 288 (3), 1489–1499. http://dx.doi.org/10.1074/jbc.M112.419788 (Jan 18).