Hydroxylases regulate intestinal fibrosis through the suppression of ERK mediated TGF-β1 signaling

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Abstract

Fibrosis is a complication of chronic inflammatory disorders such as inflammatory bowel disease (IBD), a condition which has limited therapeutic options and often requires surgical intervention. Pharmacologic inhibition of oxygen-sensing prolyl hydroxylases (PHD), which confer oxygen-sensitivity upon the hypoxia inducible factor (HIF) pathway, has recently been shown to have therapeutic potential in colitis, although the mechanisms involved remain unclear. Here, we investigated the impact of hydroxylase inhibition on inflammation-driven fibrosis in a murine colitis model. Mice exposed to dextran sodium sulfate followed by period of recovery developed intestinal fibrosis characterized by alterations in the pattern of collagen deposition and infiltration of activated fibroblasts. Treatment with the hydroxylase inhibitor dimethyloxalylglycine (DMOG) ameliorated fibrosis. TGF-β1 is a key regulator of fibrosis which acts through the activation of fibroblasts. Hydroxylase inhibition reduced TGF-β1-induced expression of fibrotic markers in cultured fibroblasts suggesting a direct role for hydroxylases in TGF-β1 signalling. This was at least in part due to inhibition of non-canonical activation of extracellular signal-regulated kinase (ERK) signalling. In summary, pharmacologic hydroxylase inhibition ameliorates intestinal fibrosis, through suppression of TGF-β1-dependent ERK activation in fibroblasts. We hypothesize that in addition to previously reported immunosupressive effects, hydroxylase inhibitors independently suppress pro-fibrotic pathways.

New and noteworthy

Here we show that hydroxylase inhibitors reduce fibrosis associated with intestinal inflammation in vivo. At the cellular level, our data suggest a new HIF independent role of hydroxylase inhibition in the regulation of TGF-β1 signalling. These data also suggest that non-canonical ERK signalling pathway is regulated by hydroxylase inhibition. Together, our results show the therapeutic potential of hydroxylase inhibitors for the treatment of intestinal fibrosis.

Keywords

Hypoxia; Inflammatory bowel disease; Intestinal fibrosis; Hydroxylase inhibition; Transforming growth factor β1 (TGF-β1) signalling.

Introduction

Intestinal fibrosis is a debilitating complication of inflammatory bowel disease (IBD)(16, 19, 58). Most approaches to therapeutic intervention in IBD are focused on the control of inflammation rather than fibrosis(58). However, surgical intervention is commonly necessary due to the formation of fibrotic tissue(58, 63). Therefore, there is an unmet clinical need for therapies that suppress pro-fibrotic pathways in IBD.

Fibrosis occurs as a result of an overactive wound healing response and is characterized by excessive deposition of extracellular matrix (ECM)(16, 47, 58). Fibrosis is a complication of multiple chronic inflammatory disorders including chronic kidney disease(12, 55), interstitial lung disease(1, 79) and chronic liver disease(38, 50) as well as IBD(16, 19, 81). At a cellular level, fibrosis occurs as the result of the over-activation of fibroblasts and other ECM-producing cells(1, 12, 16, 79). Among the factors regulating fibrosis, transforming growth factor-β1 (TGF-β1) is the key regulator of healing responses that is implicated in most fibrotic disorders(10, 16, 19, 38). The interaction of TGF-β1 with its cognate receptors on fibroblasts activates canonical (Smad-mediated) and non-canonical (Mitogen Activated Protein Kinases (MAPK)-mediated) signalling pathways that lead to the differentiation of fibroblasts into pro-fibrotic myofibroblasts(10). Through these pathways, TGF-β1 induces the expression of crucial pro-fibrotic genes such as α-smooth

muscle actin (α-SMA), collagen and matrix metalloproteinases and stimulates fibroblast migration to wounded tissue.

Hydroxylases are oxygen sensing enzymes which confer hypoxic sensitivity upon the hypoxia inducible factor (HIF) pathway. Four HIF hydroxylases have been identified to date. Three of these are prolyl hydroxylases (termed PHD1-3) which regulate HIF stability, and the fourth is an asparagine hydroxylase, termed factor inhibiting HIF (FIH), that controls HIF transcriptional activity. The PHD2 isoform is the main regulator of HIF stability through targeting HIF-alpha subunits for hydroxylation-dependent proteosomal degradation(7). These enzymes have also been identified as key players in inflammatory responses(13, 20, 53, 61). Hydroxylase inhibitors have recently been found to be well tolerated in patients in clinical trials for efficacy in anemia(9, 28). These compounds have also been found to ameliorate inflammation in multiple animal models of colitis(14, 15, 22, 36, 60, 64). However, it remains unclear whether the protective effects of these drugs extend to the amelioration of intestinal fibrosis. Furthermore, it is also unclear if their protective effects in colitis are dependent on the regulation of HIF or other pathways(36, 60, 61, 64). Moreover recent studies have highlighted a potentially important role for PHD-2 in the regulation of wound healing responses(32, 78, 80).

In this study, we investigated the effects of hydroxylase inhibition on the development of intestinal fibrosis. Developing our understanding of the

mechanisms underpinning the protective effects of hydroxylase inhibitors in intestinal inflammatory disease will enhance our understanding of their therapeutic potential.

Materials and methods

1. Animal studies

C57BL/6 mice were obtained from Charles River U.K. Prolyl hydroxylase-2 heterozygous knockout mice (PHD2+/-) of the Swiss129 background were provided by Professor Martin Schneider (University of Heidelberg, Germany). These mice have been characterised and used in several previous studies(37, 48). All *in vivo* experiments were performed in compliance with regulations of the Irish Department of Health and approved by the University College Dublin's animal research ethics committee or approved by the ethical commission of the local government (No. G263/14) and carried out at the Interfaculty Biomedical Faculty, University of Heidelberg. All experiments were carried out according to the federation of laboratory animal Science association (FELASA) guidelines. Female and male mice (ages 10-12 weeks) were used. For induction of fibrosis, mice were exposed to 2.5% or 5% DSS (MP Biomedicals, Solon, OH, U.S.A) in drinking water for five days. On day 6 mice were switched to normal drinking water and allowed to recover for 8 or 14 days as indicated. During this period, mice were treated with 8mg/mouse DMOG (i.p) every 48 hours.

2. In vitro experiments

Primary human colonic fibroblasts (CCD-18Co) were purchased from the American Type Culture Collection (ATCC, LGC Standards, Middlesex, UK). Immortalized mouse embryonic fibroblasts (MEF) were a kind gift from Dr Alexander Hoffmann (University of California, Los Angeles, U.S.A). Primary MEF of both wild type (WT) and PHD-2+/- backgrounds were isolated from murine foetuses of the appropriate background. All cells were grown in 1X Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, U.S.A) containing 4.5 g/l glucose and L-glutamine. The medium was supplemented with 10% foetal bovine serum, 50u/ml penicillin and 50 mg/ml streptomycin. In the case of primary MEFs, the medium was also supplemented with 2mM L-glutamine. For CCD-18Co cells, 0.1mM non-essential amino acids were added. For all experiments, cells were switched to serum free medium 24 hours prior to treatment. All treatments were performed in serum free medium. Cells were stimulated with TGF-β1 for the indicated time periods to induce expression of fibrotic markers and activation of TGF-β1-stimulated signalling pathways. To investigate the effects of hydroxylase inhibition on TGF- β1 mediated responses, cells were treated with DMOG or JNJ1935 for 1 or 8 hours prior to TGF-β1 stimulation as indicated. Hypoxia was achieved in a hypoxia chamber (Coy laboratories, Grass Lake, MI, USA). Steady-state atmospheric levels of oxygen inside the chamber were reduced to 1%, at a temperature of

37°C with 5% CO_2 (balance N_2) in humidified conditions. Media added to cells during hypoxic exposures was pre-equilibrated to hypoxia overnight. For studies where the effects of hydroxylase inhibitors were compared to MEK inhibitors, cells were treated with the MEK inhibitor PD186416 for 1 hour prior to TGF-β1 stimulation. For studies assessing the involvement of HIF in hydroxylase inhibitor mediated effects on fibrosis, cell were treated with Digoxin or Camptothecin and then treated with DMOG prior to TGF-β1 stimulation.

3. Estimation of disease parameters and histologic analysis

Body weight, presence of blood in feces, and stool consistency/diarrhea were recorded daily for each mouse to determine the disease activity index (DAI). These parameters were scored as previously described(64). On termination of the experiments, mice were sacrificed by cervical dislocation and approximately 1cm from the distal colon was collected, fixed in 10% formalin and embedded in paraffin. 4 μm sections were cut, mounted on slides, deparaffinized with xylene, and rehydrated in a graded series of alcohols. For assessment of tissue inflammation, sections were stained using hematoxylin and eosin (H&E). Images were taken using an Aperio scanscope XT (Aperio technologies, Vista, CA, U.S.A). Tissue inflammation was assessed in a blinded fashion by 2-3 independent experienced observers and expressed as an average between the estimations provided. The score was calculated as previously described(61).

Fibrosis was assessed as changes in the amount and pattern of collagen deposition in the colonic mucosa and submucosa using Picrosirius red staining (0.5g of direct red 80 (Sigma) and 0.5g of fast green (Sigma) in 500 ml of saturated picric acid solution) and images obtained as described above. Tissue collagen was quantified using ImageJ (National Institutes of Health, Bethesda, MD) and a quantification method developed in-house. Briefly, images were separated into different colour channels and the red channel was selected for quantification of Collagen. From images obtained using double polarized microscopy, collagen-I was quantified, whereas from images obtained using transmitted light, the total amount of collagen was quantified. The colour threshold was adjusted to obtain black and white images, where collagen structures appeared in black on a white background. A small square fitting the width of the sub-mucosal area was drawn and the percentage of black vs white was quantified in 6 random areas of the sub-mucosa for each imaged sample. Alternatively, the lamina propria was separated using a drawing tool and total mucosal collagen quantified as the percentage of black over a white background.

To analyse the presence of activated fibroblasts in the colon, immunofluorescent staining for α-SMA was performed using 4', 6-diamidino-2-phenylindol (DAPI) to counterstain nuclei (details on the primary and secondary antibodies can be found in table 1, antibodies list). Quantification of α-SMA

was developed using the total percentage of area tool in ImageJ® using a method similar to that described for collagen quantification. Briefly, images were split into color channels and the green channel was selected. The threshold of the signal was adjusted and color filter applied so the green signal appeared as black versus a white background. The total amount of black signal in the mucosal area was quantified using ImajeJ.

For assessment of the presence of phospho-ERK positive cells in fibrotic tissues, immunohistochemistry was performed using a specific phospho-ERK primary antibody (details can be found in table 1). The procedure was developed using a Vectastain Elite ABC-kit (Vector Laboratories, CA, U.S.A) following manufacturer's instructions with 3, 3'-Diaminobenzidine (DAB) used as substrate. Haematoxylin was used to counter-stain nuclei.

4. Wound healing assay

MEF cells were grown to confluence in 6 well plates. Once confluent, cells were starved overnight in serum free medium. Monolayers were treated with DMOG for 1 hour. After treatment, monolayers were scratched with a p200 tip to produce a wound, and stimulated with TGF-beta for 24 hours. Cells were then fixed in 4% p-formaldehyde and stained for alpha-SMA as described in the previous section.

5. Western blotting

Whole cell protein lysates preparation and western blot analysis were performed according to previously described methods(15). A full list of the antibodies used can be found in table 1.

6. Quantitative real time polymerase chain reaction

RNA was isolated using trizol based extraction. Complementary DNA was synthesized using standard protocols and quantitative real time polymerase chain reaction carried out as previously described(20). A full list of targets analysed and primers used can be found in table 2.

7. luciferase reporter studies

All transfections were performed following previously described methods(52). For the study of the effects of hydroxylase inhibition on TGF-beta mediated activation of Smad responses, MEF cells were transfected with the luciferase reporter SBE4-Luc which was a gift from Bert Vogelstein (addgene plasmid#16495)(75). To validate the specificity of the reporter, MEF were co-transfected with SBE4-Luc and Smad2 or Smad3 overexpression constructs, and the activation of the luciferase reporter was evaluated. pCMV5B-Flag-Smad3 was a gift from Jeff Wrana (addgene pasmid #11742)(40). pCMV5 Smad2-HA was a gift from Joan Massague (addgene plasmid #14930)(25).

8. Statistical analysis

Graph Pad Prism version 5.0 was used for all statistical analysis. One-way ANOVA followed by a Newman Keuls post-test was used for comparison of multiple groups. Student's t-test was used for individual group comparisons. Differences were considered statistically significant when the p-value was ≤ 0.05. There was a minimum of 3 experimental replicates per group.

Results

1. DMOG reduces intestinal fibrosis in DSS-induced colitis

Hydroxylase inhibitors have been shown to have beneficial effects in multiple models of experimental colitis although their effect on colitis-associated fibrosis remains unknown(15, 60, 64). Figure 1A shows representative images of mouse colon stained with H&E (reflecting tissue inflammation-upper panels), picro-sirius red (reflecting collagen deposition-middle panels) or with immunofluorescence staining for α-SMA (reflecting fibrotic fibroblast infiltration-lower panels). DSS-treatment led to mucosal inflammation, disruption of intestinal structures and submucosal edema which partially recovered following 14 days of natural recovery (Figures 1A). Treatment with the hydroxylase inhibitor DMOG reduced markers of inflammation and significantly accelerated the recovery of disease activity scores (figures 1A, 1B and 1C). Inflammation was associated with the development of fibrosis, reflected by a change in the pattern of collagen deposition characterized particularly by increased submucosal collagen (Figures 1A and 1D, white arrows point areas of higher collagen deposition). A similar pattern of collagen deposition in the submucosa in a model of DSS induced fibrosis has previously been described by Ding and colleagues(17). Fibrosis was also characterized by the presence of infiltrating α-SMA positive fibroblasts in the colonic mucosa (Figures 1A and 1E, white arrows point areas of fibroblast infiltration). Mice treated with DMOG demonstrated reduced inflammation with normal mucosal

structures, an absence of inflammatory infiltrates and a reduction in submucosal edema (figure 1A and 1C). DMOG treatment also reduced and normalized the pattern of submucosal collagen deposition, and reduced infiltration by α-SMA positive fibroblasts (Figure 1A, D and E).

2. Heterozygous deficiency of PHD2 is not protective against DSS induced intestinal fibrosis

PHD2 is the main regulator of HIF-α stability (2, 7, 68). PHD2 has been previously reported to be involved in the regulation of the wound healing response(32, 78, 80). To investigate whether the antifibrotic effects of DMOG in intestinal fibrosis were mediated by regulation of the canonical PHD2/HIF pathway, we compared the development of fibrosis in WT vs PHD2+/- mice exposed to DSS and allowed to recover for 8 days. Figure 2A shows representative images of mouse colon cross-sections stained with H&E, picro-sirius red or immunofluorescence for α-SMA, comparing WT and PHD2+/- mice. DSS caused colitis in WT mice (Figure 2A, B and D). Furthermore, intestinal inflammation was accompanied by profound fibrosis with formation of α-SMA fibroblast aggregates (Figure 2A and 2E) and increased mucosal and submucosal deposition of collagen (Figure 2A and 2F). As demonstrated previously, WT mice treated with DMOG had significantly reduced DAI scores (Figure 2B) and reduced severity of fibrosis with reduced fibroblast infiltration and mucosal collagen deposition (Figure 2A, 2E and 2F). In contrast to DMOG

285 treated mice, PHD2+/- mice were fully susceptible to DSS induced colitis with
286 severe signs of disease reflected by high DAI scores (Figure 2C), severe
287 inflammation (Figure 2A and 2D) and significant fibrosis (Figure 2A, 2E and
288 2F). These results show that reduced activity of PHD2 alone does not
289 ameliorate intestinal fibrosis, suggesting that the antifibrotic actions of DMOG
290 are likely independent of the inhibition of PHD2 and canonical activation of the
291 HIF pathway.

292 *3. Hydroxylase inhibition directly inhibits TGF-β1 induced fibroblast*
293 *activation.*

294 In order to investigate whether the effects of hydroxylase inhibition on fibrosis
295 were due to its anti-inflammatory actions or to direct anti-fibrotic effects on
296 fibroblasts, we investigated the effects of hydroxylase inhibitors on TGF-β1-
297 induced fibroblast activation. As shown in Figure 3A, human colonic CCD-
298 18Co fibroblasts stimulated with 1ng/ml of TGF-β1 for 48 hours underwent a
299 phenotypic transformation into myofibroblasts, characterised by increased
300 expression of α-SMA stress fibres. Moreover, western blot analysis revealed a
301 TGF-β1 mediated induction of both α-SMA and collagen-1(α) expression
302 (Figure 3B). Treatment with either DMOG or JNJ1935 prior to TGF-β1
303 stimulation reduced differentiation of the cells into myofibroblasts as shown by
304 reduced presence of α-SMA stress fibres (Figure 3C). Moreover, a reduction of
305 both α-SMA and collagen-1(α)-1 was observed in cells treated with the

hydroxylase inhibitors prior to TGF-β1 stimulation (Figure 3D-F). Taken together, these data suggest that the antifibrotic actions of hydroxylase inhibitors may be due to their ability to block TGF-β mediated fibroblast activation. Moreover the fact that JNJ1935, which evokes significantly lower activation of HIF than DMOG, caused an equivalent reduction of fibrotic markers to DMOG further suggests that the effects of DMOG are HIF independent(4).

4. Hydroxylase inhibition does not affect activation of the TGF-β-Smad signalling pathway

To investigate how hydroxylase inhibition suppresses the effects of TGF-β1, we analysed the effects of hydroxylase inhibitors on activation of the canonical TGF-Smad signalling pathway. To facilitate transfection studies, mouse embryonic fibroblasts (MEF) were used in these experiments. DMOG reduced the number of α-SMA positive MEF that migrated into a wound space under the influence of TGF-β1 in a wound healing assay, compared to cells that were stimulated with TGF-β1 in the absence of hydroxylase inhibition (Figure 4A). This effect correlated with the ability of DMOG to significantly reduce TGF-β1 induced expression of α-SMA mRNA, confirming that hydroxylase inhibitors exert similar effects on TGF signalling in MEF and indicating that such effects are also reflected at the transcriptional level (Figure 4B). We next tested the effect of DMOG on Smad signalling. Stimulation with TGF-β1 induced

phosphorylation of Smad2 and Smad3 at 30 to 120 minutes (Figure 4C-4E). Treatment with DMOG failed to reduce Smad2 phosphorylation at any time point, although it modestly reduced levels of pSmad3 at 2 hours (figure 4C-4E). The combination of hydroxylase and proteosome inhibition did not further affect the phosphorylation of Smad2/3, confirming that DMOG does not enhance pSmad degradation (Figure 4C-4E). To further analyse the effects of hydroxylase inhibitors on Smad pathway functionality, MEF were transfected with the Smad luciferase reporter SBE4-Luc. This construct incorporates four copies of the Smad binding element (SBE) in its promoter. Co-transfection of MEF with SBE4 and Smad2 or Smad3 overexpression plasmids showed activation of the reporter as expected (Figure 4F), with Smad3 more active than Smad2 in this regards in the absence of TGF-β1 (Figure 4F). Similarly, TGF- β1 activated the SBE4-Luc reporter in a time-dependent fashion that was maximal at 8 hours (Figure 4G). However, DMOG did not reverse the effect of TGF-β1 on Smad-dependent luciferase activity (Figure 4G). Together these results show that the effects of hydroxylase inhibitors on responses to TGF-β1 are not due to blockade of the canonical Smad signalling pathway.

5. Hydroxylase inhibitors reduce TGF-β1 dependent activation of the non-canonical extracellular regulated kinase (ERK) signalling pathway

To investigate an alternative explanation for the effects of the hydroxylase inhibitors on TGF-β1 signalling, we next examined the effects on non-canonical

signalling. Amongst MAPK pathways implicated in TGF-β1 responses, c-Jun N-terminal kinases (JNK) and ERK have been described for their activation in fibrotic disease and involvement in the expression of fibrotic markers(3, 26, 31, 38, 42, 43, 55, 72). In the present study, areas of the mucosa of both WT and PHD-2+/- mice where α-SMA positive fibroblasts are concentrated exhibited increased pERK nuclear staining, confirming the activation of the ERK pathway in fibrotic colons (Figure 5A). In vitro, DMOG did not affect TGF-β1 induced phosphorylation of JNK (data not shown). In contrast, DMOG reduced TGF-β1-induced phosphorylation of ERK (pERK) at 8 hours post stimulation (Figure 5B and 5D). Interestingly, DMOG did not affect the phosphorylation of the upstream kinase MEK at the same time point, indicating that the kinase cascade was specifically affected at the level of ERK activation (Figure 5C and 5E). Phosphorylation of the linker region of Smad2, which is mediated directly by ERK(11, 29, 69), was also moderately decreased by DMOG further confirming the effects of DMOG on phosphorylation of ERK (Figure 5B). The hydroxylase inhibitor JNJ1935 and atmospheric hypoxia were also examined for their effects on ERK activation. JNJ1935 caused a time-dependent reduction in TGF-β1-induced ERK phosphorylation that was already present at 6 hours and was profound at 8 hours post TGF stimulation (Figure 5F and 5G). Of note, hypoxia did not significantly affect the ability of TGF-β1 to activate this kinase cascade (Figure 5F and 5G). In order to further analyse the mechanism of hydroxylase inhibitor mediated reduction of ERK phosphorylation, we compared the effect

of DMOG and JNJ1935 on ERK and MEK phosphorylation to that of the MEK inhibitor PD184161 (Figure 5H). While DMOG and JNJ1935 reduced ERK phosphorylation without affecting that of MEK, the reduction of ERK phosphorylation caused by PD184161 was accompanied by a dramatic increase in phosphorylated MEK (Figure 5H). Taken together, these results show that hydroxylase inhibitors modulate TGF-ERK signalling, which is a potential mechanism whereby they exert anti-fibrotic effects.

6. The antifibrotic effect of hydroxylase inhibitors is independent of HIF

Our previous in vivo studies demonstrated no protective effects of PHD2 deficiency against fibrosis, thus providing evidence that the antifibrotic effects of DMOG are HIF independent. To further demonstrate this hypothesis, we compared the response of WT and PHD2+/- MEF to TGF-β1. Heterozygous deficiency of PHD2 did not block ERK phosphorylation in response to TGF-β1, nor did it prevent the ability of DMOG or JNJ1935 to reduce levels of pERK (Figure 6A). Moreover, stimulation of PHD2+/- cells with TGF-β1 caused an increase in production of α-SMA and CTGF mRNA (Figure 6B and 6C). The increment was significantly higher than that found in WT cells, but was nevertheless abrogated when PHD2+/- cells were treated with DMOG (Figure 6B and 6C). Next, we analysed whether reducing HIF in hydroxylase inhibitor treated cells would affect the ability of the compounds to reduce the expression of fibrotic markers. Drugs such as Digoxin and Camptothecin have been

described as potent HIF inhibitors (8, 46, 76). As has been previously described, we found treatment with either digoxin or camptothecin to reduce DMOG mediated HIF-1α protein stabilization in MEF (data not shown). However, this reduction of HIF did not affect the ability of DMOG to reduce the expression of Acta2 (α-SMA) or Ctgf RNA (Figure 6D and 6E). Together these data suggest that the antifibrotic effects of hydroxylase inhibitors are independent of HIF.

Discussion

Hydroxylase inhibitors have recently been shown in clinical trials for anemia to be well tolerated by patients and therefore represent a potentially important new class of therapeutic agents(9, 28). We and others have previously demonstrated protective effects of these reagents in multiple models of murine colitis. Therefore, the potential for repurposing hydroxylase inhibitors for the treatment of colitis is a realistic possibility. Here, we investigated whether the beneficial effects of hydroxylase inhibition in intestinal inflammation extend to inflammation-associated fibrosis, using the DSS-induced murine colitis model. We show that treatment with the pan-hydroxylase inhibitor DMOG during the post-DSS recovery period reduces intestinal fibrosis in addition to its known anti-inflammatory effects. We also show that hydroxylase inhibitors are able to block TGF-β1 mediated activation of intestinal fibroblasts as well as MEF, and that they do so at least in part via a HIF-independent manner. At the molecular level, the inhibitors reduce TGF-β1 mediated activation of the ERK signalling pathway, thus providing mechanistic insight into the anti-fibrotic actions of these compounds.

The DSS model of colitis has been used to investigate intestinal fibrosis by allowing long recovery periods after exposure to DSS(63, 71). Another approach to the DSS colitis model often used to investigate fibrosis is the so called multicycle DSS model, where the animals are exposed to multiple cycles

of DSS followed by one week recovery periods. In a study comparing the different approaches, Ding et al showed the presence of fibrosis already after the first DSS cycle of DSS and highlighted that C57BL6 mice do not show mucosal healing after DSS and progress towards chronic colitis(17). Moreover, the notion that fibrosis is also a common complication in ulcerative colitis further validates the potential for the DSS recovery model of colitis as a model for the study of intestinal fibrosis(23). As previously described, our results show that mice allowed to recover after exposure to DSS have evident signs of inflammation and fibrosis even 8 or 14 days after DSS exposure. We also noted that there are differences in disease progression between the different strains of mice used. C57BL6 were susceptible to a lower dose of DSS and rapidly developed colitis showing signs of disease already during the DSS exposure period. In contrast, Swiss 129 mice did not show signs of disease initially, even if exposed to a higher dose of DSS. However, this mice developed clear signs of colitis after the DSS period and progressed towards more severe colitis and fibrosis. In our studies, treatment of mice with DMOG during the post-DSS recovery period reduced fibrosis as shown by the reduced presence of mucosal α-SMA positive fibroblasts and reduced sub-mucosal collagen deposition. These effects were associated with reduced inflammation, showing that hydroxylase inhibitors might prove beneficial if used as treatments in established colitis. Of note, a number of recent studies have indicated that the inhibition of PHD2 positively regulates healing responses, supporting the

hypothesis that hydroxylases might be novel targets for the modulation of wound healing and therefore, fibrosis(32, 78, 80). In our studies, however, PHD2+/- mice were not protected against chronic inflammation or fibrosis induced by DSS. Furthermore, PHD2+/- MEF did not demonstrate reduced sensitivity to TGF-β1 stimulation compared to WT, suggesting that the beneficial effects of hydroxylase inhibitors in wild-type mice are likely independent of the PHD2/HIF axis. This HIF independent mechanism is also supported by the antifibrotic effects obtained with JNJ1935, a drug used at a concentration that does not inhibit FIH and thus, does not strongly activate HIF. We further tested the involvement of HIF in hydroxylase inhibitor anti-fibrotic actions by inhibiting HIF stabilization in DMOG treated cells. As previously demonstrated, digoxin and camptothecin reduced HIF-1α in DMOG treated cells(8, 46, 76). However, this did not affect the ability of DMOG to reduce the expression of α-SMA and CTGF in TGF-β1 stimulated cells. This provided further evidence that the anti-fibrotic action of hydroxylase inhibitors is HIF independent.

Fibrosis results from overactive wound healing and is mainly attributed to the profibrogenic factor TGF-β1(16, 42, 49, 67). In our studies, hydroxylase inhibitors demonstrated specific anti-fibrotic actions by targeting the ability of TGF-β1 to up-regulate the production of various key fibrosis markers in cultures of human colonic fibroblasts and MEF. However, DMOG failed to

block the phosphorylation of Smad2/3 or the activation of a Smad luciferase reporter in response to TGF-β1. Rather, hydroxylase inhibitors reduced TGF-β1 mediated activation of the ERK signalling pathway, which has been shown to play important roles in fibrosis(31, 42, 55) and which we found to be active in areas of intestinal fibrosis. Thus, we purpose that hydroxylase inhibitors target the activation of the non-canonical TGF-ERK signaling pathway to cause the inactivation of the ERK mediated fibrotic response (Figure 7). Together, these results indicate that hydroxylase inhibitors, in addition to their previously described anti-inflammatory effects, have beneficial effects against intestinal fibrosis at least in part through inhibition of the TGF-ERK signaling pathway. Of note, the inhibitory effects of hydroxylase inhibitors on ERK phosphorylation were not seen for its upstream kinase MEK. Indeed, comparison between hydroxylase inhibitors and a MEK inhibitor suggested a different mechanism for the inhibition of ERK phosphorylation between the two types of drugs. While MEK inhibitors reduced ERK phosphorylation but induced a higher level of phosphoMEK, hydroxylase inhibitors reduced phosphoERK but did not affect phosphoMEK. In order to gain further insights to the possible mechanism of this PHD inhibitor mediated regulation of ERK phosphorylation, we hypothesised that PHD inhibitors could upregulate dual specificity phosphatases (DUSP). DUSP are known to inactivate MAPK and have been shown to be regulated by hypoxia in multiple studies(5, 35, 41, 54, 56, 66). Hydroxylase inhibition did not increase the expression of DUSPs

494 investigated (data not shown). Furthermore, our results are in line with recently
495 published reports showing beneficial effects of hydroxylase inhibitors in
496 fibrosis(34, 57, 62, 73, 77). Of note, previous studies have described deleterious
497 effects for HIF activation in fibrotic pathologies(27, 70). The current study is
498 not in contradiction with this possibility, as we show that the intestinal
499 antifibrotic actions of hydroxylase inhibitors are HIF-independent.

500 The ability of hydroxylase inhibitors to regulate the stability of HIF-α subunits
501 may have not only beneficial effects, but also potential adverse effects. A
502 widely discussed side effect of hydroxylase inhibitors is the activation of HIF-
503 dependent EPO production(6, 30, 33), which is seen as an important limitation
504 to the use of these drugs. However, this ability to increase EPO is currently
505 being investigated as a therapy for anemia(6, 9, 28). In IBD, anemia is one of
506 the most common extraintestinal symptoms and it is estimated that one third of
507 IBD patients suffer from anemia(21). Moreover, anemia is reportedly predictive
508 of severe and disabling progression in IBD(39). Therefore, the activation of
509 EPO production with hydroxylase inhibition could be a secondary beneficial
510 effect in IBD patients where fibrosis and anemia are often co-incidental
511 outcomes. On the other hand, the use of targeted release forms that allow
512 specific local delivery of hydroxylase inhibitors to the colon could minimize
513 systemic exposure. In recent work, we demonstrated that the use of DMOG

514 mini-spheres formulated to target delivery to the colon achieved anti-
515 inflammatory effects while minimizing systemic side effects(65).

516 IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is increasing
517 in prevalence, especially in developed countries(45, 51). Advanced stages of
518 IBD are often associated with excessive wound healing causing fibrosis and
519 tissue scarring. While much IBD research seeks to unravel the early causes of
520 pathology, less attention has been paid to long-term complications. Despite the
521 advances achieved in the treatment of IBD with novel therapies such as anti-
522 TNF-α antibodies that help to maintain remission(14, 44), IBD is still in need of
523 improved therapeutic approaches. Intestinal fibrosis is a major indication for
524 surgery in IBD as 75-80% of CD patients are estimated to require surgical
525 intervention due to the formation of fibrotic scars leading to intestinal
526 obstructions(47, 59, 63). Further, the importance of fibrosis in a number of other
527 chronic inflammatory diseases such as chronic kidney disease or interstitial
528 pulmonary fibrosis(16, 18, 24, 59, 74) and the lack of effective means to limit
529 progression towards fibrosis suggest that treatments which target fibrotic
530 disease may represent an important therapeutic advance. We have shown that
531 hydroxylase inhibitors have promising anti-fibrotic effects, potentially due to
532 their actions on the non-canonical TGF-ERK signalling pathway.

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Author contributions

Mario Manresa, Murtaza Tambuwala, Jonathan Harnoss, Martin Schneider and Cormac Taylor participated in the design of this study. Mario Manresa, Murtaza Tambuwala, Praveen Radhakrishnan, Eric Brown, Miguel Cavadas, Ciara Keogh and Alex Cheong participated in the acquisition of data. Mario Manresa,

Murtaza Tambuwala, Eoin Cummins, Kim Barrett and Cormac Taylor participated in the analysis and interpretation of the data. Mario Manresa, Kim Barrett and Cormac Taylor participated in the drafting of this manuscript.

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Tables

Target	Supplier	Code
α-SMA	Abcam	Ab7817
Collagen-1(α)-1	Santa Cruz Biotechnology	sc-8784
HIF-1α	BD Transduction Laboratories	#610958
HIF-1α (clone H1a67)	Merck Millipore	MAB5382
pSmad2 (carboxyterminal)	Merk Millipore	AB3849
pSmad2 (linker)	Cell Signalling Technology	#3104
pSmad3	Cell Signalling Technology	#9520
TotalSmad2	Cell Signalling Technology	#3103
TotalSmad3	Cell Signalling Technology	#9513
pERK	Santa Cruz Biotechnology	sc-7383
TotalERK	Cell Signalling Technology	#9102
Alexa fluor 488 (anti-mouse)	Thermo Fisher Scientific	A-21202

Table 1. *Antibodies list*. List of antibodies used for immunostaining and western blot protein analysis.

Target	Sequence	Chemistry	Source
m-α-SMA (Acta2)	5'-TGCTGTCCCTCTATGCCTCT-3', sense		
	5'-GCAGGGCATAGCCCTCATAG-3', antisense	Sybr green	Self-designed
Ctgf	Commercially available Taqman probe	Taqman	Applied Biosystems
Eukaryotic 18S rRNA	Commercially available Taqman probe	Taqman	Applied Biosystems

Table 2. *Primer list*. List of target genes analysed and the complementary primers used for qRT-PCR. The list includes the source of the primers.

Figure legends

Figure 1. *DMOG reduces fibrosis in DSS induced colitis.* A, representative images of mouse colon stained with hematoxylin and eosin (H&E), Picrosirius red imaged with double polarized light (collagen) and immunofluorescence histochemistry (α-SMA). White arrows point areas of major collagen accumulation or α-SMA positive infiltrates. Quantification of disease activity index (DAI) (B), assessment of tissue inflammation (C), submucosal collagen deposition (D) and α-SMA positive infiltration (E). n=4, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 2. *Heterozygous deficiency of PHD2 does not protect against intestinal fibrosis in DSS-induced colitis.* A, representative images of mouse colon from WT and PHD2+/- mice stained with H&E, Picrosirius red imaged with transmited light (collagen) and immunofluorescence histochemistry (α-SMA). Quantification of disease activity index (DAI) in WT mice comparing natural recovery to IP DMOG treatment (B); Quantification of DAI comparing natural recovery between WT and PHD2+/- mice (C); Assessment of tissue inflammation (D); Quantification of α-SMA positive staining (E); Quantification of percentage of mucosal collagen (F). $n \geq 3$, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 3. *Hydroxylase inhibition reduces TGF-β1 induced human colonic fibroblast activation.* A, representative images of CCD-18Co cells stimulated with 1ng/ml TGF-β1 and stained for α-SMA using immunofluorescence histochemistry with DAPI as a nuclear counter-stain (n=2). B, representative western blot of α-SMA and collagen-1(α)-1 in CCD-18Co cells stimulated with 1ng/ml TGF-β1 (n=3); C, representative images of CCD-18Co stimulated with 1ng/ml TGF-β1 and treated with 1mM DMOG or 100μM JNJ1935, stained for α-SMA using immunofluorescence histochemistry with DAPI as a nuclear counter-stain (n=3). D, representative western blot of α-SMA and collagen-1(α)-1 in CCD-18Co cells treated with 1mM DMOG or 100μM JNJ1935 and stimulated with 1ng/ml of TGF-β1 for 48 hours (n=10). E, densitometry of α-SMA in CCD18Co treated as described in D (n=10). F, densitometry of collagen-1α in CCD18Co cells treated as described in D (n=10).

Figure 4. *Hydroxylase inhibition does not affect TGF-β1 induced Smad activation.* A, representative images of MEF monolayers, treated with 1mM DMOG for 1 hour, wounded and then stimulated for 24 hours with 10ng/ml TGF-β1, stained for α-SMA using immunofluorescence histochemistry with DAPI as a nuclear counter-stain (n=4). B, qRT-PCR of α-SMA in MEF stimulated with 1ng/ml TGF-β1 and treated with 1mM DMOG (n=3); C, representative western blot of pSmad2, pSmad3, TotalSmad2, TotalSmad3, HIF-1α and β-Actin in MEF stimulated with 1ng/ml TGF-β1 for 1/2h or 1h or 2h and treated with 1mM DMOG for 1h +/- 1/2h 10nM MG132 pre-treatment (n=6); D, densitometry of pSmad2 (n=6); E, densitometry of pSmad 3 (n=6). F, luciferase production in MEF transfected with SBE4-Luc and co-transfected with pCMV5B-Smad3-flag or pCMV5-Smad2-HA (n=4); G, luciferase production in MEF transfected with SBE4-Luc and stimulated with 1ng/ml TGF-β1 and treated with 1mM DMOG (n=4). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 5. *Hydroxylase inhibition reduces TGF-β1 induced ERK activation.* A, representative images of mouse colon cross-sections stained for α-SMA using immunofluorescence histochemistry and stained for phospho-ERK (pERK) using immunohistochemistry (n≥3). Red arrows point areas of abundant pERK positive cells, which are often coincident to areas where activated fibroblasts accumulate. B, representative western blot of pERK, TotalERK, pSmad2 linker, TotalSmad2 and β-Actin in MEF stimulated with 1ng/ml TGF-β1 and treated with 1mM DMOG (n=6); C, representative western blot of pMEK and β-Actin in MEF stimulated with 1ng/ml TGF-β1 and treated with 1mM DMOG (n=5). D, densitometry for pERK of the western blots described in B (n=6); E, densitometry for pMEK of the western blots described in C (n=5). F, representative western blot of pERK, TotalERK, pMEK, TotalMEK and β-Actin in MEF stimulated with 1ng/ml TGF-β1 and treated with 100μM JNJ1935 or 1% oxygen (n≥3); G, densitometry for pERK of the western blots described in F; H, representative western blot of pSmad2 linker, pERK, pMEK and β-Actin in MEF treated with 1mM DMOG or 100μM JNJ1935 or 0.3mM PD184161 and stimulated with 1ng/ml of TGF-β1 (n=4). * $p \leq 0.05$; ** $p \leq 0.01$.

Figure 6. *The anti-fibrotic effect of hydroxylase inhibitors is HIF independent.* A, representative western blot of pERK, TotalERK and β-Actin in WT vs PHD2+/- MEF stimulated with 1ng/ml TGF-β1 and treated with 1mM DMOG or 100μM JNJ1935 (n=4); qRT-PCR of α-SMA (B) and CTGF (C) in WT vs PHD2+/- MEF stimulated with 1ng/ml TGF-β1 and treated with 1mM DMOG (n≥3). Quantitative real time PCR of α-SMA (D) or CTGF (E) in MEF pre-treated with 100μM digoxin or 2μM camptothecin for 30 minutes, then with 1mM DMOG for 1 hour and then stimulated with 1ng/ml TGF-β1 for 8 hours (n≥3). * $p \leq 0.05$; ** $p \leq 0.01$.

Figure 7. *Purposed mechanism for hydroxylate inhibitor mediated anti-fibrotic actions.* Upper panel displays the combination of TGF-β1 activated Smad dependent (canonical) and non-canonical (ERK dependent) signaling pathways. Lower panel shows our purposed model for hydroxylase inhibition antifibrotic action. Pharmacologic inhibition of oxygen sensing hydroxylases mediates the long term dephosphorylation of the non-canonical TGF-ERK signaling pathway and reduces the crosstalk between ERK and Smad pathways. This causes a reduction of the TGF mediated fibroblast activation and ameliorates fibrosis in a mouse model of colitis.

Figure 1

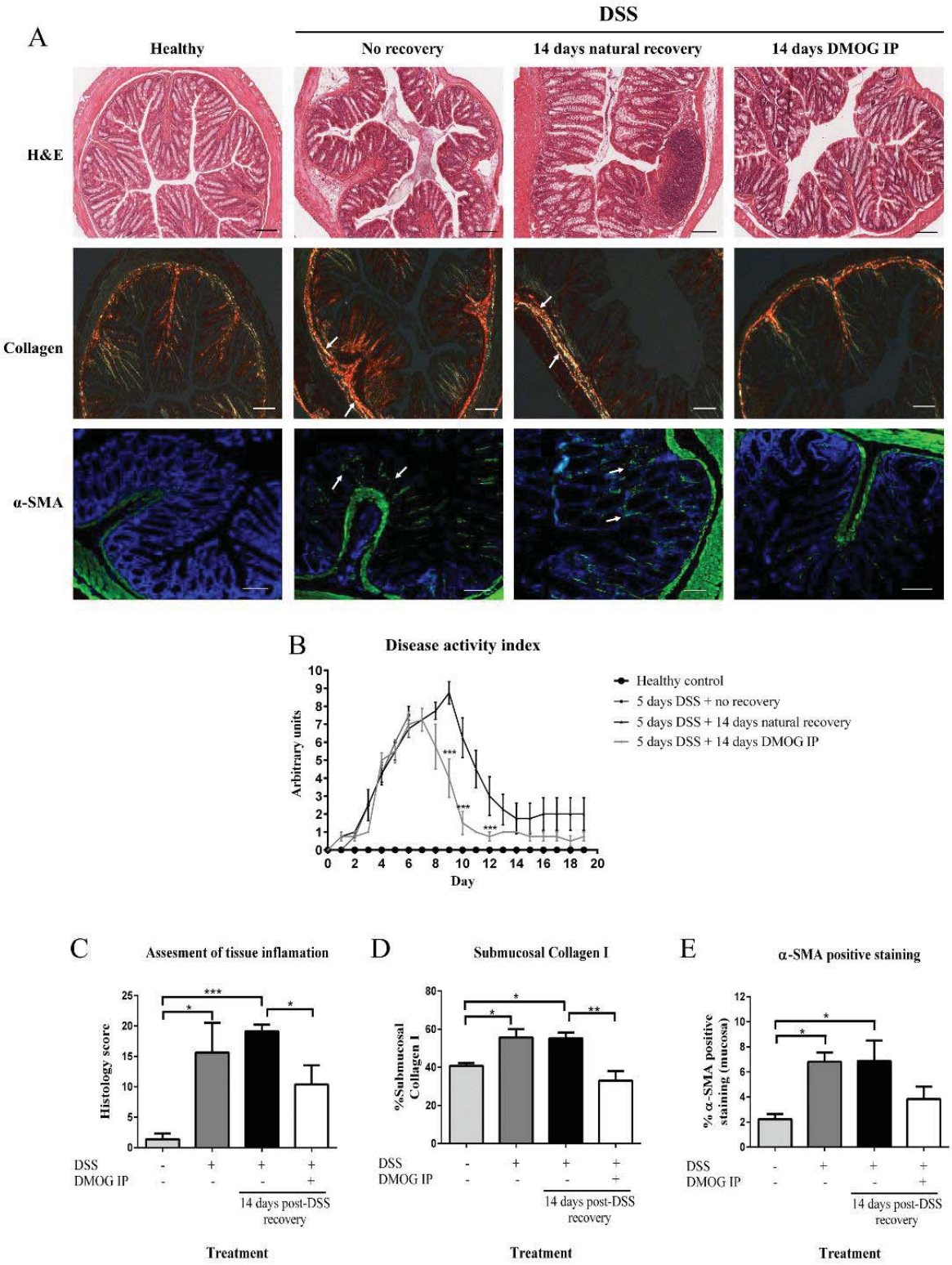

A
DSS
Healthy
No recovery
14 days natural recovery
14 days DMOG IP
H&E
Collagen
α-SMA
B
Disease activity index
Arbitrary units
Day
Healthy control
5 days DSS + no recovery
5 days DSS + 14 days natural recovery
5 days DSS + 14 days DMOG IP
C
Assesment of tissue inflamation
Histology score
D
Submucosal Collagen I
%Submucosal Collagen I
E
α-SMA positive staining
% α-SMA positive staining (mucosa)
DSS
DMOG IP
14 days post-DSS recovery
Treatment

Figure 2

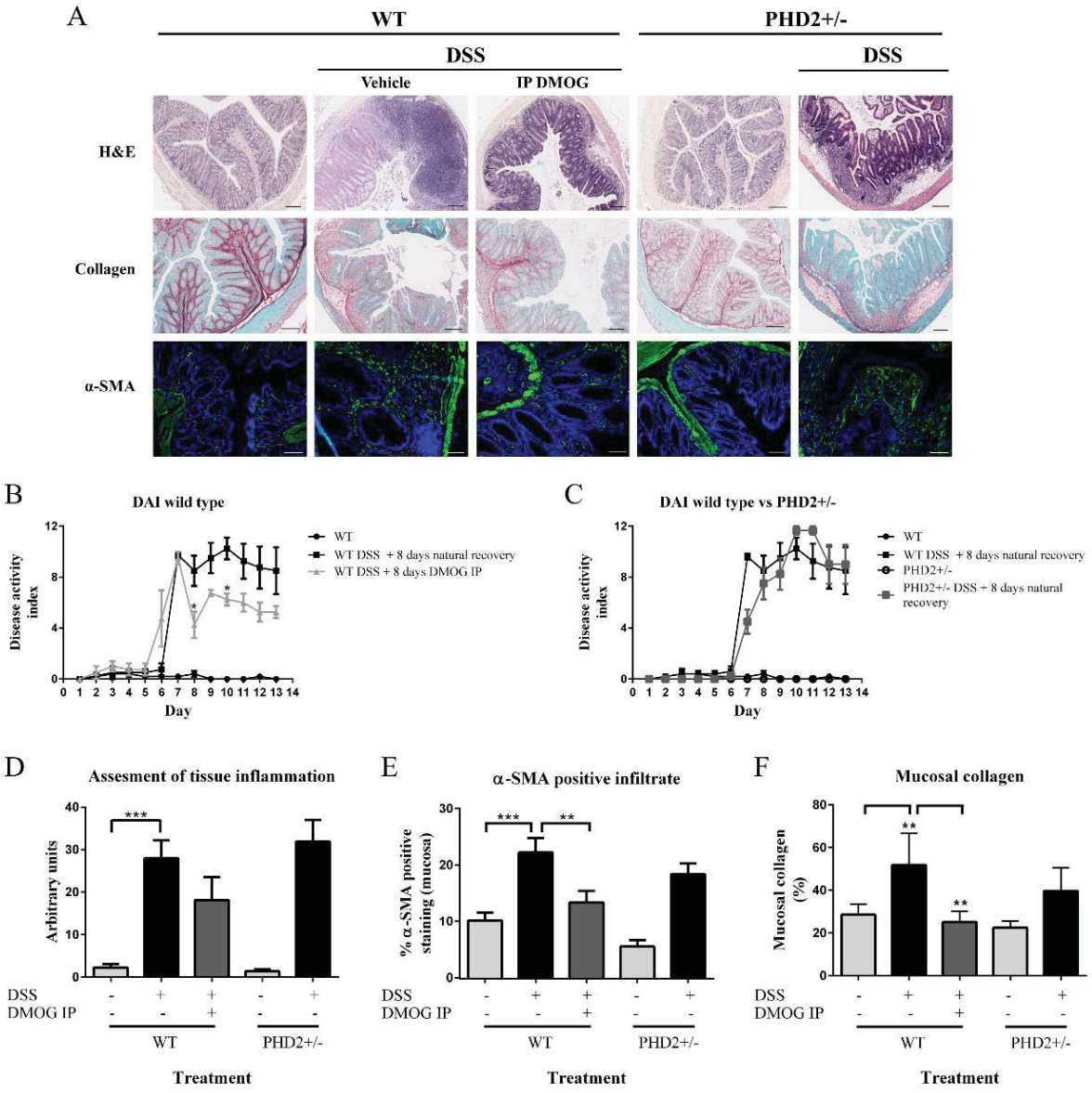

A
WT
PHD2+/-
DSS
Vehicle
IP DMOG
DSS
H&E
Collagen
α-SMA
B
DAI wild type
Disease activity index
Day
WT
WT DSS + 8 days natural recovery
WT DSS + 8 days DMOG IP
C
DAI wild type vs PHD2+/-
Disease activity index
Day
WT
WT DSS + 8 days natural recovery
PHD2+/-
PHD2+/- DSS + 8 days natural recovery
D
Assesment of tissue inflammation
Arbitrary units
DSS
DMOG IP
WT
PHD2+/-
Treatment
E
α-SMA positive infiltrate
% α-SMA positive staining (mucosa)
DSS
DMOG IP
WT
PHD2+/-
Treatment
F
Mucosal collagen
Mucosal collagen (%)
DSS
DMOG IP
WT
PHD2+/-
Treatment

Figure 3

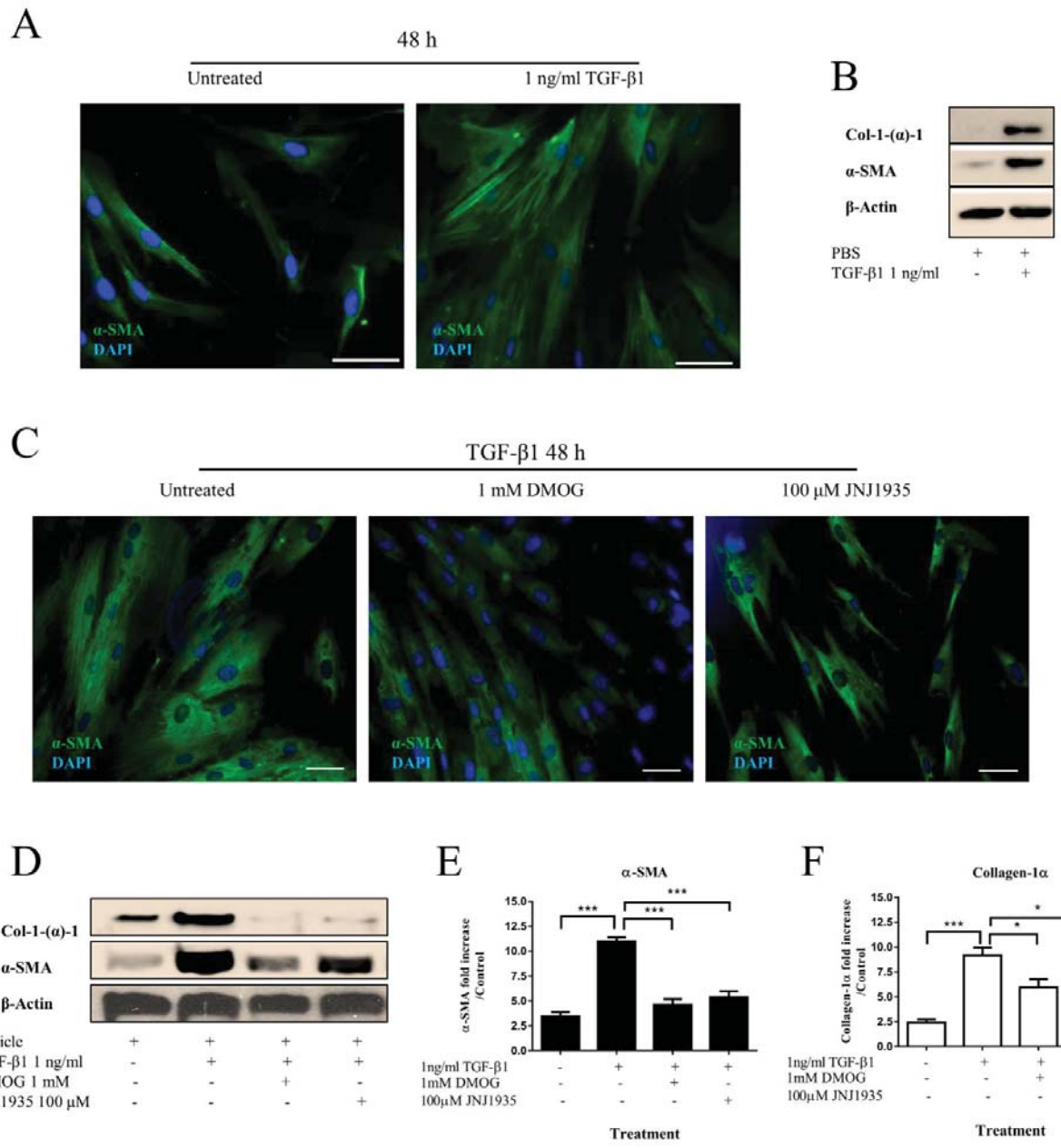

A
48 h
Untreated
1 ng/ml TGF-β1
α-SMA
DAPI
B
Col-1-(α)-1
α-SMA
β-Actin
PBS + +
TGF-β1 1 ng/ml - +
C
TGF-β1 48 h
Untreated
1 mM DMOG
100 μM JNJ1935
D
Col-1-(α)-1
α-SMA
β-Actin
Vehicle + + + +
TGF-β1 1 ng/ml - + + +
DMOG 1 mM - - + -
JNJ1935 100 μM - - - +
E
α-SMA
α-SMA fold increase /Control
1ng/ml TGF-β1 - + + +
1mM DMOG - - + -
100μM JNJ1935 - - - +
Treatment
F
Collagen-1α
Collagen-1α fold increase /Control
1ng/ml TGF-β1 - + + +
1mM DMOG - - + -
100μM JNJ1935 - - - +
Treatment

Figure 4

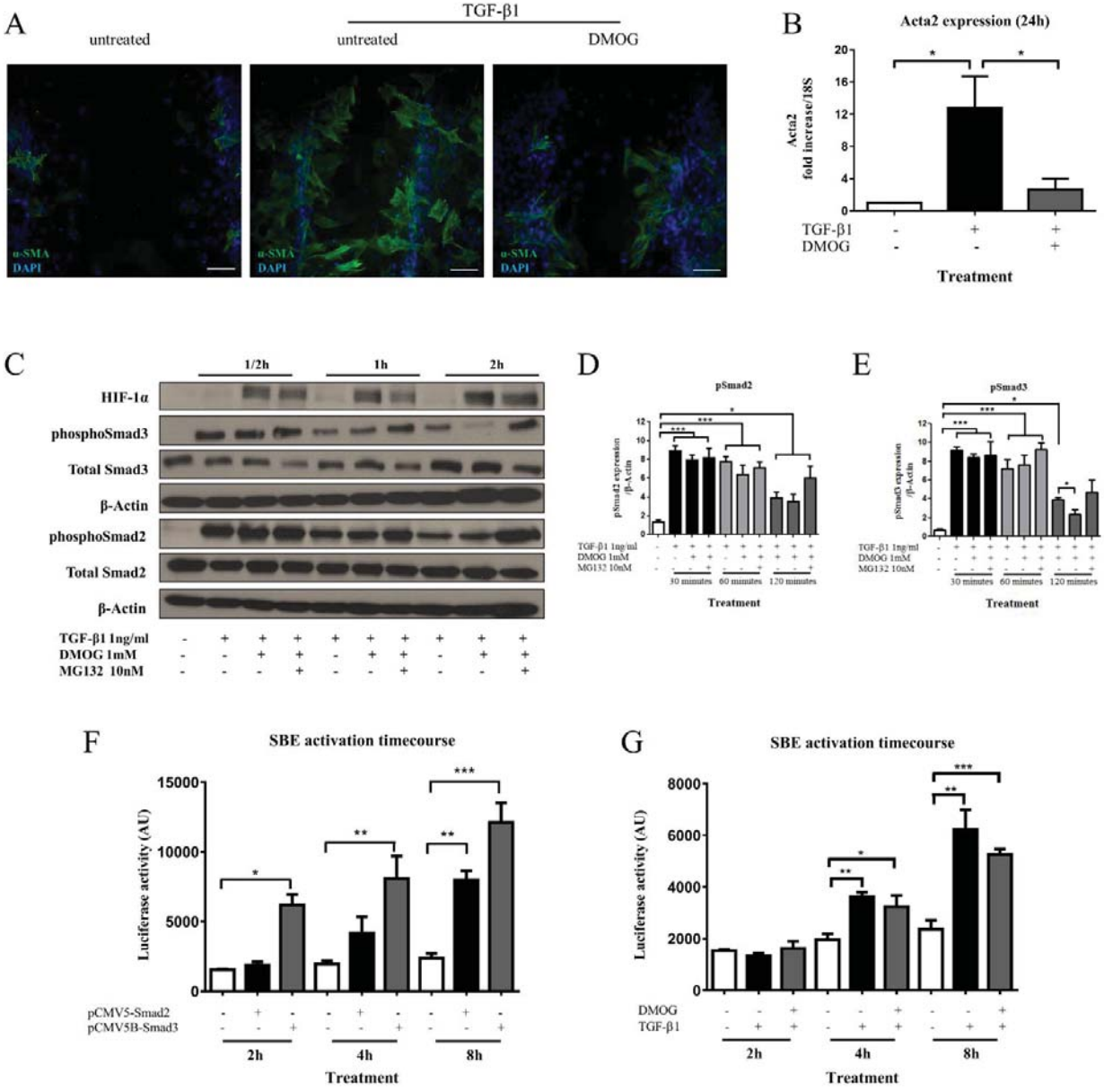

A
TGF-β1
untreated
untreated
DMOG
α-SMA
DAPI
B
Acta2 expression (24h)
Acta2 fold increase/18S
TGF-β1
DMOG
Treatment
C
1/2h
1h
2h
HIF-1α
phosphoSmad3
Total Smad3
β-Actin
phosphoSmad2
Total Smad2
β-Actin
TGF-β1 1ng/ml
DMOG 1mM
MG132 10nM
D
pSmad2
pSmad2 expression /β-Actin
30 minutes
60 minutes
120 minutes
Treatment
E
pSmad3
pSmad3 expression /β-Actin
Treatment
F
SBE activation timecourse
Luciferase activity (AU)
pCMV5-Smad2
pCMV5B-Smad3
2h
4h
8h
Treatment
G
SBE activation timecourse
Luciferase activity (AU)
DMOG
TGF-β1
2h
4h
8h
Treatment

Figure 5

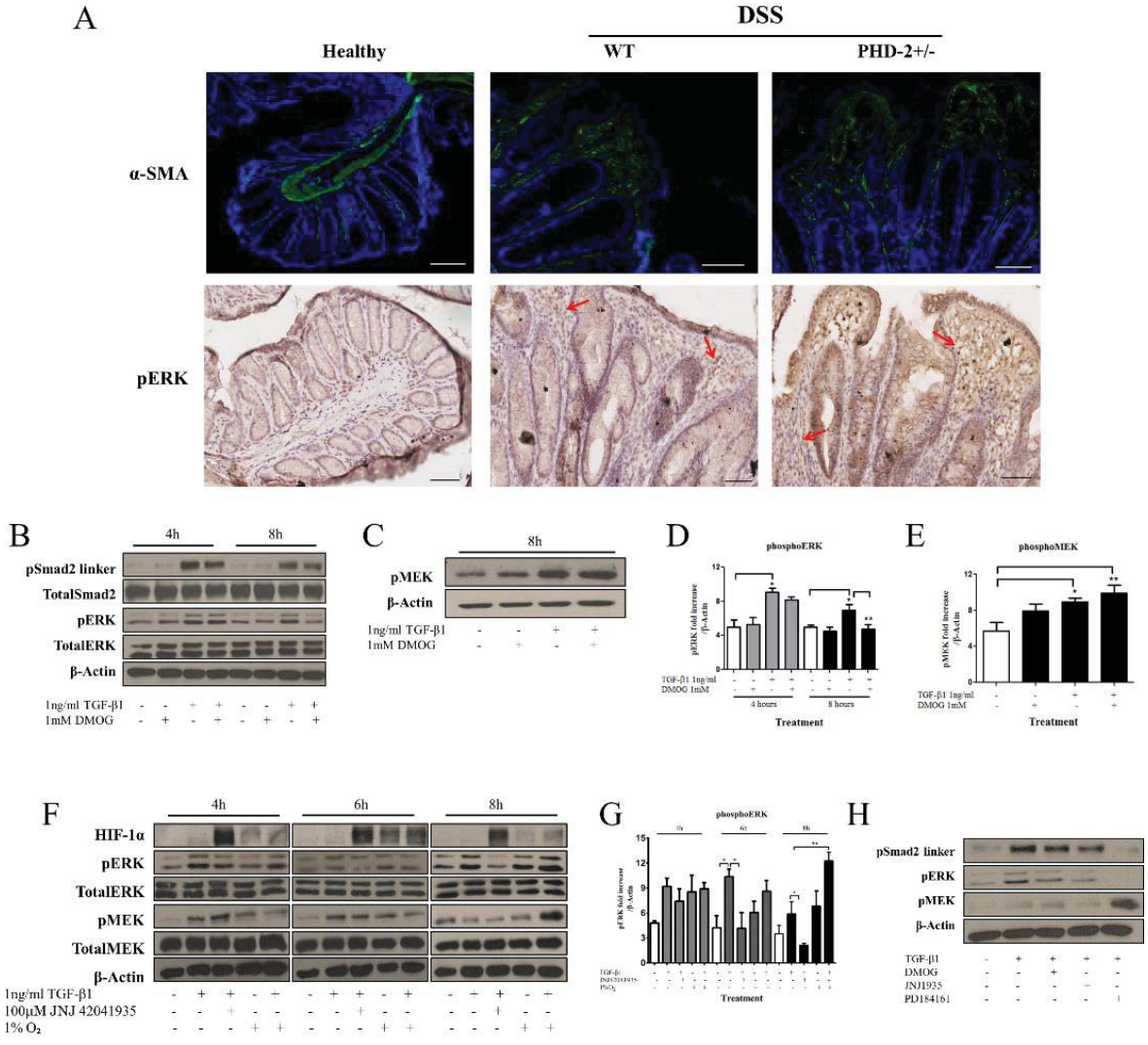

A
DSS
Healthy
WT
PHD-2+/-
α-SMA
pERK
B
4h
8h
pSmad2 linker
TotalSmad2
pERK
TotalERK
β-Actin
1ng/ml TGF-β1
1mM DMOG
C
8h
pMEK
β-Actin
1ng/ml TGF-β1
1mM DMOG
D
phosphoERK
pERK fold increase /β-Actin
TGF-β1 1ng/ml
DMOG 1mM
4 hours
8 hours
Treatment
E
phosphoMEK
pMEK fold increase /β-Actin
TGF-β1 1ng/ml
DMOG 1mM
Treatment
F
4h
6h
8h
HIF-1α
pERK
TotalERK
pMEK
TotalMEK
β-Actin
1ng/ml TGF-β1
100μM JNJ 42041935
1% O2
G
phosphoERK
pERK fold increase /β-Actin
Treatment
H
pSmad2 linker
pERK
pMEK
β-Actin
TGF-β1
DMOG
JNJ1935
PD184161

Figure 6

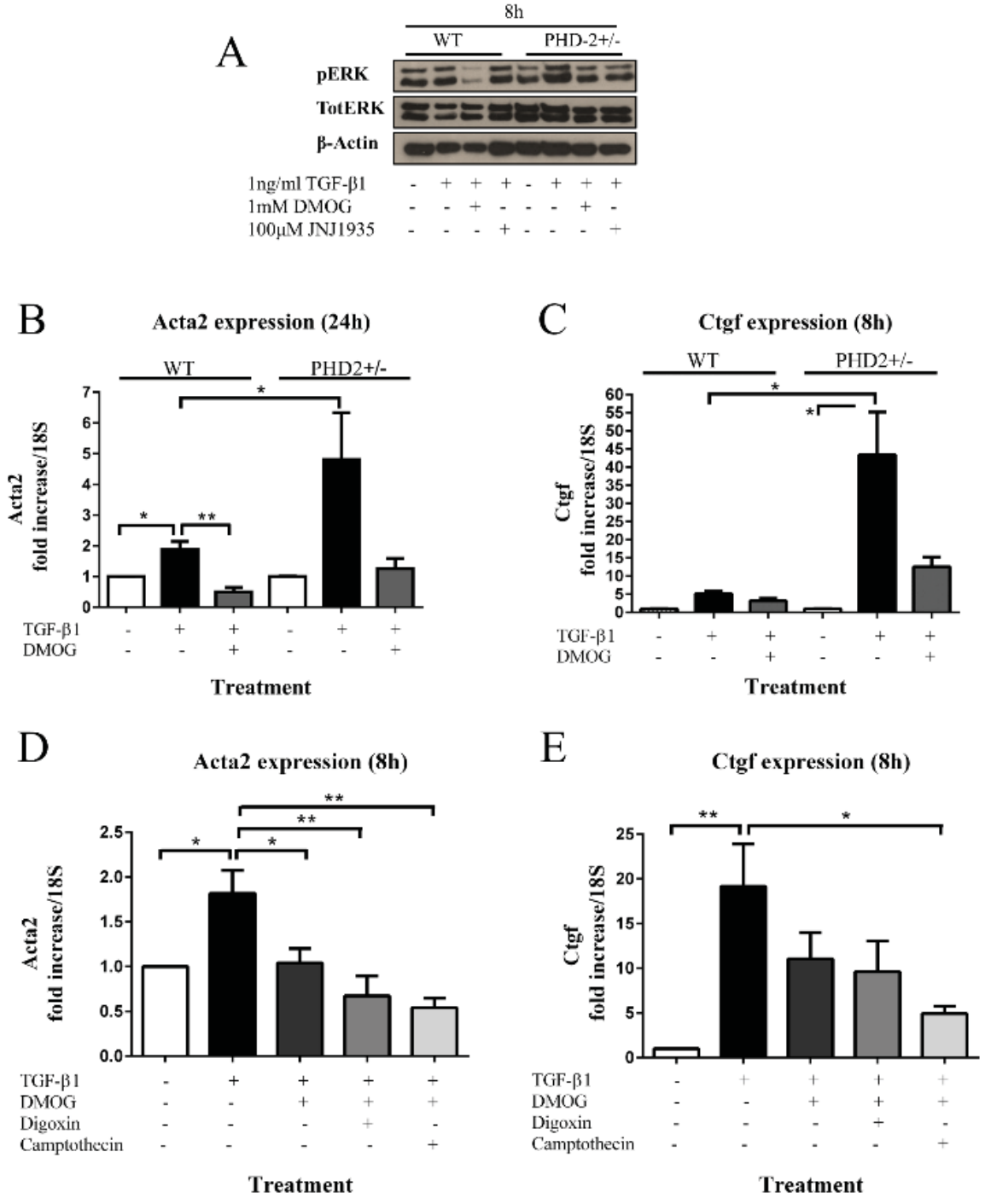
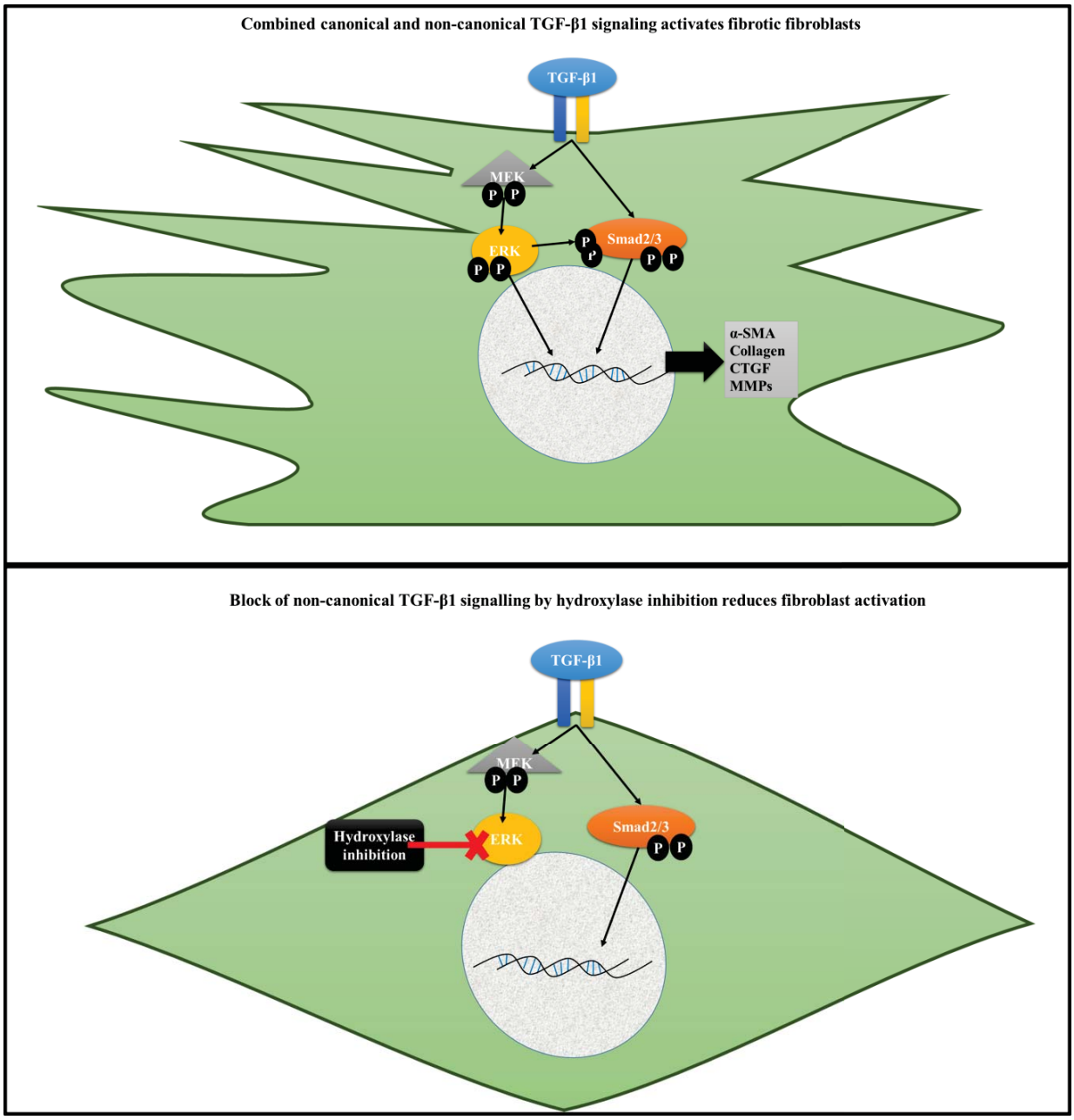

A
8h
WT
PHD-2+/-
pERK
TotERK
β-Actin
1ng/ml TGF-β1 - + + + - + + +
1mM DMOG - - + - - - + -
100μM JNJ1935 - - - + - - - +
B
Acta2 expression (24h)
WT
PHD2+/-
Acta2 fold increase/18S
TGF-β1 - + + - + +
DMOG - - + - - +
Treatment
C
Ctgf expression (8h)
WT
PHD2+/-
Ctgf fold increase/18S
TGF-β1 - + + - + +
DMOG - - + - - +
Treatment
D
Acta2 expression (8h)
Acta2 fold increase/18S
TGF-β1 - + + + +
DMOG - - + + +
Digoxin - - - + -
Camptothecin - - - - +
Treatment
E
Ctgf expression (8h)
Ctgf fold increase/18S
TGF-β1 - + + + +
DMOG - - + + +
Digoxin - - - + -
Camptothecin - - - - +
Treatment

Figure 7


Combined canonical and non-canonical TGF-β1 signaling activates fibrotic fibroblasts
TGF-β1
MEK
P P
ERK
P P
Smad2/3
P P
P P
α-SMA
Collagen
CTGF
MMPs
Block of non-canonical TGF-β1 signalling by hydroxylase inhibition reduces fibroblast activation
TGF-β1
MEK
P P
Hydroxylase inhibition
ERK
Smad2/3
P P