# Arginase 1 expression is increased during hepatic stellate cell activation and facilitates collagen synthesis

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RESEARCH ARTICLE

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# Arginase 1 expression is increased during hepatic stellate cell activation and facilitates collagen synthesis

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## Abstract

Activation of hepatic stellate cells (HSC) is a key event in the initiation of liver fibrosis. Activated HSCs proliferate and secrete excessive amounts of extracellular matrix (ECM), disturbing liver architecture and function, leading to fibrosis and eventually cirrhosis. Collagen is the most abundant constituent of ECM and proline is the most abundant amino acid of collagen. Arginine is the precursor in the biosynthetic pathway of proline. Arginine is the exclusive substrate of both nitric oxide synthase (NOS) and arginase. NOS is an M1 (proinflammatory) marker of macrophage polarization whereas arginase-1 (Arg1) is an M2 (profibrogenic) marker of macrophage polarization. Differential expression of NOS and Arg1 has not been studied in HSCs yet. To identify the expression profile of arginine catabolic enzymes during HSC activation and to investigate their role in HSC activation, primary rat HSCs were cultured-activated for 7 days and expression of iNOS and Arg1 were investigated. Nor-NOHA was used as a specific and reversible arginase inhibitor. During HSC activation, iNOS expression decreased whereas Arg1 expression increased. Inhibition of Arg1 in activated HSCs efficiently inhibited collagen production but not cell proliferation. HSC activation is accompanied by a switch of arginine catabolism from iNOS to Arg1. Inhibition of Arg1 decreases collagen synthesis. Therefore, we conclude that Arg1 can be a therapeutic target for the inhibition of liver fibrogenesis.

**KEYWORDS**

Arg1, arginase, arginine, fibrosis, hepatic stellate cell, iNOS, proline

## 1 | INTRODUCTION

Liver fibrosis is the result of an uncontrolled wound healing response in chronic liver diseases of various etiology. Liver fibrosis is characterized by the accumulation of excessive amounts of extracellular matrix (ECM) in the liver.[1] Although liver fibrosis is still reversible, advanced liver fibrosis can progress to cirrhosis and even hepatocellular carcinoma.[2] In these terminal stages, unresolved ECM deposition segments hepatic lobules, causing architectural changes that eventually cause hepatic failure with severe complications.[1] In view of the poor prognosis of cirrhosis, therapy is aimed at the stages preceding cirrhosis including (reversible) fibrosis. However, no effective medication to treat liver fibrosis is currently approved for clinical application.

Fibrogenesis is the key pathogenic mechanism of liver fibrosis and is attributed to excessive production of ECM by myofibroblasts. In chronic liver diseases, hepatic stellate cells (HSCs) are the major source of myofibroblasts.[3] The HSC is the most abundant nonparenchymal cell type in the liver. In a healthy liver, HSCs maintain a quiescent state and store retinoids. Upon continuous liver injury, the quiescent HSCs (qHSC) transdifferentiate into activated HSCs (aHSCs) and acquire the ability to proliferate, migrate, and contract. Furthermore, they secrete large amounts of various ECM components to contribute to wound repair.[4,5] In pathological conditions, ECM secretion by aHSCs is uncontrolled and ECM accumulates in the perisinusoidal space.[6] The grade of liver fibrosis correlates with the long-term prognosis of patients.[7] Preclinical evidence suggests that reversal of the activated state of aHSCs to reduce the deposition of ECM is a promising strategy to slow down or even reverse liver fibrosis.[4] Collagen is one of the major components of ECM.[8] Collagen contains a relatively high proportion of the amino acids arginine, proline and glycine and the availability of these amino acids is the limiting factor in the biosynthesis of collagen.[9] This phenomenon permits to target amino acid metabolism to reduce fibrogenesis.

The metabolism of the nonessential amino acid arginine is closely related to the metabolism of proline. L-arginine is the exclusive substrate of the enzymes nitric oxide synthase (NOS) and arginase.[9] NOS has three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) which are encoded by NOS1, NOS2, and NOS3, respectively. Arginase has two isoforms: arginase-1 (Arg1) and Arg2. Arg1 is predominantly expressed in the cytoplasm of liver cells, whereas Arg2 is localized in mitochondria of kidney cells.[10] NOS converts arginine into nitric oxide (NO) and citrulline. NO is an important gasotransmitter and activates soluble guanylate cyclase (sGC). Some studies suggest that activation of sGC attenuates fibrosis.[11] In addition, NO can serve as a scavenger of reactive oxygen species (ROS) and inhibit the proliferation of aHSCs.[12] Arginase catalyzes the reaction arginine + $H_2O$ → ornithine + urea. Ornithine can be converted by ornithine aminotransferase (OAT) into proline and by ornithine decarboxylase (ODC) into polyamines. Proline is essential for collagen synthesis. Polyamines are necessary for progression through the cell cycle and proliferation.[10] It has been demonstrated that arginine promotes the synthesis of proline and collagen in vascular smooth muscle cells, a cell type which could be considered the myofibroblast in vascular tissue.[13] Since the catabolism of arginine by NOS and arginase are likely to induce opposite effects in HSCs, the metabolic fate of arginine is hypothesized to be associated with the trans-differentiation of HSCs. However, there is currently little evidence to support the hypothesis that (increased) arginase promotes fibrosis and (increased) NOS attenuates fibrosis.

The differential expression of iNOS and Arg1 is associated with M1 and M2 polarization in macrophages, respectively.[10] It has not been elucidated whether activation of HSCs is associated with a similar metabolic shift of arginine catabolism. Our hypothesis in this study is that the activation of HSCs is accompanied by a metabolic shift in arginine catabolism. Primary rat HSCs are an established in vitro model to study the activation of HSCs and were used in this study to test the hypothesis.

## 2 | MATERIALS AND METHODS

### 2.1 | Hepatic stellate cell isolation and culture

Primary rat HSCs were isolated as previously described[14] and cultured in Iscove's Modified Dulbecco's Medium supplemented with Glutamax (Thermo Fisher Scientific), 20% heat-inactivated fetal calf serum (Thermo Fisher Scientific), 1% MEM Non Essential Amino Acids (Thermo Fisher Scientific), 1% Sodium Pyruvate (Thermo Fisher Scientific) and antibiotics: 50 μg/mL gentamycin (Thermo Fisher Scientific), 100 U/mL Penicillin (Lonza), 10 μg/mL streptomycin (Lonza) and 250 ng/mL Fungizone (Lonza) in an incubator containing 5% $CO_2$ at 37°C. HSCs were culture-activated for 7 days on tissue culture plastic.

### 2.2 | Quantitative real-time polymerase chain reaction

Total RNA was isolated by Tri-reagent (Sigma-Aldrich) according to manufacturer's protocol and then used for

preparation of cDNA. cDNA was diluted in RNAse-free water and used for real-time polymerase chain reaction on the QuantStudioTM 3 system (Thermo Fisher Scientific). All samples were analyzed in duplicate using 36b4 as housekeeping gene. The genes were quantified by TaqMan probes and primers. Relative gene expression was calculated via the $2^{-\Delta\Delta C_t}$ or $2^{-\Delta C_t}$ method. Arg2 messenger RNA (mRNA) levels were assessed by "Assay on Demand" (Thermo Fisher Scientific; #Rn01469630_m1). The primers and probes are shown in Supporting Information: Table 1.

### 2.3 | Cell proliferation assays

Cell proliferation was determined by BrdU incorporation assay (Roche Diagnostic Almere) and Real-Time xCelligence assay (RTCA DP; ACEA Biosciences, Inc.). Cells were seeded in 96-well plates and treated as described. Incorporation of BrdU was detected by chemiluminescence using Synergy-4 (Bio-Tek).

### 2.4 | Western blot analysis

Cells were seeded and treated as described. Protein lysates were prepared by scraping in cell lysis buffer (HEPES 25 mmol/L, KAc 150 mmol/L, EDTA pH 8.0; 2 mmol/L, NP-40 0.1%, NaF 10 mmol/L, PMSF 50 mmol/L, aprotinin 1 μg/μL, pepstatin 1 μg/μL, leupeptin 1 μg/μL, DTT 1 mmol/L). 10–20 μg protein was loaded on SDS-PAGE gels and transferred to nitrocellulose transfer membranes using Trans-Blot Turbo Blotting System for tank blotting. Proteins were detected using the primary antibodies listed in Supporting Information: Table 2. Protein band intensities were determined and detected using the Chemidoc MR (Bio-Rad) system. The protein expression was quantified with ImageJ software and the integrated intensity of protein bands was normalized and used for comparison.

### 2.5 | RNA sequencing data analysis

Human liver transcription raw counts data were collected from Liver hepatocellular carcinoma (LIHC) data set of The Cancer Genome Atlas (TCGA) database and the GSE176042 data set of the Gene Expression Omnibus (GEO) database.[15] RNA sequencing data were processed following a combination of limma and edgeR methods using R (4.1.3 version).[16]

### 2.6 | Statistical analysis

Data are presented as mean ± standard deviation (mean ± SD) or mean ± standard error of mean (mean ± SEM) of at least three independent experiments. Statistical significance was analyzed by Mann–Whitney test between the two groups. $p < 0.05$ was considered statistically significant. Analysis was performed using GraphPad Prism 7 (GraphPad Software).

## 3 | RESULTS

### 3.1 | Differential transcription of iNOS and Arg1 during activation of HSCs

The mRNA level of Arg1 was increased up to fivefold in aHSCs compared with qHSCs (Figure 1A), whereas mRNA level of iNOS was strongly reduced in aHSCs (Figure 1B). The ratio of Arg1 to iNOS mRNA expression was calculated and this ratio was strongly increased during activation (Figure 1C). These results demonstrate that arginine catabolizing enzymes exhibit differential transcription profiles in qHSCs and aHSCs. In addition, we performed RT-qPCR to detect mRNA expression of Arg2. The $C_t$ values of Arg2 were above 35 or undetermined, indicating that Arg2 is not expressed in HSCs. Activation of HSCs at Day 7 was confirmed by measuring mRNA level of Acta2, an established marker of HSC activation (Figure 1D).

### 3.2 | Protein expression of arginine catabolizing enzymes

In line with the mRNA levels, the protein level of iNOS was increased at Day 1 but strongly reduced at Day 7 (Figure 2A,C). Protein expression of Arg1 was increased at Day 3 and appeared to be even more increased at Day 7 (Figure 2A,C). As expected, protein levels of the activation markers collagen type 1 and α-smooth muscle actin (αSMA) were strongly induced in aHSCs. In addition, aHSCs (D7) treated with the profibrogenic cytokine TGF-β demonstrated increased expression of Arg1 (Figure 2B).

### 3.3 | Inhibition of Arg1 reduces collagen production in HSCs without affecting proliferation

Nor-NOHA, a specific inhibitor of arginase, was used to demonstrate the necessity of Arg1 for collagen production

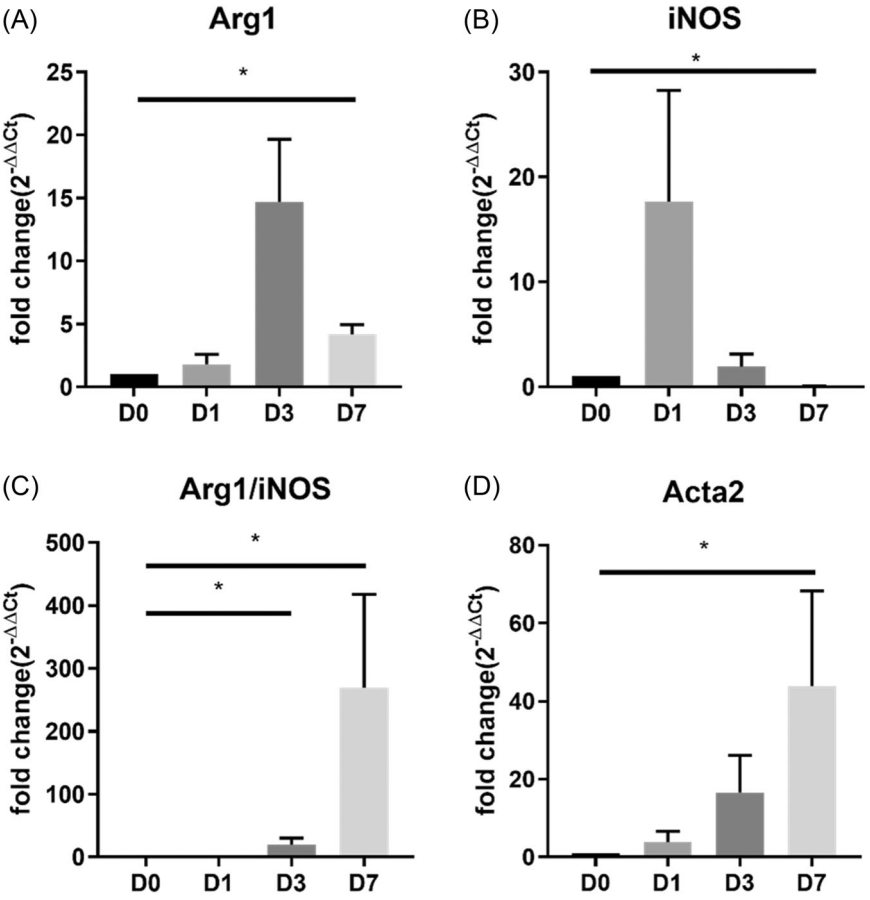


**FIGURE 1** mRNA expression of iNOS, Arg1 and Acta2 during activation of primary rat HSCs. (A) mRNA expression of Arg1. (B) mRNA expression of iNOS. (C) Ratio of mRNA expression of Arg1 to iNOS. (D) mRNA expression of Acta2. Data are shown as mean ± SEM. Arg1, Arginase-1; HSC, hepatic stellate cell; iNOS, inducible nitric oxide synthase; mRNA, messenger RNA. $n = 3$, $^*p < 0.05$.

and proliferation of aHSCs. Nor-NOHA at 50 μmol/L significantly inhibited collagen type 1 protein level, but had no effect on αSMA protein expression (Figure 3A–C). BrdU proliferation assay was performed to verify the effect of Nor-NOHA on aHSCs. As shown in Figure 3D, Nor-NOHA at concentrations ranging from 2 to 50 μmol/L did not affect HSC proliferation. These data demonstrate that arginase specifically affects collagen protein synthesis in HSCs.

We also investigated the effect of nor-NOHA on qHSCs. qHSCs (Day 1) were treated with 50 μmol/L nor-NOHA for 3 or 5 days (early-intermediate stages of activation). As shown in Figure 4, the mRNA expression of collagen type 1 and αSMA were slightly but significantly reduced by nor-NOHA in activating HSCs. The reason for the minor, but statistically significant, reduction of collagen type 1 and αSMA expression in activating HSCs is that the expression levels of these markers is still very low at these stages of activation compared with fully aHSCs.

### 3.4 | Arginase inhibition reduces arginine-stimulated proliferation of HSCs

Polyamine synthesis is dependent on arginase catalyzing the conversion of arginine into ornithine. Therefore, addition of exogenous arginine may increase the synthesis of polyamines and promote cell proliferation. To verify this hypothesis, cell proliferation was analyzed in real-time in HSCs treated with 0.2–5 mmol/L L-arginine (Figure 5A). Only 1 mmol/L arginine increased proliferation of HSCs. Since serum-containing culture medium contains significant amounts of arginine, we performed experiments using arginine starvation followed by low-serum containing culture medium (Figure 5B). After arginine starvation, aHSCs appeared to be more sensitive to exogenous arginine with 0.5 mmol/L exogenous arginine maximally increased proliferation. Next, we investigated the effect of the arginase inhibitor nor-NOHA on the proliferation of HSCs (Figure 5C). HSCs were treated with 1 mmol/L arginine with or without 100 μmol/L Nor-NOHA and the cell proliferation was analyzed. The arginase inhibitor only decreased proliferation of HSCs treated with arginine. The results indicate that exogenous arginine promotes cell proliferation of HSCs in an arginase-dependent manner.

## 4 | DISCUSSION

Activation of HSCs is a key pathogenic mechanism in liver fibrosis. It has been demonstrated that clearance and/or inactivation of aHSCs is a therapeutic mechanism to alleviate or resolve liver fibrosis.[4,17] The activation of

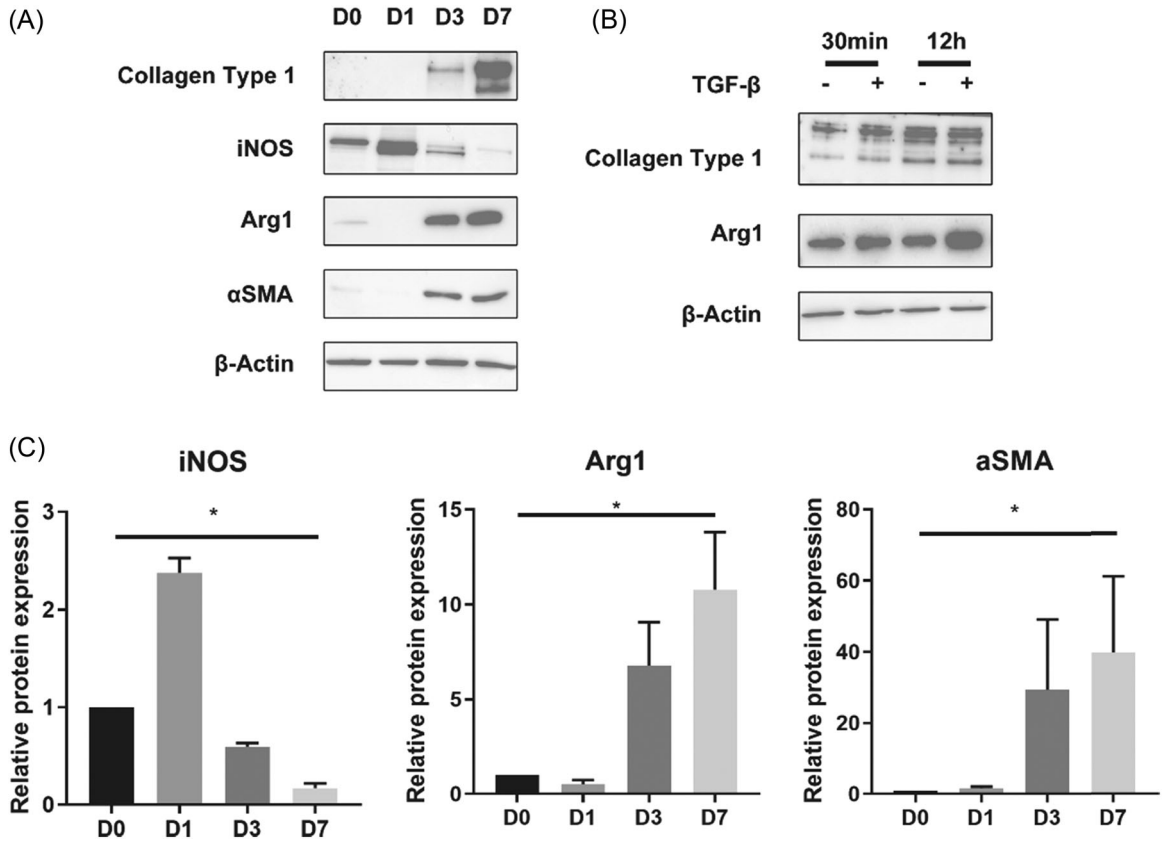


FIGURE 2 (A) Protein levels of the arginine catabolizing enzymes iNOS and Arginase- 1 (Arg1) and the HSC activation markers α-smooth muscle actin (αSMA) and collagen type 1. β-actin was used as loading control. $n = 3$. (B) Protein expression of collagen type 1 and Arg1 in activated HSCs treated with or without 5 ng/mL TGF-β. (C) Relative protein expression of iNOS, Arg1, and αSMA. Intensity of protein signals from repeated Western blot analysis was quantified and analyzed. Data are shown as mean ± SEM, $n = 3$. HSC, hepatic stellate cell; iNOS, inducible nitric oxide synthase. *$p < 0.05$.

HSCs is driven by endogenous and exogenous factors, including mitogens such as TGF-β, PDGF, and FGF.[4,18] Upon activation, HSCs acquire the ability to secrete profibrogenic mitogens and cytokines, causing a positive feedback loop in fibrogenesis.[19] Moreover, endogenous factors affect HSC activation such as organellar function, autophagy, and so forth.[20-22] Spontaneous activation of isolated primary HSCs on tissue culture plastic is a known model for HSC activation.[23] Even though the exact mechanism of spontaneous activation is not fully elucidated yet, primary aHSCs share a similar phenotype with in vivo aHSCs.[24] Increased expression of ACTA2 and COL1A1 and reduced expression of the quiescence-associated transcription factor PPARG and LRAT are characteristic for the transcription profile of aHSCs and is identical in culture aHSCs and in situ aHSCs.[3,24]

Arginine is a nonessential amino acid but it is conditionally essential during wound healing.[25] Arginine is the exclusive substrate of NOS and arginase. NOS consists of three isoforms. iNOS is an inducible NOS widely expressed in many cell types while eNOS and nNOS are specifically expressed in endothelium and neurons, respectively. Arginase has two isoforms Arg1 and Arg2. Arg1 is dominantly expressed in liver while Arg2 is dominantly expressed in kidney.[10] RNA sequencing data from human liver tissue and mouse primary HSCs demonstrated that mRNA expression of ARG1 (Arg1) was much higher than that of ARG2 (Arg2) (Supporting Information: Figure 1). These data were confirmed for rat HSCs in our study, demonstrating that rat HSCs do not express Arg2.

Increased Col1a1 expression correlated with increased Arg1 expression but not with increased Acta2 expression. In addition, ornithine metabolizing enzyme ornithine decarboxylase (Odc1) and the proline biosynthetic pyrroline-5-carboxylate reductase family member 2 (Pycr2) enzyme were significantly downregulated (Supporting Information: Figure 1). These results indicate that increased mRNA expression of Arg1 correlates closely to collagen production.

NOS and arginase play opposite roles in the polarization of macrophages, with NOS promoting the

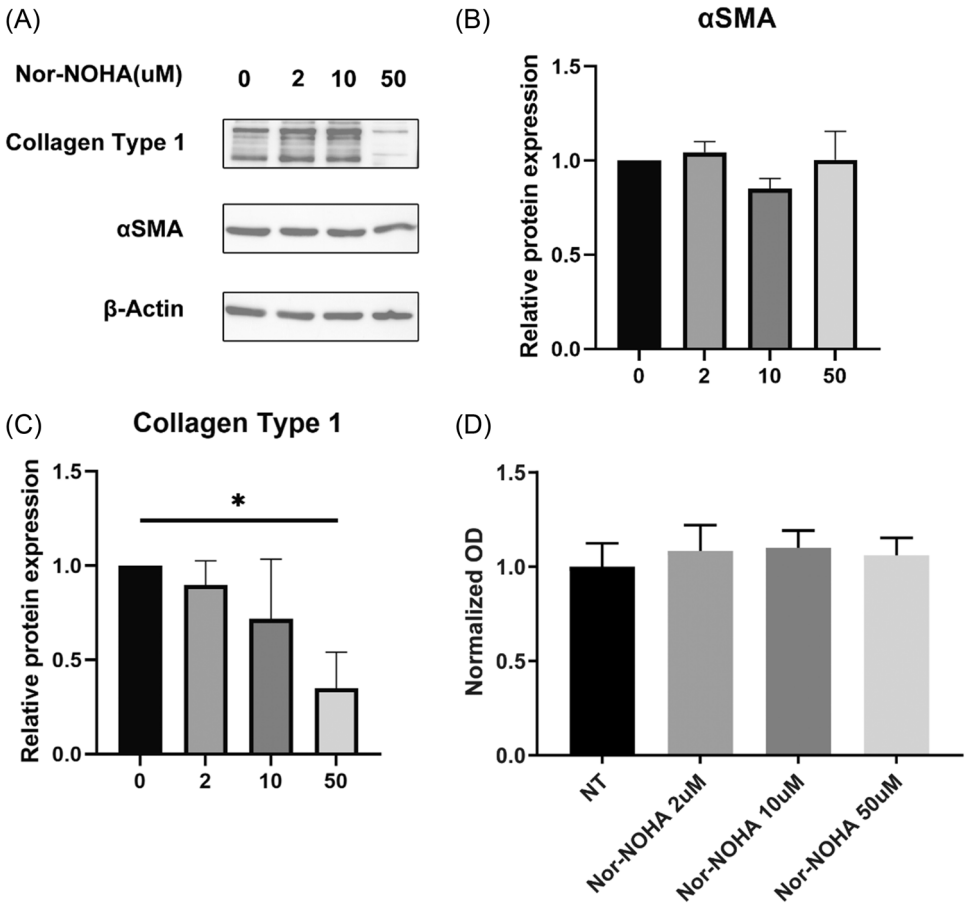


**FIGURE 3** Effect of the Arg1 inhibitor Nor-NOHA on aHSCs. (A) Representative Western blot for collagen type 1 and αSMA in aHSCs treated with Nor-NOHA for 72 h. (B) Quantification of Western blot results for αSMA normalized to β-Actin. (C) Quantification of Western blot results for collagen type 1 normalized to β-Actin. (D) BrdU incorporation into aHSCs treated with Nor-NOHA for 72 h. $n = 3$. aHSC, activated hepatic stellate cell; Arg1, Arginase-1; αSMA, α-smooth muscle actin. $^*p < 0.05$.

M1 phenotype and Arginase the M2 phenotype.[10] NO has been demonstrated to inhibit the proliferation of aHSCs and alleviate liver fibrosis.[26] Activation of sGC, the downstream target of NO, has been demonstrated to suppress activation of HSCs as well.[27] Ornithine, which is synthesized from arginine by arginase, is subsequently converted by ODC and OAT into putrescine and proline, respectively. Putrescine is the main substrate for polyamine biosynthesis and is essential for cell cycle progression.[28] Proline is the most abundant amino acid of collagen. The opposite roles of NOS and arginase on macrophage polarization is well-known. However, a shift in arginine catabolism between NOS and Arginase in the activation of HSCs has not been demonstrated before. In this study, we have demonstrated that during activation of primary HSCs, the dominant catabolic enzyme of arginine shifts from iNOS to Arg1. The differential expression of iNOS and Arg1 was demonstrated both at the mRNA and protein level. The differential expression of iNOS and Arg1 in HSC activation is in accordance with the function of proline in collagen production and polyamines in proliferation of aHSCs. Inhibition of NOS has been demonstrated to promote activation of fibroblasts and to prevent resolution of liver fibrosis.[29,30] In a rat model of wound healing induced by experimental surgical trauma, oral administration of arginine increased the systemic concentration of hydroxyproline, a marker of collagen production.[31] Likewise, inhibition of Arg1 or OAT decreased the concentration of intracellular proline and reduces collagen synthesis in vascular smooth muscle cells and colonic epithelial cell.[13,32] Based on these studies, we hypothesized that the downregulation or inhibition of iNOS and the upregulation of Arg1 induces a switch in arginine catabolism that promotes activation of HSCs.

Since arginase activity determines proline and collagen synthesis and cell proliferation, inhibition of Arg1 could be a therapeutic method to alleviate hepatic fibrosis. N(omega)-hydroxy-nor-L-arginine (nor-NOHA) is a specific arginase inhibitor without inhibitory effects

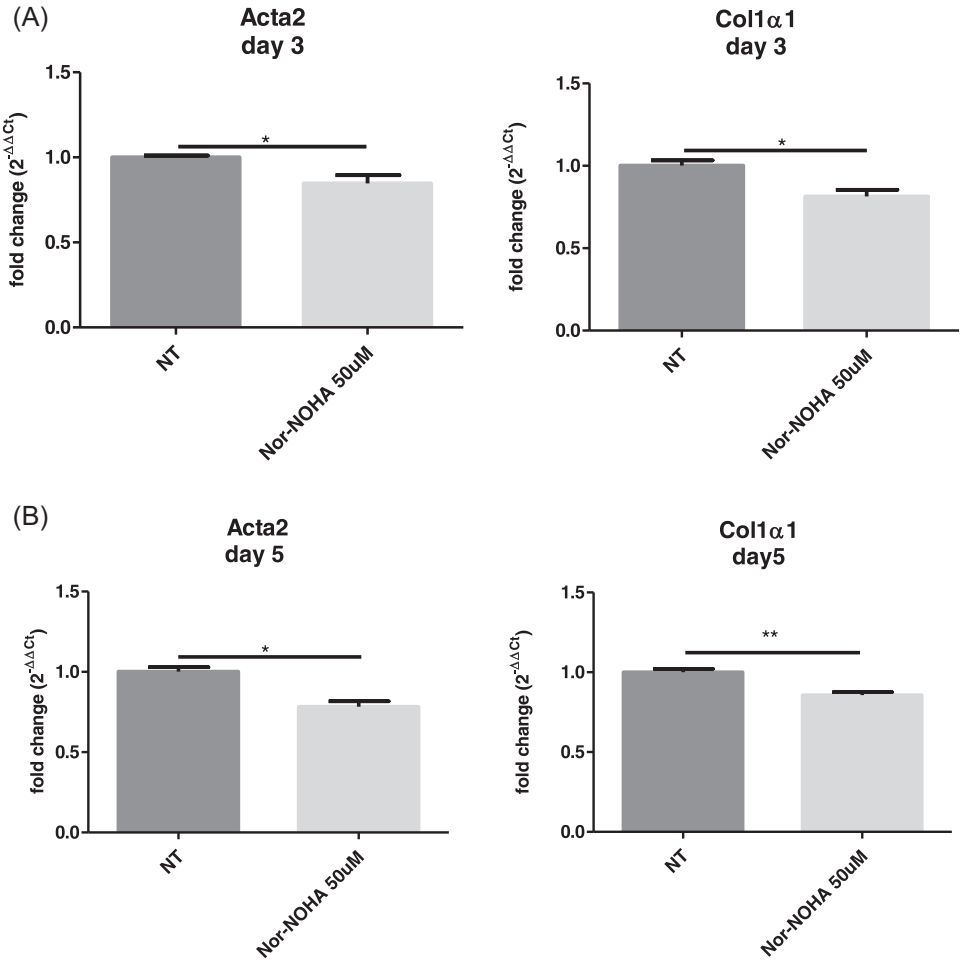


**FIGURE 4** Effect of the Arg1 inhibitor Nor-NOHA on quiescent HSCs. (A) Expression of Acta2 and Col1α1 in quiescent HSCs at Day 3 after isolation with and without Nor-NOHA treatment. (B) Expression of Acta2 and Col1α1 in quiescent HSCs at Day 5 after isolation with and without Nor-NOHA treatment. Data are shown as mean ± SEM. HSC, hepatic stellate cell. $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$.

on NOS.[33] Our results demonstrated that inhibition of Arg1 by Nor-NOHA reduced collagen production but not αSMA expression. Recent single-cell RNA sequencing data indicated that αSMA and collagen type 1 have different regulators: some collagen-producing HSCs have low expression of αSMA.[34] Moreover, we have recently demonstrated that senescent HSCs show decreased collagen synthesis without affecting αSMA expression.[35] The reason for this differential regulation may be both transcriptional as well as posttranscriptional (e.g., differences in protein half-life). Moreover, since arginine/arginase determines the supply of arginine for proline synthesis and since proline is the predominant amino acid in collagen, the effects of arginine/arginase may be specific for the regulation of collagen.

It has been shown that proliferation of some cell lines is sensitive to arginine.[36] Apart from fueling putrescine synthesis, arginine has been demonstrated to increase global protein synthesis also at low concentrations.[37] Our results show that nor-NOHA did not inhibit DNA replication, as indicated by BrdU incorporation assay performed on aHSCs cultured in normal fetal calf serum-containing medium. However, nor-NOHA did inhibit proliferation of aHSCs cultured in arginine-deficient (low FCS) medium. Supplementation of arginine did increase proliferation of HSCs and nor-NOHA inhibited cell proliferation stimulated by exogenously supplemented arginine. We assume that serum-containing culture medium contains significant amounts of arginine to preserve ornithine levels for polyamine biosynthesis. However, in arginine-deficient conditions, ornithine depletion together with nor-NOHA treatment impairs polyamine biosynthesis and eventually inhibits the proliferation of HSCs. Ornithine is an amino acid that is not present in proteins. It is synthesized from arginine by arginase. ODC is the rate-limiting enzyme of polyamine synthesis. Two proteins, an antizyme and antizyme inhibitor, form an autoregulatory loop that associates with ODC to maintain polyamine homeostasis. The antizymes bind with high affinity to the ODC

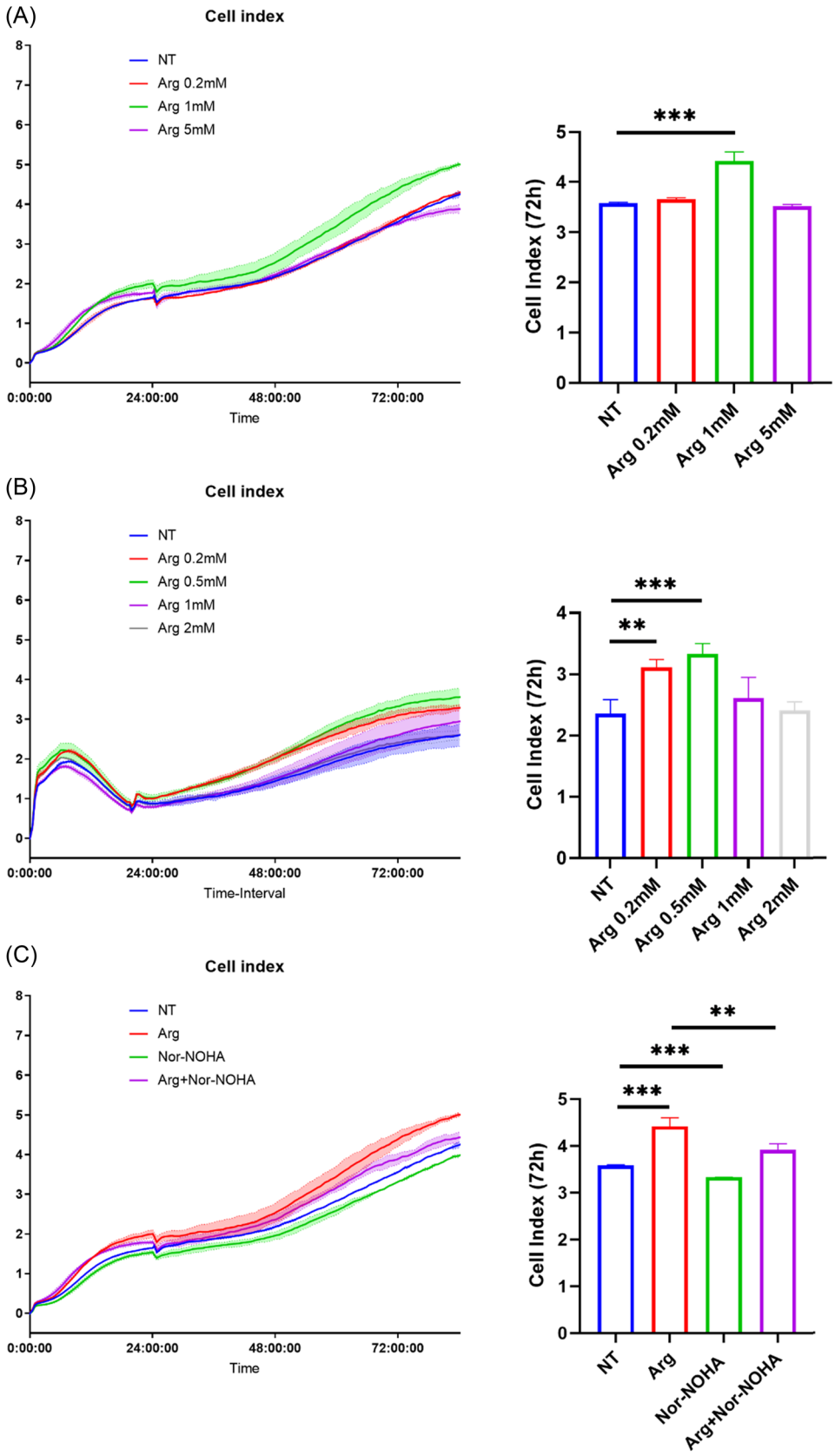


**FIGURE 5** Cell proliferation of HSCs treated with arginine and/or Nor-NOHA. (A) Real-time and 72 h cell index of HSCs treated with different concentrations of arginine. Arginine (Arg) was added 24 h after cell seeding. (B) HSCs cultured in arginine-deficient medium for 20 h followed by culture in low serum (1% FCS) medium with different concentrations of arginine. (C) Real-time and 72 h cell index of HSCs treated with arginine and/or Nor-NOHA. The concentrations of arginine and Nor-NOHA were 1 and 100 μmol/L, respectively. HSC, hepatic stellate cell. $^{**}p < 0.05$; $^{***}p < 0.01$.

protein, resulting in its degradation. The antizyme inhibitor is highly homologous to the ODC protein, but lacks ornithine decarboxylating activity. The antizyme inhibitor binds to the antizymes and prevents the degradation of the ODC protein.[38] It has been observed that polyamine synthesis remains intact even when ornithine concentrations are reduced by the arginase inhibitor nor-NOHA.[39] Therefore, we assume that the ODC activity of aHSCs is less sensitive than OAT when the intracellular concentration of ornithine is decreased by nor-NOHA. However, in arginine-deficient conditions, polyamine biosynthesis is likely to be reduced due to the lack of ornithine caused by arginase inhibition.

In conclusion, the activation of HSC is accompanied by a switch in arginine catabolism resulting from downregulation of iNOS and upregulation of Arg1. Inhibition of Arg1 can be an anti-fibrotic target for the treatment of liver fibrosis.

## AUTHOR CONTRIBUTIONS

**Mengfan Zhang and Zongmei Wu**: Conceptualization; investigation; formal analysis; methodology; writing first draft. **Sandra Serna Salas, Magnolia Martinez Aguilar, Maria Camila Trillos-Almanza**: Investigation; writing; methodology. **Manon Buist-Homan**: Investigation; methodology; validation; project administration; writing-review/editing. **Han Moshage**: Conceptualization; project administration; funding acquisition; writing-review/editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ETHICS STATEMENT

The authors state that the data that support the findings of this study were generated in accordance with the ethical guidelines outlined by the journal and that all animal experiments were conducted according to Dutch laws on the welfare of laboratory animals (The Animal act 2011) and Permission No 16778-01-002 of the Committee for the care and use of laboratory animals of the University of Groningen.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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